Journal of Chemical Ecology, Vol. 23, No. 4, 1997

ATTRACTION OF COLORADO POTATO BEETLE TO HERBIVORE-DAMAGED PLANTS DURING HERBIVORY AND AFTER ITS TERMINATION

CAROLINE J. BOLTER,[1,2] MARCEL DICKE,[1,*] JOOP J. A. VAN LOON,[1] J. H. VISSER,[3] and MAARTEN A. POSTHUMUS[4]

[1]*Department of Entomology, Wageningen Agricultural University P.O. Box 8031, 6700 EH Wageningen, The Netherlands*

[2]*Pest Management Research Centre, Agriculture and Agri-food Canada 1391 Sandford Street, London, Ontario, N5V 4T3, Canada*

[3]*Research Institute for Plant Protection IPO-DLO P.O. Box 9060, 6700 GW Wageningen, The Netherlands*

[4]*Department of Organic Chemistry, Wageningen Agricultural University Dreijenplein 8, 6703 HB Wageningen, The Netherlands*

(Received May 13, 1996; accepted November 27, 1996)

Abstract—Large, undamaged potato plants (>60 cm, 5–6 weeks old) attract the Colorado potato beetle (*Leptinotarsa decemlineata*), but small potato plants (15–25 cm high, 2–3 weeks old) do not. However, small plants become attractive to CPB when they are damaged. Mechanical damage inflicted with scissors results in short-term (lasting less than 15 min) attraction, while more severe damage with carborundum powder results in a longer lasting attraction (at least 1 hr). CPB adults are also attracted to small plants infested with CPB and *Spodoptera exigua* larvae. After the larvae had been removed for 50 min following a short duration (30 min) of feeding, CPB adults were no longer attracted to the plants. However, when CPB larvae had been removed after they had fed for 60–90 min, the plants were somewhat attractive to the beetles, although significantly less than they had been when the larvae were feeding. Attraction increased with time after feeding ceased. Furthermore, beetles were strongly attracted to plants 50 min after larvae were removed when the plants had been fed upon by larvae for 18–24 hr. Thus it appears that there are two stages of attraction, first, to volatiles released directly from the wound site, and second, to volatiles that are induced in response to herbivory. Chemical analyses of the headspace of infested potato plants show that infestation results in the emission of a mixture of chemicals that is qualitatively quite similar to that emitted by undamaged plants. The major components of the mixture are

*To whom correspondence should be addressed.

0098-0331/97/0400-1003$12.50/0 © 1997 Plenum Publishing Corporation

that emitted by undamaged plants. The major components of the mixture are terpenoids and fatty acid derivatives such as aldehydes and alcohols. The emission rate of some of these chemicals declines after removal of the beetles, while the emission rate of other chemicals increases with the duration of beetle feeding and remains at a high level even after removal of the beetles. Thus, the composition of the mixture changes temporally during and after herbivore feeding, which may explain the recorded behavior of the beetles.

Key Words—Coleoptera, Chrysomelidae, *Leptinotarsa decemlineata*, olfaction, locomotion compensator, behavior, gas chromatography–mass spectrometry, terpenoids, lipoxygenase, fatty acid derivatives.

INTRODUCTION

When herbivores feed on a plant, cell damage results and there is a release of volatiles from the wound site. In addition to such general damage-related effects, herbivory may also result in the emission of plant volatiles that are not induced by mechanical damage alone. Studies carried out in the past decade have shown that a range of plant species become highly attractive to carnivorous arthropods after damage by herbivores (reviewed by Vet and Dicke, 1992; Dicke, 1994; Turlings et al., 1995; Takabayashi and Dicke, 1996). Two different types of plant responses have been recorded: (1) Herbivory may lead to the emission of relatively large amounts of plant volatiles that are not emitted or only emitted in trace amounts by mechanically damaged plants or undamaged plants (Dicke et al., 1990, Turlings et al., 1990). The composition of the blend emitted by herbivore-infested plants may depend on the herbivore species that infests the plant (Takabayashi et al., 1991, Turlings et al., 1993). This type of response has been recorded in various plant species, such as lima bean, cucumber, corn, and soybean (for reviews see Dicke, 1994; Takabayashi et al., 1994; Turlings et al., 1995). (2) Alternatively herbivore infestation may lead to the emission of a volatile blend that is qualitatively similar to that emitted by mechanically damaged or undamaged plants. However, the amount released from herbivore-infested plants is larger than that from mechanically damaged or undamaged plants. This category is represented by cabbage plants (Blaakmeer et al., 1994; Mattiacci et al., 1994; Agelopoulos and Keller, 1994).

Studies on volatiles from herbivore-infested plants have mostly been restricted to the effects on carnivore behavior. These are considered to be adaptive responses by the plant, because an influx of carnivores often leads to the decimation of herbivore populations (Dicke and Sabelis, 1989; Dicke, 1995). The volatiles emitted by herbivore-infested plants have other adaptive effects as well. The compounds, especially the fatty acid derivatives produced through the lipoxygenase pathway, may be toxic to herbivores such as aphids (Hildebrand et al., 1993) or phytopathogenic fungi (Zeringue and McCormick, 1989; Croft

et al., 1993). Such effects also contribute to plant defense. However, the increased volatiles also make the plant stand out among its neighbors, and this may represent a risk. Herbivores are well known to use plant volatiles to locate a food plant (Visser, 1986), and if a plant increases its emission of volatiles, this may be exploited by herbivores to locate a food plant. However, the volatiles from an herbivore-infested plant represent a food source with competitors and an elevated risk of carnivore influx. Thus, it is not possible to predict whether herbivores will be attracted to these volatiles or whether they will use them to avoid infested plants. Indeed, both types of responses have been found. Adult two-spotted spider mites (*Tetranychus urticae*) were attracted to volatiles from uninfested lima bean plants but avoided volatiles from spider-mite-infested leaves unless the blend was diluted with volatiles from uninfested leaves (Dicke, 1986). Avoidance behavior was also recorded for beet armyworm caterpillars (*Spodoptera exigua*) in response to corn leaves infested by conspecifics (Turlings and Tumlinson, 1991). In contrast, adult scarabaeid beetles (*Maladera matrida* and *Popillia japonica*) were attracted to host plants infested by conspecific or by heterospecific herbivores (Harari et al., 1994; Loughrin et al., 1995). It remains unclear why some herbivore species are attracted and others avoid volatiles from herbivore-infested plants. This may be caused by differences in herbivore biology, such as an improved use of host plant tissue when living in aggregations, employment of secondary plant compounds in the defense against predators, or as a method for meeting a suitable mate (Birch, 1984; Pasteels et al., 1988). Alternatively, it may also depend on the type of plant response to herbivory, i.e., on the type of information conveyed by the plant. For instance, if the blend emitted by infested plants is the same as that from uninfested plants, the information on plant quality is more reliable than when the blends differ in composition (see above). In this paper we present data for another beetle species, the Colorado potato beetle (*Leptinotarsa decemlineata* Say) (CPB) (Coleoptera: Chrysomelidae) and demonstrate that it is attracted to small potato plants infested with conspecific or heterospecific herbivores, but not to small undamaged plants.

The behavioral response of the Colorado potato beetle to plant volatiles has been intensively studied. The beetles are attracted to volatiles from large uninfested potato plants through odor-conditioned positive anemotaxis (e.g., Visser, 1976; Visser and Thiery, 1985; Thiery and Visser, 1986, 1987). Electrophysiological experiments demonstrated that the beetle's olfactory receptors are sensitive to a range of components, especially C_6 alcohols and derivatives emitted by potato plants (Ma and Visser, 1978; Visser, 1979, 1983; de Jong and Visser, 1988a,b). Here we show that attraction to uninfested potato plants does not occur when the plants are small. However, when small plants are infested by conspecific larvae they become highly attractive to adult beetles. This also occurs after the CPB larvae have been removed from the plant. Interestingly, removal of CPB larvae from an infested plant first leads to a decreased

attraction, which is followed by a temporal rise in attraction. This phenomenon is affected by the preceding feeding duration of the infesting larvae. These data are discussed in the context of different types of plant response to herbivore infestation.

METHODS AND MATERIALS

Insects and Plants. Potato plants, *Solanum tuberosum* L. cultivar Surprise, were grown in an air-conditioned greenhouse at 21 ± 2°C and a 16-hr photoperiod. The light was supplemented with 400-W high-pressure sodium-vapor lamps hanging 1 m above pot level when daylight intensity fell below 240 W/m^2. Colorado potato beetles were reared on potato foliage at 20–25°C. Newly emerged female beetles were collected from the laboratory stock culture and placed individually in Petri dishes, where they were allowed to feed on an excised potato leaf for 4 hr. The plant material was then removed and the beetles were starved for at least 16 hr before the experiment. Beetles that did not eat were excluded from the experiment.

Recording of Beetle Behavior. Locomotory responses of the CPB were measured using a locomotion compensator in front of a low-speed (80 cm/sec) wind tunnel as described in detail by Thiery and Visser (1986). Incoming air was filtered through activated charcoal and then passed through the wind tunnel containing the potted plant material. Using a small piece of reflective tape glued to the insect's elytra, movements of the CPB were detected and compensated for by the rotation of the 50-cm-diam. sphere on which the insect was positioned. The insect effectively remained in the same position relative to the wind tunnel. The rotation of the sphere was detected by two pulse generators, which allowed the movement to be recorded and analyzed by a microprocessor. Visual surroundings were made homogeneous by the use of a horizontal circular white plate around the sphere and a black curtain surrounding the apparatus. The light intensity was set at 1750 lux by means of two high-frequency illumination units (2500 Hz) that were suspended over the sphere. Each unit contained six daylight fluorescent lamps. The beetle's position was recorded every second, and walking speed and direction calculated. Five variables were used to quantify the adult beetle's behavior: (1) walking speed (millimeters per second), (2) vector length (millimeters), the resultant displacement from the origin after 300 sec; (3) straightness, the quotient of the vector length and the total length of the walking track; (4) upwind length (millimeters), the upwind displacement after 300 sec; and (5) upwind fixation, the quotient of upwind length and the total track length (Thiery and Visser, 1986, 1987; de Jong, 1988). Upwind fixation varies between −1 (absolutely straight downwind movement) and +1 (absolutely straight upwind movement). Variables were compared by Kruskal-Wallis one-way analysis of variance, followed by Mann-Whitney U tests among treatments with Bonferroni correction for α.

When beetles are strongly attracted by volatiles blown from the wind tunnel, their walking behavior on the sphere is characterized by a longer vector, a higher straightness value, longer upwind length, and higher upwind fixation value than in a control situation and often a higher walking speed (Thiery and Visser, 1986). The most informative indicator of attraction is upwind fixation, which is a measure of the degree of direct upwind movement.

Experiments. In all treatments, two pots (5 liters) containing four tubers per pot were used. Plants were placed in a controlled environment room at 25 ± 1°C, 16-hr photoperiod, during feeding treatments. Small, young plants were placed on a platform in the tunnel so that their leaves were in the center of the airstream in the wind tunnel. The airflow was measured at the outlet of the tunnel constriction. Unless otherwise stated, plants were left in the wind tunnel for 50 min before the experiment was started to allow them to equilibrate. Ten adult beetles were used per experiment per experimental day. Individual beetles were observed for 5 min in response to a wind control, to control plants, and to treated plants. Initially experiments were conducted to compare the response of beetles to undamaged plants of different sizes and/or ages (small, 15–25 cm, 2–3 weeks old; medium, 35–45 cm, ca. 4 weeks old, and large, >60 cm, 5–6 weeks old). Medium-sized plants were stressed by shaking using a Titertek laboratory beaker shaker at speed setting 2 and the response of adult beetles monitored.

The following treatments were carried out using small plants of which 30 leaflets per pot were handled as follows: (1) leaflets damaged manually by cutting (scissors were used to cut off a 2-mm strip of tissue); (2) leaflets damaged by applying carborundum powder (180 grit) with a piece of damp cotton wool; (3) leaflets damaged by 10 early third instars of CPB per clip cage (2 cm diameter), one clip cage per leaflet—the larvae consumed all available tissue before the assay was completed; (4) leaflets damaged by 10 early second-instar CPB per clip cage, in which case they did not consume all available tissue before the experiment was completed; (5) plants fed upon by 10 early second-instar CPB per cage for 30 min (add 20 min to find the total time, i.e., 10 min for the cages to be put on the plant and 10 min for them to be removed) before the plants were placed in the wind tunnel and tested for their attractiveness after larvae were removed; (6) as for treatment 5, but the CPB larvae were allowed to feed for 60–90 min, after which they were removed. Plants were placed in the wind tunnel and tested for up to 24 hr following removal of the larvae. In between two windtunnel tests each lasting ca. 2 h, the plants were placed in a controlled environment room at 25 ± 1°C; (7) leaflets fed upon by one second-instar CPB per clip cage for 18–24 hr—thereafter the beetle larvae were removed and the plants were placed in the wind tunnel and tested for their attractiveness for up to 24 hours after removal of the larvae; and (8) as in treatment 7, but leaflets were damaged by three early second-instar *S. exigua* per clip cage. Plants of the same age that had experienced similar incubation durations were assayed

as controls. The experiments were conducted at 25 ± 2°C, and were repeated up to 10 times. All larvae used were deprived of food for 60 min prior to treatments.

Collection of Headspace Volatiles. Pots (2 liters) containing two potato plants (2–3 weeks old, 15–25 cm tall) were placed in a 20-liter stainless steel vessel with an airtight Perspex cap. Technical air, cleaned by passage through 275 g anhydrous potassium hydroxide, 175 g molecular sieves 4A and 13X (Linde), and 180 g activated charcoal, used to generate the airstream (0.5 liters/min) (Dicke et al., 1990; Blaakmeer et al., 1994) was passed through the container containing the plant to be analyzed for 70 min (24.5 liters). This was done to remove contaminated air from the container before headspace collection was started. The volatiles were trapped during the subsequent 2 hr (flow 0.5 liters/min) in a 160 × 4-mm glass tube containing 90 mg Tenas TA at the outlet of the vessel. A series of four collections was made from each of the experimental plants. This consisted of collections from: the undamaged plants, the plants with 20 clip cages each containing three early second-instar CPB, the same plants with larvae still feeding after 18 hr, and from the aforementioned plants with larvae removed for 24 hr.

Analysis of Headspace Volatiles. The collected volatiles were removed from the Tenax by heating the trap in a Thermodesorption Cold Trap Unit (Chrompack, Middelburg, The Netherlands) at 250°C for 10 min and flushing with a He flow of 10 ml/min. The compounds were cryofocused in a cold trap (0.52-mm ID deactivated fused silica) at ca. −85°C (Dicke et al., 1990). By ballistic heating of the cold trap to 220°C the volatiles were transferred to the analytical column (Supelcowax 10, 60 m × 0.25 mm ID, 0.25-μm film thickness), which was connected to a Finnigan MAT 95 mass spectrometer. The column was programmed from 40°C (3 min hold) to 270°C (4 min hold) at 4°C/min and the initial helium velocity was 30 cm/sec. The mass spectrometer was operated in the 70 eV EI ionization mode and scanning was done from mass 24 to 400 at 0.7 sec/decade. Identification of the compounds was done by comparison of the mass spectra with those in the Wiley library and in the Wageningen Mass Spectral Database of Natural Products and by comparing the retention indices.

RESULTS

Behavioral Responses of Adult CPB

No Damage. When undamaged potato plants of different size were placed in the wind tunnel, adult female beetles starved for 16 hr were significantly attracted by volatiles from potato plants more than 60 cm tall. The response to small, young plants (15–25 cm tall) was not significantly different from that to wind only, even when the wind tunnel was filled with 12 pots of plants (data

not shown). This is clear from all parameters measured. The beetles walked faster and the tracks had a longer vector length, higher straightness, longer upwind length, and higher upwind fixation when large plants (>60 cm) are placed in the wind tunnel, than when small plants (15–25 cm) or wind only were offered (Table 1, A; Figures 1A and 2A and B).

Mechanical Damage. Medium-sized plants (35–45 cm) that were not attractive to the beetles when offered in the wind tunnel became attractive when the plants were shaken throughout the experiment (Table 1, B; Figure 1B). Furthermore, small plants (15–25 cm) became attractive to the beetles after they were mechanically damaged with carborundum powder. This attraction lasted throughout an experiment with 10 beetles (ca. 60 min). Twenty-four hours after the damage was performed, the attraction was no longer present (Table 1, C; Figure 1C). Results from leaf cutting experiments (scissors) are not shown because it was found that leaves had to be cut repeatedly during the course of the experiment in order to sustain attraction of the beetles. It appeared that the wounds healed rapidly after cutting and that beetle attraction disappeared within 15 min after cutting the leaves.

Colorado Potato Beetle Damage. Adult beetles were attracted to small plants when CPB larvae were actively feeding on them (Table 1, D; Figure 2C, and 3A). However, the beetles were not attracted to plants 50 min after the larvae had been removed following a 30-min feeding duration (Table 1, E; Figure 3B). A somewhat longer feeding duration by CPB larvae, i.e., 60–90 min, resulted in a slight although significant attraction 50 min after removal of the beetle larvae, and this attraction was significantly greater 150 min after removal of the larvae (Table 1, F; Figures 2D–F, and 3C). Finally, when CPB larvae had been feeding on potato plants for 18–24 hr, the plants were highly attractive 50 min after removal of the beetles. This level of attraction was similar to that recorded 150 min after removal of beetles that had fed on a plant for 60–90 min (Figure 3C). Five to 24 hr after removal of the beetle larvae, the response of adult beetles to the damaged plants was not significantly different from their response to undamaged plants or wind only (Table 1, G; Figure 3D).

Beet Army Worm Damage. Infestation of small potato plants by *Spodoptera exigua* larvae for 18–24 hr rendered these plants highly attractive to adult CPB 50 min after removal of the caterpillars (Table 1, H; Figure 4). This level of attraction was similar to that of plants that had been infested for 18–24 hr with Colorado potato beetle larvae and from which the beetle larvae had been removed 50 min before the experiment (Table 1, G; Figure 3D).

Chemical Analyses

Undamaged potato plants released small amounts of volatiles (Table 2): a total of 146 peak units, equivalent to ca. 58 ng/2 hr were collected (depending

TABLE 1. PARAMETERS (MEAN ± STANDARD ERROR) OF WALKING TRACKS OF ADULT FEMALE COLORADO POTATO BEETLES IN RESPONSE TO VOLATILES FROM HOST PLANT[a] *S. tuberosum* AFTER VARIOUS TREATMENTS.

A: Undamaged potato plants of different size				
Wind (N = 20)	14.8 ± 0.4a[b]	1982 ± 215a	0.45 ± 0.05a	695 ± 433a
Plants 15–25 cm (N = 20)	14.8 ± 0.6a	2364 ± 244a	0.53 ± 0.05a	1180 ± 337a
Plants >60 cm (N = 20)	17.0 ± 0.5b	4368 ± 231b	0.85 ± 0.03b	4078 ± 272b
B: Shaking, undamaged plants, 35–45 cm				
Wind (N = 10)	16.9 ± 0.7a	2467 ± 448a	0.69 ± 0.06a	580 ± 606a
No shaking (N = 10)	17.8 ± 0.6ab	3112 ± 377a	0.72 ± 0.06a	1011 ± 651a
Shaking (N = 10)	18.9 ± 0.6b	5343 ± 196b	0.96 ± 0.00b	4307 ± 390b
C: Mechanical damage with carborundum				
Wind (N = 30)	16.1 ± 0.4a	2647 ± 180a	0.55 ± 0.03a	467 ± 352a
Undamaged (N = 30)	17.2 ± 0.5a	3410 ± 265a	0.65 ± 0.04a	630 ± 479a
Right after treatment (N = 30)	19.2 ± 0.5b	5189 ± 210b	0.90 ± 0.02b	5091 ± 215b
24 hr after treatment (N = 30)	15.4 ± 0.4a	2622 ± 249a	0.56 ± 0.05a	1257 ± 363a
D: CPB larvae feeding during experiment				
Wind (N = 30)	15.9 ± 0.4a	2742 ± 249a	0.56 ± 0.05a	1424 ± 402a
Undamaged potato plants (N = 30)	16.1 ± 0.4a	2739 ± 242a	0.57 ± 0.05a	1365 ± 429a
CPB on potato plants (N = 30)	17.8 ± 0.4b	4598 ± 251b	0.85 ± 0.04b	4361 ± 282b
E: removal of CPB larvae after 30 min of feeding				
Wind (N = 20)	14.2 ± 0.5a	2355 ± 229a	0.54 ± 0.04a	1009 ± 460a
Undamaged potato plants (N = 20)	14.5 ± 0.7a	2115 ± 195a	0.50 ± 0.05a	701 ± 371a
50 min after removal (N = 20)	15.8 ± 0.6a	2788 ± 234a	0.59 ± 0.04a	1733 ± 406a

F: removal of CPB larvae after 60–90 min of feeding				
Wind (N = 70)	17.5 ± 0.4ac	2812 ± 151ab	0.53 ± 0.02a	1172 ± 195a
Undamaged potato plants (N = 100)	16.1 ± 0.3a	2377 ± 118a	0.49 ± 0.02a	869 ± 178a
50 min after removal (N = 90)	16.7 ± 0.4ab	3335 ± 166bc	0.65 ± 0.03bc	2062 ± 247b
150 min after removal (N = 100)	18.0 ± 0.3bc	4504 ± 130d	0.83 ± 0.02c	4061 ± 155c
18–24 hr after removal (N = 50)	17.0 ± 0.4ab	3863 ± 195cd	0.75 ± 0.03c	1673 ± 326ab
G: removal of CPB larvae after 18–24 h of feeding				
Wind (N = 75)	17.7 ± 0.3bc	3454 ± 156a	0.64 ± 0.02a	805 ± 286ab
Undamaged potato plants (N = 75)	17.5 ± 0.3bc	3218 ± 177a	0.60 ± 0.03a	−14 ± 245a
50 min after removal (N = 60)	18.6 ± 0.3c	4862 ± 113b	0.91 ± 0.01b	4520 ± 140c
5–7 hr after removal (N = 40)	16.6 ± 0.5ab	3468 ± 259a	0.68 ± 0.04a	1789 ± 405b
18–24 hr after removal (N = 25)	16.7 ± 0.4ab	3234 ± 268a	0.65 ± 0.05a	969 ± 521ab
H: removal of *S. exigua* larvae after 18–24 hr of feeding				
Wind (N = 30)	16.2 ± 0.7a	2313 ± 200a	0.46 ± 0.03a	1262 ± 271a
Undamaged potato plants (N = 30)	18.3 ± 0.7a	3505 ± 286b	0.65 ± 0.05b	741 ± 495a
50 min after removal (N = 30)	17.6 ± 0.6a	4646 ± 204c	0.88 ± 0.02c	4184 ± 277b

[a] All plants were 15–25 cm tall unless otherwise stated.

[b] Parameter values within an experimental set that are followed by different letters are significantly different: Kruskal-Wallis one-way analysis of variance followed by Mann-Whitney U tests with Bonferroni correction of α (α = 0.05/3 = 0.017 for sections A, B, D, E and H; α = 0.05/6 = 0.0083 for section C; and α = 0.05/10 = 0.005 for sections F and G). N = number of beetles used.

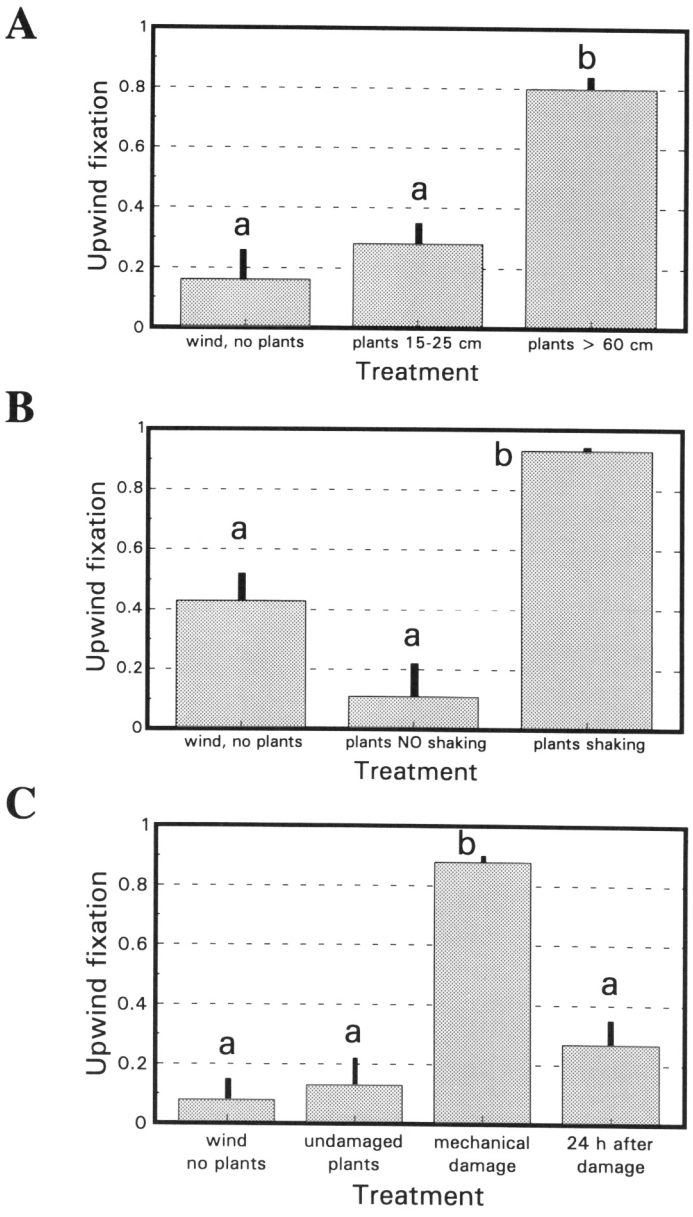


FIG. 1. Upwind fixation (mean ± SE) of beetles on the locomotion compensator in front of the wind tunnel when offered: (A) undamaged plants of different size, (B) small plants (35–45 cm) that are motionless in the wind tunnel or plants that are shaken in the wind tunnel, (C) plants with or without mechanical damage applied with carborundum powder. Different letters indicate significant differences ($P < 0.05$).

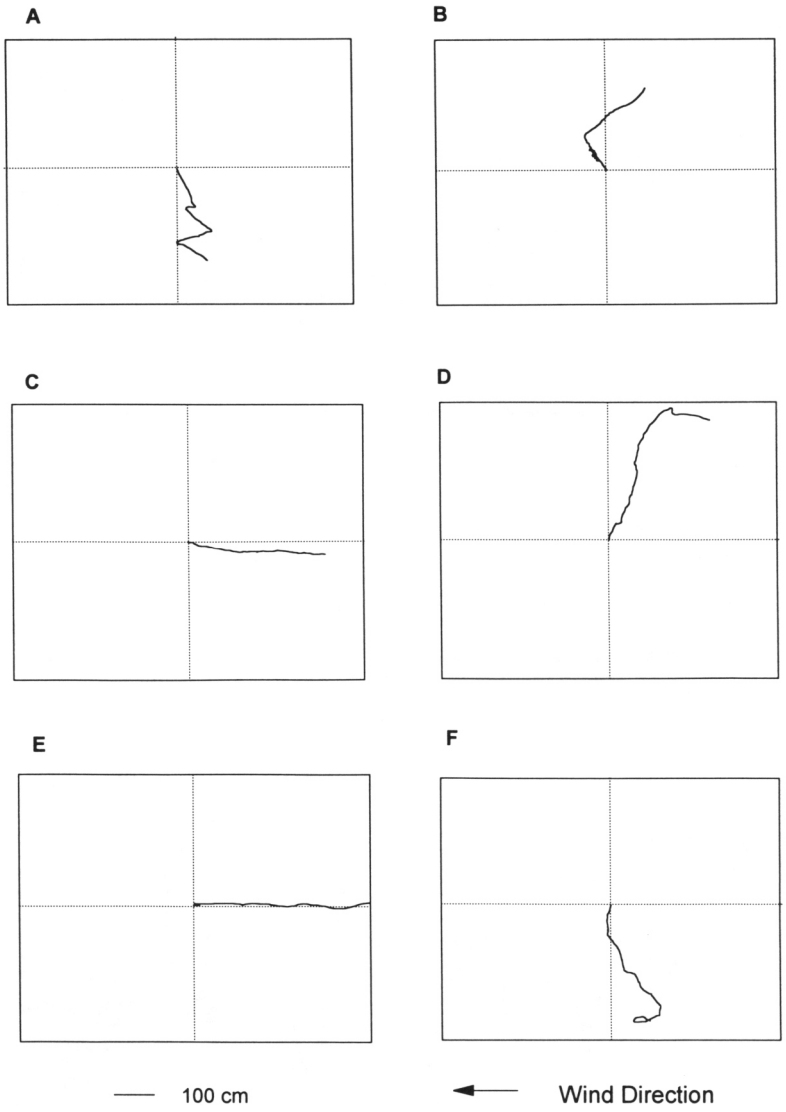


FIG. 2. Representative walking tracks of adult CPB females when offered; (A) wind, (B) undamaged small (15–25 cm) potato plants, (C) potato plants infested with CPB larvae, (D) potato plants that had been infested with CPB larvae for 60–90 min and from which the larvae had been removed 50 min prior to the test, (E) from which the larvae had been removed 150 min prior to the test, or (F) from which the larvae had been removed 18–24 hr prior to the test.

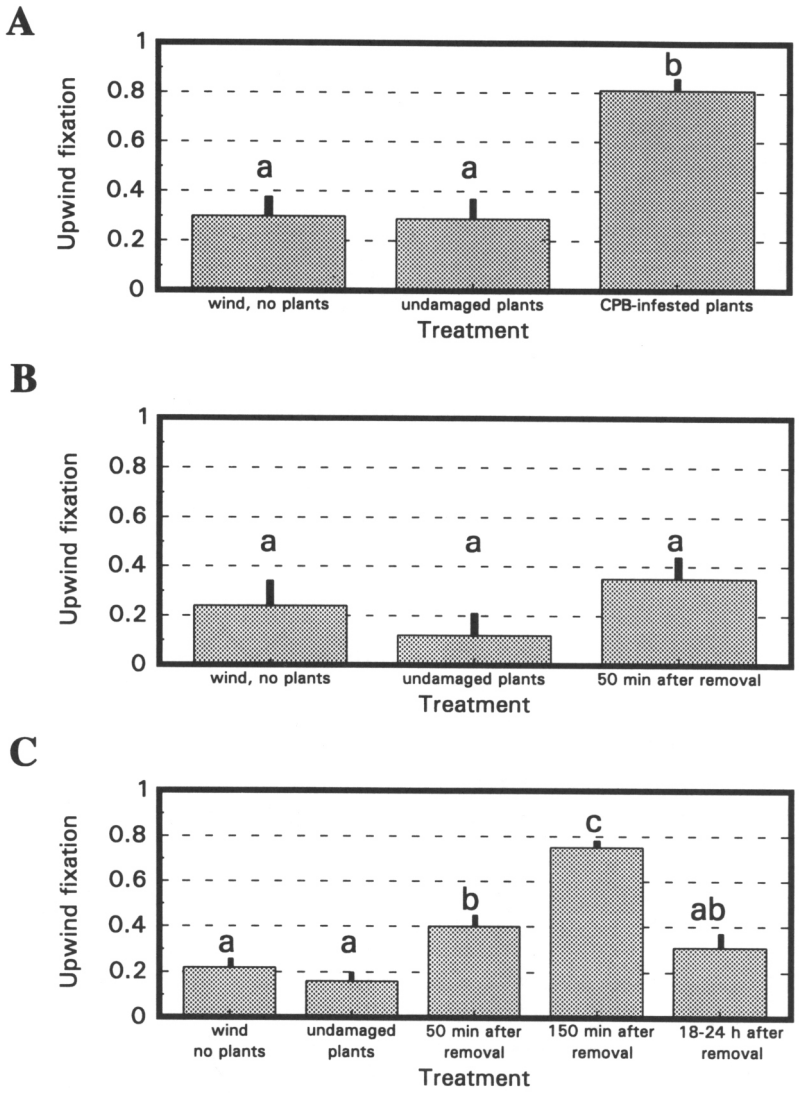


FIG. 3. Upwind fixation (mean ± SE) of beetles on the locomotion compensator in front of the wind tunnel when offered potato plants with different duration infestation by CPB larvae and plants from which the CPB larvae had been removed prior to using them as an odor source in the wind tunnel. (A) CPB feeding during test, (B) CPB had been feeding for 30 min and were then removed prior to testing the plants in the wind tunnel, (C) CPB larvae had been feeding for 60–90 min and were then removed prior to testing the plants in the wind tunnel, (D) CPB larvae had been feeding for 18–24 hr and were then removed prior to testing the plants in the wind tunnel. Different letters indicate significant differences ($P < 0.05$).

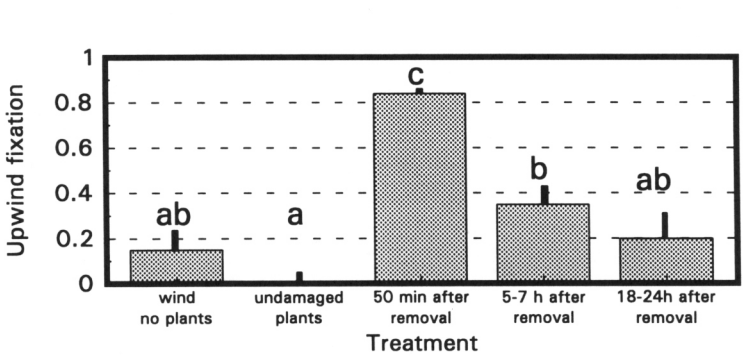


FIG. 3. Continued.

on the compound, a unit peak area represents 0.3–0.5 ng). β-Caryophyllene and β-selinene were the most abundantly emitted compounds. Infestation with CPB larvae resulted in a 7- to 10-fold increase in the amount of chemicals emitted, and this level was still recorded after the larvae have been removed for 24 hr (Table 2). Several chemicals were emitted at maximal rates as soon as after 2 hr of CPB infestation (Table 2A), while the emission rate of others increased with the duration of herbivory (Table 2B). These data show that the composition of the volatile blend emitted by potato plants changes with time.

Qualitatively, the volatile blends emitted by intact and infested potato plants were not strikingly different. Those compounds that were emitted in large amounts by infested plants were emitted in low amounts by uninfested plants,

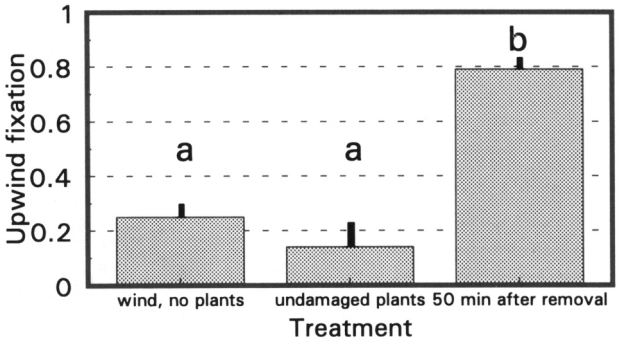


FIG. 4. Upwind fixation (mean ± SE) of beetles on the locomotion compensator in front of the wind tunnel when offered potato plants on which *Spodoptera exigua* larvae had been feeding for 18–24 hr and from which the larvae were removed prior to testing the plants in the wind tunnel. Different letters indicate significant differences ($P < 0.05$).

TABLE 2. HEADSPACE ANALYSES FROM POTATO PLANTS OF DIFFERENT TREATMENTS: PEAK AREA UNITS[a] (MEAN ± SE)

Compound	Intact plant (N = 5)	CPB infested for 2 hr (N = 5)	CPB infested for 20 hr (N = 5)	24 hr after removal of CPB (N = 4)
A: Compounds emitted in higher amounts in CPB infested for 2 hr and CPB infested for 20 hr than in intact plant and 24 hr after CPB removal treatments				
(*Z*)-3-Hexen-1-ol	4.0 ± 4.0^{+b}	10.8 ± 5.2^{+++++}	33.8 ± 18.3^{+++}	0
(*Z*)-3-Hexen-1-yl butyrate	0	3.2 ± 2.0^{++}	9.6 ± 5.7^{+++}	0
Heptanal	0	1.2 ± 1.2^{+}	3.6 ± 2.4^{++}	0
Octanal	0	5.6 ± 3.4^{++}	6.0 ± 6.0^{+}	0
Nonanal	2.4 ± 2.4^{+}	29.2 ± 13.3^{+++}	55.8 ± 30.1^{+++}	13.8 ± 13.8^{+}
Decanal	8.6 ± 8.6^{+}	22.6 ± 14.3^{++}	12.0 ± 12.0^{+}	0
Linalool	1.4 ± 0.9^{+++}	49.2 ± 25.6^{+++++}	57.8 ± 16.8^{+++++}	13.3 ± 8.8^{+++}
Longifolene	0	6.8 ± 3.1^{+++}	15.0 ± 10.9^{++}	0
B: Compounds emitted in higher amounts in CPB infested for 20 hr and 24 hr after removal of CPB treatments than in intact plant treatment and in similar or higher amounts than in CPB infested for 2 hr treatment				
4,8-Dimethyl-1,3(*E*),7-nonatriene	3.8 ± 1.7^{+++}	5.6 ± 3.9^{++}	46.2 ± 22.3^{++++}	43.5 ± 17.1^{++++}
1,4,8-Trimethyl-1,3(*E*),7(*E*),11-decatetraene	0	0	15.0 ± 4.6^{++++}	16.3 ± 6.1^{+++}
β-Caryophyllene	36.8 ± 24.5^{+++}	564 ± 76^{+++++}	421 ± 85^{+++++}	470 ± 324^{++++}
α-selinene	0.8 ± 0.8^{+}	5.4 ± 5.4^{+}	41.0 ± 13.2^{++++}	16.0 ± 16.0^{+}
β-Selinene	23.8 ± 14.3^{+++}	39.2 ± 11.3^{++++}	440 ± 61^{++++}	377 ± 169^{++++}
γ-Selinene (tent. ident.)	0	0	19.2 ± 13.4^{++}	6.5 ± 6.5^{+}
3-Pentanone	0	3.6 ± 3.6^{+}	11.0 ± 5.7^{+++}	3.0 ± 3.0^{+}
Sabinene	0	1.1 ± 0.7^{++}	1.8 ± 1.8^{+}	1.0 ± 1.0^{+}
Myrcene	0.6 ± 0.6^{+}	7.0 ± 4.5^{++}	13.2 ± 3.5^{++++}	14.3 ± 4.9^{+++}
Limonene	6.6 ± 2.1^{++++++}	22.6 ± 8.9^{++++}	54.2 ± 21.2^{++++++}	30.5 ± 8.3^{++++}
Methyl salicylate	0	0	4.6 ± 4.6^{+}	2.8 ± 1.9^{++}

C: Compounds emitted in highest amounts in 24 hr after CPB removal treatment, in lower amounts in CPB infested for 2 hr and CPB infested for 20 hr treatments, and still lower in intact plant				
Ledol	3.0 ± 3.0^{+}	6.6 ± 4.1^{++}	5.8 ± 3.6^{++}	26.3 ± 19.9^{++}
α-Cubebene	0	3.6 ± 3.6^{+}	2.8 ± 2.8^{++}	8.3 ± 5.0^{++}
α-Copaene	0	4.8 ± 3.0^{++}	8.8 ± 3.9^{+++}	26.0 ± 8.9^{++++}
β-Elemene	0	9.8 ± 2.9^{++++}	24.2 ± 16.9^{++}	35.5 ± 30.4^{++}
α-Humulene	0	10.4 ± 4.7^{+++}	11.6 ± 5.8^{++}	32.5 ± 32.5^{+}
Germacrene D	0	10.0 ± 5.1^{+++}	15.2 ± 9.8^{+++}	60 ± 60^{+}
δ-Cadinene	5.0 ± 5.0^{+}	16.2 ± 10.8^{++}	14.8 ± 13.6^{++}	37.0 ± 18.3^{++++}
γ-Cadinene	1.4 ± 1.4^{+}	9.2 ± 7.2^{++}	5.4 ± 5.4^{+}	23.8 ± 17.5^{++}
D: Other compounds				
(*Z*)-3-Hexen-1-yl acetate	0	0	11.2 ± 7.0^{+++}	0
β-Sesquiphellandrene	0	16.0 ± 7.1^{+++}	6.4 ± 4.8^{++}	16.3 ± 16.3^{+}
(*E*)-α-Bergamotene	0	3.8 ± 2.0^{+++}	0	2.0 ± 2.0^{+}
Indole	0	0.4 ± 0.4^{+}	3.2 ± 2.1^{++}	0.5 ± 0.5^{+}
1,8-Cineole	0	5.6 ± 5.6^{+}	0	4.8 ± 4.8^{+}
Furfural	0	0	2.6 ± 2.6^{+}	5.3 ± 5.3^{+}
γ-Muurolene	0	2.4 ± 2.4^{+}	2.6 ± 2.6^{+}	2.8 ± 2.8^{+}
ar-Curcumene	0	1.4 ± 1.4^{+}	0.4 ± 0.4^{+}	0
Tricyclene	0	14.0 ± 9.5^{++}	0	0
(*E*)-β-Farnesene	0	5.4 ± 3.7^{++}	0	12.8 ± 10.8^{++}
α-Pinene	0.6 ± 0.6^{+}	2.7 ± 1.8^{++}	1.2 ± 1.2^{+}	2.0 ± 1.2^{++}
Total	146 ± 52	1012 ± 128	1462 ± 250	1401 ± 663

[a] One peak unit equals 0.3–0.5 ng (depending on the compound).
[b] The number of pluses indicates the number of samples in which the compound was recorded, which is an indicator of the consistency with which a compound was recorded.

while those compounds that were emitted in low amounts by infested plants were not recorded in the headspace of intact plants. This is probably because these compounds were emitted at a rate below the detection limit of the GC-MS. Among the volatiles recorded, terpenes were the dominant category. Among these are two homoterpenes 4,8-dimethyl-1,3(*E*),7-nonatriene and 1,4,8-trimethyl-1,3(*E*),7(*E*),11-tetradecaene, that are emitted by a several other plant species when infested by herbivores (Dicke, 1994). In addition, several fatty acid derivatives were recorded.

DISCUSSION

Wound-Induced Attraction and Time Lag in Postwounding Attraction. We have demonstrated that undamaged small potato plants are not attractive to adult female CPB. Disturbing the plants by shaking or by mechanical damage makes the plants attractive to the beetles. Previous studies on the response of CPB to undamaged potato plants have used mainly older and larger (>60 cm high) plants and also consistently found attraction (e.g., Visser, 1976; Visser and Thiery, 1985; Thiery and Visser, 1986, 1987). First indications were that older plants are more attractive than younger plants (Visser, 1976), which is confirmed by our study.

From the experiments with wounding by scissors, it is clear that the plants heal rapidly and that in this case adult beetles are attracted to volatiles released directly from the wound site. It seems likely that the same phenomenon is observed when the larvae are allowed to feed for 30 min. When the larvae cease feeding, the wound site heals and volatiles are no longer released, resulting in no attraction by the adult. When larvae are allowed to feed for 60–90 min, there is some attraction to the plant after removal of the larvae. Attraction is observed to increase significantly approximately 150 min after removal, indicating a secondary release of volatiles that presumably have been induced in response to wounding. Beetles were highly attracted to plants 50 min after larvae were removed following 18–24 hr of feeding, again suggesting that attractive wound-induced volatiles were being released from the plant. Thus, it appears that there are two stages of attraction, first, to volatiles released directly from the wound site, and second to volatiles that are induced in response to herbivory.

From chemical analyses of the headspace volatiles from plants prior to the infestation with CPB larvae, during infestation, and after removal of the beetles, different categories of chemicals can be discerned on the basis of their emission patterns. The emission pattern of the chemicals from group A (Table 2) seems to correlate best with behavioral analyses. This group contains aldehydes, two terpenes, and an alcohol and its butyl ester. Of these compounds three, (*Z*)-3-hexen-1-ol, heptanal, and linalool, are known to be perceived by the beetles' olfactory chemoreceptors (Visser, 1979). The chemicals in group B (Table 2)

may also play a role, but if they are important in the attraction, a longer period of attraction after removal of the beetles would be expected. Two chemicals from this group are known to be perceived by the beetles: limonene and methyl salicylate (Visser, 1979; Ma and Visser, 1978). No information is available on the effect of the chemicals in group C on CPB olfaction, while in the miscellaneous group (group D, Table 2), (Z)-3-hexenyl acetate yields distinct electroantennogram responses from beetles (Visser, 1979).

Headspace Volatile Composition from Potato Plants. A marked difference with previous analyses of odor composition of pototo plants is that those analyses almost exclusively recorded fatty acid derivatives (green odors) when applying steam distillation extraction (Visser et al., 1979), but headspace sampling similar to that done here (Visser, 1983) showed that besides these compounds, sesquiterpenes are markedly present in the air over cut, fully grown potato plants.

Different Plant Responses to Herbivory: Their Effect on Herbivore and Carnivore Behavior. The characteristics of attraction of CPB to infested potato plants are different from the attraction of *P. japonica* beetles to infested apple plants (Loughrin et al., 1995). The differences between the data for CPB–potato interactions and *P. japonica*–apple interactions may be compared with the two classes of plant–carnivore interactions (see Introduction). Attraction to apple foliage of *P. japonica* beetles is effected by long-term herbivore damage, but not by mechanical damage or fresh herbivore damage (Loughrin et al., 1995). Furthermore, long-term (overnight) herbivore damage but not short-term (2 hr) herbivore damage leads to the emission of eight chemicals that are not emitted by undamaged leaves. Thus, apple resembles plants such as lima bean, cucumber, corn, and soybean (Dicke et al., 1990, Turlings et al., 1990; Dicke, 1994; Takabayashi et al., 1994), and this is supported by studies on headspace composition from apple foliage infested with spider mites (Takabayashi et al., 1991). These plant species attract carnivorous arthropods when damaged by herbivores, but not when damaged mechanically or after fresh herbivore damage (Sabelis and van de Baan, 1983; Dicke et al., 1990; Turlings et al., 1990). They respond to long-term herbivore damage with the production of several chemicals that are not emitted in response to mechanical damage or short-term feeding damage (Dicke et al., 1990; Turlings et al., 1990; Takabayashi et al., 1994). In contrast, potato plants become attractive as soon as CPB larvae start feeding on them. The chemical blend emitted by infested potato plants does not differ qualitatively between freshly damaged (2 hr infestation) and long-term damaged (24 hr infestation) plants (Table 2). Thus, the response of potato to herbivory resembles that of cabbage plants. Cabbage plants that are infested by caterpillars (*Pieris* species) become highly attractive to the parasitic wasps *Cotesia glomerata* and *Cotesia rubecula* that use these caterpillars as hosts (Steinberg et al., 1992, 1993; Blaakmeer et al., 1994; Mattiacci et al., 1994; Agelopoulos and Keller, 1994; Geervliet et al., 1994, 1997). Moreover, cabbage plants also become attractive to wasps when damaged mechanically, but this effect quickly

disappears if damage is not continued (Steinberg et al., 1993; Geervliet et al., 1994; Agelopoulos and Keller, 1994).

Herbivore Biology as a Factor Determining Attraction or Avoidance to Volatiles from Infested Plants. Herbivore biology most likely determines whether an herbivore is attracted or repelled by volatiles from plants infested by conspecifics. Aspects that are likely to play a role are whether the herbivore has an aggregated life-style and whether the investigated herbivore stage is the one that makes the foraging decision leading to such a life-style (e.g., the adult female butterfly instead of the caterpillars; see Turlings and Tumlinson, 1991). Another aspect that is likely to be important is whether the herbivore has many natural enemies that may be attracted by the infested plant's volatiles and whether the herbivore has an effective defense to attracted predators. For instance, chrysomelid beetles are well known for their chemical defenses, and many chrysomelid species live in large aggregations that can do substantial damage to their host plant (Pasteels et al., 1988). Some predators of CPB are known, such as *Coleomegilla maculata* (Coleoptera: Coccinellidae) (Giroux et al., 1995) and *Perillus bioculatus* (Heteroptera: Pentatomidae) (Hough-Goldstein and Whalen, 1993). It remains unclear whether these predators exert a strong selection pressure on CPB. It will be interesting to investigate the response of CPB predators to potato plants infested by CPB in a comparable way.

CONCLUSION

We have demonstrated that the CPB employs volatiles from herbivore-infested potato plants and that herbivore removal is followed by a short reduction, subsequent increase, and final decrease in attractiveness. Plant species differ in the way they respond to herbivory, which determines the characteristics of the signal that herbivores as well as their natural enemies have at their disposal during foraging behavior. It will be interesting to investigate the behavioral response of herbivores to conspecific and heterospecific herbivore damage in other plant–herbivore systems as well as to compare these with responses of carnivores to the same volatile blends.

Acknowledgments—We thank W. H. Frentz, J. Holman, J. van den Wege, and D. Diederik (WAU) for help with the locomotion compensator; F. van Aggelen for rearing the beetles; and R. J. Jansen (IPO-DLO) for rearing of potato plants.

REFERENCES

AGELOPOULOS, N. G., and KELLER, M. A. 1994. Plant-natural enemy association in the tritrophic system, *Cotesia rubecula–Pieris rapae*–Brassicaceae (Cruciferа): III. Collection and identification of plant and frass volatiles. *J. Chem. Ecol.* 20:1955–1967.

BLAAKMEER, A., GEERVLIET, J. B. F., VAN LOON, J. J. A., POSTHUMUS, M. A., VAN BEEK, T. A., and DE GROOT, A. E. 1994. Comparative headspace analysis of cabbage plants damaged by two species of *Pieris caterpillars*: Consequences for in-flight host location by *Cotesia* parasitoids. *Entomol. Exp. Appl.* 73:175–182.

BIRCH, M. C. 1984. Aggregation in bark beetles, pp. 331–353, *in* W. J. BELL and R. T. CARDÉ (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

CROFT, K. P., JUTTNER, F., and SLUSARENKO, A. J. 1993. Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv *phaseolicola*. *Plant Physiol.* 101:13–24.

DE JONG R. 1988. Host odour recognition by the Colorado potato beetle. PhD thesis. Wageningen Agricultural University, 65 pp.

DE JONG, R., and VISSER, J. H. 1988a. Integration of olfactory information in the Colorado potato beetle brain. *Brain Res.* 447:10–17.

DE JONG, R., and VISSER, J. H., 1988b. Specificity-related suppression of responses to binary mixtures in olfactory receptors of the Colorado potato beetle. *Brain Res.* 447:18–24.

DICKE, M. 1986. Volatile spider-mite pheromone and host-plant kairomone, involved in spaced-out gregariousness in the spider mite *Tetranychus urticae*. *Physiol. Entomol.* 11:251–262.

DICKE, M. 1994. Local and systemic production of volatile herbivore-induced terpenoids: Their role in plant-carnivore mutualism. *J. Plant Physiol.* 143:465–472.

DICKE, M. 1995. Why do plants 'talk'? *Chemoecology* 5/6: 159–165.

DICKE, M., and SABELIS, M. W. 1989. Does it pay plants to advertize for bodyguards? Towards a cost–benefit analysis of induced synomone production, pp. 341–358, *in* H. Lambers, M. L. Cambridge, H. Konings and T. L. Pons (eds.). Variation in Growth rate and Productivity of Higher Plants. SPB Academic Publishing, The Hague, The Netherlands.

DICKE, M., VAN BEEK, T. A., POSTHUMUS, M. A., BEN DOM, N., VAN BOKHOVEN, H., and DE GROOT, A. E. 1990. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions. Involvement of host plant in its production. *J. Chem. Ecol.* 16:381–396.

GEERVLIET, J. B. F., VET, L. E. M., and DICKE, M. 1994. Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid *Cotesia rubecula*. *Entomol. Exp. Appl.* 73:289–297.

GEERVLIET, J. B. F., VREUGDENHIL, A. I., DICKE, M., and VET, L. E. M. 1997. Learning to discriminate between infochemicals from different plant-host complexes by the parasitoids *Cotesia glomerata* and *C. rubecula*. Submitted.

GIROUX, S., DUCHESNE, R.-M., and CODERRE, D. 1995. Predation of *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) by *Coleomegilla maculata* (Coleoptera: Coccinellidae): Comparative effectiveness of predator developmental stages and effect of temperature. *Environ. Entomol.* 24:748–754.

HARARI, A. R., BEN-YAKIR, D., and ROSEN, D. 1994. Mechanism of aggregation behavior in *Maladera matrida* Argaman (Coleoptera: Scarabeidae). *J. Chem. Ecol.* 20:361–371.

HILDEBRAND, D. F., BROWN, G. C., JACKSON, D. M., and HAMILTON-KEMP, T. R. 1993. Effects of some leaf-emitted volatile compounds on aphid population increase. *J. Chem. Ecol.* 19:1875–1887.

HOUGH-GOLDSTEIN, J., and WHALEN, J. 1993. Inundative release of predatory stink bugs for control of Colorado potato beetle. *Biol. Control* 3:343–347.

LOUGHRIN, J. H., POTTER, D. A., and HAMILTON-KEMP, T. R., 1995. Volatile compounds induced by herbivory act as aggregation kairomones for the Japanese beetle (*Popilia japonica* Newman). *J. Chem. Ecol.* 21:1457–1467.

MA, W.-C., and VISSER, J. H. 1978. Single unit analysis of odour quality coding by the olfactory antennal receptor system of the Colorado beetle. *Entomol. Exp. Appl.* 24:520–533.

MATTIACCI, L., DICKE, M., and POSTHUMUS, M. A. 1994. Induction of parasitoid attracting synomone in brussels sprouts plants by feeding of *Pieris brassicae* larvae: Role of mechanical damage and herbivore elicitor. *J. Chem. Ecol.* 20:2229–2247.

PASTEELS, J. M., ROWELL-RAHIER, M., and RAUPP, M. J. 1988. Plant-derived defense in chrysomelid beetles, pp. 235–272, *in* P. Barbosa, and D. K. Letourneau (eds.). Novel Aspects of Insect–Plant Interactions. Wiley, New York.

SABELIS, M. W., and VAN DE BAAN, H. E. 1983. Location of distant spider mite colonies by phytoseiid predators: Demonstration of specific kairomones emitted by *Tetranychus urticae* and *Panonychus ulmi*. *Entomol. Exp. Appl.* 33:303–314.

STEINBERG, S., DICKE, M., and VET, L. E. M. 1993. Relative importance of infochemicals from first and second trophic level in long-range host location by the larval parasitoid *Cotesia glomerata*. *J. Chem. Ecol.* 19:47–59.

STEINBERG, S., DICKE, M., VET, L. E. M., and WANNINGEN, R. 1992. Response of the braconid parasitoid *Cotesia* (=*Apanteles*) *glomerata* to volatile infochemicals: Effects of bioassay set-up, parasitoid age and experience and barometric flux. *Entomol. Exp. Appl.* 63:163–175.

TAKABAYASHI, J., and DICKE, M. 1996. Plant-carnivore mutualism through herbivore-induced carnivore attractants. *Trends Plant Sci.* 1:109–113.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M. A. 1991. Variation in composition of predator-attracting allelochemicals emitted by herbivore-infested plants: Relative influence of plant and herbivore. *Chemoecology* 2:1–6.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M. A. 1994. Volatile herbivore-induced terpenoids in plant-mite interactions: Variation caused by biotic and abiotic factors. *J. Chem. Ecol.* 20:1329–1354.

THIERY, D., and VISSER, J. H. 1986. Masking of host plant odour in the olfactory orientation of the Colorado potato beetle. *Entomol. Exp. Appl.* 41:165–172.

THIERY, D., and VISSER, J. H. 1987. Misleading the Colorado potato beetle with an odor blend. *J. Chem. Ecol.* 13:1139–1146.

TURLINGS, T. C. J., and TUMLINSON, J. H. 1991. Do parasitoids use herbivore-induced plant chemical defenses to locate hosts? *Fla. Entomol.* 74:42–50.

TURLINGS, T. C. J., TUMLINSON, J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250:1251–1253.

TURLINGS, T. C. J., WÄCKERS, F. L., VET, L. E. M., LEWIS, W. J., and TUMLINSON, J. H. 1993. Learning of host-finding cues by Hymenopterous parasitoids, pp. 51–78, *in* D. R. Papaj and A. C. Lewis (eds.). Insect Learning. Chapman & Hall, New York.

TURLINGS, T. C. J., LOUGHRIN, J. H., MCCALL, P. J., ROSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. U.S.A.* 92:4169–4174.

VET, L. E. M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

VISSER, J. H. 1976. The design of a low-speed wind tunnel as an instrument for the study of olfactory orientation in the Colorado beetle (*Leptinotarsa decemlineata*). *Entomol. Exp. Appl.* 20:275–288.

VISSER, J. H. 1979. Electroantennogram responses of the Colorado beetle, *Leptinotarsa decemlineata*, to plant volatiles. *Entomol. Exp. Appl.* 25:86–97.

VISSER, J. H. 1983. Differential sensory perceptions of plant compounds by insects, pp. 215–230, *in* P. A. Hedin. (ed.). A.C.S. Symposium Series No. 208 Plant Resistance to Insects. American Chemical Society, Washington, DC.

VISSER, J. H. 1986. Host odor perception in phytophagous insects. *Annu. Rev. Entomol.* 31:121–144.

VISSER, J. H., and THIERY, D. 1985. Behavioral responses of the Colorado potato beetle to stimulation by wind and plant odors. *Bull. Mass. Agric. Exp. Stn.* 704:117–125.

VISSER, J. H., STRATEN, S. VAN, and MAARSE, H. 1979. Isolation and identification of volatiles in the foliage of potato, *Solanum tuberosum*, a host plant of the Colorado beetle, *Leptinotarsa decemlineata*. *J. Chem. Ecol.* 5:13–25.

ZERINGUE, H. J., and MCCORMICK, S. P. 1989. Relationships between cotton leaf-derived volatiles and growth of *Aspergillus flavus*. *JAOCS* 66:581–585.

Journal of Chemical Ecology, Vol. 23, No. 4, 1997

TRAIL PHEROMONE OF MYRMICINE ANT *Pristomyrmex pungens*[1]

EDELGARD JANSSEN,[2] BERT HÖLLDOBLER,[3] FRIEDRICH KERN,[2] HANS JÜRGEN BESTMANN,[2,*] and KAZUKI TSUJI[3,4]

[2]*Institut für Organische Chemie der Universität Erlangen-Nürnberg Henkestr. 42, D-91054 Erlangen, Germany*

[3]*Theodor Boveri Institut Lehrstuhl für Verhaltensphysiologie und Soziobiologie der Universität Würzburg am Hubland, D-97074 Würzburg, Germany*

[4]*Department of Biology Faculty of Science, Toyama University Toyama, 930 Japan*

(Received June 3, 1996; accepted November 27, 1996)

Abstract—The myrmicine ant *Pristomyrmex pungens* marks recruitment trails with poison gland secretions. The effective trail pheromone compound is 6-*n*-pentyl-2-pyrone. Poison gland components were identified by means of gas chromatographic coupled mass spectrometry. The biological activity was examined in trail-following experiments as well as in gas chromatographic coupled electroantennograms. In addition to 6-*n*-pentyl-2-pyrone, a number of monoterpenes were found in the poison gland secretion, i.e., α-pinene, camphene, β-pinene, myrcene, α-phellandrene, α-terpinene, and limonene. The terpenoid compounds increased the trail-following response only slightly when offered together with 6-*n*-pentyl-2-pyrone. In contrast to the latter component, synthetic monoterpenes elicited no orientation behavior in trail-following bioassays.

Key Words—Ants, Myrmicinae, poison gland, trail pheromone, 6-*n*-pentyl-2-pyrone, monoterpenes.

INTRODUCTION

The unusual social organization of the myrmicine ant *Pristomyrmex pungens* Mayr recently has attracted special attention of sociobiologists. In this ant spe-

*To whom correspondence should be addressed.

[1] Pheromones 106; Pheromones 105: U. Haak, B. Hölldobler, H. J. Bestmann, and F. Kern. 1996. Species specifity in trail pheromones and Dufour's gland contents of the formicine ants *Camponotus atriceps* and *Camponotus floridanus* (Hymenoptera: Formicidae). *Chemoecology*, 7:85–93.

0098-0331/97/0400-1025$12.50/0 © 1997 Plenum Publishing Corporation

cies, a queen caste does not exist, and males are rare and probably useless because workers reproduce by thelytokous parthenogenesis (Itow et al., 1984; Tsuji, 1988a). The colonies consist of several thousand workers with their brood, and they reproduce by colony fission (Itow et al., 1984; Tsuji 1988a,b), i.e., colony fragments emigrate along chemically marked trails and establish new colonies some distance from the stock colony. Several evolutionary aspects of the sociobiology of *P. pungens* recently have been studied in great detail (Tsuji, 1994, 1995), and the communication system involved in colony emigration has also been investigated. Hayashi and Komae (1976) reported that *P. pungens* mark their trails with a blend of nine carboxylic acids. However, results of trail-following experiments were not given in detail, and the source of the trail pheromone remained unknown.

Here we report the results from our investigations of trail communication in *P. pungens* revealing that poison gland secretions are employed for trail marking in this ant species. We were unable to confirm the chemical results of Hayashi and Komae (1976), but instead, discovered a hitherto unknown trail pheromone compound in ants.

METHODS AND MATERIALS

Pristomyrmex pungens ants were collected by K. Tsuji in Japan and brought to the laboratory in Würzburg. The ants were cultured as described in Tsuji (1994). We separated from the stock colony four groups, each of which consisted of approximately 400 workers. Each group was housed in a shallow plaster nest (5 × 10 × 0.5 cm) covered by red glass plates. Each nest had access to a foraging arena (40 × 25 cm). For the behavioral tests of glandular secretions, we dissected, under water, the body parts from *P. pungens* workers that were killed by placing them in a freezer for a few minutes. Dufour's gland, the poison gland, hindgut, and pygidial gland from the 6th and 7th abdominal tergites were used for tests. In a series of pilot tests, the freshly dissected organs of single workers were smeared with the tips of hardwood applicator sticks over a sheet of paper placed on the arena floor to create artificial trails 20 cm in length. We also made trails with crushed gasters from which we had removed the above-mentioned organs. During the experiments, a test trail and a control trail drawn with water or with one of the other glands were offered simultaneously, both starting at the same spot at the nest exit, but then deviating so that they were approximately 10 cm apart at the end of the trail. The sides of the trail were regularly alternated, and for each test a new paper was used. The number of ants that left the nest entrance and followed the trail during 2-min intervals was recorded.

Trail-Following Experiments. The best behavioral response to trail pheromones was obtained when we tested the secretions during colony emigration. Emigrations could be elicited relatively easily by lifting the red glass plate that covered the nest chambers, and providing a new dark nest in an adjacent arena. The old and new arenas were connected by a 23-cm-long cardboard bridge. After the emigration process had started, we replaced this bridge with a V-shaped bridge with two branches. On each branch of the bridge a trail was drawn with one of the test substances (0.2 ng/cm) or pure solvent as control. The number of ants crossing from the nest to the arena over one or the other branch of the bridge was recorded during a 2-min interval, after the ants had adjusted to the new situation, which took them 3–5 min.

Sample Preparation. For each chemical analysis of poison gland contents, worker ants of *P. pungens* were dissected, and the poison glands or the whole poison apparatus were extracted in hexane or sealed in glass capillary tubes. The samples were subjected to gas chromatography–mass spectrometry (GC-MS) with splitless injection. The glandular samples were introduced as solvent extracts or sealed glass capillaries crushed with a solid sampling device (Morgan and Wadhams, 1972).

Gas Chromatography (GC). Gas chromatography was carried out on a Hewlett-Packard 5890 instrument equipped with a 25-m × 0.22-mm fused silica capillary column coated with SE-52. The oven was held at 60°C for 4 min and programmed to increase 6°C/min to 260°C. For analysis of poison glands, the sample was heated for 2 min in the injector area (220°C) before crushing.

Preparative Gas Chromatography. Preparative gas chromatography was performed on a Hewlett-Packard 427 instrument fitted with a 25-m × 0.22-mm fused silica capillary column (FSCC) coated with SP-2340 or SE-52. Using SP-2340, the oven was kept at 70°C for 2 min and increased 3°C/min to 195°C. The temperature program for SE-52 entailed a 60°C isothermal for 4 min increasing 6°C/min to 260°C. The effluent was split 99:1 (trap-FID) by an all-glass splitter. Different fractions of the *Pristomyrmex* poison gland extract were trapped by the method described by Attygalle and Morgan (1988). Trapped material was washed out with pentane and directly used for bioassays. For better location of the active compound, an internal standard of hydrocarbons (C_8–C_{26}) was added to each sample.

Gas Chromatography–Mass Spectrometry (GC-MS). Hexane extracts or solid samples were examined by GC-MS on a Finnigan MAT 90 instrument linked with a Varian 3400 GC utilizing stationary phases and time programs as described above. Helium was used as the carrier gas at a flow rate of 1 ml/min and 70 eV EI spectra were recorded at a rate of 1 sec/scan.

Chemicals. Structural assignments were based on comparisons of mass spectra and GC retention times of authentic standards with those of glandular

constituents. Synthetic samples were obtained from commercial sources, i.e., α-fenchene, 6-*n*-pentyl-2-pyrone (Aldrich), camphene and limonene (Merck); myrcene, α-pinene, β-pinene, α-phellandrene and α-terpinene (Aldrich).

Microreactions. Silyl derivation of carboxylic acids was conducted with 30 *P. pungens* poison glands. The sample was extracted in 15 μl pyridine and mixed with 1 μl of bis(trimethylsilyl) trifluoracetamide (BSTFA). The solution was heated for several hours at 50–60°C (Knapp, 1979) in a reaction vial with a Teflon-faced rubber disc. After this procedure, the mixture was freed from (BSTFA) by preparative GC and directly used for bioassays.

Methyl ester derivatives of fatty acids were obtained by treatment with diazomethane, which was prepared from 100 μg *N*-nitroso-*N*-methylurea by addition of 60% aqueous KOH. Diazomethane was extracted with diethyl ether and 5 μl of the solution were added to 50 μl of poison gland extract. The mixture was allowed to stand for 5 min before it was cleaned by preparative GC and used for bioassay.

Electroantennography (EAG). Electrophysiological responses were recorded with *P. pungens* worker antennae by an electroantennogram technique previously described by Kern and Bestmann (1993). The head of an ant was excised, and one glass capillary electrode filled with insect Ringer was inserted into the laceration at the base of the head. The very sharp recording electrode was inserted carefully into the soft tip of the antenna. For a gas chromatography–electroantennography (GC-EAG), the effluent of the fused silica column (SP-2340) was split 1 : 1 between the flame ionization detector and the airstream towards the ant antenna. The effluent to the biological detector was introduced in a glass tube with a continuous airflow of 0.5 liters/min. The ant antenna was positioned approximately 10 cm after insert of the oven outlet at the narrowing end (4 mm diameter) of the glass tube. For each GC run (1 min at 80°C, 5°C/min, 195°C hold), six poison glands from workers were prepared, extracted, and introduced by splitless injection, and the GC-EAG was repeated three times under the same conditions. The data were acquired in analog mode with a high pass filter, digitally converted, and processed using a personal computer. In order to improve the signal-to-noise ratio, the electrophysiological data from each individual measurement were pooled by addition. The gas chromatography coupled electroantennogram data presented here represent the sum of three individual measurements using different *P. pungens* worker antennae.

RESULTS

The tests of the glandular secretions were conclusive. Only extracts from poison glands released clear trail-following behavior in *P. pungens*. In seven independent tests 10.7 ± 6.8 ($X \pm$ SD) ants followed the trails drawn with one

crushed poison gland. No ants followed trails drawn with crushed Dufour glands, pygidial glands, rectal bladders, or gasters from which the sting glands had been removed. Our subsequent analysis therefore focused on the contents of the poison gland.

Gas chromatography of the poison gland compounds of *P. pungens* revealed the presence of five major and five minor peaks (Figure 1). GC-MS analysis of the volatile constituents of 15 poison glands showed that the mass spectra and retention times of the major components matched those of the hydrocarbon monoterpenes α-pinene (**1**), camphene (**2**), β-pinene (**4**) and α-terpinene (**7**) (Figure 2). Minor compounds of the early eluting section were α-fenchene (**3**), myrcene (**5**), α-phellandrene (**6**), and limonene (**8**) (Figure 2).

In order to quantify each component in the glands, we used synthetic poison gland compounds. One individual gland reservoir contained approximately 260 pg of 6-*n*-pentyl-2-pyrone (**9**) and several terpenes in higher concentrations, i.e., β-pinene (**4**, 120 ng/gland), camphene (**2**, 52 ng/gland), α-pinene (**1**, 48 ng/gland), and α-terpinene (**7**, 16 ng/gland). In trace quantities we detected limonene (**8**, 420 pg/gland), α-fenchene (**3**, 280 pg/gland), α-phellandrene (**6**, 200 pg/gland), and myrcene (**5**, 110 pg/gland).

A later eluting minor component, **9** (Figure 1), showing no terpenoid characteristics, was identified by retention time and mass spectral data as 6-*n*-pentyl-2-pyrone (Figure 2). This poison gland compound **9** exhibited a molecular ion at *m/z* 166 (M+, 37%), 138 (15), 123 (5), 110 (49), 95 (100), 81 (58), 82

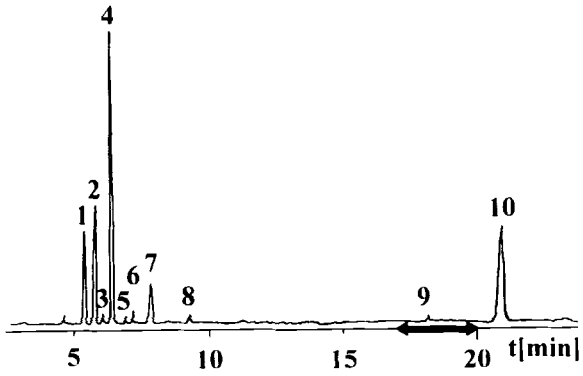


FIG. 1. Reconstructed gas chromatogram of a GC-MS analysis from injection of 15 poison glands of *P. pungens* workers by solid-sampling technique (SE-52, 60°C for 4 min, 6°C/min, 260°C hold). **1**, α-pinene; **2**, camphene; **3**, α-fenchene; **4**, β-pinene; **5**, myrcene; **6**, α-phellandrene; **7**, α-terpinene; **8**, limonene; **9**, 6-*n*-pentyl-2-pyrone. Thick double-headed arrows indicate the region corresponding to the GC fraction that elicited trail-following behavior.

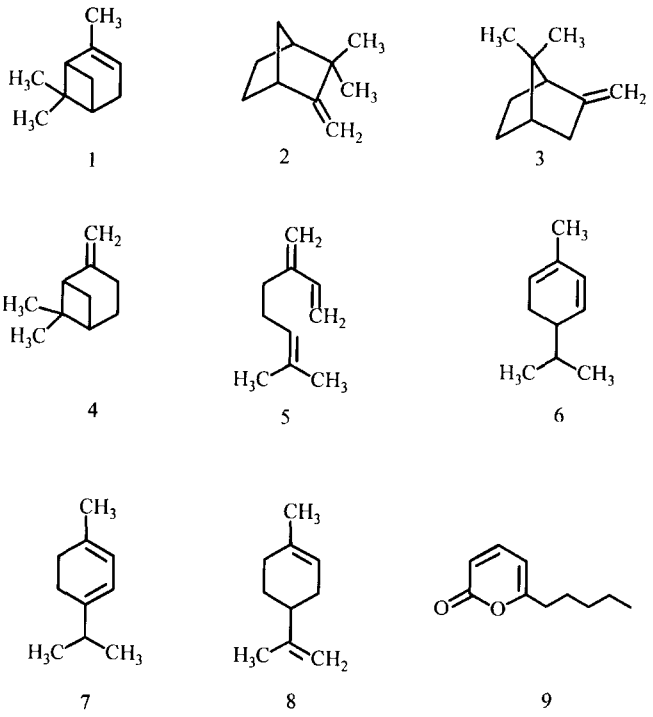


FIG. 2. Structures of the poison gland compounds of *Pristomyrmex pungens:* **1,** α-pinene; **2,** camphene; **3,** α-fenchene; **4,** β-pinene; **5,** myrcene; **6,** α-phellandrene; **7,** α-terpinene; **8,** limonene; **9,** 6-*n*-pentyl-2-pyrone.

(53), 68 (23), 55 (8), 53 (13), 41 (15), 39 (60) (see Figure 3). Peak **10** (Figure 1) was found to be an artifact that apparently resulted from column bleeding.

We employed preparative GC in order to fractionate the poison gland extract, each fraction of which was submitted to a behavioral test. Only one fraction (double-headed arrow, Figure 1), trapped between 18–20 min and corresponding to the retention time range of C_{24}–C_{25}, elicited trail-following responses in worker ants of *P. pungens*. All compounds of the poison gland volatiles eluting within this time range were analyzed by GC-MS. We found only one major component within this fraction. The mass spectral and retention time characteristics matched those obtained from synthetic standard of 6-*n*-pentyl-2-pyrone **9**. (Figure 3).

Microreactions. In three of 15 poison gland samples, we detected fatty acids (hexadecanoic, octadecanoic, and octadecenoic acid), probably contaminations from cell tissue of the sting apparatus of *P. pungens*. To make sure that

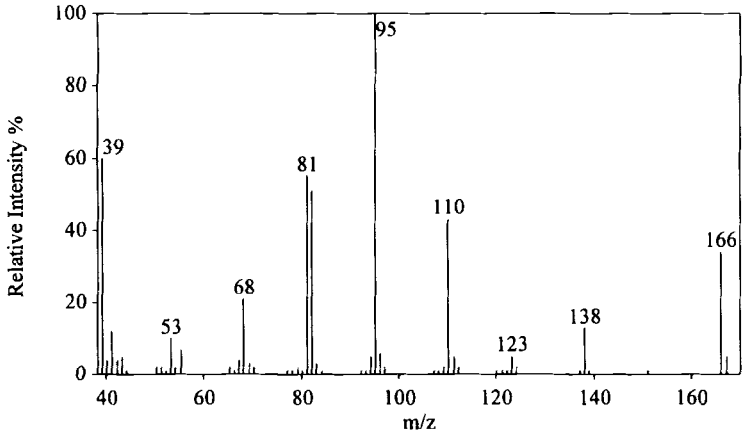


FIG. 3. Mass spectrum of poison gland compound **9** (6-*n*-pentyl-2-pyrone) from *P. pungens* workers.

fatty acids are not biologically active components eliciting trail-following behavior, we performed derivatization reactions for the fatty acids, such as esterification and silylation, with the total poison gland extract. Behavioral responses were the same before and after derivatization; i.e., after derivatization, we did not observe a disappearance or reduction of trail-following activity.

Gas Chromatography-Electroantennography (GC-EAG). The GC of poison gland secretions of *P. pungens* showed the presence of many volatile components (Figure 1). The antennae of *P. pungens* workers were used to detect biological compounds contained in the poison gland by GC-EAG. The EAGs were digitized and the responses of three electrophysiological measurements added, which improved the signal-to-noise proportion (Figure 4A). A significant and reproducible major response in every single measurement was observed at the retention time of 20.8 min using a FSCC SP-2340. This poison gland compound eliciting an electrophysiological response from *P. pungens* workers antennae matched retention time characteristics of synthetic 6-*n*-pentyl-2-pyrone, used subsequently for comparison in further GC analyses. In addition, an authentic sample of 6-*n*-pentyl-2-pyrone (0.001 ng) also showed electrophysiological activity when injected in GC-EAG experiments. In contrast, no significant electrophysiological responses were observed for any other identified volatiles from extracts of the *P. pungens* gland.

Trail-Following Experiments. Each gland contains about 250 pg of 6-*n*-pentyl-2-pyrone **9**. For trail-following experiments, we applied 5 μl of a 100 ng/ml solution to the trail, which approximates two gland equivalents of compound **9**. Although the response of the ants during the emigration tests varied

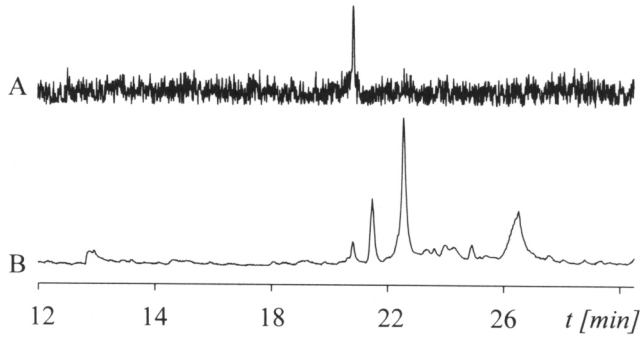


FIG. 4. Pooled electroantennogram responses from three *P. pungens* worker antennae (A) using digitally converted data summed from three individual measurements. The gas chromatogram of a hexane extract of six poison glands (B) was obtained with a coupled GC-EAG technique (FSCC SP-2340; temperature program: 80°C for 1 min, 5°C/min, 195°C hold).

greatly, the results were unambiguous: 23.2 ± 15.2 ($\bar{X}$ ± SD; range 7–56; N = 12) ants followed the branch of the V bridge on which a trail was drawn with 5 μl of the solvent pentane or with two gland equivalents of 240 ng β-pinene **4**, 96 ng α-pinene **1**, 104 ng camphene **2**, and 900 pg limonene **8**. These terpenes, which previously were mentioned as alarm pheromones (Hayashi and Komae, 1976), elicited some excitement in the ants that was expressed by faster and more erratic running. We suspected, therefore, that they might have a synergistic effect when presented together with 6-*n*-pentyl-2-pyrone **9** on the same trail. We offered alternatively trails drawn with two gland equivalents of 6-*n*-pentyl-2-pyrone **9** and trails drawn with a mixture of two gland equivalents of compound **9** and the four terpenes mentioned above. Whereas 11 ± 3.9 ($\bar{X}$ ± SD) ants followed the first type of trails, 16 ± 9.9 ants followed the second type. The difference is not significant (one-way ANOVA; $F = 2.61$; $P = 0.122$) and does not unequivocally support the hypothesis that the poison gland compounds **1, 2, 4,** and **8** have a synergistic effect in trail communication. We cannot rule out the possibility, however, that terpenoid compounds may aid in trail initiation.

DISCUSSION

Hayashi and Komae (1976) reported that the trail pheromone of *P. pungens* consisted of a mixture of nine carboxylic acids. In their study, the extract of entire bodies from workers was subjected to chemical analysis and behavior experiments. Samples prepared in this way obviously contain a large variety of

substances, but only some are derived from pheromone glands, the main target of their study. It is most likely that the positive trail-following response observed by Hayashi and Komae was not elicited by the blend of fatty acids, but by undetected minor compounds contained in whole-body extracts.

After we identified the poison gland as the source of the trail pheromone, we focused on the chemical analysis of the secretions of this organ. In three of 15 poison gland samples, we detected hexadecanoic, octadecanoic, and octadecenoic acids, probably contaminations from the cell tissues of the sting apparatus of *P. pungens*. Synthetic samples of the acids were tested in trail-following experiments, but neither a mixture nor the individual compounds elicited trail-following response in these ants.

Monoterpenes occur as major compounds in the secretion of *P. pungens* poison glands, the source of the trail pheromone. Hayashi and Komae (1976) claimed α-pinene **1**, β-pinene **4**, camphene **2**, and limonene **8** had alarm pheromone function, but no detailed behavioral tests were reported. We observed an increased locomotory activity when terpenes were presented. This might be due to an alarm response. Although it occurs only in picogram quantities, only with 6-*n*-pentyl-2-pyrone was a clear, unambiguous electroantennogram response recorded. This result is matched by the behavioral tests in which only 6-*n*-pentyl-2-pyrone released unambiguous trail-following behavior in *P. pungens*. None of the terpenoid compounds elicited orientation behavior in trail-following experiments when offered in individual trails with synthetic substances. Since there was a slightly better response to 6-*n*-pentyl-2-pyrone-trails when a mixture of the terpenes in nanogram quantities was added, it is possible that some of the poison gland terpenes contribute to the whole trail pheromone blend. The main trail pheromone component is, however, 6-*n*-pentyl-2-pyrone, and to our knowledge this is the first pyrone identified as a trail substance in ants (see Jackson and Morgan, 1993).

Acknowledgments—We thank the Deutsche Forschungsgemeinschaft for financial support. K. Tsuji was supported by a stipend from the Alexander von Humboldt-Foundation (Germany).

REFERENCES

ATTYGALLE, A. B., and MORGAN, E. D. 1988. Pheromones in nanogram quantities: Structure determination by combined microchemical and gas chromatographic methods. *Angew. Chem.* 100:475–494, *Int. Engl. Ed.* 27:460–478.

HAYASHI, N., and KOMAE, H. 1976. The trail and alarm pheromones of the ant, *Pristomyrmex pungens* Mayr. *Experientia* 33:424–425.

ITOW, T., KOBAYASHI, K., KUBOTA, M., OGATA, K., IRNAI, H. T., and CROZIER, R. H. 1984. The reproductive cycle of the queenless ant *Pristomyrmex pungens*. *Insectes Soc.* 31:87–102.

JACKSON, B. D., and MORGAN, E. D. 1993. Insect communication: Pheromones and exocrine glands of ants. *Chemoecology* 4:125–144.

KERN, F., and BESTMANN, H. J. 1993. Antennal electrophysiological responsiveness of the Ponerine ant *Leptogenys diminuta* to trail and recruitment pheromones and its structure analogs. *Naturwissenschaften* 80:424–427.

KNAPP, D. R. 1979. Handbook of Analytical Derivatization Reactions. John Wiley & Sons, New York, pp. 177–178.

MORGAN, E. D., and WADHAMS, L. J. 1972. Gas chromatography of volatile compounds in small samples of biological material. *J. Chromatogr. Sci.* 10:528–529.

TSUJI, K. 1988a. Obligate parthenogenesis and reproductive division of labor in the Japanese queenless ant, *Pristomyrmex pungens:* A comparison of intranidal and extranidal workers. *Behav. Ecol. Sociobiol.* 23:247–255.

TSUJI, K. 1988b. Nest relocation in the Japanese queenless ant *Pristomyrmex pungens* Mayr (Hymenoptera: Formicidae). *Insectes Soc.* 35:321–340.

TSUJI, K. 1994. Inter-colonial selection for the maintenance of cooperative breeding in the ant *Pristomyrmex pungens:* A laboratory experiment. *Behav. Ecol. Sociobiol.* 35:109–113.

TSUJI, K. 1995. Reproductive conflicts and levels of selection in the ant *Pristomyrmex pungens:* Contextual analysis and partitioning of covariance. *Am. Nat.* 146:586–607.

Journal of Chemical Ecology, Vol. 23, No. 4, 1997

AMBROSIA BEETLE HOST SELECTION AMONG LOGS OF DOUGLAS FIR, WESTERN HEMLOCK, AND WESTERN RED CEDAR WITH DIFFERENT ETHANOL AND α-PINENE CONCENTRATIONS

RICK G. KELSEY[1,*] and GLADWIN JOSEPH[2]

[1]*USDA Forest Service, Pacific Northwest Research Station*
3200 Jefferson Way, Corvallis, Oregon 97331
[2]*Department of Forest Science, Oregon State University*
Corvallis, Oregon 97331

(Received April 4, 1996; accepted November 28, 1996)

Abstract—Logs from Douglas fir, *Pseudotsuga menziesii*; western hemlock, *Tsuga heterophylla*; and western red cedar, *Thuja plicata*, were left in the forest through winter. In April, segments from these logs were removed and randomly positioned adjacent to one another allowing ambrosia beetles to select their preferred host. In early June the tissues of Douglas fir and western hemlock logs contained significantly higher ethanol concentrations and ambrosia beetle (*Trypodendron lineatum* and *Gnathotrichus* spp.) densities than logs of western red cedar. Low beetle attack densities in western red cedar were probably a consequence of low ethanol concentrations. Although Douglas fir tissues produced significantly higher ethanol concentrations than western hemlock, the beetles did not effectively discriminate between these two conifer species. Ethanol and α-pinene were significant covariates for the ambrosia beetle densities. α-Pinene concentrations were highest in the phloem of western red cedar, intermediate in Douglas fir, and nearly absent in western hemlock. α-Pinene did not synergize the beetle's response to ethanol or to ethanol + pheromone during host selection, and it may have functioned as a deterrent.

Key Words—Host selection, kairomones, ambrosia beetles, *Trypodendron lineatum*, *Gnathotrichus* spp., *Pseudotsuga menziesii*, *Tsuga heterophylla*, *Thuja plicata*.

INTRODUCTION

Host selection by the ambrosia beetles *Trypodendron lineatum* (Oliv.), *Gnathotrichus sulcatus* (LeConte), and *G. retusus* (LeConte) (Coleoptera: Scolytidae)

*To whom correspondence should be addressed.

0098-0331/97/0400-1035$12.50/0 © 1997 Plenum Publishing Corporation

in the spring or summer is characterized by a distinct preference for logs that have aged through the winter over logs freshly felled in the spring (Prebble and Graham, 1957; Bletchly and White, 1962; Dyer and Chapman, 1965; Pulliainen, 1983; Lindelöw et al., 1992; Kelsey, 1994a). Graham (1968) demonstrated that *T. lineatum* were attracted to fresh western hemlock, *Tsuga heterophylla* (Raf.) Sarg., sapwood only after subjecting it to anaerobic conditions. Ethanol was found to accumulate in live conifer tissues under anaerobic conditions and was identified as a kairomone in aged logs under attack by *T. lineatum* (Moeck, 1970) and *G. sulcatus* (Cade et al., 1970).

Trypodendron lineatum is attracted to ethanol released alone from artificial sources (Moeck, 1970, 1971; Nijholt and Shönherr, 1976; Klimetzek et al., 1986; Schroeder, 1988; Liu and McLean, 1989; Schroeder and Lindelöw, 1989; Salom and McLean, 1990), and in Europe the trap catches increase as the ethanol release rates increase (Klimetzek et al., 1986; Schroeder, 1988; Schroeder and Lindelöw, 1989). A comparable response has not been observed in North American populations of this species (Moeck, 1970; Salom and McLean, 1988).

Also in Europe, the release of ethanol with α-pinene causes a synergistic increase in the trap catches of *T. lineatum* compared to each compound released alone (Bauer and Vité, 1975; Nijholt and Shönherr, 1976; Borden et al., 1982; Schroeder, 1988; Schroeder and Lindelöw, 1989). A synergistic response was observed when the ethanol–α-pinene release rate was 10:1 but not at 1:1 (Schroeder and Lindelöw, 1989). In western North America, Nijholt and Schönherr (1976) are the only investigators to observe a synergistic response from *T. lineatum* with the binary release of ethanol + α-pinene.

In North America and Europe the combined release of ethanol + pheromone, or the ternary combination of ethanol + α-pinene + pheromone, may trap more *T. lineatum* than traps releasing only the pheromone (Vité and Bakke, 1979; Borden et al., 1982; Shore and McLean, 1983). Similar responses have been observed for the *Gnathotrichus* spp. (Borden et al., 1980, 1982; Shore and McLean, 1983, 1985). In some experiments, however, the combination of pheromone + kairomone(s) did not enhance the beetle capture over the pheromone alone (Borden et al., 1980, 1982; Salom and McLean, 1988). In Europe, increasing the release rate of α-pinene from low to high in ternary mixtures, without changing the release rates of the pheromone (lineatin) or ethanol, caused a decrease in trap catches of *T. lineatum* (Bakke, 1983).

Aside from the initial studies by Moeck (1970) and Cade et al. (1970), only a few experiments have focused on the kairomone chemistry of logs during host selection by these ambrosia beetles. Ethanol and acetaldehyde concentrations accumulate in logs of Norway spruce, *Picea abies* (L.) Karst., felled in November and left on the ground through winter (Lindelöw et al., 1992). Injured (tops removed) Scots pine trees, *Pinus sylvestris* L., attacked by *T. lineatum*

contained more acetaldehyde and ethanol than injured trees that were not attacked (Sjödin et al., 1989).

Recently it was shown that ambrosia beetle preference for Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco, logs felled on different dates is related to the tissue ethanol concentrations (Kelsey, 1994a). Logs felled in November synthesized and accumulated more ethanol than those felled in January and March, and subsequently they were more heavily attacked. In a related study, delimbed Douglas fir logs produced more ethanol and were more heavily attacked by beetles than logs with branches attached (Kelsey, 1994b).

These beetles also prefer to attack particular conifer species. For instance, logs of Douglas fir and western hemlock are preferred over those of western red cedar, *Thuja plicata*, Donn ex D. Don, and Pacific silver fir, *Abies amabilis* Dougl. ex Forbes, (Prebble and Graham, 1957; Chapman, 1961, 1963; Johnson, 1958; Zhong and Schowalter, 1989). In Europe, *T. lineatum* attacks Scots pine logs more heavily than Norway spruce, (Pulliainen, 1983). However, very little is known about interspecific kairomones and their relationships to ambrosia beetle host preference.

Considering the effect of ethanol on ambrosia beetle behavior it seems reasonable to suspect that their preference for certain conifer species may be caused, in part, by the synthesis or release of different quantities of ethanol from each conifer species. Under laboratory conditions, ethanol synthesis among conifer species varied significantly when foliage (Kimmerer and MacDonald, 1987) or stems (Kelsey, 1996) were subjected to anaerobic conditions. The objective of this study was to measure and compare ethanol, α-pinene, and acetaldehyde concentrations with ambrosia beetle attack densities in Douglas fir, western hemlock, and western red cedar logs exposed to similar environmental conditions in the field.

METHODS AND MATERIALS

Study Site. This study was conducted on the Oregon State University, College of Forestry, Blodgett Tract (46°05′30″N, 123°20′06″W) in the Coast Range of northwestern Oregon, near the Columbia River. The experiment was set up on a ridge top, adjacent to the forest edge at the upper boundary of a 23-ha clear-cut. The cutting unit faced north with some components facing east and west. Elevation of the unit ranged from 311 to 390 m. Logs for this experiment were taken from trees harvested in the clear-cut during September and October 1992.

Log Preparation. After felling and limbing, the logs (approximately 10 m long) were yarded and decked near the experimental site on the ridge top. In February 1993, nine logs from each species were removed from the decks, sorted

by species and set aside. On April 13, log segments 1.83 m long, with minimal bark damage and similar diameters, were cut from each 10-m log. Their mean diameters were 30.2 ± 1.5 (SE), 32.2 ± 1.0, and 30.9 ± 1.7 cm ($P = 0.685$) for Douglas fir, western hemlock, and western red cedar, respectively.

The top of each log was marked to ensure it remained upright and then placed into one of three blocks. Within blocks, the logs were randomly positioned 0.5–1.5 m apart, with their sides parallel and the cut ends facing east/west. Each block contained three logs from each species. The blocks were parallel to one another with the log ends separated by about 1–2 m. The remaining pieces of the original 10-m logs were left on site less than 50 m from the blocks.

Tissue Sampling. Log tissues were sampled for chemical analysis on June 2, 1993. Separate samples of phloem and sapwood (1.0 cm depth) were removed with an increment borer (5 mm ID) along the top of each log at 30, 91, and 152 cm from the west end. The north and south sides of each log were sampled in the same manner, yielding a total of nine samples of each tissue per log. Each sample was sealed in a small vial (15 × 45 mm), frozen with Dry Ice for transport to the laboratory, and stored at −36°C until prepared for analysis. After thawing for a few minutes on ice, each sample was weighed into a preweighed headspace vial (22 × 75 mm, 22 ml volume) and sealed with a septum. The sealed vials were heated for 30 min at 100°C to deactivate the enzymes. Sample analysis was completed within three weeks after collection.

Headspace Analysis. The chemical analysis was performed with a Perkin Elmer HS40 headspace autosampler connected to a Hewlett-Packard 5890 gas chromatograph with a J&W DB-Wax column, 0.32 mm ID × 30 m, and 0.25-μm film thickness. Other operating conditions and calculations were the same as previously reported (Kelsey, 1994b), except for a sample thermostat temperature of 90°C instead of 100°C on the HS40. Furthermore, chemical concentrations were expressed on a fresh weight basis rather than dry weight, because ethanol is water-soluble and nonionic so it can mix and move with water through the tissues. Fresh weight concentrations may represent more accurately the actual ethanol concentrations experienced by ambrosia beetles excavating the tissues.

Ambrosia Beetle Measurements. All logs were free of beetle attacks on April 13 when arranged into blocks. Ambrosia beetle attack densities were estimated on June 3 and 4, 1993, by removing the bark and counting gallery entrance holes in a 15- × 15-cm quadrat centered around each tissue sampling hole (nine quadrats per log). If the bark was damaged, the quadrat was moved to the nearest undamaged position. Entrance holes of *T. lineatum* were separated from those of *Gnathotrichus* spp. by checking the diameter of each hole with the end of a No. 53 wire gauge drill bit (Kinghorn, 1957a). When sampling

was complete, a few beetles were excavated from their galleries for identification by the Entomology Extension Service at Oregon State University.

Statistical Analysis. The data were analyzed as a split-plot design with randomized complete blocks in the whole plot and strip-plots in the subplot. Tree species was the whole plot treatment, whereas tissue and position on logs (north side, south side, and top) were the subplot treatments.

For the analysis of variance (ANOVA), concentrations of each chemical component were averaged across the three logs of a species to give a single species value for each tissue and position within each block. The averages were weighted by the number of observations because values from one of the Douglas fir logs were discarded as outliers. Its tissues contained no ethanol, low α-pinene and acetaldehyde concentrations, and a water content about half the amount in the other Douglas fir logs. This log might have been taken from a dying tree at the time of felling. The SAS (SAS Institute Inc., 1989) General Linear Model (GLM) procedure with type III sums of squares was used for the ANOVA of each chemical constituent, the attack densities of *T. lineatum*, and the combined attack densities of the *Gnathotrichus* species. Fisher's protected LSD test (α = 0.05) was used for preplanned comparisons of means. The values for beetle densities, acetaldehyde, and α-pinene required natural log transformation to ensure normality and homogeneity of variances. Geometric means with back-transformed standard errors are presented for the transformed variables.

To determine the relative influence of individual chemical constituents on the variation in beetle densities, the data were subjected to an analysis of covariance (Steel and Torrie, 1980) run on mean values for each log using the GLM procedure of SAS (SAS Institute Inc., 1989). Means for each log were obtained by averaging across tissues and the side positions, but excluding values from the tops. Data from log tops were not included because beetle attacks on the top and sides were probably confounded by a photonegative response (Graham, 1959) (see Discussion).

To identify the significant covariates explaining beetle densities, an iterative GLM procedure was used (Thomas Sabin, Oregon State University, personal communication). Each of the covariates was independently included in an initial model of the form: y (beetle density) = covariate (ethanol, etc.) $\times$ treatment (species). Ethanol was identified as the only significant covariate at $P \leq 0.05$ with the corresponding type III sums of squares. Ethanol was then paired with each of the other covariates and analyzed as separate models using GLM procedures (type I sums of squares) to determine the significance of the other covariates after adjusting for the effect of ethanol. Finally, ethanol and the other covariates (in the order of significance from the paired analyses with ethanol) were combined in a final model (with type I sums of squares) to determine their relative contributions to the variation in beetle densities.

RESULTS

Ethanol concentrations were dependent on the species, tissue, and position on the log (Table 1), illustrating how heterogeneous the source of this kairomone is in logs. Nevertheless, for each tissue and position, the ethanol concentrations were significantly different among species (Figure 1). On average, the ethanol concentrations in tissues of Douglas fir were 2.1 times greater than in western hemlock and 3.3–4.0 times greater than in western red cedar. Comparing tissues within species, ethanol concentrations in the phloem were significantly greater than in the sapwood on the sides of Douglas fir and western hemlock logs, whereas the opposite was observed in western red cedar (Figure 1). Western hemlock was the only species with a significant difference in ethanol concentration between sapwood and phloem on the tops of logs. Overall, within each species the differences in ethanol concentrations between tissues were not large at any position. Comparing positions within species, the tissues with the highest ethanol concentrations were usually from the tops of log, although they were not always significantly greater than the concentrations in the corresponding tissues from the sides (Figure 1). Western hemlock exhibited the greatest difference between tops and sides, for both tissue types. Tissues from the north side of Douglas fir contained greater ethanol concentrations than the tissues collected from the south side, whereas the opposite was observed for western red cedar (Figure 1). In general, ethanol concentrations in a tissue differed more among positions than they did between tissues from the same position.

Acetaldehyde concentrations were dependent on the species and tissue (Table 1), but only in western red cedar were the tissue concentrations significantly different (Figure 2). Concentrations among species exhibited a trend similar to ethanol, with highest quantities in the sapwood and phloem of Douglas fir and the lowest quantities in the sapwood from western red cedar. The acetaldehyde concentrations, however, were much lower, at only 1–4% of the ethanol concentrations (Figures 1 and 2). There were no significant differences in acetaldehyde concentrations among positions (Table 1).

α-Pinene concentrations were dependent on the species and tissue (Table 1) because of large concentration differences between the phloem and sapwood within western red cedar, but no differences in concentrations between these tissues for the other two species (Figure 2). α-Pinene concentrations were highest in the phloem of western red cedar, but not significantly greater than the concentrations in Douglas fir sapwood (Figure 2). The phloem and sapwood of western hemlock contained the lowest quantities of α-pinene (Figure 2). α-Pinene was detected in only two of the nine hemlock logs, at very low concentrations in three tissue samples of one log and in four tissue samples of another. There were no differences in α-pinene concentrations among positions on the logs (Table 1).

TABLE 1. ANALYSIS OF VARIANCE FOR ETHANOL, ACETALDEHYDE, α-PINENE, AND WATER IN LOGS OF DOUGLAS FIR, WESTERN HEMLOCK, AND WESTERN RED CEDAR[a]

Source of variation	*df*	Ethanol		Acetaldehyde		α-Pinene		Water	
		MS	*P*	MS	*P*	MS	*P*	MS	*P*
Block (B)	2	2,241,952		14.28		31.26		10,399	
Species (S)	2	579,634,018	0.002	102.75	0.004	979.62	0.001	159,844	0.005
Error (a)	4	12,840,322		3.42		17.23		6,020	
Tissue (T)	1	1,458,594	0.120	13.99	0.002	77.63	0.003	456,346	0.000
S × T	2	2,569,395	0.040	21.02	0.000	161.04	0.000	17,419	0.032
Error (b)	6	445,445		0.55		3.50		2,711	
Position (P)	2	34,072,379	0.007	5.87	0.148	0.50	0.952	9,132	0.030
S × P	4	9,334,849	0.146	4.80	0.187	10.71	0.421	437	0.917
Error (c)	12	4,482,034		2.62		10.16		1,913	
T × P	2	538,524	0.019	0.76	0.108	2.82	0.218	4,528	0.012
S × T × P	4	704,894	0.003	0.19	0.615	1.96	0.358	926	0.311
Error (d)	12	96,457		3.38		1.62		690	

[a]The acetaldehyde and α-pinene values were $\log_e(x + 1)$-transformed before analysis. MS: mean square.

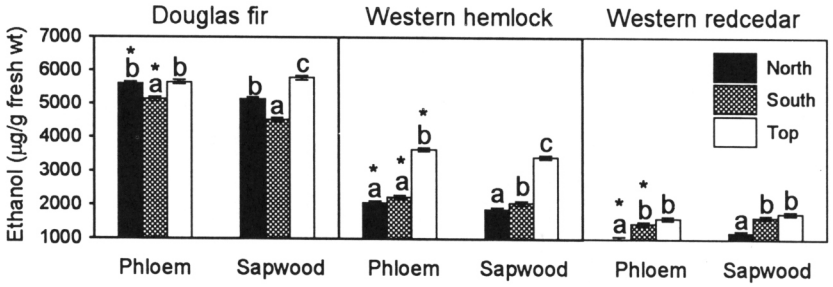


FIG. 1. Mean (±SE) ethanol concentrations in the phloem and sapwood at three different positions on logs of Douglas fir, western hemlock, and western red cedar that were being attacked by ambrosia beetles. For each tissue and position, the differences between each of the species were significant ($P \leq 0.05$). Within each species and tissue, log positions with different letters are significantly different. Asterisks indicate a significant difference between the phloem and sapwood at a particular position within each species.

Water content was dependent on the species and tissue (Table 1), but in all three species the sapwood contained significantly less water than the phloem (Figure 2). Among species, western red cedar phloem and sapwood contained the highest water content of each tissue (Figure 2). The water contents of western hemlock and Douglas fir tissues were not different. Although the water content was also dependent on the tissue and log position (Table 1), this was probably of little biological significance because at all three log positions the sapwood contained 65.2–71.9% of the water in the phloem. The water content of phloem on the tops of logs was 90.1% of the water in phloem from the sides. For sapwood, there were no differences in the water contents among log positions.

In addition to *T. lineatum*, both *Gnathotrichus retusus* and *G. sulcatus* were extracted from galleries and identified. The attack densities of *T. lineatum* on Douglas fir and western hemlock (Table 2; Figure 3) were greater than on western red cedar. There were 1.4 times more *T. lineatum* attacks on Douglas fir than on western hemlock logs, but the difference was not significant ($P = 0.090$). The densities of *Gnathotrichus* spp. on both Douglas fir and western hemlock were greater than on western red cedar logs (Table 2; Figure 3). There were no differences ($P = 0.508$) in *Gnathotrichus* spp. densities between Douglas fir and western hemlock. Both beetle genera avoided attacking the tops of logs in favor of either side, with no preference between sides (Table 2; Figure 4).

To examine further the relationship between beetle densities and ethanol concentrations, the means of both variables were calculated for each log within a species. Logs with the lowest ethanol concentrations had the lowest beetle

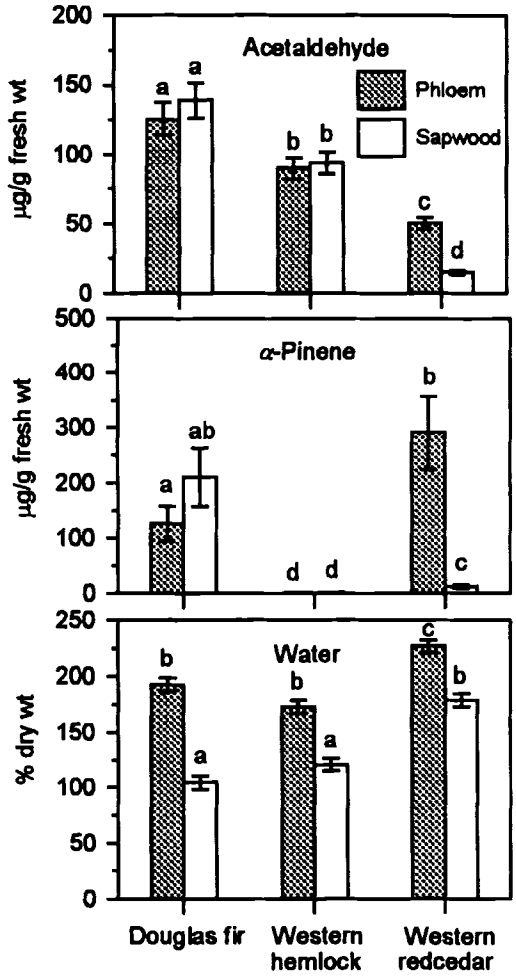


FIG. 2. Mean (±SE) acetaldehyde, α-pinene, and water contents of tissues from Douglas fir, western hemlock, and western red cedar logs that were being attacked by ambrosia beetles.

densities in each species. However, at the higher ethanol concentrations, the beetle densities were not always correspondingly higher.

Covariate analysis indicated that ethanol and α-pinene were the only two significant covariates (Table 3), accounting for 61% of the variation in *T. lineatum* densities and 52% of the variation in the *Gnathotrichus* spp. densities.

TABLE 2. ANALYSIS OF VARIANCE FOR BEETLE DENSITIES IN LOGS OF DOUGLAS FIR, WESTERN HEMLOCK, AND WESTERN RED CEDAR[a]

Source of variation	*df*	*T. lineatum*		*Gnathotrichus* spp.	
		MS	*P*	MS	*P*
Block	2	0.73		4.88	
Species	2	6.24	0.013	7.61	0.049
Error (a)	4	0.41		1.08	
Position	2	17.83	0.000	35.33	0.000
Species × position	4	0.05	0.963	0.93	0.293
Error (b)	12	0.34		0.67	

[a]Data were $\log_e(x + 1)$-transformed before analysis. MS: mean square.

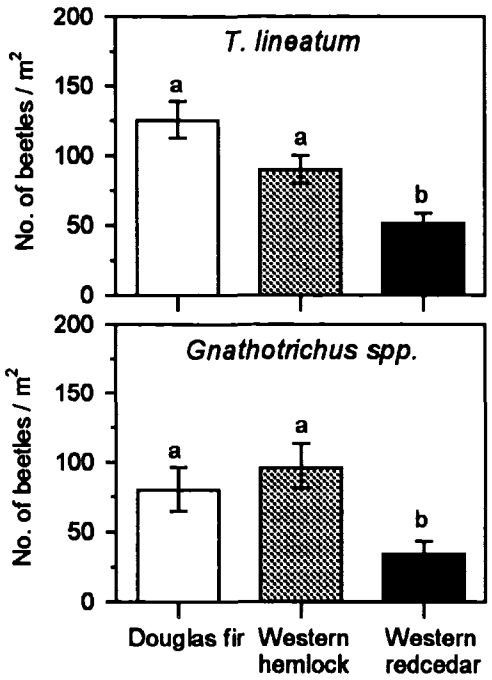


FIG. 3. Mean (±SE) densities of *T. lineatum* and the *Gnathotrichus* spp. in logs of Douglas fir, western hemlock, and western red cedar in early June.

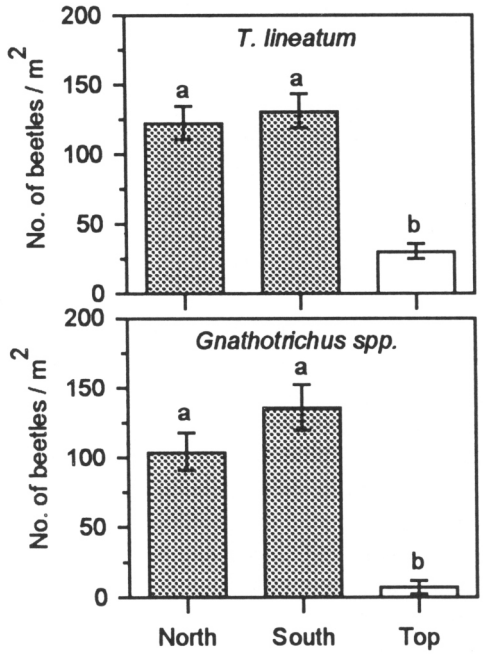


FIG. 4. Mean (±SE) densities of *T. lineatum* and the *Gnathotrichus* spp. in the sides and tops of Douglas fir, western hemlock, and western red cedar logs in early June.

TABLE 3. ANALYSIS OF COVARIANCE FOR ETHANOL, α-PINENE, ACETALDEHYDE, AND WATER AMONG CONIFER SPECIES WITH VARYING DENSITIES OF BEETLE ATTACK

		T. lineatum		*Gnathotrichus* spp.	
Source of variation	*df*	MS	*P*	MS	*P*
Covariate					
Ethanol	1	53.388	0.000	27.020	0.013
α-Pinene	1	7.698	0.054	25.054	0.016
Acetaldehyde	1	3.566	0.179	0.302	0.775
Water	1	2.594	0.249	0.148	0.841
Treatment					
Species	2	4.481	0.317	8.267	0.338
Error	19	1.834		3.600	

[a] MS: mean square.

Ethanol concentrations explained more variation in attack densities of *T. lineatum* than it did for *Gnathotrichus* spp. The effect of α-pinene was just the opposite. Estimates of regression coefficients for each variable in the model indicated a positive relationship between beetle densities and ethanol concentrations and a negative relationship between beetle densities and α-pinene concentrations.

DISCUSSION

During the winter and spring months the tissues of Douglas fir and western hemlock logs synthesized and accumulated much higher ethanol concentrations than the tissues of western red cedar. Their ethanol concentrations were not correlated with their tissue water contents, which agrees with previous observation for Douglas fir (Kelsey, 1994a,b). Logs of western red cedar, with low ethanol, were more lightly attacked by *T. lineatum* and the *Gnathotrichus* spp. than logs of Douglas fir and western hemlock, with higher ethanol concentrations. For individual logs within each conifer species, the relationship between low beetle densities and low ethanol concentrations paralleled that observed among species. Finding ethanol as the most significant contributor to the variation in beetle attack densities is consistent with ethanol attracting ambrosia beetles to artificial traps (Moeck, 1970, 1971; Nijholt and Shönherr, 1976; Klimetzek et al., 1986; Schroeder, 1988; Liu and McLean, 1989; Schroeder and Lindelöw, 1989; Salom and McLean, 1990).

Although the chemical concentrations in our logs were not measured immediately after the beetle attacks began, they were sampled soon enough to represent accurate concentrations that most beetles would have experienced. The majority of attacks probably occurred in May (Daterman et al., 1965) (we observed no attacks on April 13), only 30–35 days preceding our sampling date. Furthermore, the relative differences in ethanol concentrations among November-, January-, and March-felled Douglas fir logs were the same when sampled in March (Kelsey, 1994a), or May (Kelsey, unpublished data). Therefore, in this experiment it is unlikely that relative ethanol concentrations among species changed between the initial beetle attacks and the time we sampled the logs.

These ambrosia beetles, however, showed no preference between Douglas fir and western hemlock even though the Douglas fir logs contained 2.1 times (averaged across positions) more ethanol. Zhong and Schowalter (1989) also observed equal ambrosia beetle attack densities when these two conifer species where positioned near one another. Explanations could be a reduced beetle response to ethanol or repellence above some threshold concentration. There is evidence to support these responses (Montgomery and Wargo, 1983; Kelsey,

1994a). In particular, during olfactometer bioassays, Moeck (1970) noted that high ethanol concentrations were strongly attractive to *T. lineatum* initially, but the beetles then lost their ability to discriminate odors in subsequent runs.

It is also possible that the ethanol release rates from the exposed surfaces of Douglas fir and western hemlock logs were not as different as the ethanol concentrations measured in their tissues, because of interfering factors. The outer bark of these two species differs in thickness and morphology, and this might influence both the quantity and dispersal of the ethanol released. Differences in release rates could be most important during the initial phases of the attack, becoming less important as beetle entrance holes open up the tissues and enhance vaporization.

An ethanol-pheromone interaction also could have interfered with the beetles' response to Douglas fir and hemlock. The initial pioneering beetles might have responded more strongly to the high ethanol concentrations in Douglas fir logs when pheromone concentrations were low, but, as the attack densities and the subsequent release of pheromone increased, their ability to discriminate high ethanol concentrations could have been modified. In western North America, a high ethanol-to-lineatin release ratio (250,000 : 1 per day) significantly decreased the trap catches of *T. lineatum* compared to traps baited with lineatin alone, while in England, traps with a lower ethanol-to-lineatin release ratio (25,000 : 1 per day) caught more beetles than traps with just the pheromone (Borden et al., 1982).

α-Pinene in Douglas fir did not enhance the beetles' preference for these logs over western hemlock, so apparently α-pinene was not functioning as an essential kairomone or as a synergist with ethanol or ethanol + pheromone during host selection in this experiment. There are several examples where the release of α-pinene with ethanol + pheromone either did not increase trap catches of these ambrosia beetles or resulted in decreased trap catches compared to traps baited with ethanol + pheromone (Borden et al., 1980, 1982; Salom and McLean, 1988; Liu and McLean, 1989). In Europe, a synergistic response by *T. lineatum* to ethanol + α-pinene is dependent on the ratio of their release rates (Schroeder, 1988; Schroeder and Lindelöw, 1989). The relative release rates of ethanol and α-pinene from our logs may have been influenced by the wet and cold spring weather. The volatilization of α-pinene would probably be reduced more by these conditions because of a lower water solubility and lower vapor pressure than ethanol. If the release of α-pinene from Douglas fir was minimal, there would be no synergistic beetle response.

The high concentrations of α-pinene and other terpenoids (Snajberk et al., 1974; Snajberk and Zavarin, 1976; Kelsey unpublished data) in the tissues of Douglas fir and western red cedar might have reduced the beetles' response to these species. In olfactory bioassays *T. lineatum* was repelled by α-pinene vapors (Moeck, 1970). The application of terpenoid mixtures, such as turpentine oil

(Nijholt, 1973) or pine oil (Nijholt, 1980; Dubbel, 1992), on log surfaces can delay attack and significantly reduce attack densities.

It is unlikely that acetaldehyde was functioning as a kairomone. In a previous experiment, acetaldehyde concentrations were higher than ethanol concentrations in November-felled Douglas fir logs when sampled in May (Kelsey, 1994b), and the analysis suggested it might have contributed to the variation in *G. retusus* attack densities. Here, acetaldehyde exhibited essentially the same relationship as ethanol among the conifer species, but the ethanol concentrations were 26–101 times higher. Secondly, from our covariate analysis, none of the variation in ambrosia beetle attack densities was explained by the acetaldehyde concentrations. Furthermore, acetaldehyde failed to attract any scolytid beetles to traps in the field (Montgomery and Wargo, 1983), or to attract *T. lineatum* in olfactometer bioassays (Moeck, 1970).

The logs used in this experiment were relatively short and provided the beetles with a limited area to attack. Consequently, the beetle densities in these logs could potentially reach saturation levels and not increase further, even though additional beetles were available to attack. If this were the cause of similar beetle densities on Douglas fir and western hemlock logs, these values may not accurately estimate kairomone-mediated preference. However, the beetle populations in these logs were not at saturation levels, since others have reported much higher attack densities for both *T. lineatum* (Kinghorn, 1957b; Dyer, 1963; Lindgren et al., 1982; Zhong and Schowalter, 1989) and *Gnathotrichus* spp. (McLean and Borden, 1977; Liu and McLean, 1993; Kelsey, 1994a,b).

The ambrosia beetles preferred to attack the sides of logs in each conifer species, although the ethanol concentrations on log tops were equal to or greater than on the sides. Freshly emerged *T. lineatum* are so strongly photopositive they do not respond to host kairomones (Graham, 1959), but during flight they switch to a photonegative response and become chemosensitive (Graham, 1959; Bennett and Borden, 1971). In our experiment the photonegative response dominated the attraction to kairomones on the log tops. *Trypodendron lineatum* avoid the tops of logs when the logs are exposed to light (Dyer, 1963; Lindgren et al., 1982), but they are less discriminating when the logs are in the shade of a forest canopy (Dyer, 1963; Kelsey, 1994a,b). Both *G. retusus* and *G. sulcatus* have been reported to avoid log tops (McLean and Borden, 1977; Lindgren et al., 1982; Kelsey, 1994a,b).

In conclusion, when Douglas fir, western hemlock, and western red cedar logs were left in the forest through winter and spring, they synthesized and accumulated different concentrations of ethanol. Lower ambrosia beetle attack densities on western red cedar, compared with Douglas fir and western hemlock, were most likely a consequence of lower ethanol concentrations in western red cedar. The ambrosia beetles were unable to effectively discriminate between the

high and moderate ethanol concentrations in Douglas fir and western hemlock, respectively, for a number of possible reasons. α-Pinene did not appear to function as an ambrosia beetle kairomone under the conditions of this experiment. Beetles did not respond synergistically to logs containing α-pinene in combination with ethanol or ethanol + pheromone, and α-pinene may have functioned as a deterrent during host selection.

Acknowledgments—We thank Oregon State University, College of Forestry, for supplying the logs and allowing access to the Blodgett study site. We thank M. Rector for his assistance in finding the site and securing the logs, E. Gerson for technical assistance in the field and laboratory, T. Sabin for advice on the statistical analysis, and G. Parsons for beetle identification. We also thank Dr. C. Niwa and Dr. S. M. Salom for reviewing the manuscript. The use of trade names is for the information and convenience of the reader and does not constitute official endorsement or approval by the US Department of Agriculture.

REFERENCES

BAKKE, A. 1983. Dosage response of the ambrosia beetle *Trypodendron lineatum* (Oliver) (Coleoptera, Scolytidae) to semiochemicals. *Z. Angew. Entomol.* 95:158-161.

BAUER, J., and VITÉ, J. P. 1975. Host selection by *Trypodendron lineatum*. *Naturwissenschaften* 62:539.

BENNETT, R. B., and BORDEN, J. H. 1971. Flight arrestment of tethered *Dendroctonus pseudotsugae* and *Trypodendron lineatum* (Coleoptera:Scolytidae) in response to olfactory stimuli. *Ann. Entomol. Soc. Am.* 64:1273-1286.

BLETCHLY, J. D., and WHITE, M. G. 1962. Significance and control of attack by the ambrosia beetle *Trypodendron lineatum* (Oliv.) (Col. Scolytidae) in Argyllshire forests. *Forestry* 35:139-163.

BORDEN, J. H., LINDGREN, B. S., and CHONG, L. 1980. Ethanol and α-pinene as synergists for the aggregation pheromones of two *Gnathotrichus* species. *Can. J. For. Res.* 10:290-292.

BORDEN, J. H., KING, C. J., LINDGREN, S., CHONG, L., GRAY, D. R., OEHLSCHLAGER, A. C., SLESSOR, K. N., and PIERCE, H. D., JR. 1982. Variation in response of *Trypodendron lineatum* from two continents to semiochemicals and trap form. *Environ. Entomol.* 11:403-408.

CADE, S. C., HRUTFIORD, B. F., and GARA, R. I. 1970. Identification of a primary attractant for *Gnathotrichus sulcatus* isolated from western hemlock logs. *J. Econ. Entomol.* 63:1014-1015.

CHAPMAN, J. A. 1961. A note on felling date in relation to log attack by the ambrosia beetle *Trypodendron*. *Can. Dep. For., For. Entomol. Pathol. Branch Bi-Mon. Prog. Rep.* 17:3-4.

CHAPMAN, J. A. 1963. Field selection of different log odors by scolytid beetles. *Can. Entomol.* 95:673-676.

DATERMAN, G. E., RUDINSKY, J. A., and NAGEL, W. P. 1965. Flight patterns of bark and timber beetles: Associated with coniferous forest of western Oregon. Oregon State University Agriculture Bulletin 87.

DUBBEL, V. 1992. The effectiveness of pine oil as a repellent against the stripped ambrosia beetle *Trypodendron lineatum* (Col., Scolytidae). *J. Appl. Entomol.* 114:91-97.

DYER, E. D. A. 1963. Distribution of *Trypodendron* attacks around the circumference of logs. *Can. Dep. For., For. Entomol. Pathol. Branch Bi-Mon. Prog. Rep.* 19:3-4.

DYER, E. D. A., and CHAPMAN, J. A. 1965. Flight and attack of the ambrosia beetle, *Trypodendron lineatum* (Oliv.) in relation to felling date of logs. *Can. Entomol.* 97:42-57.

GRAHAM, K. 1959. Release by flight exercise of a chemotrophic response from photosensitive domination in a scolytid beetle. *Nature* 184:283–284.

GRAHAM, K. 1968. Anaerobic induction of primary chemical attractancy for ambrosia beetles. *Can. J. Zool.* 46:905–908.

JOHNSON, N. E. 1958. Ambrosia beetle infestation of coniferous logs on clearcuttings in northwestern Oregon. *J. For.* 56:508–511.

KELSEY, R. G. 1994a. Ethanol synthesis in Douglas-fir logs felled in November, January, and March and its relationship to ambrosia beetle attack. *Can. J. For. Res.* 24:2096–2104.

KELSEY, R. G. 1994b. Ethanol and ambrosia beetles in Douglas fir logs with and without branches. *J. Chem. Ecol.* 20:3307–3319.

KELSEY, R. G. 1996. Anaerobic induced ethanol synthesis in the stems of greenhouse-grown conifer seedlings. *Trees* 10:183–188.

KIMMERER, T. W., and MACDONALD, R. C. 1987. Acetaldehyde and ethanol biosynthesis in leaves of plants. *Plant Physiol.* 84:1204–1209.

KINGHORN, J. M. 1957a. Two practical methods of identifying types of ambrosia beetle damage. *J. Econ. Entomol.* 50:213.

KINGHORN, J. M. 1957b. An induced differential bark-beetle attack. *Can. Dep. Agric., For. Biol. Div. Bi-Mon. Prog. Rep.* 13:3–4.

KLIMETZEK, D., KÖHLER, J., VITÉ, J. P., and KOHNLE, U. 1986. Dosage response to ethanol mediates host selection by "secondary" bark beetles. *Naturwissenschaften* 73:270–272.

LINDELOW, Å., RISBERG, B., and SJÖDIN, K. 1992. Attraction during flight of scolytids and other bark- and wood-dwelling beetles to volatiles from fresh and stored spruce wood. *Can. J. For. Res.* 22:224–228.

LINDGREN, B. S., BORDEN, J. H. GRAY, D. R. LEE, P. C., PALMER, D. A., and CHONG, L. 1982. Evaluation of two trap log techniques for ambrosia beetles (Coleoptera: Scolytidae) in timber processing areas. *J. Econ. Entomol.* 75:577–586.

LIU, Y.-B., and MCLEAN, J. A. 1989. Field evaluation of responses of *Gnathotrichus sulcatus* and *G. retusus* (Coleoptera: Scolytidae) to semiochemicals. *J. Econ. Entomol.* 82:1687–1690.

LIU, Y.-B., and MCLEAN, J. A. 1993. Observations on the biology of the ambrosia beetle *Gnathotrichus retusus* (Leconte) (Coleoptera: Scolytidae). *Can. Entomol.* 125:73–83.

MCLEAN, J. A., and BORDEN, J. H. 1977. Attack by *Gnathotrichus sulcatus* (Coleoptera: Scolytidae) on stumps and felled trees baited with sulcatol and ethanol. *Can. Entomol.* 109:675:686.

MOECK, H. A. 1970. Ethanol as the primary attractant for the ambrosia beetle *Trypodendron lineatum* (Coleoptera: Scolytidae). *Can. Entomol.* 102:985–995.

MOECK, H. 1971. Field test of ethanol as a scolytid attractant. *Can. Dep. Fish For. Bi-Mon. Res. Notes* 27:11–12.

MONTGOMERY, M. E., and WARGO, P. M. 1983. Ethanol and other host-derived volatiles as attractants to beetles that bore into hardwoods. *J. Chem. Ecol.* 9:181–190.

NIJHOLT, W. W. 1973. Ambrosia beetle attack delayed by turpentine oil. *Environ. Can. For. Serv. Bi-Mon. Res. Notes* 29:36.

NIJHOLT, W. W. 1980. Pine oil and oleic acid delay and reduce attacks on logs by ambrosia beetles (Coleoptera: Scolytidae). *Can. Entomol.* 112:199–204.

NIJHOLT, W. W., and SHÖNHERR, J. 1976. Chemical response behavior of scolytids in West Germany and western Canada. *Environ. Can. For. Serv. Bi-Mon. Res. Notes* 32:31–32.

PREBBLE, M. L., and GRAHAM, K. 1957. Studies of attack by ambrosia beetles in softwood logs on Vancouver Island, British Columbia. *For. Sci.* 3:90–112.

PULLIAINEN, E. 1983. Infestation by *Trypodendron lineatum* (Oliv.) (Coleoptera, Scolytidae) of spruce and pine felled in different years and months near the subarctic timberline. *Entomol. Fenn.* 49:45–48.

SALOM, S. M., and MCLEAN, J. A. 1988. Semiochemicals for capturing the ambrosia beetle, *Trypodendron lineatum*, in multiple-funnel traps in British Columbia. *J. Entomol. Soc. B. C.* 85:34–39.

SALOM, S. M., and MCLEAN, J. A. 1990. Flight and landing behavior of *Trypodendron lineatum* (Coleoptera: Scolytidae) in response to different semiochemicals. *J. Chem. Ecol.* 16:2589–2604.

SAS INSTITUTE INC. 1989. SAS/STAT User's Guide, Volume 2, Version 6, 4th ed. SAS Institute, Cary, North Carolina.

SCHROEDER, L. M. 1988. Attraction of the bark beetle *Tomicus piniperda* and some other bark- and wood-living beetles to the host volatiles α-pinene and ethanol. *Entomol. Exp. Appl.* 46:203–210.

SCHROEDER, L. M., and LINDELOW, Å. 1989. Attraction of scolytids and associated beetles by different absolute amounts and proportions of α-pinene and ethanol. *J. Chem. Ecol.* 15:807–817.

SHORE, T. L., and MCLEAN, J. A. 1983. A further evaluation of the interactions between the pheromones and two host kairomones of the ambrosia beetles *Trypodendron lineatum* and *Gnathotrichus sulcatus* (Coleoptera: Scolytidae). *Can. Entomol.* 115:1–5.

SHORE, T. L., and MCLEAN, J. A. 1985. A survey for the ambrosia beetles *Trypodendron lineatum* and *Gnathotrichus retusus* (Coleoptera: Scolytidae) in a sawmill using pheromone-baited traps. *Can. Entomol.* 117:49–55.

SJÖDIN, K., SCHROEDER, L. M., EIDMANN, H. H., NORIN, T., and WOLD, S. 1989. Attack rates of scolytids and composition of volatile wood constituents in healthy and mechanically weakened pine trees. *Scand. J. For. Res.* 4:379–391.

SNAJBERK, K., and ZAVARIN, E. 1976. Mono- and sesqui-terpenoid differentiation of *Pseudotsuga* of the United States and Canada. *Biochem. Syst. Ecol.* 4:159–163.

SNAJBERK, K., LEE, C. J., and ZAVARIN, E. 1974. Chemical composition of volatiles from cortical oleoresin of *Pseudotsuga menziesii*. *Phytochemistry* 13:185–188.

STEEL, R. G. D., and TORRIE, J. H. 1980. Principles and Procedures of Statistics, 2nd Ed. McGraw-Hill, New York.

VITÉ, J. P., and BAKKE, A. 1979. Synergism between chemical and physical stimuli in host colonization by an ambrosia beetle. *Naturwissenschaften* 66:528–529.

ZHONG, H., and SCHOWALTER, T. D. 1989. Conifer bole utilization by wood-boring beetles in western Oregon. *Can. J. For. Res.* 19:943–947.

Journal of Chemical Ecology, Vol. 23, No. 4, 1997

CUTICULAR HYDROCARBONS AS CHEMOTAXONOMIC CHARACTERS OF PINE ENGRAVER BEETLES (*Ips* spp.) IN THE *grandicollis* SUBGENERIC GROUP

MARION PAGE,[1,*] LORI J. NELSON,[1] GARY J. BLOMQUIST,[2] and STEVEN J. SEYBOLD[2]

[1]*Pacific Southwest Research Station*
USDA Forest Service
Albany, California 94710

[2]*Department of Biochemistry*
University of Nevada
Reno, Nevada 89557-0014

(Received April 15, 1996; accepted November 29, 1996)

Abstract—Cuticular hydrocarbons were extracted, identified, and evaluated as chemotaxonomic characters from all species of adult *Ips* pine engraver beetles in the *grandicollis* subgeneric group. The *grandicollis* group consists of *Ips grandicollis* (Eichhoff), *I. cribricollis* (Eichhoff), *I. lecontei* Swaine, *I. montanus* (Eichhoff), *I. paraconfusus* Lanier, *I. confusus* (LeConte), and *I. hoppingi* Lanier. In order to provide outgroups for a phylogenetic analysis, cuticular hydrocarbons were also analyzed from *Orthotomicus caelatus* (Eichhoff), *I. latidens* (LeConte) (*latidens* subgeneric group), and *I. pini* (Say) (*pini* subgeneric group). Two hundred forty-eight hydrocarbon components were identified by gas chromatography–mass spectrometry. The members of the *grandicollis* group provided 206 of these compounds. The components represented eight classes: *n*-alkanes, alkenes, alkadienes, terminally branched methylalkanes, internally branched methylalkanes, dimethylalkanes, trimethylalkanes, and tetramethylalkanes. Different populations of *O. caelatus*, *I. grandicollis*, *I. lecontei*, *I. montanus*, *I. paraconfusus*, *I. confusus*, and *I. hoppingi* provided no evidence for interpopulational variation in cuticular hydrocarbons. Single populations only were analyzed for *I. latidens*, *I. pini*, and *I. cribricollis*. Sexual dimorphism in cuticular hydrocarbons occurred only in *I. lecontei* where females produced eight unique components with a pentatriacontane parent chain. Several phylogenetic analyses based on hydrocarbon phenotypes agreed in general with the established morphologically based system of relatedness and with published phylogenies reconstructed from pro-

*To whom correspondence should be addressed.

0098-0331/97/0400-1053$12.50/0 © 1997 Plenum Publishing Corporation

tein and nucleic acid characters. Nearly all hydrocarbon analyses suggested a close relationship between *I. grandicollis* and *I. cribricollis*; between *I. lecontei* and *I. montanus*; and among the sibling species *I. paraconfusus*, *I. confusus*, and *I. hoppingi*. The presence or absence of specific *n*-alkanes (*n*-docosane, *n*-triacontane); certain dimethylalkanes (terminally branched with octacosane and triacontane parent chains and internally branched with heptacosane, hentriacontane, and docotriacontane parent chains); and 3,7,11-; 3,7,15-trimethylheptacosane permit facile discrimination of *I. paraconfusus*, *I. confusus*, and *I. hoppingi*. These three sibling species are difficult to resolve by external morphology. These data support the species status of *I. hoppingi* rather than it being considered a host race of the *I. confusus* complex. They also support the species status of *I. cribricollis* rather than it being considered part of *I. grandicollis*. In contrast to other published phylogenies reconstructed from molecular data, phylogenies reconstructed from cuticular hydrocarbons repeatedly place *I. lecontei* as an integral part of the *grandicollis* subgeneric group. Thus, cuticular hydrocarbon and pheromone alcohol composition of *I. lecontei* support its inclusion in the *grandicollis* subgeneric group.

Key Words—Scolytidae, *Ips*, *Orthotomicus*, *Pinus*, evolution, cuticular hydrocarbons, chemotaxonomy, methyl-branched hydrocarbons, mass spectrometry.

INTRODUCTION

Species identification is fundamental for understanding the biology and ecology of insect populations and their impacts on resources. In particular, forest insect populations are often comprised of sibling species or species complexes with uncertain identities and subtle ecological differences (Bright and Stock, 1982; Harvey, 1985; Powell, 1995; Powell and DeBenedictis, 1995a,b). Failure to separate these entities and to recognize their different biological adaptations can create serious problems in managing pestiferous forest insects and sustaining healthy forests.

Classical taxonomic methods based on differences in morphological character sets and biosystematic studies that confer biological species status have been and continue to be powerful tools for revealing cryptic species. The phenotype of an organism, however, also includes a wealth of chemical character sets. They range from nucleic acid and protein sequences for information storage, catalysis, and structure, to isomeric blends of lipids as communicative exocrine compounds or antidesiccant surface coatings. These character sets provide additional information for unraveling complex taxonomic relationships [e.g. see Bright (1993) for a discussion of the status of molecular taxonomy in the Scolytidae, a significant family of forest insects]. In particular, chemical analysis of cuticular hydrocarbons offers a nondestructive and reliable chemotaxonomic method (e.g., Carlson and Service, 1980; Carlson and Bolten, 1984; Carlson

and Brenner, 1988; Castner and Nation, 1986; Clement et al., 1985; 1987; Haverty et al., 1988, 1989, 1990a,b, 1991a,b; Howard, 1993; Howard et al., 1978, 1982; Jacob, 1977, 1978, 1979; Lockey, 1979, 1981, 1984a,b, 1991b, 1992; Page et al., 1990a,b; de Renobales et al., 1991; Ross et al., 1987; Vander Meer, 1986).

The pine and spruce engraver beetles in the genus *Ips* are economically important forest insects that are suspected to alter forest stand structure and species diversity (Furniss and Carolin, 1977). Hopping (1963) divided *Ips* DeGeer into 10 subgeneric groups (I–X) of North American species. After Wood (1966, 1968) transferred *Ips latidens* (LeConte) and *I. sabinianae* [now *spinifer* (Eichhoff)] from *Orthotomicus* Ferrari to *Ips*, Lanier and Cameron (1969) designated an eleventh subgeneric group (0) for these two species. The only North American species of *Orthotomicus* currently recognized is *O. caelatus* (Eichhoff) (Wood, 1982). One of the largest and perhaps most economically important group of *Ips*, IX (Hopping, 1965), contained *I. grandicollis* (Eichhoff), *I. confusus* (LeConte), *I. montanus* (Eichhoff), *I. cribricollis* (Eichhoff), and *I. lecontei* Swaine. Based on controlled matings, karyology, and morphometric analyses, Lanier (1970) split *I. confusus* into *I. paraconfusus* Lanier, *I. hoppingi* Lanier, and *I. confusus* (LeConte). Wood (1982) designated this group the *grandicollis* subgeneric group and recognized six species: *I. grandicollis*, *I. lecontei*, *I. montanus*, *I. paraconfusus*, *I. confusus*, and *I. hoppingi*. Lanier (1987) provided strong evidence for the validity of *I. cribricollis*, restoring it to the *grandicollis* group. This group, a Nearctic endemic, currently contains the most *Ips* species in North America, although the *perturbatus* and *plastographus* groups have more and equal numbers, respectively, if the Palaearctic fauna are included (Wood, 1982).

A robust phylogeny for a group allows comparative ecological, behavioral, and biogeographic studies to be carried out (Brooks and McLennan, 1991). Cane et al. (1990b) proposed a hypothetical phylogeny of seven species of *Ips* based on the migration of selected metabolic enzymes in an electrophoretic gel. Their analysis included five of the seven members of the *grandicollis* group; *I. montanus* and *I. cribricollis* were not studied. Cognato et al. (1995) have also reconstructed a phylogeny of the complete *grandicollis* group based on the migration of random amplified polymorphic DNA (RAPD) fragments in an electrophoretic gel.

In this paper we report mass spectral characterization of the surface hydrocarbons from populations of the seven species of the *grandicollis* group and three outgroup species, *O. caelatus*, *I. latidens*, and *I. pini* (Say). The presence, absence, and relative quantities of these cuticular hydrocarbons represent characters for the surface chemical phenotype of these organisms, and we use these characters diagnostically to supplement the currently accepted morphological discrimination of the species (Lanier, 1987; Wood, 1982).

METHODS AND MATERIALS

Insects. Adults of the seven species in the *grandicollis* subgeneric group and related taxa were reared from infested host material (Browne, 1972) or obtained from cooperators. Prior to hydrocarbon extraction, live specimens were refrigerated (5°C) while dead specimens were frozen (−20°C) or held at ambient temperatures in vials. After extraction of surface lipids, specimens were dried, pinned, and deposited as vouchers in the California Academy of Sciences Entomology Collection, San Francisco, California; the Canadian National Collection, Ottawa, Canada; and the Combined United States Forest Service Collection, Oregon State University, Corvallis, Oregon. Collection source and host associations for each population examined are summarized in Table 1.

Hydrocarbon Analysis. Cuticular hydrocarbons were extracted with hexane from groups of 50 and 100 individuals, separated from other lipid components, and analyzed by gas chromatography–mass spectrometry (GC-MS) as described for other scolytids (Page et al., 1990a,b). We experimented with various sample sizes (1–200 individuals) of *Ips* spp. Based on these experiments, we determined that samples of 100 individuals allowed for maxium detectability of hydrocarbons, while samples of 50 individuals optimized resolution of peaks. Integration of the total ion chromatogram was performed using the HP Chemstation software (HP59974J Rev. 3.1.2). GC-MS peak areas were converted to percentages of the total hydrocarbon fraction. Summary statistics for percentages of each hydrocarbon for each taxon or geographical location of a taxon were computed using SAS (1990).

We use a shorthand nomenclature to describe hydrocarbons. This nomenclature uses a descriptor (XX) for the total number of carbons in the hydrocarbon component. The number of double bonds (Y) follows a colon ($C_{XX:Y}$), and the location of methyl groups uses the descriptor (X-Me). Thus heptacosane becomes *n*-C_{27}; heptacosene becomes $C_{27:1}$; 5-methylnonacosane becomes 5-MeC_{29}; 11,15-dimethylnonacosane becomes 11,15-DimeC_{29}; 3,7,15-trimethylhentriacontane becomes 3,7,15-TrimeC_{31}; and 3,7,11,15-tetramethylhentriacontane becomes 3,7,11,15-TetrameC_{31}. Hydrocarbons are presented by class in Table 2 for each taxon in the order of elution on our GC-MS system.

n-Alkanes, alkenes, alkadienes, and methyl-branched alkanes were identified by their mass spectral fragmentation patterns (Blomquist et al., 1987b; Nelson, 1993) and their relative elution times. Alkenes and alkadienes were separated from alkanes by liquid chromatography as described in Howard et al. (1988) except that the column packing was 20% $AgNO_3$/Bio-Sil A silica gel (100–200 mesh, Bio-Rad Laboratories, Richmond, California) and the hexane–diethylether gradient involved 3.5%, 5%, and 30% diethylether. Double-bond positions in mono- (5% diethylether fractions) and diunsaturated (30% diethylether fractions) compounds were determined by preparing dimethyl disul-

TABLE 1. SOURCE OF *Orthotomicus caelatus* AND *Ips* SPECIES FOR HYDROCARBON ANALYSIS OF *grandicollis* SUBGENERIC GROUP

Species	Collection site	Host (*Pinus* spp.)	Collection date[a]
O. caelatus (Eichhoff)	a. Kellogg Forest, Augusta, Kalamazoo County, Michigan	*P. strobus* L.	VI-93
	b. Morgan Hill State Forest, Onondaga County, New York	*P. resinosa* Ait.	VI-4-94
	c. Morgan Hill State Forest, Onondaga County, New York	*P. resinosa* Ait.	VIII-14-94
I. latidens (LeConte)	Meadow Valley, Plumas County, California	*P. ponderosa* Laws.	VI-7-89
I. pini (Say)	Ward Spring, 17 km E. Hat Creek, Shasta County, California, S28&29, R6E, T34N	*P. jeffreyi* Grev. & Balf.	V-16-92
I. grandicollis (Eichhoff)	a. Arena, Sauk County, Wisconsin	*P. strobus* L.	XII-23-88
	b. 15 km SW Auburn, Lee County, Alabama	*P. taeda* L.	I-21-89
	c. Durham County, North Carolina	*P. taeda* L.	VII-10-89
	d. 4.8 km S. Pollock, Grant Parish, Louisiana	Pheromone trapped	VI and VII-93
I. cribricollis (Eichhoff)	16 km E. Cloudcroft, Hwy 82, Otero County, New Mexico	*P. ponderosa* Laws.	VIII-18-90
I. lecontei Swaine	a. 3.2 km S. Mingus Mtn., Prescott National Forest, Yavapai County, Arizona	*P. ponderosa* Laws.	III-6-88
	b. Point of Pines, S11, R25E, T1S, Graham County, Arizona	*P. ponderosa* Laws.	VI-10-93
I. montanus (Eichhoff)	a. 6.5 km E. Benton Meadow, Warner Mtns., Modoc County, California	*P. monticola* Dougl.	IX-4-88
	b. 1.6 km N. Benton Meadow, Warner Mtns., Modoc County, California	*P. monticola* Dougl.	VII-25-94
I. paraconfusus Lanier	a. University of California Blodgett Forest, El Dorado County, California	*P. lambertiana* Dougl.	XII-8-88
	b. Shake Creek, S18, R2W, T2N, San Bernardino County, California	*P. coulteri* D. Don	IX-13-88

TABLE 1. Continued

Species	Collection site	Host (*Pinus* spp.)	Collection date[a]
I. paraconfusus Lanier Continued	c. 0.2 km SE Bon Tempe Reservoir, Marin County, California	*P. radiata* D. Don	XII-9-88
	d. 3869 Greenwood Ave., Contra Costa County, California	*Picea abies* (L.) Karst.	II-22-90
	e. Orinda, Contra Costa County, California	*P. radiata* D. Don	II-93
	f. University of California Gill Tract, Albany, Alameda County, California	*P. radiata* D. Don	II-3-94
I. confusus (LeConte)	a. San Bernardino Mtns., San Bernardino County, California	*P. monophylla* Torr. & Fr.	VI-83
	b. Baldwin Lake, S31, R2E, T3N, San Bernardino County, California	*P. monophylla* Torr. & Fr.	IX-12-88
	c. 3.1 km SE Carter Springs, Hwy 395, S15, R21E, T11N Douglas County, Nevada	*P. monophylla* Torr. & Fr.	II-23-94
I. hoppingi Lanier	a. Chiricahua Mtns., S35, R30E, T17S, Cochise County, Arizona	*P. discolor* Bailey & Hawksworth	VIII-87
	b. Rt. 118, NE McDonald Observatory, Davis County, Texas	*P. cembroides* Zucc.	VIII-16-87
	c. McDonald Observatory, Mt. Locke, Davis Mtns., Davis County, Texas	*P. cembroides* Zucc.	VIII-15-90
	d. Las Crucitas, Mpio. Galeana, 2470 m, Nuevo Leon, Mexico	*P. cembroides* Zucc.	X-25-93

[a]Dates are given as month (Roman numbered), day, and year.

fide (DMDS) derivatives. The resulting linear dithiomethylethers and sulfur heterocycles were analyzed by GC-MS and interpreted as described by Francis and Veland (1981), Howard et al. (1988), Howard (1993), Takano et al. (1989), and Vicenti et al. (1987). Monounsaturated alkenes (e.g., $C_{25:1}$, $C_{27:1}$, and $C_{29:1}$) with double bonds at carbons 7 or 9 were interpreted when DMDS treatment resulted in thiomethylether addition to carbons 7 and 8 or 9 and 10, respectively. For example, addition of thiomethylether to carbons 7 and 8 of heptacosene

TABLE 2. RELATIVE QUANTITIES OF CUTICULAR HYDROCARBONS IDENTIFIED FROM *Ips* SPECIES IN *grandicollis* SUBGENERIC GROUP[a]

Hydrocarbon[b]	Species						
	grandicollis	*cribricollis*	*montanus*	*paraconfusus*	*confusus*	*hoppingi*	*lecontei*
1. C_{22}	0	tr	0	0	+	0	0
2. C_{23}	tr	++	tr	tr	+	tr	tr
3. C_{24}	tr	+	tr	tr	+	tr	tr
4. C_{25}	+++	+++	+++	+++	+++	+++	+++
5. C_{26}	++	++	++	++	++	++	++
6. C_{27}	+++	+++	+++	+++	+++	+++	+++
7. C_{28}	tr	++	tr	+	+	++	+
8. C_{29}	+++	+++	+	++	++	+++	+
9. C_{30}	+	+	+	++	++	++	+
10. C_{31}	++	0	+	+	++	0	+
11. C_{33}	0	0	+	tr	tr	0	tr
12. C_{35}	0	0	0	0	0	0	+[c]
13. $C_{25:1}$ + 3-MeC_{24}	++[d]	tr	tr[d]	++[d]	+[d]	++[d]	+[d]
14. $C_{25:1}$	tr[e]	tr	tr[e]	tr[e]	tr[e]	tr	tr
15. $C_{26:1}$	+	++	0	+[f]	++[f]	++[f]	0
16. $C_{27:1}$	++[d]	++[d] 3-MeC_{26}	++[d] 3-MeC_{26}	++[d] 3-MeC_{26}	3-meC25 ++[d] 3-MeC_{26}	3-meC25 ++[d] 3-MeC_{26}	++[d] 3-MeC_{26}
17. $C_{27:1}$	+[e]	tr	tr[e]	tr[e]	tr[e]	tr[e]	tr[e]
18. $C_{28:1}$	tr	tr	tr	tr	tr	tr	tr
19. $C_{29:1}$ + 3-MeC_{28}	+[d]	+	++[d]	+[d]	+[d]	+[d]	+[d]
20. $C_{29:1}$	+++[e]	tr 6,18-DimeC_{28}	+[e] 6,18-DimeC_{28}	++[e] 6,18-DimeC_{28}	+[e] 6,18-DimeC_{28}	tr[e] 6,18-DimeC_{28}	+[e]
21. $C_{30:1}$	tr	tr	tr[f]	tr	tr	tr	tr

TABLE 2. Continued

Hydrocarbon[b]	Species						
	grandicollis	*cribricollis*	*montanus*	*paraconfusus*	*confusus*	*hoppingi*	*lecontei*
22. $C_{31\ 1}$	++[d]	+	++[d] 3-MeC_{30}	tr[d]	tr[d] 3-MeC_{30}	+[d]	+[d]
23. $C_{31\ 1}$	tr 5,17-$DimeC_{30}$	0	+[e] 5,17-$DimeC_{30}$	tr	tr[e] 5,17-$DimeC_{30}$	0	tr
24. $C_{33\ 1}$	0	0	tr[g]	tr	0	0	tr[d]
25. $C_{35\ 1}$	0	0	tr	0	0	0	0
26. $C_{25,2}$	tr	0	0	0	0	0	0
27. (6Z,9Z)$C_{27\ 2}$	+++	+++ 2-MeC_{26}	+++ 2-MeC_{26}	+++ 2-MeC_{26}	+++ 2-MeC_{26}	+++ 2-MeC_{26}	+++ 2-MeC_{26}
28. $C_{29\ 2}$	tr	tr	+ 2- or 4-MeC_{28}	+ 2- or 4-MeC_{28} 11,15-; 12,16-$DimeC_{28}$	+ 2- or 4-MeC_{28}	tr	0
29. $C_{31\ 2}$	0	0	tr	0	0	0	0
30. $C_{31\ 2}$	0	0	tr	0	0	0	0
31. $C_{33\ 2}$	0	0	tr	0	0	0	0
32. 9-; 11-; 13-MeC_{25}	0	tr	0	0	tr	0	+
33. 11-; 12-; 13-MeC_{26}	0	0	0	tr	0	0	tr
34. 9-; 11-; 13-MeC_{27}	0	+	+	++	tr	tr	++
35. 10-; 11-;12; 13-; 14-MeC_{28}	0	0	tr	++	tr	0	+
36. 9-; 11-; 13-; 15-MeC_{29}	++	tr	++	++	++	+	++
37. 10-; 11-; 12-; 13-; 14-MeC_{30}	tr	0	tr	+	tr	tr	tr
38. 9-; 11-; 13-; 15-MeC_{31}	++	+	+	++	+	tr	tr

39. 10-; 11-; 12-; 13-; 15-; 16-MeC_{32}	0	0	tr	0	0	0	0
40. 9-; 11-; 13-; 15-; 17-MeC_{33}	0	tr	tr	0	0	0	+
41. 11-; 13-; 15-; 17-MeC_{35}	0	0	tr	0	0	0	+[c]
42. 7-MeC_{25}	0	0	0	0	tr	0	0
43. 7-MeC_{26}	0	0	0	0	0	0	tr
44. 7-MeC_{27}	tr	tr	tr	+	++	tr	++
45. 7-MeC_{28}	tr	0	0	0	+	tr	0
46. 7-MeC_{29}	+++	tr	++	++	++	++	tr
47. 7-MeC_{31}	+	+	tr	tr	tr	tr	tr
48. 7-MeC_{33}	0	0	tr	0	0	0	0
49. 7-MeC_{35}	0	0	+	0	0	0	0
50. 5-MeC_{25}	tr	0	0	tr	+	tr	tr
51. 5-MeC_{26} (*I. pini*)	0	0	0	0	0	0	0
52. 5-MeC_{27}	+	+	++	+++	++	++	++
53. 5-MeC_{28}	0	0	0	tr	+	tr	0
54. 5-MeC_{29}	++	tr	++	+	++	++	++
55. 5-MeC_{31}	++	0	tr	tr	tr	tr	tr
56. 5-MeC_{33}	0	0	tr	0	0	0	0
57. 6-MeC_{26}	0	0	0	0	tr	0	tr
58. 6-MeC_{28}	+	0	tr	tr	+	tr	tr
59. 6-MeC_{30}	tr	0	0	0	0	0	0
60. 6-MeC_{24} + $C_{25:1}$	tr	tr	tr	tr	tr	tr	tr
61. 3-MeC_{25}	+	++	++	++	++ $C_{26:1}$	++ $C_{26:1}$	+
62. 3-MeC_{26} + $C_{27:1}$	0	++	++	++	++	++	++
63. 3-MeC_{27}	tr	++	+++	++	+	+	+++
64. 3-MeC_{28} + $C_{29:1}$	+++	++	++	+	++	++	tr
65. 3-MeC_{29}	+++	++	+++	++	++	++	++
			7,19-$DimeC_{29}$	7,19-$DimeC_{29}$	7,19-$DimeC_{29}$	7,19-$DimeC_{29}$	7,15-$DimeC_{29}$

TABLE 2. Continued

Hydrocarbon[b]	Species						
	grandicollis	*cribricollis*	*montanus*	*paraconfusus*	*confusus*	*hoppingi*	*lecontei*
66. 3-MeC$_{30}$	0	0	tr	0	tr	0	0
			31:1		31:1		
67. 3-MeC$_{31}$	+ +	+	+ +	+	+	+	tr
	7,19-DimeC$_{31}$		7,19-DimeC$_{31}$	7,19-DimeC$_{31}$	7,19-DimeC$_{31}$	7,19-DimeC$_{31}$	7,19-DimeC$_{31}$
68. 3-MeC$_{33}$	0	0	tr	0	0	0	0
69. 2-MeC$_{26}$ + C$_{27:2}$	0	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
70. 2- or 4-MeC$_{28}$	0	0	+	+	tr	tr	+ + +
			C$_{29:2}$	C$_{29:2}$	C$_{29:2}$		
71. 4-MeC$_{30}$	tr	0	tr	0	0	0	0
72. 9,13-; 11,15-; 13,17-DimeC$_{25}$	0	0	0	0	0	0	+
73. 9,13-; 11,15-; 13,17-DimeC$_{27}$	0	0	+	+ +	0	0	+
74. 11,15-12,16-DimeC$_{28}$	0	0	0	tr	0	0	0
				C$_{29:2}$			
75. 9,13-; 11,15-; 13,17-DimeC$_{29}$	0	tr	tr	+ +	tr	tr	+
76. 11,15-; 12,16-DimeC$_{30}$	0	0	0	tr[h]	0	0	0
77. 9,13-; 11,15-; 13,17-DimeC$_{31}$	0	0	tr	+	0	0	tr
78. 9,19-; 9,21-; 11,19-DimeC$_{31}$ (*O. caelatus*, *I. pini*)	0	0	0	0	0	0	0

79. 11,17-; 12,16-; 13,17-DimeC_{32} (*O. caelatus*, *I. latidens*)	0	0	0	0	0	0	0
80. 9,13-; 11,15-; 11,21-; 13,17-DimeC_{33}	0	0	tr (13,17-)[f]	tr (9,13-)[f]	tr (9,13-)[f]	tr (9,13-)[f]	tr (9,13-; 11,15-)[f]
81. 9,13-DimeC_{35}	0	0	0	0	0	0	+[e]
82. 11,21-; 11,23-; 13,19-; 15,19-DimeC_{35} (*O. caelatus*)	0	0	0	0	0	0	0
83. 7,11-; 7,15-; 7,17-; 7,19-DimeC_{29}	0	0	+++ 3-MeC_{29}	++ 3-MeC_{29}	++ 3-MeC_{29}	++ 3-MeC_{29}	++ 3-MeC_{29}
84. 7,15-; 7,17-; 7,19-DimeC_{31}	++ 3-MeC_{31}	0	++ 3-MeC_{31}	+ 3-MeC_{31}	+ 3-MeC_{31}	+ 3-MeC_{31}	tr 3-MeC_{31}
85. 7,15-; 7,17-; 7,19-DimeC_{33}	0	0	tr	tr	tr	tr	tr
86. 7,19-DimeC_{35}	0	0	0	0	0	0	tr
87. 5,9-DimeC_{25}	0	0	0	0	0	0	tr
88. 5,15-; 5,17-; 5,19-DimeC_{27}	0	++	++	+++	++	++	++
89. 5,17-DimeC_{28}	0	0	0	tr	0	0	0
90. 5,15-; 5,17-; 5,19-DimeC_{29}	+++	+	++	++	++	+	++
91. 5,17-DimeC_{30}	tr $C_{31:1}$	0	+ $C_{31:1}$	0	tr $C_{31:1}$	0	0
92. 5,17-; 5,19-DimeC_{31}	+++	tr	++	+	++	+	tr
93. 5,17-DimeC_{32}	0	0	0	0	+	0	0
94. 5,17-DimeC_{33}	++	0	+	+	++	+	0
95. 5,13-; 5,17-DimeC_{35}	0	0	tr	0	0	0	tr[d]
96. 6,18-DimeC_{28}	0	tr $C_{29:1}$	++ $C_{29:1}$	++ $C_{29:1}$	+ $C_{29:1}$	tr $C_{29:1}$	0

TABLE 2. Continued

Hydrocarbon[b]	Species						
	grandicollis	*cribricollis*	*montanus*	*paraconfusus*	*confusus*	*hoppingi*	*lecontei*
97. 6,18-DimeC_{30} (*I. pini*)	0	0	0	0	0	0	0
98. 4,14-; 4,16-; 4,18-DimeC_{28}	0	0	0	+	tr	0	+
99. 4,14-; 4,16-; 4,18-DimeC_{30}	+	0	tr	tr	+	tr	0
100. 4,14-; 4,16-; 4,18-DimeC_{32}	0	0	0	tr	tr	tr	0
101. 3,11-; 3,13-; 3,17-DimeC_{25}	0	0	0	tr	0	0	0
102. 3,7-; 3,9-DimeC_{25}	0	0	+ +	+ +	+ +	+ +	+ +
103. 3,11-; 3,13-; 3,17-DimeC_{27}	0	0	+ +	+ +	+ +	+ +	+ +
104. 3,7-; 3,9-DimeC_{27}	0	+ +	+ +	+ +	+ +	+ +	+ +
105. 3,7-DimeC_{28}	0	0	0	tr	tr	0	0
106. 3,11-; 3,13-; 3,15-; 3,17-DimeC_{29}	+ +	0	+ +	+ +	+ +	+ +	+
107. 3,7-DimeC_{29}	+ + + 3,5-DimeC_{29}	+ +	+ + 3,5-DimeC_{29}	+ +	+ +	+ +	+ 3,5-DimeC_{29}
108. 3,5-DimeC_{29}	+ + + 3,7-DimeC_{29}	0	+ + 3,7-DimeC_{29}	0	0	0	+ 3,7-DimeC_{29}
109. 3,11-; 3,15-DimeC_{30}	0	0	0	0	+ +	0	0
110. 3,7-DimeC_{30}	0	0	0	tr	tr	tr	0
111. 3,11-; 3,13-; 3,15-; 3,17-DimeC_{31}	+ +	0	tr	+	+ +	+ +	0

112. 3,7-DimeC_{31}	++	tr	tr	++	++	++	0
113. 3,13-; 3,15-; 3,17-; 3,19-DimeC_{33}	0	0	tr	tr	tr	tr	tr
114. 3,7-DimeC_{33}	0	0	tr	tr	tr	tr	0
115. 3,7,11-; 3,7,13-TrimeC_{25} (*I. latidens*)	0	0	0	0	0	0	0
116. 4,8,12-TrimeC_{26} (*I. latidens*)	0	0	0	0	0	0	0
117. 3,7,11-; 3,7,15-TrimeC_{27}	0	+	tr	+	0	0	+
118. 9,13,17-TrimeC_{27}	0	tr	0	0	0	0	0
119. 4,10,14-; 4,12,16-TrimeC_{28} (*I. latidens*)	0	0	0	0	0	0	0
120. 3,7,11-; 3,7,13-; 3,7,15-; 3,7,19-TrimeC_{29}	++	tr	++	++	++	++	tr
121. 7,11,17-; 7,13,17-TrimeC_{29} (*O. caelatus*)	0	0	0	0	0	0	0
122. 11,15,19-TrimeC_{29} (*I. latidens*, *I. pini*)	0	0	0	0	0	0	0
123. 9,13,17-TrimeC_{29} (*O. caelatus*, *I. latidens*)	0	0	0	0	0	0	0
124. 4,8,12-TrimeC_{30}	0	0	0	tr	0	0	0
125. 3,7,11-; 3,7,15-; 3,7,17-TrimeC_{31}	+	tr	++	+	++	+	0
126. 11,15,19-TrimeC_{31} (*O. caelatus*, *I. latidens*, and *I. pini*)	0	0	0	0	0	0	0

TABLE 2. Continued

Hydrocarbon[b]	Species						
	grandicollis	*cribricollis*	*montanus*	*paraconfusus*	*confusus*	*hoppingi*	*lecontei*
127. 9,13,19-; 9,15,21-; 9,13,21-TrimeC$_{31}$ (*O. caelatus*)	0	0	0	0	0	0	0
128. 7,11,19-TrimeC$_{31}$ (*O. caelatus*)	0	0	0	0	0	0	0
129. 5,11,15-TrimeC$_{31}$ (*O. caelatus*)	0	0	0	0	0	0	0
130. 10,14,18-; 10,16,20-; 12,16,20-TrimeC$_{32}$ (*O. caelatus*)	0	0	0	0	0	0	0
131. 3,7,17-TrimeC$_{33}$	0	0	0	tr	tr	tr	0
132. 3,7,15-; 3,7,21-TrimeC$_{33}$ (*I. pini*)	0	0	0	0	0	0	0
133. 9,17,21-; 11,15,21-; 11,17,21-TrimeC$_{33}$ (*O. caelatus*)	0	0	0	0	0	0	0
134. 7,11,19-TrimeC$_{33}$ (*O. caelatus*)	0	0	0	0	0	0	0
135. 13,17,21-TrimeC$_{33}$ (*O. caelatus*)	0	0	0	0	0	0	0
136. 11,15,23-; 11,17,23-TrimeC$_{35}$ (*O. caelatus*)	0	0	0	0	0	0	0
137. 3,7,11,15-TetrameC$_{27}$ (*I. latidens*)	0	0	0	0	0	0	0

138. 3,7,11,15-; 3,7,13,17-TetrameC$_{29}$ (*I. latidens*)	0	0	0	0	0	0	0
139. 3,7,11,15-TetrameC$_{31}$	0	0	0	tr	0	0	0

[a] tr: trace (<0.5%); +: 0.5–1.0%, ++: 1.0%–5.0%; and +++: >5.0%.
[b] Hydrocarbons that coelute are listed together in the table. If all seven species in the *grandicollis* group had the coelutants, they are listed together in the first column. If coeluting components were not found in all seven species, they were listed in the body of the table in the appropriate columns. The amount given for each entry corresponds to the first hydrocarbon listed in cases of coelution. Hydrocarbons unique to a given outgroup species (*Orthotomicus caelatus*, *Ips latidens*, or *Ips pini*) are followed by the name of the relevant outgroup species in parentheses.
[c] Female *Ips lecontei* only.
[d] Double bond position for this isomer is 9.
[e] Double bond position for this isomer is 7.
[f] Double bond positions for these coeluting isomers are 8 and 9.
[g] Double bond positions for these coeluting isomers are 7 and 9.
[h] 10,14-; 10,18-; 13,17-DimeC$_{30}$ coeluted with 11,15-; 12,16-DimeC$_{30}$ in *O. caelatus*.
[i] Only these isomers were present in these species.

results in mass ion fragments at *m/z* 145 and 327, while addition to carbons 9 and 10 of heptacosene results in mass ion fragments at *m/z* 173 and 299 (Figure 1). After following the above procedure, one abundant diene ($C_{27:2}$) with double bonds at carbons 6 and 9 was interpreted as (6Z,9Z)-$C_{27:2}$ by comparison with DMDS derivatization and analysis of a standard isolated from *Periplaneta americana* (Blomquist et al., 1980b).

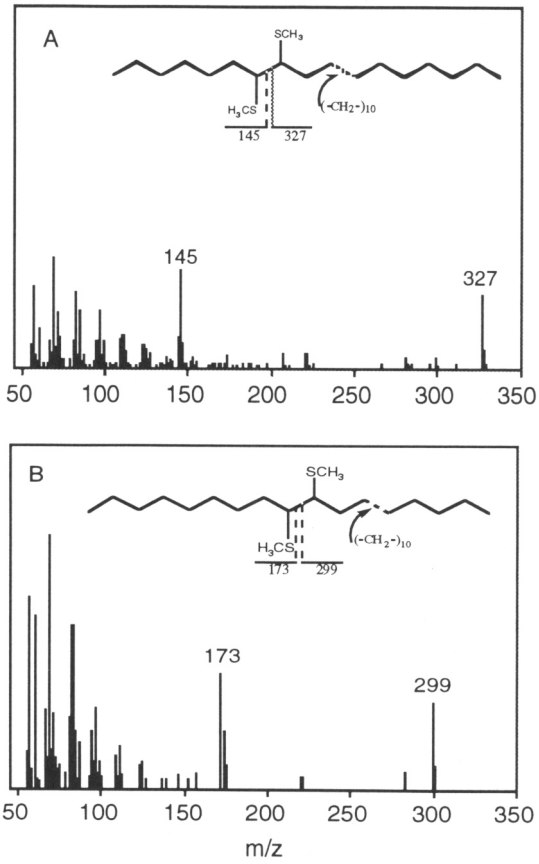


FIG. 1. Mass spectra containing (A) fragmentation ions at *m/z* 145 and 327 arising from thiomethylether addition to carbons 7 and 8 of 7-heptacosene from *I. paraconfusus* and (B) fragmentation ions at *m/z* 173 and 299 arising from thiomethylether addition to carbons 9 and 10 of 9-heptacosene from *I. paraconfusus*. The 50 females used to generate the sample for each analysis were collected from *P. radiata* in Albany, Alameda County, California. For purposes of illustration, the stereochemistry of heptacosene is indicated as *trans* in the figure; however, we did not determine the stereochemistry of this alkene.

Monomethylalkanes with a branch on carbon 9–17 have strong *m/z* ion pairs, indicating cleavage internal to the methyl branch. These compounds all elute approximately 0.7 carbon units before the *n*-alkane with the same number of total carbons (= corresponding *n*-alkane). The signal intensities for the odd and even fragments in the *m/z* ion couplets for each of the daughter ions from these monomethylalkanes are nearly equivalent, indicative of a single methyl branch remaining on the daughter ion following a cleavage (Blomquist et al., 1987b; Nelson, 1993). For example, 9-methylalkanes and 10-methylalkanes have strong 140/141 and 154/155 ion pairs, respectively. Mass spectra with ion couplets at *m/z* 112/113 and elution order approximately 0.6 carbon units before the corresponding *n*-alkane indicate a single methyl branch at carbon 7. 6-Methylalkanes elute immediately after the 7-methylalkanes and give an ion couplet at *m/z* 98/99. 5-Methylalkanes elute approximately 0.5 carbon units before the corresponding *n*-alkane with an ion pair at *m/z* 84/85. 2- and 4-Methylalkanes elute approximately 0.4 carbon units before the corresponding *n*-alkane and both display a strong $(M\text{-}43)^+$ ion. The 4-methylalkanes display an ion pair at *m/z* 70/71, although the intensity may vary. Finally, 3-methylalkanes elute approximately 0.3 carbon units before the corresponding *n*-alkane and give spectra with a strong $(M\text{-}29)^+$ ion and a weak $(M\text{-}57)^+$ ion.

Elution orders and mass spectra of dimethylalkanes were interpreted as described in Page et al. (1990a). In general, 11,15- and 13,17-dimethylalkanes coelute. The 7,17-; 7,15-; and 7,19-isomers coelute, and they elute after 11,15- and 13,17- isomers with the same parent chain. In some cases (e.g., C_{29}, C_{30}, and C_{31}), these 7,X-isomers coelute with 3-methylalkanes of the same parent chain. The 5,X-dimethylalkanes elute immediately after the 3-methylalkanes of the same parent chain. Finally, the 3,X-dimethylalkanes elute with or immediately after the *n*-alkane with one more carbon than the parent chain of the dimethylalkane.

Trimethylalkanes with branches at 3, 7 and 13, 15, or 17 were interpreted as described by Blomquist et al. (1987b). These isomers elute approximately 1.75 carbon units before the corresponding *n*-alkane and yield an $(M\text{-}29)^+$ ion, suggesting trimethylalkanes with one terminal branch at carbon 3. For example, the mass spectra of 3,7,15- and 3,7,17-trimethylhentriacontane ($TrimeC_{31}$) yield an $(M\text{-}29)^+$ ion of *m/z* 449. They also have *m/z* couplets at 252/253 and 224/225, respectively, with an abundance ratio for the even and odd ions of approximately 1:1 (Figure 2A). This indicates a cleavage internal to the methyl-branched carbons 15 and 17. In each of the two trimethylalkane isomers, couplets with significant odd-mass ion fragments of *m/z* 127, 253, and 281 arise from a cleavage external to methyl-branched carbons 7, 15, and 17, respectively. The couplets with a significant odd-mass ion fragment of *m/z* 379 arise from a cleavage internal to methyl-branched carbon 7 (Figure 2A). The even-to-odd

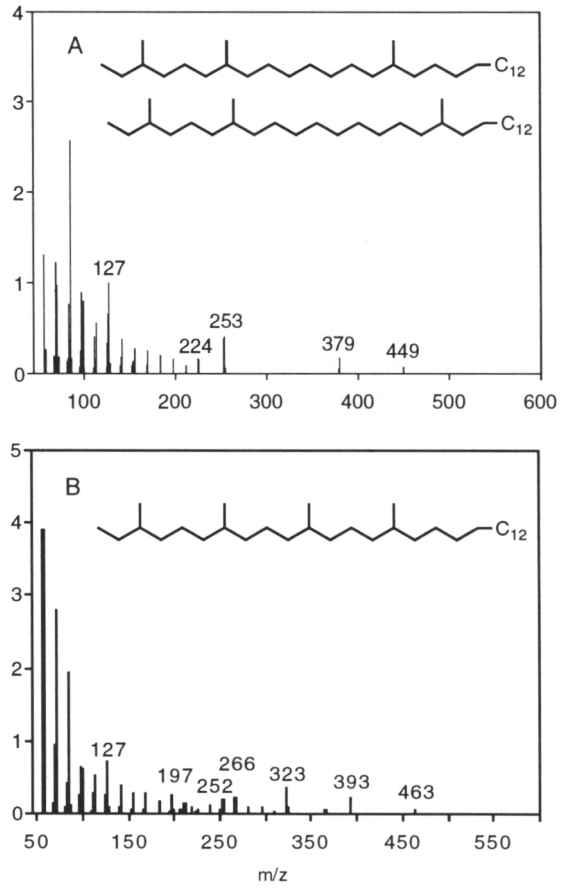


FIG. 2. (A) Mass spectrum of the GC peak containing 3,7,15- and 3,7,17-trimethylhentriacontane from 50 male *I. hoppingi* collected from *P. cembroides* near Las Crucitas, Nuevo Leon, Mexico. (B) Mass spectrum of the GC peak containing 3,7,11,15-tetramethylhentriacontane from 100 male *I. paraconfusus* collected from *P. radiata* in Albany, Alameda County, California.

ratios in these cases strongly favor the odd mass fragments, indicative of daughter fragments with multiple methyl branches.

Tetramethylalkanes were interpreted similarly to trimethylalkanes. For example, 3,7,11,15-tetramethylhentriacontane (TetrameC_{31}) (Figure 2B) elutes approximately 2.4 carbon units before the corresponding *n*-alkane. It also has an $(M\text{-}29)^+$ ion (in this case *m/z* 463) and an *m/z* odd-mass ion fragment at 127, suggesting a tetramethylalkane with one terminal branch at carbon 3 and a second branch at carbon 7. The couplet at 252/253 with an abundance ratio

of approximately 1 : 1 for the even and odd ions indicates a cleavage internal to methyl-branched carbon 15. Couplets with significant odd-mass ion fragments of *m/z* 127, 197, and 267 indicate a cleavage external to the methyl-branched carbons 7, 11, and 15. Couplets with significant odd-mass ion fragments of *m/z* 323 and 393 indicate a cleavage internal to the methyl-branched carbons 11 and 7. All of these fragmentation patterns and abundances suggest daughter fragments with multiple methyl branches.

Phylogenetic Analysis. Analyses of the relatedness among the seven species in the *grandicollis* group were performed using Phylogenetic Analysis Using Parsimony (PAUP) [MAC version 3.1.1 (Swofford, 1993)]. With the small data set of hydrocarbon characters and 7–10 taxa, the exhaustive search algorithm was used to find the most parsimonious tree (MPT) for each analysis. Previous studies of the relatedness of insects based on cuticular hydrocarbon phenotypes have generally employed cluster analyses [e.g. Bartelt et al., 1986; Lockey and Metcalfe, 1988; Golden et al., 1992; reviewed in Lockey, 1991b; Howard, 1993]. To our knowledge, only one other study of insect hydrocarbons has employed a parsimony-based phylogenetic analysis (Kaib et al., 1991). We chose parsimony analysis for two reasons. We hypothesized that discrete lineages exist between the species of *Ips* in the *grandicollis* group, and we anticipated that parsimony analysis would allow us to explore the phylogenetic relationships among these lineages. Parsimony has been shown to be a reasonable method of phylogenetic inference under a broad range of conditions (Mishler, 1994). Secondly, we hypothesized that cuticular hydrocarbons were homologous characters among the taxa in this group and that these compounds also represent independent characters with discrete states. It is conceivable that some of these compounds are biosynthetically related, but for the purposes of our analysis, we regarded them as independent characters.

Characters used in our analysis consisted of 139 hydrocarbon components coded as not detected (0) or present in the following relative quantities: $<0.5\%$ (1); 0.5–1.0% (2); 1.0–5.0% (3); or $>5.0\%$ (4). Thus, coeluting compounds were considered one character for the purposes of this analysis. In preliminary analyses we coded the hydrocarbon data as binomial [not detected (0) or present (1)]. Under these conditions, each analysis generated more than one MPT, and the taxa were poorly resolved in consensus trees. These pilot studies suggested that the loss of quantitative information through binomial coding may have impacted the resolving power of the analysis.

As it has been employed with *Odontotermes* spp. termites (Kaib et al., 1991), coding the hydrocarbons as discrete, relative quantitative characters appears to be an appropriate strategy for evaluating relationships of these *Ips* spp. We recognize that applying these four discrete character states to continuous, relative quantitative characters may not reflect natural character states of cuticular hydrocarbons; however, the natural character states are presently

unknown. Furthermore, our approach did not utilize character weighting because little is known about ancestral states of cuticular hydrocarbons among beetles or insects in general.

We selected *O. caelatus*, a species in a closely related genus, *I. latidens*, and *I. pini* as outgroup species. We hypothesized that they represented true outgroup species, not highly bizarre forms of ingroup species. Morphologically, the genus *Orthotomicus* intergrades with *Ips* (Wood, 1982). Furthermore, *I. latidens* has general morphological features of *Orthotomicus* (Wood, 1982). Thus, based on the known morphological relationships, we hypothesized that *O. caelatus* and *I. latidens* would represent possible outgroups. *Ips pini* was included as an outgroup and paired with *I. latidens* for comparison with the phylogenetic study of Cane et al. (1990b), which also used populations of both species from western North America as outgroups. Cognato et al. (1995) also used *I. latidens* from western North America and *I. pini* from eastern North America as outgroups for their phylogenetic study. Populations of *I. pini* from eastern and western North America have different pheromone biologies (Seybold et al., 1995) and might not be entirely equivalent as outgroups for molecular analyses of phylogeny. Wood (1982) suggests an American origin of *Ips*. All three outgroup species have closely related Palearctic counterparts, while the *grandicollis* group is entirely Nearctic with no Palearctic counterparts (Wood, 1982). Thus, it seems plausible that the *grandicollis* group may be more recently evolved than our outgroup species.

PAUP analyses were performed with each outgroup individually, with all binary combinations and with the ternary combination. An analysis was also performed only with the ingroup species. The outgroup species were included in all possible binary combinations and in the ternary combination to test the integrity of the ingroup. In these cases, all non-*grandicollis* group taxa were desginated as outgroups for the analysis. Thus, analyses were performed with 7–10 taxa, depending on the combination. Outcomes of each analysis were summarized as the single MPT or as a strict consensus tree from multiple trees of equivalent parsimony. Support for each cladogram was assessed by computing decay indices (Mishler et al., 1991). This method evaluates the relative support for clades by relaxing parsimony one step at a time and calculating strict consensus trees of all trees one step longer than the MPT, then two steps longer, etc. The decay index for a clade is the number of steps that can be added before a clade loses its support. Thickness of branches in cladograms indicates the degree of decay value support (Mishler, 1994; Mishler et al., 1994).

RESULTS

Hydrocarbon Analysis. Two hundred forty-eight hydrocarbon components were identified by GC-MS from the surface lipids of adult *Ips* species in the

grandicollis group and from *O. caelatus*, *I. latidens*, and *I. pini* (Table 2). No individual species contained all 248 components. Because some compounds had identical retention times under our chromatographic conditions, the 248 hydrocarbons were represented by 139 peaks (Table 2, Figures 3 and 4). The classes of hydrocarbons characterized were *n*-alkanes (12 components), alkenes (16 components), alkadienes (6 components), and methyl-branched alkanes (214 components) (Table 2).

The outgroup species provided 42 hydrocarbon components that did not occur in the cuticular lipids of members of the *grandicollis* group. Thus, the seven members of the group provided 206 different compounds for the analysis. Nearly all (33) of the additional components from the outgroup species were present uniquely in the individual outgroup species [*O. caelatus* (21), *I. latidens* (8), and *I. pini* (4)], while six dimethylalkanes (9,19-; 9,21-; 11,19-DimeC_{31} and 11,17-; 12,16-; 13,17-DimeC_{32}), and three trimethylalkanes (11,15,19-TrimeC_{29}; 9,13,17-TrimeC_{29}; and 11,15,19-TrimeC_{31}) were produced by various combinations of the three outgroup species. The unique components from the outgroup species were generally trimethylalkanes, except for one monomethylalkane from *I. pini*, four and one dimethylalkanes from *O. caelatus* and *I. pini*, respectively, and three tetramethylalkanes from *I. latidens* (Table 2).

Extracts from both sexes of *O. caelatus*, *I. latidens*, *I. pini*, *I. grandicollis*, *I. cribricollis*, *I. montanus*, *I. paraconfusus*, *I. confusus*, and *I. hoppingi* provided no evidence of sexual dimorphism in the cuticular hydrocarbon phenotype. However, extracts from both sexes of *I. lecontei* indicated qualitative dimorphism (Table 2, Figure 5). Female *I. lecontei* had eight more cuticular hydrocarbon components than males including *n*-C_{35}, 11-; 13-; 15-; and 17-MeC_{35}; 9,13-DimeC_{35}; and 5,13-; 5,17-DimeC_{35}. There was no evidence of differences in the cuticular hydrocarbon phenotypes for different populations of *O. caelatus* (2), *I. grandicollis* (4), *I. lecontei* (2), *I. montanus* (2), *I. paraconfusus* (6), *I. confusus* (3), or *I. hoppingi* (4) examined in this study.

The *n*-alkanes occurred as a generally continuous series from *n*-C_{22} to *n*-C_{35}. Only *n*-C_{32} and *n*-C_{34} were not represented. *n*-C_{22} was present only in *I. confusus* and *I. cribricollis*, while the *n*-alkanes inclusive of *n*-C_{23} to *n*-C_{30} were present in all seven species in the *grandicollis* group (Table 2). However, as a fraction of total hydrocarbon present, the percentage of each *n*-alkane varied from species to species (Table 2). Of the *n*-alkanes, only *n*-C_{22}, *n*-C_{31} (absent in *I. cribricollis* and *I. hoppingi*), *n*-C_{33} (absent in *I. grandicollis*, *I. cribricollis*, and *I. hoppingi*), and *n*-C_{35} (present in female *I. lecontei*) appeared to have diagnostic utility (Table 2) (Haverty et al., 1989).

The monounsaturated alkenes were not equally represented across species. The most abundant alkene was $C_{27:1}$ (double bond at carbon 9), and generally the series from $C_{25:1}$ to $C_{35:1}$ was present. Only $C_{32:1}$, and $C_{34:1}$ were not represented. Among the 16 components detected in this class of hydrocarbons, we

identified two isomers with double bonds at carbons 7 and 9, respectively, for the C_{25}, C_{27}, C_{29}, C_{31}, and C_{33} alkenes (Table 2, Figure 1). There were also two isomers with double bonds at carbons 8 and 9, respectively, for the C_{26} and C_{30} alkenes (Table 2). In the cases of $C_{26:1}$, $C_{30:1}$, and $C_{33:1}$, the positional isomers coeluted and did not receive individual listings in Table 2. We were unable to characterize the position of the double bond in $C_{35:1}$, which occurred in trace quantities in *I. montanus*, or in $C_{28:1}$, which occurred in trace quantities in all species. $C_{25:1}$, $C_{27:1}$, $C_{29:1}$, and $C_{31:1}$ were fairly abundant in nearly all species in the *grandicollis* group. In these cases, the 9-positional isomer tended to predominate, although the 7-positional isomer was also abundantly repre-

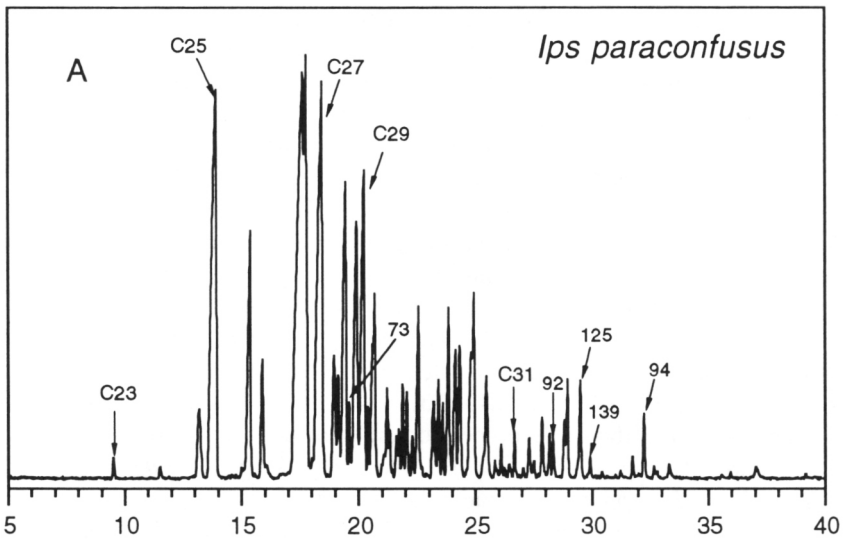
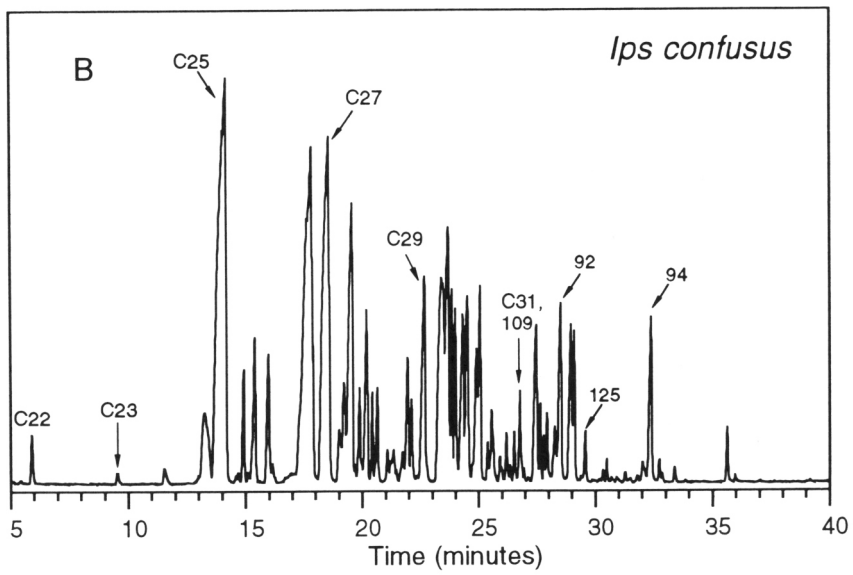


FIG. 3. Total ion chromatograms of the cuticular hydrocarbons of the sibling species (A) *Ips paraconfusus*, (B) *I. confusus*, and (C) *I. hoppingi*. The 100 females of each species were collected as follows: From *Pinus radiata* in Albany, Alameda County, California (*I. paraconfusus*); from *P. monophylla* near Carter Springs, Douglas County, Nevada (*I. confusus*); and from *P. cembroides* near Las Crucitas, Nuevo Leon, Mexico (*I. hoppingi*). In each chromatogram, *n*-C_{23}, *n*-C_{25}, *n*-C_{27}, *n*-C_{29}, *n*-C_{31} (except *I. hoppingi*), 92 (5,17-; 5,19-DimeC_{31}), 94 (5,17-DimeC_{33}), and 125 (3,7,11-; 3,7,15-; 3,7,17-TrimeC_{31}) are labeled for reference. Selected diagnostic compounds 73 (9,13-; 11,15-; 13,17-DimeC_{27}) and 139 (3,7,11,15-TetrameC_{31}) for *I. paraconfusus* and 109 (3,11-; 3,15-DimeC_{30}) for *I. confusus* are labeled on the respective chromatograms with peak numbers from Table 2.



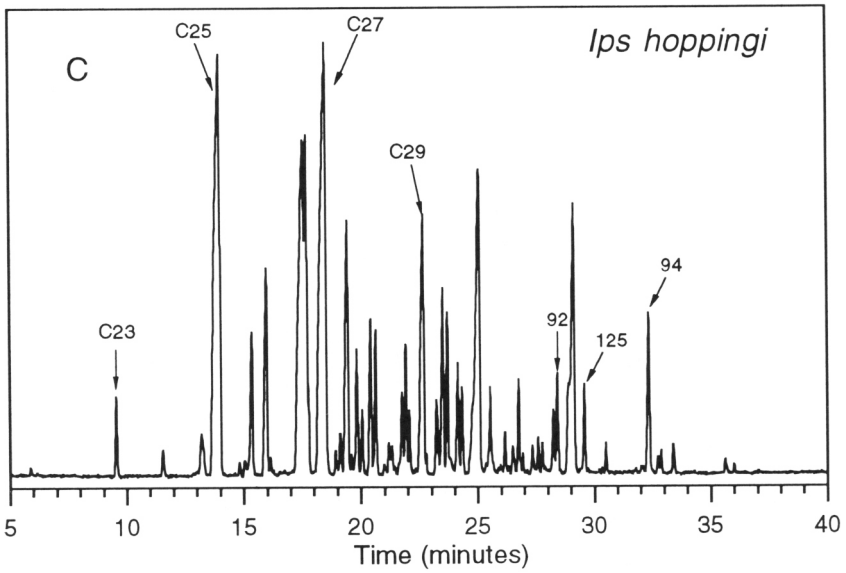


FIG. 3. Continued.

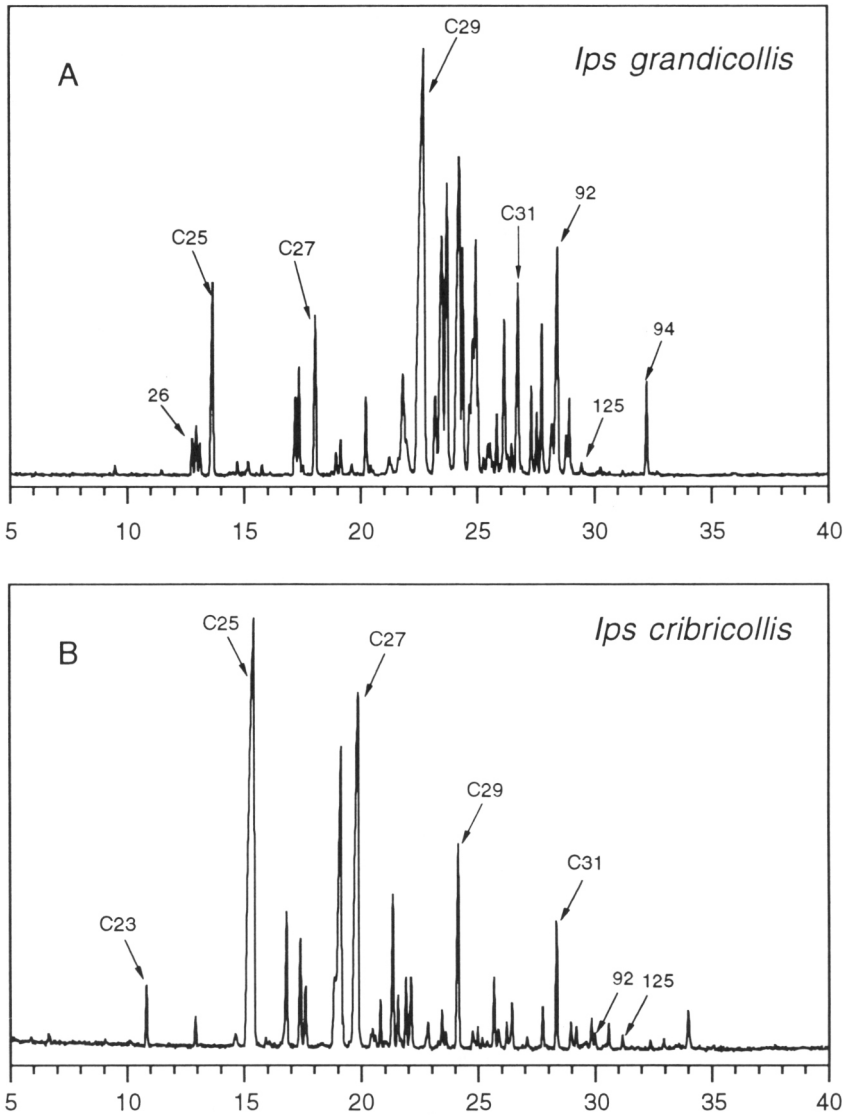


FIG. 4. Total ion chromatograms of the cuticular hydrocarbons of (A) *Ips grandicollis*, (B) *I. cribricollis*, (C) *I. lecontei*, and (D) *I. montanus*. The females of each species were collected as follows: from pheromone traps near Pollock, Grant Parish, Louisiana (50 *I. grandicollis*); from *Pinus ponderosa* near Cloudcroft, Otero County, New Mexico (41 *I. cribricollis*); from *P. ponderosa* near Point of Pines, Graham County, Arizona (50 *I. lecontei*); and from *P. monticola* near Benton Meadow, Warner Mountains, Modoc County, California (25 *I. montanus*). In each chromatogram, *n*-C_{23}, *n*-C_{25}, *n*-C_{27}, *n*-C_{29}, *n*-C_{31}, 92 (5,17-; 5,19-DimeC_{31}), 94 (5,17-DimeC_{33}), and 125 (3,7,11-; 3,7,15-; 3,7,17-

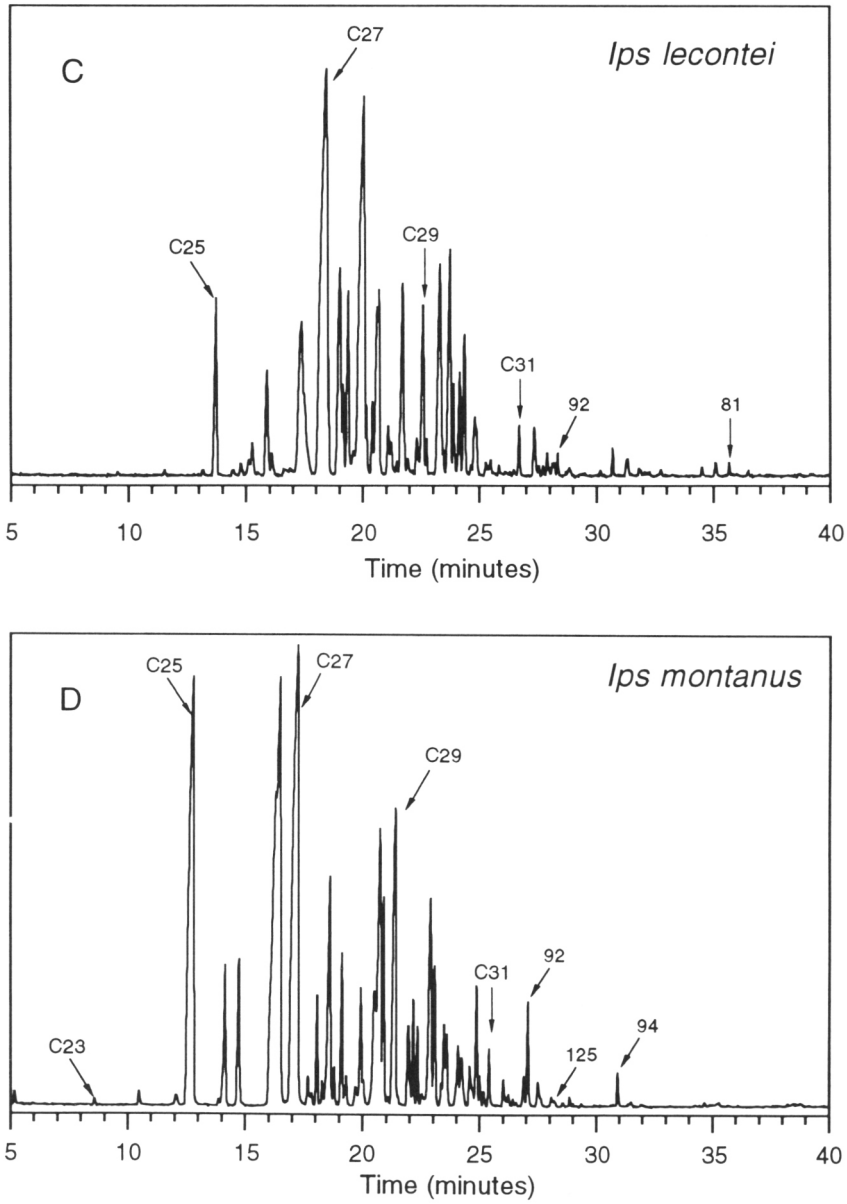


FIG. 4. Continued.

TrimeC$_{31}$) are labeled for reference. Selected diagnostic compounds 26 (C$_{25:2}$) for *I. grandicollis* and 81 (9,13-DimeC$_{35}$) for female *I. lecontei* are labeled on the respective chromatograms with peak numbers from Table 2.

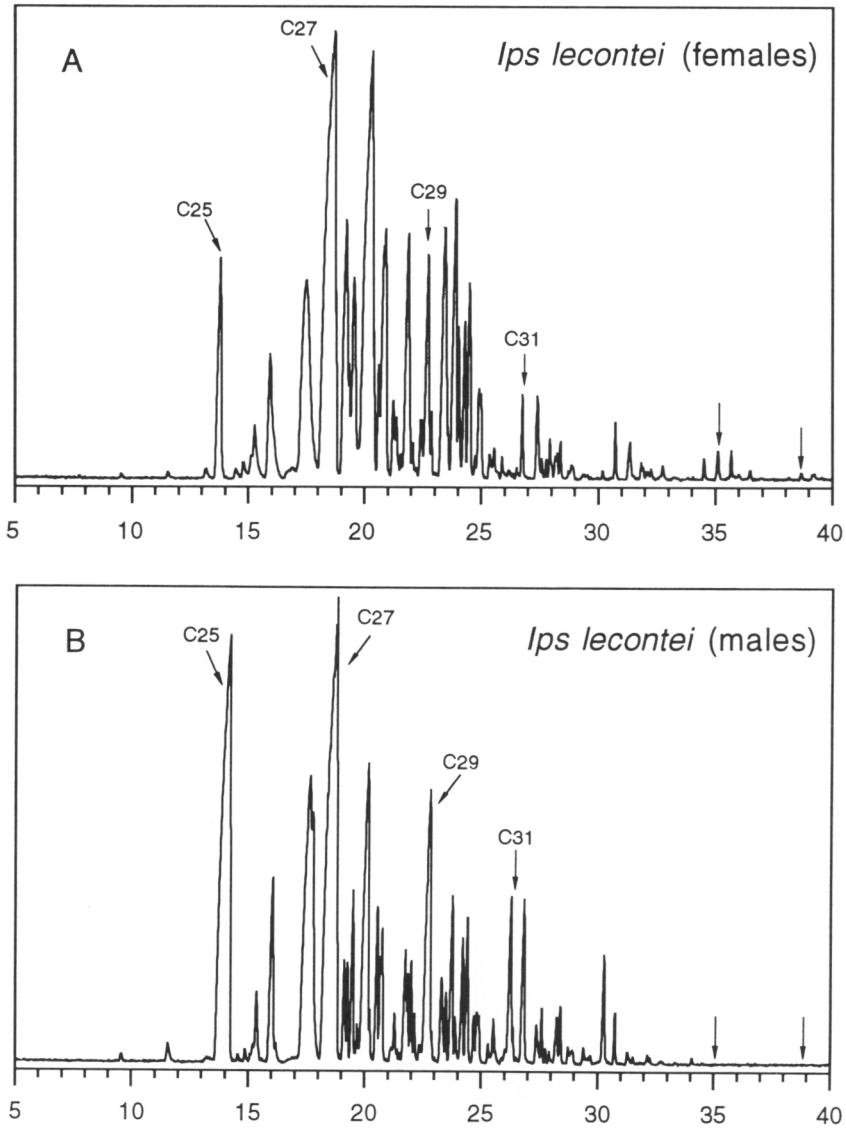
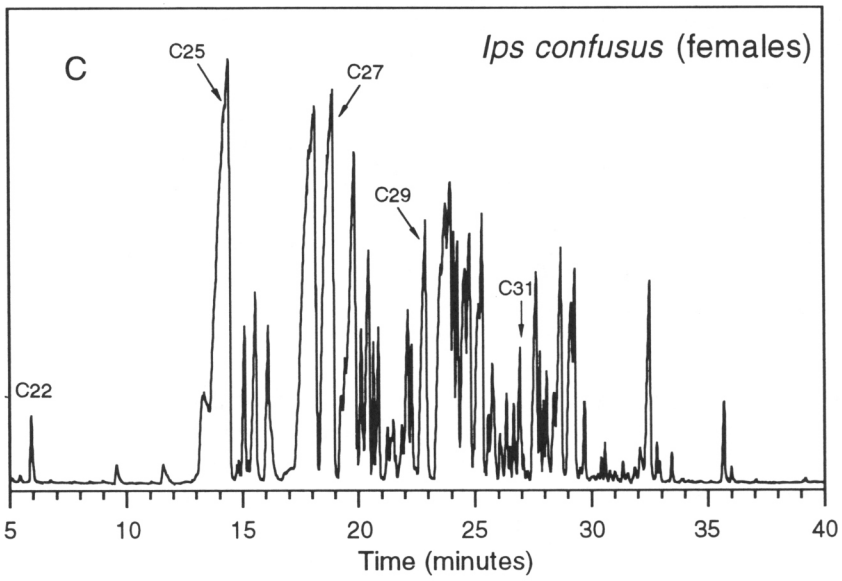


FIG. 5. Total ion chromatograms showing cuticular hydrocarbon sexual dimorphism in *Ips lecontei* (A and B) and absence of dimorphism in *I. confusus* (C and D). The 100 male and female *I. lecontei* were collected from *Pinus ponderosa* near Point of Pines, Graham County, Arizona, and the 100 male and female *I. confusus* were collected from *P. monophylla* near Carter Springs, Douglas County, Nevada. In each chromatogram, *n*-C_{25}, *n*-C_{27}, *n*-C_{29} and *n*-C_{31} are labeled for reference. Arrows indicate region of chromatogram where *I. lecontei* female-specific pentatriacontane derivatives elute.



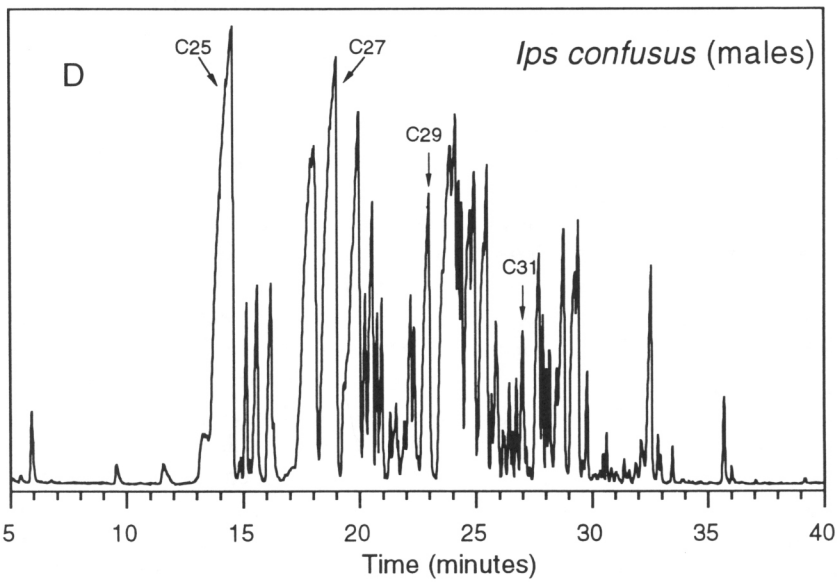


FIG. 5. Continued.

sented for $C_{29:1}$ in certain species (Table 2). The absence of $C_{26:1}$ from the profiles of *I. lecontei* and *I. montanus*, as well as a fairly strong peak for 7-$C_{31:1}$ in the profile of *I. montanus* have diagnostic value (Table 2).

We found six alkadienes in our analysis. Pentacosadiene ($C_{25:2}$) was diagnostic in trace quantities for *I. grandicollis*, while $C_{27:2}$ was extremely abundant in all species (Table 2). Because of the large quantities of the latter compound, we were able to determine the double-bond positions and stereochemistries as 6*Z*,9*Z* in all species by analysis and comparison to a standard isolated from *P. americana*. Nonacosadiene ($C_{29:2}$) was absent in *I. lecontei* and present in trace amounts in *I. grandicollis*, *I. cribricollis*, and *I. hoppingi*. Two undetermined isomers of $C_{31:2}$ and one of $C_{33:2}$ were present in trace quantities only in *I. montanus* (Table 2).

The 214 methylalkanes identified included monomethyl-, dimethyl-, trimethyl-, and tetramethylalkanes. Among the monomethylalkanes were 12 terminally branched methylalkanes (i.e., 2-, 3-, and 4-methylalkanes). For most species in the *grandicollis* group, 3-methylalkanes formed a homologous series of 3-MeC_{24} through 3-MeC_{29}; however *I. grandicollis* was anomalous because it did not produce 3-MeC_{26} (Table 2). All species had 3-MeC_{31} in their cuticular lipids, and *I. grandicollis* and *I. montanus* contained this isomer in large amounts (1.0–5.0% of the respective hydrocarbon profiles). Few species had 3-MeC_{30} or 3-MeC_{33} in their cuticular lipids (Table 2). There were only three instances of 2- or 4-methylalkanes, and they all occurred on even-numbered parent carbon chains (2-MeC_{26}, 4-MeC_{30}, and a component that was either 2- or 4-MeC_{28}).

In addition to the terminally branched monomethylalkanes, there were 61 internally branched monomethylalkanes with methyl branches on carbons 5–17 and parent chains ranging from 25 to 35 carbons (Table 2). The 5- and 7-methylalkanes were represented in most species by the homologous series of C_{27}, C_{29}, and C_{31}. The methyl-branched isomers on longer or shorter parent chains were variously present in the group. *Ips confusus* had only a trace of 7-MeC_{25}, while 5-MeC_{25} was absent in *I. cribricollis* and *I. montanus* and present in low quantities in all other *Ips* species (Table 2). 7-MeC_{29} was abundant in most species except *I. cribricollis* and *I. lecontei* (both sexes). No species in our study produced 5-MeC_{35}, but 7-MeC_{35} was diagnostic for *I. montanus*. The outgroup species *I. pini* was the only taxon in this analysis that produced 5-MeC_{26} (Table 2). The remaining internally branched monomethylalkanes occurred as mixtures of isomers with branches at 9, 11, 13, 15, and 17 carbons with odd-numbered parent chains or branches at 10, 11, 12, 13, 14, 15, and 16 carbons with even-numbered parent chains.

We identified 98 dimethylalkanes, and most of them occurred as coeluting isomeric mixtures (Table 2). Among these isomeric blends were components with odd-numbered parent chains whose first methyl branch was located on odd-numbered carbons 3–13, and whose second methyl was located on odd-num-

bered carbons 7–23. Those components that had branches positioned on even-numbered carbons (e.g., 4,14-; 4,16-; 4,18-; 6,18-; or 12,16-) always had a parent chain with an even number of carbons. However, there were several instances of components with branches on odd-numbered carbons, yet even-numbered parent chains (e.g., 3,7-DimeC_{28}/C_{30}, 3,11-; 3,15-DimeC_{30}; 5,17-DimeC_{28}/C_{30}/C_{32}; 11,15-DimeC_{28}/C_{30}; and 11,17-; 13,17-DimeC_{32}). Of these isomers, 3,11-; 3,15-DimeC_{30} was diagnostic for *I. confusus*.

The 3,X- and 5,X-dimethylalkanes were fairly abundant in the *grandicollis* group, although all members of the group did not have every homolog (Table 2). The absence of several of the shorter chain 3,X- and 5,X-dimethylalkanes in *I. grandicollis* was diagnostic, and in two cases of coeluting isomers (3,7-; 3,9-DimeC_{25} and 3,11-; 3,13-; 3,17-DimeC_{27}), *I. cribricollis* shares this trait. Most species produced $>1.0\%$ of 7,X-DimeC_{29}, but these isomers were also absent in *I. grandicollis* and *I. cribricollis*. Also conspicuous by their absence from our extracts of *I. cribricollis* were 3,11-; 3,13-; 3,15-; 3,17-DimeC_{29}. Another commonly occurring dimethylalkane group contained isomers with 9,13; 11,15; and 13,17 branch positions. Male and female *I. lecontei* were the only species to produce any of this blend with a 25-carbon parent chain, while female *I. lecontei* alone produced 9,13-DimeC_{35}. *Ips grandicollis*, *I. cribricollis*, *I. confusus*, and *I. hoppingi* had at most traces of any 9,13-; 11,15-; or 13,17-dimethylalkanes (Table 2).

We identified 39 trimethylalkane hydrocarbon components in this study (Table 2). Only 12 of these occurred in the cuticular lipids of species in the *grandicollis* group. Although there were a few exceptions with the dimethylalkanes, with the trimethylalkanes all even-numbered parent chains had even-numbered branch positions. Furthermore all trimethylalkanes with odd-numbered parent chains had odd-numbered branch positions. One trimethylalkane (9,13,17-TrimeC_{27}) was present as a trace for *I. cribricollis* (Table 2). One isomeric mixture of 3,7,X-TrimeC_{29} was present in every species in the *grandicollis* group, while a second isomeric mixture of 3,7,X-TrimeC_{31} was present in every species except *I. lecontei*. Mass spectral evidence for 3,7,15-; 3,7,17-TrimeC_{31} in *I. hoppingi* is given in Figure 2A. In general, the outgroup species, particularly *O. caelatus*, were rich in trimethylalkanes.

Tetramethylalkanes were rare in our survey, occurring in only four instances. Both sexes of *I. paraconfusus* produced 3,7,11,15-TetrameC_{31}, while both sexes of *I. latidens* produced 3,7,11,15-TetrameC_{27} and 3,7,11,15-; 3,7,13,17-TetrameC_{29}. Mass spectral evidence for 3,7,11,15-TetrameC_{31} in *I. paraconfusus* is given in Figure 2B.

Since there are few criteria to discriminate the sibling species *I. paraconfusus*, *I. confusus*, and *I. hoppingi*, it is of taxonomic significance to focus on their cuticular hydrocarbon phenotypes. Although the overall phenotypes are quite similar, an examination of the individual components shows that important

TABLE 3. DIAGNOSTIC HYDROCARBONS FOR SIBLING SPECIES *Ips paraconfusus*, *Ips confusus*, AND *Ips hoppingi* IN *grandicollis* SUBGENERIC GROUP[a]

Hydrocarbon	*paraconfusus*	*confusus*
C_{22}	0	+
9,13-; 11,15-; 13,17-DimeC_{27}	+ +	0
7-MeC_{28}	0	+
3,11-; 3,15-DimeC_{30}	0	+ +
9,13-; 11,15-; 13,17-DimeC_{31}	+	0
5,17-DimeC_{32}	0	+
3,7,11-; 3,7,15-TrimeC_{27}	+	0
3,7,11,15-TetrameC_{31}	tr	0
	confusus	*hoppingi*
C_{22}	+	0
C_{31}	+ +	0
3,11-; 3,15-DimeC_{30}	+ +	0
5,17-DimeC_{32}	+	0
	paraconfusus	*hoppingi*
C_{31}	+	0
10-; 11-; 12-; 13-; 14-MeC_{28}	+ +	0
9,13-; 11,15-; 13,17-DimeC_{27}	+ +	0
4,14-; 4,16-; 4,18-DimeC_{28}	+	0
9,13-; 11,15-; 13,17-DimeC_{31}	+	0
3,7,11-; 3,7,15-TrimeC_{27}	+	0
3,7,11,15-TetrameC_{31}	tr	0

[a]tr: trace (<0.5%); +: 0.5-1.0%; and + +: 1.0-5.0%.

diagnostic differences do exist (Table 3). *Ips paraconfusus* and *I. confusus* can be clearly discriminated based on the presence or absence of 14 compounds (Table 3). *Ips confusus* produces *n*-C_{22}; 7-MeC_{28}; 3,11-; 3,15-DimeC_{30}; and 5,17-DimeC_{32}, while *I. paraconfusus* does not. *Ips paraconfusus* produces 9,13-; 11,15-; 13,17-DimeC_{27}; 9,13-; 11,15-; 13,17-DimeC_{31}; and 3,7,11-; 3,7,15-TrimeC_{27}, while *I. confusus* does not. In addition, *I. paraconfusus* produces a trace of 3,7,11,15-TetrameC_{31}, while *I. confusus* does not produce this compound.

There are fewer substantial differences in the quantitative and qualitative representation of hydrocarbons in the *I. confusus–I. hoppingi* couplet than in the *I. paraconfusus–I. confusus* couplet (Tables 2 and 3). There are 11 instances where *I. hoppingi* produces a component in trace quantities, while *I. confusus* produces the component at a level between 0.5 and 1.0% of the total hydrocarbon complement (Table 2). The reverse is true in one case ($C_{31:1}$), and in

another case (7-MeC_{27}) *I. confusus* produces 1.0–5.0% of a component that occurs as only a trace in *I. hoppingi*. However, the production of *n*-C_{22}; *n*-C_{31}; 3,11-; 3,15-DimeC_{30}; and 5,17-DimeC_{32} by *I. confusus* and their absence from *I. hoppingi* are diagnostic.

In the *I. paraconfusus–I. hoppingi* couplet, the species can be discriminated based on the presence of 18 compounds in the cuticular lipids of *I. paraconfusus* that are absent in the same of *I. hoppingi* (Table 3). Two isomeric blends, 10-; 11-; 12-; 13-; 14-MeC_{28} and 9,13-; 11,15-; 13,17-DimeC_{27}, are present in quantities between 1.0 and 5.0% of the cuticular lipids components of *I. paraconfusus*. In addition, there are ten instances where *I. hoppingi* produces a component in trace amounts, while *I. paraconfusus* produces it at levels above 0.5% (Table 2). In only one case ($C_{31:1}$) does *I. hoppingi* produce a component at a level greater than 0.5%, while *I. paraconfusus* produces it in trace amounts. It is noteworthy that *I. hoppingi* does not produce a single unique component that is absent in either *I. paraconfusus* or *I. confusus*.

Phylogenetic Analysis. PAUP analyses with various combinations of outgroups provided fairly consistent versions of hydrocarbon-based relatedness for species in the *grandicollis* subgeneric group (Figures 6 and 7). All analyses suggested a close relationship between *I. grandicollis* and *I. cribricollis* and between *I. lecontei* and *I. montanus*. With the exception of the analysis where *I. pini* was the only outgroup (Figure 6D), all analyses also indicated a close relationship among the sibling species *I. paraconfusus*, *I. confusus*, and *I. hoppingi*. The analyses with individual outgroups and with the ingroup alone each produced one most parsimonious tree (MPT) (Figure 6). When no outgroup was included (a preferred topology because it is an unpolarized network rather than a tree), *I. lecontei* and *I. montanus* were adjacent and possible sister taxa, while all other species were paraphyletic (Figure 6A). The same relationship resulted from an analysis with *O. caelatus* as the outgroup, except that the decay analysis indicated less support for the relatedness of various taxa (Figure 6B). When *I. latidens* was included as the outgroup, the analysis indicated that *I. confusus* and *I. hoppingi* were adjacent and possible sister taxa, while all other species were paraphyletic (Figure 6C). When *I. pini* was designated as the outgroup, the analysis strongly supported sister relationships between *I. grandicollis* and *I. cribricollis* (decay index = 3 steps) as well as between *I. lecontei* and *I. montanus* (decay index = 2 steps) (Figure 6D). Goodness-of-fit statistics, consistency index (CI), and retention index (RI) are also reported in Figures 6 and 7. Consistency index (Kluge and Farris, 1969) measures the level of support for each tree, and retention index (Farris 1989) measures the congruency of characters to each other and the tree. Consistency index will equal 1 when a data set explains the tree as well as possible. A retention index value of 1 indicates that the characters in a data set are totally congruent with each other and the tree.

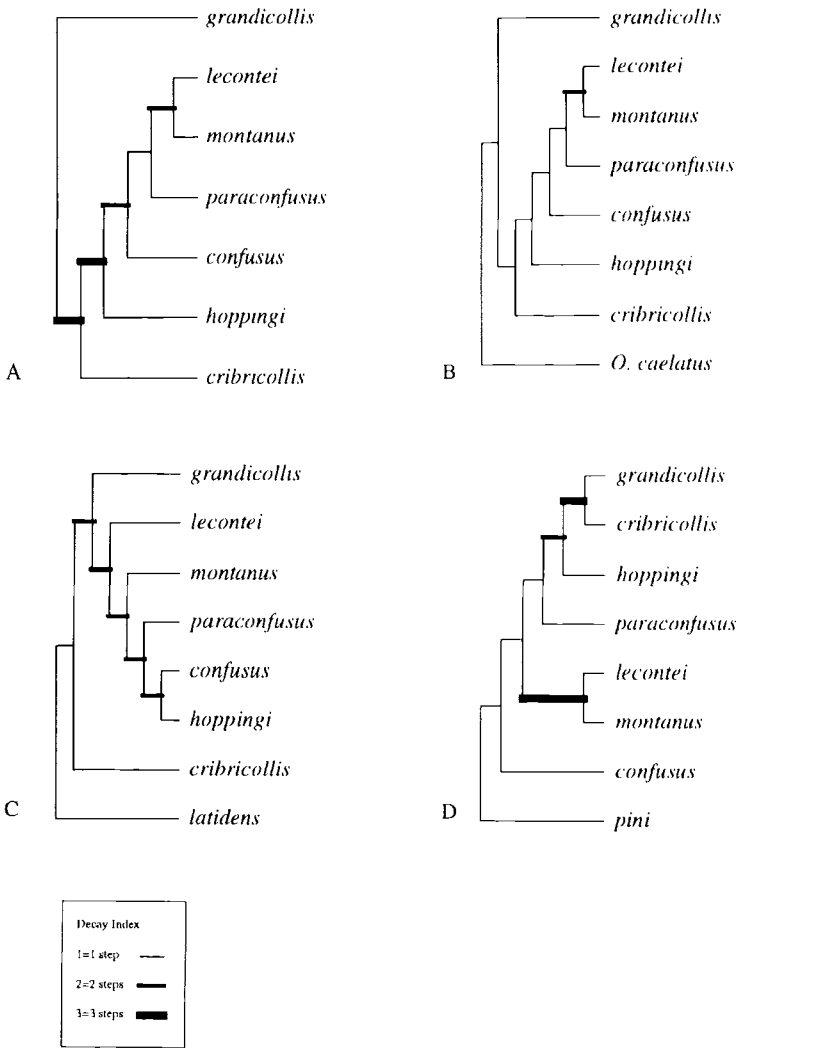


FIG. 6. The most parsimonious trees (MPT) for *Ips* species in the *grandicollis* subgeneric group from phylogenetic analyses of cuticular hydrocarbon phenotypes with: (A) Ingroup alone (MPT length = 221 steps; consistency index (CI) = 0.783; retention index (RI) = 0.429); (B) *Orthotomicus caelatus* as the outgroup (MPT length = 280 steps; CI = 0.701; RI = 0.469); (C) *I. latidens* as the outgroup (MPT length = 276 steps; CI = 0.739; RI = 0.410); and (D) *I. pini* as the outgroup (MPT length = 274 steps; CI = 0.723; RI = 0.387). The decay indices, the number of steps that parsimony was relaxed before a particular clade lost its support, are indicated by branch thicknesses. The thick branches are better supported.

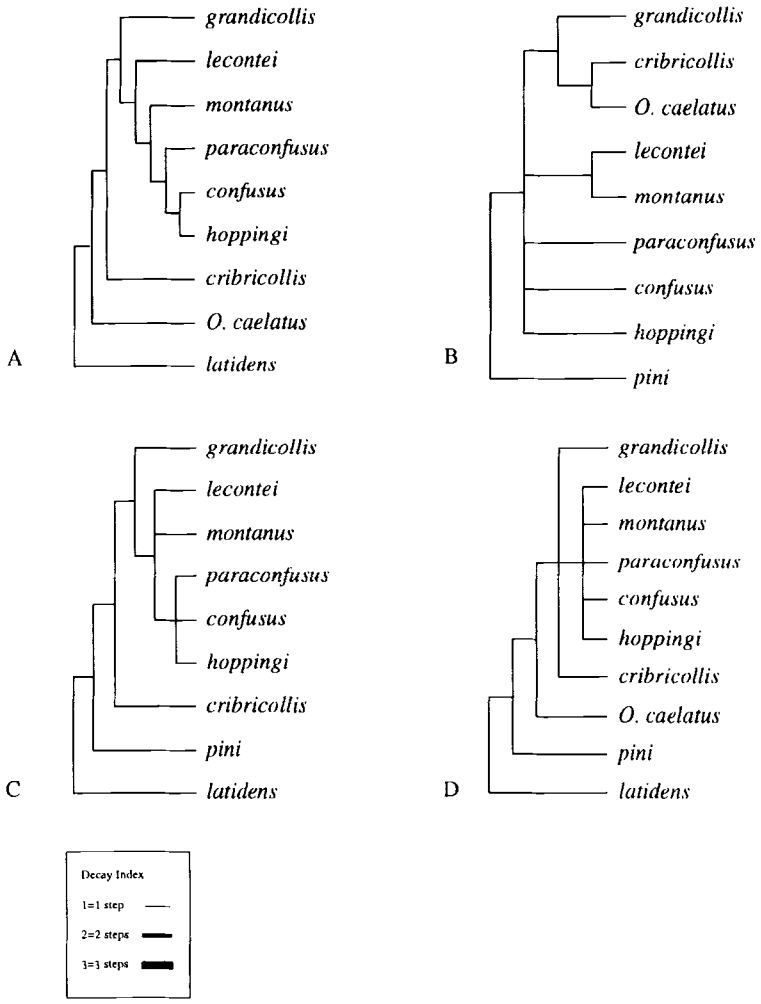


FIG. 7. The most parsimonious trees (MPT) or strict consensus (SC) of MPT for *Ips* species in the *grandicollis* subgeneric group from phylogenetic analyses of cuticular hydrocarbon phenotypes with: (A) *Orthotomicus caelatus* and *I. latidens* as the outgroups (MPT length = 330 steps; consistency index (CI) = 0.736; retention index (RI) = 0.442); (B) *O. caelatus* and *I. pini* as the outgroups (SC of 3 MPT; MPT length = 334 steps; CI = 0.710; RI = 0.410); (C) *I. latidens* and *I. pini* as the outgroups (SC of 3 MPT; MPT length = 324 steps; CI = 0.605; RI = 0.387); and (D) *O. caelatus*, *I. latidens*, and *I. pini* as the outgroups (SC of 5 MPT; MPT length = 382 steps: CI = 0.668; RI = 0.392). The decay indices, the number of steps that parsimony was relaxed before a particular clade lost its support, are indicated by branch thicknesses. The thick branches are better supported.

In three of four instances, analyses with binary combinations of the three outgroups and the ternary combination supported the integrity of the *grandicollis* ingroup (Figure 7). Only when *O. caelatus* and *I. pini* were utilized as paired outgroups did one of the taxa (*O. caelatus*) disturb the integrity of the *grandicollis* group (Figure 7B). In this case, the PAUP algorithm indicated that the *grandicollis* ingroup could not be rooted as an ingroup because of the insertion of *O. caelatus* as a sister taxon to *I. cribricollis*. Surprisingly, all analyses involving *I. latidens* (Figure 7A,C,D) always placed this species furthest away from the *grandicollis* group. This was even the case when *O. caelatus* was included in the analysis (Figure 7A and D). When multiple outgroups were utilized in the analysis, only the analysis involving *O. caelatus* and *I. latidens* yielded a single MPT (Figure 7A). The three other cases yielded strict consensus trees based on three or five MPT (Figure 7B–D).

DISCUSSION

Studies of the cuticular hydrocarbons from species of two other scolytid genera, *Conophthorus* spp. (Page et al., 1990a) and *Dendroctonus* spp. (Page et al., 1990b), have shown that there are only minor quantitative differences in cuticular hydrocarbons isolated from specimens from different populations or different sexes. This trend is supported in this study by analyses of multiple populations of *O. caelatus*, *I. grandicollis*, *I. lecontei*, *I. montanus*, *I. paraconfusus*, *I. confusus*, and *I. hoppingi*, as well as analyses of both sexes of *O. caelatus*, *I. latidens*, *I. pini*, *I. grandicollis*, *I. cribricollis*, *I. montanus*, *I. paraconfusus*, *I. confusus*, and *I. hoppingi*. However, *I. lecontei* provides the first example of a scolytid with a sexually dimorphic cuticular hydrocarbon phenotype.

There are many differences in hydrocarbon phenotype among these three genera of Scolytidae. Seven of the eight classes of hydrocarbons represented in this survey of the *grandicollis* group of *Ips* were all represented in *Conophthorus* spp. (Page et al., 1990a). Members of this cone-infesting genus do not appear to produce tetramethylalkanes. Only five of the classes were present in *Dendroctonus* spp., which do not appear to produce alkadienes, trimethylalkanes, or tetramethylalkanes (Page et al., 1990b). While *Dendroctonus* spp. and these *Ips* spp. possess hydrocarbons that range from C_{21} to C_{37} and C_{22} to C_{35}, respectively, *Conophthorus* spp. have hydrocarbons that range from C_{20} to C_{43} (Page et al., 1990a,b). In many species of *Conophthorus* the hydrocarbons between C_{37} and C_{43} comprise between 1% and 5% of the total hydrocarbon complement, and in some species these long-chain components comprise greater than 5%. The *n*-alkanes represent a substantial proportion of the hydrocarbon phenotype for all three scolytid genera. In all cases the shortest and longest *n*-alkanes are generally least abundant. For the four *Dendroctonus* spp. previ-

ously studied, this decline in abundance of long-chain *n*-alkanes begins with *n*-C_{28}, while for *Ips* spp. and *Conophthorus* spp. this trend begins with *n*-C_{33} and *n*-C_{32}, respectively. These *Ips* spp. and *Dendroctonus* spp. have a relative abundance of internally branched 5- and 7-methylalkanes; *Conophthorus* spp. do not. Conspicuous by its relative absence in both *Ips* spp. and *Conophthorus* spp. is 7-methylpentacosane, which is present in all four species of *Dendroctonus* surveyed by Page et al. (1990b). In the *grandicollis* group it is produced only in trace quantities by *I. confusus*.

The scolytids analyzed to date, and most strikingly the *Ips* spp. in the *grandicollis* group, have demonstrated a richness of methyl-branched alkanes in their cuticular lipids that has great utility for chemotaxonomy. Indeed, 214 of 248 components identified were methyl-branched alkanes. More chemotaxonomic information awaits the discovery of chromatographic techniques to determine the enantiomeric composition of the naturally occurring compounds (Howard, 1993). For the most part, the methyl-branched structures are typical of insects (Nelson, 1993). Somewhat unusual are the 3-methylalkanes with even-numbered parent carbon chains [e.g., 3-MeC_{24}, 3-MeC_{26}, and 3-MeC_{28} present in all species (3-MeC_{26} is absent in *I. grandicollis*); 3-MeC_{30} present in *I. montanus* and *I. confusus*]. Also noteworthy in various species are the 4-methylalkanes, the 4,X-dimethylalkanes, the general abundance of di- and trimethylalkanes, and the tetramethylalkanes identified in *I. latidens* and *I. paraconfusus*. All 2- and 4-methylalkanes identified in this study have an even number of carbons in the parent chain. The likely biosynthetic origin of these methylalkanes would appear to dictate an even-numbered parent chain (Blomquist et al., 1980a, 1987b). However, 2-methylalkanes with an odd number of carbons in the parent chain have been identified in termites (Haverty et al., 1988, 1990a, 1991a,b), and there are several reports of other insect taxa that have 4-methylalkanes with an odd number of carbons in the parent chain (Nelson, 1993).

Some of the dimethylalkanes are important diagnostic characters for sibling species (e.g., 3,11-; 3,15-DimeC_{30} for *I. confusus* and 9,13-; 11,15-; and 13,17-DimeC_{27} for *I. paraconfusus*). All three sibling species have 3,7,11-; 3,7,15-; 3,7,17-TrimeC_{31} isomers. Internally or terminally branched trimethylalkanes in Coleoptera are relatively rare (Blomquist et al., 1987b; Dubis et al., 1987; Golden et al., 1992; Jacob, 1978, 1979; Lockey, 1981, 1984a, 1988, 1991a, 1992; Lockey and Metcalfe, 1988; Page et al., 1990a,b). However, terminally branched trimethylalkanes (specifically those that have the first methyl branch on carbon 3) occur quite frequently in the *Ips grandicollis* group and the three outgroup species (Table 2). Indeed, *I. latidens*, *I. paraconfusus*, *I. amitinus* (Eichhoff), *I. perturbatus* (Eichhoff), and *I. woodi* Thatcher (the three latter species from the *perturbatus* subgeneric group) even possess terminally branched tetramethylalkanes (Table 2) (Page et al., unpublished data). Other insects with

terminally branched trimethylalkanes include *Atta columbica*, *A. sexdens*, and *A. cephalotes isthmicola* (Hymenoptera) (Martin and MacConnell, 1970); *Glossina* spp. (Diptera) in the *palpalis* and *fusca* species groups (Nelson et al., 1988); *Leptinotarsa decemlineata* (Coleoptera) (Dubis et al., 1987) (first methyl branch on carbon 2); and perhaps *Zophosis gracilipes* (Coleoptera) (Lockey, 1984a). Terminally branched tetramethylalkanes have been identified among the cuticular hydrocarbons from *Glossina brevipalpis* (Nelson et al., 1988) and perhaps from *L. decemlineata* (Dubis et al., 1987).

The presence of certain methyl-branched alkanes such as 3,11-; 3,15-DimeC_{30}, 3,7,X-TrimeC_{31}, and 3,7,11,15-TrameC_{31} may be derived characters for these insects. The available data on methylalkane biosynthesis in insects (Blomquist et al., 1987b; Nelson, 1993; Nelson and Blomquist, 1995) suggest specific variations from normal acetate-based chain elongation for the biosynthesis of mono-, di-, tri-, and tetramethylalkanes. In insects studied to date, internal methyl branches in alkanes with multiple methyl branches arise from the insertion of the carbons from propionate in the form of a methylmalonyl-CoA elongation unit (Blomquist et al., 1975, 1979; Dwyer et al., 1981; Nelson, 1993). Depending on the species the elongation unit is derived from branched-chain amino acids (Dillwith et al., 1982), succinate (Blomquist et al., 1980a), or potentially from odd-chain fatty acids (Voet and Voet, 1990) (all reviewed in Nelson, 1993). The species specificity in spacing between methyl branches in di-, tri-, and tetramethylalkanes occurs when a given species controls when propionate is incorporated into the elongating hydrocarbon chain in place of acetate (Nelson, 1993). In the German cockroach, *Blattella germanica*, this high degree of species-specific biosynthetic control appears to be regulated by an enzymatic system involving a microsomal fatty acid synthase in integumental tissue (Juarez et al., 1992). The enzyme system is apparently different even in closely related insects (Blomquist et al., 1987b).

The taxonomic structure of the *grandicollis* subgeneric group of *Ips* as defined by the classical methods of Hopping (1963, 1965), Lanier (1970, 1987), and Wood (1982) has been substantiated by this study of the cuticular hydrocarbons. This is obvious when we compare the *grandicollis* group with other subgeneric groups (Page et al., unpublished data). Furthermore, this study has demonstrated that it is practical to identify and separate all species in the *grandicollis* group by GC-MS analysis of their cuticular hydrocarbon profiles.

The study of Cane et al. (1990b) based on characteristic electrophoretic migration of metabolic enzymes revealed that *I. lecontei* is less related to four members of the *grandicollis* group (*grandicollis*, *paraconfusus*, *confusus*, and *hoppingi*) than is a member of another subgeneric group, *I. pini* (*pini* group). Based on isozyme data, Cane et al. (1990b) also found that the three sibling species, *I. paraconfusus*, *I. confusus*, and *I. hoppingi*, are more closely related to each other than they are to *I. grandicollis*. In an analysis of the entire *gran-*

dicollis group based on the electrophoretic migration of random amplified polymorphic DNA (RAPD) fragments, Cognato et al. (1995) largely concurred. However, unlike Cane et al. (1990b), Cognato et al. (1995) did not place *I. lecontei* as an earlier derivative of *I. pini*.

In a third chemosystematic approach, Seybold (1992) determined the presence, absence, and enantiomeric composition of the male-produced aggregation pheromone components, ipsenol and ipsdienol, as potential sources of behavioral variation among the species in the *grandicollis* group. A study by Lanier and Wood (1975) provided the biological background for the study. They found that *I. lecontei* was the least cross-attractive of the *Ips* in the *grandicollis* group. Although *I. lecontei* produces nearly 100% (*S*)-(−)-ipsenol, as do the other members of the group, it is unique because it produces ~50% (*R*)-(−)-ipsdienol (Seybold, 1992). The reduced cross-attraction of other members of the group to *I. lecontei* may be due to the enantiomeric composition of its ipsdienol. The sensitivity of members of this group to the enantiomeric composition of ipsdienol was illustrated by Light and Birch (1979), who demonstrated in field studies that 92% (*R*)-(−)-ipsdienol interrupts the response of *I. paraconfusus* to its male-produced attractant. *I. paraconfusus* produces ~91–92% (*S*)-(+)-ipsdienol as part of its aggregation pheromone (Seybold, 1992). The absence of ipsdienol in *I. grandicollis* may support its phylogenetic distance from the sibling species which all produce ipsdienol (Seybold, 1992).

Like morphological features and metabolic enzyme structure, hydrocarbon components are genetically fixed and represent a unique, species-specific phenotype (Kaib et al., 1991; Lockey, 1991b; Coyne et al., 1994). The presence, absence, or abundance of cuticular hydrocarbons is related to the presence, absence, or kinetics of the biosynthetic enzymes. Although the regulation of hydrocarbon biosynthesis may result from genetically programmed physiological processes, species-specific hydrocarbon phenotypes most likely result from variation in the genes that code directly for the enzymes.

Phylogenetic analyses of our hydrocarbon data utilizing various combinations of outgroup species generally indicate that *I. grandicollis* and *I. cribricollis* are closely related and distinct from the remaining species in the group (Figures 6 and 7). This is consistent with prior morphological studies (Wood, 1982; Lanier, 1987). In addition, *I. paraconfusus*, *I. confusus*, and *I. hoppingi* appear to be closely related to one another using this analytical technique (Figures 6 and 7). This sibling relationship is also consistent with previous studies (Lanier, 1970; Wood, 1982). Based on hydrocarbon phenotype, the core group of sibling species appears to be related in order to *I. montanus* and then *I. lecontei*. The latter two species appear as sister taxa in many of our analyses (Figures 6 and 7).

When coupled with the discovery of sexual dimorphism, the cuticular hydrocarbon phenotype of *I. lecontei* clearly distinguishes it from sibling species

and *I. montanus* within the *grandicollis* group. A direct comparison with the isozyme study of Cane et al. (1990b) is difficult because that study did not include *I. cribricollis* or *I. montanus*. Nonetheless, in contrast to the suggestions of Cane et al. (1990b) and Cognato et al. (1995), our data do not indicate that *I. lecontei* should be excluded. From this feature, *I. lecontei* more clearly resembles the core members of the group than it does *I. pini* or other outgroup species. Furthermore, *I. grandicollis* and *I. cribricollis* appear to have hydrocarbon phenotypes that are intermediate in relatedness between *I. lecontei* and the outgroup species. From the standpoint of semiochemistry, the presence of racemic ipsdienol in male *I. lecontei* is no more unique than is the absence of ipsdienol in male *I. grandicollis*. For the most part, however, the results of this study support the chemosystematic work of Cane et al. (1990b), Seybold (1992), and Cognato et al. (1995); the morphological and cytological studies of Lanier (1970, 1987); and the morphological work of Wood (1982). The phylogenetic analyses emphasize the distance of *I. latidens* as an outgroup from the members of the *grandicollis* group. This is consistent with the taxonomic similarities of the members of the *latidens* group to the genus *Orthotomicus* (Wood, 1966, 1968, 1982). Independent of the outgroup employed for phylogenetic analysis, all hydrocarbon-based analyses strongly suggest that *I. hoppingi* and *I. cribricollis* merit separate species status. This is further supported by RAPD analysis (Cognato et al., 1995). The next logical step would be to integrate cuticular hydrocarbon and semiochemical characters with protein, nucleic acid, and morphological characters.

Lanier (1970) and Wood (1982) found that *I. paraconfusus*, *I. confusus*, and *I. hoppingi* are closely related and separated with difficulty based on morphology. Indeed, Wood (1982) remained doubtful of the species status of *I. hoppingi*. These three sibling species occur in parapatric couplets (Lanier and Burkholder, 1974). *I. paraconfusus* and *I. confusus* have a zone of sympatry in southern California (Fox et al., 1991; Merrill, 1991), while *I. confusus* and *I. hoppingi* appear to have zones of sympatry in the desert mountains of Arizona, New Mexico, and Texas (Cane et al., 1990a; Merrill, 1991). *I. paraconfusus* and *I. hoppingi* have allopatric distributions. There is no evidence of hybridization between the three species (Merrill, 1991). Separation of the three sibling species had been based on two destructive methods: scanning electron microscope (SEM) analysis of the interstrial distance in the female *pars stridens* (Lanier, 1970; Merrill, 1991) and cytological analysis (Lanier, 1970). However, Merrill (1991) has found that the distribution of the mean interstrial distance overlaps between *I. confusus* and *I. hoppingi* as well as between *I. paraconfusus* and *I. confusus*. Until recently, only host association, cytological analysis, or cross-breeding could be used to achieve absolute species discrimination.

However, using RAPD analysis, Cognato et al. (1995) recently demonstrated a diagnostic DNA banding pattern for each species. Despite evidence of

variation in banding pattern between individuals and populations, they reported three diagnostic bands to discriminate *I. hoppingi* from *I. confusus*; 11 to discriminate *I. paraconfusus* from *I. confusus*; and 11 to discriminate *I. paraconfusus* from *I. hoppingi*. The technique has merit because it can be applied to individuals; however, it apparently only works optimally on tissue frozen alive at −80°C.

In a practical sense, we have found that sibling species can be discriminated easily by analysis of their cuticular hydrocarbons (Table 3), based on a similar number of diagnostic characters as described by Cognato et al. (1995). Living or deep frozen tissue is not required, and there appears to be little interpopulational variation in phenotype. Although both techniques require a considerable investment in technology, diagnostic hydrocarbons and diagnostic RAPD banding patterns provide the "discrete distinguishing characters" noted to have been absent for the sibling species (Cane et al., 1990b).

Evolution of variation in the cuticular hydrocarbon complement for *Ips* in the *grandicollis* group may have been an important early step in speciation. Long-distance behavioral discrimination related to host finding and response to con- and heterospecific aggregation pheromones has been well studied (Lanier and Wood, 1975; Cane et al., 1990a,c; Fox et al., 1991). Close-range discrimination in *Ips* may be related to sonic transmission (Barr, 1969), although recent evidence (Lewis and Cane, 1992) clearly suggests otherwise. While female-produced vibratory signals of the sibling *Ips* spp. may have different quantitative characteristics, in laboratory (Lewis and Cane, 1992) and field (Fox et al., 1991) studies they are apparently not associated with an absolute communication barrier for premating isolation of heterospecific sexes. Close-range heterospecific discrimination in other insects has been found to involve contact detection of hydrocarbons [e.g., *Camponotus floridanus* (Morel et al., 1988); *Camponotus vagus* (Bonavita-Cougourdan et al., 1987), *Pseudomyrmex* spp. (Mintzer, 1989), *Drosophila* spp. (Cobb and Jallon, 1990), other taxa reviewed in Howard (1993)]. *Ips* species may utilize hydrocarbon components as part of the close-range heterospecific/conspecific recognition process.

Besides possible close-range communication, another biological significance of cuticular hydrocarbons is as a physical barrier to water loss. *I. confusus* and *I. hoppingi*, which have relatively few differences in their hydrocarbon profiles (Table 3), are both found in extremely xeric climates. Perhaps similar climatic conditions have provided selection pressure for similar hydrocarbon phenotypes. However, abundance of different hydrocarbons may simply allow for redundancy in functionality in the water barrier. Certain components may be isofunctional vis-à-vis water loss and hence interchangeable. Furthermore, the species-specific complement of cuticular hydrocarbons may be related to species-specific host or environmental relationships. For example, in their zone of sympatry, *I. confusus* colonizes *Pinus monophylla* Torr. & Fr., while *I.*

paraconfusus colonizes *Pinus coulteri* D. Don. The different biochemical environment presented by each host species may have necessitated correspondingly different exterior biochemical coatings for each of these endophytic insect species.

Sexual dimorphism in cuticular hydrocarbon phenotype is normally associated with insects that utilize these hydrocarbons as pheromones (Howard, 1993). This has been documented in the Diptera (Blomquist et al., 1987a; Carlson et al., 1971; Nelson and Carlson, 1986; Nelson et al., 1988), primarily the Drosophilidae (Cobb and Jallon, 1990; Jackson and Bartelt, 1986; Jallon, 1984; Oguma et al., 1992a,b; Coyne et al., 1994). Sex-dependent, quantitative differences in certain cuticular hydrocarbons of beetles have been reported in *Rhagonycha fulva* (Cantharidae) (Jacob, 1978); *Leptinotarsa decemlineata* (Chrysomelidae) (Dubis et al., 1987); and *Diabrotica* species (Chrysomelidae) (Golden et al., 1992). Peschke and Metzler (1987) demonstrated a male behavioral response from two female-specific cuticle-based monoenes [(*Z*)-7-heneicosene and (*Z*)-7-tricosene] produced by *Aleochara curtula* (Coleoptera: Staphylinidae).

Female *I. lecontei* also possess an ensemble of sex-specific cuticular hydrocarbons that may play a role in courtship behavior. In limited studies of close-range discrimination behavior, Lewis and Cane (1992) found that female *I. lecontei* were unanimously rejected by males of two heterospecifics and nearly unanimously accepted by conspecific males. Thus, the female-specific compounds may play a role in proximal species recognition as well as courtship.

Behavioral assays that employ crude cuticular lipid extracts or unique components may help us to elucidate the process of sex-specific (*I. lecontei*) or heterospecific (all species) close-range discrimination. Cuticular hydrocarbon transfer techniques recently described for rhinotermitid termites may be useful (Takahashi and Gassa, 1995). The absence of sexual dimorphism in cuticular hydrocarbons for the remaining *Ips* species suggests that sex-specific discrimination and other mating-related behaviors are not likely to be mediated by hydrocarbons in these species. The possibility remains that there is latent sexual dimorphism related to the enantiomeric composition of the methyl-branched alkanes. By analogy, however, three members of the *melanogaster* subgroup of *Drosophila* species display marked sexual dimorphism in cuticular hydrocarbons, while five other species in the group do not. Nonetheless, behavioral assays indicate that interspecific differences in drosophilid cuticular hydrocarbons appear sufficient to regulate both conspecific mate recognition and courtship stimulation (Coyne and Oyama, 1995), even in cases where sexual dimorphism does not exist (Cobb and Jallon, 1990).

We have demonstrated that a new suite of characters, cuticular hydrocarbons, corroborates existing taxonomic identities of all seven species of *Ips* in the *grandicollis* group. Our reconstructed phylogenies based on hydrocarbon

phenotypes are consistent with morphological concepts of relatedness. Organizing insects according to their cuticular hydrocarbon phenotypes does not always produce an outcome that mirrors the accepted taxonomic picture. For example, Bartelt et al. (1986), working on *Drosophila* spp. in the *virilis* group, and Lockey and Metcalfe (1988), both used cluster analyses to evaluate their hydrocarbon data, but found that their dendrograms were not perfectly correlated with established taxonomic relationships. Kaib et al. (1991) found that phylogenetic analysis of cuticular hydrocarbon profiles produced results different from the morphometrical data that divided *Odonotermes* into two groups. They concluded, however, that hydrocarbon profiles originating from workers and size-correlated morphological data taken from soldiers yielded basically congruent groupings. Cases where correspondence is not optimal should not, however, minimize the utility of cuticular hydrocarbons for discriminating insect species, elucidating evolutionary relationships, or uncovering the chemical basis of proximal behavioral interactions.

Acknowledgments—This manuscript is dedicated to the late Paul Tilden, our dear friend and colleague who provided valuable assistance in collecting *Ips* species and shared our interests in the Scolytidae. We are grateful to D. L. Wood, University of California at Berkeley for suggesting this project and for his critical review of the manuscript. This work would not have been possible without the original curated specimens of *I. paraconfusus* and *I. confusus* provided by J. H. Cane, Auburn University. We are indebted to Donald E. Bright, Biosystematics Research Centre, Agriculture Canada, Ottawa, Ontario, Canada, for verifying our identifications of *Ips* species. We thank R. W. Howard, United States Department of Agriculture, Agricultural Research Service, for his invaluable technical assistance in interpretation of double bond locations in DMDS derivatized alkenes. Our cooperators, S. P. Cook, formerly at North Carolina State University (*I. grandicollis*), R. A. Haack, USDA Forest Service, East Lansing, Michigan (*O. caelatus*), J. Flores Lara and C. Garza Quintanilla, Universidad Autonoma de Nuevo Leon (*I. hoppingi*), L. D. Merrill, USDA Forest Service, Forest Pest Management, San Bernardino, California (*I. hoppingi*), B. L. Strom, USDA Forest Service, Pineville, Louisiana (*I. grandicollis*), and S. A. Teale and A. I. Cognato, State University of New York, Syracuse, New York (*O. caelatus*) kindly provided us with some of the insects for this study. Finally, this manuscript was greatly improved by S. A. Teale, who initially suggested that we utilize PAUP to analyze the data and, especially, by B. D. Mishler, University of California at Berkeley, who guided our use and interpretation of PAUP and introduced us to decay analysis.

REFERENCES

BARR, B. A. 1969. Sound production in Scolytidae (Coleoptera) with emphasis on the genus *Ips*. *Can. Entomol.* 101:636–672.

BARTELT, R. J., ARMOLD, M. T., SCHANER, A. M., and JACKSON, L. L. 1986. Comparative analysis of cuticular hydrocarbons in the *Drosophila virilis* species group. *Comp. Biochem. Physiol.* 83B:731–742.

BLOMQUIST, G. J., MAJOR, M. A., and LOK, J. B. 1975. Biosynthesis of 3-methylpentacosane in the cockroach *Periplaneta americana*. *Biochem. Biophys. Res. Commun.* 64:43–50.

BLOMQUIST, G. J., HOWARD, R. W., and MCDANIEL, C. A. 1979. Biosynthesis of the cuticular hydrocarbons of the termite *Zootermopsis angusticollis* (Hagen). Incorporation of propionate into dimethylalkanes. *Insect Biochem.* 9:371–374.

BLOMQUIST, G. J., CHU, A. J., NELSON, J. H., and POMONIS, J. G. 1980a. Incorporation of [2,3-^{13}C]succinate into methyl-branched alkanes in a termite. *Arch. Biochem. Biophys.* 204:648–650.

BLOMQUIST, G. J., HOWARD, R. W., MCDANIEL, C. A., REMALEY, S., DWYER, L. A., and NELSON, D. R. 1980b. Application of methoxymercuration–demercuration followed by mass spectrometry as a convenient microanalytical technique for double-bond location in insect-derived alkenes. *J. Chem. Ecol.* 6:257–269.

BLOMQUIST, G. J., DILLWITH, J. W., and ADAMS, T. S. 1987a. Biosynthesis and endocrine regulation of sex pheromone production in Diptera, pp. 217–250, *in* G. D. Prestwich and G. J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, New York.

BLOMQUIST, G. J., NELSON, D. R., and DE RENOBALES, M. 1987b. Chemistry, biochemistry, and physiology of insect cuticular waxes. *Arch. Insect Biochem. Physiol.* 6:227–265.

BONAVITA-COUGOURDAN, A., CLEMENT, J. L., and LANGE, C. 1987. Nestmate recognition: The role of cuticular hydrocarbons in the ant *Camponotus vagus Scop. J. Entomol. Sci.* 22:1–10.

BRIGHT, D. E. 1993. Systematics of bark beetles, pp. 23–36, *in* T. D. Schowalter and G. M. Filip (eds.). Beetle-Pathogen Interactions in Conifer Forests. Academic Press, London.

BRIGHT, D. E., and STOCK, M. W. 1982. Taxonomy and geographic variation, pp. 46–73, *in* J. B. Mitton and K. B. Sturgeon (eds.). Bark Beetles in North American Conifers. University of Texas Press, Austin.

BROOKS, D. R., and MCLENNAN, D. A. 1991. Phylogeny, Ecology, and Behavior. University of Chicago Press, Chicago.

BROWNE, L. E. 1972. An emergence cage and refrigerated collector for wood-boring insects and their associates. *J. Econ. Entomol.* 65:1499–1501.

CANE, J. H., MERRILL, L. D., and WOOD, D. L. 1990a. Attraction of a pinyon pine bark beetle, *Ips hoppingi*, to conspecific and *I. confusus* pheromones (Coleoptera: Scolytidae). *J. Chem. Ecol.* 16:2791–2798.

CANE, J. H., STOCK, M. W., WOOD, D. L., and GAST, S. J. 1990b. Phylogenetic relationships of *Ips* bark beetles (Coleoptera: Scolytidae): Electrophoretic and morphometric analyses of the *grandicollis* group. *Biochem. Syst. Ecol.* 18:359–368.

CANE, J. H., WOOD, D. L., and FOX, J. W. 1990c. Ancestral semiochemical attraction persists for adjoining populations of sibling *Ips* bark beetles (Coleoptera: Scolytidae). *J. Chem. Ecol.* 16:993–1013.

CARLSON, D. A., and BOLTEN, A. B. 1984. Identification of Africanized and European honey bees, using extracted hydrocarbons. *Bull. Entomol. Soc. Am.* 30:32–35.

CARLSON, D. A., and BRENNER, R. J. 1988. Hydrocarbon-based discrimination of three North American *Blattella* cockroach species (Orthoptera: Blattellidae) using gas chromatography. *Ann. Entomol. Soc. Am.* 51:711–723.

CARLSON, D. A., and SERVICE, M. W. 1980. Identification of mosquitoes of *Anopheles gambiae* species complex A and B by analysis of cuticular components. *Science* 207:1089–1091.

CARLSON, D. A., MAYER, M. S., SILHACEK, D. L., JAMES, J. D., BEROZA, M., and BIERL, B. A. 1971. Sex attractant pheromone of the house fly: Isolation, identification, and synthesis. *Science* 174:76–78.

CASTNER, J. L., and NATION, J. L. 1986. Cuticular lipids for species recognition of mole crickets (Orthoptera: Gryllotaplidae) *Scapteriscus didactylus*, *Scapteriscus imitatus*, and *Scapteriscus vicinus*. *Fla. Entomol.* 67:155–160.

CLEMENT, J. L., LANGE, C., BLUM, M. S., HOWARD, R., and LLOYD, H. 1985. Chemiosystemique du genre *Reticulitermes* (Isoptera) aux U.S.A. et en Europe. *Actes Coll. Insectes Soc.* 2:123–132.

CLEMENT, J. L., BONAVITA-COUGOURDAN, A., and LANGE, C. 1987. Nestmate recognition and

cuticular hydrocarbons in *Camponotus vagus*, pp. 473–474, *in* J. Eder and H. Rembold (eds.). Chemistry and Biology of Social Insects. Verlag J. Peperny, Munich.

COBB, M., and JALLON, J. M. 1990. Pheromones, mate recognition and courtship stimulation in the *Drosophila melanogaster* species sub-group. *Anim. Behav.* 39:1058–1067.

COGNATO, A. I., ROGERS, S. O., and TEALE, S. A. 1995. Species diagnosis and phylogeny of the *Ips grandicollis* group (Coleoptera: Scolytidae) using random amplified polymorphic DNA. *Ann. Entomol. Soc. Am.* 88:397–405.

COYNE, J. A., and OYAMA, R. 1995. Localization of pheromonal sexual dimorphism in *Drosophila melanogaster* and its effect on sexual isolation. *Proc. Natl. Acad. Sci. U.S.A.* 92:9505–9509.

COYNE, J. A., CRITTENDEN, A. P., and MAH, K. 1994. Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. *Science* 265:1461–1464.

DE RENOBALES, M., NELSON, D. R., and BLOMQUIST, G. J. 1991. Cuticular Lipids, pp. 240–251, *in* K. Binnington and A. Retnakaran (eds.). Physiology of the Insect Epidermis. CSIRO, Melbourne, Australia.

DILLWITH, J. W., NELSON, J. H., POMONIS, J. G., NELSON, D. R., and BLOMQUIST, G. J. 1982. A ^{13}C-NMR study of methyl-branched hydrocarbon biosynthesis in the housefly. *J. Biol. Chem.* 257:11305–11314.

DUBIS, E., MALINSKI, E., DUBIS, A., SZAFRANEK, J., NAWROT, J., POPLAWSKI, J., and WROBEL, J. T. 1987. Sex-dependent composition of cuticular hydrocarbons of the Colorado potato beetle, *Leptinotarsa decemlineata* Say. *Comp. Biochem. Physiol.* 87A:839–843.

DWYER, L. A., BLOMQUIST, G. J., NELSON, J. H., and POMONIS, J. G. 1981. A ^{13}C-NMR study of the biosynthesis of 3-methylpentacosane in the American cockroach. *Biochem. Biophys. Acta* 663:536–544.

FARRIS, J. S. 1989. The retention index and the rescaled consistency index. *Cladistics* 5:417–419.

FOX, J. W., WOOD, D. L., and CANE, J. H. 1991. Interspecific pairing between two sibling *Ips* species (Coleoptera: Scolytidae). *J. Chem. Ecol.* 17:1421–1435.

FRANCIS, G. W., and VELAND, K. 1981. Alkylthiolation for the determination of double bond positions in linear alkenes. *J. Chromatogr.* 219:379–384.

FURNISS, R. L., and CAROLIN, V. M. 1977. Western forest insects. US Department of Agriculture Miscellaneous Publication No. 1339, 654 pp.

GOLDEN, K. L., MEINKE, L. J., and STRANLEY-SAMUELSON, D. W. 1992. Cuticular hydrocarbon discrimination of *Diabrotica* (Coleoptera: Chrysomelidae) sibling species. *Ann. Entomal. Soc. Am.* 85:561–570.

HARVEY, G. T. 1985. The taxonomy of the coniferophagous *Choristoneura* (Lepidoptera: Tortricidae): A review, pp. 16–48, *in* C. J. Sanders, R. W. Stark, E. J. Mullins, and J. Murphy (eds.). Recent Advances in Spruce Budworms Research, Proceedings of the CANUSA Spruce Budworms Research Symposium, Bangor Maine, September 16–20, 1984.

HAVERTY, M. I., PAGE, M., NELSON, L. J., and BLOMQUIST, G. J. 1988. Cuticular hydrocarbons of the dampwood termites, *Zootermopsis*: Intra- and intercolony variation and potential as taxonomic characters. *J. Chem. Ecol.* 14:1035–1058.

HAVERTY, M. I., PAGE, M., and BLOMQUIST, G. J. 1989. Value of cuticular hydrocarbons for identifying morphologically similar species of pine cone beetles, G. E. Miller (compiler). pp. 50–63, in Proceedings, 3rd Cone and Seed Insects Working Party Conference, Working Party S2.07-01, International Union of Forestry Research Organizations. June 26–30, 1988, Victoria, British Columbia, Canada.

HAVERTY, M. I., NELSON, L. J., and PAGE, M. 1990a. Cuticular hydrocarbons of four populations of *Coptotermes formosanus* Shiraki in the United States: Similarities and origins of introductions. *J. Chem. Ecol.* 16:1635–1647.

HAVERTY, M. I., THORNE, B. L., and PAGE, M. 1990b. Surface hydrocarbon components of two species of *Nasutitermes* from Trinidad. *J. Chem. Ecol.* 16:2441–2450.

HAVERTY, M. I., PAGE, M., THORNE, B. L., and ESCOUBAS, P. 1991a. Cuticular hydrocarbons: Species and population-level discrimination in termites. USDA Forest Service General Technical Report PSW-128, pp. 15–23.

HAVERTY, M. I., NELSON, L. J., THORNE, B. L., COLLINS, M. S., DARLINGTON, J. P. E. C., and PAGE, M. 1991b. Cuticular hydrocarbons for species determination of tropical termites. USDA Forest Service General Technical Report PSW-129, 9 pp.

HOPPING, G. R. 1963. The natural groups of species in the genus *Ips* De Geer (Coleoptera: Scolytidae) in North America. *Can. Entomol.* 95:508–516.

HOPPING, G. R. 1965. North American species in group IX of *Ips* De Geer (Coleoptera: Scolytidae). *Can. Entomol.* 97:422–434.

HOWARD, R. W. 1993. Cuticular lipids and chemical communication, pp. 179–226, *in* D. W. Stanley-Samuelson and D. R. Nelson (eds.). Insect Lipids: Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln.

HOWARD, R. W., MCDANIEL, C. A., and BLOMQUIST, G. J. 1978. Cuticular hydrocarbons of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar) (Isotpera: Rhinotermitidae). *J. Chem. Ecol.* 4:233–245.

HOWARD, R. W., MCDANIEL, C. A., NELSON, D. R., BLOMQUIST, G. J., GELBAUM, L. T., and ZALKOW, L. H. 1982. Cuticular hydrocarbons as possible species and caste-recognition cues in *Reticulitermes* spp. *J. Chem. Ecol.* 8:1227–1239.

HOWARD, R. W., KUWAHARA, Y., SUZUKI, H., and SUZUKI, T. 1988. Pheromone study on acarid mites. XII. Characterization of the hydrocarbons and external gland morphology of the opisthonotal glands of six species of mites (Acari: Astigmata). *Appl. Entomol. Zool.* 23:58–66.

JACKSON, L. L., and BARTELT, R. J. 1986. Cuticular hydrocarbons of *Drosophila virilis*: Comparison by age and sex. *Insect Biochem.* 16:433–439.

JACOB, J. 1977. The cuticular lipids of the beetle *Phyllobius maculicornis*. Hoppe-Seyler's Z. *Physiol. Chem.* 368:1375–1377.

JACOB, J. 1978. Sex-dependent composition of cuticular lipids from the beetle *Rhagonycha fulva*. *Hoppe-Seyler's Z. Physiol. Chem.* 359:653–656.

JACOB, J. 1979. Chemotaxonomic investigations on the cuticular lipids of beetles. *Biochem. Syst. Ecol.* 7:141–145.

JALLON, J. M. 1984. A few chemical words exchanged by *Drosophila* during courtship and mating. *Behav. Genet.* 14:441–478.

JUAREZ, P., CHASE, J., and BLOMQUIST, G. J. 1992. A microsomal fatty acid synthetase from the integument of *Blattella germanica* synthesizes methyl branched fatty acid precursors to hydrocarbon and contact sex pheromone. *Arch. Biochem. Biophys.* 293:333–341.

KAIB, M., BRANDL, R., and BAGINE, R. K. N. 1991. Cuticular hydrocarbon profiles: A valuable tool in termite taxonomy. *Naturwissenschaften* 78:176–179.

KLUGE, A. G., and FARRIS, J. S. 1969. Quantitative phyletics and the evolution of anurans. *Syst. Zool.* 18:1–32.

LANIER, G. N. 1970. Biosystematics of North American *Ips* (Coleoptera: Scolytidae) Hopping's group IX. *Can. Entomol.* 102:1139–1163.

LANIER, G. N. 1987. The validity of *Ips cribricollis* (Eich.) (Coleoptera: Scolytidae) as distinct from *I. grandicollis* (Eich.) and the occurrence of both species in Central America. *Can. Entomol.* 119:179–187.

LANIER, G. N., and BURKHOLDER, W. E. 1974. Pheromones in speciation of Coleoptera, pp. 161–189, *in* M. C. Birch (ed.). Pheromones. North Holland, Amsterdam.

LANIER, G. N., and CAMERON, E. A. 1969. Secondary sexual characters in the North American species of the genus *Ips* (Coleoptera: Scolytidae). *Can. Entomol.* 101:862–870.

LANIER, G. N., and WOOD, D. L. 1975. Specificity of response to pheromones in the genus *Ips* (Coleoptera: Scolytidae). *J. Chem. Ecol.* 1:9–23.

LEWIS, E. E., and CANE, J. H. 1992. Inefficacy of courtship stridulation as a premating ethological barrier for *Ips* bark beetles (Coleoptera: Scolytidae). *Ann. Entomol. Soc. Am.* 85:517–524.

LIGHT, D. M., and BIRCH, M. C. 1979. Inhibition of the attractive pheromone response in *Ips paraconfusus* by *R*-(−)-ipsdienol. *Naturwissenschaften* 66:159–160.

LOCKEY, K. H. 1979. Cuticular hydrocarbons of adult *Alphitophagus bifasciatus* (Say) and *Alphitophagus diaperinus* (Panz.) (Coleoptera: Tenebrionidae). *Comp. Biochem. Physiol.* 64B:47–56.

LOCKEY, K. H. 1981. Cuticular hydrocarbons of adult *Cylindrinotus laevioctostriatus* (Goeze) and *Phylan gibbus* (Fabricius) (Coleoptera: Tenebrionidae). *Insect Biochem.* 11:549–561.

LOCKEY, K. H. 1984a. Hydrocarbons of adult *Zophosis* (*Gyrosis*) species and *Zophosis* (*Onychosis*) *gracilipes* (Deyrolle) (Coleoptera: Tenebrionidae). *Insect Biochem.* 14:645–656.

LOCKEY, K. H. 1984b. Hydrocarbons of *Metriopus depressus* (Haag) and *Renatiella scrobipennis* (Haag) (Coleoptera: Tenebrionidae). *Insect Biochem.* 14:65–75.

LOCKEY, K. H. 1988. Lipids of the insect cuticle: Origin, composition, and function. *Comp. Biochem. Physiol.* 89B:595–645.

LOCKEY, K. H. 1991a. Cuticular hydrocarbons of adult *Onymacris bicolor* (Haag) and *Onymacris boschimana* (Peringuey) (Coleoptera: Tenebrionidae). *Comp. Biochem. Physiol.* 98B:151–163.

LOCKEY, K. H. 1991b. Insect hydrocarbon classes: Implications for chemotaxonomy. *Insect Biochem.* 21:91–97.

LOCKEY, K. H. 1992. Insect hydrocarbon chemotaxonomy: Cuticular hydrocarbons of adult and larval *Epiphysa* species Blanchard and adult *Onymacris unguicularis* (Haag) (Coleoptera: Tenebrionidae). *Comp. Biochem. Physiol.* 102B:451–470.

LOCKEY, K. H., and METCALFE, N. B. 1988. Cuticular hydrocarbons of adult *Himatismus* species and a comparison with 21 other species of adult tenebrionid beetle using multivariate analysis. *Comp. Biochem. Physiol.* 91B:371–382.

MARTIN, M. M., and MACCONNELL, J. G. 1970. The alkanes of the ant, *Atta columbica*. *Tetrahedron* 26:307–319.

MERRILL, L. D. 1991. Biological barriers to hybridization in closely related species of *Ips* (Coleoptera: Scolytidae). PhD thesis. University of California at Berkeley, 121 pp.

MINTZER, A. C. 1989. Incompatibility between colonies of *Acacia* ants: Genetic models and experimental results, pp. 163–182, *in* M. D. Breed and R. E. Page (eds.). Westview Studies in Insect Biology: The Genetics of Social Evolution, VIII. Westview Press, Boulder.

MISHLER, B. D. 1994. Cladistic analysis of molecular and morphological data. *Am. J. Phys. Anthropol.* 94:143–156.

MISHLER, B. D., DONOGHUE, M. J., and ALBERT, A. 1991. The decay index as a measure of relative robustness within a cladogram. Hennig X, Toronto, Ontario (abstract).

MISHLER, B. D., LEWIS, L. A., BUCHHEIM, M. A., RENZAGLIA, K. S., GARBURY, D. J., DELWICHE, C. F., ZECHMAN, F. W., KANTZ, T. S., and CHAPMAN, R. L. 1994. Phylogenetic relationships of the "green algae" and "bryophytes." *Ann. Mo. Bot. Gard.* 81:451–483.

MOREL, L., VANDER MEER, R. K., and LAVINE, B. K. 1988. Ontogeny of nestmate recognition cues in the red carpenter ant *Camponotus floridanus*: Behavioral and chemical evidence for the role of age and social experience. *Behav. Ecol. Sociobiol.* 22:175–184.

NELSON, D. R. 1993. Methyl-branched lipids in insects, pp. 271–315, *in* D. W. Stanley-Samuelson and D. R. Nelson (eds.). Insect Lipids: Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln.

NELSON, D. R., and BLOMQUIST, G. J. 1995. Insect waxes, pp. 1–90, *in* R. J. Hamilton (ed.). Waxes: Chemistry, Molecular Biology and Functions. W. W. Christie, The Oily Press, Ltd., Dundee, United Kingdom.

NELSON, D. R., and CARLSON, D. A. 1986. Cuticular hydrocarbons of the tsetse flies *Glossina morsitans morsitans*, *G. austeni*, and *G. pallidipes*. *Insect Biochem.* 16:403–416.

NELSON, D. R., CARLSON, D. A., and FATLAND, C. L. 1988. Cuticular hydrocarbons of tsetse flies

II: *Glossina fuscipes fuscipes*, *G. palpalis palpalis*, *G. p. gambiensis*, *G. tachinoides*, and *G. brevipalpis*. *J. Chem. Ecol.* 14:963–987.

OGUMA, Y., NEMOTO, T., and KUWAHARA, Y. 1992a. (Z)-11-Pentacosene is the major sex pheromone component in *Drosophila virilis* (Diptera). *Chemoecology* 3:60–64.

OGUMA, Y., NEMOTO, T., and KUWAHARA, Y. 1992b. A sex pheromone study of a fruit fly *Drosophila virilis* Sturtevant (Diptera: Drosophilidae): Additive effect of cuticular alkadienes to the major sex pheromone. *Appl. Entomol. Zool.* 27:499–505.

PAGE, M., NELSON, L. J., HAVERTY, M. I., and BLOMQUIST, G. J. 1990a. Cuticular hydrocarbons of eight species of North American cone beetles, *Conophthorus* Hopkins. *J. Chem. Ecol.* 16:1173–1198.

PAGE, M., NELSON, L. J., HAVERTY, M. I., and BLOMQUIST, G. J. 1990b. Cuticular hydrocarbons as chemotaxonomic characters for bark beetles: *Dendroctonus ponderosae*, *D. jeffreyi*, *D. brevicomis* and *D. frontalis* (Coleoptera: Scolytidae). *Ann. Entomol. Soc. Am.* 83:892–901.

PESCHKE, K., and METZLER, M. 1987. Cuticular hydrocarbons and female sex pheromones of the rove beetle, *Aleochara curtula* (Goeze) (Coleoptera: Staphylinidae). *Insect. Biochem.* 17:167–178.

POWELL, J. A. 1995. Introduction: Historical review, taxonomic problems, and research approaches, pp. 1–19, *in* J. A. Powell (ed.). Biosystematic Studies of Conifer-feeding *Choristoneura* (Lepidoptera: Tortricidae) in the Western United States. University of California Publications in Entomology, Berkeley, No. 115.

POWELL, J. A., and DE BENEDICTIS, J. A. 1995a. Biological relationships: Host tree preferences and isolation by pheromones among allopatric and sympatric populations of western *Choristoneura*, pp. 21–68, *in* J. A. Powell (ed.). Biosystematic Studies of Conifer-feeding *Choristoneura* (Lepidoptera: Tortricidae) in the Western United States. University of California Publications in Entomology, Berkely, No. 115.

POWELL, J. A., and DE BENEDICTIS, J. A. 1995b. Evolutionary interpretation, taxonomy, and nomenclature, pp. 217–275, *in* J. A. Powell (ed.). Biosystematic Studies of Conifer-feeding *Choristoneura* (Lepidoptera: Tortricidae) in the Western United States. University of California Publications in Entomology, Berkeley, No. 115.

ROSS, K. G., VANDER MEER, R. K., FLETCHER, D. J., and VARGO, E. L. 1987. Biochemical phenotypic and genetic studies of two introduced fire ants and their hybrid (Hymenoptera: Formicidae). *Evolution* 4:280–293.

SAS. 1990. SAS/STAT Users Guide, Version 6, 4th ed., Vol. 1. SAS Institute, Cary, North Carolina, 890 pp.

SEYBOLD, S. J. 1992. The role of chirality in the olfactory-directed aggregation behavior of pine engraver beetles in the genus *Ips* (Coleoptera: Scolytidae). PhD thesis. University of California at Berkeley, 355 pp.

SEYBOLD, S. J., OHTSUKA, T., WOOD, D. L., and KUBO, I. 1995. The enantiomeric composition of ipsdienol: A chemotaxonomic character for North American populations of *Ips* spp. in the *pini* subgeneric group (Coleoptera: Scolytidae). *J. Chem. Ecol.* 21:995–1016.

SWOFFORD, D. L. 1993. PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.1. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

TAKANO, I., KUWAHARA, Y., HOWARD, R. W., and SUZUKI, T. 1989. Location of double bond positions in conjugated alkadienes by electron impact mass spectrometry of 3,4-dimethylthio-substituted thiolane derivatives. *Agric. Biol. Chem.* 53:1413–1415.

TAKAHASI, S., and GASSA, A. 1995. Roles of cuticular hydrocarbons in intra- and interspecific recognition behavior of two Rhinotermitidae species. *J. Chem. Ecol.* 21:1837–1845.

VANDER MEER, R. K. 1986. Chemical taxonomy as a tool for separating *Solenopsis* spp., pp. 316–326, *in* C. S. Lofgren and R. K. Vander Meer (eds.). Fire Ants and Leaf-cutting Ants: Biology and Management. Westview Press, Boulder.

VICENTI, M., GUGLIELMETTI, G., CASSANI, G., and TONINI, C. 1987. Determination of double bond position in diunsaturated compounds by mass spectrometry of dimethyl disulfide derivatives. *Anal. Chem.* 59:694–699.

VOET, D., and VOET, J. G. 1990. Biochemistry. John Wiley & Sons, New York, 1223 pp.

WOOD, S. L. 1966. New synonymy in the Platypodidae and Scolytidae (Coleoptera). *Great Basin Nat. Mem.* 26:17–33.

WOOD, S. L. 1968. New record and species of neotropical bark beetles (Scolytidae: Coleoptera). *Great Basin Nat. Mem.* 28:1–15.

WOOD, S. L. 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. *Great Basin Nat. Mem.* No. 6, 1359 pp.

Journal of Chemical Ecology, Vol. 23, No. 4, 1997

USE OF MIXTURES OF ALLELOCHEMICALS TO COMPARE BIOASSAYS USING RED MAPLE, PIN CHERRY, AND AMERICAN ELM

HÉLÈNE VÉRONNEAU,[1] ANN FRANCINE GREER,[1] STÉPHANE DAIGLE,[1] and GILLES VINCENT[1,2,*]

[1]*Institut de recherche en biologie végétale, Université de Montréal*
[2]*Jardin botanique de Montréal*
4101 Rue Sherbrooke Est
Montréal, Québec, H1X 2B2, Canada

(Received May 15, 1996; accepted November 29, 1996)

Abstract—Toxicity threshold levels for five different bioassays (germination in combination with radicle length, seedling growth, callus growth, plating, and cell suspension growth) were determined on three invasive arborescent species [American elm (*Ulmus americana*), red maple (*Acer rubrum*), and pin cherry (*Prunus pensylvanica*)] using two different mixtures of allelochemicals. Thresholds for the duckweed (*Lemna minor*) bioassay and lettuce (*Lactuca sativa*) seed germination bioassay were also determined for comparison with the same mixtures. All five bioassays showed a sensitivity similar to those of duckweed and lettuce bioassays. The lowest sensitivity was in mixtures containing about 5000 μg/liter of each chemical, but the species tested and the mixture applied influenced the outcome. Some bioassays could not be used with red maple (plating and cell suspension) and pin cherry (germination and seedling growth). Cell suspension growth was the most sensitive in *in vitro* culture, whereas callus culture was the least sensitive. Cell suspension growth in multiwell culture plates should be favored in allelopathy studies because of its sensitivity and the need for relatively small volumes of sample. Detection of phytotoxicity using whole plant systems (germination and seedling growth) and tissue culture appeared not to be directly correlated. We conclude that using many bioassays produces the most conclusive evidence of allelopathic activity.

Key Words—Allelopathy, phenolic acid mixture, bioassays, *Lemna minor*, germination, seedling growth, in vitro culture.

*To whom correspondence should be addressed.

0098-0331/97/0400-1101$12.50/0 © 1997 Plenum Publishing Corporation

INTRODUCTION

Control of arborescent vegetation growth under power lines is expensive. Cutting the vegetation and use of herbicides are two approachs currently employed, but new ones are being investigated. These include the use of allelochemicals produced by plant species compatible with power-line operation that could inhibit germination or growth of undesirable species. Bioassays were developed to assess the phytotoxicity of allelochemicals against three invasive arborescent species: American elm (*Ulmus americana* L.), red maple (*Acer rubrum* L.), and pin cherry (*Prunus pensylvanica* L.f.).

Bioassays are defined as the assessment of the potency of a compound via its response caused by the application of the compound to the subject (Govindarajulu, 1988). They are necessary in each step of the isolation, purification, and identification processes of allelopathic compounds (Leather and Einhellig, 1986). Numerous bioassays have been employed to check the activity of putative allelopathic compounds. The most widely used is seed germination carried out in Petri dishes (see Leather and Einhellig, 1986). However, this test is rarely applied to target species; in fact, lettuce (*Lactuca* sp.) seed has been used extensively as a test because of its fast germination and high sensitivity (Yu and Matsui, 1994; Ohwaki et al., 1993; Macias et al., 1993a,b; Zhang, 1993; Li et al., 1993b). Duckweed (*Lemna minor*) has also been used as test organism, because it is sensitive and easy to manipulate (Einhellig et al., 1985; Jenner and Janssen-Mommen, 1993; Wang and Williams, 1990). Seedling growth bioassays also have been employed with various species (Bradow, 1993; Blum et al., 1993; Gallet, 1994; Lehman et al., 1994). Plant cell and tissue cultures have been considered cost-effective and rapid ways of screening for phytotoxins (Gressel, 1984; Grossmann and Jung, 1984), and results can reflect whole plant sensitivity (Zilkah and Gressel, 1977; Zilkah et al., 1977).

In the present study, five types of bioassay were employed using American elm, red maple, and pin cherry as the test species to evaluate two mixtures of phenolic and carboxylic acids. The bioassays were germination, seedling growth, and tests with in vitro cultures (callus growth, plating, and cell suspension growth). The objectives were: (1) to develop bioassays applicable to our target species, (2) to assess the usefulness of each bioassay in detecting phytotoxic activity, and (3) to determine if levels of phytotoxity detected vary among the different bioassays. Various criteria were taken into consideration: (1) the amount of test solution needed to apply the bioassay, (2) the sensitivity of these bioassays compared with standard biological tests using duckweed and lettuce, and (3) the availability of vegetal material.

METHODS AND MATERIALS

Plant Materials

Seed Source and Seedling Growth Conditions. Seeds of red maple and American elm were collected in June 1994 in the Montreal area (Quebec, Canada). Pin cherry seeds collected in 1992 at Brigham (Quebec, Canada) were provided by the Botanical Gardens of Montreal. All seeds were air-dried and stored at 4°C. Seeds of lettuce (*Lactuca sativa*, Black-seeded Simpson) were obtained from a horticultural company. Red maple and American elm were germinated in vermiculite in a growth chamber (Conviron) under a 16-hr photoperiod, fluorescent and incandescent lights (200–300 μmol photons/m^2/sec) at 23°C day and 20°C night with 65–70% humidity. Seedlings were watered daily and fertilized with full-strength Hoagland's solution once a week.

Initiation of Callus Culture. Callus cultures were initiated from leaves of 15-day-old red maple and American elm seedlings and from stems of in vitro grown seedlings of pin cherry. Explant pieces were transferred to 100- × 15-mm Petri dishes (sealed with parafilm) containing Murashige and Skoog (1962) basal medium (MS) supplemented with 3% sucrose and 0.7% agar (Difco Bacto). The pH was adjusted to 5.7 prior to autoclaving for 20 min. Callus was induced and maintained on a medium containing benzylaminopurine (BA) and α-naphthaleneacetic acid (NAA) (1.0 and 0.1 mg/ml for red maple and American elm and 0.5 and 1 mg/liter for pin cherry). Cultures were maintained in an in vitro culture room at 24°C in the dark. Subcultures were carried out every three to four weeks.

Initiation of Cell Suspension Culture. Friable callus derived from young leaf explant of American elm and pin cherry was used to initiate suspension cultures. Cultures were kept on a gyratory shaker (100 rpm) at 24°C in the dark. Every six to eight days, approximately 8 ml were transferred to 125-ml flasks containing 25 ml of culture medium. The medium was the same as that used for the callus culture, except that concentration of BA was increased to 1.0 mg/liter for the pin cherry cell suspension. For both species, the medium was supplemented with coconut water (20 ml/liter) and casein hydrolysate (250 mg/liter). Since it was impossible to establish isolated cells or small aggregate suspension culture from callus of red maple, this species was omitted from both experiments that required cell suspension (Véronneau et al., 1993).

Duckweed Culture. Aseptic stock cultures of duckweed (7935) were provided by Dr. L. Vasseur (Biology Department, McGill University). Stock cultures were maintained by transferring 15 fronds with a sterile loop to a foam-stopped 250-ml Erlenmeyer flask containing 150 ml of autoclaved modified Hoagland solution (Beaumont et al., 1976) without sucrose at pH 5.8. Cultures were kept in an incubator at 23°C under continuous light (100 μmol photons/m^2/sec) and were transferred to fresh medium every two to three weeks.

Bioassays

Chemicals. Two different mixtures of known allelopathic compounds were used. Fraction 5 (F5) was a mixture of five different acids (saligenin, *p*-coumaric, *t*-cinnamic, *p*-hydroxybenzoic, and picolonic), while fraction 12 (F12) was a mixture of F5 plus seven other substances (amygdalin, esculin, L-proline, theophylline, caffeine, thymol, and tryptophan). The mixture was made of an equal amount of each compound and its concentration (micrograms per liter) represented that quantity of each compound. For all bioassays, pH of the mixture was adjusted and cold-filter sterilized (0.22 μm). Dilutions were prepared by adding cooled (50°C) autoclaved (20 min, 121°C) nutrient solution.

Seed Germination Experiment. Tests were conducted on seeds of red maple, American elm, and lettuce. Pin cherry was excluded because of the long period of stratification needed for germination and the low percentage obtained (Laidlaw, 1987). Maple and elm seeds were dewinged and pericarps removed. Prior to treatment, cleaned maple seeds were kept under cold running water overnight. Seeds were surface sterilized for 1 min in 1% sodium hypochlorite solution and subsequently rinsed three times with sterile bidistilled water. Germination was evaluated in parafilmed (100 × 15 mm) Petri dishes with 4.5 ml sterile bidistilled water with a known concentration of mixture (pH 6) that saturated two layers of Whatman No. 1 filter paper. Lettuce and elm germination was carried out at 24°C, 8-hr photoperiod (light intensity 30 μmol photons/m^2 sec). For maple, conditions were 20°C day and 30°C night with an 8-hr photoperiod. Germination (root output > 2 mm from seed shell) was recorded daily (5 days for lettuce, 10 for elm, and 15 for maple). On the final day, root length was measured. A setup of 20 elm, 20 lettuce, and 30 maple seeds for each concentration and mixture formed an experimental block. The experiment consisted of five blocks randomly distributed within the incubator.

Seedling Growth Experiment. Two-leaf-stage, 4-week-old red maple seedlings and 2-week-old American elm seedlings at the two-leaf stage were selected on the basis of uniformity and weight. Selected seedlings weighed 140–210 mg (maple) and 90–130 mg (elm). Roots were cleaned with distilled water and seedlings transferred to 25-ml glass scintillation vials containing 15 ml Hoagland's solution (Jones, 1983) with a known concentration of mixture. Dilutions were prepared in a 2:5 ratio of Hoagland's solution and autoclaved bidistilled water. The pH of all solutions was adjusted to 6.0. Small pieces of cotton were placed in the mouths of the vials to limit evaporation. Experiments were carried out in a growth chamber under the conditions previously described. Seedlings were harvested 14 days after treatment, dried at 60°C, and weighed after 48 hr. The experiment consisted of four blocks of three replicates.

Callus Growth Experiment. Callus growth experiments were done with pin cherry, red maple, and American elm. Preliminary tests demonstrated that an

initial weight of 100 mg (fresh weight) of callus resulted in a good proliferation rate (Véronneau et al., 1993). Five pieces of callus weighting 100 ± 4 mg, cultured for 24 days, which corresponded to late exponential phase of growth (Véronneau et al., 1993), were transferred to 100- × 15-mm Petri dishes sectioned in half. One half contained 13 ml test medium with F5, while the other half had the same concentration with F12. The pH of all solutions was adjusted to 5.7. After 24 days in culture (24°C, dark), the calli were dried for 48 hr at 60°C and weighed. The experiment consisted of 10 blocks.

Plating Growth Experiment. Plating experiments were carried out on pin cherry and American elm cell suspensions. Tests were done following a modified method of the double filter paper technique (Horsch et al., 1980) and the filter paper growth assay (Horsch and Jones, 1980). Tests were set up in 60- × 15-mm Petri dishes containing 13 ml test medium. A filter paper (Whatman No. 1) was fitted over the medium of the Petri dish and a second preweighed filter paper (Whatman No. 2, 4.5 cm) was placed on top of the first. Cultures were asceptically filtered under vacuum onto a 20-μm nylon filter, and cells were transferred to a beaker containing the appropriate medium for each species (same medium use for cell suspension cultures). The mixture was stirred continuously. The slurry was prepared to obtain a concentration of 100 mg of cells when 350 μl of the slurry was pipetted. Preliminary tests indicated that an initial weight of 100 mg of inoculated cells and 16 days of culture were optimal for a good proliferation rate (Véronneau et al., 1993). Cells (350 μl of slurry) were distributed evenly over the top filter. After 16 days (dark, 24°C), dry weights of the top filter holding cells were taken after 48 hr at 60°C. The experiment consisted of four blocks of three replicates.

Cell Suspension Growth Experiment. Pin cherry and American elm cells were collected by filtering suspension cultures under gentle suction onto a 20-μm nylon mesh. Cells were weighed and placed in a beaker containing cell suspension medium adequate for each species with known concentrations of the mixture. Dilutions for the test solution were prepared as for the plating experiment, without the agar. The cell density was adjusted to 10 mg/ml. Under gentle agitation, 3 ml of prepared suspension in solution was pipetted into each well of a 12-well plate. Plates were kept on a gyratory shaker at 100 rpm. After eight days, cells were transferred to Eppendorf tubes, centrifuged at 10,000 rpm for 10 min, the supernant discarded, and dry weights measured after 48 hr at 60°C. The experiment consisted of four block of three replicates.

Duckweed Experiment. The duckweed bioassay was carried out as described by Einhellig et al. (1985). Colonies with three similar-sized fronds void of blemishes were selected. One colony was transferred to each well of a 24-well tissue culture cluster plate containing 1.5 ml of culture medium (modified Hoagland's solution; Beaumont et al., 1976) with the appropriate concentration of the allelochemical mixture. The pH was adjusted to 6 prior to autoclaving for

the Hoagland's solution and prior to cold filtration sterilization for the allelochemical mixture. Each plate was composed of six concentrations and four replicates. After seven days in culture (23°C, 100 μmol photons/m^2 sec), the total number of fronds per well was counted. Experiment consisted of five blocks.

Experimental Design and Statistical Analysis. Each bioassay was designed as a split-plot model with concentration as the main plot factor and fraction and species as subplots. Blocks were fully repeated setups randomly distributed within the incubator or growth chamber. Analysis of variance (ANOVA) was computed on growth parameters (number or fronds, percentage of germination, root length, dry weight). The ANOVA was followed by Tukey's multiple-range tests at the 0.05 level of probability. Statistical analysis was performed with subprogram GLM of SAS/PC package version 6.03 (SAS, 1989).

RESULTS

Seed Germination Experiment. No significant phytotoxic germination effects were noticed (ANOVA $P > 0.05$ for each day; results presented only for last day). On the last day, percent germination differed significantly among species (Table 1). Mean percentages were 52.9% for red maple, 83.8% for American elm, and 98.6% for lettuce (Table 2). In terms of radicle growth, all main effects and interactions were significant (Table 1). The significant interaction fraction × concentration indicated that each fraction at the different concentrations had different effects on radicle growth. The significant interaction species × concentrations indicated that each species has a different level of sensitivity. Root growth of red maple was not affected by any fractions. For American elm, inhibition of root elongation was significant from 1000 μg/liter for F12 and 5000 μg/liter for F5. For lettuce, low concentrations (0.01 and 10 μg/liter) of F12 seemed to promote the growth of the radicle from 10 μg/liter, while for both fractions, growth was inhibited from 5000 μg/liter.

Seedling Growth Experiment. At concentrations below 10 mg/liter, no phytotoxic effects were detected during a preliminary experiment (ANOVA $P > 0.05$). Results of the experiment performed with higher concentrations are presented in Tables 1 and 3. The significant effects are species, concentration, and the interaction fraction × concentration (Table 1). No significant differences in seedling dry weight were observed with F5, while F12 exhibited significant phytotoxicity at concentrations higher than 1000 μg/liter (Table 3). At a concentration of 10,000 μg/liter, leaves began to wilt, and the first signs of leaf decoloration appeared in both red maple and American elm.

Callus Growth Experiment. The growth of callus of 100 mg (fresh weight) in culture for 24 days was first tested with concentrations of 0.01, 10, and 1000

TABLE 1. SUMMARY OF ANALYSIS OF VARIANCE FOR GROWTH PARAMETERS FOR SEEDLING GROWTH AND GERMINATION BIOASSAYS[a]

	Bioassays					
	Seedling growth		Germination			
	Dry weight		Percentage		Radicle growth	
Source of variation	*df*	*P* > *F*	*df*	*P* > *F*	*df*	*P* > *F*
S	1	0.0001	2	0.0001	2	0.0001
B (S)	6	0.8189	12	0.0002	12	0.0001
FRAC	1	0.5035	1	0.5162	1	0.0413
S × FRAC	1	0.3412	2	0.1895	2	0.5191
B × FRAC(S)	6	0.3610	12	0.0537	12	0.0005
CONC	4	0.0001	6	0.3512	6	0.0001
S × CONC	4	0.1182	12	0.5378	12	0.0001
B × CONC (S)	24	0.5342	72	0.0382	72	0.0001
FRAC × CONC	4	0.0016	6	0.0118	6	0.0001
S × FRAC × CONC	4	0.0860	12	0.3470	12	0.0002
B × FRAC × CONC (S)	24	0.6731		[b]	72	0.0001
R^2		0.76802		0.96784		0.59649

[a]The effects included in the analysis were: species, S; blocks within species, B(S); fractions, FRAC; concentrations of the fractions, CONC; and their interactions.
[b]Not included in the model.

μg/liter for F12 and F5. No significant differences were detected ($P > 0.05$) when compared to the control for final dry weight (data not presented). For higher concentrations (5000, 10,000, and 20,000 μg/liter), the sources of variation were related to species, concentration, and the interaction species × concentration (Table 4). Interactions involving fraction were not significant. Final dry weights of pin cherry calli did not differ when compared against the control, even at the highest concentration (20,000 μg/liter), while for maple and American elm, changes in dry weight, regardless of the fraction, were significant from 10,000 μg/liter and 20,000 μg/liter, respectively (Table 5).

Plating Growth Experiment. Concentration and the interaction species × concentration were the major sources of variation (Table 4). Both fractions produced a strong inhibition on plate cells of American elm from concentrations of 5000 μg/liter (Table 6), while the pin cherry phytotoxic effect was significant only at the 20,000 μg/liter concentration. After 16 days of incubation, differences in growth were apparent and affected cells became darker in color.

Cell Suspension Growth Experiment. The most important sources of vari-

TABLE 2. EFFECT OF DIFFERENT CONCENTRATIONS OF FRACTIONS F12 AND F5 ON SEED GERMINATION[a] AND RADICLE GROWTH[b] OF ELM, MAPLE, AND LETTUCE[c]

Concentrations (μg/liter)	F12				F5			
	Germ. (%)	% of control	Root length (mm)	% of control	Germ. (%)	% of control	Root length (mm)	% of control
Elm								
0 (control)	83 ± 10a	100	26 ± 13a	100	83 ± 10a	100	26 ± 13a	100
0.01	82 ± 6a	99	27 ± 11a	103	79 ± 13a	95	23 ± 12a	86
10	89 ± 9a	107	27 ± 14a	104	78 ± 10a	94	27 ± 12a	102
1,000	88 ± 7a	106	19 ± 8b	71	87 ± 8a	105	26 ± 13a	100
5,000	92 ± 11a	111	8 ± 4c	32	86 ± 10a	104	12 ± 7b	47
10,000	87 ± 6a	105	9 ± 7c	34	80 ± 11a	96	9 ± 4b	34
20,000	80 ± 6a	96	7 ± 6c	28	80 ± 6a	96	8 ± 6b	32
Maple								
0 (control)	56 ± 3a	100	14 ± 12a	100	56 ± 3a	100	14 ± 12ab	100
0.01	49 ± 15a	87	18 ± 13a	126	53 ± 10a	94	15 ± 12ab	108
10	63 ± 12a	113	13 ± 11a	93	48 ± 13a	86	15 ± 11ab	107
1,000	47 ± 14a	84	14 ± 11a	97	52 ± 5a	93	15 ± 11ab	106
5,000	54 ± 9a	96	20 ± 14a	139	53 ± 4a	94	10 ± 10b	68
10,000	53 ± 11a	94	12 ± 12a	87	53 ± 9a	94	21 ± 15a	148
20,000	47 ± 4a	84	13 ± 10a	93	57 ± 4a	101	18 ± 14ab	124
Lettuce								
0 (control)	99 ± 2a	100	36 ± 13b	100	99 ± 2a	100	36 ± 13a	100
0.01	94 ± 8a	95	40 ± 14ab	111	100 ± 0a	101	37 ± 12a	103
10	98 ± 3a	99	46 ± 14a	128	98 ± 3a	99	40 ± 14a	112
1,000	99 ± 2a	100	33 ± 9b	92	100 ± 0a	101	42 ± 13a	116
5,000	99 ± 2a	100	19 ± 4c	52	100 ± 0a	101	26 ± 5b	72
10,000	96 ± 4a	97	11 ± 3cd	32	99 ± 2a	100	17 ± 3c	47
20,000	100 ± 0a	101	8 ± 2d	23	99 ± 2a	100	11 ± 3c	31

[a] Each value is the mean of five replicates ± SD.
[b] Each value is the mean of the number of germinated seeds ± SD.
[c] Mean values in columns followed by the same letters are not significantly different at $P \leq 0.05$ in Tukey's multiple-ragne test.

TABLE 3. EFFECT OF DIFFERENT CONCENTRATIONS OF FRACTIONS F12 AND F5 ON DRY WEIGHT OF SEEDLINGS OF ELM[a] AND MAPLE[b,c]

Concentrations (μg/liter)	F12		F5	
	Dry weight (mg)	% of control	Dry weight (mg)	% of control
Elm				
0 (control)	30.8 ± 11.3a	100	25.5 ± 10.8a	100
1,000	28.9 ± 13.2a	94	28.7 ± 12.2a	112
5,000	20.3 ± 4.6b	66	23.6 ± 8.7a	92
10,000	19.4 ± 3.5b	63	23.9 ± 10.1a	94
20,000	19.1 ± 3.5b	62	19.1 ± 2.7a	75
Maple				
0 (control)	68.2 ± 14.8a	100	52.6 ± 10.2a	100
1,000	62.5 ± 11.8a	92	51.1 ± 11.0a	97
5,000	44.7 ± 9.1b	65	44.7 ± 9.9a	85
10,000	43.3 ± 15.8b	64	48.8 ± 11.9a	93
20,000	36.7 ± 5.5b	54	45.3 ± 11.0a	87

[a] Initial dry weight = 17.4 ± 2.6 (N = 52).
[b] Initial dry weight = 35.7 ± 6.4 (N = 48).
[c] Each value is the mean of 12 replicates ± SD. Mean values in columns followed by the same letters are not significantly different at $P \leq 0.05$ in Tukey's multiple-range test.

ation were concentration and the interaction of fraction × concentration. Species main effect and its interaction with other factors are nonsignificant (Table 4). Regardless of the species or fractions, decreases in growth compared to the control were observed at concentrations of 5000 μg/liter and higher (Table 7). For these concentrations, cells became dark by the second day of growth.

Duckweed Experiment. The most significant effect on duckweed growth was concentration, while fraction and the interaction fraction × concentration were not significant (Table 8). Both fractions induced significant phytotoxicity at concentrations higher then 1000 μg/liter in terms of number of fronds produced (Table 9).

For the majority of the tests, a very high percentage of the variability (R^2) was explained by the model used for the analysis of the variance (Tables 1, 4, and 8). The lowest R^2 in the radicle and callus growth analyses were associated with a high coefficient of variation (CV) among control replicates (40–85% and 30–70%, for the radicle length and callus growth, respectively). The CVs obtained for the controls of most other tests were low: 25% for germination, plating, cell suspension, and duckweed bioassays and 20–42% for the seedling bioassay.

TABLE 4. SUMMARY OF ANALYSIS OF VARIANCE FOR GROWTH PARAMETERS OF THREE IN VITRO BIOASSAYS[a]

	Bioassays					
	Callus growth		Plating		Cell growth	
	Dry weight		Dry weight		Dry weight	
Source of variation	*df*	*P*	*df*	*P*	*df*	*P*
B	9	0.0001	2	0.2061	3	0.0001
S	2	0.0001	1	0.8628	1	0.1144
B × S	18	0.0001	2	0.0001	3	0.0001
FRAC	1	0.3420	1	0.4410	1	0.9219
B × FRAC	9	0.6104	2	0.5469	3	0.8881
S × FRAC	2	0.4270	1	0.7261	1	0.0935
B × S × FRAC	18	0.2074	2	0.0049	3	0.0077
CONC	3	0.0001	6	0.0001	6	0.0001
B × CONC	27	0.0001	12	0.0001	18	0.0001
S × CONC	6	0.0856	6	0.0001	6	0.3705
B × S × CONC	54	0.0001	12	0.0001	18	0.0001
FRAC × CONC	3	0.4851	6	0.6675	6	0.0135
B × FRAC × CONC	27	0.0004	12	0.0004	18	0.0293
S × FRAC × CONC	6	0.4872	6	0.7654	6	0.1853
B × S × FRAC × CONC	54	0.2822	12	0.2129	18	0.1190
R^2		0.62173		0.94222		0.91696

[a]The effects included in the analysis were: blocks, B; species, S; fractions, FRAC; concentrations of the fractions, CONC; and their interactions.

DISCUSSION

Except for percent germination, all bioassays detected phytotoxic activity from mixtures of phenolic and carboxylic acids. The threshold of inhibition for the majority of tests was 5000 μg/liter for each compound. This sensitivity is comparable to tests using duckweed (Devare and Bahdir, 1994; Einhellig et al., 1985; Tanaka et al., 1993) and lettuce (Baud-Grasset et al., 1993; Hulzebos et al., 1993; Lehman et al., 1994, Li et al., 1992a,b, 1993a,b). Nevertheless, detection levels differed depending on the test used, the species chosen, and the fraction tested.

Activity differed from one test to another. Radicle and seedling growth were inhibited, while percent germination appeared insensitive to phytotoxins. The difference between percent germination and radicle growth does not seem to be due to an impression in the parameter measured, since germination per-

TABLE 5. EFFECT OF DIFFERENT CONCENTRATIONS OF FRACTIONS F12 AND F5 ON CALLUS DRY WEIGHT OF ELM, MAPLE, AND PIN CHEERY[a]

Concentrations (μg/liter)	F12		F5	
	Dry weight (μg)	% of control	Dry weight (μg)	% of control
Elm				
0 (control)	17 ± 12a	100	15 ± 11a	100
5,000	18 ± 17a	105	13 ± 14a	85
10,000	12 ± 15a	71	12 ± 13a	75
20,000	4 ± 4b	24	5 ± 10b	31
Maple				
0 (control)	22 ± 8a	100	24 ± 10a	100
5,000	19 ± 7ab	85	20 ± 8ab	84
10,000	17 ± 7b	78	19 ± 7b	79
20,000	17 ± 6b	75	16 ± 6b	68
Pin cherry				
0 (control)	23 ± 12a	100	22 ± 10a	100
5,000	23 ± 9a	98	21 ± 9a	95
10,000	20 ± 8a	88	20 ± 8a	91
20,000	20 ± 10a	87	24 ± 16a	113

[a]Each value is the mean of 45-55 replicates ± SD. Mean values in columns followed by the same letters are not significantly different at $P \leq 0.05$ in Tukey's multiple-range test.

centage had a much lower CV than did the radicle length. It may be due to a difference in the physiological process involved, as has been shown in previous studies (Leather and Einhellig, 1986; Li et al., 1993a; Wink and Twardowski, 1992).

Although all three in vitro bioassays performed (callus growth, plating, cell suspension growth) measure changes occurring at the cellular level, variations in sensitivity were noticed among them. Callus growth was less sensitive to phytotoxins. That difference may be related to the degree of contact between the cells and the phytotoxin-containing medium; callus stands on top of the medium, while in cell suspension the cells are completely and uniformly dispersed in the test solution.

The effect of mixtures differed according to the plant species tested. Caution should be used in the interpretation of these results, because when using seeds collected in the wild (e.g., American elm and red maple), germination rates can be low and variable (Leather and Einhellig, 1986). However, our results indicate low coefficients of variations in the percentage germination between one Petri dish and another for all species, suggesting that differences in inhibition thresh-

TABLE 6. EFFECT OF DIFFERENT CONCENTRATIONS OF FRACTIONS F12 AND F5 ON DRY WEIGHT OF PLATE CELL OF ELM AND PIN CHERRY[a]

Concentrations (μg/liter)	F12		F5	
	Dry weight (μg)	% of control	Dry weight (μg)	% of control
Elm				
0 (control)	167 ± 25a	100	151 ± 20a	100
0.01	168 ± 18a	100	163 ± 33a	108
10	164 ± 17a	99	162 ± 16a	107
1,000	147 ± 20a	88	153 ± 24a	102
5,000	39 ± 32b	24	49 ± 24b	33
10,000	25 ± 12b	15	24 ± 7b	16
20,000	15 ± 4b	9	19 ± 3b	13
Pin cherry				
0 (control)	128 ± 23a	100	121 ± 16a	100
0.01	119 ± 16a	93	112 ± 18a	92
10	119 ± 17a	93	122 ± 16a	101
1,000	114 ± 18a	89	127 ± 15a	105
5,000	111 ± 19a	87	123 ± 11a	102
10,000	97 ± 16a	76	93 ± 35a	77
20,000	30 ± 20b	24	22 ± 6b	18

[a] Each value is the mean of 12 replicates ± SD. Mean values in columns followed by the same letters are not significantly different at $P \leq 0.05$ in Tukey's multiple-range test.

old observed among species are probably related to species sensitivity. In vitro cultures also showed differences among the species. All three species showed different levels of sensitivity for callus growth and, of the two species tested for plating, elm was the most sensitive. Differences observed among species using in vitro culture, especially for callus growth experiments and plating, may be due to the type of callus produced. Callus produced from different species may differ on the basis of color, texture, water content, and compactness (Pierik, 1987). These properties can influence growth in the presence of test compounds.

Variations in the levels of sensitivity among the bioassays also were dependent on the fraction used. Both fractions induced significant inhibitory effects at concentrations ≥5000 μg/liter for the majority of bioassays. However, seedling growth was found to be inhibited only by F12, and this fraction was found to be more inhibitory (at lower concentrations) than F5 when *Lemna minor* and radicle length bioassays were employed. These results indicate that F12 is more toxic, likely due to the greater number of compounds in this fraction, which increases the total amount of phytotoxic material, thus favoring additive

TABLE 7. EFFECT OF DIFFERENT CONCENTRATIONS OF FRACTIONS F12 AND F5 ON DRY WEIGHT OF CELL CULTURE OF ELM AND PIN CHERRY[a]

Concentrations (μg/liter)	F12		F5	
	Dry weight (μg)	% of control	Dry weight (μg)	% of control
Elm				
0 (control)	9.1 ± 1.9a	100	9.0 ± 2.3a	100
0.01	9.3 ± 1.8a	102	8.0 ± 2.4a	98
10	8.7 ± 1.9a	95	9.1 ± 2.4a	101
1,000	9.4 ± 2.1a	104	7.2 ± 1.8a	80
5,000	4.3 ± 1.5b	47	3.5 ± 1.0b	39
10,000	2.8 ± 1.0c	31	3.2 ± 1.1bc	35
20,000	1.8 ± 0.9d	19	2.6 ± 1.4c	29
Pin cherry				
0 (control)	9.7 ± 0.8a	100	9.1 ± 1.1a	100
0.01	9.6 ± 1.6a	99	9.0 ± 1.0a	99
10	9.3 ± 1.3a	96	9.3 ± 1.0a	103
1,000	8.5 ± 1.4a	88	8.3 ± 1.2a	92
5,000	5.8 ± 0.9b	60	7.3 ± 1.3b	80
10,000	3.8 ± 1.1c	39	5.3 ± 1.1bc	88
20,000	1.3 ± 0.7d	14	2.7 ± 0.7c	29

[a]Each value is the mean of 12 replicates ± SD. Mean values in columns followed by the same letters are not significantly different at $P \leq 0.05$ in Tukey's multiple-range test.

or synergic effects, as it has been found with many mixtures (Zhu and Mallik, 1994; Liu and Lovett, 1993; Gerig and Blum, 1991).

Selection of Bioassay. Since extraction, isolation, and purification of allelopathic substances can be difficult, it is important to consider the quantity of substance that will be needed for a bioassay. The manipulations and availability of vegetal material also are important considerations. Finally, the closer the bioassay is to its ecological representation, the greater the chance that the conclusions drawn will be observed under natural conditions.

Germination tests and cell suspension growth in multiwell plates are the tests that required the smallest amount of test substance, while the others (plating, callus, seedling) required five times more test substance. Among all bioassays performed, seedling growth is the one that required the greatest volume of solution to be tested.

Germination tests require high rates of germination, which is not always possible, as was observed with pin cherry in this study. In some species, seed germination is only possible for a few months after collection, as in the case of

TABLE 8. SUMMARY OF ANALYSIS OF VARIANCE FOR GROWTH PARAMETER OF DUCKWEED BIOASSAY[a]

Source of variation	*Lemna minor* number of fronds	
	df	*P*
B	4	0.0001
FRAC	1	0.4393
B × FRAC	4	0.0001
CONC	5	0.0001
B × CONC	20	0.0271
FRAC × CONC	5	0.1078
B × FRAC × CONC	20	0.0653
R^2		0.71202

The effects included in the analysis were: blocks, B; fractions, FRAC; concentrations of the fractions, CONC; and their interactions.

red maple. Seedling growth bioassays are sensitive and representative of nature, but problems occur when not enough seedlings can be produced and only small volumes of test solution are available.

Vegetal material needed for in vitro culture techniques can be produced in large quantities at any season. Cellular suspensions have several advantages

TABLE 9. EFFECT OF DIFFERENT CONCENTRATIONS OF FRACTIONS F12 AND F5 ON NUMBER OF FRONDS[a] OF DUCKWEED

Concentration (μg/liter)	F12		F5	
	Number of fronds	% of control	Number of fronds	% of control
0 (control)	27.8 ± 4.4a	100	24.6 ± 4.8a	100
0.01	28.1 ± 4.4a	101	25.4 ± 4.5a	104
10	27.2 ± 4.2a	98	25.2 ± 5.1a	103
1,000	24.7 ± 4.9a	89	22.9 ± 3.4a	93
5,000	19.5 ± 4.6b	70	20.7 ± 3.8b	84
10,000	18.0 ± 3.6b	64	19.2 ± 4.7b	78

[a]Each value is the mean of 20 replicates ± SD (initial number of fronds = 3). Means values in a column followed by the same letters are not significantly different at $P \leq 0.05$ in Tukey's multiple-range test.

over callus cultures; they are more sensitive, easier to handle, show little variability, and require less material than that needed for callus culture experiments. Cell suspensions are simple, cost effective in the long term, and a rapid means of screening of phytotoxins. Establishment of cell suspensions from some species, however, can be difficult or impossible.

Zilkah et al. (1977) found a good correlation between the effects of phytotoxic compounds observed with callus and with seedling growth. In our study, this correlation was not demonstrated. For American elm, bioassays using whole plant (radicle and seedling growth) found a difference in toxicity between the two test substances, while the one using tissue culture demonstrated equal toxicity. Both fractions were toxic for red maple tissue culture (callus), while no activity was detected using radicle growth, and only one fraction was toxic using the seedling growth test.

If not as the only bioassay, in vitro techniques can be used in addition to other bioassays to screen potential allelopathic compounds for activity prior to testing on whole plant systems. If bioassays using cell and tissue cultures cannot detect all the different modes of actions, they can nonetheless help to verify if action is taking place at the cellular or plant level. Choice of an appropriate bioassay depends on the test species, the amount of substance available, and time limitations. As species sensitivity is an integral part of any bioassay, the target species should be used whenever possible. Accurate estimates of phytotoxicity of potential allelopathic compounds may require the use of more than one bioassay for conclusive results. In that regard, the development of various bioassays using the same target species is the optimal approach.

Acknowledgments—This research was carried out under contract by the Institut de recherche en biologie végétale (Hydro-Québec grant). We wish to thank Nathalie Campeau and Geneviève Thibeault for their technical assistance. Finally, we want to acknowledge the members of the Vice-président Environment, Hydro-Québec, for their support.

REFERENCES

BAUD-GRASSET, F., BAUD-GRASSET, S., and SAFFERMAN, S. I. 1993. Evaluation of the bioremediation of a contaminated soil with phytotoxicity tests. *Chemosphere* 26:1365–1374.

BEAUMONT, G., BASTIN, R., and THERRIEN, H. P. 1976. Effets physiologiques de l'atrazine à doses sublétales sur *Lemna minor* L. 1. Influence sur la croissance, la teneur en chlorophylle, en protéines et en azote soluble et total. *Nat. Can.* 10:527–533.

BLUM, U., GERIG, T. M., WORSHAM, A. D., and KING, L. D. 1993. Modification of allelopathic effects of *p*-coumaric acid on morning-glory seedling biomass by glucose, methionine, and nitrate. *J. Chem. Ecol.* 19:2791–2811.

BRADOW, J. M. 1993. Inhibitions of cotton seedling growth by volatile ketones emitted by cover crop residues. *J. Chem. Ecol.* 19:1085–1108.

DEVARE, M., and BAHDIR, M. 1994. Ecotoxicological assessment of inorganic waste disposal in salt mines. Part II: Phytotoxicity tests. *Fresenius Environ. Bull.* 3:119–126.

EINHELLIG, F. A., LEATHER, G. R., and HOBBS, L. L. 1985. Use of *Lemna minor* L. as a bioassay in allelopathy. *J. Chem. Ecol.* 11:65–72.

GALLET, C. 1994. Allelopathic potential in bilberry–spruce forests: Influence of phenolic compounds on spruce seedlings. *J. Chem. Ecol.* 20:1009–1024.

GERIG, T. M., and BLUM, U. 1991. Effects of mixtures of four phenolic acids on leaf area expansion of cucumber seedlings grown in Portsmouth B_1 soil material. *J. Chem. Ecol.* 17:29–40.

GOVINDARAJULU, Z. 1988. Statistical Techniques in Bioassay. Karger, New York.

GRESSEL, J. 1984. Plant tissue systems for screening of growth regulators: Hormones, herbicides, and natural phytotoxins. *Adv. Cell Cult.* 3:93–181.

GROSSMANN, K., and JUNG, J. 1984. A new micro-method for testing plant growth retardants in cell suspension cultures. *Plant Cell Rep.* 3:156–158.

HORSCH, R. B., and JONES, G. E. 1980. A double filter technique for plating cultured plant cells. *In Vitro* 16:103–108.

HORSCH, R. B., KING, J., and JONES, G. E. 1980. Measurement of cultured plant cell growth on filter paper discs. *Can. J. Bot.* 58:2402–2406.

HULZEBOS, E. M., ADEMA, D. M. M., DIRVEN-VAN BREEMEN, E. M., HENZEN, L., VAN DIS, W. A., HERBOLD, H. A., HOEKSTRA, J. A., BAERSELMAN, R., and VAN GESTEL, C. A. M. 1993. Phytotoxicity studies with *Lactuca sativa* in soil and nutrient solution. *Environ. Toxicol. Chem.* 12:1079–1094.

JENNER, H. A., and JANSSEN-MOMMEN, J. P. M. 1993. Duckweed *Lemna minor* as a tool for testing toxicity of coal residues and polluted sediments. *Arch Environ. Contam. Toxicol.* 25:3–11.

JONES, J. B., JR. 1983. A Guide for the Hydroponic and Soilless Culture Grower. Timber Press, Portland, Oregon.

LAIDLAW, T. F. 1987. Drastic temperature fluctuation: The key to efficient germination of pin cherry. *Tree Planter's Notes.* Summer:30–32.

LEATHER, G. R., and EINHELLIG, F. A. 1986. Bioassays in the study of allelopathy, pp. 133–145, *in* A. Putnam, and C. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

LEHMAN, M. E., BLUM, U., and GERIG, T. M. 1994. Simultaneous effects of ferulic and *p*-coumaric acids on cucumber leaf expansion in split-root experiments. *J. Chem. Ecol.* 20:1773–1782.

LI, H.-H., NISHIMURA, H., HASEGAWA, K., and MISUTANI, J. 1992a. Allelopathy of *Sasa Cernua*. *J. Chem. Ecol.* 18:1785–1796.

LI, H.-H., URASHIMA, M., AMANO, M., LAJIDE, L., NISHIMURA, H., HASEGAWA, K., and MIZUTANI, J. 1992b. Alleopathy of barnyardgrass (*Echinochloa crus-galli* L. Beauv. var. *crus-galli*) *Weed Res.* 37:146–152.

LI, H.-H., INOUE, M., NISHIMURA, H., MIZUTANI, J., and TSUZUKI, E. 1993a. Interactions of *trans*-cinnamic acid, its related phenolic allelochemicals, and abscisic acid in seedling growth and seed germination of lettuce. *J. Chem. Ecol.* 19:1775–1787.

LI, H.-H., NISHIMURA, H., HASEGAWA, K., and MISUTANI, J. 1993b. Some physiological effects and the possible mechanism of action of Juglone in plants. *Weeds Res.* 38:1–9.

LIU, D. L., and LOVETT, J. V. 1993. Biologically active secondary metabolites of barley. II. Phytotoxicity of barley allelochemicals. *J. Chem. Ecol.* 19:2231–2244.

MACIAS, F. A., GALINDO, J. C. G., MASSANET, G. M., RODRIGUEZ-LUIS, F., and ZUBIA, E. 1993a. Allelochemicals from *Pilocarpus goudotianus* leaves. *J. Chem. Ecol.* 19:1371–1379.

MACIAS, F. A., VARELA, R. M., TORRES, A., and MOLINILLO, J. M. G. 1993b. Potential allelopathic guaianolides from cultivar snowflower leaves, var SH-222. *Phytochemistry* 34:669–674.

MURASHIGE, T., and SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473–497.

OHWAKI, Y. J., OGINO, K., and SHIBANO. 1993. 3-Hydroxy-5-methoxystilbene-2-carboxylic acid, a phytotoxic compound isolated from methanolic extracts of pigeonpea (*Cajanus cajan* Mill sp.) leaves. *Soil Sci. Plant Nutr.* 39:55–61.

PIERIK, R. L. M. 1987. In Vitro Culture of Higher Plants. Martinus Nijhoff, Boston.

SAS. 1989. SAS/STAT User's Guide, Version 6. SAS Institute Inc., Cary, North Carolina.

TANAKA, T., ABBAS, H. K., and DUKE, S. O. 1993. Structure-dependent phytotoxicity of fumonisins and related compounds in a duckweed bioassay. *Phytochemistry* 33:779–785.

VÉRONNEAU, H., GREER, A. F., and VINCENT, G. 1993. Mise au point de tests biologiques sur cellules végétales pour l'étude des substances à potentiel allélopathique. Rapport final pour le service Santé environnementale, Vice-présidence Environnement, Hydro-Québec, par l'Institut de recherche en biologie végétale.

WANG, W., and WILLIAMS, J. M. 1990. The use of phytotoxicity tests (common duckweed, cabbage, and millet) for determining effluent toxicity. *Environ. Monit. Assess.* 14:45–58.

WINK, M., and TWARDOWSKI, T. 1992. Allelochemical properties of alkaloids. Effects on plants, bacteria and protein biosynthesis, pp. 129–150, *in* S. J. H. Rizvi and V. Rizvi (eds.). Allelopathy: Basic and Applied Aspects. Chapman and Hall, New York.

YU, J. Q., and MATSUI, Y. 1994. Phytotoxic substances in root exudates of cucumber (*Cucumis sativus* L.). *J. Chem. Ecol.* 20:21–31.

ZHANG, Q. S. 1993. Potential role of allelopathy in the soil and the deocmposing root of Chinese-fir replant woodland. *Plant Soil* 151:205–210.

ZILKAH, S., and GRESSEL, J. 1977. Cell culture vs. whole plants for measuring phytotoxicity I. The establishment and growth of callus and suspension cultures: Definition of factors affecting toxicity on calli. *Plant Cell Physiol.* 18:641–655.

ZILKAH, S., BOCION, P. F., and GRESSEL, J. 1977. Cell cultures vs. whole plants for measuring phytotoxicity II. Correlations between phytotoxicity in seedlings and calli. *Plant Cell Physiol.* 18:657–670.

ZHU, H., and MALLIK, A. U. 1994. Interactions between *Kalmia* and black spruce: Isolation and identification of allelopathic compounds. *J. Chem. Ecol.* 20:407–421.

Journal of Chemical Ecology, Vol. 23, No. 4, 1997

SEX PHEROMONE COMPONENTS OF THE APPLE LEAFMINER, *Lyonetia prunifoliella*

REGINE GRIES,[1] GERHARD GRIES,[1,*] G. G. SKIP KING,[2] and CHRIS T. MAIER[3]

[1]*Centre for Pest Management, Department of Biological Sciences*
[2]*Department of Chemistry*
Simon Fraser University
Burnaby, British Columbia V5A 1S6, Canada
[3]*Department of Entomology*
The Connecticut Agricultural Experiment Station
New Haven, Connecticut 06504

(Received May 2, 1996; accepted December 1, 1996)

Abstract—Three methylated hydrocarbons, 10,14-dimethyloctadec-1-ene (10Me14Me-1-ene-18Hy = 5Me9Me-17-ene-18Hy), 5,9-dimethyloctadecane (5Me9Me-18Hy), and 5,9-dimethylheptadecane (5Me9Me-17Hy), are synergistic sex pheromone components of the leafminer *Lyonetia prunifoliella*. Compounds extracted from female pheromone glands were identified by coupled gas chromatographic-electroantennographic detection (GC-EAD), and one compound, 10Me14Me-1-ene-18Hy, also by coupled GC-mass spectrometry. In field trapping experiments, 10Me14Me-1-ene-18Hy, 5Me9Me-18Hy, and 5Me9Me-17Hy singly were unattractive to males but in ternary combination attracted numerous male moths. Attractiveness of the three-component blend significantly exceeded that of two-component blends. No attraction of males to pheromone lures without 10Me14Me-1-ene-18Hy indicates that this compound is essential for pheromone communication of *L. prunifoliella*. Common C-5 and C-9 methyl branches in lyonetiid pheromone hydrocarbons suggest a common biosynthetic pathway; the presence of 5Me9Me-17Hy and 5Me9Me-18Hy in pheromone blends of *L. prunifoliella* and *Leucoptera malifoliella* provides evidence for phylogeny of lyonetiid chemical communication. Determination of the stereoisomeric composition is required to completely describe the pheromone blend of *L. prunifoliella* and to support the hypothesis of phylogenetically related sex pheromones.

Key Words—Lepidoptera, Lyonetiidae, *Lyonetia prunifoliella*, *Perileucoptera coffeella*, *Lyonetia clerkella*, *Leucoptera malifoliella*, sex pheromone,

*To whom correspondence should be addressed.

0098-0331/97/0400-1119$12.50/0 © 1997 Plenum Publishing Corporation

synergism, 10,14-dimethyloctadec-1-ene, 5,9-dimethyloctadecane, 5,9-dimethylheptadecane.

INTRODUCTION

Lyonetia prunifoliella (Hübner) [formerly *L. speculella* (Schmitt et al., 1996)] (Lepidoptera: Lyonetiidae) is a small polyphagous leafminer. Host plants include five rosaceous species in three genera, two *Betula* spp. (Betulaceae) and possibly two *Ceanothus* spp. (Rhamnaceae) (Maier, 1995). Females insert eggs singly into the lower surface of new leaves. After hatching, larvae form serpentine mines in the upper half of leaves. Second instars enlarge their mines and third instars occupy full-depth blotch mines (Maier, 1988). Within blotches, larvae consume all leaf tissue between the upper and lower epidermis (Maier, 1988). Pupation occurs in a silken cocoon suspended from the underside of a leaf in a silken hammocklike structure constructed by the larvae (Frost, 1924). There are several generations per year, each commencing with eggs deposited in young expanding leaves (Brown, 1989). In fruit orchards in Connecticut and West Virginia, *L. prunifoliella* injures apple, *Malus domestica* (Borkh.), and, to a lesser extent, other species of fruit trees (Brown, 1989; Maier, 1995). Heavily damaged leaves of apple may abscise prematurely (Maier, 1988; and C.T.M., unpublished data).

Three other lyonetiid species, *L. prunifoliella malinella* (Matsumura), *Leucoptera malifoliella* (Costa) (formerly *scitella* Zeller) and *Lyonetia clerkella* L. are pests of fruit trees in Europe and Asia (Kremer, 1963; Esmaili, 1971; Naruse, 1978; Sekita and Yamada, 1979; Seprõs, 1985). Because of the pestiferous reputations of these close relatives and increasing abundance and damage by *L. prunifoliella* in eastern West Virginia (Brown, 1989) and Connecticut (Maier, 1988), *L. prunifoliella* is considered a pest of apple in eastern North America (Brown, 1989). Abundant populations in 1995 provided the opportunity to study the pheromone blend of this leafminer. We report the identification and field testing of pheromone components of *L. prunifoliella.*

METHODS AND MATERIALS

Experimental Insects and Pheromone Analysis

Apple leaves with *L. prunifoliella* pupae were field-collected at Lewis Farms, Southington, Hartford County, Connecticut, sent to Simon Fraser University, and kept at 20°C, 70% relative humidity, and a 14L : 10D photoperiod. Emergent adults were placed individually into filter-paper-lined Petri dishes to avoid mating and subsequent decline of pheromone titers. Pheromone glands of

2-day-old, calling females were removed 0.5 hr into the photophase and extracted for 10 min in HPLC-grade hexane.

Aliquots of one female equivalent (FE) of pheromone extracts were subjected to gas chromatographic analyses [Hewlett Packard (HP) 5890A] with both flame ionization and electroantennographic detection (GC-EAD) (Arn et al., 1975), employing three fused silica columns (DB-5, DB-210, and DB-23; 30 m × 0.25 or 0.32 mm ID; J&W Scientific, Folsom, California). Coupled GC–mass spectrometry (MS) in electron impact (70 eV) mode employed a HP 5985B fitted with the DB-5 column referred to above.

General Procedures

Nuclear magnetic resonance (NMR) spectroscopy was conducted on a Bruker AMX-400 spectrometer at 400 Mhz in deuteriochloroform solution. ^1H chemical shifts are reported in parts per million (ppm, δ) and relative to TMS (0.00 ppm). Mass spectra were obtained on a Varian Saturn II Ion Trap GC-MS system fitted with a DB-5-coated fused silica column (30 m × 0.25 mm ID; J&W Scientific). GC analyses were carried out on a HP 5880 fitted with a DB-1-coated fused silica column (30 m × 0.25 mm ID). THF was freshly distilled from sodium–benzophenone ketyl. Recovered hexane–ether solvent mixtures were distilled from P_2O_5 via a Dufton fractionating column. Chemicals from commercial sources were used without further purification. 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) (Still et al., 1978).

Syntheses

*Ethyl 4-methyloctanoate (**1**; Figure 1).* This compound was synthesized as previously reported (Gries et al., 1994).

*4-Methyloctanol (**2a**; Figure 1).* Ester **1** was dissolved in ether, lithium aluminum hydride (0.21 g, 5.5 mmol) was added, and the mixture refluxed overnight. The reaction was quenched by dropwise addition of 5% NaOH (5 ml). A granular precipitate formed that was removed by filtration and washed three times with ether. Evaporation of solvent gave 0.55 g (3.8 mmol, 75% yield) of **2a**; ^1H NMR ($CDCl_3$); δ 3.61 (2H, t, J = 7 Hz), 1.6–1.1 (12H, m), 0.85 (6 H, m).

*1-Bromo-4-methyloctane (**2b**; Figure 1).* Alcohol **2a** (0.55 g, 3.8 mmol) was dissolved in CH_2Cl_2. To this was added triphenylphosphine (1.4 g, 5.4 mmol), followed by dropwise addition of bromine (0.9 g, 5.6 mmol) until excess Br_2 was present. After stirring 30 min, the solution was quenched with methanol. The mixture was diluted with mixed solvent (100 ml), washed (water; 5% $Na_2S_2O_3$; sat. brine), and dried. After solvent evaporation, the residue was

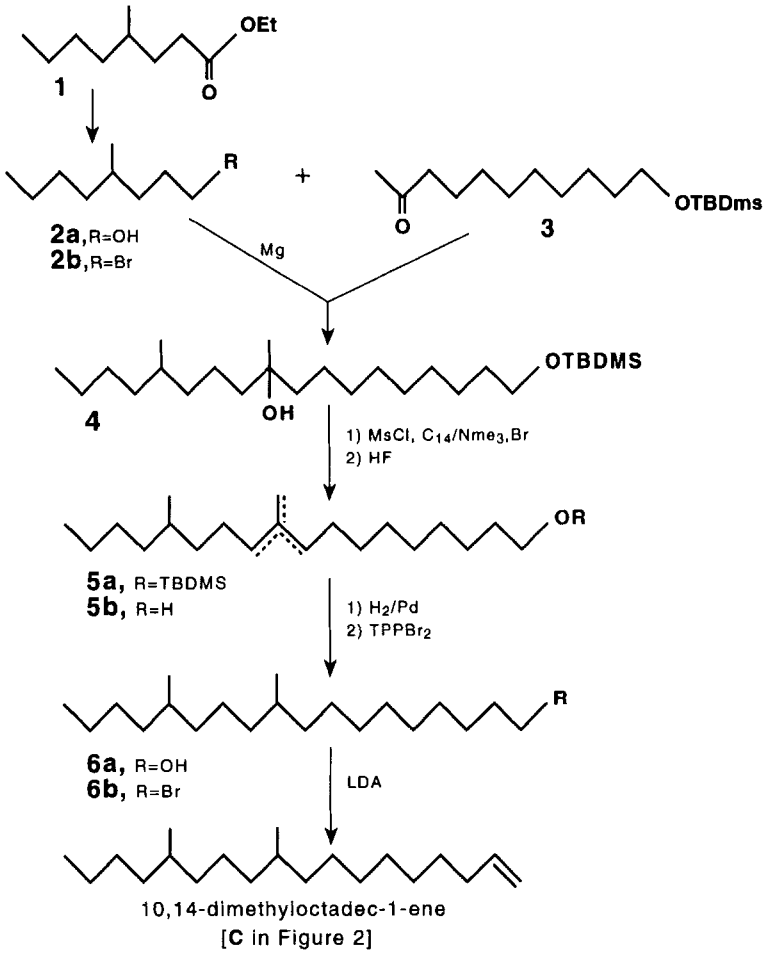


FIG. 1. Scheme for the synthesis of 10,14-dimethyloctadec-1-ene.

passed through silica gel with hexane as eluent to provide 0.8 g (3.8 mmol, 100% yield) of **2b**; ^{1}H NMR ($CDCl_3$); δ 3.40 (2H, t, J = 7 Hz), 1.8 (2H, m,), 1.4 (2H, m,), 1.25 (6H, m), 1.10 (1H, m), 0.88 (6H, m).

1-tert-Butyldimethylsilyloxy-10-undecanone (3; Figure 1). This compound was synthesized according to Nonaka et al. (1984).

1-tert-Butyldimethylsilyloxy-10,14-dimethyl-10-octadecanol (4; Figure 1). A Grignard reagent was prepared from **2b** (0.8 g, 3.8 mmol) and Mg (0.24 g, 10 mmol) in refluxing ether. After 1 hr, the mixture was cooled and **3** (1.2 g, 4 mmol) added. After stirring for several hours, the mixture was quenched with

aqueous NH_4Cl, diluted with mixed solvent, and the aqueous layer removed. The organic layer was washed (brine) and dried. Evaporation and chromatography gave 1 g (2.3 mmol, 60% yield) of **4**. ^1H NMR ($CDCl_3$); δ 3.60 (2H, t, J = 7 Hz), 2.35 (1H, s), 1.5–1.3 (29H, m), 1.15 (3H, s), 0.90 (12H, bs), 0.87 (3H, d, J = 7 Hz), 0.04 (6H, s).

*10,14-Dimethyloctadecanol (**6a**; Figure 1).* Compound **4** was dissolved in THF (15 ml) containing triethylamine (3 ml). To this was added MsCl (0.8 g), and 1 hr later trimethyltetradecylammonium bromide (1 g) (Corey and Snider, 1972). The mixture was stirred overnight, diluted with mixed solvent, and filtered. Chromatography provided 0.8 g of **5a** as a mixture of isomers.

This isomeric mixture was dissolved in CH_3CN (40 ml) containing 2 ml of 40% HF (Newton et al., 1979). After 2 hr, starting material was not detectable by TLC. Water was added, the mixture neutralized with 5% NaOH, and extracted. Evaporation provided 0.4 g of **5b**. GC-MS showed identical spectra for all three isomeric compounds [*m/z* (relative intensity)]: 296 (3), 277 (2), 239 (4), 169 (15), 151 (20), 123 (21), 109 (41), 95 (83), 81 (80), 69 (84), 55 (100).

The isomeric mixture of unsaturated **5b** was dissolved in hexane, 5% catalytic Pd/C (200 mg) was added, and the mixture hydrogenated overnight at 1 atmosphere. Following catalyst removal by filtration and solvent evaporation, the crude product was chromatographed to give 0.3 g (1.0 mmol, 44% yield from **4**) of pure **6a.** ^1H NMR ($CDCl_3$); δ 3.60 (2H, t, J = 7 Hz), 1.90 (2H, quintet, J = 7 Hz), 1.3 (25H, m), 1.1 (4H, m), 0.85 (9H, m); MS [*m/z* (relative intensity)]: 152 (4), 151 (4), 125 (16), 111 (34), 97 (79), 83 (58), 69 (75), 55 (100).

*1-Bromo-10,14-dimethyloctadecane (**6b**; Figure 1).* Bromination reagent was prepared from triphenylphosphine (0.55 g, 2 mmol) and bromine (0.34 g, 2.1 mmol) in CH_2Cl_2. Alcohol **6a** (1 mmol) was added and the mixture stirred 0.5 hr. The reaction was diluted with hexane, washed (water; 2 × 50% aqueous MeOH; brine) and dried. Evaporation and chromatography gave 0.35 g (0.97 mmol, 96% yield) of **6b**. ^1H NMR ($CDCl_3$); δ 3.40 (2H, t, J = 7 Hz), 1.85 (2H, quintet, J = 7 Hz), 1.25 (24H, m), 1.05 (4H, m), 0.85 (9H, m).

10,14-Dimethyloctadec-1-ene (Figure 1). LDA (3.8 mmol) was prepared in THF (5 ml) from diisopropylamine (0.42 g) and 2.4 M of *n*-butyl lithium (1.6 ml). After 5 min, **6b** (0.35 g) was added in THF (5 ml). The solution was stirred overnight, then diluted with hexane, washed (10% H_2SO_4; brine), dried, and evaporated. After chromatography, 0.2 g (0.72 mmol, 71% yield) of the target compound was obtained. ^1H NMR ($CDCl_3$); δ 5.80 (1H, m), 4.99 (1H, d, J = 18 Hz), 4.91 (1H, d, J = 12 Hz), 1.9 (2H, m), 1.4–1.2 (22H, m), 1.10 (4H, m), 0.85 (9H, m).

5,9-Dimethylheptadecane and 5,9-dimethyloctadecane, previously synthesized by Francke et al. (1988) and Riba et al. (1990), were prepared by methods

analogous to the synthesis of 3,13-dimethylheptadecane (Gries et al., 1993). After purification by HPLC [Waters LC 625 high performance liquid chromatograph equipped with a Waters 486 variable wavelength UV-visible detector, a HP 3396 Series II integrator and a Nova-Pak C_{18} (3.9 × 300 mm) column], compounds were >97% chemically pure.

Field Experiments

Field experiments (August 30–October 16, 1995) in apple orchards in Guilford, New Haven County, Connecticut, were set up in randomized complete blocks with traps and blocks at 20-m intervals. Pherocon 1 CP traps (Trécé Inc., Salinas, California) were suspended (north–south-oriented) from trees 2 m above ground and 1–2 m away from the main trunk. The lures were grey sleeve stoppers (The West Company, Lionville, Pennsylvania) that were impregnated with candidate pheromone components in HPLC-grade hexane and suspended on colored pins from trap tops. The first experiment tested 10Me14Me-1-ene-18Hy (25 μg), 5Me9Me-18Hy (25 μg), and 5Me9Me-17Hy (25 μg) singly and in ternary combination. The second and third experiments tested 10Me14Me-1-ene-18Hy, 5Me9Me-18Hy, and 5Me9Me-17Hy in all binary and ternary combinations at 25 μg (experiment 2) or 35 μg (experiment 3) per compound.

Despite transformation, data from male moths captured in all three field experiments were not normally distributed and were therefore subjected to nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means (Scheffé test, $P < 0.05$) (Zar, 1984; SAS Institute Inc., Cary North Carolina, Propietary Software Release 6.10).

RESULTS AND DISCUSSION

GC-EAD analysis of female *L. prunifoliella* pheromone extract on three GC columns consistently revealed three antennal responses (Figure 2). Compounds A and B, with <10 pg/FE, were not FID detectable, whereas compound C occurred at >50 pg/FE and was the most EAD active. Retention indices (Van den Dool and Kratz, 1963) of all EAD-active compounds on DB-5, DB-23, and DB-210 columns were indicative of methylated hydrocarbons. Based on these indices and previously identified methylated hydrocarbon pheromone components in the Lyonetiidae (Figure 3), compounds A and B were hypothesized to be 5Me9Me-17Hy and homologous 5Me9Me-18Hy. Identical retention and comparable EAD characteristics of synthetic 5Me9Me-17Hy and female-produced A, and 5Me9Me-18Hy and female-produced B on all three columns referred to above confirmed these structural assignments.

Most EAD-active C eluted later on DB-23 and DB-210 and earlier on DB-5 than did 5Me9Me-18Hy, indicative of at least one unsaturation in the

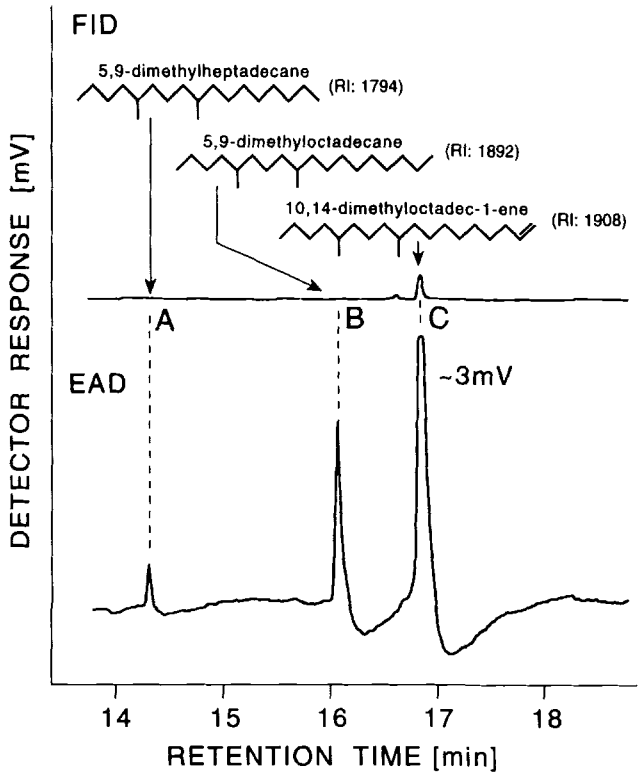


FIG. 2. Flame ionization detector (FID) and electroantennographic detector (EAD: male *L. prunifoliella* antenna) responses to one female equivalent of pheromone gland extract. Chromatography: splitless injection, injector and FID detector: 240°C; DB-23 column: temperature program: 50°C for 1 min, then 20°C/min to 70°C, then 5°C/min to 200°C; RI = retention index.

molecule. The mass spectrum of C also indicated unsaturation (Figure 4), and fragmentation ions *m/z* 85 and *m/z* 153 suggested methyl branches at C-5 and C-9. Because a methylated octadecene with terminal unsaturation [(14*S*)-methyloctadec-1-ene] is a pheromone component in *L. clerkella* (L.) (Sugie et al., 1984; Sato et al., 1985), and C-5 and C-9 are common methyl branches in lyonetiid hydrocarbon pheromones (Figure 3), C was hypothesized to be 10Me14Me-1-ene-18Hy (5Me9Me-17-ene-18Hy). Consistent mass spectra, identical retention characteristics on three fused silica columns (DB-23, DB-5, and DB-210), and comparable EAD activity of female-produced C and synthetic 10Me14Me-1-ene-18Hy confirmed the identification.

In field experiments, the ternary blend of 10Me14Me-1-ene-18Hy,

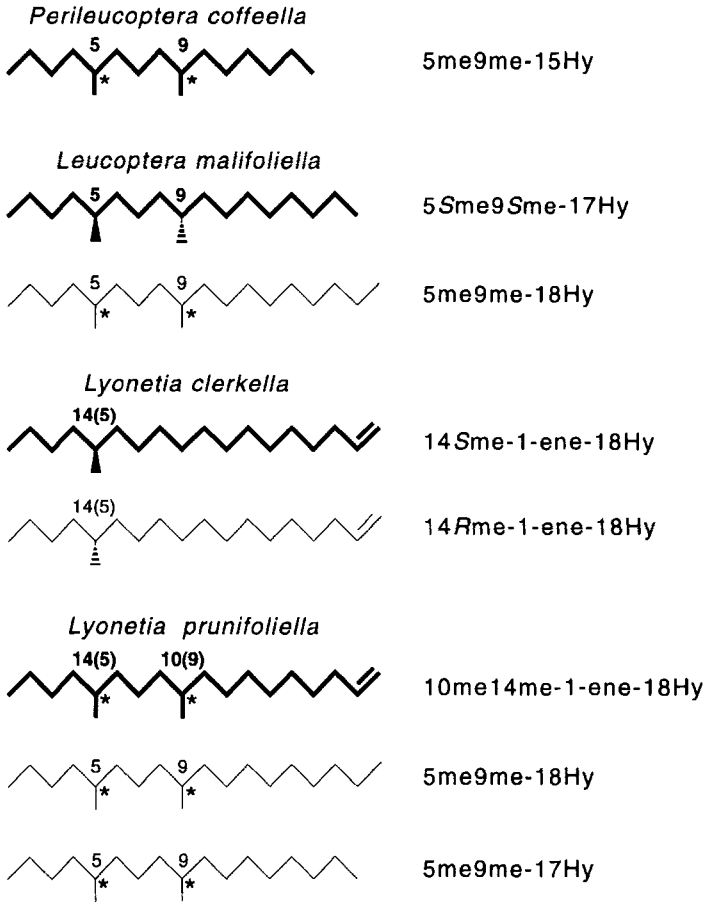


FIG. 3. Methylated hydrocarbon pheromone components of lyonetiids *Perileucoptera coffeela* (Francke et al., 1988), *Leucoptera malifoliella* (formerly scitella) (Francke et al., 1987; Tóth et al., 1989; Riba et al., 1990), *Lyonetia clerkella* (Sugie and Kumakura, 1984; Sato et al., 1985), and *Lyonetia prunifoliella* (this study). *Stereoisomers attractive to insects not yet determined.

5Me9Me-18Hy, and 5Me9Me-17Hy was strongly attractive, whereas none of the three components singly attracted a significant number of *L. prunifoliella* males (Figure 5, experiment 1). Attractiveness of the ternary 1 : 1 : 1 blend exceeded that of binary 1 : 1 blends (Figure 6, experiments 2 and 3), indicating that all three compounds are part of the pheromone blend. Greatest abundance and antennal activity of 10Me14Me-1-ene-18Hy in GC-EAD recordings (Figure 2), and no field attraction of males to lures without 10Me14Me-1-ene-18Hy

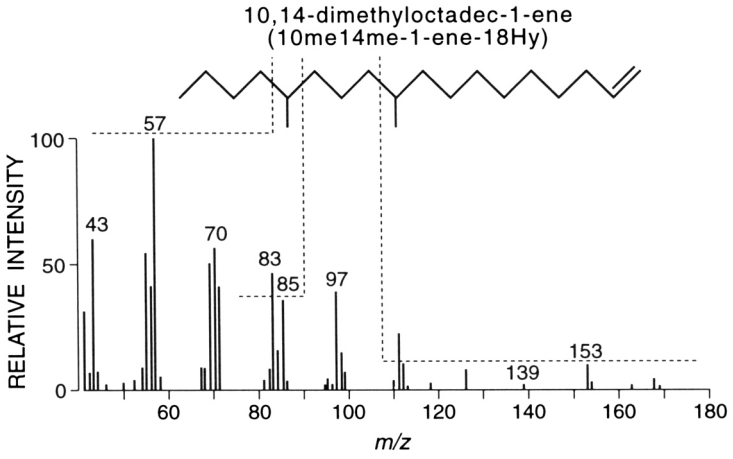


FIG. 4. Electron impact (70 eV) mass spectrum of synthetic 10,14-dimethyloctadec-1-ene. Fragmentation ions *m/z* 85 and *m/z* 153 are diagnostic of methyl branch positions C-14 (5) and C-10 (9).

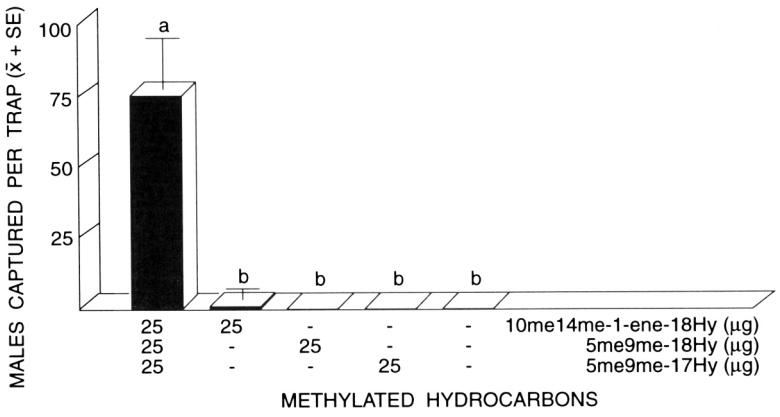


FIG. 5. Captures of *L. prunifoliella* males (experiment 1) in Pherocon-1 CP traps baited with 10,14-dimethyloctadec-1-ene (10Me14Me-1-ene-18Hy), 5,9-dimethyloctadecane (5Me9Me-18Hy), and 5,9-dimethylheptadecane (5Me9Me-17Hy) singly and in ternary combination at 25 µg each); Guilford, New Haven County, Connecticut, August 30–September 6, 1995; 10 replicates. Bars with the same superscript are not significantly different; nonparametric analysis of variance by ranks (Friedman's test) followed by comparison of means (Scheffé test), $P < 0.05$.

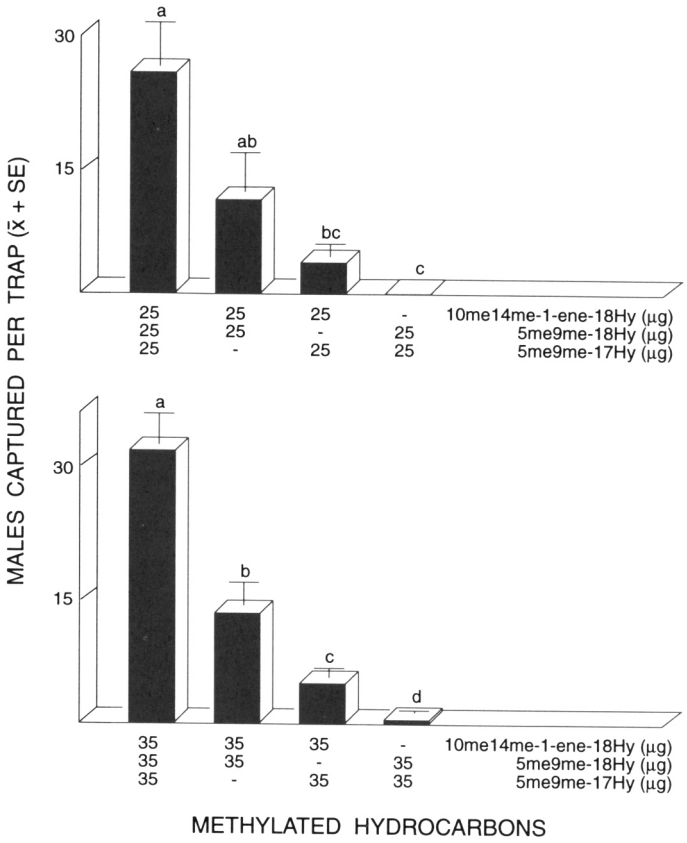


FIG. 6. Captures of *L. prunifoliella* males in Pherocon-1 CP traps baited with 10,14-dimethyloctadec-1-ene (10Me14Me-1-ene-18Hy), 5,9-dimethyloctadecane (5Me9Me-18Hy), and 5,9-dimethylheptadecane (5Me9Me-17Hy) in all binary and ternary combinations at 25 µg (experiment 2) and 35 µg (experiment 3) per compound; Guilford, New Haven County, Connecticut; experiment 2: September 18–25, 1995; 10 replicates; experiment 3: October 6–16, 1995; 8 replicates. For each experiment, bars with the same superscript are not significantly different; nonparametric analyses of variance by ranks (Friedman's test), followed by comparison of means (Scheffé test), $P < 0.05$.

(Figure 6), strongly suggest that this compound is essential for *L. prunifoliella* pheromone communication. It is the first unsaturated, dimethylated hydrocarbon identified as a pheromone component in the Lepidoptera.

Sex pheromone components of other lyonetiid moths also comprise methylated hydrocarbons (Figure 3). Structural similarities between pheromone components of congeners support taxonomic classifications. For instance, congeneric

Lyonetia clerkella and *L. prunifoliella* both produce a methylated octadecene, which is not part of the pheromone blend in heterogeneric *Perileucoptera coffeella* (Guérin-Meneville) or *Leucoptera malifoliella*. Occurrence of methyl-branches at C-5 and C-9 in all identified lyonetiid pheromone components (Figure 3) implies phylogeny of lyonetiid chemical communication and a common biosynthetic pathway, possibly from propionate (methylmalonate) and acetate units (Francke et al., 1987). Determination of all pheromone stereoisomers, such as (5*S*,9*S*)-5,9-dimethylheptadecane in *L. malifoliella* (Figure 3) (Tóth et al., 1989), is required to completely describe the pheromone blend of *L. prunifoliella* and to support the hypothesis of phylogenetically related sex pheromones.

Acknowledgments—We thank Greg Owen for mass spectrometry, Prof. Dr. Wittko Francke and one anonymous colleague for constructive review of the manuscript, and Jonathan Bishop and M. Stewart Ramsay for allowing us to conduct pheromone research in their orchards. The research was supported in part by a research grant of the Natural Sciences and Engineering Research Council of Canada to G.G.

REFERENCES

ARN, H., STÄDLER, E., and RAUSCHER, S. 1975. The electroantennographic detector—a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. *Z. Naturforsch.* 30c:722–725.

BROWN, M. W. 1989. Spatial dynamics and sampling of *Lyonetia speculella* (Lepidoptera: Lyonetiidae), a new leafminer of apple. *Environ. Entomol.* 18:875–880.

COREY, E. J., and SNIDER, B. B. 1972. A synthesis of (±)-fumagillin. *J. Am. Chem. Soc.* 1972:2549.

ESMAILI, M. 1971. Four species of leaf miners attacking deciduous fruit trees in Iran's central province. *Z. Angew. Entomol.* 69:407–415.

FRANCKE, W., FRANKE, S., TÓTH, M., SZÖCS, G., GUERIN, P., and ARN, H. 1987. Identification of 5,9-dimethylheptadecane as a sex pheromone of the moth *Leucoptera scitella*. *Naturwissenschaften* 74:143–144.

FRANCKE, W., TÓTH, M., SZOCS, G., KRIEG, W., ERNST, H., and BUSCHMANN, E. 1988. Identifizierung und Synthese von Dimethylalkanen als Sexuallockstoff weiblicher Miniermotten (Lyonetiidae). *Z. Naturforsch.* 43c:787–789.

FROST, S. W. 1924. Two little known leaf-miners of apple (Lepid.: Tineidae; Col.: Curculionidae). *Entomol. News* 35:132–134.

GRIES, G., KING, G. G. S., GRIES, R., WIMALARATNE, P. D. C., GRAY, T. G., SHEPHERD, R. F., LI, J., SLESSOR, K. N., and KHASKIN, G. 1993. 3,13-Dimethylheptadecane: Major sex pheromone component of the western false hemlock looper, *Nepytia freemani* Munroe (Lepidoptera: Geometridae). *J. Chem. Ecol.* 19:1501–1510.

GRIES, G., GRIES, R., PEREZ, A. L., OEHLSCHLAGER, A. C., GONZALES, L. M., PIERCE, H. D., JR., ZEBEYOU, M., and KOUAME, B. 1994. Aggregation pheromone of the African rhinoceros beetle, *Oryctes monoceros* (Olivier) (Coleoptera: Scarabaeidae). *Z. Naturforsch.* 49c:363–366.

KREMER, W. F. 1963. Major leaf miner species occuring in the South Tirolean fruit-farming region, and their control. *Pflanzenschutz-Nachr.* 16:1–6.

MAIER, C. T. 1988. A new leafminer, *Lyonetia speculella*, in Connecticut apple orchards. *Rep. Conn. Pomol. Soc.* 1987:6–12.

MAIER, C. T. 1995. Hosts and density of *Lyonetia* spp. (Lepidoptera: Lyonetiidae) mining the foliage of woody ericaceous and rosaceous plants. *Ann. Entomol. Soc. Am.* 88:739–747.

NARUSE, H. 1978. Defoliation of peach tree caused by the injury of the peach leaf-miner, *Lyonetia clerkella* L. I. Influence of larval density. *Jpn. J. Appl. Entomol. Zool.* 22:1–6.

NEWTON, R. F., REYNOLDS, D. P., FINCH, M. A. W., KELLY, D. R., and ROBERTS, S. M. 1979. An excellent reagent for the removal of the *t*-butyldimethylsilyl protecting group. *Tetrahedron Lett.* 41:3981–3982.

NONAKA, T., KANEMOTO, S. OSHIMA, K., NOZAKI, H. 1984. Pyridinium flurochromate or benzyltrimethylammonium chlorochromate for selective oxidation of alcohols. *Bull. Chem. Soc. Jpn.* 57:2019–2020.

RIBA, M., ROSELL, J. A., EIZAGUIRRE, M., CANELA, R., and GUERRERO, A. 1990. Identification of a minor component of the sex pheromone of *Leucoptera malifoliella* (Lepidoptera, Lyonetiidae). *J. Chem. Ecol.* 16:1471–1483.

SATO, R., ABE, N., SONNET, P., SUGIE, H., and TAMAKI, Y. 1985. Biological activity of (*R*)- and *S*)-14-methyl-1-octadecene, as the chiral component of the sex pheromone of the peach leafminer moth, *Lyonetia clerkella* Linné. *Appl. Entomol. Zool.* 20:411–415.

SEKITA, N., and YAMADA, M. 1979. Life history of *Lyonetia prunifoliella* Hübner subspecies *malinella* (Matsumura) (Lepidoptera: Lyonetiidae). *Appl. Entomol. Zool.* 14:285–292.

SCHMITT, J. J., BROWN, M. W., and DAVIS, D. R. 1996. Taxonomy, morphology, and biology of *Lyonetia prunifoliella* (Lepidoptera: Lyonetiidae), a leafminer of apple. *Ann. Entomol. Soc. Am.* 89:334–345.

SEPRŐS, I. 1985. Fontosabb Fitofáag Mikrolepidopterák. Aknázómolyok. Mém. Növényvédelmi és Agrokémiai Központja. Budapest.

STILL, W. C., KAHN, M., and MITRA, A., 1978. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* 43:2923–2925.

SUGIE, R., and KUMAKURA, M. 1984. Sex pheromone of the peach leafminer moth, *Lyonetia clerkella* Linné: Isolation and identification. *Appl. Entomol. Zool.* 19:323–330.

TÓTH, M., HELMCHEN, G., LEIKAUF, U., SZIRAKI, GY., and SZÖCS, G. 1989. Behavioural activity of optical isomers of 5,9-dimethylheptadecane, the sex pheromone of *Leucoptera scitella* L. (Lepidoptera: Lyonetiidae). *J. Chem. Ecol.* 15:1534–1543.

VAN DEN DOOL, H., and KRATZ, P. 1963. A generalization of the retention index system including linear temperature programmed gas liquid partion chromatography. *J. Chromatogr.* 2:463–471.

ZAR, J. H. 1984. Biostatistical Analysis, Prentice-Hall, Englewood Cliffs, New Jersey, 718 pp.

Journal of Chemical Ecology, Vol. 23, No. 4, 1997

EFFECT OF *Centaurea maculosa* ON SHEEP RUMEN MICROBIAL ACTIVITY AND MASS IN VITRO

BRET E. OLSON[1,*] and RICK G. KELSEY[2]

[1]*Department of Animal and Range Sciences*
Montana State University
Bozeman, Montana 59717

[2]*Forestry Sciences Laboratory*
3200 SW Jefferson Way
Corvallis, Oregon 97331

(Received June 10, 1996; accepted December 1, 1996)

Abstract—Spotted knapweed, *Centaurea maculosa*, a herbaceous weed from Eurasia, is altering the composition of native rangeland communities across western North America. Herbivore use of this plant is limited, possibly because glandular trichomes covering the epidermal surfaces of aerial tissues produce cnicin, a biologically active sesquiterpene lactone. We determined the concentrations of cnicin in plant parts from different growth stages (initial, mature, regrowth) of *C. maculosa* and the effects of these plant parts on sheep rumen microbial activity and mass (in vitro), when mixed in different proportions with grass hay. Leaves had higher crude protein and lower neutral and acid detergent fiber than stems or grass hay. Cnicin concentrations were highest in leaves, intermediate in flower buds, and lowest in stems. Cnicin concentrations in leaves increased from June to July, but decreased in stems. Regrowth had slightly lower cnicin concentrations that mature growth. High percentages (70% and 100%) of mature and regrowth leaves and flowers of *C. maculosa* in the mixtures depressed the rate and amount of microbial activity, whereas high percentages of stems from initial growth enhanced the rates of microbial activity. Microbial activity was more responsive to the different mixtures, plant parts, and growth stages than microbial mass, possibly because microbial populations cannot adjust rapidly to changes in diet. After cnicin was extracted from leaves, microbial activity was greater from these leaves than from grass hay. In contrast, after cnicin was extracted from flower buds, microbial activity from these flower buds was still depressed, indicating other compounds or the remaining cnicin were still affecting microbial activity. In summary, sheep rumen microbial activity was reduced significantly by mature and regrowth leaves that contained high concentrations of cnicin. Since most herbivores selectively graze leaves, the bitter-tasting cnicin could deter large

*To whom correspondence should be addressed.

0098-0331/97/0400-1131$12.50/0 © 1997 Plenum Publishing Corporation

ruminant feeding of *C. maculosa* by altering their behavior and/or by affecting rumen function.

Key Words—Cnicin, spotted knapweed, sesquiterpene lactone, ruminant, rumen, herbivory, secondary compounds, weeds, sheep, *Centaurea maculosa*.

INTRODUCTION

Spotted knapweed, *Centaurea maculosa* Lam, an herbaceous biennial or short-lived perennial from Eurasia, is altering the composition of native rangeland communities across western North America. Its invasion of rangelands appears undeterred by herbivory or disease. Domestic ungulates and wild ruminants will consume *C. maculosa* (Kelsey and Mihalovich, 1987; Cox, 1989; Robertson, 1989; Miller, 1990; Thompson 1996; Olson et al., 1997; Wright and Kelsey, unpublished data), but only in limited amounts despite its high nutritive value and digestibility in vitro (Kelsey and Mihalovich, 1987).

Glandular trichomes covering the epidermal surfaces of *C. maculosa* aerial tissues produce cnicin, a biologically active sesquiterpene lactone (Locken and Kelsey, 1987) that inhibits growth of gram-negative bacteria (Cavallito and Bailey, 1949; Suchý and Herout, 1962). Antimicrobial properties of cnicin have been verified (Vanhaelen-Fastré, 1972; Vanhaelen-Fastré and Vanhaelen, 1976), and there is evidence of cytotoxicity toward the growth of cancerous cells (Gonzalez et al., 1978). Other sesquiterpene lactones are toxic to many groups of herbivores, including insects, voles, and domestic livestock; they also deter feeding by insects, rabbits, voles, and deer (cited in Gershenzon and Croteau, 1991). Cnicin is the only compound that has been isolated from aerial tissues of *C. maculosa* from North America (Locken and Kelsey, 1987), whereas cnicin (Landau et al., 1994) and two long-chain aldehydes have been isolated from aerial tissues of European populations (Bohlmann et al., 1966). Other classes of potentially active compounds isolated from species in the *Centaurea* genus include aromatic amines, chromenes, phenols, nonterpenoid lactones, lignans, and triterpenes (Huneck et al., 1986; Marco et al., 1992).

Ruminants may avoid grazing plants with secondary compounds because the compounds deter herbivory by taste or smell or cause toxicosis to the animal or rumen microbial populations (Striby et al., 1987; Kronberg and Walker, 1993; Provenza, 1995). These factors are not mutually exclusive and may be intimately related via aversive postingestive feedback (Provenza, 1996). With its antimicrobial activity, the bitter tasting cnicin (Suchý and Herout, 1962) may limit ruminant consumption of *C. maculosa* by deterrence, toxicity or some combination of the two. By limiting consumption, cnicin protects aboveground tissues of *C. maculosa* from macroherbivores, providing a competitive advan-

tage for this exotic over native plants (Locken and Kelsey, 1987; Landau et al., 1994).

We hypothesized that effects of different plant parts of *C. maculosa* on sheep rumen microbial activity and mass would vary depending on the concentration of cnicin. Our objectives were to determine the concentrations of cnicin in different plant parts (leaves, flowers, stems) from different stages of growth (initial, mature, regrowth) of *C. maculosa* and the effects of different plant parts from these stages on sheep rumen microbial activity and mass (in vitro), when mixed with different percentages of grass hay. In vitro microbial gas production was used as an indicator of microbial activity, whereas microbial purine (nitrogen) accumulation was used as an indicator of microbial mass.

METHODS AND MATERIALS

Field Site and Plant Material. The field site was 45 km west of Bozeman, Montana in Madison County (45°35′N, 111°37′W). The soil is a loamy sand, classified in the mixed frigid Ustic Torripsamments family. Randomly selected plots dominated by *C. maculosa* were clipped on June 12, 1995. Material collected from these plots was considered initial growth. Regrowth on these plots and mature growth on adjacent plots were clipped on July 10, 1995. Most of the mature *C. maculosa* plants were in the flower-bud stage at this time.

All *C. maculosa* material collected from these three stages (initial, regrowth, mature) was air-dried at room temperature for seven days. Leaves, stems, and flower buds were separated and then composited by plant part. Flower buds were not present on initial growth; stems and flower buds were not present on regrowth. All plant parts were ground to pass through a 1-mm screen in a Wiley mill. Crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) were determined by standard methods (Goering and Van Soest, 1970; AOAC, 1980).

Tissue Extraction. Triplicate subsamples (100–200 mg) from each ground *C. maculosa* tissue were weighed into screw-cap tubes and covered with 10.0 ml of high performance liquid chromatography (HPLC)-grade methanol. The tubes were shaken for 1 hr at room temperature, then centrifuged for 5 min at 3000 rpm. Each supernatant was decanted and filtered through a 0.2-μm nylon 66 membrane filter. Because of low cnicin concentrations, the mature stem tissue was reextracted as above with 2 ml of methanol. The extracts from all leaf tissues were diluted 1 : 1 before analysis. Duplicate subsamples were dried at 50°C for 16 hr, desiccated while cooling to room temperature, and then weighed.

Cnicin Analysis. Cnicin concentrations in the methanol extracts were measured by HPLC. The chromatograph was fitted with a 5-μm, C18 Hypersil col-

umn, 250 × 4.6 mm, preceded by a guard column, and maintained at 28°C. The solvent system was isocratic methanol–water (1 : 1) at a flow rate of 1.0 ml/min. Extracts were injected by an autosampler fitted with a 10-μl fixed loop and the compounds detected with a UV diode array detector set at 225 nm. Cnicin concentrations were calculated from a standard curve using the external standard method. Verification of cnicin in the extracts was achieved by spiking an extract with standard cnicin and by HPLC–mass spectrometry (MS). The cnicin standard was obtained by large-scale tissue extraction and column chromatography, after Kelsey and Locken (1987). Its identity was verified by ^{1}H and ^{13}C NMR.

The HPLC-MS analysis was conducted with a Sciex API III conspray mass spectrometer fitted with a 5-μm, Spherosorb ODS-2 column, 250 × 2.2 mm. Solvent system A was water with 0.1% trifluoracetic acid (TFA), and B was acetonitrile with 0.1% TFA programmed as a gradient from 10% B to 100% B in 30 min at a flow rate of 200 μl/min. The mass range was 100–450, scanned every 3 sec.

In Vitro Microbial Activity and Mass. All plant parts from the different stages were mixed with grass hay (7.5% CP) in the following *C. maculosa*–grass hay mixtures: (1) 100:0, (2) 70:30, (3) 30:70, and (4) 0:100. The grass hay was also ground to pass through a 1-mm screen in a Wiley mill. Thus, treatments consisted of these mixtures of separate *C. maculosa* plant parts from three growth stages.

Microbial gas production from these treatments was measured with a modified in vitro gas production system that we have used with leafy spurge (*Euphorbia esula* L.) (Thomas et al., 1994; Roberts and Olson, unpublished data). For each treatment, duplicate 250-ml flasks were prepared containing 2.0 g of material, 100 ml McDougall's buffer, and 50 ml strained sheep rumen fluid. Rumen fluid was collected from cannulated sheep maintained on the same grass hay before and during the trial. Flasks were placed in a 39°C shaking water bath. Microbial gas production was measured by the amount of displaced water in inverted burettes. Readings were taken at 2, 3, 4, 6, 12, 18, and 24 hr. These readings were used to determine the rate of microbial gas production and cumulative gas production. After the 24-hr reading, contents of the flasks were filtered to separate residue from the fluid fraction. Residues were dried at 60°C for 48 hr and weighed to determine in vitro dry matter disappearance (IVDMD). The purine concentration of attached bacteria (microbial nitrogen) was also determined from the residues (Zinn and Owens, 1986).

To assess whether cnicin may be one compound affecting microbial response in vitro, leaves (mature, regrowth) and flower buds were rinsed briefly (60 sec, twice) with chloroform to extract cnicin (Kelsey and Mihalovich, 1987) and other epidermal compounds including waxes (Mazliak, 1968). In vitro microbial

TABLE 1. PLANNED LINEAR CONTRASTS OF MICROBIAL GAS PRODUCTION (RATE AND CUMULATIVE), MICROBIAL N, AND IVDMD OF STEMS (S), LEAVES (L), AND FLOWER BUDS (F) FROM INITIAL (I), MATURE (M), AND REGROWTH (R) OF *C. maculosa*[a]

Planned contrasts	Microbial gas production		Microbial N	IVDMD
	Rate	Cumulative		
Stems-I vs. stems-M	S-I**	S-I***	S-I*	S-I***
Leaves-I vs. leaves-M	NS	L-I***	L-I**	L-I***
Leaves-I vs. stems-I	S-I***	S-I***	NS	L-I***
Leaves-M vs. leaves-R	NS	NS	NS	L-M*
Flower-M vs. leaves-M	L-M**	L-M*	NS	L-M***
Flower-M vs. stems-M	S-M*	S-M*	NS	F-M***

[a]Plant part stage indicated in columns 2–5 indicate treatments that had greater response of the treatments contrasted. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS: not significant, $P > 0.05$.

gas production from 100% mixtures of extracted materials was compared with microbial gas production from grass hay.

Statistical Analyses. Each treatment combination was replicated three times (three runs). Rate (1–24 hr) of in vitro microbial gas production was analyzed with repeated-measures ANOVA, with time as the within-subject factor; plant part, growth stage, *C. maculosa*–grass hay mixture (100:0, 70:30, 30:70), and their interactions were analyzed as between-subject factors (SAS, 1988). Cumulative (24-hr) microbial gas production, microbial N, and IVDMD were analyzed with ANOVA; the model included plant part, growth stage, and mixture of *C. maculosa* as main effects and their interactions. A 0:100 *C. maculosa*–grass hay mixture was included in each run. Microbial responses to this mixture were used as covariates to adjust for run effect. Planned linear contrasts were used to compare cumulative microbial gas production, microbial N, and IVDMD of different plant parts from different growth stages (Table 1). Orthogonal contrasts were used to compare responses to the three mixtures (100:0, 70:30, 30:70).

RESULTS

Nutritive Value. Centaurea maculosa leaves from all three stages had higher crude protein and lower neutral and acid detergent fiber than *C. maculosa* stems or the grass hay (Table 2). Flowers had similar crude protein levels as leaves from mature growth, but higher neutral and acid detergent fiber concentrations. Cnicin concentrations were highest in leaves, intermediate in flower buds, and

TABLE 2. CRUDE PROTEIN (CP), NEUTRAL DETERGENT FIBER (NDF), ACID DETERGENT FIBER (ADF), AND CNICIN CONCENTRATIONS OF *C. maculosa* PLANT PARTS FROM DIFFERENT STAGES OF GROWTH, AND OF GRASS HAY

Plant and stage	Part	CP (%)	NDF (%)	ADF (%)	Cnicin (% dry wt)
C. maculosa					
Initial	Stem	6.3	41.4	30.1	0.68
	Leaf	10.8	20.6	14.3	3.35
Mature	Stem	3.2	60.6	45.4	0.12
	Leaf	11.2	20.3	15.0	3.86
	Flower	11.2	42.1	28.5	1.95
Regrowth	Leaf	12.8	26.8	19.6	3.61
Grass hay		7.5	53.1	31.4	NA[a]

[a]NA: not applicable.

lowest in stems. Cnicin concentrations in leaves increased from June to July, but decreased in stems. Regrowth leaves had slightly lower cnicin concentrations than mature leaves collected at the same time.

In Vitro Microbial Activity. The rate of microbial gas production over the 24-hr period varied depending on the plant growth stage and the mix of *C. maculosa* and grass hay (plant growth stage × mix × time, $P < 0.001$; Figure 1). At the 70% and 100% mixtures of leaves (initial, mature, regrowth) and flowers (mature) of *C. maculosa*, rate of microbial gas production was greatly reduced compared with the 30% mixture. In contrast, the 70% and 100% mixtures of stems from initial growth had higher rates of gas production than the 30% mixture. Increasing the percentage of stems from mature *C. maculosa* plants had minimal effect on rate of microbial gas production.

Microbial gas was produced faster from stems than leaves from initial growth and stems from mature growth (Table 1). Microbial gas was produced faster from leaves and stems from mature growth than flowers from mature growth.

Cumulative (24-hr) microbial gas production varied depending on plant growth stage and mix of *C. maculosa* and grass hay (plant growth stage × mix, $P < 0.001$; Figure 2a). As the percentage of stems from initial growth increased in the mixture, microbial gas production increased. In contrast, as the percentage of leaves, stems, and flowers from mature growth and of leaves from regrowth increased, microbial gas production decreased. Increasing the amount of leaves from initial growth had no effect on microbial gas production.

More microbial gas was produced from stems from initial growth than leaves from initial growth and stems from mature growth (Table 1). More micro-

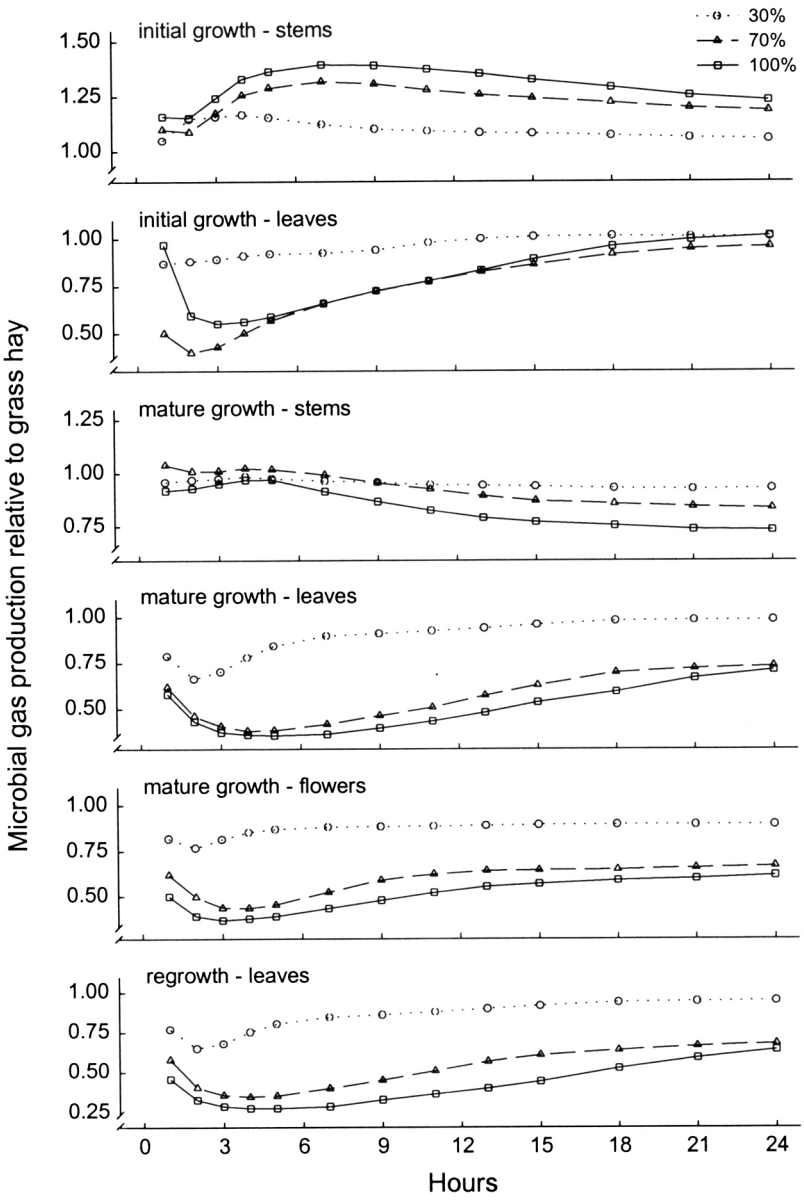


FIG. 1. Microbial gas production over the 24-hr fermentation period for mixtures (*C. maculosa*-grass hay; 100:0, 70:30, 30:70) of stems, leaves, and flower buds from initial, mature, and regrowth of *C. maculosa*, relative to microbial gas production from 100% grass hay in each run. Note that scales of *y* axes differ slightly.

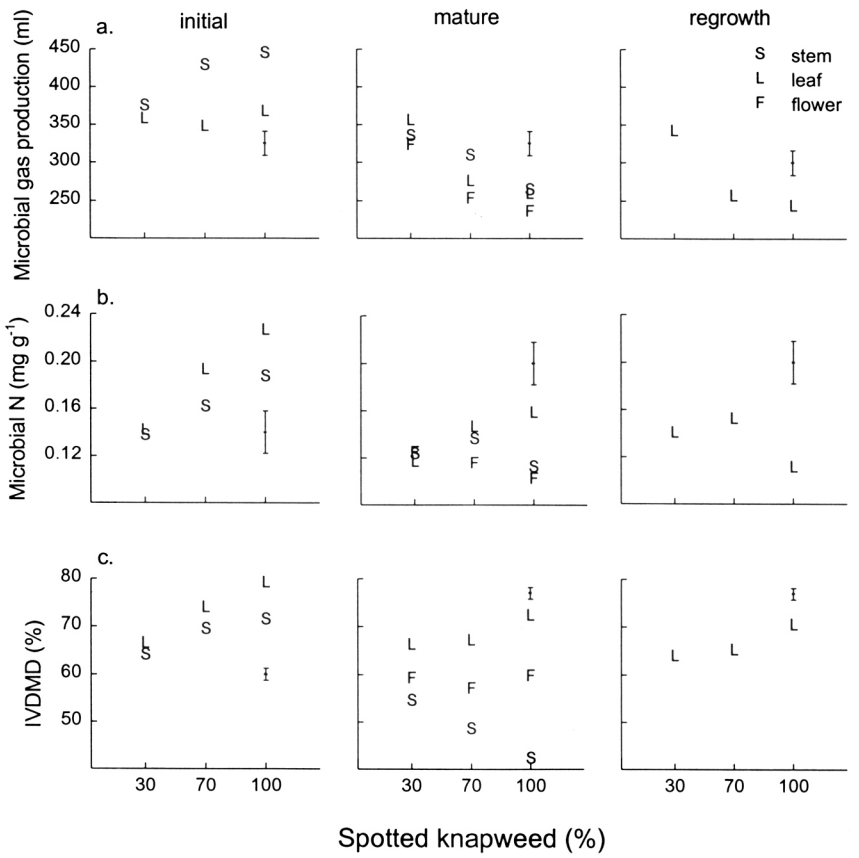


FIG. 2. Cumulative microbial gas production (a), microbial N (b), and in vitro dry matter disappearance (c) of mixtures (*C. maculosa*-grass hay; 100:0, 70:30, 30:70) of stems (S), leaves (L), and flower buds (F) from initial, mature, and regrowth of *C. maculosa*. Error bars indicate standard errors of least square means.

bial gas was produced from leaves from initial growth than leaves from mature growth. More microbial gas was produced from leaves and stems from mature growth than flowers from mature growth.

Extracting cnicin from mature and regrowth leaves reduced cnicin concentrations from 3.86 and 3.61% (dry wt) to 0.59 and 0.36% (dry wt), respectively. More microbial gas was produced from extracted leaves than from grass hay (mature leaves 438 ml, regrowth leaves 410 ml, grass hay 361 ml). Extracting cnicin from flower buds reduced cnicin concentrations from 1.95% to 0.51% (dry wt). In contrast to the microbial response to extracted leaves, less microbial

gas was produced from extracted flower buds than from grass hay (flower buds 278 ml).

In Vitro Microbial Mass. Microbial N varied depending on plant growth stage and mix of *C. maculosa* and grass hay (plant growth stage × mix, $P < 0.13$; Figure 2b). As the percentage of leaves and stems from initial growth increased, microbial N increased. Microbial N associated with the other plant part-growth stages did not respond consistently to increasing percentages of *C. maculosa.* Microbial N was greater from stems and leaves from initial growth than from mature growth (Table 1).

In Vitro Dry Matter Disappearance. In vitro dry matter disappearance varied depending on plant growth stage and mix of *C. maculosa* (plant growth stage × mix, $P < 0.001$; Figure 2c). As the percentage of leaves (initial, mature, regrowth) and stems from initial growth increased in the mixture, IVDMD increased. In contrast, as the percentage of stems from mature *C. maculosa* plants increased, IVDMD decreased. The percentage of flower buds from mature stems in the mixture had no effect on IVDMD.

In vitro dry matter disappearance was greater for leaves than stems from initial growth (Table 1). It was greater for leaves and stems from initial growth than leaves and stems from mature growth. In vitro dry matter disappearance was greater for leaves from mature growth than leaves from regrowth or flowers from mature growth. It was greater for flowers than stems from mature growth.

DISCUSSION

Based on traditional measures of crude protein and fiber, *C. maculosa* is a nutritious mid-summer forage for ruminants. Our crude protein and fiber values were similar to those of plants sampled in western Montana (Kelsey and Mihalovich, 1987); however, their values represented a composite of entire plants, whereas we separated plants into leaves, flower buds, and stems. Many herbivores selectively remove leaves and flower buds, while avoiding stems (Watson and Renny, 1974; Arnold and Dudzinski 1978). From one of their sites, Kelsey and Mihalovich (1987) collected plants with 18.2% CP, which they attributed to enhanced soil fertility. The soil on our study site was an infertile loamy sand, which would explain our lower CP levels.

Cnicin concentrations were much higher than those reported from other *C. maculosa* populations in Montana (1.8% from small stem leaves collected in August) (Locken and Kelsey, 1987). Cnicin concentrations in leaves from our site ranged from 3.35% to 3.86% depending on growth stage. Cnicin concentrations in our flower buds were intermediate to leaves and stems, whereas Locken and Kelsey (1987) did not detect cnicin in flower buds in plants from their population. Stems from our site contained low concentrations of cnicin,

whereas stems from *C. maculosa* in western Montana did not contain any cnicin (Locken and Kelsey, 1987). Our relatively high cnicin concentrations could reflect low fertility or soil moisture holding capacity of the loamy sand on the site. Production of secondary metabolites is often enhanced by nutrient, light, water, or defoliation stress (Bryant et al., 1983; Gershenzon, 1984; Tuomi et al., 1984; Mihaliak and Lincoln, 1989).

Regrowth leaves had a slightly lower cnicin concentration than mature leaves, which was similar to results from a western Montana population (Kelsey and Mihalovich, 1987). This indicates that defoliation does not stimulate the production of cnicin as a defense against herbivory, which is consistent with physiological constraints on terpenoid biosynthesis in mature leaves (Fahn, 1979; Gershenzon, 1994). Since cnicin is located in glands on the epidermal surface, the seasonal decrease in stem concentrations most likely reflects a dilution effect because the surface to internal biomass ratios of stems are lower than leaves as they mature.

The effects of terpenoids are often dosage-dependent (Harborne, 1991). High percentages (70% and 100%) of flower buds and mature and regrowth leaves in the mixtures depressed the rate and amount of microbial gas production. Thirty percent mixtures had a minimal effect on rate and amount of microbial gas production, indicating a threshold for microbial activity somewhere between 30% and 70%.

Flower buds and mature and regrowth leaves were higher in crude protein and lower in fiber than stems, which should have maximized microbial activity. Presumably, high cnicin concentrations in these parts depressed microbial response. When given a choice, goats select less-nutritious older twigs of *Coleogynne ramosissima* over the current year's growth because older twigs have lower condensed tannin concentrations than the current year's growth (Provenza and Malechek, 1984). The goats' avoidance of the current years' growth was attributed more to effects of high condensed tannin levels on palatability than on digestibility. In a later study, acquired aversion of goats to the current year's growth was partially attributed to the volume ingested, although they did not distinguish whether the acquired aversion reflected the effect of condensed tannins on the animal itself or on rumen microbial activity (Provenza et al., 1994).

Microbial gas was produced faster from leaves and stems than from flowers, even though flower buds were intermediate in nutritive value and cnicin concentrations compared with mature leaves and stems. These flower buds may have contained other secondary compounds (Langenheim, 1994) that depressed microbial activity. After we extracted cnicin and other epidermal constituents from mature leaves and stems, in vitro microbial activity and mass from these materials were similar to that of grass hay, indicating that cnicin probably was the compound depressing rumen microbial activity. In contrast, after we extracted

cnicin from flower buds, in vitro microbial activity and mass were still depressed, indicating that other secondary defense compounds or the remaining cnicin in the tissue in flower buds were affecting microbial response. Given the importance of seed production for the fitness of this nonclonal plant, it would not be surprising that *C. maculosa* produces a suite of chemical defenses.

In vitro microbial N only increased as the percentage of leaves and stems from initial growth increased in the mixtures, possibly indicating that our fermentation period (24 hr) was not long enough for microbial mass to increase when exposed to mature growth and regrowth. Microbial populations do not adjust rapidly to changes in diet (Yokoyama and Johnson, 1988). In addition, we used rumen fluid from sheep fed grass hay to evaluate the immediate response of sheep exposed to an area infested with *C. maculosa*. Presumably, microbial mass would have responded more readily to rumen fluid from sheep fed a mixed diet of *C. maculosa* and grass hay prior to the trial. This would mimic a situation where microbial populations of the host animal have time to adjust to this plant. In contrast to microbial mass, in vitro microbial activity was highly responsive to the different *C. maculosa*–grass hay mixtures, which was an expected short-term response.

As the percentage of leaves (initial, mature, regrowth) and stems from initial growth increased, IVDMD increased, presumably reflecting high crude protein–low fiber content of leaves offsetting the influence of high cnicin concentrations. The reduced IVDMD at high percentages of mature stems of *C. maculosa* agrees with reduced in vitro microbial gas production at those mixtures. Apparently, the low crude protein–high fiber content of stems at this stage dominated microbial response.

We cannot unequivocally state that cnicin is the only compound responsible for reduced microbial activity in vitro, because we did not directly test the effects of pure cnicin on their activity. Our results imply that it was cnicin because: (1) tissues high in cnicin (leaves) generally depressed microbial activity more than those low in cnicin (stems), (2) removing cnicin (and other epidermal compounds) with chloroform increased microbial activity, and (3) this type of antimicrobial activity is consistent with activity reported for cnicin (Cavallito and Bailey, 1949; Vanhaelen-Fastré, 1972; Vanhaelen-Fastré and Vanhaelen, 1976).

CONCLUSIONS

Leaves of *C. maculosa* containing cnicin have a strong depressant effect on sheep rumen microbial activity and mass in vitro. Flower buds appear to contain other compounds in addition to cnicin that depress microbial activity

and mass. Apparently, for unadjusted microbial populations there is a threshold somewhere between 30% and 70% of *C. maculosa* in the diet. Cnicin concentrations were not higher in regrowth than mature growth, indicating physiological or environmental constraints on cnicin synthesis. On the other hand, cnicin concentrations were consistently high in all leaf material, and an inducible defense may not be necessary for *C. maculosa* on this site.

Acknowledgments—We thank M. Kuchar for collecting the plant materials and determining in vitro microbial gas production, E. Gerson for assisting with isolating the standard cnicin and reviewing the manuscript, and K. Olson-Rutz, R. Sheley, and R. Wallander for also reviewing the manuscript. This study was supported by the Montana Department of Agriculture Noxious Weed Trust Fund [94-44] and the Montana Agricultural Experiment Station. This is published with the approval of the Director, Montana Agricultural Experiment Station as J-5053.

REFERENCES

ARNOLD, G. W., and DUDZINSKI, M. L. 1978. Ethology of Free Ranging Domestic Animals. Elsevier, New York.

AOAC. 1980. Official Methods of Analysis, 10th ed. Association of Official Agricultural Chemists, Washington, D.C.

BOHLMANN, F., RODE, K. M., and ZDERO, C. 1966. Neue polyine der Gattung *Centaurea* L. *Chem. Ber.* 99:3544–3551.

BRYANT, J. P., CHAPIN, F. S., III, and KLEIN, D. R. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368.

CAVALLITO, C. J., and BAILEY, J. H. 1949. An antibacterial principle from *Centaurea maculosa*. *J. Bacteriol.* 57:207–212.

COX, J. W. 1989. Observations, experiments and suggestions for research on the sheep-spotted knapweed relationship, pp. 79–82, *in* P. K. Fay and J. R. Lacey (eds.). Proceedings, 1989 Knapweed Symposium, Montana State University EB45, Bozeman, Montana.

FAHN, A. 1979. Secretory Tissues in Plants. Academic Press, London.

GERSHENZON, J. 1984. Changes in the levels of plant secondary metabolites under water and nutrient stress. *Recent Adv. Phytochem.* 18:273–320.

GERSHENZON, J. 1994. Metabolic costs of terpenoid accumulation in higher plants. *J. Chem. Ecol.* 20:1281–1328.

GERSHENZON, J., and CROTEAU, R. 1991. Terpenoids, pp. 165–219, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores, Their Interactions with Secondary Metabolites, Vol. 1, The Chemical Participants. Academic Press, New York.

GOERING, H. K., and VAN SOEST, P. J. 1970. Forage fibre analysis (apparatus, reagents, procedures, and some applications). ARS, USDA Agriculture Handbook 379.

GONZALEZ, A. G., DARIAS, V., ALONSO, G., BOADA, J. N., and FERIA, M. 1978. Cytostatic activity of sesquiterpene lactones from Compositae of the Canary Islands. *Planta Med.* 33:356–359.

HARBORNE, J. B. 1991. The chemical basis of plant defense, pp. 46–59, *in* R. T. Palo and C. T. Robbins (eds.) Plant Defenses against Mammalian Herbivory. CRC Press, London.

HUNECK, S., JAKUPOVIC, J., and SCHUSTER, A. 1986. Weitere inhaltsstoffe aus *Centaurea stoebe* (Further compounds from Centaurea stoebe). *Planta Med.* 5:343–532.

KELSEY, R. G., and LOCKEN, L. J. 1987. Phytotoxic properties of cnicin, a sesquiterpene lactone from *Centaurea maculosa* (spotted knapweed). *J. Chem. Ecol.* 13:19–33.

KELSEY, R. G., and MILHALOVICH, R. D. 1987. Nutrient composition of spotted knapweed (*Centaurea maculosa*). *J. Range Manage.* 40:277–281.

KRONBERG, S. L., and WALKER, J. W. 1993. Ruminal metabolism of leafy spurge in sheep and goats: A potential explanation for differential foraging on spurge by sheep, goats, and cattle. *J. Chem. Ecol.* 19:2007–2017.

LANDAU, I., MULLER-SCHARER, H., and WARD, P. I. 1994. Influence of cnicin, a sesquiterpene lactone of *Centaurea maculosa* (Asteraceae), on specialist and generalist insect herbivores. *J. Chem. Ecol.* 20:924–942.

LANGENHEIM, J. H. 1994. Higher plant terpenoids: A phytocentric overview of their ecological roles. *J. Chem. Ecol.* 20:1223–1280.

LOCKEN, L. J., and KELSEY, R. G. 1987. Cnicin concentrations in *Centaurea maculosa*, spotted knapweed. *Biochem. Syst. Ecol.* 15:313–320.

MARCO, J. A., SANZ, J. F., SANCENON, SUSANNA, A., FUSTAIYAN, A., and SABERI, M. 1992. Sesquiterpene lactones and lignans from *Centaurea* species. *Phytochemistry* 31:3527–3530.

MAZLIAK, P. 1968. Chemistry of plant cuticles. *Prog. Phytochem.* 1:49–111.

MIHALIAK, C. A., and LINCOLN, D. E. 1989. Plant biomass partitioning and chemical defense: Response to defoliation and nitrate limitation. *Oecologia* 80:122–126.

MILLER, V. 1990. Knapweed as forage for big game in the Kootenays. *Knapweed* 4:3.

OLSON, B. E., WALLANDER, R. W., and LACEY, J. R. 1997. Effects of sheep grazing on a spotted knapweed-infested Idaho fescue community. *J. Range Manage.* In press.

PROVENZA, F. D. 1995. Postingestive feedback as an elementary determinant of food selection and intake in ruminants. *J. Range Manage.* 48:2–17.

PROVENZA, F. D. 1996. Acquired aversions as the basis for varied diets of ruminants foraging on rangelands. *J. Anim. Sci.* 74:2010–2020.

PROVENZA, F. D., and MALECHEK, J. C. 1984. Diet selection by domestic goats in relation to blackbrush twig chemistry. *J. Appl. Ecol.* 21:831–841.

PROVENZA, F. D., LYNCH, J. J., BURRITT, E. A., and SCOTT, C. B. 1994. How goats learn to distinguish between novel foods that differ in postingestive consequences. *J. Chem. Ecol.* 20:609–624.

ROBERTSON, K. 1989. Living with spotted knapweed in the Bitterroot Valley, pp. 33–36, *in* P. K. Fay and J. R. Lacey (eds.). Proceedings, 1989 Knapweed Symposium, Montana State University EB45, Bozeman, Montana.

SAS. 1988. SAS/STAT User's Guide: Volume 2, Version 6, 4th ed. SAS Institute, Cary, North Carolina.

STRIBY, K. D., WAMBOLT, C. L., KELSEY, R. G., and HAVSTAD, K. M. 1987. Crude terpenoid influence on in vitro digestibility of sagebrush. *J. Range Manage.* 40:244–248.

SUCHÝ, M., and HEROUT, V. 1962. Identity of the bitter principle from *Centaurea stoebe* (L.) Sch. et Thell. with cnicin. *Coll. Czech. Chem. Commun.* 27:1510–1512.

THOMAS, V. M., CLARK, C. K., KOTT, R. W., and OLSON, B. E. 1994. Influence of leafy spurge on ruminal digestion and metabolism and blood metabolite profiles in sheep. *Sheep Goat Res. J.* 10:168–172.

THOMPSON, M. J. 1996. Winter foraging response of elk to spotted knapweed removal. *Northwest Sci.* 70:10–19.

TUOMI, J. P., NIEMELA, P., HAUKIOJA, E., and NEUVONEN, S. 1984. Nutrient stress: An explanation for plant anti-herbivore responses to defoliation. *Oecologia* 61:208–210.

VANHAELEN-FASTRE, R. 1972. Activities antibiotique et cytotoxique de la cnicine, isolee de *Cnicus benedictus* L. *J. Pharm. Belg.* 27:683–688.

VANHAELEN-FASTRÉ, R., and VANHAELEN, M. 1976. Active antibiotique et cytotoxique de la cnicine et de ses produits d'hydrolyse. *Planta Med.* 29:179–189.

WATSON, A. K., and RENNEY, A. J. 1974. The biology of Canadian weeds. 6. *Centaurea diffusa* and *C. maculosa*. *Can. J. Plant Sci.* 54:687–701.

YOKOYAMA, M. T., and JOHNSON, K. A. 1988. Microbiology of the rumen and intestine, pp. 125–144, *in* D. C. Church (ed.). The Ruminant Animal: Digestive Physiology and Nutrition. Prentice-Hall, Englewood Cliffs, New Jersey.

ZINN, R. A., and OWENS, F. N. 1986. A rapid procedure for purine measurements and its use for estimating net ruminal protein synthesis. *Can. J. Anim. Sci.* 66:157.

Journal of Chemical Ecology, Vol. 23, No. 4, 1997

SYNTHESIS, ANALYSIS, AND FIELD ACTIVITY OF SORDIDIN, A MALE-PRODUCED AGGREGATION PHEROMONE OF THE BANANA WEEVIL, *Cosmopolites sordidus*

SEETHARAMAN JAYARAMAN,[1] ISAIAH O. NDIEGE,[1] ALLAN C. OEHLSCHLAGER,[1,*] LILLIANA M. GONZALEZ,[2] DENNIS ALPIZAR,[3] MARIO FALLES,[3] WILLIAM J. BUDENBERG,[4] and PETER AHUYA[4]

[1]*Department of Chemistry, Simon Fraser University Burnaby, British Columbia V5A 1S6, Canada*

[2]*Chem Tica Internacional, S.A. Apdo. 159-2150 San José, Costa Rica*

[3]*Ministerio de Agricultura Guapiles, Costa Rica*

[4]*International Center of Insect Physiology and Ecology P.O. Box 30772, Nairobi, Kenya*

(Received June 18, 1996; accepted December 7, 1996)

Abstract—An efficient synthesis of the diastereoisomers of sordidin (**1**), a male-produced aggregation pheromone of *Cosmopolites sordidus*, has been developed from commercially available 4-methylpent-4-en-2-ol (*2*). Preparation of *exo*-β-sordidin (**1a**) and *endo*-β-sordidin (**1b**) is via the *anti* epoxide, **4d,** which is derived via iodocarbonation of **2.** The *endo*-α-sordidin (**1c**) and *exo*-α-sordidin (**1d**) are prepared from the corresponding *syn* epoxide, which is available via stereo-controlled epoxidation of the triisopropylsilyloxy derivative, **3b.** Silyloxy derivatives of the epoxides, **4,** efficiently alkylate the anions of *N*-cyclohexyldiethylketimine (**6a**) and 3-pentanone *N,N*-dimethylhydrazone (**6b**). Acidic work-up of these alkylation reactions promotes cyclization to give **1.** Gram quantities of **1a–1d, 1a + 1b** and **1c + 1d** have been prepared by this route. In field tests in a banana plantation in Costa Rica, compounds **1a–1d** significantly increased capture rates of standard pseudostem traps. Comparable numbers of adults were attracted to pseudostem traps baited with the major naturally occurring isomers of sordidin **1c + 1d** or **1a–1d.** Although addition of the minor naturally occurring isomers of sordidin (**1a** and **1b**) to pseudostem traps increased capture rates above

*To whom correspondence should be addressed.

0098-0331/97/0400-1145$12.50/0 © 1997 Plenum Publishing Corporation

controls, these compounds did not increase attraction of pseudostem traps to the same extent as **1a–1d.** In comparison tests with conventional pseudostem traps, significantly more adults were trapped in water-containing pitfall traps baited with **1a–1d.**

Key Words—*Cosmopolites sordidus*, banana weevil, aggregation pheromone, sordidin, 2,8-dioxa-1-ethyl-3,5,7-trimethylbicyclo[3.2.1]octane synthesis, field attraction, pitfall trap.

INTRODUCTION

Cosmopolites sordidus (Germar) is considered among the most serious pests of banana. Female *C. sordidus* prefer to lay eggs below the periderm of the rhizome of older plant material, and larvae burrow into corm providing entry for rot-promoting organisms and physically weakening the plant. With a generation time of 4.5–5.5 months, and a relatively long-lived adulthood (two to three months) (Jirasurat, 1989) damage can be especially high in plantations with poor trash management, longer cropping cycle, and slower plant growth (Stanton, 1994).

Because removal of breeding sites is often impractical in large-scale plantations, the main management strategy has traditionally been application of insecticides or nematocides with insecticidal properties. In recent years, traps constructed of banana leaf sections sandwiched between two short (10- to 20-cm) pieces of pseudostem (Ogenga-Latigo and Bakyalire 1993) have been used to monitor adult activity. Management via inoculation of stems of harvested plants with entomopathogenic nematodes has been shown to be effective in Australia, but the number and distribution of baited sites are limited by the pattern of harvesting (Treverrow, 1994). The development of a pheromone lure could increase attractiveness of baiting sites and improve the economics of entomopathogenic nematode application. Since *C. sordidus* are long-lived, mass trapping could overcome their reproductive capacity and lead to a successful management strategy.

Evidence for a male-produced aggregation pheromone was first provided by Budenberg et al. (1993). Gas chromatography–electroantennal detection (GC-EAD) subsequently revealed six antennally active components, the major one of which, named sordidin (**1**), has been identified and synthesized (Beauhaire et al., 1995). Due to the low amount (>2 mg) of material realized from synthesis (Beauhaire and Ducrot, 1996), laboratory bioassay and field testing were not undertaken. Similarly, synthesis of (1*S*,3*R*,5*R*,7*R*)-**1c** and (1*S*,3*R*,5*R*,7*S*)-**1d** (Figure 1 below) have recently been reported and shown to be the naturally produced isomers but field activity was not assessed (Mori et al., 1996). We have recently reported an efficient synthesis that yielded mixtures of all four diastereoisomers of sordidin (**1**). Lures containing these quaternary mixtures

significantly increased attraction of *C. sordidus* to stem traps and were effective with or without banana stem in Giblin-Davis pitfall traps (Ndiege et al., 1996). We now report the preparation and field activity of binary mixtures of sordidin (**1a** + **1b** and **1c** + **1d**) and the development of an efficient food-free trap for *C. sordidus*.

METHODS AND MATERIALS

Instruments and General Chemical Procedures

Infrared spectra were recorded on neat films using a Perkin-Elmer 1600 FTIR. Nuclear magnetic resonance (NMR) spectroscopy was conducted on a Bruker AMX-400 spectrometer at 400.13 and 100.62 MHz for ^1H and ^{13}C NMR spectra, respectively. ^1H chemical shifts are reported in parts per million (ppm, δ) and relative to TMS (0.00 ppm). ^{13}C chemical shifts are referred to $CDCl_3$ (77.0 ppm) or C_6D_6. Gas chromatographic analyses were performed on Hewlett-Packard 5880A and 5890 instruments equipped with flame ionization detection. Fused silica columns used were: methylsilicone, 50 m × 0.32 mm ID and 15 m × 0.25 mm ID; carbowax 20M 50 m × 0.21 mm ID; and Cyclodex B, 30 m × 0.25 mm ID (J&W Scientific). Preparative GC was on a Varian 1200 instrument equipped with a 3-m × 0.31-cm-ID, SP-1000 steel column at 100°C. GC-MS data were obtained on a HP5785B instrument using a DB-5 column (15 m × 0.21 mm ID). Elemental analyses were performed using a Carlo Erba model 1106 elemental analyzer. Diethyl ether (Et_2O) and tetrahydrofuran (THF) were freshly distilled from sodium benzophenone–ketyl. Methylene chloride and DMF were purchased and stored over heat-activated 4 Å molecular sieves. Unless otherwise indicated chemicals obtained from commercial sources were used without further purification. All moisture- and air-sensitive reactions were conducted under argon. Column chromatography refers to the procedure of Still et al. (1978) using Silica Gel 60 (230–400 mesh E Merck, Darmstadt) or neutral alumina.

Sordidin Synthesis

*2-(4-Methyl-4-pentyl)*tert-*butyldimethylsilyl Ether (**3a**).* To a cold (0°C) solution of 15.4 g (150 mmol) of **2** (Wiley Organics, Columbus, Ohio) and 30.6 g (445 mmol) of imidazole in 60 ml of dry DMF was added with stirring, 33.9 g (225 mmol) of (*tert*-butyl)dimethylsilyl chloride in 100 ml of dry DMF. The mixture was warmed to room temperature and stirred for 18 hr, then quenched with 400 ml of water and extracted with Et_2O (5 × 100 ml). The extract was washed with satd. NaCl solution (2 × 100 ml), then dried over Na_2SO_4 and concentrated in vacuo. Distillation gave 29.3 g (91%) of **3a** as a colorless oil

(bp: 77–80°C (15 mm Hg). IR (neat): 3076, 2957, 2930, 2898, 2858, 1649, 1472, 1462, 1374, 1255, 1129, 1086, 1036, 1004, 971, 939, 890, 835, and 774 cm^{-1}; ^1H NMR ($CDCl_3$): 4.75 (1H, d, J = 2.3 Hz), 4.69 (1H, d, J = 2.3 Hz), 3.95 (1H, sex, J = 6.1 Hz), 2.21 (1H, dd, J = 6.1, 13.2 Hz), 2.07 (1H, dd, J = 6.1, 13.2 Hz), 1.72 (3H, s), 1.12 (3H, d, J = 6.1 Hz), 0.88 (9H, s), and 0.04 (6H, s); ^{13}C NMR (C_6D_6) δ: −4.6, −4.3, 18.3, 23.1, 23.7, 26.1, 48.6, 67.7, 112.9, and 143.1; El-MS *m/z* (relative intensity): 171 (3), 159 (22), 158 (6), 157 (36), 117 (9), 116 (5), 115 (49), 114 (6), 113 (51), 103 (15), 101 (10), 99 (5), 85 (17), 77 (7), 76 (1), 75 (100), 74 (7), 73 (62), 59 (10), 57 (5), 56 (6), 55 (5), 45 (5), 44 (7), and 41 (8). Anal. calcd. for $C_{12}H_{26}SiO$: C, 67.22; H, 12.22. Found: C, 67.18; H, 12.19.

*2-(4-Methyl-4-pentenyl)triisopropylsilyl Ether (**3b**).* To a stirred solution of imidazole (6.8 g, 100 mmol) and triisopropylsilyl chloride (7.7 g, 40 mmol) in dry DMF (100 ml) was added alcohol **2** (4 g, 40 mmol) via syringe. Stirring was continued at room temperature for 28 hr. Work-up, as for **3a,** gave a liquid that was distilled (80–85°C/0.5 mm Hg) to give 9.3 g, 91% yield. IR (neat): 1647, 1462, 1375, 1247, 1214, 1126, 1099, 1069, 1036, 1002 cm^{-1}; ^1H NMR (C_6D_6) δ: 1.10–1.23 (24 H, m), 1.72 (3H, s), 2.20 (1H, dd, J = 7.9, 13.3 Hz), 2.43 (1H, dd, J = 4.9, 13.3 Hz), 4.08–4.16 (1H, m), 4.84 (1H, s), and 4.87 (1H, s); ^{13}C NMR (C_6D_6) δ: 12.9, 18.4, 23.1, 23.4, 48.9, 67.7, 113.1, and 143.5; Cl-MS *m/z* (relative intensity): 255 (1), 215 (2), 214 (9), 213 (50), 203 (9), 202 (34), 201 (100), 171 (11), 169 (4), 159 (3), 157 (3), and 127 (2). Anal. calcd. for $C_{15}H_{32}SiO$; C, 70.24; H, 12.50. Found: C, 70.15; H, 12.61.

*2-(4,5-Epoxy-4-methyl-2-pentanyl)*tert*-butyldimethylsilyl Ether (**4c + 4d**).* To a solution of 15 g (70.1 mmol) of **3a** in 100 ml of CH_2Cl_2 at 0°C was added slowly with stirring 18.04 g (67% pure, 70 mmol) of *m*-CPBA. The reaction was kept at 0°C for 2 hr, then warmed to 25°C and stirred for 12 hr. The mixture was cooled in ice, filtered, and the precipitate washed with cold CH_2Cl_2 (2 × 20 ml). The filtrate was washed with saturated $NaHCO_3$ solution (2 × 100 ml), saturated sodium bisulfite (2 × 100 ml), and brine (2 × 50 ml). Drying (anhyd. Na_2SO_4), filtration, concentration in vacuo, and distillation gave 15.2 g (93%) of **4c** and **4d,** bp: 95–98°C (15 mm Hg). Analysis of the epoxide on a Cyclodex B column at 100°C revealed a 2:1 ratio of **4c–4d,** which was confirmed by ^1H NMR. IR (neat): 3038, 2857, 2930, 2857, 1472, 1389, 1360, 1256, 1138, 1082, 1003, 932, 835, 808, and 774 cm^{-1}; ^1H NMR (C_6D_6): 3.76–3.97 (1H, m, H-2), 2.46 (0.67H, d, H-5, J = 5.3 Hz) and 2.34 (0.33H, d, H-5, J = 5.2 Hz), 2.32 (0.67H, d, H-5, J = 5.3 Hz) and 2.22 (0.33H, d, H-5, J = 5.2 Hz), 1.60 (0.67H, dd, H-3, J = 4.5, 13.8 Hz) and 1.87 (0.33H, dd, H-3, J = 6.2, 13.9 Hz), 1.50 (0.67H, dd, H-3, J = 8.1, 13.8 Hz) and 1.42 (0.33H, dd, H-3, J = 6.3, 13.9 Hz), 1.18 (2H, s, CH_3-) and 1.17 (1H, s, CH_3-), 1.06 (2H, d, H-1, J = 6.1 Hz) and 1.05 (1H, d, H-1, J = 6.1 Hz), 0.97 [3H, s, $(CH_3)_2Si$-] and 0.94 [6H, s, $(CH_3)_3$-], 0.09 [~2H, s, $(CH_3)_3$-] and

0.02 [~4H, s, $(CH_3)_2Si$-]; ^{13}C NMR (C_6D_6) δ (for **4c**): −4.6, −4.2, 18.1, 21.3, 24.6, 26.0, 47.4, 54.1, and 66.6. In addition, signals due to **4d** were observed. El-MS *m/z* (relative intensity): 174 (2), 173 (16), 171 (7), 159 (11), 155 (6), 129 (31), 117 (7), 115 (14), 103 (15), 101 (10), 75 (100), and 73 (24). Anal. calcd. for $C_{12}H_{26}SiO_2$; C, 62.55; H, 11.37. Found: C, 62.53; H, 11.26.

2-(4,5-Epoxy-4-methylpentanyl)triisopropylsilyl Ether (4e + 4f). To a stirred and cooled (ice-salt bath) solution of **3b** (9.1 g, 35.5 mmol) in CH_2Cl_2 (150 ml) was added *m*-CPBA (67%, 9.2 g, 35.5 mmol). The reaction was allowed to warm to room temperature over 5 hr. Work-up as for **4c + 4d** gave a liquid (bp: 105–110°C/1 mm Hg; 7.8 g, 82% yield). IR (neat): 1464, 1382, 1301, 1247, 1206, 1114, 1055, and 1007 cm^{-1}; ^1H NMR (C_6D_6) δ: 4.14–3.99 (m, 1H), 2.41–2.07 (m, 2H), 1.80–1.49 (m, 2H), 1.27–1.05 (m, 27 H). The *syn/anti* ratio was 90:10. ^{13}C NMR (C_6D_6) δ (for *syn* isomer): 66.9, 54.3, 53.8, 47.4, 24.5, 21.5, 21.5, 18.3, and 12.9; Cl-MS (isobutane, relative intensity) *m/z*: 273 (8), 255 (2), 231 (2), 230 (6), 229 (33), 203 (10), 202 (36), 201 (100), 199 (2), 188 (1), 187 (9), 186 (2), 185 (9), 175 (6), and 159 (6). Anal. calcd. for $C_{15}H_{32}SiO_2$; C, 66.11; H, 11.84. Found: C, 66.28; H, 11.89.

3-(Iodomethyl)-3,5-dimethyl-2,6-dioxacyclohexanones (5a and 5b). This preparation was executed in 84% yield according to the procedure of Bongini et al. (1982). The *trans* isomer, **5a,** could be recrystallized (60%) from ethyl acetate, mp: 107°C [lit. mp: 109°C (Bongini et al., 1982)]. Due to instability, **5a** was immediately hydrolyzed to **4b** or **4h.**

anti-*4,5-Epoxy-4-methyl-2-pentanol (4b).* To a stirred and cooled (0°C) solution of iodocarbonate **5a** (15.5 g, 57 mmol) in THF (300 ml) and water (100 ml) was added 2 M NaOH solution (100 ml) dropwise during 0.5 hr. Stirring was continued for 1 hr. Then the reaction was saturated with solid NaCl, and the organic layer separated. The aqueous layer was extracted with ether (2 × 50 ml). The combined organic extracts were washed with brine (50 ml) and dried (Na_2SO_4). Solvent removal gave a colorless liquid, and GC analysis revealed a purity of 100% (6.52 g, 98% yield). The *anti* epoxy alcohol was used for the following reaction without further purification. ^1H NMR (C_6D_6): 3.92 (1H, m, H-2), 2.26 (1H, d, H-5, J = 4.9 Hz), 2.16 (1H, d, H-5, J = 4.9 Hz), 1.40 (1H, dd, H-3, J = 3.5, 14.2 Hz), 1.32 (1H, dd, H-3, J = 9.0, 14.2 Hz), 1.08 (3H, s, CH_3-) and 1.06 (3H, d, H-1), J = 6.2 Hz); ^{13}C NMR (C_6D_6): 65.1, 55.8, 53.4, 45.0, 24.1, and 21.3. ^{13}C assignments were confirmed by DEPT experiments. Spectral data were in agreement with reported values (Bongini et al., 1982).

*2-(*anti-*4,5-Epoxy-4-methyl-2-pentanyl)*tert-*butyllimethylsiyl Ether (4d).* Reaction of **4b** with TBDMS-Cl, following a reported procedure (Corey and Venkateswarlu, 1972) gave **4d** in 50% yield after distillation (bp: 96°C/15 mm hg). IR (neat): 3035, 2957, 2930, 2857, 1472, 1389, 1360, 1256, 1138, 1114, 1082, 1051, 1004, 940, 835, and 775 cm^{-1}; ^1H NMR (C_6D_6): 3.31 (1H, sex,

J = 6.2 Hz), 2.35 (1H, d, J = 5.2 Hz), 2.22 (1H, d, J = 5.2 Hz), 1.87 (1H, dd, J = 6.2, 13.9 Hz), 1.42 (1H, dd, J = 6.2, 13.9 Hz), 1.17 (3H, s), 1.06 (3H, d, J = 6.2 Hz), 0.96 (9H, s), and 0.03 (6H, s); ^{13}C NMR (C_6D_6): 66.9, 54.6, 53.0, 46.9, 26.1, 24.3, 22.3, 18, and −4.4; El-MS *m/z* (relative intensity): 174 (1), 173 (8), 159 (6), 129 (21), 115 (10), 103 (13), 101 (8), 77 (7), 76 (8), 75 (100), 73 (24), and 41 (7); GC analyses on DB-1 and Cyclodex B columns confirmed that this compound was identical with the minor component (**4d**) in the mixture (**4c** and **4d;** see above)

*2-(*anti*-4,5-Epoxy-4-methyl-2-pentanyl)methyl Carbonate (4h).* Rexyn R 203 (62 g) was stirred under nitrogen with 200 ml of 3 N NaOH for 1 hr, then filtered and washed with water (2 × 100 ml) and methanol (100 ml). To the generated resin was added **5a** (14.2 g, 52.6 mmol) in 300 ml methanol and the mixture stirred for 48 hr. The mixture was filtered and the resin washed with methanol (2 × 100 ml). The solvent was removed and the aqueous residue extracted with ether (4 × 50 ml). The combined ether extracts were dried over Na_2SO_4 and concentrated in vacuo to give 9.3 g of crude product from which 1 g of unreacted **5a** crystallized. Distillation gave 5.82 g (70%) of **4h,** bp: 105–107°C (15 mm Hg). IR (neat): 2984, 2961, 1746, 1444, 1386, 1357, 1277, 1218, 1138, 1084, 1036, 996, 923, and 905 cm^{-1}; ^{1}H NMR (C_6D_6): 4.91 (1H, m), 3.40 (3H, s), 2.30 (1H, d, J = 4.8 Hz), 2.15 (1H, d, J = 4.8 Hz), 1.89 (1H, dd, J = 7.5, 14.5 Hz), 1.39 (1H, dd, J = 5.4, 14.5 Hz), 1.13 (3H, s), and 1.10 (3H, d, J = 6.3 Hz); ^{13}C NMR (C_6D_6): 155.70, 72.3, 54.0, 52.4, 42.6, 21.9, and 20.4. ^{13}C NMR assignments were confirmed by DEPT. EI-MS *m/z* (relative intensity): 133 (1), 112 (7), 110 (11), 99 (10), 98 (22), 97 (12), 96 (16), 95 (8), 89 (5), 85 (12), 83 (100), 82 (6), 81 (7), 77 (22), 71 (17), 69 (50), 68 (17), 67 (35), 65 (6), 59 (37), 55 (28), 44 (37), 43 (43), and 42 (13). Anal. calcd. for $C_8H_{14}O_4$; C, 55.14; H, 8.10. Found: C, 55.20; H, 8.12.

N-Cyclohexyldiethylketimine (**6a**) (Mori, 1976) and 3-pentanone *N,N*-dimethylhydrazone (**6b**) (Newkome and Fishel, 1988) were prepared by reported procedures.

Alkylation of 6a by 4c + 4d. To a cold (0°C) solution of ethyl magnesium bromide (38.6 ml of 1 M THF soln., Aldrich Chemical Co., Milwaukee, Wisconsin) was added 6.45 g (38.6 mmol) of **6a** in 20 ml of dry THF. The mixture was refluxed for 3 hr, then cooled to 0°C and 4.2 g (18.2 mmol) of **4c–4d** (2 : 1) in 10 ml of dry THF was added slowly (15–20 min). The mixture was refluxed for 18 hr, cooled to 0°C and 24 ml of 2 N HCl added slowly. The mixture was warmed to 25°C and stirred for 48 hr, then cooled to 0°C, quenched with saturated $NaHCO_3$, extracted with ether (5 × 100 ml), dried over Na_2SO_4 and the solvent removed to give 6.8 g of crude product. Chromatography on neutral alumina gave 3.0 g of the **1a–1d.** Distillation gave 2.7 g (85%), bp: 70–72°C (15 mm Hg). GC analysis revealed **1a–1b–1c–1d** in a ratio of 22 : 11 : 28 : 39.

This mixture was subjected to preparative GC to give the pure components, and the ^{1}H and ^{13}C NMR spectral details of these are reported (Ndiege et al., 1996).

exo-*β-Sordidin (**1a**).* EI-MS *m/z*: 184 (7), 169 (2), 155 (4), 142 (13), 127 (5), 113 (50), 95 (100), 85 (12), and 83 (14). CI-MS *m/z* (isobutane, relative intensity): 185 (100), 167 (6), 155 (1), 141 (15), 127 (3), and 110 (6).

endo-*β-Sordidin (**1b**).* EI-MS *m/z* 184 (7), 169 (1), 155 (3), 142 (11), 127 (4), 113 (42), 95 (100), 85 (13), and 83 (17). CI-MS *m/z* (isobutane, relative intensity): 185 (100), 167 (6), 155 (3), 141 (30), 127 (11), and 110 (13).

endo-*α-Sordidin (**1c**).* EI-MS *m/z*: 184 (2), 169 (2), 155 (3), 142 (21), 127 (6), 113 (32), 95 (100), 85 (16), and 83 (20). CI-MS *m/z* (isobutane, relative intensity): 185 (100), 167 (8), 155 (1), 142 (5), 127 (2), and 110 (2).

exo-*α-Sordidin (**1d**).* EI-MS *m/z*: 184 (3), 169 (2), 155 (3), 142 (22), 127 (5), 113 (34), 95 (100), 85 (16), and 83 (27). CI-MS *m/z* (isobutane, relative intensity): 185 (100), 167 (4), 155 (1), 142 (15), 127 (3), and 110 (4).

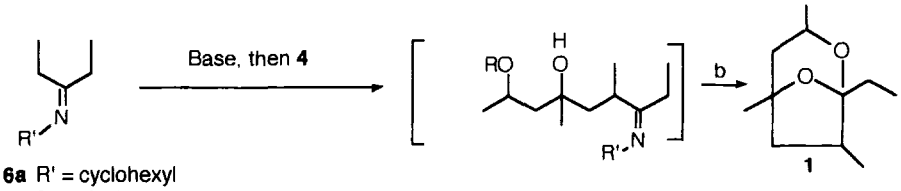
*Alkylation of **6b** by **4c** + **4d**.* A solution of LDA (120 mmol) was prepared at 0°C using diisopropylamine (16.8 ml, 120 mmol), *n*-BuLi (*n*-butyllithium purchased from Aldrich as a 2.5 M solution in hexane, 120 mmol, 48 ml) and THF (250 ml). To this was added a solution of **6b** (13.9 g, 110 mmol) in THF (25 ml) via cannula. Stirring was continued for 2 hr, at which time a deep-yellow color developed. A solution of a mixture of **4c** and **4d** (18.6 g, 80 mmol) in THF (25 ml) was then added via cannula. After stirring for 2 hr at 0°C, a pale-yellow color was observed. The mixture was allowed to warm to room temperature for 14 hr, then cooled in an ice-bath, and a saturated NH_4Cl solution was added (100 ml). The organic layer was separated and the aqueous layer extracted with ether (2 × 100 ml). The combined organic extracts were washed with water (2 × 100 ml) and brine (50 ml). Drying (anhyd. $MgSO_4$) and removal of solvent in vacuo gave a liquid whose capillary GC analysis revealed complete disappearance of **4c** and **4d.** All volatiles were removed in vacuo (1 mm) to give a pale yellow liquid (78.2 g). This was stirred with IR 120 A resin (120 g), anhyd. $MgSO_4$ (78.82 g), and THF (commercial grade, 400 ml) at room temperature for 92 hr. The **1a–1b** ratio remained unchanged during this period, but the **1d–1c** ratio changed from 0.73 : 1 to 1.2 : 1. The mixture was filtered and the residue washed with ether (200 ml). The ethereal solution was then washed with saturated $NaHCO_3$ soln. (100 ml) and brine (100 ml). Drying (anhyd. $MgSO_4$) and concentration at atmospheric pressure gave a liquid that distilled (80–84°C/15 mm Hg) to give a mixture of **1a, 1b, 1c** and **1d** (13.6 g, 92% yield; ratio: 22 : 12 : 30 : 36; 95% pure, Table 1 below).

*Alkylation of **6b** by **4e** + **4f**.* Adaptation of the above procedure gave the pheromone mixture **1a, 1b, 1c,** and **1d** in a ratio of 9 : 4 : 78 : 9 (80% yield, Table 1 below) after distillation. Pure **1c** (>95%) can be obtained from this mixture by chromatography on silica gel.

Preparation of Binary Mixture of 1a and 1b. Derivative **4d** was alkylated using the magnesium salt of *N*-cyclohexyldiethylketimine as described for the mixture **4c** and **4d.** Subsequent acid treatment furnished the diastereoisomeric mixture (**1a** and **1b**), which was purified by chromatography on silica gel (65% yield; 98% purity; the **1a–1b** ratio was 67:33, Table 1 below).

Preparation of Binary Mixture of 1c and 1d. The lithium salt of **6b** (65 mmol), prepared as described above, was alkylated using the diastereoisomeric mixture of epoxides, **4c** and **4d** (43 mmol) prepared as described above. The crude product was dissolved in CH_2Cl_2 (250 ml) and then refluxed with *p*-toluenesulfonic acid monohydrate (20 g, 105 mmol) for 15 hr. This mixture was washed with water (2 × 100 ml), saturated $NaHCO_3$ soln. (2 × 100 ml), water (100 ml), and dried over anhyd. Na_2SO_4. Solvent was removed in vacuo

TABLE 1. ALKYLATION OF ANIONS OF **6a** AND **6b** WITH DERIVATIVES OF **4**



6a R' = cyclohexyl
6 b R' = Me_2N

Entry	R'	Base	4	Alkylation conditions	Work-up	Overall yield (%)	Ratio 1a–1b	Ratio 1c–1d
1	c-Hex	[a]	**4c:4d** 2:1	[c]	2N HCl[e]	85	22:11	28:39
2	Me_2N	[b]	**4c:4d** 2:1	[d]	Amberlite Resin	92	22:12	30:36
3	Me_2N	[b]	**4c:4d** 2:1	[d]	PTSA, reflux	45	nil	41:59
4	Me_2N	[b]	**4e:4f** 90:10	[d]	Amberlite Resin	80	9:4	78:9
5	c-Hex	[a]	**4c:4d** 1:99	[c]	K_2CO_3/MeOH: H_2O then HCl[e]	65	67:33	nil
6	c-Hex	[a]	**4g:4h** 1:99	[c]	K_2CO_3/MeOH: H_2O then HCl[e]	25	67:33	nil

[a] EtMgBr, THF, 0°C, 20 min, reflux 3 hr.
[b] LDA, 0°C, 2hr.
[c] 0°C, 20 min, reflux 12 hr.
[d] 0°C to room temperature, 14 hr.
[e] 0–25°C, 48 hr.

to give a liquid that was chromatographed on silica gel and eluted with a ether–hexanes mixture (5:95). Further purification by distillation under reduced pressure (85–90°C/20 mm Hg) yielded **1c** and **1d** (3.6 g, 45% yield; 100% pure; ratio, 41:59, Table 1 below).

Collection of Adult Volatiles and Electrophysiological Analysis. Adult *C. sordidus* (ca. 2000) were collected from small banana fields in Oyugis Division, Nyanza Province, Kenya. A water aspirator was used to draw charcoal-filtered air at 1–2 liters/min through a 6-liter chamber containing 100 freshly collected insects of mixed or individual sexes and then through a trap containing 100 mg of activated charcoal to collect insect-produced volatiles. Aerations were conducted for 18 hr. Volatiles were eluted from charcoal traps with 2 ml of double-distilled CH_2Cl_2. Eluates were combined and concentrated under a stream of N_2 to 400 μl for analysis and semipreparative GC. Captured volatiles were subjected to gas chromatographic–mass spectrometric analysis using a HP 5790 GC equipped with a 50-m × 0.21-mm fused silica column coated with Carbowax 20 M and a VG MassLab MS (Figure 3 below, semipreparatively separated extract showing only EAD active components). Gas chromatographic electroantennodetection was conducted using a HP5890 unit with the same column. Simultaneous detection by FID and EAD used a SG detector splitter operating at a 1:1 split ratio and an interface card installed in a PC (Syntex, Netherlands, Harvard Professional Computer, American Megatrends Inc.). EAD recordings used female antennae (Figure 4 below). In both GC-MS and GC-EAD analyses, the column temperature was programmed from 60°C (2 min) to 220°C at 10°C/min and held at the latter temperature for 20 min using an EAD-THC-3 temperature control unit.

Field Activity of Sordidin Isomers. Three experiments were conducted in a mature commercial banana plantation near Cariari, Costa Rica. Pseudostem traps used in experiments were constructed of a ca. 20 cm × 20 cm square of banana leaf with a pheromone lure, sandwiched between two freshly cut 20 cm sections of pseudostem. Pseudostems in experiments two and three were soaked in a solution containing insecticide (10 g carbaryl A.I. in 4 liters of water) immediately before placement. Pheromone was released from membrane devices (Chem Tica Internacional, San José, Costa Rica) at ca. 1.0, 0.5, and 1.0 mg/day (under laboratory conditions at 25°C) in experiments 1, 2, and 3, respectively.

In the first experiment (Figure 5 below) pseudostem traps baited with a mixture of **1a, 1b, 1c, and 1d** (22:12:30:36) were compared with unbaited pseudostem traps. In experiment 2 (Figure 6 below) the relative attractancy of pseudostem traps baited with **1a, 1b, 1c,** and **1d** (22:12:30:36); **1a** and **1b** (67:33) or **1c** and **1d** (41:59) were compared with an unbaited control traps.

The third experiment (Figure 7 below) examined the relative attractiveness of pseudostem traps and pitfall traps [black (16 cm ID × 16 cm deep) or white

(14 cm ID × 12 cm deep) plastic containers| baited with **1a, 1b, 1c,** and **1d** (22:12:30:36). The pitfall traps were buried at ground level and contained 3–4 cm detergent-laced (3%) water. A complete randomized block design with 20–30 m spacing between treatment and blocks was used for all experiments. Data were tested for heteroscadiscity and, if necessary, transformed to achieve homogeneity (Zar, 1984). Data were analyzed using Systat 5.2.1.

RESULTS AND DISCUSSION

Sordidin Synthesis. Retrosynthetic analysis revealed diastereoisomers of **1** can be prepared via acyclic precursor (Figure 1, A). Relative C-3 stereochemistry is determined by the *syn*:*anti* stereochemistry of hydroxy epoxide intermediate (Figure 1, B). A *syn* intermediate is required for preparation of **1c** and **1d,** while synthesis of **1a** and **1b** require the *anti* isomer. Stereochemistry at C-7 is set during alkylation of B by C (Figure 1). Since C-7 is α to a carbonyl equivalent, C-7 substituents are easily equilibrated to the more thermodynamically stable stereochemistry found in **1b** and **1d** (Beauhaire et al., 1995).

An initial synthetic goal was selective generation of *syn* and *anti* epoxide intermediates (B). Epoxidation [$V(AcAc)_2$] of commercially available homoallylic alcohol, **2,** gave poor (~25%) yields of a 2:1 mixture of *syn* and *anti* epoxides **4a** and **4b,** respectively (Figure 2) (Tanaka et al., 1974). The yield, but not the *syn*:*anti* ratio, was improved by *m*-CPBA epoxidation of the *tert*-butyldimethylsilyl ether (Corey and Venkateswarlu, 1972) (**3a,** 91%) of **2** (**4c–4d,** 2:1) but was significantly improved by epoxidation of the TIPS derivative, **3b** (**4e–4f,** 90:10).

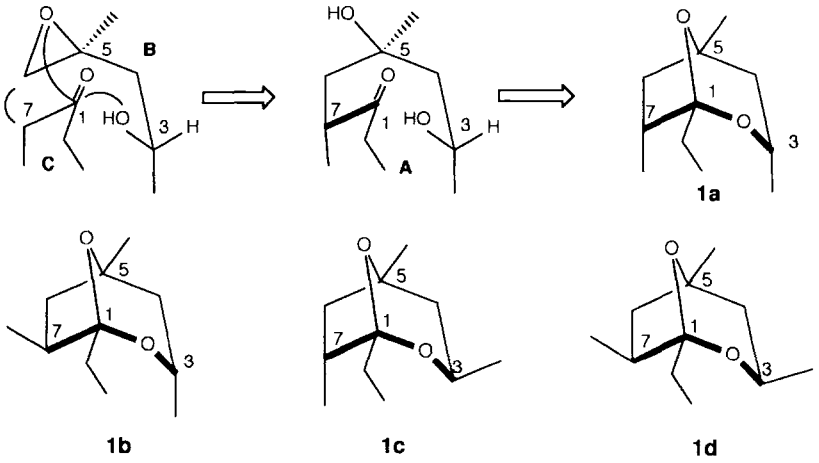


FIG. 1. Retrosynthetic analysis of sordidin.

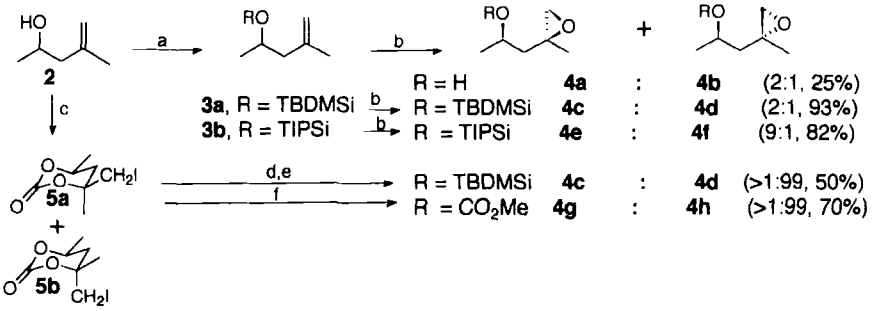


FIG. 2. Synthesis of derivatives of **4.** (a) TBDMSCI or TIPSCI, imidazole, DMF, 0–25°C, 18 hr; (b) *m*-CPBA, CH_2Cl_2, 0°C, 2 hr–15°C, 5 hr; (c) BuLi, THF, 0°C, 1 hr (ii) CO_2, 0°C, 1 hr–25°C, 1 hr (iii) I_2, 0–25°C, 12 hr; (d) 3.3 eq 1N NaOH, THF : H_2O (3 : 1), 0°C, 0.5 hr; (e) TBDMSCl, imidazole, DMF, 0–25°C, 12 hr; (f) Rexyn R 203 resin, MeOH, 48 hr.

Diastereoselective synthesis of the *anti*-epoxide, **4d,** was achieved by iodocarbonation (Bongini et al., 1982; (Figure 2). Addition of *n*-butyllithium to **2** in THF, followed by CO_2, then iodine, gave 84% of a 8 : 1 mixture of **5a** and **5b.** These were easily differentiated by ^1H NMR (Bongini et al., 1982). Iodocarbonate **5a** was recrystallized from ethyl acetate to >99% de. The *anti*-epoxy alcohol, **4b**, was generated almost quantitatively by base hydrolysis of **5a** (Bongini et al., 1982). The *tert*-butyldimethylsilyl ether (**4d**) was obtained in 50% yield by standard procedure (Corey and Venkateswarlu, 1972). The *anti*-epoxide could also be obtained (70%) as methylcarbonate, **4h,** by treatment of **5a** with Rexyn R-203, a mild basic resin (Bartlett and Meyerson, 1978).

Because alkylation of epoxides with metallodimethylhydrazones and imines is more efficient than alkylation via enolates (Harvey and Tarbell, 1967; Stork and Dowd, 1963), we prepared both the cyclohexylimine (**6a**) and dimethylhydrazone (**6b**) derivatives of 3-pentanone for reaction with derivatives of **4.** Deprotonation of **6a** with ethyl magnesium bromide followed by alkylation with **4c–4d** (2 : 1) and treatment with dilute HCl (Mori, 1976) gave all four diastereoisomers of **1** (Table 1, entry 1). GC analysis revealed the **1a** + **1b** to **1c** + **1d** ratio to be ~1 : 2. This conforms with the expectation that **1a** and **1b** are formed from *anti*-epoxide, **4d,** while **1c** and **1d** are derived from *syn*-epoxide, **4c.** The **1a–1b** ratio was 2 : 1 and the **1c–1d** ratio was ~1 : 1.4. Similar results were obtained when the lithium derivative of **6b** was alkylated with the same 2 : 1 mixture of **4c–4d** and worked up using Amberlite IR-120 resin as the cyclizing agent (Table 1, entry 2). In the latter reaction, the initial ratio of **1d–1c** increases from 0.73 : 1 to 1.2 : 1 during the course of this reaction, suggesting equilibration. Alkylation of **6b** with **4c–4d** (2 : 1) followed by refluxing with PTSA gave three unidentified more polar products, no **1a** or **1b,** and a 45% yield of **1c** and **1d.** We presume that under these forcing conditions precursors

of and/or **1a** and **1b** undergo preferential decomposition leaving only **1c** and **1d.**

Alkylation of **6b** with the TIPS protected *syn*-epoxy alcohol **4e** *gave* **1c** with improved diastereoselectivity (Table 1, entry 4). Alkylation of **6a** with **4d,** derived via iodocarbonation, gave a 2:1 mixture of **1a** and **1b** in good yield (Table 1, entry 5) while alkylation of **6a** with the *anti*-epoxy carbonate (**4h**) followed by removal of the carbonate (Meyers et al., 1978) deprotection of the ketone and cyclization gave the same mixture of **1a** and **1b** in low yield (Table 1, entry 6). Purification of the product from the latter reaction could not be achieved due to minor closely eluting products.

Individual components of **1a–1d** were isolated by preparative GC and structural assignments confirmed by ^{1}H and ^{13}C NMR (Ndiege et al., 1996).

Electroantennal Activity. It has been previously reported that there are six EAD active components in *C. sordidus* male-produced volatiles (Beauhaire et al., 1995). Analyses of natural and synthetic diastereoisomers of **1** by GC revealed that all four isomers of sordidin are present in the natural extract and that the natural ratio of **1a, 1b, 1c,** and **1d** was ~ 1:4:4:44. GC/EAD analysis revealed that all four stereoisomers of **1** are antennally active (Figure 4). Although **1a** is present in much lower amounts than **1d,** it evoked an EAD response comparable to that of **1d.** Two additional as yet unidentified EAD active components are highlighted in Figure 3 near scans 180 and 200.

Field Activity. Previous work (Ndiege et al., 1996) established that attraction of *C. sordidus* to pitfall traps containing pseudostem and **1a–1d** was statistically equivalent to attraction to traps containing only **1a–1d.** This result should be contrasted to the claim of Mori et al. (1996) that in field tests in Venezuela, K. Jaffé found that (±)-1 was not attractive unless presented with banana plant tissue. In the present work an initial experiment (Figure 5) established that addition of **1a–1d** to pseudostem traps increased capture rates about fivefold over the one-month term of the experiment. The recovery of approximately equal numbers of males and females from the pheromone-baited traps in

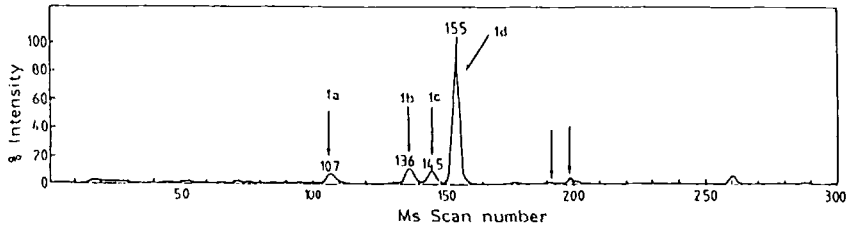


FIG. 3. Ion current chromatogram of semipreparatively purified weevil-produced EAD active components (50 × 0.20 mm ID Carbowax 20 M fused silica column).

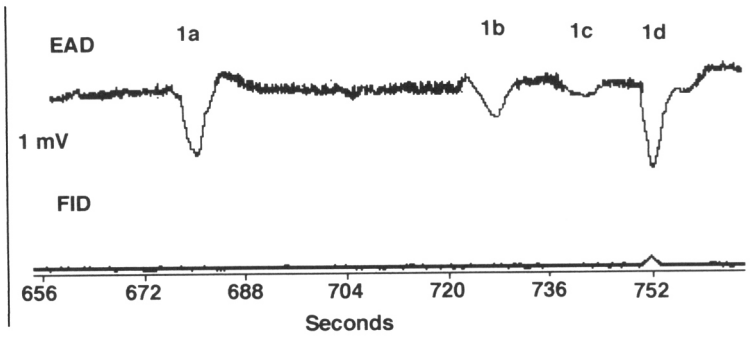


FIG. 4. Representative GC-EAD recording of a female *Cosmopolites sordidus* antenna responding to sordidin diastereoisomers **1a–1d** (50 m × 0.20 mm ID Carbowax 20 M fused silica column).

this experiment suggests **1** behaves as an aggregation pheromone. Male-produced volatiles of *C. sordidus* from which **1** was obtained were attractive to both males and females in laboratory bioassays (Budenberg et al. 1993).

A second experiment (Figure 6) demonstrated that mixtures of **1a** + **1b**

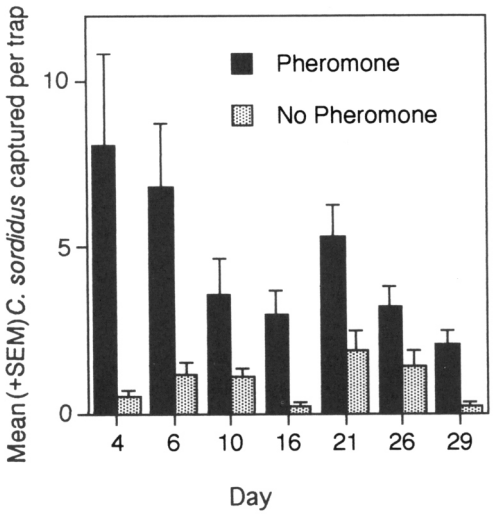


FIG. 5. Mean number (+SEM) of adult *C. sordidus* captured in pseudostem traps with or without **1a–1d.** Test was conducted October 21–November 19, 1995. Captured insects were removed and stem traps were replaced at each counting date. ANOVA ($N = 10$) followed by Bonferonni t test gave significant differences ($P < 0.05$) for pheromone-baited vs. unbaited traps for each date.

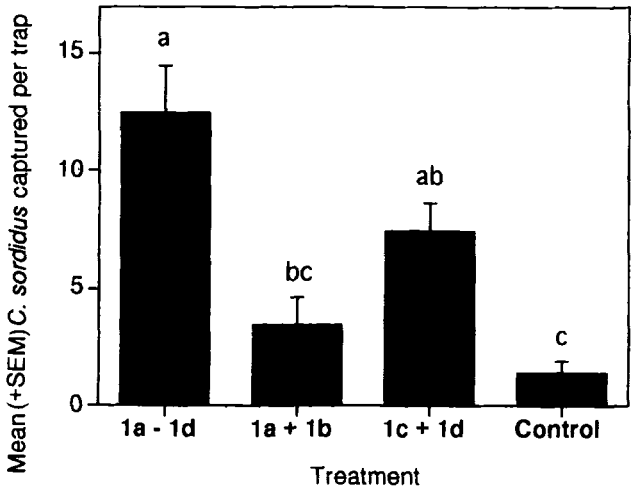


FIG. 6. Mean number (+SEM) of adult *C. sordidus* captured in insecticide-laced pseudostem traps baited with **1a + 1b, 1c + 1d,** or **1a–1d** or without pheromone. Test was conducted November 20–27, 1995. ANOVA ($N = 10$) on log ($X + 1$)-transformed data gave $F = 14.83$; $df = 3,35$; $P < 0.001$. Means are presented untransformed. Means topped by the same letter are not significantly different by Bonferonni t test ($P < 0.05$).

and **1c + 1d** significantly increased attraction to pseudostem traps. Mixtures of the major naturally occurring isomers **1c** and **1d** increased attraction to the same extent as **1a–1d.** Mixtures of the minor isomers **1a** and **1b** produced by males increased attraction to pseudostem traps but not to the same extent as **1a–1d.** All four diastereoisomers of **1** are naturally produced and the small amount of **1a** in the natural extract gives a large antennal response in proportion to its presence in the naturally produced mixture of diastereoisomers (see above). Sordidin is structurally related to the bicyclic ketal pheromones commonly named brevicomins, frontalins, and multstriatins. It is known that natural sordidin is enantiomerically enriched in one isomer for each of **1a–1d** (Beauhaire et al., 1995). A recent synthesis of (1*S*,3*R*,5*R*,7*R*)-**1c** and (1*S*,3*R*,5*R*,7*S*)-**1d** (enantiomers shown in Figure 1) confirmed the chirality of the naturally produced isomers without assessment of field activity (Mori et al., 1996). The mixture of **1a–1d** prepared and used in this work is a mixture of four sets of enantiomers. Responses of insects that employ bicyclic ketals as pheromones are rarely inhibited by the presence of enantiomers and diastereoisomers of their natural enantiomerically and diastereoisomerically enriched semiochemicals (Borden, 1985).

Dissection of the pseudostem trap to determine the number of captured

insects is time consuming and risks exposure of workers to high levels of phenolics and insecticides. Furthermore, we observed that in insecticide-treated pseudostem traps used in the second experiment, ~20% of the insects were still alive after only one week of use. A third experiment (Figure 7) revealed that water-containing white pitfall traps baited with **1a–1d** were more attractive than baited pseudostem traps. Since *C. sordidus* does not fly and spends most of its life in the moist environment of the corm it was expected that pheromone-baited, water-containing pitfall traps would be attractive.

The pheromone-baited pitfall trap is an attractive alternative even to pheromone-baited pseudostem traps because the former does not contain pseudostem or insecticide and is lethal to all arriving insects. In similarly replicated experiments, we found that *C. sordidus* was not captured by pheromone-baited (**1a–1d**), Stickem-coated wire mesh traps (1/4 in. mesh, 10-cm-diameter cylinder, 30-cm long) suspended ~1.5 m above the ground or by Stickem-coated milk cartons (1 liter) on the ground. We are examining the use of pheromone-baited (**1a–1d**) water-containing pitfall traps to monitor for and mass trap *C. sordidus* in commercial banana plantations.

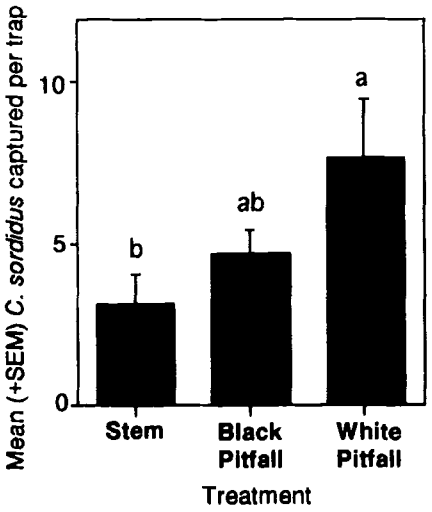


FIG. 7. Mean number (±SEM) of adult *C. sordidus* captured in insecticide-laced pseudostem traps, buried black or white plastic containers (containing detergent-laced water) baited with **1a–1d.** Test was conducted December 17–24, 1995. ANOVA ($N = 10$) on log ($X + 0.5$)-transformed data gave $F = 20.15$; $df = 3,33$; $P < 0.001$. Means are presented untransformed. Means topped by the same letter are not significantly different by Bonferonni t test ($P < 0.05$).

Acknowledgments—The authors would like to thank D. O. Otieno, K. Sum, and F. W. Karago (ICIPE) for collection of insects, trapping of volatiles; K. B. Wanyama (ICIPE) for GC-EI-MS analysis; H. Pierce (SFU) for preparative GC; M. Tracey (SFU) for NMR analyses; and R. M. McDonald for statistical analyses. The financial support from the Royal Norwegian Ministry of Research Co-operation & Development, NSERC of Canada, and the President's Research Grant Committee of SFU is highly appreciated.

REFERENCES

BARTLETT, P. A., and MEYERSON, J. 1978. Stereoselective epoxidation of acyclic olefinic carboxylic acids via iodolactonization. *J. Am. Chem. Soc.* 100:3950–3952.

BEAUHAIRE, J., and DUCROT, P.-H. 1996. On the regioselectivity of the Baeyer-Villiger reaction of 2,6-dialkyl cyclohexanones: Application to the synthesis of sordidin, a male pheromone emitted by *Cosmopolites sordidus*. *Bioorg. Med. Chem.* 4:413–418.

BEAUHAIRE, J., DUCROT, P.-H., MALOSSE, C., ROCHAT, D., NDIEGE, I. O., and OTIENO, D. O. 1995. Identification and synthesis of sordidin, a male-produced pheromone emitted by *Cosmopolites sordidus*. *Tetrahedron Lett.* 36:1043–1046.

BONGINI, A., CARDILLO, G., ORENA, M., PORZI, G., and SANDRI, S. 1982. Regio- and stereocontrolled synthesis of epoxy alcohols and triols from allylic and homoallylic alcohols via iodo carbonates. *J. Org. Chem.* 47:4626–4633.

BORDEN, J. H. 1985. Aggregation pheromones, pp. 257–285, *in* G. A. Kerkut and L. I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 9. Pergamon Press, Oxford.

BUDENBERG, W. J., NDIEGE, I. O., and KARAGO, F. W. 1993. Evidence for volatile male-produced pheromone in banana weevil, *Cosmopolites sordidus*. *J. Chem. Ecol.* 19:1905–1916.

COREY, E. J., and VENKATESWARLU, A. 1972. Protection by hydroxyl groups as *tert*-butyldimethylsilyl ethers. *J. Am. Chem. Soc.* 94:6190–6191.

HARVEY, W. E., and TARBELL, D. S. 1967. The reaction of the magnesium salt of *N*-cyclohexylcyclohexylimine with epoxides. *J. Org. Chem.* 32:1679–1681.

JIRASURAT, M. 1989. Study of biology of banana corm borer weevil. Annual Report of the Entomology and Zoology Division, Department of Agriculture, Bangkok, Thailand. 5 pp. Cited by W. Vittayaruk, W. Wattanachaiyingcharoen, T. Chuaycharoen, and D. Wattanachaibingcharoen (eds.) International Network for the Improvement of Banana and Plantain, Proceedings of Conference-Workshop on Nematodes and Weevil Borers Affecting Bananas in Asia and the Pacific, Serdang, Selangor, Malaysia, pp. 106–107.

MEYERS, A. I., TOMIOKA, K., ROLAND, D. M., and COMINS, D. 1978. Progress toward the total synthesis of maytansinoids. An efficient route to two major precursors (western–southern zone). *Tetrahedron Lett.* 19:1375–1378.

MORI, K. 1976. Synthesis of (1*S*:2*R*:4*S*:5*R*)-(−)-α-multistriatin. The pheromone in the smaller European elm bark beetle, *Scolytus multistriatus*. *Tetrahedron* 32:1979–1981.

MORI, K., NAKAYAMA, T., and TAKIKAWA, H. 1996. Synthesis and absolute configuration of sordidin, the male-produced aggregation pheromone of the banana weevil, *Cosmopolites sordidus*. *Tetrahedron Lett.* 37:3741–3744.

NDIEGE, I. O., JAYARAMAN, S., OEHLSCHLAGER, A. C., GONZALEZ, L. M., ALPIZAR, D., and FALLAS, M. 1996. Convenient synthesis and field activity of a male-produced aggregation pheromone of *Cosmopolites sordidus*. *Naturwissenschaften* 83:280–282.

NEWKOME, G. R., and FISHEL, D. L. 1988. Preparation of hydrazones: Acetophenone hydrazone. *Org. Synth. Coll. Vol.* 6:12–13.

OGENGA-LATIGO, M. W., and BAKYALIRE, R. 1993. Use of pseudostem traps and coefficient of infestation (PCI) for assessing banana infestation and damage by *Cosmopolites sordidus* Germar. *Afr. Crop Sci.* 1:31–38.

STANTON, J. M. 1994. Status of nematode and weevil borer problems affecting banana in Australia. Annual Report of the Entomology and Zoology Division, Department of Agriculture, Bangkok, Thailand. 5 pp. Cited by W. Vittayaruk, W. Wattanachaiyingcharoen, T. Chuaycharoen, and D. Wattanachaibingcharoen (eds.). International Network for the Improvement of Banana and Plantain, Proceedings of Conference-Workshop on Nematodes and Weevil Borers Affecting Bananas in Asia and the Pacific, Serdang, Selangor, Malaysia, pp. 48–56.

STILL, W. C., KAHN, M., and MITRA, A. 1978. Flash chromatography—a convenient method of rapid chromatography. *J. Org. Chem.* 43:2923–2926.

STORK, G., and DOWD, S. R. 1963. A new method for the alkylation of ketones and aldehydes: The C-alkylation of the magnesium slats of N-substituted imines. *J. Am. Chem. Soc.* 85:2178–2180.

TANAKA, S., YAMAMOTO, H., NOZAKI, H., SHARPLESS, K. B., MICHAELSON, R. C., and CUTTING, J. D. 1974. Stereoselective epoxidations of acyclic alcohols by transition metal-hydroperoxide reagents. Synthesis of *dl*-C_{18} *Cecropia* juvenile hormone from farnesol. *J. Am. Chem. Soc.* 96:5254–5255.

TREVERROW, N. L. 1994. Control of the banana weevil borer, *Cosmopolites sordidus* with entomopathogenic nematodes. W. Vittayaruk, W. Wattanachaiyingcharoen, T. Chuaycharoen, and D. Wattanachaibingcharoen (eds.). International Network for the Improvement of Banana and Plantain, Proceedings of Conference-Workshop on Nematodes and Weevil Borers Affecting Bananas in Asia and the Pacific, Serdang, Selangor, Malaysia, pp. 125–137.

ZAR, J. H. 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, New Jersey.

Journal of Chemical Ecology, Vol. 23, No. 4, 1997

REACTIONS OF *Gammarus lacustris* TO CHEMICAL STIMULI FROM NATURAL PREDATORS AND INJURED CONSPECIFICS

KAREN WUDKEVICH, BRIAN D. WISENDEN, DOUGLAS P. CHIVERS, and R. JAN F. SMITH*

Department of Biology, University of Saskatchewan
112 Science Place, Saskatoon, Saskatchewan, Canada, S7N 5E2

(Received June 3, 1996; accepted December 8, 1996)

Abstract—We exposed the freshwater amphipod *Gammarus lacustris*, to chemical stimuli from injured conspecifics and to chemical stimuli from two types of natural predators: dragonfly larvae (*Aeshna eremita*) and northern pike (*Esox lucius*). Exposure to all three stimuli caused *G. lacustris* to reduce significantly its level of activity relative to activity recorded in response to a distilled water control. The similarity in responses to chemicals associated with predators and to injured conspecifics suggests the presence of an alarm pheromone within the body tissues of *G. lacustris*. In response to chemical stimuli from pike, *G. lacustris* tended to reduce its time in the water column and spend more time near the bottom of the test aquaria. However, no such trend was apparent in response to chemical stimuli from dragonfly larvae. The differences in response to chemical stimuli from pike and larval dragonflies suggest that *G. lacustris* does not have a rigid behavioral response to predation risk; instead, antipredator behavior may be modified to maximize avoidance of predators that are active in different microhabitats.

Key Words—*Gammarus lacustris*, alarm pheromone, kairomone, predator avoidance, antipredator behavior, *Esox lucius*, *Aeshna eremita*.

INTRODUCTION

Visibility in aquatic habitats is often limited due to low light levels, turbidity, and dense vegetation. Perhaps because of this, many aquatic organisms use chemical cues, such as alarm pheromones and/or kairomones to assess their risk of predation. Alarm pheromones from injured individuals are chemical signals

*To whom correspondence should be addressed.

0098-0331/97/0400-1163$12.50/0 © 1997 Plenum Publishing Corporation

which elicit the same, or a similar, response in conspecifics as stimuli associated with predators (Smith, 1992). Alarm pheromones have been identified in various groups of fish (see Smith, 1992 for a review) and numerous other aquatic organisms, including amphibians (e.g., Hews and Blaustein, 1985), gastropods (Snyder, 1967), echinoderms (e.g., Snyder and Snyder, 1970; Parker and Shulman, 1986), sea anemones (Howe and Sheikh, 1975), insect larvae (Sih, 1986; Scrimgeour et al., 1994; Chivers et al., 1996), crayfish (Hazlett, 1994), and one species of amphipod (Williams and Moore, 1985). Kairomones, however, are chemical signals that produce a behavioral change in heterospecific receivers (Brown et al., 1970). The ability to detect predator kairomones and respond in a way that reduces predator success has been studied in numerous and diverse aquatic organisms (see Weldon, 1990; and Dodson et al., 1994 for reviews).

The detection of an alarm pheromone by an individual indicates that a conspecific has recently been captured or injured by a predator in the immediate vicinity. Exposure to alarm pheromone can increase the probability of fathead minnows (*Pimephales promelas*) surviving an encounter with a northern pike (*Esox lucius*) by increasing the latency to capture (Mathis and Smith, 1993a). For larval western toads (*Bufo boreas*) an increase in activity in response to alarm pheromone decreases the capture efficiency of predatory larval dragonflies (*Aeshna umbrosa*) (Hews, 1988). Hews suggests the decrease in capture efficiency is possibly the result of an increase in the physical difficulty of prey capture, an increase in the confusion effect, or an increase in prey awareness and vigilance. Detection and avoidance of predator kairomones may increase prey survival simply by decreasing the probability an individual will occupy the foraging habitat of a potential predator (Brodie et al., 1991).

Previous work on gammarids (Crustacea: Amphipoda: Gammaridae), small freshwater and marine amphipods, has shown that *Gammarus pseudolimnaeus* respond to kairomones of fish predators by decreasing their activity and drift rate (Williams and Moore, 1982, 1985) and increasing the latency to precopulatory pair formation (Mathis and Hoback, 1997). *G. minus* decrease activity in response to chemical stimuli from green sunfish (*Lepomis cyanellus*) (Holomuzki and Hoyle, 1990). When Andersen et al. (1993) added predatory brown trout (*Salmo trutta*) to two previously fishless streams, they noted an immediate reduction in the nighttime drift rate of *G. pulex*. The authors attribute this reduction in activity to chemical detection of trout by the gammarid. *G. pulex* reduce activity in response to the presence of caged predatory sculpins (*Cottus gobio*), but do not react when the sculpins are visible in sealed glass jars (Andersson et al., 1986). This suggests that chemical, rather than visual cues, are important to gammarids for predator detection and, presumably, predator avoidance.

In their review of 22 published studies of the antipredator behavior of stream prey in the presences of predators, Wooster and Sih (1995) found that, in general, prey increase drift in response to invertebrate predators and decrease

activity in response to vertebrate predators. In this study, we examined the response of *G. lacustris*, a common North American and European gammarid amphipod (Bousfield, 1958), to stimuli from injured conspecifics and to chemical stimuli (kairomones) from two natural predators that are active in different components of the *G. lacustris* habitat: benthic dragonfly larvae (*Aeshna eremita*), and an open-water predatory fish, the northern pike. Gammarids can compose a large portion of the diet of larval dragonflies (Pritchard, 1964) and pike (Chapman et al., 1989; Chapman and Mackay, 1990; Sammons et al., 1994), especially where gammarids are abundant and alternative invertebrate prey are scarce.

METHODS AND MATERIALS

Experimental Analysis. In May 1995, mature *G. lacustris* (mean = 0.071 g, SD = 0.007 g, N = 25) were collected from Pike Lake, Saskatchewan (51°54′N, 106°49′W), using dipnets, transferred to the laboratory and held in 175-liter aerated aquaria (22°C, 15.5L:8.5D photoperiod). Both northern pike and larval dragonflies occur naturally in Pike Lake; therefore, the test population of gammarids had previous experience with both experimental predators. The gammarids were identified using keys in Pennak (1978). In experiment 1, only male *G. lacustris* were used to avoid the possibility of sex differences in behavioral responses to predation risk during the breeding season (Boates et al., 1995; Mathis and Hoback, 1997). The breeding period of Canadian *G. lacustris* begins in April and continues into July (Bousfield, 1958; Clifford, 1969; De March, 1982), with maximal reproduction occurring when the water temperature reaches 18°C (Smith, 1973). No natural population of *G. lacustris* has been observed in precopula in late summer or autumn (De March, 1982), including the Pike Lake population (personal observation).

In August 1995, smaller, immature *G. lacustris* (mean = 0.026 g, SD = 0.004 g, N = 25) were collected from Pike Lake for use in experiment 2. These *G. lacustris* were immediately transferred to 37-liter study aquaria (room temperature of 22°C, 14L:10D photoperiod). The expected life-span of *G. lacustris* is about 15 months (Bousfield, 1958); the majority of mature individuals die in June or July after breeding (Hynes, 1955; Clifford, 1969; Menon, 1969). Menon (1969) estimated that in August, immatures compose 99% of the total *G. lacustris* population in Big Island Lake, Alberta. Since August is not the breeding season for *G. lacustris*, individuals of both sexes were tested.

Stimuli Preparation. Chemical stimuli from a single pike (fork length = 18.2 cm) were used in experiment 1. Use of multiple pike probably would not have affected the results, as several studies with fishes have shown that chemical stimuli from different individual pike elicit similar responses (Gelowitz et al.,

1993; Mathis and Smith, 1993b,c; Brown et al., 1995). Alarm pheromones of fathead minnows ingested by a pike can be released or excreted by the pike and detected by other prey (Mathis and Smith, 1993b,c; Brown et al., 1995). To ensure the pike stimulus contained only cues from the pike without residual alarm pheromones, the pike from which the stimulus was collected was maintained on a diet of mealworm larvae (*Tenebrio molitor*). Mealworms are terrestrial coleopteran larvae not normally encountered by pike or *G. lacustris*; therefore, even if *T. molitor* possesses an alarm pheromone, neither pike nor *G. lacustris* should recognize it. The pike received four mealworms per day for three days. Approximately 6 hr after the final feeding, the pike was rinsed in dechlorinated tap water and placed into an aerated, unfiltered collection chamber ($26 \times 8 \times 8$ cm) containing 1200 ml of dechlorinated water and held there for three days without food. The chamber was unfiltered to ensure that chemical stimuli produced by the pike were not removed from the water. The pike was then removed and the chamber water frozen at $-20°C$ in 60-ml aliquots and thawed as required immediately prior to use.

Larval dragonflies (*A. eremita*) (mean body length = 4.41 cm, SD = 0.37 cm, $N = 7$) were caught with a dipnet from Pike Lake in July 1995 for use in experiment 2. The larvae were identified using books by Merritt and Cummins (1984) and Walker (1958). Chemical stimuli from the larval dragonflies were collected in the same manner as the pike stimulus. However, prior to being placed in the collection chamber, all seven dragonflies were held in a 37-liter aquarium without food for one week to clear their digestive tract of residual alarm pheromones and prey consumed before capture. Dragonfly larvae can survive for extended periods without food, due to their low level of activity and low metabolic requirements (Corbet, 1962).

Experimental Protocols and Statistical Analysis. Thirty 37-liter study aquaria, with a substrate of silica sand 1 cm deep, were filled with dechlorinated water to a depth of 25 cm and aerated with a single airstone located at the back of each tank. A few strands of pondweed (*Potamogeton perfoliatus*), watermilfoil (*Myriophyllum*, spp.), and coontail (*Ceratophyllum demersum*), all collected from Pike Lake, were placed on the bottom of each tank such that the vegetation remained below a horizontal line drawn on the tank front 9 cm above the base of the aquarium's floor. The area above the horizontal line was designated as the "water column." The vegetation provided *G. lacustris* with both food and refuge during the experiments. Plastic airline tubing opening near the airstone served as a stimulus injection line. Each of the aquaria held a single, arbitrarily selected *G. lacustris*. Individuals were allowed 12–14 h to acclimate to laboratory conditions before data collection began.

In experiment 1, *G. lacustris* were exposed to one of three stimuli: (1) 20 ml of distilled water (control), (2) 20 ml of pike stimulus, or (3) 20 ml of distilled water containing two crushed mature *G. lacustris*. To test for an injury-

released alarm pheromone, *G. lacustris* were crushed using a mortar and pestle, similar to the way in which a predator might macerate its prey and release alarm pheromone.

In experiment 2, conducted in August, immature *G. lacustris* were exposed to one of three different stimuli: (1) 60 ml of distilled water (control), (2) 60 ml of larval dragonfly stimulus, or (3) 60 ml of distilled water containing three crushed immature *G. lacustris*. Experimental procotols and statistical analyses were identical to those used in experiment 1, except that in experiment 2 three *G. lacustris* were used to prepare the conspecific stimulus to compensate for the smaller size of the *G. lacustris* collected in August compared to those used in the conspecific stimulus of experiment 1.

To clear the stimulus injection line of any stagnant water, 60 ml of tank water was drawn through the line by a syringe just prior to testing and discarded. An additional 60 ml were then drawn and retained. Each experimental trial was composed of two 5-min observation periods separated by a 1-min interval. During the 1-min interval, the test stimulus was slowly injected into the injection line and then flushed into the tank using the previously collected 60 ml of tank water. Water currents created by the airstone quickly dispersed the stimulus throughout the aquaria. Dye tests conducted in previous experiments have shown that stimuli injected in this way are uniformly distributed throughout the tank in about 13 sec (Mathis and Smith, 1993b,c).

During each experimental trial, we quantified the total time each individual *G. lacustris* was active before and after the introduction of a stimulus. An individual was described as active if it was swimming through the water column, swimming or crawling among the vegetation, or crawling along the substratum. Change in activity was calculated by subtracting the time each individual spent active before stimulus injection from time spent active after injection. During each trial we also recorded the time spent above the horizontal line (i.e., in the water column) before and after stimulus injection and calculated the change in time spent in the water column in the same manner as for time active. A Kruskal-Wallis one-way analysis of variance test with nonparametric multiple comparisons (Siegel and Castellan, 1988) was used to test for significant differences in the change in total time active and the change in time spent in the water column in response to the three different chemical stimuli.

RESULTS

Experiment 1: Responses of Gammarus lacustris *to Chemical Stimuli from Northern Pike and Injured Conspecifics.* There was a significant overall difference in the change in time *G. lacustris* spent active among the three different treatment conditions ($KW = 8.86$, $P < 0.05$). In response to chemical stimuli

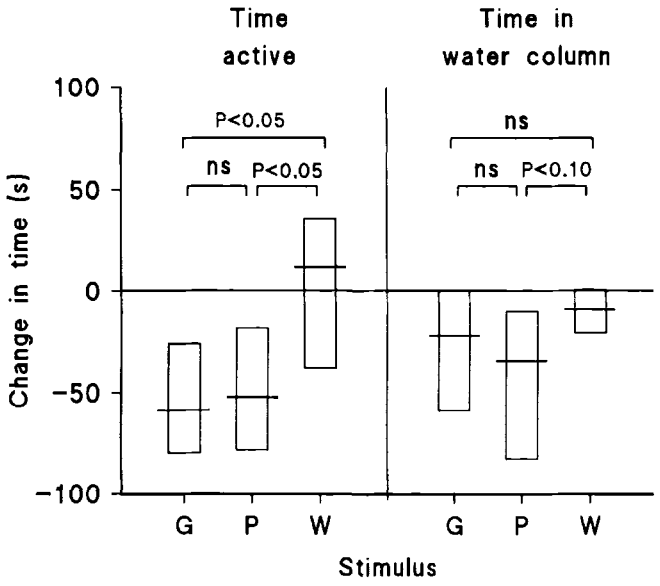


FIG. 1. Median values (±25% quartiles) reflecting a change in the time *G. lacustris* spent active (swimming or crawling) and a change in the time spent in the water column before and after injection of various stimuli. Level of significance ($P < 0.05$, $P < 0.10$) calculated by post-hoc nonparametric multiple comparisons of a Kruskal-Wallis ANOVA (ns denotes $P > 0.10$). G = distilled water containing crushed conspecifics (*G. lacustris*), P = chemical stimuli from northern pike, W = distilled water control.

from injured conspecifics and from pike, *G. lacustris* significantly decreased their time spent active when compared to the distilled water control ($P < 0.05$ for both stimuli, Figure 1). There was no significant difference in the change in time spent active between the response of *G. lacustris* exposed to injured conspecifics and those exposed to chemical stimuli from pike ($P > 0.10$, Figure 1).

The overall difference among the treatment conditions in the change in time *G. lacustris* spent in the water column was not significant, although a trend was evident ($KW = 4.64$, $0.05 < P < 0.10$). *G. lacustris* tended to reduce the amount of time spent in the water column in response to the pike stimulus compared to distilled water (Figure 1).

Experiment 2: Responses of Gamma lacustris *to Chemical Stimuli from Dragonfly Larvae and Injured Conspecifics.* There was a significant overall difference in the change in time *G. lacustris* was active ($KW = 12.72$, $P < 0.05$). Compared to distilled water, *G. lacustris* significantly decreased activity

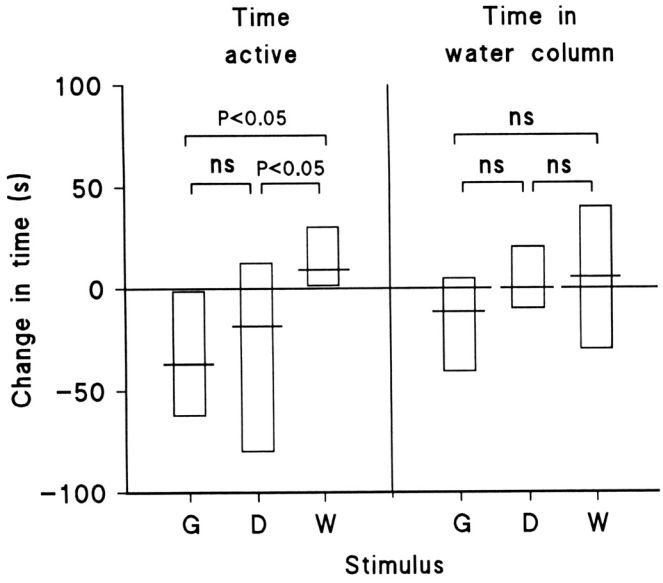


FIG. 2. Median values (±25% quartiles) reflecting a change in the time *G. lacustris* spent active (swimming or crawling) and a change in the time spent in the water column before and after injection of various stimuli. Level of significance ($P < 0.05$) calculated by post-hoc nonparametric multiple comparisons of a Kruskal-Wallis ANOVA (ns denotes $P > 0.05$). G = distilled water containing crushed conspecifics (*G. lacustris*), D = chemical stimuli from dragonfly larvae (*Aeshna eremita*), W = distilled water control.

in response to stimuli from injured conspecifics and to stimuli from larval dragonflies ($P < 0.05$ for both stimuli, Figure 2). In terms of reduced time active, there was no significant difference between the response of *G. lacustris* exposed to injured conspecifics and those exposed to chemical stimuli from dragonfly larvae ($P > 0.10$, Figure 2). There was no overall significant difference among treatments in the time *G. lacustris* spent in the water column ($KW = 2.15$, $P > 0.10$, Figure 2).

DISCUSSION

G. lacustris displayed the ability to detect and respond to predator kairomones from pike and from a larval dragonfly. In response to kairomones from both predators, *G. lacustris* significantly decreased activity. Both larval dragonflies (Corbet, 1962; Pritchard, 1965) and pike (Savino and Stein, 1989) are visually oriented, ambush predators, remaining immobile until they perceive

movement by a prey organism. By reducing activity, *G. lacustris* can possibly reduce visual detection and avoid attack, especially from larval dragonflies, which can perceive moving prey at distances of up to 20 cm; however, motionless prey can only be detected at distances of up to 5 cm (Corbet, 1962). Motion has also been shown to increase the distance at which fish (Wright and O'Brien, 1982) and bird (Martel and Dill, 1995) predators can detect their potential prey. The ability to detect and respond adaptively to kairomones from predatory invertebrates and fish has also been found in *G. pseudolimnaeus* (Williams and Moore, 1982, 1985; Mathis and Hoback, 1997), *G. minus* (Holomuzki and Hoyle, 1990), and *G. pulex* (Andersson et al., 1986; Andersen et al., 1993).

The reduction in activity displayed by *G. lacustris* when exposed to crushed conspecifics was similar to its response to chemical stimuli from both predators. This indicates that some component of the body tissues of *G. lacustris* alerts conspecifics to respond in the same manner as to chemicals associated with danger from predators. Therefore, *G. lacustris* possesses an alarm pheromone.

Wooster and Sih (1995) found that, in general, prey increase drift in response to invertebrate predators and decrease activity in response to vertebrate predators. *G. lacustris* tended to move out of the water column towards the bottom of the test aquaria in response to chemical stimuli from pike, predators that remain in or near the shallow vegetated regions of the littoral zone (Chapman and Mackay, 1984). However, there was no such tendency in response to chemical stimuli from larval dragonflies, benthic predators that live among submerged vegetation (Pritchard, 1965). This qualitative difference in antipredator response suggests that *G. lacustris* is capable of recognizing and reacting with variable antipredator behavior to stimuli from different types of predators. Variable antipredator behavior has previously been observed in *G. pseudolimnaeus* (Williams and Moore, 1985). In response to the slow-moving, tactile, benthic, predatory stonefly larvae, *Phasganophora capitata*, *G. pseudolimnaeus* increases activity and drift; however, to visually oriented predators, such as the crayfish, *Cambarus robustus*, *G. pseudolimnaeus* significantly reduce their activity level.

Mature individuals of a closely related species, *G. pseudolimnaeus*, react to the non-contact presence of numerous fish and invertebrate predators regardless of season (Williams and Moore, 1985). All sizes of *G. pseudolimnaeus* respond to trichopteran predators, which only prey on very small amphipods (Williams and Moore, 1985). In both experiments, *G. lacustris* decreased activity in response to conspecific cues (a general antipredator response) independent of the time of year and the age class of test gammarids. Therefore, it is unlikely that the different times of season and age classes of test animals used in this study are responsible for the variation in antipredator behavior of *G. lacustris*. Besides fish and dragonfly larvae, amphipods are potential prey to many other predators, such as ducks (Winfield and Winfield, 1994), shorebirds (Boates et

al., 1995), crayfish (Williams and Moore, 1985), and numerous small predatory invertebrates (e.g., Dytiscidae, Notonectidae). Being vulnerable to so many different types of predators, each with its own unique hunting style, possession of variable antipredator behavior that allows individuals to react to each potential threat as it occurs would be of great benefit to *G. lacustris*. Variability in antipredator behavior allows prey individuals an opportunity to maximize survival against multiple predators that are active in different microhabitats or that possess different hunting styles.

Acknowledgments—We thank Dr. D. Lehmkuhl and Dr. C. Gillott for advice concerning the biology of dragonfly larvae. R. Mirza assisted with the collection of study specimens, and Dr. G. Brown and Dr. J. Schmutz provided helpful comments on earlier drafts of this manuscript. Financial support for this research was provided by the Natural Sciences and Engineering Research Council of Canada and the University of Saskatchewan.

REFERENCES

ANDERSEN, T. H., FRIBERG, N., HANSEN, H. O., IVERSEN, T. M., JACOBSEN, D., and KRØJGAARD, L. 1993. The effects of introduction of brown trout (*Salmo trutta* L.) on *Gammarus pulex* L. drift and density in two fishless Danish streams. *Arch. Hydrobiol.* 126:361–371.

ANDERSSON, K. G., BRÖNMARK, C., HERRMANN, J., MALMQVIST, B., OTTO, C., and SJÖRSTROM, P. 1986. Presence of sculpins (*Cottus gobio*) reduces drift and activity of *Gammarus pulex* (Amphipoda). *Hydrobiologia* 133:209–215.

BOATES, J. S., FORBES, M., ZINCK, M., and MCNEIL, N. 1995. Male amphipods (*Corophium volutator* [Pallas]) show flexible behaviour in relation to risk of predation by sandpipers. *Ecoscience* 2:123–128.

BOUSFIELD, E. L. 1958. Fresh-water amphipod crustaceans of glaceated North America. *Can. Field Nat.* 72:55–113.

BRODIE, E. D., JR., FORMANOWICZ, D. R., JR., and BRODIE, E. D., III. 1991. Predator avoidance and antipredator mechanisms: distinct pathways to survival. *Ethol. Ecol. Evol.* 3:73–77.

BROWN, G. E., CHIVERS, D. P., and SMITH, R. J. F. 1995. Localized defecation by pike: A response to labelling by cyprinid alarm pheromone? *Behav. Ecol. Sociobiol.* 36:105–110.

BROWN, W. L., EISNER, T., and WHITTAKER, R. H. 1970. Allomones and kairomones: Transpecific chemical messengers. *BioScience* 20:21–22.

CHAPMAN, C. A., and MACKAY, W. C. 1984. Direct observation of habitat utilization by northern pike. *Copeia* 1984:255–258.

CHAPMAN, L. J., and MACKAY, W. C. 1990. Ecological correlates of feeding flexibility in northern pike (*Esox lucius*). *J. Freshwater Ecol.* 5:313–322.

CHAPMAN, L. J., MACKAY, W. C., and WILKINSON, C. W. 1989. Feeding flexibility in northern pike (*Esox lucius*): Fish versus invertebrate prey. *Can. J. Fish. Aquat. Sci.* 46:666–669.

CHIVERS, D. P., WISENDEN, B. D., and SMITH, R. J. F. 1996. Damselfly larvae learn to recognize predators from chemical cues in the predator's diet. *Anim. Behav.* 52:315–320.

CLIFFORD, H. F. 1969. Limnological features of a northern brown-water stream with special reference to the life histories of the aquatic insects. *Am. Midl. Nat.* 82:578–597.

CORBET, P. S. 1962. A Biology of Dragonflies. Quadrangle Books, Illinois.

DE MARCH, B. G. E. 1982. Decreased day length and light intensity as factors inducing reproduction in *Gammarus lacustris lacustris* Sars. *Can. J. Zool.* 60:2962–2965.

DODSON, S. I., CROWL, T. A., PECKARSKY, B. L., KATS, L. B., COVICH, A. P., and CULP, J. M. 1994. Non-visual communication in freshwater benthos: An overview. *J. North Am. Benthol. Soc.* 13:268–282.

GELOWITZ, C. M., MATHIS, A., and SMITH, R. J. F. 1993. Chemosensory recognition of northern pike (*Esox lucius*) by brook stickleback (*Culaea inconstans*): Population differences and the influence of predator diet. *Behaviour* 127:105–118.

HAZLETT, B. A. 1994. Alarm responses in the crayfish *Orconectes virilis* and *Orconectes propinquus*. *J. Chem. Ecol.* 20:1525–1535.

HEWS, D. K. 1988. Alarm response in larval western toads, *Bufo boreas*: release of larval chemicals by a natural predator and its effect on predator capture efficiency. *Anim. Behav.* 36:125–133.

HEWS, D. K., and BLAUSTEIN, A. R. 1985. An investigation of the alarm response in *Bufo boreas* and *Rana cascadae* tadpoles. *Behav. Neural Biol.* 43:47–57.

HOLOMUZKI, J. R., and HOYLE, J. D. 1990. Effect of predatory fish presence on habitat use and diel movement of the stream amphipod, *Gammarus minus*. *Freshwater Biol.* 24:509–517.

HOWE, N. R., and SHEIKH, Y. M. 1975. Anthopleurine: A sea anemone alarm pheromone. *Science* 189:386–388.

HYNES, H. B. N. 1955. The reproductive cycle of some British freshwater Gammaridae. *J. Anim. Ecol.* 24:352–387.

MARTEL, G., and DILL, L. M. 1995. Influence of movement by coho salmon (*Oncorhynchus kisutch*) parr on their detection by common mergansers (*Mergus merganser*). *Ethology* 99:139–149.

MATHIS, A., and HOBACK, W. W. 1997. The influence of chemical stimuli from predators on precopulatory pairing by the amphipod, *Gammarus pseudolimnaeus*. *Ethology* 103:33–40.

MATHIS, A., and SMITH, R. J. F. 1993a. Chemical alarm signals increase the survival time of fathead minnows (*Pimephales promelas*) during encounters with northern pike (*Esox lucius*). *Behav. Ecol.* 4:260–265.

MATHIS, A., and SMITH, R. J. F. 1993b. Chemical labeling of northern pike (*Esox lucius*) by the alarm pheromone of fathead minnows (*Pimephales promelas*). *J. Chem. Ecol.* 19:1967–1979.

MATHIS, A., and SMITH, R. J. F. 1993c. Fathead minnows, *Pimephales promelas*, learn to recognize northern pike, *Esox lucius*, as predators on the basis of chemical stimuli from minnows in the pike's diet. *Anim. Behav.* 46:645–656.

MENON, P. S. 1969. Population ecology of *Gammarus lacustris* Sars in Big Island Lake. I. Habitat preference and relative abundance. *Hydrobiologia* 33:14–32.

MERRITT, R. W., and CUMMINS, K. W. 1984. An Introduction to the Aquatic Insects of North America, 2nd ed. Kendall/Hunt Publishing, Dubuque, Iowa.

PARKER, D. A., and SHULMAN, M. J. 1986. Avoiding predation: alarm responses of Caribbean sea urchins to simulated predation on conspecific and heterospecific sea urchins. *Mar. Biol.* 93:201–208.

PENNAK, R. W. 1978. Freshwater Invertebrates of the United States, 2nd ed. John Wiley & Sons, New York.

PRITCHARD, G. 1964. The prey of dragonfly larvae (Odonata; Anisoptera) in ponds in northern Alberta. *Can. J. Zool.* 42:785–800.

PRITCHARD, G. 1965. Prey capture by dragonfly larvae (Odontata; Anisoptera). *Can. J. Zool.* 43:271–289.

SAMMONS, S. M., SCALET, C. G., and NEUMANN, R. M. 1994. Seasonal and size-related changes in the diet of northern pike from a shallow prairie lake. *J. Freshwater Ecol.* 9:321–329.

SAVINO, J. F., and STEIN, R. A. 1989. Behavior of fish predators and their prey: Habitat choice between open water and dense vegetation. *Env. Biol. Fish.* 24:287–293.

SCRIMGEOUR, G. J., CULP, J. M., and CASH, K. J. 1994. Antipredator responses of mayfly larvae to conspecific and predator stimuli. *J. North Am. Benthol. Soc.* 13:299–309.

SIEGEL, S., and CASTELLAN, N. J. 1988. Nonparametric Statistics for the Behavioral Sciences, 2nd ed. McGraw-Hill, New York.

SIH, A. 1986. Antipredator responses and the perception of danger by mosquito larvae. *Ecology* 67:434–441.

SMITH, R. J. F. 1992. Alarm signals in fishes. *Rev. Fish Biol. Fish.* 2:33–63.

SMITH, W. E. 1973. Thermal tolerance of two species of *Gammarus*. *Trans. Am. Fish. Soc.* 102:431–433.

SNYDER, N. F. R. 1967. An alarm reaction of aquatic gastropods to intraspecific extract. Cornell University, Agricultural Experiment Station, New York State College of Agriculture, Ithaca, NY, Memoir 403, pp. 1–122.

SNYDER, N., and SNYDER, H. 1970. Alarm response of *Diadema antillarum*. *Science* 168:276–278.

WALKER, E. D. 1958. The Odonata of Canada and Alaska, Vol. II. University of Toronto Press, Ontario.

WELDON, P. J. 1990. Responses by vertebrates to chemicals from predators, pp. 500–521, *in* D. W. MacDonald, D. Muller-Schwarze, and S. E. Natynczuk (eds.). Chemical Signals in Vertebrates 5. Oxford University Press, Oxford.

WILLIAMS, D. D., and MOORE, K. A. 1982. The effect of environmental factors on the activity of *Gammarus pseudolimnaeus* (Amphipoda). *Hydrobiologia* 96:137–147.

WILLIAMS, D. D., and MOORE, K. A. 1985. The role of semiochemicals in benthic community relationships of the lotic amphipod *Gammarus pseudolimnaeus*: A laboratory analysis. *Oikos* 44:280–286.

WINFIELD, I. J., and WINFIELD, D. K. 1994. Feeding ecology of the diving ducks pochard (*Aythya ferina*), tufted duck (*A. fuligula*), scaup (*A. marila*) and goldeneye (*Bucephala clangula*) overwintering on Lough Neagh, Northern Ireland. *Freshwater Biol.* 32:467–477.

WOOSTER, D., and SIH, A. 1995. A review of the drift and activity responses of stream prey to predator presence. *Oikos* 73:3–8.

WRIGHT, D. I., and O'BRIEN, W. J. 1982. Differential location of *Chaoborus* larvae and *Daphnia* by fish: the importance of motion and visible size. *Am. Midl. Nat.* 108:68–73.

Journal of Chemical Ecology, Vol. 23, No. 4, 1997

CHEMICAL AND BIOLOGICAL ASSAYS TO EVALUATE BACTERIAL INHIBITION BY TANNINS

K. E. NELSON,[1] A. N. PELL,* P. H. DOANE, B. I. GINER-CHAVEZ,[2] and P. SCHOFIELD

Department of Animal Science
Cornell University
Ithaca, New York 14853

(Received September 5, 1996; accepted December 8, 1996)

Abstract—The interactions among purified tannins from three different plants with five strains of ruminal bacteria were investigated. Tannins were purified from crude quebracho (*Schinopsis balansae* spp.), desmodium (*Desmodium ovalifolium*), and myrtle (*Mirtus communis*). The ruminal bacteria studied were *Streptococcus bovis* JB1, *Ruminococcus albus* 8, *Fibrobacter succinogenes* S85, *Prevotella ruminicola* H_14, and a recently isolated tannin-tolerant bacterium. Bacterial binding to tannins, rate of tannin binding, and the inhibitory concentrations for each bacterium to each tannin were evaluated. The effects of tannins on glucose utilization and fermentation product formation also were studied. Tannin binding was rapid with little additional binding occurring 10 min after tannin addition. There was variation among bacteria in the amount of tannins bound and in the amount of tannin required to inhibit growth. Condensed tannins from myrtle and desmodium had the highest binding capacity and were most inhibitory to microbes. Absorbance readings from the total phenolics assay were highest for myrtle and lowest for quebracho. Similarly, readings for condensed tannins were highest for desmodium, which also had the largest polymer size. Myrtle and quebracho gave the largest precipitate rings in the radial diffusion assay, a biological assay that measures precipitation of bovine serum albumin by tannins. The relative absorbance values obtained from the total phenolics assay were the most useful predictor of tannin inhibition of microbial growth. The acid–butanol assay and the radial diffusion assay using bovine serum albumin as the precipitable protein were much less useful predictors of tannin biological activity against the bacteria studied.

Key Words—Condensed tannin, quebracho, rumen, proanthocyanidin, inhibition.

*To whom correspondence should be addressed.
[1]Currently at the Institute for Genome Research, Rockville, Maryland.
[2]Currently at Eli Lilly, Mexico City, Mexico.

0098-0331/97/0400-1175$12 50/0 © 1997 Plenum Publishing Corporation

INTRODUCTION

Tannins are a diverse group of naturally occurring plant polyphenolic compounds that readily form hydrophobic and hydrogen bonds to proteins resulting in tannin–protein complexes. Condensed tannins interact with microorganisms in a number of artificial and natural environments, including sewage sludge (Field et al., 1988), wastewaters derived from fruit and vegetable operations (Field and Lettinga, 1987), forest litter (Deschamps, 1982), and the rumen (Mueller-Harvey et al., 1988). The condensed tannins of sainfoin (*Onobrychis viciifolia* Scop.) inhibited the cell-associated proteolytic activity of the ruminal bacteria *Butyrivibrio fibrisolvens* A38 and *Streptococcus bovis* 45S1 by 48% and 92%, respectively, when 25 μg/liter of the condensed tannin was included in the growth medium (Jones et al., 1994). The condensed tannins bound to the cell-coat polymers of these strains. The endoglucanase activity of *Fibrobacter succinogenes* was reduced by the condensed tannins of birdsfoot trefoil (*Lotus corniculatus* L.) at concentrations as low as 25 μg/ml (Bae et al., 1993).

There is limited information on the inhibitory effects of tannins on microorganisms, and on the best methods for the accurate estimation of tannin activity in biological systems. Many chemical and protein precipitation assays have been used to predict tannin biological activity, but correlations between these assays and actual inhibition of microbial activity have not been reported. The two main objectives of this study were to determine the effects of purified tannins from various plant sources on several species of ruminal bacteria and to evaluate some of the available chemical tannin analytical methods as predictors of tannin antimicrobial activity. Protein binding and chemical assays, two methods available for quantifying tannins, were used to study the relationships between tannins and microbes. The capacity of bacteria to bind tannins also was evaluated as a predictor of tannin antimicrobial activity.

METHODS AND MATERIALS

Reagents. All reagents were analytical grade. Agarose, bovine serum albumin (BSA), gallic acid, and catechin were obtained from Sigma Chemical Co. (St. Louis, Missouri). Cyanidin chloride was purchased from Extrasynthese (B.P. 69726, Genay, Cedex, France).

Isolation of Condensed Tannins. The condensed tannins used in this study were purified from crude quebracho (*Schinopsis balansae* spp.), desmodium (*Desmodium ovalifolium*), and myrtle (*Mirtus communis*). Desmodium is a tropical forage legume species, and myrtle is a browse species fed to livestock in the Mediterranean region. Quebracho is commonly used as a standard in many tannin assays (Waterman and Mole, 1994).

Spray-dried quebracho was purchased from Trask Chemical Company, Marietta, Georgia. Desmodium and myrtle were harvested in Honduras and Sardinia, respectively, and frozen within 1 hr of harvest. This frozen plant material was lyophilized within 2 hr, and the lyophilized material was ground to pass a 1-mm screen in a Wiley mill (Arthur Thomas, Philadelphia, Pennsylvania). The ground lyophilized material was stored refrigerated in the dark at 4°C. To extract condensed tannins from the lyophilized plant material, 2 g of plant tissue were placed in a 50-ml screw cap centrifuge tube with 20 ml of 70% aqueous acetone and sonicated in a Bransonic 521 sonicator (Shelton, Connecticut) for 30 min at 4°C. Samples were filtered through a sintered glass funnel, and the filtrate was stored at 4°C. This aqueous acetone extraction was repeated three times on the same 2-g sample. Acetone was removed from the composited extracts under vacuum in a water bath at 29°C, and the resulting fraction was applied to a Sephadex LH-20 column for purification of the condensed tannins. The method of Asquith and Butler (1985), as modified by Hagerman and Butler (1994), was used for this purification.

Tannin Studies. Total phenolics were determined by the Prussian-blue assay (Price and Butler, 1977). Condensed tannins were measured by the acid–butanol assay of Porter et al. (1986), and the vanillin assay of Price et al. (1978). The radial diffusion assay was used for the estimation of the amount of BSA precipitated by tannin (Hagerman, 1987). Hydrolyzable tannins were measured by the method of Inoue and Hagerman (1988) for gallotannin determination.

Choice of Standards. Standards were chosen depending on the chemical structures being measured. Cyanidin was used as the standard in the acid–butanol assay. Cyanidin, an anthocyanidin, may be an appropriate standard for proanthocyanidins because when flavonoid subunits are oxidatively cleaved from proanthocyanidins, predominantly anthocyanidins are formed (Hagerman and Butler, 1989). However, cyanidin is a less appropriate standard for condensed tannins that yield delphinidins when cleaved (Waterman and Mole, 1994).

The vanillin assay, which is specific for flavonols, is based on the condensation of vanillin with the phloroglucinol structure of flavon-3-ols and proanthocyanidins under acidic conditions in methanol or in ethanol. Catechin was used as the standard in the vanillin assay to represent the phloroglucinol structure. One of the drawbacks of using catechin as a standard is that catechin and condensed tannins do not react with vanillin at the same rate (Spielman and Bennick, 1989). Gallic acid was used as the standard for the total phenolics and the hydrolyzable tannin assays.

In addition to these external standards, internal standards were generated for each plant for each assay. This was achieved by purifying sufficient quantities of tannins from each plant source as previously described so that the same tannin extracts could be used in all experiments. Different specified quantities of the

purified tannins were used to construct standard curves for the total phenolics, vanillin, and acid-butanol assays.

Relative Degree of Polymerization. The ratio of the absorbance measurements obtained from the acid-butanol and the vanillin methods was used to determine the relative degree of polymerization of the condensed tannins (Waterman and Mole, 1994).

Culture Technique and Media. The bacteria used in this study included *Streptococcus bovis* JB1, *Ruminococcus albus* 8, *Fibrobacter succinogenes* S85, *Prevotella ruminicola* $H_1$4, and the recently isolated KN1, a member of the streptococci and a near relative of *S. bovis* (Nelson, 1996). This gram-positive diplococcus is able to tolerate high levels of both hydrolyzable and condensed tannins (Nelson et al., 1995). It was isolated from a Colombian goat eating a diet high in tannins (Nelson et al., 1995). *Streptococcus bovis* JB1, *P. ruminicola* $H_1$4, and *F. succinogenes* S85 were from the culture collection of J. B. Russell, Cornell University. *Ruminococcus albus* 8 was originally from the University of Illinois culture collection.

The anaerobic culture techniques of Hungate (1950) with modifications (Bryant, 1972) were used for all incubations. Ruminal fluid medium (Bryant and Burkey, 1953), which was used for routine maintenance of cultures and to measure tannin binding to cells, included 0.3% cellobiose as the carbon source. Filter-sterilized carbohydrates were added after autoclaving. The ruminal fluid used in the medium was from a nonlactating cow fed medium-quality grass hay that did not contain tannins. The ruminal fluid was clarified aerobically by centrifugation at 20,000*g* for 20 min at 4°C. Medium was transferred in 9.7-ml quantities to butyl-rubber-stoppered roll tubes purged with oxygen-free CO_2. Tubes were autoclaved at 120°C for 15 min. The pH after autoclaving was between 6.5 and 6.7. Incubations were performed at 39°C. Basal medium (Russell, 1987) from which resazurin was omitted was used for all microbial growth and uptake studies conducted in the presence of purified condensed tannins.

Tannin-Microbial Binding. To measure binding of tannins to bacteria, 5-ml aliquots of mid-exponential-phase cultures of the five bacteria grown in the absence of tannins were exposed to five levels of tannins for 30 min. Tannins were added to the microbial cultures when the absorbance of the growth medium at 600 nm (A_{600}) was 0.75. Purified tannins in phosphate buffer (pH 6.8) (Goering and Van Soest, 1970) were added to the growth medium to yield final concentrations of 20, 100, 200, 300, and 400 μg/ml. Tannins were absent from control tubes. After 30 min, exposed cultures were centrifuged at 6000*g* for 10 min. The supernatant was discarded and the pellet was resuspended in 5 ml of pH 6.8 phosphate buffer solution (Goering and Van Soest, 1970) by vortexing and was spun again at 6000*g* for 10 min at 4°C. This was repeated, and the pellets were subsequently lyophilized. Each bacterial pellet was analyzed for proanthocyanidins by the acid-butanol method. Lyophilization of the pellet was necessary to prevent interference caused by water (Porter et al., 1986).

For the timed binding experiments, 500 μg of tannins were added to 5-ml aliquots of mid-log-phase cultures grown without tannins, and the cultures were incubated for 30 sec, 1 min, 5 min, 10 min, 30 min, and 2 hr. Cultures were treated as previously described in the tannin binding experiment, and the lyophilized pellets were analyzed for proanthocyanidins by the acid–butanol method.

As the five strains of bacteria differ in size, shape, and in the relationship between absorbance and cell number, it was necessary to examine the relationship between tannin binding and surface area per milliliter of cells for all the species tested. The dimensions of *S. bovis* JB1, *R. albus* 8, *F. succinogenes* S85 *P. ruminicola* H_I4, and KN1 were measured with a confocal scanning microscope during the mid-exponential phase of growth. Cell numbers for all bacterial species were counted with a Neubauer chamber (Philadelphia, Pennsylvania). Six fields were counted for each culture, and this was repeated three times. Standard curves relating cell number to A_{600} were constructed, and these numbers were used to calculate numbers of cells per milliliter. The dimensions of the bacteria used to calculate total microbial cell surface area were: *S. bovis* JB1, 0.85 μm; *R. albus* 8, 0.84 μm; *F. succinogenes* S85, 0.25 × 1.5 μm; *P. ruminicola* H_I4, 0.84 × 1.5 μm; and KN1, 0.82 μm. *Streptococcus bovis*, *R. albus*, and KN1 were assumed to be spherical, and *F. succinogenes* and *P. ruminicola* were assumed to be cylindrical in surface area calculations.

Growth Inhibition Studies with Condensed Tannins. To study the effects of purified condensed tannins on growth of five ruminal bacteria, 9.7 ml of basal medium with cellobiose (control) and 9.7 ml of basal medium plus cellobiose and added purified tannins from myrtle, desmodium, or quebracho were inoculated with 0.3 ml of mid-exponential-phase cultures of the respective ruminal bacteria grown in basal medium. The purified tannins were dissolved in phosphate buffer (pH 6.8) (Goering and Van Soest, 1970) and added by filter sterilization to yield final concentrations ranging from 10 to 1200 μg/ml in the medium. The pH of the medium was not altered by addition of these compounds. The effect on growth rate was monitored hourly by measuring changes in absorbance (A_{600}) of the culture. Uninoculated medium served as the control. Duplicate readings were taken in a Spectronic 601 spectrophotometer (Milton Roy, Rochester, New York). The inhibitory tannin concentration was defined as the amount of purified tannin at which there was no change in A_{600} when inoculated cultures were compared to a tannin-containing blank.

Streptococcus bovis JB1, *P. ruminicola* H_I4, and KN1 were selected for further evaluation of the effects of tannins on growth, glucose utilization, and end-product formation. *Streptococcus bovis* and *P. ruminicola* represented gram-positive and gram-negative bacteria. The gram-positive isolate KN1 was included in the analyses as it can tolerate high levels of tannins (Nelson et al., 1995).

The three bacteria were grown in pure culture in 10 ml of basal medium from which resazurin had been omitted and with glucose as the carbon source. During the incubation, 0.5-ml samples were removed hourly with a hypodermic

syringe, immediately placed on ice, and the A_{600} and pH of the cultures recorded. Uninoculated medium served as the control. Samples were centrifuged at 6000 *g* for 10 min at 4°C and the cell free supernatants stored at −20°C. At A_{600} of 0.4, cultures of each of the three bacteria were exposed to concentrations of purified desmodium tannins previously shown to inhibit growth of the respective organisms.

Glucose utilization and volatile fatty acid production were analyzed by HPLC as previously described (Nelson et al., 1995). The frozen aliquots were thawed and mixed with 25 mg of polyvinylpolypyrrolidone (PVPP) to remove residual tannins. Samples were vortexed, allowed to stand at room temperature for 60 min, and centrifuged at 6000*g* for 10 min. Aliquots of 40 μl were analyzed on a Rainin HPLC (Macrode, Woburn, Massachusetts) with a Knauer differential refractometer, a Varian model 9176 recorder, and a Bio-Rad Aminex HPX-87H fast acid column (10 cm × 7.8 mm). The solvent was 1.5 mM H_2SO_4 at a flow rate of 2.4 ml/min. A mixture of acetic, propionic, isobutyric, butyric, fumaric, succinic, and lactic acids was included as an external calibration standard in all analyses. Glucose was used as a separate standard for calibration.

Growth rate changes were calculated by plotting the natural logarithm of absorbance against time and determining the changes in growth rates in the control and inoculated cultures following addition of tannins to the cultures.

Statistical Analysis. Differences among the five bacteria in the quantity of tannin bound, and among the three purified tannins in their binding to bacteria were determined by a two-way analysis of variance (SigmaStat ver. 1.0; Jandel Scientific, San Rafael, California). If the overall analysis of variance indicated that there were significant differences among treatments ($P < 0.05$), the Student-Newman-Keuls test was used to determine differences among the means. Correlation analysis was used to explore the relationship among the results of the BSA precipitation, total phenolics, and the acid–butanol assays with the concentrations of purified tannins inhibitory to growth (Minitab Inc., ver. 10, State College, Pennsylvania). In addition, correlation analysis was used to describe the relationship between the amount of purified tannin that a bacterium could bind and the inhibition of that bacterium by the tannin. Stepwise regressions were performed to determine the variation in bacterial inhibition that could be explained by total phenolics, condensed tannins, ability to precipitate protein, or ability of bacteria to bind tannins.

RESULTS

Tannin Measurement. Purification of tannins from quebracho, desmodium, and myrtle resulted in yields of approximately 6%, 17%, and 13%, respectively, of the initial material on a dry matter basis.

The relative absorbance values for the total phenolics assay with either

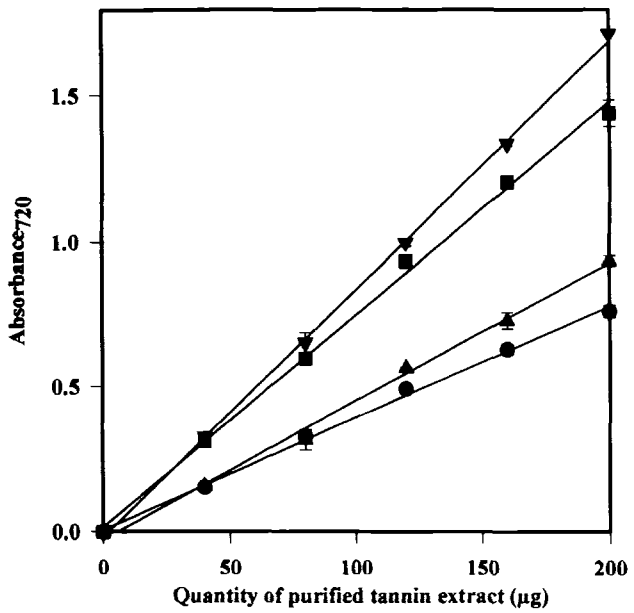


FIG. 1. Total phenolics standard curves of three purified tannins and gallic acid. Symbols: ▼, gallic acid; ●, quebracho; ■, desmodium; ▲, myrtle.

gallic acid as the standard or with internal standard curves for the individual tannins are presented in Figure 1. Problems associated with the use of external standards are evident here. If purified quebracho tannins had been the standard of choice in this assay, we would conclude that approximately 150 μg of the purified desmodium tannin was present at an A_{720} reading of 0.6. However, 80 μg of desmodium tannin actually generated this reading. This would be an overestimation of almost 88% of the total phenolics concentration in the purified desmodium. Similarly, at the same absorbance, gallic acid slightly underestimated the quantity of total phenolics in the desmodium extract by 10%. For this reason, we have used the internal standards in all of our calculations.

Hydrolyzable tannins, or gallic acid equivalents, as measured by the method of Inoue and Hagerman (1988), were absent from the purified desmodium and quebracho extracts. The myrtle extract had 7 mg gallic acid equivalents per gram of purified extract.

The regression values for the standard curves generated from the acid-butanol assay were desmodium, $y = 0.00906x + 0.0155$, $R^2 = 0.997$; myrtle, $y = 0.00144x - 0.00414$, $R^2 = 0.997$; quebracho, $y = 0.00106x - 0.00360$, $R^2 = 0.995$; and cyanidin $y = 0.000821x + 0.00305$, $R^2 = 0.994$ where y is absorbance (A_{720}) and x is tannins (mg). The vanillin assay was used

primarily for estimation of the relative polymer sizes of the three purified tannins. The absorbance values obtained from the vanillin assay for 100 μg of the three purified tannins were used with the absorbance values obtained from 100 μg of the purified tannin in the acid–butanol assay to generate this value (Waterman and Mole, 1994). The acid–butanol assay involves depolymerization and measurement of the resulting anthocyanins. Thus, when we use this assay, we must assume that the efficiency with which the interflavan bonds are cleaved is unaffected by structure and that the differences in the spectra and absorption maxima will not distort the results. The vanillin reacts with the flavonoid A ring at C-6 to form a complex (Waterman and Mole, 1994). Two primary limitations affect results from this assay: (1) it is sensitive to temperature and timing and (2) the interflavan bonding pattern affects the results as the vanillin cannot bind in the presence of C-4 → C-8 linkages. The ratio of the results from the two assays provides an approximation of the relative polymer sizes. The three relative polymer sizes were 3.27, 2.82, and 0.56 for the desmodium, myrtle, and quebracho, respectively.

In the radial diffusion assay, purified tannins from myrtle and quebracho precipitated more BSA than purified tannins from desmodium (Figure 2).

Microbial Binding. Microbial binding to all purified tannin extracts was rapid for all combinations of tannins and bacteria tested, with little additional binding taking place after 10 min of exposure to tannins. The time course of

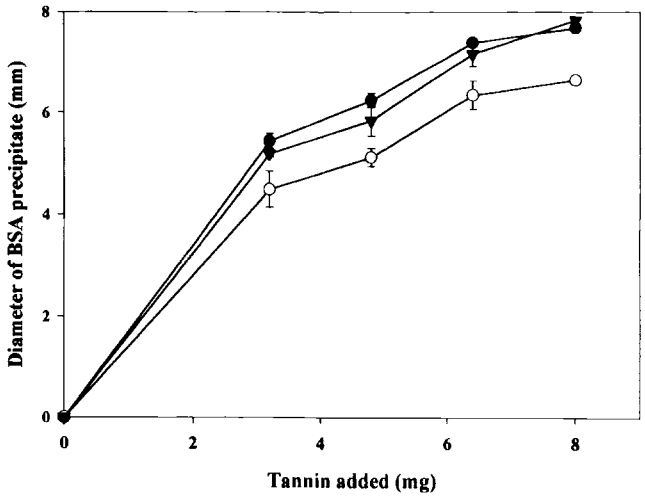


FIG. 2. Precipitation of bovine serum albumin (BSA) in the radial diffusion assay with increasing concentrations of three purified tannins. ●, quebracho; ○, desmodium; ▼, myrtle.

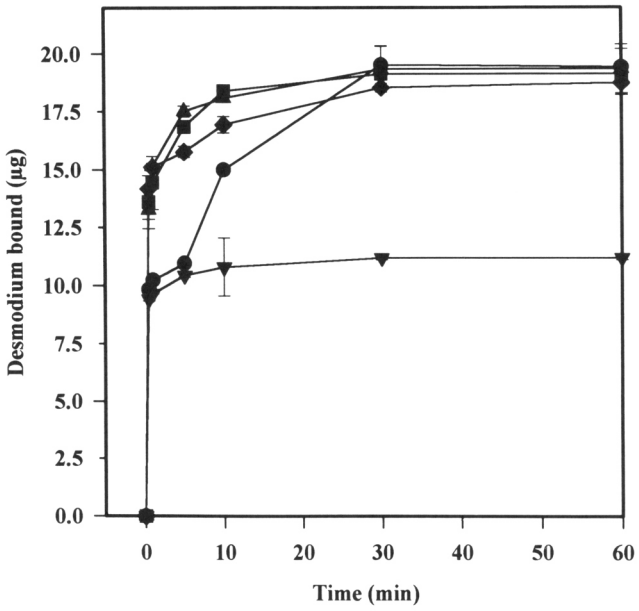


FIG. 3. Rate of timed binding of purified desmodium tannins (100 µg/ml to five ruminal bacteria (per milliliter of cells). Symbols: ●, KN1; ■, *S. bovis*; ▲, *R. albus*; ♦, *F. succinogenes*; ▼, *P. ruminicola*.

binding of the bacteria to purified desmodium tannin is presented as an example (Figure 3). For all bacteria and all tannins tested, there was a saturation point beyond which no additional binding of tannins to bacteria was detected, regardless of the quantity of tannins included in the growth medium.

Table 1 contains unadjusted and adjusted tannin binding values for the five species of ruminal bacteria. As the five bacteria vary in size, shape, and the relationship of absorbance to cell number, cell surface area adjustments were necessary to relate the maximum quantity of tannins bound to the total available surface. A higher ratio indicates a greater capacity for the purified tannin by the bacterium in question.

On an unadjusted cell surface area basis, there were no differences among *R. albus*, *S. bovis*, and KN1 with respect to the maximum amounts of the three purified tannins bound. *Fibrobacter succinogenes* bound slightly higher quantities of quebracho than the other bacteria, but bound similar amounts of purified desmodium and myrtle compared to the three gram-positive cocci. *Prevotella ruminicola* bound less myrtle and desmodium tannin than the other bacteria tested but did not differ from the other bacteria except for *F. succinogenes* in its capacity to bind quebracho.

TABLE 1. MAXIMUM QUANTITY OF THREE PURIFIED TANNIN BOUND–CELL SURFACE AREA RATIOS FOR THREE TANNINS AND FIVE RUMINAL BACTERIA AT A_{600} OF 0.75[a]

Bacteria	Cell surface area[b]	Quebracho		Desmodium		Myrtle	
		Bound/ml of cells[c]	Bound/cell surface[d]	Bound/ml of cells[c]	Bound/cell surface[d]	Bound/ml of cells[c]	Bound/cell surface[d]
S. bovis	8.62	4.63	5.37	20.00	23.20	18.29	21.23
KN1	7.81	6.50	8.33	20.00	25.57	19.32	24.72
R. albus	8.20	5.94	7.24	17.83	21.75	18.76	22.90
F. succinogenes	5.65	10.82	19.20	19.44	34.40	17.15	30.30
P. ruminicola	11.80	7.74	6.58	8.69	7.40	12.44	10.60

[a] Cell surface was calculated from the following dimensions. *S. bovis* JB1, 0.85 μm; *R. albus* 8, 0.84 μm; KN1, 0.82 μm; *F. succinogenes* S85, 0.25 × 1.5 μm; *P. ruminicola* $H_1$4, 0.84 × 1.5 μm. *S. bovis*, *R. albus*, and KN1 were assumed to be spheres. *P. ruminicola* and *F. succinogenes* were assumed to be cylinders. Cell numbers per ml at A_{600} of 0.75 were *S. bovis* JB1, 3.8×10^9; *R. albus* 8, 3.7×10^9; KN1, 3.7×10^9; *F. succinogenes* S85, 4.8×10^9; *P. ruminicola* $H_1$4, 3.0×10^9.

[b] Units for cell surface area are μm^2/ml × 10^{10}.

[c] Units for tannin bound/ml of cells are μg.

[d] Units for tannin bound:cell surface area ratios are $g/m^2 \times 10^{10}$.

When surface area adjustments were made, consistently more tannins were bound by *F. succinogenes* than by the other bacteria. *Prevotella ruminicola* ranked last or second to last in its ability to bind tannins. The three gram-positive cocci (*S. bovis*, KN1, and *R. albus*) bound similar amounts of condensed tannins.

Effects of Tannins on Growth. Experiments using NMR on tannin–dextran binding (Williamson et al., 1995) have showed that there is little interaction between tannins and glucose residues. Therefore, glucose availability should not have been reduced in the presence of tannins. The effects on growth of adding increasing amounts of three purified tannins to the growth medium of five strains of ruminal bacteria are presented in Table 2. These data must be interpreted with caution as bacteria grown in the presence of tannins may be smaller than those grown in tannin-free medium (Nelson et al., 1995) and daughter cells may fail to separate completely (Jones et al., 1994). Bacteria varied in their abilities to tolerate tannins in the growth medium. *Streptococcus bovis* and *F. succinogenes* were the bacteria most affected by tannins, and the gram-positive isolate KN1 was the least susceptible. *Prevotella ruminicola* and *R. albus* also demonstrated a high level of tolerance to these purified tannins. Of the three tannins tested, quebracho was the least and myrtle the most inhibitory. Increasing tannin concentrations in the growth medium were always associated with increased time required for cultures to achieve maximum optical density as measured by changes in A_{600} (Table 2). For all of the bacteria tested, there was no growth at 1400 μg/ml of quebracho, 1000 μg/ml desmodium, or 400 μg/ml myrtle.

The effects of different levels of purified desmodium tannin addition to cultures of *S. bovis*, KN1, and *P. ruminicola* are shown in Figures 4, 5, and 6, respectively. The changes that occur after addition of tannins, approximately 2 hr into the fermentation, will be used to describe the effect of adding purified tannins to the growing cultures. Following the addition of 100 μg/ml of purified desmodium tannins to the *S. bovis* culture, the doubling time increased from 20 min to 24 min. Glucose continued to disappear for only 2 hr in the control tubes but for 4 hr when 100 μg/ml of the tannins were added. When 200 μg/ml of the tannins were added, glucose disappearance and lactate production were evident for only 1 hr afterward. In the control and the 100 μg/ml tubes, glucose disappearance ceased with the achievement of a maximum growth level (A_{600} of approximately 1.1), but, at the higher tannin concentration, glucose metabolism was affected. Growth of the gram-positive isolate KN1 was slightly inhibited when 200 μg/ml of purified desmodium tannins were added to the growth medium at an A_{600} of 0.4. Glucose utilization and lactate production were changed little by this level of tannin supplementation. When 400 μg/ml of purified desmodium was added to the cultures, the final A_{600} was lower and the

TABLE 2. MAXIMAL ABSORBANCE (A_{600}) OF FIVE STRAINS OF RUMINAL BACTERIA GROWN IN PRESENCE OF INCREASING CONCENTRATIONS OF THREE PURIFIED TANNINS[a]

Strain	Maximum absorbance (A_{600} ± SD) with increasing amounts of tannins (μg/ml)				
	0	200	400	800	1200
A. Quebracho					
S. bovis	1.13 ± 0.067 (6)	1.03 ± 0.076 (18)	0.79 ± 0.077 (21)	0	0
KN1	1.30 ± 0.014 (7)	1.28 ± 0.011 (14)	1.27 ± 0.002 (14)	1.25 ± 0.002 (50)	1.27 ± 0.011 (72)
R. albus	1.30 ± 0.008 (8)	1.18 ± 0.028 (10)	1.18 ± 0.030 (12)	0.38 ± 0.020 (48)	0
F. succinogenes	1.50 ± 0.022 (12)	0.67 ± 0.097 (13)	0	0	0
P. ruminicola	1.38 ± 0.010 (7)	1.34 ± 0.040 (9)	1.30 ± 0.122 (9)	0	0
	2	100	200	400	800
B. Desmodium					
S. bovis	0.87 ± 0.041 (9)	0	0	0	0
KN1	1.13 ± 0.174 (7)	1.02 ± 0.066 (7)	1.10 ± 0.010 (10)	1.10 ± 0.102 (12)	0.53 ± 0.069 (17)
R. albus	1.23 ± 0.067 (7)	1.22 ± 0.071 (7)	1.16 ± 0.068 (9)	0.71 ± 0.098 (14)	0.51 ± 0.118 (20)
F. succinogenes	0.90 ± 0.026 (16)	0	0	0	0
P. ruminicola	1.31 ± 0.084 (7)	1.30 ± 0.023 (10)	1.21 ± 0.111 (10)	0.78 ± 0.024 (14)	0
	10	50	100	200	
C. Myrtle					
S. bovis	1.03 ± 0.083 (17)	0.51 ± 0.088 (24)	0	0	
KN1	1.19 ± 0.077 (11)	0.89 ± 0.009 (17)	1.16 ± 0.059 (17)	0.50 ± 0.089 (24)	
R. albus	1.19 ± 0.009 (12)	0.93 ± 0.002 (24)	0.79 ± 0.019 (24)	0.53 ± 0.007 (96)	
F. succinogenes	1.51 ± 0.163 (15)	1.51 ± 0.040 (15)	0	0	
P. ruminicola	1.27 ± 0.054 (7)	1.19 ± 0.073 (8)	0.99 ± 0.156 (12)	0.53 ± 0.066 (24)	

[a]Numbers in parenthesis represent hours required to reach maximum A_{600}.

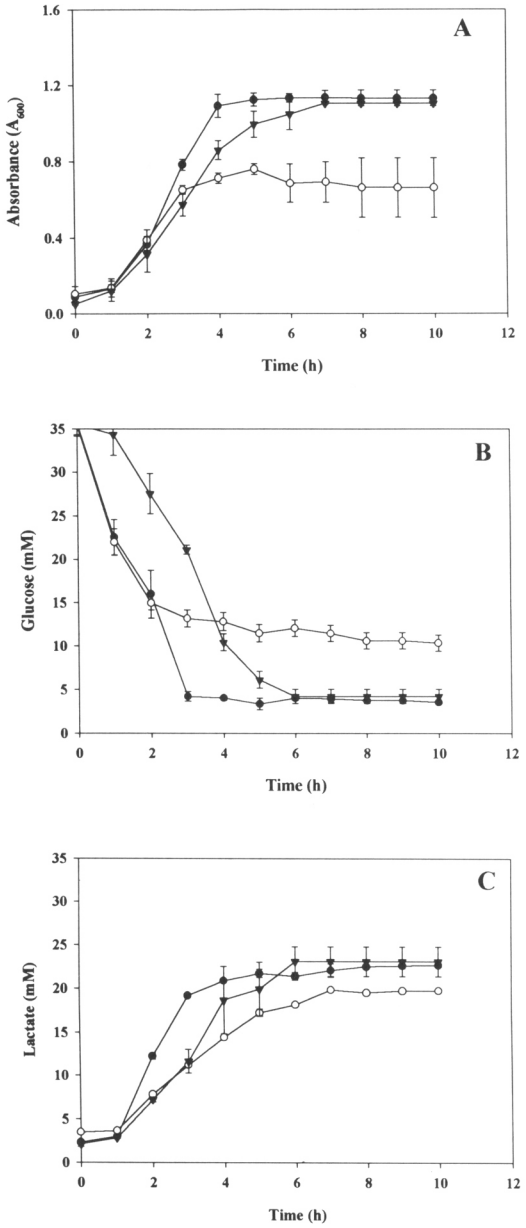


FIG. 4. Growth rate (A), glucose utilization (B), and lactate production (C) of *S. bovis* exposed to 0 (●), 100 (▼), and 200 (○) μg/ml of purified desmodium tannin. Tannins were added at approximately 2 hr when A_{600} reached 0.40.

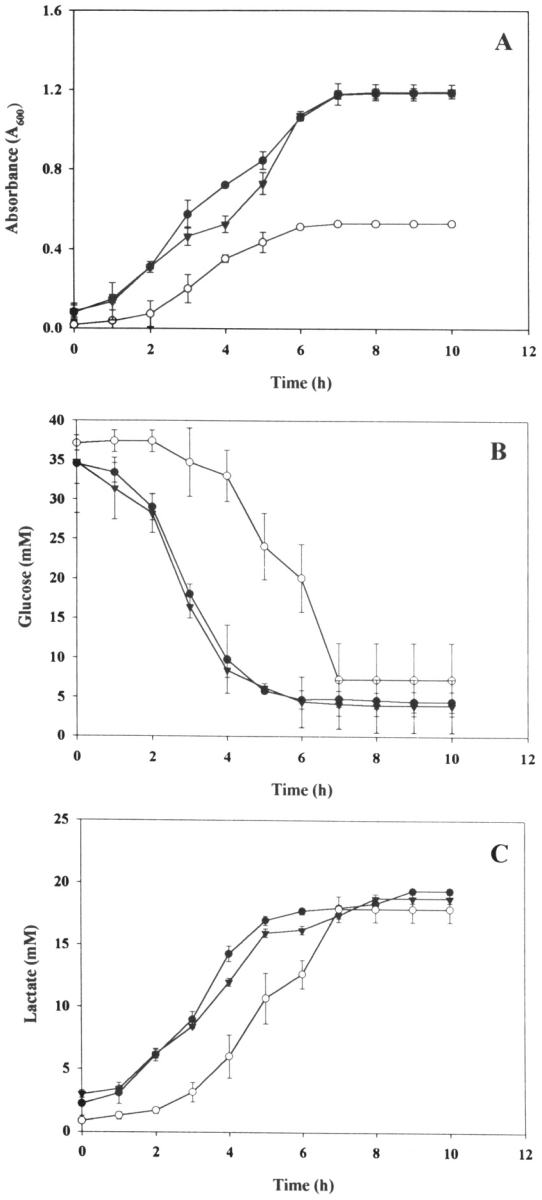


FIG. 5. Growth rate (A), glucose utilization (B), and lactate production (C) of KN1 exposed to 0 (●), 200 (▼), and 400 (○) μg/ml of purified desmodium tannin. Tannins were added at approximately 2.5 hr when A_{600} reached 0.40.

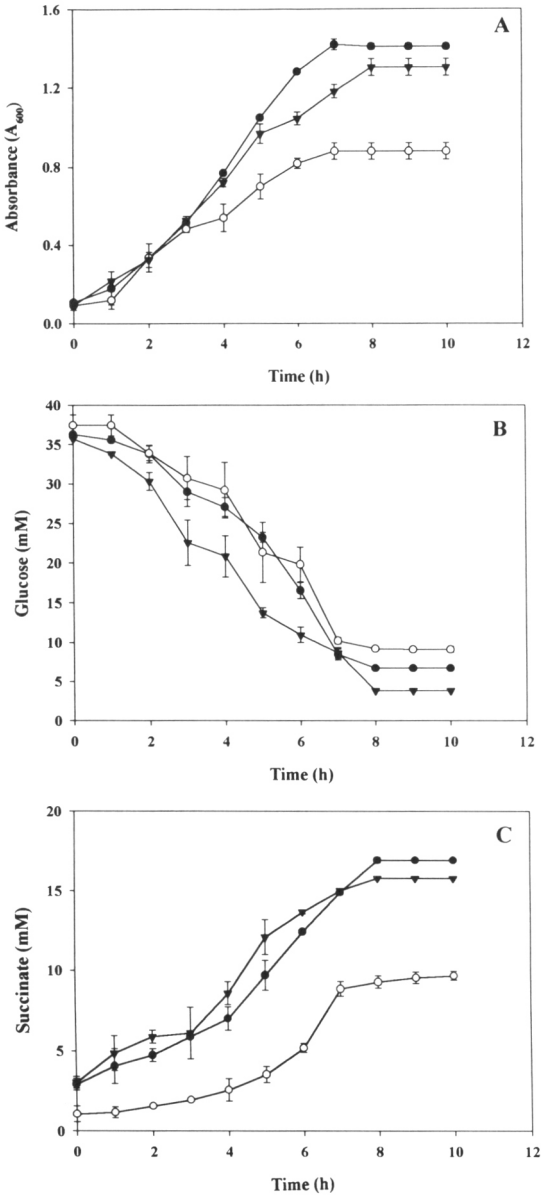


FIG. 6. Growth rate (A), glucose utilization (B), and succinate production (C) of *P. ruminicola* exposed to 0 (●), 200 (▼), and 400 (○) μg/ml of purified desmodium tannin. Tannins were added at approximately 2.5 hr when A_{600} reached 0.40.

rates of glucose utilization and lactate production were reduced compared to the tannin-free medium. The maximum amount of glucose used in 10 ml of medium was 0.30 mmol both in the absence and presence of 400 μg/ml of purified desmodium. The maximum A_{600} achieved in the KN1 culture in the presence of 400 μg/ml was only 0.53, suggesting that energy utilization and growth were uncoupled (Figure 5).

But the growth rate and the maximum absorbance (1.4 vs. 0.9) of *P. ruminicola* were lower when 400 μg/ml of desmodium tannin was added compared to bacteria grown in medium without tannins (Figure 6). The rate at which glucose was utilized changed little, and there was a reduction in the final quantity of succinate produced by this bacterium (0.097 mmol vs. 0.17 mmol for the control in 10 ml of medium) when 400 μg/ml of purified desmodium tannins were added to the medium. The quantities of acetate that this bacterium produced did not change when purified tannins were added to the medium (data not included in graphs). Adding 200 μg/ml of purified desmodium did not affect this bacterium with respect to the maximum amounts of glucose it used or of succinate it produced.

DISCUSSION

Tannins are naturally occurring secondary plant compounds whose biological activity is associated with an ability to bind and precipitate proteins and other macromolecules from solution. Tannins in forage protect plants from herbivory (Clausen et al., 1992). The complexing of tannins with proteins is postulated to be the most significant of their toxic effects, although they also bind carbohydrates and other macromolecules (Hagerman and Butler, 1981). Tannin–protein interactions are likely responsible for the negative effects of tannins on ruminants through an inhibition of gut enzyme activity (Barry, 1989; Van Leeuwen et al., 1995; Bae et al., 1993). The inhibitory effects of tannins on bacteria have been associated with the binding to extracellular polymers, inhibition of cell membrane and enzyme activity, and substrate and metal ion deprivation (Scalbert, 1991). Not all of the nutritional effects of tannins are negative; they reduce the incidence of bloat (Tanner et al., 1995), they may alter the site of digestion and efficiency of use of protein by ruminants (Barry and Manley, 1984), and they may inhibit pathogenic bacteria such as *Clostridium perfringens* (Schrägle and Müller, 1990).

In the preceding experiments, chemical and biological assays were used to evaluate the inhibition of bacteria by purified tannins. There has been much discussion about the suitability of different standards for tannin assays. Our choice of standards was based on the chemical structures that were being tested. In addition, internal standard curves were constructed for each tannin for each

assay. The standard curves for the total phenolics assay (Figure 1) demonstrate some of the errors that can arise from the incorrect choice of external standards. Although quebracho has been used regularly as a standard in tannin assays, this study shows that purified quebracho is not a good model for prediction of the biological activity of other tannins. Purified quebracho tannin inhibited bacterial growth less and it had a lower capacity to bind bacteria than did either myrtle or desmodium. This is likely a result of both structural differences among the tannins and processing artifacts.

The significance of the structural variations in tannins is evident when the absorbance readings and biological reactivities in the various assays are compared. Although all three extracts fit the definition of tannins because they bind protein, the chemical and biological activity of these plant extracts varied widely in these experiments, reflecting the structural variations in the tannin molecules. Among the most important structural variations are size of the polymer, hydroxylation patterns of the A and B rings, and sites for interflavan bonds (4 → 6 or 4 → 8).

The radial diffusion assay is a biological assay of the ability of a tannin to precipitate BSA. The ability to precipitate BSA is related to the molecular weight, hydroxylation pattern, interflavan binding, and other structural characteristics of a tannin. This assay did not correlate well with tannin antimicrobial activity. Quebracho and myrtle precipitated similar amounts of BSA but quebracho was much less inhibitory to microbial growth than myrtle. These results confirm those of Giner-Chavez et al. (1997), who found a poor correlation between the radial diffusion assay and other chemical methods.

The acid–butanol assay was designed to measure proanthocyanidin content, but it does not include all phenolic components with antimicrobial activity. Although myrtle had a lower proanthocyanidin absorbance reading than desmodium, the nonproanthocyanidin components in the purified myrtle extracts, which were detected in both the total phenolics and hydrolyzable tannin assays, resulted in this extract having an antimicrobial effect similar to desmodium. When we compared rankings of the results from the acid–butanol assay with microbial inhibition data, there was not complete agreement because of these nonproanthocyanidin components.

Of the assays evaluated, the total phenolics assay was the best predictor of tannin antimicrobial activity. High levels of total phenolics in the purified tannin extracts were strongly correlated with antimicrobial activity. Across all five bacteria, the correlation between tannin total phenolic content and antimicrobial activity was -0.77. It is surprising that the total phenolics assay was more strongly correlated with antimicrobial activity than the acid–butanol assay because we were using purified condensed tannins. Neither the desmodium nor the quebracho contained hydrolyzable tannins so nonproanthocyanidin phenolics should not have been present. It is more likely that differences in the chemical

bases of these assays were responsible for these results (oxidation-reduction for the total phenolics assay compared to depolymerization for the acid-butanol). When the total phenolics data were combined with the acid-butanol assay, the correlation with antimicrobial activity did improve to −0.88. Thus, the acid-butanol provides some additional information with respect to the antimicrobial activity of these three purified tannins.

Tannin-bacterial interactions were evident with all combinations of bacteria and purified tannins tested. *Fibrobacter succinogenes* had the highest capacity to bind the three purified tannins and was one of the bacteria most inhibited by tannins. *Prevotella ruminicola*, which had the lowest binding capacity of the bacteria tested, was second to KN1 in its ability to tolerate tannins in the growth medium. These data would seem to suggest that tannin binding capacity and tolerance were correlated. However, KN1, the most tannin-tolerant bacterium, bound similar amounts of tannin to the amount bound by *S. bovis*, one of the least tolerant bacteria.

In the glucose utilization assay, KN1 and *P. ruminicola* continued to use glucose in the presence of 200 μg/ml of purified desmodium tannins, whereas *S. bovis* ceased using glucose within 1 hr of exposure to this concentration of tannins. At a final concentration of 400 μg/ml of supplemented tannins, KN1 and *P. ruminicola* both grew more slowly than at lower tannin concentrations and reached lower maximum absorbance values, although both continued to use glucose.

These data suggest that different mechanisms are used by different bacteria to cope with tannins. Low tannin binding capacity may protect *P. ruminicola* from the effects of tannins, but this is not a strategy used by KN1. Cell wall structure may affect tannin tolerance, but gram stain results do not reflect this. *Streptococcus bovis* and KN1 are closely related members of the genus *Streptococcus* (Nelson, 1996), but KN1 tolerates high levels of tannins while *S. bovis* is inhibited at low tannin concentrations.

Others have found differences in the ability of bacteria to tolerate tannins: *P. ruminicola* H_14 was better able to tolerate condensed tannins from sainfoin than *Butryrivibrio fibrisolvens* A38 and *S. bovis* 45S1 (Jones et al., 1994). Sainfoin tannins had more effect on the growth and protease activity of *B. fibrisolvens* and *S. bovis* than on *P. ruminicola*. Differences were shown for tolerance of carob pod tannins and tannic acid among *Bacillus subtilis*, *Sporocytophaga myxococcoides*, and *Cellvibrio fulvus* (Henis et al., 1964). In these studies, as in our own, the exact causes of these differences in bacterial sensitivity were not identified.

These experiments demonstrate that the properties measured in chemical tannin assays differ from the properties involved in tannin toxicity and that bacteria vary in their ability to tolerate secondary compounds. As a result, many tannin assays do not accurately predict the biological activity of tannins. We

must be cautious in the use of model tannins to predict tannin activity because tannins from various sources often behave differently under similar conditions. In addition, the process used to purify the tannins may alter their biological activity. The lack of correlation between microbial inhibition and the results of the chemical assays may be partly explained by structural differences that are not measured by these assays.

Acknowledgments—The authors thank Dave Hinman, James B. Russell, and Mike Thonney for advice on experiments; Antonello Cannas for supplying myrtle samples from Sardinia; and La Escuela Agricola Panamericana (E.A.P.) in Tegucigalpa (Honduras) for desmodium samples. In addition, we appreciate the efforts of Dr. Carlos Lascano and his staff at the Centro Internacional de Agricultura Tropical in Cali, Colombia, who provided the ruminal fluid from which KN1 was isolated.

REFERENCES

ASQUITH, T. N., and BUTLER, L. G. 1985. Use of dye-labeled protein as spectrophotometric assay for protein precipitants such as tannin. *J. Chem. Ecol.* 11:1535–1544.

BAE, H. D., MCALLISTER, T. A., YANKE, J., CHENG, K.-J., and MUIR, A. D. 1993. Effects of condensed tannins on endoglucanase activity and filter paper digestion by *Fibrobacter succinogenes* S85. *Appl. Environ. Microbiol.* 59:2132–2138.

BARRY, T. N. 1989. Condensed tannins: their role in ruminant protein and carbohydrate digestion and possible effects upon the rumen ecosystem, pp. 153–169, *in* J. V. Nolan, R. A. Leng, and D. I. Demeyer (eds.). The Roles of Protozoa and Fungi in Ruminant Digestion. Penambul Books, Armidale, New South Wales.

BARRY, T. N., and MANLEY, T. R. 1984. The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 2. Quantitative digestion of carbohydrates and proteins. *Br. J. Nutr.* 51:493–504.

BRYANT, M. P. 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. *Am. J. Clin. Nutr.* 25:1324–1328.

BRYANT, M. P., and BURKEY, L. A. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *J. Dairy Sci.* 36:205–217.

CLAUSEN, T. P., REICHARDT, P. B., BRYANT, J. P., and PROVENZA, F. 1992. Condensed tannins in plant defense: A perspective on classical theories, pp. 639–651, *in* R. W. Hemingway and P. E. Laks (eds.). Plant Polyphenols: Synthesis, Properties, Significance. Plenum Press, New York.

DESCHAMPS, A. M. 1982. Nutritional capacities of bark and wood decaying bacteria with particular emphasis on condensed tannin degrading strains. *Eur. J. For. Pathol.* 12:252–257.

FIELD, J. A., and LETTINGA, G. 1987. The methanogenic toxicity and anaerobic degradability of a hydrolyzable tannin. *Water Res.* 21:367–374.

FIELD, J. A., LEYENDECKERS, M. J. H., ALVAREZ, R. S., LETTINGA, G., and HABETS, L. H. A. 1988. The methanogenic toxicity of bark tannins and the anaerobic biodegradability of water soluble bark matter. *Water Sci. Technol.* 20:219–240.

GINER-CHAVEZ, B., VAN SOEST, P. J., ROBERTSON, J. B., LASCANO, C. E., and PELL, A. N. 1997. Comparison of the precipitation of protein from alfalfa leaf and bovine serum albumin by tannins in the radial diffusion method. *J. Sci. Food Agric.* In press.

GOERING, H. K., and VAN SOEST, P. J. 1970. Forage Fiber Analysis (Apparatus, Reagents, Procedures, and Some Applications). Handbook No. 379. ARS-USDA, Washington, D.C.

HAGERMAN, A. E. 1987. Radial diffusion method for determining tannin in plant extracts. *J. Chem. Ecol.* 13:437–449.

HAGERMAN, A. E., and BUTLER, L. G. 1981. The specificity of proanthocyanidin–protein interactions. *J. Biol. Chem.* 256:4494–4497.

HAGERMAN, A. E., and BUTLER, L. G. 1989. Choosing appropriate methods and standards for assaying tannin. *J. Chem. Ecol.* 15:1795–1810.

HAGERMAN, A. E., and BUTLER, L. G. 1994. Assay of condensed tannins or flavonoid oligomers and related flavonoids in plants. *Methods Enzymol.* 234:429–437.

HENIS, Y., TAGARI, H., and VOLCANI, R. 1964. Effect of water extracts of carob pods, tannic acid and their derivatives on the morphology and growth of microorganisms. *Appl. Microbiol.* 12:204–209.

HUNGATE, R. E. 1950. The anaerobic mesophilic cellulolytic bacteria. *Bacteriol. Rev.* 14:1–49.

INOUE, K. H., and HAGERMAN, A. E. 1988. Determination of gallotannin with rhodanine. *Anal. Biochem.* 169:363–369.

JONES, G. A., MCCALLISTER, T. A., MUIR, A. D., and CHENG, K.-J. 1994. Effects of sainfoin (*Onobrychis viciifolia* Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. *Appl. Environ. Microbiol.* 60:1374–1378.

MUELLER-HARVEY, I., MCALLAN, A. B., THEODOROU, M. K., and BEEVER, D. E. 1988. Phenolics in fibrous crop residues and their effects on the digestion and utilisation of carbohydrates and proteins in ruminants. pp. 97–132, *in* J. D. Reed, B. S. Capper and P. H. H. Neate (eds.). Plant Breeding and the Nutritive Value of Crop Residues. ILCA, Addis Ababa.

NELSON, K. E. 1996. Tannins and Ruminal Bacteria. PhD dissertation. Cornell University, Ithaca, New York.

NELSON, K. E., PELL, A. N., SCHOFIELD, P., and ZINDER, S. 1995. Isolation and characterization of an anaerobic hydrolyzable tannin degrading bacterium. *Appl. Environ. Microbiol.* 61:3293–3298.

PORTER, L. J., HRSTICH, L. N., and CHAN, B. G. 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* 1:223–230.

PRICE, M. L., and BUTLER, L. G. 1977. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *J. Agric. Food Chem.* 25:1268–1277.

PRICE, M. L., VAN SCOYOC, S., and BUTLER, L. G. 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *J. Agric. Food Chem.* 26:1214–1218.

RUSSELL, J. B. 1987. A proposed mechanism of monensin action in inhibiting ruminal bacterial growth: Effects on ion flux and protonmotive force. *J. Anim. Sci.* 64:1519–1525.

SCALBERT, A. 1991. Antimicrobial properties of tannins. *Phytochemistry* 30:3875–3883.

SCHRÄGLE, R., and MÜLLER, W. 1990. The influence of selected tannin-containing plant species on the tenacity of pathogenic bacteria in an in vitro rumen system. *J. Vet. Med. B* 37:181–186.

SPIELMAN, A. I., and BENNICK, A. 1989. Isolation and characterization of six proteins from rabbit parotid saliva belonging to a unique family of proline-rich proteins. *Arch. Oral Biol.* 34:117–130.

TANNER, G. J., MOATE, P. J., DAVIS, L. H., LABY, R. H., YUGUANG, L., and LARKIN, P. J. 1995. Proanthocyanidins (condensed tannin) destabilise plant protein foams in a dose dependent manner. *Aust. J. Agric. Res.* 46:1101–1109.

VAN LEEUWEN, P., JANSMAN, A. J. M., WIEBENGA, J., KONINKX, J. F. J. G., and MOUWEN, J. M. V. 1995. Dietary effects of faba-bean (*Vicia faba* L.) tannins on the morphology and function of the small-intestinal mucosa of weaned pigs. *Br. J. Nutr.* 73:31–39.

WATERMAN, P. G., and MOLE, S. 1994. Analysis of Phenolic Plant Metabolites. Blackwell, London.

WILLIAMSON, M. P., TREVITT, C., and NOBLE, J. M. 1995. NMR studies of dextran oligomer interactions with model polyphenols. *Carbohydr. Res.* 266:229–235.

Journal of Chemical Ecology, Vol. 23, No. 4, 1997

ROLES OF OXALIC AND MALIC ACIDS IN CHICKPEA TRICHOME EXUDATE IN HOST-PLANT RESISTANCE TO *Helicoverpa armigera*

MITSURU YOSHIDA,* SUSAN E. COWGILL, and JOHN A. WIGHTMAN

International Crop Research Institute for the Semi-Arid Tropics (ICRISAT)
Patancheru, 502 324, Andhra Pradesh, India

(Received November 6, 1995; accepted December 11, 1996)

Abstract—Effects of malic acid and oxalic acid on oviposition of *Helicoverpa armigera* were investigated in a laboratory cage choice experiment. Malic acid stimulated oviposition at a concentration of 0.6 μmol/cm^2 but inhibited it at 3.4 μmol/cm^2. Oxalic acid showed neither stimulation nor inhibition of oviposition at 0.25–1.7 μmol/cm^2. Correlations between the amount of these acids in trichome exudate on leaf and pod surface and *H. armigera* populations and pod damage were investigated in a field experiment using 14 chickpea genotypes. Malic acid on the leaves stimulated oviposition during the vegetative and flowering stages, when its concentration was 0.1–0.7 μmol/cm^2. Later, during the podding stage there was no significant correlation between either egg density or pod damage and malic acid levels. However, there was a significant negative correlation between pod damage and oxalic acid levels. Oxalic acid, which had been reported to have an antibiotic effect on *H. armigera* larvae, has an important role in resistance to this pest in chickpea. The length of the podding period was also a factor influencing the extent of pod damage; a longer podding period resulted in prolonged exposure to *H. armigera* attack and more pod damage.

Key Words—*Helicoverpa armigera*, *Cicer arietinum*, chickpea, host-plant resistance, trichome, exudate, oxalic acid, malic acid, oviposition, antibiosis.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is a grain legume adapted to dry and cool environments in southern and western Asia, northern and eastern Africa, Central

*To whom correspondence should be addressed at National Institute of Agro-Environmental Sciences, Kannondai 3-1-1, Tsukuba, Ibaraki 305, Japan.

0098-0331/97/0400-1195$12.50/0 © 1997 Plenum Publishing Corporation

and South America, and southern Europe. India produces more than 65% of the world's chickpeas (FAO, 1994), and the crop is an important source of protein for the local population. *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae), the gram pod borer, is the major pest of chickpea and causes a serious production loss in most places where this crop is grown (Reed et al., 1987). *H. armigera* lays eggs on chickpea leaves, and the larvae feed preferentially on flowers and pods. They also eat leaves during the vegetative stage and when the reproductive structures have been destroyed. The methods for the management of the pest include manipulation of sowing and harvesting time, intercropping, and application of pesticide (Lal et al., 1986; Reed et al., 1987). The development of insecticide resistance in *H. armigera* populations in the Indian subcontinent (Armes et al., 1992b, 1996), coupled with an increasing awareness of the possible detrimental effects of intensive insecticide use, has stimulated interest in the development of integrated methods of pest control that reduce pesticide inputs and produce a more sustainable farming system.

Exploitable levels of resistance to *H. armigera* exist within the chickpea germ plasm (Dias et al., 1983; Lateef, 1985; Lateef and Sachan, 1990). Resistant genotypes frequently suffer lower pod damage compared with susceptible checks. Acidic exudate from trichomes on the surface of chickpea plants, in which oxalic acid and malic acid are major acidic components (Rembold et al., 1990a; Rembold and Weigner, 1990; Yoshida et al., 1995), has been correlated with reduced pod damage (Rembold, 1981; Rembold and Winter, 1982; Srivastava and Srivastava, 1989; Rembold et al., 1990a,b). The concentration of oxalic acid is higher on the leaf surface of resistant genotypes than on susceptible ones, and this acid retards the growth of *H. armigera* larvae (Yoshida et al., 1995). Material from resistant genotypes has an antibiotic effect on *H. armigera* (Srivastava and Srivastava, 1990). The inhibition of larval growth by oxalic acid in the trichome exudate, therefore, appears to be a component of host-plant resistance in chickpea. Malic acid has not been shown to affect larval growth.

Ovipositional antixenosis has also been reported as a mechanism of resistance to *H. armigera* in chickpea (Lateef, 1985; Srivastava and Srivastava, 1989; Cowgill and Lateef, 1996). However, a chemical factor for the antixenosis has not been identified. The aim of this study was to evaluate the effects of malic acid and oxalic acid on oviposition preference of *H. armigera* adults in a laboratory experiment and to investigate in a field experiment the effects of these compounds on egg and larval density of *H. armigera* and the pod damage caused by the pest.

METHODS AND MATERIALS

Insect Culture. All *H. armigera* adults used in cage experiments were obtained from a laboratory culture maintained at ICRISAT Asia Center, Patan-

cheru, Andhra Pradesh, India. It was established from and occasionally supplemented with field-collected eggs. Larvae were reared on a chickpea-based diet (Armes et al., 1992a) at 27°C. Several pairs of adults were kept in a cage at 25°C with 10% honey solution on absorbent cotton for purpose of collecting eggs.

Oviposition Cage Experiment. A sheet of tissue paper (34.5 × 24.0 cm) (One-Way Nappy Liners, Boots Co., Nottingham, England) was dipped in oxalic acid or L-malic acid solution, squeezed, and dried in an oven at 60°C for 1 hr. The amount of the acid retained on the tissue paper (micromoles per square centimeter) was calculated from the increase in dry weight of the paper after dipping and drying. The tissue paper was cut into three strips and hung on the wall of a transparent cylindrical plastic cage (23 cm diameter × 30 cm high). Control papers were identical except that they were treated with distilled water. They were hung between the acid-treated strips. A gravid female adult and a male adult, which had been kept in a cage for two days for mating, were released in the cage. Absorbent cotton soaked in 10% honey solution was placed in the cage for feeding of the moths. Oviposition was allowed to continue for 40 hr at 25°C under a photocycle of 2L:12D:12L:12D:2L hr. Individual pairs of moths were replicates, and eight replicates were used for each concentration of the acid treatments.

Design of Field Experiment. The field experiment was carried out at ICRISAT Asia Center, Patancheru, Andhra Pradesh, India, after the 1994 rainy season. A randomized block design with four replicates of 14 treatments (chickpea genotypes) was used. Each replicate consisted of four rows 9 m long with 60 cm between adjacent rows and 30 cm between plants within rows. Of the 14 chickpea genotypes, eight were *H. armigera*-resistant genotypes, five were susceptible checks, and one was a resistant × susceptible cross. They were divided into two groups, short-duration and medium-duration, according to the number of days from sowing to maturity (Table 1). The genotypes were sown on October 12, 1994. No pesticide was applied to the crop during the growing season.

Sampling of H. armigera *in the Field.* Ten chickpea plants in the second row of each replicate were examined for *H. armigera* eggs and larvae at weekly intervals from 28 days after sowing (DAS) to 91 DAS (short-duration genotypes) or to 112 DAS (medium-duration genotypes). Larvae were recorded as small (first to second instars), medium (third to fourth instars), and large (fifth to sixth instars). Medium and large larvae cause most of the damage to chickpea, while small larvae mainly scrape the surface of pods and leaves. As a result, analyses of larval density data were restricted to those of medium and large larvae only.

Five plant samples were collected from the first or fourth row of each replicate on the same day as the pest count. The plants were cut off at the roots, dried in an oven at 60°C for five days, and weighed. The egg and larval densities

TABLE 1. GROWTH OF 14 CHICKPEA GENOTYPES[a]

Genotype	Flowering (WAS)	Pod setting (WAS)	Seed maturity (WAS)
Short-duration			
Annigeri (S)	6	8	13
ICCV 2 (S)	5	7	12
ICCV 37 (S)	6	8	13
ICCX 730266 (S)	6	9	13
ICCV 93122 (S × R)	7	8	14
ICCV 7 (R)	7	8	13
ICC 506 (R)	6	8	13
ICCL 86101 (R)	6	8	13
ICCL 86102 (R)	6	8	13
ICCL 86111 (R)	7	8	14
Medium-duration			
ICC 3137 (S)	8	8	16
ICC 4935 (R)	9	10	16
ICCL 86106 (R)	10	11	16
ICCX 730041 (R)	8	10	16

[a]WAS: week after sowing; S: susceptible check; R: genotype selected for *H. armigera* resistance; S × R: cross between Annigeri and ICC 506.

are expressed on a gram plant dry weight (DW) basis to give a more precise estimate of their distribution among the 14 genotypes, which varied in plant size.

Analysis of Oxalic Acid and Malic Acid in Leaf and Pod Exudate. A branch with green leaves was collected from each of five plants in the third row of each replicate every two weeks from 28 to 70 DAS on the same day as the pest count. Two extra samples were taken for medium-duration genotypes 84 and 105 DAS. At the first sampling, when the plants were seedlings, whole plants were collected instead of branches. All the leaves were detached and washed with distilled water to collect the exudate. The exudate samples were filtered through a Millipore Filter (HVLP, pore size 0.45 μm) and injected in a Shimadzu LC-6A liquid chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a Supelcogel C610-H column (7.8 mm ID × 30 cm) (Supelco Inc., Bellefonte, Pennsylvania), a SCL-6A system controller, and a C-R7A data analyzer. The mobile phase was 0.01 N H_2SO_4 at a flow rate of 0.4 ml/min. The elutes were monitored at 210 nm on a SPD-6AV UV-VIS spectrophotometric detector. Oxalic acid and malic acid were identified from their retention times of 12.0 and 17.0 min, respectively, and quantified from the area of the

peaks, calibrated with authentic samples injected separately. The area of the washed leaves was measured with an LI-3100 area meter (LI-COR, Inc., Lincoln, Nebraska). The leaves were then dried in an oven at 60°C for two days, and the dry weight was measured. Concentration of the acids in the leaf exudate was calculated on the basis of the leaf area (micromoles per square centimeter) and dry weight (micromoles per gram dry weight).

A green tender pod was collected from each of 10 plants in the third row of each replicate. The pods were collected twice for each genotype at 49 and 63 DAS for genotype ICCV 2, at 63 and 77 DAS for other short-duration genotypes, and at 91 and 111 DAS for medium-duration genotypes. Green tender pods with small seeds were collected in the first sampling, and those with big seeds in the second sampling. Pods were washed with distilled water, and the exudate samples were analyzed for oxalic acid and malic acid as described above. The washed pods were opened, and seeds were discarded. The pod samples were dried in an oven at 60°C for two days, and the dry weight was measured. Concentration of the acids in the pod exudate was calculated on the basis of the pod dry weight (micromoles per gram dry weight).

Pod Damage Assessment. On January 25, 1995 (105 DAS), 25 plants were collected from the second row of each short-duration replicate, except ICCV 93122 and ICCL 86111. The sampling for the other genotypes was on February 13, 1995 (124 DAS). All the pods were removed from each sampled plant and placed individually into labeled paper bags. The pods were subsequently examined, and the number of pods with and without *H. armigera* damage was recorded.

Statistical Analysis. Oviposition preference between acid treated and control papers in cage experiments was evaluated by *t* test (10% significance level) on the angular transformed percentage of the eggs laid on the acid-treated papers by analyzing whether the percentage was different from 50%. The mean number of eggs (per gram dry weight), the mean number of medium and large larvae (per gram dry weight), and mean acid concentration (micromoles per gram dry weight) on the leaves were analyzed by a factorial ANOVA (GENSTAT Version 4.03) using date and chickpea genotype as factors. The data of the short-duration genotypes were analyzed as one group, and those of the medium-duration genotypes were analyzed separately. The angular transformed pod damage, mean concentration of the acids on the pods (micromoles per gram dry weight), and cumulative medium and large larval days were analyzed by SYSTAT one-way ANOVA (Wilkinson, 1990). For the one-way ANOVA, data of the short- and medium-duration genotypes were pooled. Means were separated at the 5% level using Fisher's least significant difference (LSD) test (Wilkinson, 1990). Results of Bartlett's test showed that variances of the data sets to which ANOVA was applied were homogeneous. The significance in correlation of *H. armigera* pop-

ulations, acid concentrations, and angular transformed pod damage was evaluated by the F test of the regression coefficient at the 1% level.

RESULTS AND DISCUSSION

Oviposition Cage Experiment. The fecundity of *H. armigera* females was highly variable. The total number of eggs laid per cage ranged from 109 to 1272. As a result, the percentage of the total number of eggs laid on acid-treated papers was calculated and used in subsequent analyses instead of egg number. When the concentration of malic acid on the papers was 0.6 μmol/cm^2, significantly more than 50% of the total number of eggs were laid on the acid-treated papers (Figure 1). At 3.4 μmol/cm^2 significantly less than 50% of the total number of eggs were laid on the acid treated papers. These results indicate that malic acid stimulates oviposition at the lower concentration but inhibits it at the higher concentration.

When the concentration of oxalic acid on the papers was 0.25–1.7 μmol/cm^2, neither stimulation nor inhibition of oviposition was observed (Figure 1).

Growth of Chickpea Genotypes and H. armigera *Populations in the Field.* The growth stages of the 14 chickpea genotypes used in this study are listed in

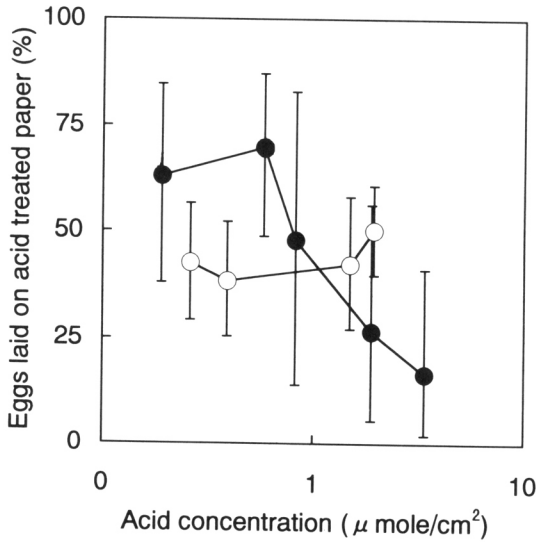


FIG. 1. Effect of malic acid and oxalic acid on oviposition of *H. armigera* in laboratory cage experiment. Bars show 95% confidence interval (t test); ●: malic acid; ○: oxalic acid.

Table 1. Short-duration chickpea genotypes started flowering 6–7 weeks after sowing (WAS), set pods 8–9 WAS, and matured 13–14 WAS, except for ICCV 2, which was one to two weeks more advanced (extra-short duration) than the other short-duration genotypes. Medium-duration genotypes, with the exception of ICC 3137, started flowering 8–10 WAS and set pods 10–11 WAS. ICC 3137 had tender pods 8 WAS. Seed maturity of the medium-duration genotypes was 16 WAS.

There was significant variation in egg density (per gram dry weight) among the genotypes in the two duration groups (Table 2: short-duration group: $F = 5.35$; $df = 9, 270$; $P < 0.001$; medium-duration group: $F = 2.98$; $df = 3, 117$; $P < 0.05$). There was also a significant date-genotype interaction in the short-duration group ($F = 1.67$; $df = 81, 270$; $P < 0.005$) because relative egg density was not constant during the sampling period. The egg density on ICCV 2, ICCV 37, ICCV 93122, and ICCV 7 peaked at 49 DAS, late flowering

TABLE 2. MEAN POPULATION OF *H. armigera* AND MEAN CONCENTRATION OF OXALIC ACID AND MALIC ACID ON LEAVES OF CHICKPEA GENOTYPES[a]

Genotype	Egg density (per g DW)	Larval density[b] (per g DW)	Oxalic acid (μmol/g DW)	Malic acid (μmol/g DW)
Short-duration				
Annigeri (S)	0.406 ab	0.212 bcd	44.6 c	67.3 e
ICCV 2 (S)	0.145 e	0.188 cd	33.8 d	80.0 cde
ICCV 37 (S)	0.455 a	0.226 bcd	28.9 d	122.3 b
ICCX 730266 (S)	0.368 abc	0.285 ab	54.6 b	86.9 c
ICCV 93122 (S × R)	0.429 a	0.275 abc	31.6 d	175.9 a
ICCV 7 (R)	0.288 bcd	0.226 bcd	63.6 a	69.9 de
ICC 506 (R)	0.156 de	0.153 d	64.1 a	76.3 cde
ICCL 86101 (R)	0.327 abc	0.256 abc	66.9 a	81.4 cde
ICCL 86102 (R)	0.260 cde	0.186 cd	63.7 a	71.3 de
ICCL 86111 (R)	0.401 ab	0.328 a	63.8 a	83.7 cd
SEM	0.047	0.033	2.7	5.1
Medium-duration				
ICC 3137 (S)	0.340 b	0.388 a	32.0 a	115.9 c
ICC 4935 (R)	0.399 b	0.276 b	34.4 a	134.4 b
ICCL 86106 (R)	0.569 a	0.210 b	25.0 b	182.1 a
ICCX 730041 (R)	0.445 ab	0.223 b	30.7 a	169.1 a
SEM	0.057	0.033	1.3	5.9

[a] Means within a duration group within a column followed by the same letter are not significantly different at the 5% level (LSD test). S, susceptible check; R, genotype selected for *H. armigera* resistance; S × R, cross between Annigeri and ICC 506; SEM, standard error of mean.
[b] Medium and large larvae.

stage, while on the other short-duration genotypes peak egg density occurred at 56 DAS, early podding stage. Peak egg density on the medium-duration genotypes occurred 49–56 DAS. The peak egg density was largest on the medium-duration genotypes and smallest on the *H. armigera*-resistant short-duration genotypes and ICCV 2. A second, smaller oviposition peak was observed approximately 70 DAS for both duration groups. At this time the medium-duration genotypes had tender pods.

There was significant variation in the density of medium and large larvae (per gram dry weight) among the genotypes in both duration groups (Table 2: short-duration group: $F = 2.65$; $df = 9, 270$; $P < 0.01$; medium duration group: $F = 6.06$, $df = 3, 117$; $P < 0.001$). There was a significant date–genotype interaction in the medium-duration group ($F = 2.03$; $df = 36, 117$; $P < 0.005$). Although the larval density generally declined along with the growth of the crop, a peak in the larval density occurred during the flowering–early podding stage of the short-duration genotypes. With the medium-duration genotypes a second peak was also observed during their pod-setting stage. With the exception of ICC 3137, larval density at the second peak was lower than that at the first peak. ICC 3137 had the highest second peak among the medium-duration genotypes, although the first peaks of ICC 3137, ICC 4935, and ICCX 730041 were similar. ICCL 86106 had the lowest larval density at these peaks.

Oxalic Acid and Malic Acid Concentration on Leaves. There was significant variation in oxalic acid concentration on a plant dry weight basis (micromoles per gram dry weight) among the short-duration genotypes from vegetative to podding stage (Table 2: $F = 32.73$; $df = 9, 108$; $P < 0.001$), and a significant date–genotype interaction ($F = 5.63$; $df = 27, 108$; $P < 0.001$). The level of oxalic acid was highest at 56 DAS, except for ICCV 93122. It decreased as the leaves senescenced during the podding stage. ICCV 93122 retained flowers and green leaves approximately one week longer than the other short-duration genotypes and had the maximum oxalic acid concentration at 70 DAS. The resistant genotypes retained significantly higher concentrations of oxalic acid on the leaf surface than the susceptible genotypes throughout the sampling period.

There was significant variation in the amount of oxalic acid (micromoles per gram dry weight) among the medium-duration genotypes (Table 2: $F = 8.85$; $df = 3, 54$; $P < 0.001$), and a significant data-genotype interaction ($F = 3.16$; $df = 15, 54$; $P < 0.001$). The pattern of change in the oxalic acid level was similar to that of the short-duration genotypes; the maximum level was observed at 70 DAS (flowering–early podding stage), and the concentration decreased towards senescence. The oxalic acid level on ICCL 86106 was significantly lower than those on the other medium-duration genotypes in the vegetative stage, but the levels were similar for all the genotypes in the podding stage. There was no significant difference in oxalic acid levels between the

susceptible check, ICC 3137, and the resistant genotypes, except ICCL 86106, which exhibited lower oxalic acid levels.

Significant variation was also observed in malic acid level (micromoles per gram dry weight) among the short-duration group (Table 2: $F = 43.80$, $df = 9, 108$; $P < 0.001$) and medium-duration group (Table 2: $F = 26.95$, $df = 3, 54$; $P < 0.001$). A significant date-genotype interaction was found in the former group ($F = 2.53$; $df = 27, 108$; $P < 0.001$). The malic acid level was highest at 70 DAS for all the genotypes, regardless of their duration. A decrease in the level of malic acid was observed in the podding stage of the medium-duration genotypes. The malic acid levels on ICCV 93122 and ICCV 37 were significantly higher than those on the other short-duration genotypes throughout the sampling period. There was no clear difference in the malic acid level between the resistant and susceptible short-duration genotypes. Within the medium-duration genotypes, significantly more malic acid was detected on ICCL 86106 and ICCX 730041 than on ICC 4935 and ICC 3137.

Relationship Between Leaf Malic Acid Level and H. armigera *Population Density.* Analysis of the relationship between mean malic acid level on a leaf area basis (micromoles per square centimeter) and mean egg density (per gram dry weight) during the vegetative and flowering stage, 28–49 DAS, showed a significant correlation ($r = 0.906$) between the two variables for all 14 chickpea genotypes (Figure 2). Hence malic acid on the chickpea leaves in the field acts as an oviposition stimulant for *H. armigera* at a concentration of 0.1–0.7 μmol/

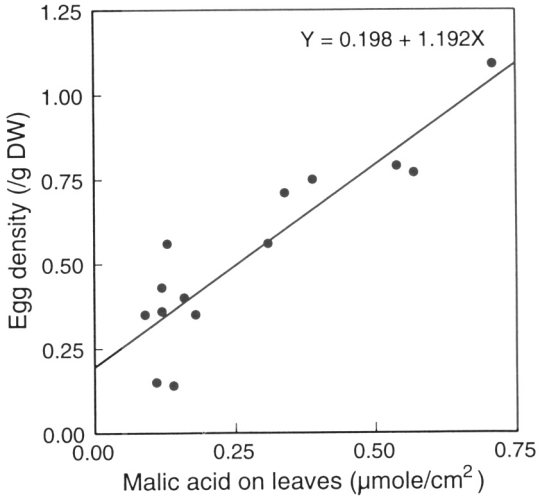


FIG. 2. Correlation between concentration of malic acid on leaves and *H. armigera* egg density during vegetative and flowering stage of chickpea genotypes.

cm^2. This observation is consistent with the result from the laboratory cage experiment (Figure 1). In the podding stage in the field, when malic acid concentration was 1–2 $\mu mol/cm^2$, there was no significant correlation between the malic acid level and the egg number, although malic acid tended to inhibit oviposition around this range of concentration in the cage experiment (Figure 1). This discrepancy may be due to other factors that may affect oviposition, e.g., the presence of leaf surface volatiles (Rembold et al., 1990a).

There was no significant correlation between the level of malic acid on the leaves (micromoles per gram dry weight) and the density of medium and large larvae (per gram dry weight).

Relationship Between Leaf Oxalic Acid Level and H. armigera *Population Density.* There was a significant negative correlation ($r = -0.840$) between mean leaf oxalic acid level (micromoles per square centimeter) and mean egg density (per gram dry weight) in the vegetative–flowering stage, when the oxalic acid concentration was 0.05–0.25 $\mu mol/cm^2$. This negative correlation is not considered to be due to oviposition antixenosis by oxalic acid because the correlation was not observed later in the podding stage, when the oxalic acid level was higher (0.25–0.75 $\mu mol/cm^2$). Even at the higher concentration of 1.5–1.7 $\mu mol/cm^2$, oxalic acid did not have a significant effect on oviposition in the laboratory cage experiment (Figure 1). It was not possible to test concentrations lower than 0.25 $\mu mol/cm^2$ in the laboratory study because the amount of acid on the tissue paper was too small to be detected. There was a significant negative correlation ($r = -0.877$) between the levels of leaf malic acid and leaf oxalic acid in the vegetative–flowering stage of chickpea; genotypes with higher malic acid levels had lower oxalic acid levels. The negative correlation between the leaf oxalic acid level and the egg number was, therefore, considered to be a result of the stimulation of oviposition by the leaf malic acid.

A negative correlation ($r = -0.614$, $P = 0.05$) between the leaf oxalic acid level (micromoles per gram dry weight) and the density of medium and large larvae (per gram dry weight) was observed 56 DAS, corresponding to pod setting and late vegetative–flowering stage in the short- and medium-duration genotypes, respectively. Before and after this date there was no correlation between the two variables. This is surprising as oxalic acid has been reported to have an antibiotic effect on *H. armigera* larvae (Yoshida et al., 1995). The frequency of sampling may have been insufficient to detect such a relationship. Alternatively, relative larval density may have been underestimated during the late podding stage as many larvae were concealed inside the pods.

Oxalic Acid and Malic Acid Concentration on Pods. There was significant variation in mean oxalic acid level (micromoles per gram dry weight) on the pods on the two sampling dates among the 14 genotypes (Table 3; $F = 23.46$; $df = 13, 55$; $P < 0.001$). The medium-duration genotypes had significantly lower oxalic acid levels on the pods than the short-duration genotypes, except

TABLE 3. MEAN CONCENTRATION OF OXALIC ACID AND MALIC ACID ON PODS, CUMULATIVE LARVAL DAYS OF PODDING STAGE, AND POD DAMAGE OF CHICKPEA GENOTYPES[a]

Genotype	Oxalic acid (μmol/g DW)	Malic acid (μmol/g DW)	Cumulative larval days[b]	Pod damage (%)
Short-duration				
Annigeri (S)	59.2 de	249.1 cd	2.75 cde	31.2 b
ICCV 2 (S)	77.8 b	278.5 abc	3.92 bcd	25.9 bcd
ICCV 37 (S)	55.2 e	300.2 a	3.93 bcd	28.8 bc
ICCX 730266 (S)	66.3 cd	282.1 abc	2.78 cde	25.7 bcd
ICCV 93122 (S × R)	51.1 ef	283.7 ab	2.59 bc	22.2 cd
ICCV 7 (R)	75.1 bc	233.9 de	4.13 cde	13.7 e
ICC 506 (R)	77.9 b	291.4 a	1.98 de	12.7 ef
ICCL 86101 (R)	77.5 b	275.4 abc	1.37 e	10.9 ef
ICCL 86102 (R)	90.3 a	289.3 a	1.18 e	6.9 f
ICCL 86111 (R)	66.7 cd	255.7 bcd	2.97 bcde	19.7 de
Medium-duration				
ICC 3137 (S)	43.6 f	208.3 e	10.20 a	64.4 a
ICC 4935 (R)	44.1 f	252.0 bcd	4.87 b	32.8 b
ICCL 86106 (R)	44.5 f	268.4 abc	2.34 cde	28.3 bc
ICCX 730041 (R)	45.0 f	253.8 bcd	3.13 cde	34.2 b
SEM	3.2	11.6	0.71	2.8

[a]Means within a column followed by the same letter are not significantly different at the 5% level (LSD test). S, susceptible check; R, genotype selected for *H. armigera* resistance; S × R, cross between Annigeri and ICC 506; SEM, standard error of mean.
[b]Medium and large larvae.

ICCV 93122. Among the short-duration genotypes, the three *H. armigera*-susceptible checks, Annigeri, ICCV 37, and ICCX 730266, had significantly lower levels of oxalic acid than the resistant genotypes, except ICCL 86111 and ICCV 7. ICCV 2 had an oxalic acid concentration comparable to that of the short-duration resistant genotypes.

Mean malic acid levels (micromoles per gram dry weight) on the pods were much higher than the mean pod oxalic acid levels (Table 3). There was significant variation in the malic acid level among the genotypes ($F = 4.73$; $df = 13, 55$; $P < 0.001$), but the genotypic variation was smaller than that of oxalic acid. There was no significant difference in the malic acid level between the *H. armigera* resistance and susceptible genotypes.

Pod Damage. There was significant variation in the angular transformed percentage pod damage among the 14 genotypes (Table 3; $F = 22.83$; $df = 13, 55$; $P < 0.001$). Highest pod damage was recorded on ICC 3137, a medium-

duration *H. armigera*-susceptible check. Other medium-duration genotypes exhibited significantly lower pod damage than ICC 3137, but the levels of the damage were similar to those in the *H. armigera*-susceptible short-duration genotypes. The *H. armigera*-resistant short-duration genotypes, except ICCL 86111, had significantly lower pod damage than the corresponding susceptible checks. These pod damage data confirmed the reduced damage in the genotypes that had previously been selected for *H. armigera* resistance. ICCV 93122, which is a new variety obtained from a cross between Annigeri, a *H. armigera*-susceptible genotype, and ICC 506, a *H. armigera*-resistant genotype, showed pod damage intermediate between the two parents.

The density of medium and large larvae (per gram dry weight) was plotted against time (DAS), and the area under the curve was calculated to provide an index of the intensity of larval attack, i.e., cumulative larval days, for each genotype from pod setting to maturity. There was significant variation in the cumulative larval days among the genotypes (Table 3; $F = 9.55$, $df = 13, 55$; $P < 0.001$). There was also a significant correlation between the cumulative larval days and the angular transformed percentage pod damage ($r = 0.879$) (Figure 3). Wightman et al. (1995) showed a negative correlation between cumulative larval days and grain yield and a strong negative correlation between grain yield and pod damage of chickpea in southern India. Our results confirm that pod and seed damage are mainly caused by medium and large *H. armigera*

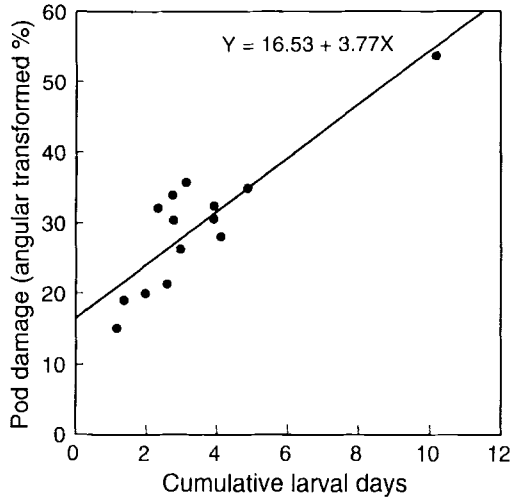


FIG. 3. Correlation between cumulative larval days for medium and large larvae of *H. armigera* during podding stage and pod damage of chickpea genotypes.

larvae in the podding stage and that their control should result in lower pod damage and higher yield in chickpea (Fitt, 1989).

In the present study genotypes that had high numbers of eggs did not necessarily have high numbers of larvae. This suggests that the factor(s) that stimulate(s) or inhibit(s) oviposition is different from the factor(s) that affect(s) larval growth and survival. In this study more oxalic acid (micromoles per gram dry weight) was detected, in general, on the leaves and pods of the short-duration *H. armigera*-resistant genotypes than on the susceptible checks or on the medium-duration genotypes (Tables 2 and 3). Angular transformed percentage pod damage was negatively correlated with the mean oxalic acid level on the pods ($r = -0.841$), when the data of ICC 3137 were excluded (Figure 4). The mean leaf oxalic acid level, which was correlated with the pod oxalic acid level ($r = 0.800$), was also negatively correlated with the angular transformed percentage pod damage ($r = -0.815$), when ICC 3137 was excluded from analysis (Figure 5). Oxalic acid has been reported to have an antibiotic effect on larvae (Yoshida et al., 1995), and it is possible that the antibiotic properties of oxalic acid may negate differences due to ovipositional antixenosis and determine the size of the larval population, and therefore pod damage, on a particular genotype.

The pod damage of ICC 3137, a susceptible check for medium-duration genotypes, was extremely high despite the levels of oxalic acid on both the leaves and pods being similar to those of other medium-duration genotypes.

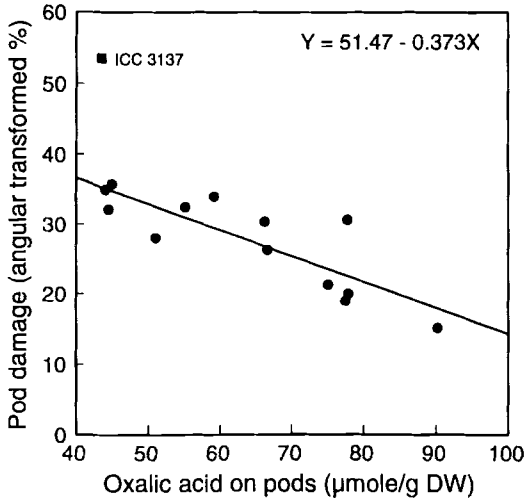


FIG. 4. Correlation between concentration of oxalic acid on pods and *H. armigera* pod damage of chickpea genotypes.

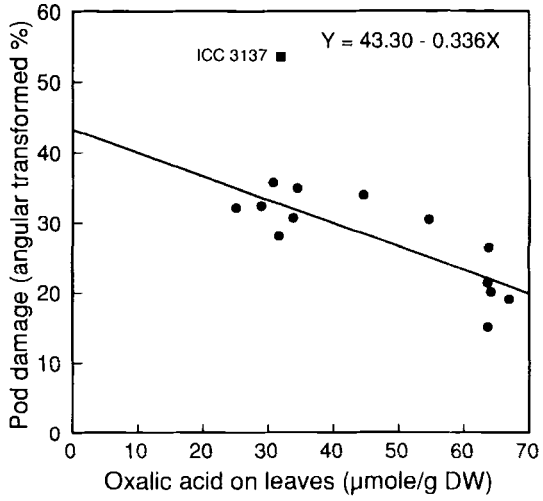


FIG. 5. Correlation between concentration of oxalic acid on leaves and *H. armigera* pod damage of chickpea genotypes.

This observation indicates that, in addition to oxalic acid, another factor also determines *H. armigera* susceptibility in chickpea. ICC 3137 began podding earlier than the other medium-duration genotypes but retained green leaves and tender pods for as long as the other genotypes (Table 1) and, as a result, had a more prolonged exposure to *H. armigera* attack than the other genotypes. The length of the podding period is, therefore, also an important factor in resistance to *H. armigera*.

There was no significant correlation between the pod damage and mean pod malic acid level (micromoles per gram dry weight). Although previous studies proposed malic acid in the chickpea leaf exudate to be a *H. armigera* resistance factor (Rembold, 1981; Rembold and Winter, 1982; Rembold et al., 1990a,b), we did not find any evidence that malic acid acts as a resistance factor in the field experiment.

The results of our study have practical implications for chickpea germ plasm enhancement programs. Oxalic acid levels could be used to select material for further screening. Leaves in the flowering–early podding or tender pod stage would be the most appropriate sample unit, as the differences in the oxalic acid level between resistant and susceptible genotypes are most marked at this time. In addition, the duration of the podding period could also be used as a selection criterion. This would be particularly useful for medium-duration genotypes where plants with shorter podding periods should be selected to minimize the period of exposure to the pest.

Acknowledgments—We are grateful to O. Singh (ICRISAT) for his advice on selection of chickpea genotypes, V. Subramanian and U. Singh (ICRISAT) for their assistance in HPLC analysis, and V. R. Bhagwat, L. Mohan, and the Legumes Entomology staff (ICRISAT) for their assistance in the experiments. Approved as Journal Article No. JA1836 by International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, Andhra Pradesh, India.

REFERENCES

ARMES, N. J., BOND, G. S., and COOTER, R. J. 1992a. The laboratory culture and development of *Helicoverpa armigera*. Natural Resources Institute Bulletin 57. Natural Resources Institute, Chatham, United Kingdom, pp. 20–21.

ARMES, N. J., JADHAV, D. R., BOND, G. S., and KING, A. B. S. 1992b. Insecticide resistance in *Helicoverpa armigera* in south India. *Pestic. Sci.* 34:355–364.

ARMES, N. J., JADHAV, D. R., and LONERGAN, P. A. 1994. Insecticide resistance in *Helicoverpa armigera*: Status and prospects for its management in India, pp. 522–533, *in* Proceedings of World Cotton Research Conference-1, February 13–17, 1994. Brisbane, Australia.

COWGILL, S. E., and LATEEF, S. S. 1996. Identification of antibiotic and antixenotic resistance to *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea. *J. Econ. Entomol.* 89:224–229.

DIAS, C. A. R., LAL, S. S., and YADAVA, C. P. 1983. Differences in susceptibility of certain chickpea cultivars and local collections to *Heliothis armigera* (Hubner). *Indian J. Agric. Sci.* 53:842–845.

FAO, 1994. FAO Yearbook, Production. Vol. 47. FAO, Rome, Italy, 104 pp.

FITT, G. P. 1989. The ecology of *Heliothis* species in relation to agroecosystems. *Annu. Rev. Entomol.* 34:17–52.

LAL, S. S., YADAVA, C. P., and SACHAN, J. N. 1986. Strategies for the development of an integrated approach to control gram pod borer, *Heliothis armigera* (Hubner) infesting chickpea. *Pesticides* May:39–51.

LATEEF, S. S. 1985. Gram pod borer (*Heliothis armigera*) (Hub.) resistance in chickpeas. *Agric. Ecosyst. Environ.* 14:95–102.

LATEEF, S. S., and SACHAN, J. N. 1990. Host-plant resistance of *Helicoverpa armigera* in different agroecological contexts, pp. 181–189, *in* Chickpea in the Nineties: Proceedings of the Second International Workshop on Chickpea Improvement, December 4–8, 1989, ICRISAT Center, India. ICRISAT, Patancheru, Andhra Pradesh, India.

REED, W., CARDONA, C., SITHANANTHAM, S., and LATEEF, S. S. 1987. Chickpea insect pests and their control, pp. 283–318, *in* M. C. Saxena and K. B. Singh (eds.). The Chickpea. CAB International, Oxon, UK.

REMBOLD, H. 1981. Malic acid in chickpea exudate—a marker for *Heliothis* resistance. *Int. Chickpea Newsl.* 4:18–19.

REMBOLD, H., and WEIGNER, CH. 1990. Chemical composition of chickpea, *Cicer arietinum*, exudate. *Z. Naturforsch.* 45c:922–923.

REMBOLD, H., and WINTER, E. 1982. The chemist's role in host-plant resistance studies, pp. 241–250, *in* Proceedings of the International Workshop on Heliothis Management, November 15–20, 1981, ICRISAT Center, India. ICRISAT, Patancheru, Andhra Pradesh, India.

REMBOLD, H., SCHROTH, A., LATEEF, S. S., and WEIGNER, CH. 1990a. Semiochemical and host-plant selection by *Helicoverpa armigera*: Basic studies in the laboratory for the field, pp. 23–26, *in* Summary proceedings of the First Consultative Group Meeting on the Host Selection Behavior of *Helicoverpa armigera*, March 5–7, 1990, ICRISAT Center, India. ICRISAT, Patancheru, Andhra Pradesh, India.

REMBOLD, H., WALLNER, P., KÖHNE, A., LATEEF, S. S., GRUNE, M., and WEIGNER, CH. 1990b. Mechanisms of host-plant resistance with special emphasis on biochemical factors, pp. 191–194, *in* Chickpea in the Nineties: Proceedings of the Second International Workshop on Chickpea Improvement, December 4–8, 1989, ICRISAT Center, India. ICRISAT, Patancheru, Andhra Pradesh, India.

SRIVASTAVA, C. P., and SRIVASTAVA, R. P. 1989. Screening for resistance to gram pod borer, *Heliothis armigera* (Hubner), in chickpea (*Cicer arietinum* L.) genotypes and observation on its mechanism of resistance in India. *Insect Sci. Applic.* 10:225–258.

SRIVASTAVA, C. P., and SRIVASTAVA, R. P. 1990. Antibiosis in chickpea (*Cicer arietinum* L.) to gram pod borer, *Heliothis armigera* (Hubner) (Noctuidae: Lepidoptera) in India. *Entomon* 15:89–93.

WIGHTMAN, J. A., ANDERS, M. M., RAMESHWAR RAO, V., and MOHAN REDDY, L. 1995. Management of *Helicoverpa armigera* (Lepidoptera: Noctuidae) on chickpea in southern India: Thresholds and the economics of host plant resistance and insecticide application. *Crop Prot.* 14:37–46.

WILKINSON, L. 1990. SYSTAT: The system for statistics. SYSTAT Inc., Evanston, Illinois.

YOSHIDA, M., COWGILL, S. E., and WIGHTMAN, J. A. 1995. Mechanism of resistance to *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea: The role of oxalic acid in leaf exudate as an antibiotic factor. *J. Econ. Entomol.* 88:1783–1786.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

Letters to the Editors

RELEASE DYNAMICS OF PHEROMONE FROM A PHEROMONE DISPENSER

A worldwide effort is in progress to develop practical insect control programs based on the use of sex pheromones to disrupt mating. One of the critical areas in the development of this technology is the performance of pheromone dispensers. In a recent issue of *Journal of Chemical Ecology*, Bradley et al. (1995) proposed a predictive model for evaporative release of pheromones from a Shin-Etsu polyethylene-tube dispenser. Their model was proposed as an expression of the physical principles governing evaporative release. Their model may have some value in predicting approximate performance in field experiments, but I believe the principles they proposed are incorrect.

I wish to challenge their assertions that: (1) air movement has no effect on evaporation rate, (2) evaporation rate is controlled by diffusion through the polyethylene wall rather than by vapor pressure, (3) a Clausius-Clapeyron type of relationship (logarithm of evaporation rate is inversely proportional to absolute temperature) is an incorrect description of the effect of temperature on evaporation rate, (4) there is no known relationship for the effect of temperature on the rate of diffusion through the polyethylene wall, and (5) degree-days is the correct concept for modeling the effect of temperature.

The following analysis shows that the factors that govern evaporation from the polyethylene wall of the Shin-Etsu dispensers are the same factors that govern evaporation from rubber septa dispensers. The evaporation rate, E, of pheromone from rubber septa is the sum of three processes as given in the following equation (McDonough, 1991):

$$E = -dP_r/dt = k_1'P_{rs} - k_2P_v + k_3P_v \quad (1)$$

Here, P_r is the pheromone concentration in the rubber septum; P_{rs} is the pheromone concentration in the surface of the rubber septum; P_v is the pheromone concentration in the vapor over the septum; k_1' is the rate constant for the movement of pheromone from the septum into the vapor; k_2 is the rate constant for condensation back into the septum; and k_3 is the rate constant for movement of pheromone to positions in space where it cannot condense into the septum.

If all three terms in equation 1 are of comparable magnitude, then the

0098-0331/97/0500-1211$12.50/0 © 1997 Plenum Publishing Corporation

relationship of evaporation rate to time and dose is quite complex. However, a priori, there are two limiting cases, depending on the relative magnitudes of k_2 and k_3, which could apply and lead to simpler relationships. The processes involving k_2 and k_3 compete for P_v. In one limiting case, k_3 is much greater than k_2, and condensation back into the septum is essentially completely suppressed (i.e., $k_2P_v = 0$). Then:

$$-dP/dt = k_1'P_{rs}$$

The rate-determining step would be diffusion through the dispenser, and there would be no simple relationship between P_r and P_{rs}. The concentration of pheromone within the septum would be highest at the greatest distance from the surface and would be lowest in the surface (Roseman and Cardarelli, 1980). Then the rate of evaporation would be proportional to $t^{-1/2}$, where t is time (Zeoli et al., 1982). This relationship was successfully applied to controlled release devices releasing active agent into a liquid medium (Roseman and Cardarelli, 1980; Zeoli et al., 1982). It was thought that the same model could be applied to controlled release devices such as rubber septa releasing pheromone into air (Zeoli et al., 1982). However, extensive data on evaporation of pheromones from septa show that this relationship does not apply to rubber septa (McDonough, 1991).

In the other limiting case, k_3 is much less than k_2. Then P_v and P_{rs} are essentially in equilibrium with each other, and $k_1'P_{rs} = k_2P_v$. Therefore,

$$-dP_r/dt = k_3P_v$$

Because the pheromone would be evenly distributed throughout the septum, $k_1P_r = k_1'P_{rs}$; therefore, $k_1P_r = k_2P_v$, and:

$$P_v = k_1P_r/k_2 = P_r/K$$

Therefore

$$-dP_r/dt = k_3P_r/K \tag{2}$$

Equation 2 is the equation of a first-order evaporative loss and is consistent with extensive data for rubber septa (McDonough, 1991). The experimentally observed rate constant, k, is equal to k_3/K.

An important point here is that the mechanism of release is independent of the dispenser, and equation 2 will apply to any dispenser based on evaporation of pheromone from a molecular matrix. The mechanism is determined by the relative values of k_2, which depends on the inherent tendency of a pheromone molecule to condense, and k_3, which depends on the rate of diffusion of the pheromone and on air movement. Both k_2 and k_3 are independent of the dispenser. Changing the dispenser could change k_1, which would change the value of K and therefore the rate of evaporation, but not the mechanism of evaporation, i.e., the rate-determining step (the slow step) is the step involving k_3. The k_3

term in equation 2 shows, contrary to the assertion of Bradley et al. (1995), that air movement must affect evaporation rate, not only from septa, but from any dispenser based upon evaporation of pheromone from a molecular matrix.

Furthermore, the K term in equation 2 shows that evaporation rates are determined by vapor pressure, since K is inversely proportional to vapor pressure. Therefore, the Clausius-Clapeyron equation (logarithm of vapor pressure is inversely proportional to absolute temperature) applies. Bradley et al. (1995) claimed that the evaporation rate was controlled by diffusion through the polyethylene wall rather than by vapor pressure, and no relationship for the effect of temperature on diffusion rate was known. However, the logarithm of polymer permeability is inversely proportional to absolute temperature (O'Neill, 1980). Therefore, even if the rate-determining step were diffusion through the polyethylene wall, the logarithm of the evaporation rate would still be inversely proportional to absolute temperature.

Equation 2 can be developed into a full predictive model encompassing air speed and temperature. The same model can be applied to the Shin-Etsu dispenser once it is modified to take into account the effect of the well.

The value of k_3 is determined by the rate of removal of pheromone from the vapor space around the dispenser. If one assumes that removal of pheromone by diffusion is slow compared to removal by air movement, then:

$$k_3 = m'S + n'$$

where m' and n' are constants and S is air speed. Substitution into equation 2 gives:

$$-dP_r/dt = (m'S + n')P_r/K \tag{3}$$

K values can not be determined experimentally for equation 3, but $t_{1/2}$ values can be used in place of K if a reference air speed is chosen. Since:

$$k_3/K = k = \ln 2/t_{1/2}$$

Then

$$K = k_3 t_{1/2}/\ln 2$$

Because $k_3/\ln 2$ is a constant at a given air speed:

$$K = a^{20}t_{1/2}$$

where a is the constant and ${}^{20}t_{1/2}$ is the half-life when the air speed is 20 cm/sec. Let $m'/a = m$ and $n'/a = n$ and then equation 3 becomes:

$$-dP_r/dt = (mS + n)P_r/{}^{20}t_{1/2} \tag{4}$$

Equation 4 shows that evaporation rate is directly proportional to air speed, and the effect of air speed on evaporation rates is greater the higher the concentration of pheromone (P_r) and the lower the half-life (${}^{20}t_{1/2}$); m and n are

universal constants applicable to any compound. The effect of temperature on half-life has been determined at an air speed of 20 cm/sec for many of the common types of Lepidoptera sex pheromones (McDonough, 1991; McDonough et al., 1989) and is:

$$^{20}t_{1/2} = \exp(\delta H/RT + y_o)$$

Here δH is the enthalpy of vaporization, R is the gas constant, T is absolute temperature, and y_o is a constant. Therefore, the complete predictive model for rubber septa is:

$$-dP_r/dt = (mS + n)P_r/\exp(\delta H/RT + y_o) \quad (5)$$

For field studies, equation 5 cannot be integrated because air speed and temperature do not vary in a predictable manner with time. However, if the parameters m, n, δH, and y_o are known, field performance could be predicted by monitoring temperature and air speed and assuming these quantities are constant over short time intervals such as 1 hr. Then the value of the rate constant, k, could be calculated for each hour:

$$k = (mS + n)/\exp(\delta H/RT + y_o)$$

and the pheromone concentration could be calculated from one hour to the next by the integral equation for a first-order evaporation and the rate constant in units of h^{-1}:

$$P = P_0 \exp(-k)$$

where P_0 is the pheromone concentration at the beginning of the hour, and P is the concentration at the end of the hour. The evaporation rate at any time is equal to kP. δH and y_o are known for many pheromones (McDonough, 1991; McDonough et al., 1989).

Previously it was shown that the evaporation rate of pheromone components from the Shin-Etsu polyethylene-tube dispensers obeyed the following equation (McDonough et al., 1992):

$$-dP/dt = k_a k_b P$$

where P is the amount of pheromone in the well; k_b is the ratio of the evaporation rate of the pheromone to the amount in the dispenser wall; and k_a is the ratio of the amount of pheromone in the wall to that in the well. Therefore, k_b is the same as the rate constant, k, for rubber septa and is equal to k_3/K, which is equal to $(mS + n)/\exp(\delta H/RT + y_o)$. k_a is an equilibrium constant and is dependent on temperature as follows (Adamson, 1986):

$$k_a = k_{a,ref} \exp(-\delta E/RT)$$

Here $k_{a,ref}$ is k_a at a reference temperature, k_a is the value at temperature T, and

δE is the molar enthalpy difference between pheromone in the wall and in the well. Therefore, the complete predictive model for the Shin Etsu dispenser is:

$$-dP/dt = k_{a,ref}(mS + n)P/\exp[(\delta E + \delta H)/RT + y_o)] \quad (6)$$

δE for partitioning of codlemone between the polyethylene wall and the dispenser well should be 1–1.5 kcal, which is much smaller than δH, and therefore the temperature dependence of evaporation rate from the Shin-Etsu dispensers should be mainly determined by δH. Then one would expect the $\delta H + \delta E$ values for Shin-Etsu dispensers to be similar in magnitude to the δH values for rubber septa. For dodecanol, tetradecanol, and codlemone, respectively, the values of δH in kilocalories per mole are 17.19, 19.47, and 17.63 in rubber septa (McDonough, 1991; McDonough, unpublished), and the $\delta H + \delta E$ values are 18.04, 19.11, and 18.08 in Shin-Etsu dispensers (Brown et al., 1992). Therefore, the temperature dependence of evaporation rates has the magnitude expected from a vapor pressure controlled evaporation.

Bradley et al. (1995) criticized the work of Brown et al. (1992) for the use of the Clausius-Clapeyron temperature relationship, and criticized the work of McDonough et al. (1992) for not using temperature in the model for codlemone. The reason for not using temperature was that the main mechanism of loss of codlemone from the Shin-Etsu (Isomate-C) dispenser was chemical decomposition, and the rate of decomposition depended mainly on the amount of light and oxygen. Data from several years, which included hot and cool seasons, showed that the half-life for loss of codlemone from these dispensers was essentially invariant (ca. 32–35 days). The half-life of codlemone could be used to determine the pheromone residue, P, in the dispenser at any time, and the values of k_a and $k_b(k_a k_b = 0.000127/\text{hr})$ could be used to determine the evaporation rate according to $E = k_a k_b P$ at a temperature of 20°C and a wind speed of 20 cm/sec. If the evaporation rate were needed at another temperature, the data of Brown et al. (1992) could be used. Thus, our model provided the information needed to satisfactorily predict the temperature and time performance of these dispensers in orchards.

Although the degree-day concept used by Brown et al. (1992) and Bradley et al. (1995) has proven valuable in studies of plant and insect development, this concept can not correctly be applied to controlled release dispensers such as the Shin-Etsu dispenser. The relationships determining evaporative release from the Shin-Etsu dispensers are contained in equation 6.

Leslie M. McDonough
Yakima Agricultural Research Laboratory
Agricultural Research Service, USDA
5230 Konnowac Pass Road
Wapato, Washington 98951-9651

REFERENCES

ADAMSON, A. W. 1986. A Textbook of Physical Chemistry, 3rd ed. Academic Press College Division, Harcourt Brace Janovich, San Francisco, p. 247, equation 7-31.

BRADLEY, S. J., SUCKLING, D. M., MCNAUGHTON, K. G., WEARING, C. H., and KARG, G. 1995. A temperature-dependent model for predicting release rates of pheromone from a polyethylene tubing dispenser. *J. Chem. Ecol.* 21:745–760.

BROWN, D. F., KNIGHT, A. L., HOWELL, J. F., SELL, C. R., KRYSAN, J. L., and WEISS, M. 1992. Emission characteristics of a polyethylene pheromone dispenser for mating disruption of codling moth (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 85:910–917.

MCDONOUGH, L. M. 1991. Controlled release of insect sex pheromones from a natural rubber substrate, *in* P. A. Hedin (ed.). Naturally Occurring Pest Bioregulators. ACS Symposium Series 449. American Chemical Society, Washington, D.C.

MCDONOUGH, L. M., BROWN, D. F., and ALLER, W. C. 1989. Insect sex pheromones: Effect of temperature on evaporation rates of acetates from rubber septa. *J. Chem. Ecol.* 15:779–790.

MCDONOUGH, L. M., ALLER, W. C., and KNIGHT, A. L. 1992. Performance characteristics of a commercial controlled-release dispenser of sex pheromone for control of codling moth (*Cydia pomonella*) by mating disruption. *J. Chem. Ecol.* 18:2177–2189.

O'NEILL, W. P. 1980. Membrane systems, p. 241, *in* A. F. Kydonieus (ed.). Controlled Release Technologies: Methods, Theory, and Applications. CRC Press, Boca Raton, Florida.

ROSEMAN, T. J., and CARDARELLI, N. F. 1980. Monolithic polymer devices, *in* A. F. Kydoneius (ed.). Controlled Release Technologies: Methods, Theory, and Applications. CRC Press, Boca Raton, Florida.

ZEOLI, L. T., KYDONIEUS, A. F., and QUISUMBING, A. R. 1982. Controlled release technologies, p. 138, *in* A. F. Kydonieus and M. Beroza (eds.). Insect Suppression with Controlled Release Pheromone Systems. CRC Press, Boca Raton, Florida.

RESPONSE BY D. M. SUCKLING ET AL.

We thank Dr. McDonough for his interest in our paper. McDonough's comments show that we disagree on how the release of pheromone from a Shin Etsu polyethylene tubing pheromone dispenser should be modeled. We contend that the rate of pheromone release is controlled solely by the active length of the dispenser—the length of capillary filled by the pheromone oil—and the conductance of the walls of the tubing dispenser. This basic scheme is thoroughly consistent with the conventional view of the control of pheromone release. For example Zeoli et al. (1982) state "All C[ontrolled] R[elease] processes are in one way or another controlled by the diffusion of the active agent through a polymer barrier or by an inward diffusion of an environmental fluid. . . ." The alternative view is that release rate is controlled by factors that influence the transport of vapor from the outer surfaces of the dispenser to the atmosphere. If this were correct, then release rate would depend on air movement and the

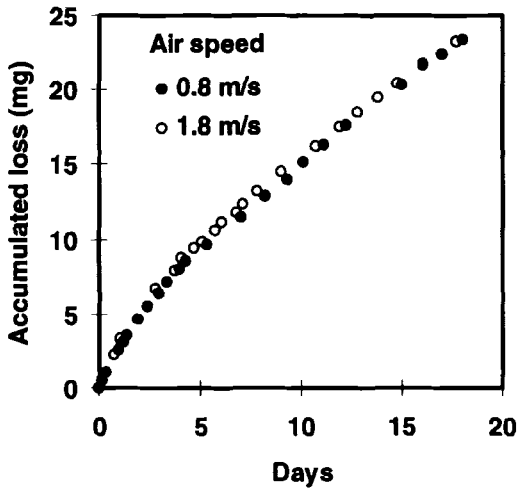


FIG. 1. Accumulated loss of pheromone from Shin Etsu polyethylene tubing dispensers exposed to air speeds of 0.8 (open circles) and 1.8 m/sec (solid circles).

vapor pressure of the pheromone at the outer wall surface of the dispenser tubes. This latter view is not consistent with our experimental evidence.

In our paper (Bradley et al., 1995) we cited the unpublished work of T. Bellas as support for our claim that release rate does not depend on air speed. We now present the original observations. Figure 1 shows the loss of pheromone from Shin Etsu polyethylene tube dispensers held in air streams of 0.8 and 1.8 m/sec. Capillary lengths in the two tubes were nearly equal, at 134 $\pm$ 1 mm. The different air speeds were achieved by hanging the dispensers under the closure of a fume cupboard, with the opening adjusted to achieve the required air speed as measured with a thermistor anemometer. The experiment was performed at normal laboratory temperatures, which varied from about 20 to 24°C, with a mean that should be near 22°C. The results show that pheromone loss rate from the two dispensers is very similar—certainly less than the 10% increase at the higher speed suggested by McDonough in his letter, and very much less than the 46% increase predicted by standard transport theory for the case where transport through the boundary layer is the rate-limiting step (see below). A fuller account of this experimental work is in preparation.

Another experiment confirms this lack of sensitivity to wind speed. Shin Etsu dispensers were exposed on a tower outdoors at six levels from 0.7 to 20 m above ground. Loss of pheromone was recorded twice over nine-week periods. Wind speeds were recorded by cup anemometers at five levels, from 1.5 to 20 m. Wind speeds increased more than fivefold between 1.5 and 20 m, from 0.36

to 1.91 m/sec and from 0.41 to 2.29 m/sec, yet there were no significant increases in rate of pheromone loss with height.

In each experiment, pheromone release was found to be insensitive to wind speed. This puts our basic data in direct conflict with McDonough's model, so it is as well we look carefully at the processes governing transport of pheromone from the capillary reservoir in the dispenser, through the polyethylene walls and out through the air boundary layer surrounding the dispenser to the ambient air beyond.

We do not share McDonough's view that evaporation from a Shin Etsu pheromone dispenser is the sum of three processes in the fashion shown in his equation 1. Rather we view the release as a catenary process, with the pheromone passing first through the dispenser wall and then through the air boundary layer surrounding the dispenser. We thus model the transport purely as a transmission process, from the capillary reservoir to the ambient air, with changes in concentration within the dispenser walls being negligibly small except during the first two weeks of exposure, while steady concentration profiles are being established. We ignored this initial period in our analysis. That is, for the Shin Etsu dispenser we ignore the term dP_r/dt that is the subject for McDonough's equation 1. Instead we introduce a time dependence by assuming that only the pheromone-filled portion of the capillary dispenser is effective.

In our model the flux of pheromone through the capillary walls necessarily equals the flux through the air boundary layer surrounding the capillary; chemical degradation is ignored. As befits a steady diffusion process, the flux through the wall is proportional to the concentration difference of pheromone within the polyethylene matrix at the inner and outer surfaces of the tube. The coefficient of proportionality is called the conductance. The inner concentration is the saturation value for the pheromone oil/polyethylene combination while the outer concentration depends on how efficiently the material is transported through the boundary layer surrounding the dispenser to the ambient air. We describe this using the equation

$$F = g_w(c_i - c_o)$$

where F is the flux of pheromone per unit filled length of capillary, g_w is the conductance of the wall, and c_i and c_o are the concentrations in the inner and outer surfaces of the wall, respectively. The concentrations c_i and c_o are in units of mass of pheromone per unit volume of wall. On leaving the wall, the pheromone diffuses out through the laminar boundary layer surrounding the tube at a rate proportional to the gradient of vapor density in air. Thus

$$F = g_b(r_o - r_a)$$

Here $r_o \gg r_a$ so we can set $r_a = 0$ in this transport equation.

Usually one or another of these stages is limiting, so only one of the

equations above need be considered. If the wall conductance limits, then $(r_o - r_a) \approx 0$ and the concentration in the outer wall falls very close to zero, whereupon $F = g_w c_i$. In this case pheromone release is independent of wind speed. This is the case considered in our model. If, on the other hand, the boundary-layer conductance limits then $(c_i - c_o) \approx 0$ and r_o is close to the saturation value, $r_{o,s}(T)$, which depends on temperature in a manner described by the Clausius-Clapeyron equation. For the Reynolds numbers appropriate to outdoor airflows past a narrow tube, such as the Shin Etsu dispenser, standard texts (e.g., Monteith and Unsworth, 1992) indicate that the boundary-layer conductance is proportional to wind speed raised to the 0.47 power. Increasing wind speed by a factor of 1.8/0.8 should therefore increase evaporation by the factor of $2.25^{0.47}$ or 46%. This was not observed in our experiments.

Mixed cases are possible, but these are less common and more complex to deal with. To solve them we need an additional relationship to link the concentration in the outer surface of the tube to the vapor concentration in the air adjacent to that surface, i.e., to link c_o to r_o. The usual way to develop such a relationship is to find the relationship between pheromone concentration within the plastic wall material and the Gibbs free energy function for the pheromone oil within that material, $\phi(c_o)$. The pheromone vapor pressure of the air in equilibrium with a surface at this concentration is then given by

$$r_o = r_{o,s}(T) \exp[\phi(c_o)/RT]$$

Since $r_a = 0$, we have $r_a = F/g_b$, and this last equation gives

$$c_o = \phi^{-1}[RT \ln(F/g_b r_{o,s})]$$

where $\phi^{-1}[\ldots]$ is the inverse function of $\phi[\ldots]$. This value of c_o is then substituted into the wall-conduction equation to give

$$F = g_w\{c_i - \phi^{-1}[RT \ln(F/g_b r_{o,s})]\}$$

We have not pursued information on the function $\phi(c_o)$ in the literature because we do not believe this case to be important. What can be said is that this equation for the pheromone flux has an implicit form, unlike McDonough's equation 1. We do not believe that McDonough's equation 1 describes well the processes important for the release of pheromone from the Shin Etsu polyethylene tubing dispensers. Since his objections 2 and 3 are based on developments from his equation 1 rather than on empirical evidence, we reject his challenge on those points. Instead we offer the analysis above. We reassert the correctness of the model presented in our paper and the deductions we have made from it. The success of our model when dealing with field data supports (but does not prove) our claims.

McDonough's fourth challenge is to our "assertion" that "there is no

known relationship of the effect of temperature on the rate of diffusion through the polyethlyene wall." Our paper is guilty of ambiguity here. We meant that we did not know the relationship. We thank Dr. McDonough for bringing it to our attention. With the more exact relationship, a new equation could be written to replace our equation 1, and this equation could then be integrated to produce more accurate but more complex alternatives to our equations 2 and 3. The advantage of the linearized form is that we obtain a linear model suitable for use in regression analysis. It is a practical alternative with accuracy consistent with field use. Knowledge of the exact relationship does not alter our analysis.

McDonough's fifth and last challenge is to our supposed "assertion" that "degree-days is the correct concept for modeling the effect of temperature." Careful reading will show that we made no such global assertion. What we did show is that our model, using the linearized expression for the dependence of wall conductance on temperature, led to an equation where the effect of temperature could be expressed in a degree-day term. We dealt with the degree-day "concept" as such because many orchardists and researchers are familiar with it. Had we included a more exact representation of this temperature dependence, then quadratic and higher-order temperature terms would have appeared in our equations and degree-days would not have been a correct representation. We agree that there is nothing fundamental about degree-days per se in our analysis, but we continue to believe our equation 3 is a useful model with accuracy consistent with field requirements.

Finally, we would like to comment on the universality of our model. There is good evidence that pheromone release from some types of polyethylene tubing dispensers does depend on wind speed. For example, Van der Kraan and Ebbers (1990) found that the rate of evaporation of tetradecenyl acetate from tubes of low-density polythene depended on wind speed raised to a power close to 0.5, as expected where transport across the air boundary layer limits release. Clearly the limiting layer depends on the particular dispenser/pheromone combination. We do not claim our model is appropriate for all pheromone dispensers, just that it describes well the Shin Etsu polyethylene tubing dispensers that we have studied.

D. M. Suckling
K. G. McNaughton
S. J. Bradley
G. Karg
C. H. Wearing
HortResearch, New Zealand
T. Bellas
Division of Entomology, CSIRO
Canberra, Australia

REFERENCES

BRADLEY, S. J., SUCKLING, D. M., MCNAUGHTON, K. G., WEARING, C. H., and KARG, G. 1995. A temperature-dependent model for predicting release rates of pheromone from a polyethylene tubing dispenser. *J. Chem. Ecol.* 21:745-760.

MONTEITH, J. L., and UNSWORTH, M. H. 1990. Principles of Environmental Physics. Edward Arnold, London, 291 pp.

VAN DER KRAAN, C., and EBBERS, A. 1990. Release rates of tetradecen-1-ol acetates from polymeric formulations in relation to temperature and air velocity. *J. Chem. Ecol.* 16:1041-1058.

ZEOLI, L. T., KYOIEUS, A. F., and QUISUMBING, A. R. 1982. Controlled release technologies, pp. 132-144, *in* A. F. Kydonnieus and M. Beroza (eds.). Insect Supression with Controlled Release Pheromone Systems. CRC Press, Boca Raton, Florida.

RESPONSE BY LESLIE M. McDONOUGH

In response to the reply to my letter submitted by Dr. Suckling, I would like to make a few more comments.

Suckling et al. presented a graph showing that the loss of pheromone from Shin Etsu dispensers was the same at two different air speeds over a period of 18 days. I believe that this result was obtained because the mechanism of release is different during the first three weeks. In the manufacturing of these dispensers, the Shin Etsu Co. heats them in order to supersaturate the dispenser wall. Consequently, the mechanism during the first three weeks is one of dissolution. In our earlier study (McDonough et al., 1992) we found that dodecanol, codlemone, and tetradecanol were all lost at the same rate during the first three weeks, but that dodecanol was lost at a rate 6.5 times that of tetradecanol over the subsequent 126 days. Therefore, it appears that the dissolution process overrode the differences in evaporation rates due to volatility differences of the compounds as well as differences in evaporation rates due to air speed differences in the case of the Bradley et al. dispensers.

Suckling et al. disagree with equation 1 of my previous letter. I think there is a misunderstanding here. There can be no argument that the steps that occur are: (1) pheromone passes from the liquid well into the dispenser wall, (2) pheromone leaves the wall, and (3) is carried away by air movement. The question is: is there a predominantly slow step among these processes, and if so which step is the slow step? Equation 1 summarizes the processes that determine the answer to this question. Equation 1 is consistant with the evaporation rate either being controlled by the rate of diffusion of pheromone through the dispenser wall [proposal of Bradley et al. (1995)], or by air speed (my proposal), or an intermediate and more complex case. The extensive data on rubber septa show that material within the dispenser is evenly distributed (it would not be

evenly distributed if the evaporation rate were controlled by diffusion), there is a near-equilibrium established vapor pressure, removal of pheromone by air movement is the rate controlling step, and the factors that determined this situation are independent of the dispenser material and whether or not there is a well of pheromone within the dispenser.

In his letter, Dr. Suckling cites the work of Van der Kraan and Ebbers (1990). In that publication, they reported that air speed affects evaporation rates for a variety of dispensers according to the equation: $\log E = a \log S + b$, where E is evaporation rate, S is air speed, and a and b are constants. Some of the dispensers were open capillaries, which release by a different mechanism. The average value of a for all dispensers was approximately 0.5. Closed polyethylene tubes releasing tetradecyl acetate was one of the dispensers measured. Since a dependence of evaporation rate on air speed was found for this dispenser, these data are clearly in contradiction to the model of Bradley et al. (1995); however, these data are not necessarily in contradiction to our model, which proposes a linear relationship between air speed and evaporation rate. For example, suppose the linear equation were: $E = 0.1S + 3.0$. If one takes points from the regression line of this equation from $S = 10$ to $S = 100$, and performs a regression analysis according to the equation of Van der Kraan and Ebbers (1990), one obtains a value of $a = 0.50$ and a correlation, R^2, of 0.98.

Leslie M. McDonough
Yakima Agricultural Research Laboratory
Agricultural Research Service, USDA
Wapato, Washington 98951

REFERENCES

BRADLEY, S. J., SUCKLING, D. M., MCNAUGHTON, K. G., WEARING, C. H., and KARG, G. 1995. A temperature-dependent model for predicting release rates of pheromone from a polyethylene tubing dispenser. *J. Chem. Ecol.* 21:745–760.

MCDONOUGH, L. M., ALLER, W. C., and KNIGHT, A. L. 1992. Performance characteristics of a commercial controlled-release dispenser of sex pheromone for control of codling moth (*Cydia pomonella*) by mating disruption. *J. Chem. Ecol.* 18:2177–2189.

VAN DER KRAAN, C. and EBBERS, A. 1990. Release rates of tetradecen-1-ol acetates from polymeric formulations in relation to temperature and velocity. *J. Chem. Ecol.* 16:1041–1058.

RESPONSE BY D. M. SUCKLING AND K. G. McNAUGHTON

In his second letter, Dr. McDonough challenges both our experimental results and our interpretation of those results, offering instead the model described in his first letter. We reject both his challenges and his model.

Firstly, Dr. McDonough claims that our experimental system was flawed because our Shin Etsu polyethylene dispensers were operated in the initial, desaturation phase of their release. This is not true, either in the laboratory or the field experiments. We are well aware of the higher rates of pheromone release in this initial phase; we mentioned it in our paper, and we avoided it in our experiments. Pheromone release from Shin Etsu dispensers was found to be independent of wind speed after the initial desaturation phase was complete. During the initial phase, by contrast, we expect that the rate of pheromone release would depend on wind speed. This is because the pheromone is, initially, readily available at the outer surface of the dispenser and only the wind-speed-dependent conductance of the boundary layer would limit loss. Such a dependence would diminish and become undetectable during the initial two or three weeks of exposure. That is, our lack of an observed dependence on wind speed is good evidence that we did not make our measurements during the initial desaturation phase.

Next Dr. McDonough reasserts that his equation 1 is correct and our disagreement must be due to a misunderstanding. He says "There can be no argument that the three steps are. . ." and provides us a list. But this list omits the step of transmission through the wall, which, we argue, is rate-limiting for the Shin Etsu dispenser. We cannot account for the structure of Dr. McDonough's equation 1 and instead offer the theory outlined in our response to his first letter.

Then Dr. McDonough reargues his case that transport across the aerodynamic boundary layer surrounding a rubber septum dispenser is the rate-limiting step in pheromone release. We accept this. Dr. McDonough has made measurements on this system while we have not. But, and this is the point, a rubber septum is not a Shin Etsu polyethylene tube dispenser. Seemingly, they behave quite differently. Indeed, we have acknowledged that some dispensers behave quite differently than our dispensers—even some types of polyethylene tube dispensers can behave differently. We referenced the work of Van der Kraan and Ebbers (cited in our previous letter) to highlight this point in our letter, but differences between systems do not constitute a contradiction, as argued by Dr. McDonough in his final point, simply a difference in system properties. It is interesting to ask how the properties of the wall matrix and impregnating fluid combine to set the wall conductance, but such an enquiry is beyond the scope of our work.

D. M. Suckling
K. G. McNaughton
HortResearch, New Zealand

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

INSECTICIDAL LIMONOIDS FROM *Swietenia humilis* AND *Cedrela salvadorensis*

A. JIMENEZ,[1] R. MATA,[1] R. PEREDA-MIRANDA,[1] J. CALDERON,[1] M. B. ISMAN,[2] R. NICOL,[3] and J. T. ARNASON[3,*]

[1]*Departamento de Farmacia and Instituto de Quimica, Facultad de Quimica Universidad Nacional Autonoma de Mexico Coyoacan, 04510 D.F., Mexico*

[2]*Department of Plant Science, University of British Columbia Vancouver, British Columbia V6T 1Z4, Canada*

[3]*Department of Biology University of Ottawa Ottawa, Ontario K1N 6N5, Canada*

(Received June 25, 1996; accepted December 9, 1996)

Abstract—Four limonoids, humilinolides A–D from *Swietenia humilis* and cedrelanolide from *Cedrela salvadorensis*, were evaluated for their effect on the European corn borer, *Ostrinia nubilalis* in comparison with toosendandin, a commercial insecticide derived from *Melia azedarach*. When incorporated into artificial diets of neonates at 50 ppm, all compounds caused larval mortality as well as growth reduction and increased the development time of survivors in a concentration-dependent manner. Humilinolide C also reduced growth and survivorship at 5 ppm. Additional effects observed in many of the limonoid-treated groups included a significant delay in time to pupation and adult emergence. The compounds showed comparable activity to toosendanin, a commercial insecticide. Furthermore, the cytotoxicity of the humilinolides to three human cell lines was low.

Key Words—Humilinolides, cedrelanolide, limonoids, insecticides, European corn borer, cytotoxicity.

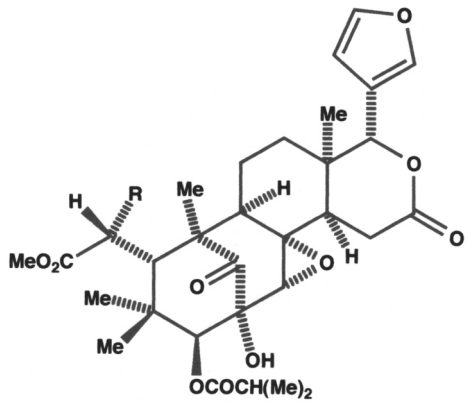
INTRODUCTION

Many limonoids of the Meliaceae and Rutaceae families possess antifeedant, toxic, or growth-reducing properties to different species of insects (Arnason et al., 1993; Champagne et al., 1989, 1992; Isman et al., 1995; Mikolajczak and Reed, 1987). Two of these compounds, azadirachtin from seeds of the neem

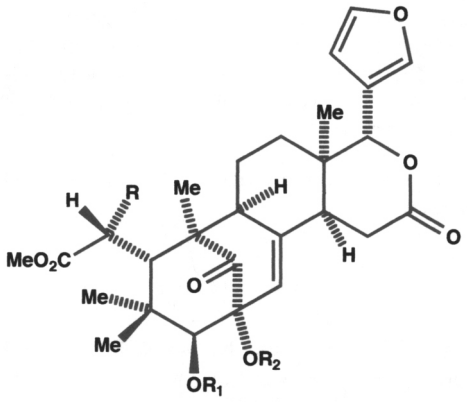
*To whom correspondence should be addressed.

0098-0331/97/0500-1225$12.50/0 © 1997 Plenum Publishing Corporation

tree of India, *Azadirachta indica*, and toosendanin from the bark of *Melia toosendan* and *Melia azedarach* of China have been commercialized for use as practical pest control agents (Chiu, 1995). In both cases the parent natural product has generally better efficacy than derivatives. Limonoids are valued as well for their low mammalian toxicity, nonneurotoxic mode of action, and low persistence, which enhance their value as botanical pesticides.



humilinolide A: R=OH
humilinolide B: R=OAc

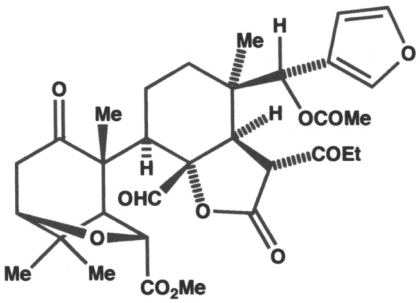


humilinolide C: R=H; R1=COC(Me)=CHMe; R2=Ac
humilinolide D: R=OAc; R1=Ac; R2=H

FIG. 1. Humilinolides A–D.

Although the Meliaceae are well represented in the neotropics and are known as natural sources of insecticides (Pennington, 1981), no insecticide has been commercialized from the neotropical Meliaceae. In an effort to find practical insecticides from the Americas, several groups have screened extracts of Meliaceae for effects against lepidopteran larvae. The significantly active extracts included species of the common American genera, *Swietenia* and *Cedrela*. For example, it was found that leaf extracts of *Swietenia humilis* Zucc. inhibited growth (50% effective concentration, EC_{50} = 100 ppm) and deterred feeding (EC_{50} = 23 ppm) by the variegated cutworm *Peridroma saucia*. Phytochemical investigation led to the isolation of seven limonoids, including humilinolides A–D (Figure 1) from seeds (Okorie and Taylor, 1970; Segura-Correa et al., 1993). *S. humilis* is commonly known in Mexico as "zopilote" and "cobano." It grows in the low to mid-elevation of subtropical regions of Mexico including the states of Guerrero, Michoacan, Colima, Sinaloa, and Chiapas and extends along the Pacific slope into Central America as far south as Guanacaste province, Costa Rica (Standley, 1920–1926). *Cedrela salvodorensis* is a tree growing at mid-elevation (500–1500 m) in the same geographical range. Recently the new limonoid cedrelanolide (Figure 2) was isolated and identified from the bark of this species (Segura-Correa et al., 1994).

In the present paper, humilinolides A–D and cedrelanolide were examined for their effects on the European corn borer (ECB), *Ostrinia nubilalis* Hübner. *O. nubilalis* is a polyphagous pest of corn, and the economic losses due to this insect on sweet corn in Minnesota alone were estimated to exceed $5,000,000 annually (Noetzel et al., 1985). It also causes damage to potatoes (Stewart, 1994), winter wheat (Buntin 1992), and bell peppers (Fran et al., 1992). Toosendandin was included both as a reference standard and because its effects on ECB had not been examined. Toosendanin at 20 ppm is known to deter feeding by the asiatic corn borer, *Ostrinia furnicalis* (Chiu, 1995). In order to assess



Cedrelanolide

FIG. 2. Cedrelanolide.

the potential for nontarget effects, the cytotoxicity of several of the limonoids to human cell lines was determined.

METHODS AND MATERIALS

Plant Extractions. Swietenia humilis was collected in the state of Guerrero, Mexico, and was identified by Dr. Robert Bye, Jardin Botanico, Instituto de Biologia, UNAM. The air-dried seeds (1 kg) were ground into powder, defatted with hexane, and then exhaustively extracted by maceration with $CHCl_3$ at room temperature. The resulting $CHCl_3$ extract was concentrated under reduced pressure to give a residue (125 g). The concentrated residue was chromatographed on silica gel, eluting with benzene–EtOAc–MeOH using a step gradient of increased polarity to yield three major fractions (FI–FIII). Fraction II, eluting with EtOAc was rechromatographed on silica gel using hexane–EtOAc (mixtures of increasing polarity) as eluents to yield humilinolides B (14.28 mg) and C (29.0 mg). Further column chromatography over silica gel of FIII (eluted with EtOAc–MeOH 7:3) using hexane–EtOAc (6:4) allowed the isolation of humilinolides A (171.4 mg) and D (21.4 mg). In all cases, final purification was achieved by preparative TLC on silica gel using benzene–EtOAc (1:1) as the eluent. IR, MP, and NMR data of the isolated humilinolides A–D (1–4) were identical to those of authentic samples (Segura-Correa et al., 1993). Toosendanin was a gift from Professor Chiu, and cedrelanolide was isolated from *Cedrela salvadorensis* as described previously (Segura-Correa et al., 1994).

Bioassays with ECB. Larvae used for the experiments were obtained from the culture at the University of Ottawa, which was maintained under previously described conditions (Arnason et al., 1987). All test materials were dissolved in 95% ethanol and added to the artificial diet at one of two concentrations (5 or 50 ppm) or control (1 ml 95% ethanol). Neonate larvae were placed collectively for nine days in glass vials containing a cube of the appropriately treated diet. Thirty larvae were then transferred to separate vials with corresponding diet cubes. Larvae were weighed approximately every four days, at which time the old diet was replaced with fresh stock. Larval weight gains and mortality were recorded the last day before the first larva pupated (approximately after 20 days). Other life-cycle measurements were recorded, such as time to pupation and adulthood, weight of pupae and adults, mortality of larvae, and adult deformities. All treatments were effected in a controlled environment chamber with an 18L:6D photoperiod, a 25°C day and 19°C night temperature regime, and a relative humidity of approximately 80%.

Cytotoxicity Assays. Cytotoxicity against human solid tumor cells was measured at the Purdue Cell Culture Laboratory, Purdue Cancer Center, in a seven-day NTT assay for MCF-7 breast carcinoma, HT-29 colon adenocarci-

noma, and A-549 lung carcinoma with adryamicin as the positive control (Anderson et al., 1991).

Data Analysis. Data analyses for all the live insect bioassays were statistically analyzed using SAS ANOVA and GLM procedures. (SAS Institute, 1982).

RESULTS AND DISCUSSION

At the larval stage, all the compounds tested inhibited growth compared to controls when incorporated into diets at 50 ppm (Figures 3 and 4). At 20 days this growth reduction is clearly significant in the 50 ppm group (Table 1), but only humilinolide C, cedrelanolide, and toosendanin showed significant inhibition at 5 ppm (Table 1). When expressed as the percentage of respective controls, toosendanin was the best larval growth inhibitor with humilinolide C producing comparable inhibition (Figure 5). Toosendanin and cedrelanolide at both 5 and 50 ppm induced only moderate larval mortalities ($<37\%$), but the humulinolides generally produced higher mortalities ($>36\%$ at both concentrations).

The percentage of larvae that reached pupation decreased in all tested compound groups in comparison to the control groups (Table 2). The most important

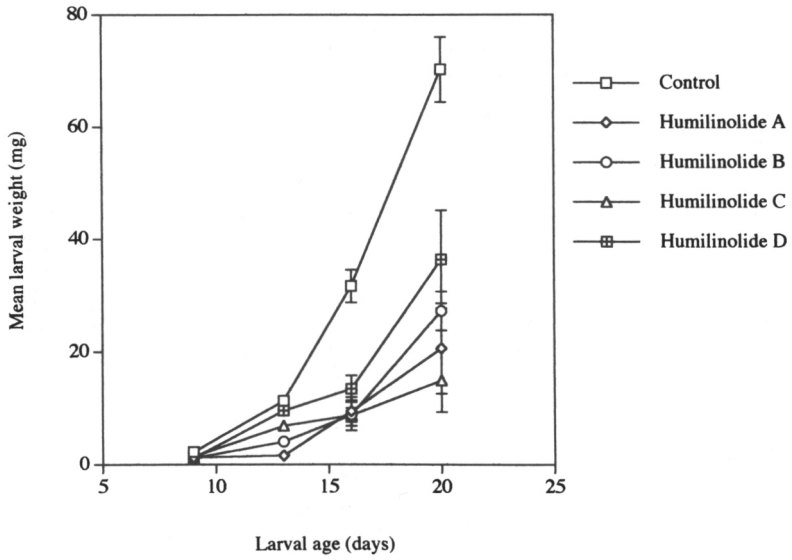


FIG. 3. Growth of ECB larvae fed control diets or diets treated with 50 ppm humilinolides A–D.

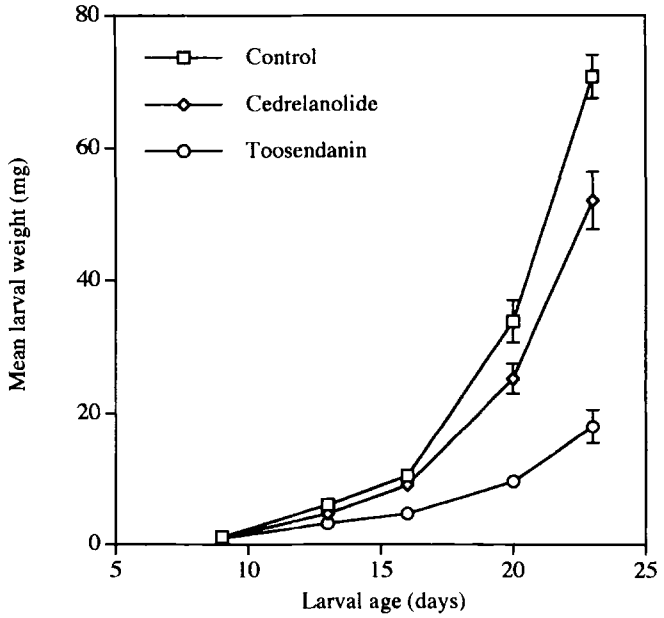


FIG. 4. Growth of ECB larvae fed control diets or diets treated with 50 ppm cedrelanolide or toosendanin.

TABLE 1. ACTIVITY OF LIMINOIDS ON LARVAL GROWTH PARAMETERS OF *O. nubilalis*

Treatment	Concentration (ppm)	Mean weight gained (mg)	Larval mortality (%)
Control	—	69.7 ± 3.2 a[a]	13.3
Cedrelanolide	5	55.7 ± 4.9 b	23.3
	50	41.6 ± 5.9 b	13.3
Toosendanin	5	51.1 ± 4.1 b	26.7
	50	17.0 ± 2.5 c	36.7
Control		68.0 ± 5.7 a	3.3
Humilinolide A	5	55.5 ± 8.0 a, b	36.6
	50	19.6 ± 3.3 b	43.3
Humilinolide B	5	80.1 ± 7.9 a	36.6
	50	25.8 ± 3.4 b	50.0
Humilinolide C	5	36.1 ± 6.4 b	43.3
	50	17.1 ± 5.6 b	50.0
Humilinolide D	5	80.3 ± 6.1 a	40.0
	50	38.8 ± 8.9 b	63.3

[a]Means followed by the same letter within a column, are not significantly different in a Student-Newman-Keuls (SNK) test at $P < 0.05$ (treatments are compared to their respective controls only, e.g., toosendanin is not compared to humilinolide A). Means are ± standard error.

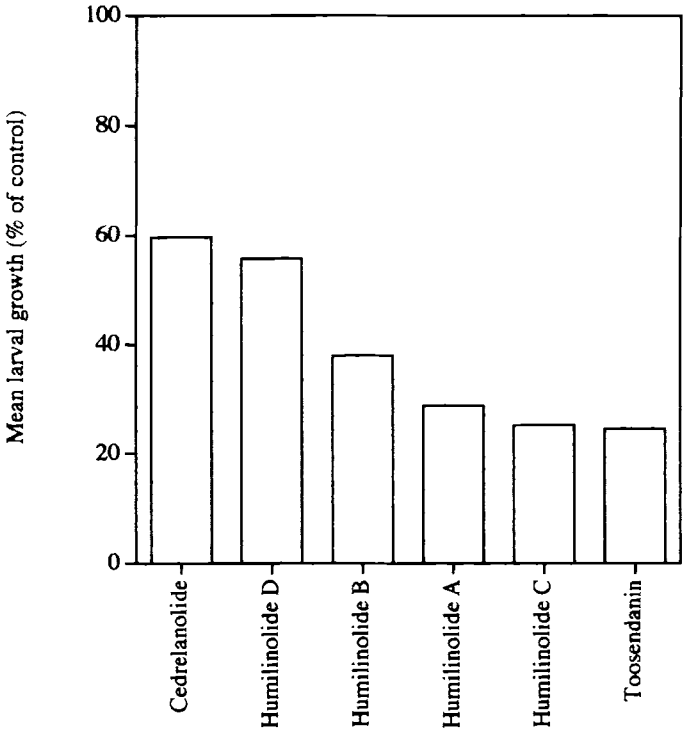


FIG. 5. Growth of ECB larvae reared on treated diets at 20 days, as a percent of respective control.

effect was observed with humilinolides D and C, which at 50 ppm resulted in only 10% and 13% pupation, respectively. Significant delays in time to pupation were observed in 5 ppm cedrelanolide-treated males, 50 ppm treated females, 5 ppm toosendanin-treated males, and both males and females at 50 ppm. Development of males was delayed in 50 ppm treatments with humilinolide B. Of all the limonoids tested, only toosendanin significantly reduced pupal weights of males and females at 50 ppm. In several of the humilinolide C groups there were too few survivors for statistical analysis.

Survival to adult stage, as compared to the pupal stage, showed no further reductions with toosendanin or cedrelanolide (Table 2). However, the humilinolides produced additional mortality and lower survivorship to the adult stage for most compounds and concentrations. Mean adult weights were significantly reduced (male and female) with 50 ppm toosendanin treatments. Significant delays in mean time to the adult stage were seen in many of the treatments.

The cytotoxic activity of humilinolides A–D was determined against three

TABLE 2. EFFECTS OF TWO CONCENTRATIONS OF LIMINOIDS ON DEVELOPMENT OF EUROPEAN CORN BORER LARVAE TO PUPAL AND ADULT STAGES[a]

Compound and concentration (ppm)	Mean time to pupation (days)		Mean pupal weight (mg)		Survival to pupattion (%)	Mean time to adult stage (days)		Mean adult weight (mg)		Survival to adult stage (%)
	Male	Female	Male	Female		Male	Female	Male	Female	
Control	28.6 ± 0.2	28.7 ± 0.8	65.5 a ± 2.1	85.9 a ± 3.5	80.0	36.1 ± 0.3	36.7 ± 0.9	24.9 a ± 1.5	45.4 a ± 2.2	80.0
5 ppm C	29.8 ± 0.5[b]	30.5 ± 0.9	62.2 a ± 2.6	83.0 a ± 1.7	63.3	38.4 ± 0.8[b]	39.1 ± 1.4	21.9 a ± 0.5	42.1 a ± 2.4	63.3
50 ppm C	29.2 ± 0.4	33.4 ± 1.2[b]	61.4 a ± 2.4	77.5 a ± 4.8	60.0	38.7 ± 0.8[b]	41.5 ± 1.2[b]	24.6 a ± 2.4	44.2 a ± 3.0	60.0
5 ppm T	29.8 ± 0.9	31.1 ± 1.0[b]	63.5 a ± 2.1	81.2 a ± 1.0	63.3	39.0 ± 0.9[b]	41.0 ± 1.6	23.0 a ± 1.4	43.0 a ± 1.9	63.3
50 ppm T	33.4 ± 1.3[b]	35.9 ± 1.2[b]	48.9 b ± 3.9	65.7 b ± 4.1	50.0	42.4 ± 1.8[b]	45.8 ± 0.7[b]	17.6 b ± 0.8	34.4 b ± 2.2	50.0
Control	26.6 ± 1.4	28.5 ± 1.6	67.0 a ± 1.4	91.3 b ± 2.1	80.0	34.9 ± 1.1	34.3 ± 1.4	32.9 a ± 1.4	57.0 a ± 2.2	80.0
5 ppm H-A	27.9 ± 1.0	29.0 ± 2.4	64.8 a ± 2.9	86.7 b ± 5.2	56.6	36.1 ± 1.0	38.0 ± 2.7	29.2 a ± 1.3	44.8 b ± 3.1	46.7
50 ppm H-A	31.0 ± 0.0	34.0 ± 1.0	60.1 a ± 0.0	76.8 b ± 5.6	40.0	35.0 ± 0.0	42.0 ± 1.0[b]	28.4 a ± 0.0	36.1 b ± 1.7	13.3
5 ppm H-B	25.4 ± 0.5	25.5 ± 1.6	67.9 a ± 1.0	108.0 a ± 4.0	56.6	33.3 ± 0.6	35.3 ± 1.6	32.4 a ± 0.9	61.5 a ± 0.6	50.0
50 ppm H-B	32.4* ± 1.4	31.5 ± 1.2	74.9 a ± 4.6	87.6 b ± 6.4	46.6	40.8* ± 0.8	38.8 ± 1.3[b]	36.7 a ± 1.9	55.1 a ± 4.6	36.7
5 ppm H-C	26.0 ± 0.0		57.3 a ± 0.0		16.6	33.0 ± 0.0		29.1 a ± 0.0		3.3
50 ppm H-C		37.0 ± 0.0		87.9 b ± 0.0	13.3		44.0 ± 0.0[b]		40.2 a ± 0.0	3.3
5 ppm H-D	23.7 ± 0.6	27.0 ± 0.5	69.3 a ± 1.0	89.1 b ± 2.2	53.3	32.6 ± 0.5	34.0 ± 0.6	29.1 a ± 1.3	48.0 a ± 2.2	50.0
50 ppm H-D	27.0 ± 0.0	31.5 ± 2.5	76.9 a ± 0.0	87.9 b ± 7.5	10.0	37.0 ± 0.0	37.0 ± 0.0	33.3 a ± 0.0	51.5 a ± 4.9	10.0

[a]C = cedrelanolide, T = toosendanin, H-A = Humilinolide A, H-B = Humilinolide B, H-C = Humilinolide C, H-D = Humilinolide D. Means followed by the same letter within a column are not significantly different in a Student-Newman-Keuls (SNK) test at $P < 0.05$ (treatments are compared by concentration, to their respective controls only, e.g., toosedanin is not compared to humilinolide A). Means are ± standard error.

[b]Means within a column are significantly different from control in a Kruskal-Wallis chi-squared approximation test at $P < 0.005$.

TABLE 3. CYTOXIC ACTIVITY OF HUMILINOLIDES A–D TO THREE HUMAN CARCINOMA CELL LINES[a]

	ED_{50} (μg/ml)		
	A-549	MCF-7	HT-29
Humilinolide A	64.4	79.5	59.6
Humilinolide B	>100	>100	81.1
Humilinolide C	37.7	94.1	>100
Humilinolide D	60.6	65.0	53.6
Ardriamycin	7.9×10^{-3}	3.2×10^{-3}	3.5×10^{-2}

[a]A-549, lung carcinoma; MCF-7, breast carcinoma; and HT-20, colon adenocarcinoma.

human solid tumor cell lines, lung carcinoma (A-549), breast carcinoma (MCF-7), and colon adenocarcinoma (HT-29). The tested limonoids showed low but measurable cytotoxic effects at concentrations several orders of magnitude higher than adriamycin (Table 3). While more formal in vivo toxicological assessments are required, these in vitro results are promising in light of the need for a selective insecticide with low mamalian toxicity if further development were to be considered.

The effect of the humilinolides and cedrelanolide on reducing insect growth, increasing development time and mortality of ECB is similar to that of other limonoids (Arnason et al., 1987; Champagne et al., 1992). The mode of action of these compounds is being investigated and may be due to a combination of antifeedant action and postdigestive toxicity, as found for other limonoids (Isman et al., 1995). The activity of these neotropical limonoids is comparable to the commercial insecticide toosendanin, which suggests potential for further development of these materials. However, no neotropical limonoid has been found with the outstanding activity of azadirachtin.

Acknowledgments—This work was partially supported by the following grants to R. Mata: Proyecto DGAPA IN206795 (Dirección General de Asuntos del Personal Académico) and Proyecto PADEP No. 005361 (Coordiantcion General de Estudios Postgraduo, UNAM). A Jiménez also acknowledges a fellowship from Consejo Nacional de Ciencia y Tecnología (CONACyT). Support to J. Arnason was provided by the Natural Sciences and Engineering Research Council of Canada. We are grateful to Dr. J. L. McLaughlin, Purdue University, Lafayette, Indiana, who kindly arranged for the cytotoxicity assays and to N. Donskov, University of Ottawa, for technical work during the insect bioassays. R. Pereda-Miranda was the recipient of a Visiting Researcher Award form the Natural Sciences and Engineering Research Council of Canada and a sabbatical fellowship awarded by DGAPA, UNAM (1994-1995).

REFERENCES

ANDERSON, J. E., GOETZ, C. M., MCLAUGHLIN, J. L., and SUFFNESS, M. 1991. A blind comparison of simple bench-top bioassays and human tumor cell cytotoxicities as antitumor prescreen. *Phytochem. Anal.* 2:107–111.

ARNASON, J. T., PHILOGÈNE, B. J. R., DONSKOV, N., and KUBO, I. 1987. Limonoids from the Meliaceae and Rutaceae reduce feeding, growth and development of *Ostrinia nubilalis*. *Entomol. Exp. Appl.* 43:221–226.

ARNASON, J. T., MACKINNON, S., DURST, A., PHILOGÈNE, B. J. R., HASBUN, C., SÁNCHEZ, P., POVEDA, I., SAN ROMAN, L., ISMAN, M. B., SATASOOK, C., TOWERS, G. H. N., WIRIYACHITRA, P., and MCLAUGHLIN, J. L. 1993. Insecticides in tropical plants with non-neurotoxic modes of action. *Recent Adv. Phytochem.* 27:107–131.

BUNTIN, G. D. 1992. Damage by the European corn borer (Lepidoptera: Pyralidae) to winter wheat. *J. Entomol. Sci.* 27:361–365.

CHAMPAGNE, D. E., ISMAN, M. B., and TOWERS, G. H. N. 1989. Insecticidal activity of phytochemicals and extracts of the Meliaceae, pp. 95–109, *in* Arnason, J. T., B. J. R. Philogène, and P. Morand (eds.). Insecticides of Plant Origin. ACS Symposium 387, American Chemical Society, Washington, D.C.

CHAMPAGNE, D. E., KOUL, O., ISMAN, M. B., SCUDDER, G. G. E., and TOWERS, G. H. N. 1992. Biological activity of limonoids from the *Rutales*. *Phytochemistry* 31:377–394.

CHIU, S.-F. 1995. Experiments on the practical application of chinaberry, *Melia azedarach*, and other naturally occurring insecticides in China, pp. 661–668, *in* H. Schmutterer and K. R. S. Ascher (eds.). Natural Pesticides from the Neem Tree and Other Tropical Plants: Sources of Unique Natural Products for Integrated Pest Management, Medicine and Other Purposes. GTZ, Eschborn, Germany.

FRAN, J. R., SCWARTZ, P. H., JR., and W. E. POTTS. 1992. Modeling the effects of weed interference periods and insects on bell peppers (*Capsicum annum*). *Weed Sci.* 40:308–312.

ISMAN, M. B.; ARNASON, J. T., and TOWERS, G. H. N. 1995. Chemistry and biological activity of ingredients of other species of Meliaceae, pp. 652–666, *in* H. Schmutterer, K. R. S. Ascher, M. B. Isman, M. Jacobson, C. M. Ketkar, M. Kraus, H. Rembold, and R. C. Saxena (eds.). The Neem Tree *Azadirachta indica* A. Juss. and other Meliaceous Plants: Sources of Unique Natural Products for Integrated Pest Management, Medicine and Other Purposes. GTZ, Eschborn, Germany.

MIKOLJCZAK, K. L., and REED, D. K. 1987. Extractives of seeds of the Meliaceae: Effects on *Spodptera frugiperda*, *Acalymma vittatum*, and *Artemia salina*. *J. Chem. Ecol.* 13:99–111.

NOETZEL, D. M., CUTKOMP, L. K., and HAREIN, P. K. 1985. Estimated annual losses due to insects in Minnesota, 1981–1983. University of Minnesota Extension Service Publ. AG-B4-2541.

OKORIE, D. A., and TAYLOR, D. H. A. 1970. Limonoids from *Swietenia humilis*. *Phytochemistry* 10:469.

PENNINGTON, T. D. 1981. Meliaceae. Flora Neotropica. Monograph No. 28. New York Botanical Garden, Brooklyn pp. 389–395.

SAS Institute. 1982. SAS User's Guide: Statistics. SAS Institute, Cary, North Carolina.

SEGURA-CORREA, R., MATA, R., ANAYA, A. L., HERNÁNDEZ, B., VILLENA, R., SORIANO-GARCÍA, R. J., BYE, R., and LINARES, E. 1993. New tetranortriterpenoids from *Swietenia humilis*. *J. Nat. Prod.* 56:1567–1575.

SEGURA-CORREA, R., CALDERON, J., TOSCANA, R., GUTIERREZ, A., and MATA, R. 1994. Cedrelanolide, a new limonoid from *Cedrela salvadorensis*. *Tetrahedron Lett.* 35:3427–3440.

STANDLEY, P. C. 1920–1926. Trees and Shrubs of México, United States National Herbarium, pp. 560.

STEWART, J. G. 1994. Monitoring adult European corn borer (Lepidoptera: Pyralidae) in potatoes on Prince Edward Island. *Environ. Entomol.* 23:1124–1128.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

SCENT MARKING, SCENT ORIGIN, AND SPECIES SPECIFICITY IN MALE PREMATING BEHAVIOR OF TWO SCANDINAVIAN BUMBLEBEES

PETER BERGMAN* and GUNNAR BERGSTRÖM

Department of Chemical Ecology
Botanical Institute, Göteborg University
Carl Skottsbergs Gata 22, S-413 19
Göteborg, Sweden

(Received May 21, 1996; accepted December 16, 1996)

Abstract—The marking behavior and the scent-marking compounds in males of two Scandinavian bumblebee species *Bombus pratorum* and *Bombus lapidarius* were studied. There are characteristic differences in the behavior and in the chemistry of the two species. Males of *B. pratorum* scent marked small areas of the edges of birch leaves in a quick and unpredictable manner, depositing farnesol, geranylgeranyl acetate, farnesyl acetate, (*Z*)-11-octadecanol, hexadecanol, and a pentacosadiene. In *B. lapidarius*, scent marking on birch leaves was much slower and the entire edge of the leaf was marked with (*Z*)-9-hexadecenol and hexadecanol. These compounds were also detected in the cephalic portion of the labial gland in males. Thus, males of *B. lapidarius* produce a blend of fatty acid derivatives while *B. pratorum* males, in addition to fatty acid derivatives, also produce sesqui- and diterpenes. The total amount of scent compounds per milligram of labial glands was 0.2 μg in *B. pratorum* and 40 μg in *B. lapidarius*. Farnesol and farnesyl acetate were detected via headspace collection in the air around leaves marked by *B. pratorum* and (*Z*)-9-hexadecenol was detected around leaves marked by *B. lapidarius*.

Key Words—Labial gland, male mating behavior, marking pheromone, *Bombus pratorum*, *Bombus lapidarius*, Hymenoptera, Apidae, GC-MS, isoprenoids, fatty acid derivatives.

INTRODUCTION

The majority of bumblebee species worldwide (about 300) have annual colonies that originate from a single mated and hibernated queen (Prŷs-Jones and Corbet,

*To whom corespondence should be addressed.

0098-0331/97/0500-1235$12.50/0 © 1997 Plenum Publishing Corporation

1987). After producing workers, the numbers of which depend on the latitude and altitude, the queen devotes her reproductive efforts to producing new queens and males. Individuals from these two sexual castes face different fates. The young queens usually mate only once, with the exception of the species *Bombus hypnorum* (Röseler, 1973; Estoup et al., 1995). The mated queens must find a suitable hibernation site and enough nourishment to survive the hibernation, which in Scandinavia lasts for about seven months. The males do not hibernate, instead dedicating their lifetime of approximately three to four weeks to reproduction. Males of many bumblebee species exhibit a conspicuous premating behavior with two distinct behavioral components: scent marking and patrol flying.

Patrol flying by male bumblebees was first described by Newman (1851) and Darwin (1886). They described how males flew patrol routes and paid regular visits without alighting at special objects (called "buzzing places" by Darwin) in their environment surroundings. The areas in which these patrol routes occurred were similar in successive years and some of the buzzing places were exactly the same from year to year. Sladen (1912) proposed that the patrol-flying bumblebee males emitted a scent at the buzzing places. He thought that it was "extremely likely that in doing so they attract not only one another, but the queens." Another idea put forward by this author was that males from each species emit a species-specific scent that most probably originates from their heads. Later, Haas (1952) suggested that the scent components are produced in the mandibular glands of the bumblebee males. This was questioned by Kullenberg et al. (1973), who claimed that the secretions originate from the cephalic portion of the labial glands.

Since then, several studies have been conducted to describe and explore the scent marking and patrolling behaviors of different European bumblebee species (Frank 1941; Haas, 1946, 1949a, 1967; Krüger, 1951; Kullenberg, 1956, 1973; Kullenberg et al., 1973; Stein, 1956; Awram, 1970; Bringer, 1973; Svensson, 1979a, 1980; Cederberg et al., 1984). Most of these studies have in common the objectives of investigating species differences concerning the kind of habitat for male premating behavior, spatial distribution within habitats, and the kind of objects used for scent marking. The present consensus of premating behavior in bumblebee males is that they start first thing in the morning to scent mark objects in their surroundings. Different bumblebee species scent mark different objects (such as small twigs, leaf edges, grass, tree trunks) at different heights from ground level up to tree tops (Bringer, 1973). After repeated scent marking for 2–3 hr, an individual male-specific flight route has been established. This route will then be patrolled during the rest of the day, and in fact, at least parts of it will be used during the whole life of the male. During patrol flying the male flies in a regular manner and in the same direction along the route and

visits, now without alighting, the earlier repeatedly scent-marked sites. The scent markings are thought to attract unmated, young queens and are thus likely to be important for species recognition and in avoiding interspecific mating (Svensson, 1980). Furthermore, the scent is an important guidepost for the male himself early in the day before the flight path has been memorized visually (Awram, 1970).

There are 29 species of *Bombus* (Løken, 1973; Svensson, 1979b) and 9 species of *Psithyrus* (Løken, 1984; Andersson, 1992) in Scandinavia. So far, the chemical composition of labial gland secretion has been analyzed in all but one species of *Bombus*, i.e., *Bombus ruderatus* (Bergström et al., 1973, 1981; Cederberg et al., 1984; Svensson et al., 1984; Lanne et al., 1987; Appelgren et al., 1991; Bergman et al., 1996). All these chemical analyses are based on labial gland preparations. However, the compounds males actually deposit during scent-marking behavior is known only for *B. hortorum* (Kullenberg et al., 1973).

The two bumblebee species used in this study, *Bombus pratorum* (L.) and *Bombus lapidarius* (L.), belong to the subgenera *Pyrobombus* and *Melanobombus*, respectively. While males of *B. pratorum* emerge early in the season in June and July, males of *B. lapidarius* emerge later in the season in July and August. The objectives of the present study were: (1) to compare the scent-marking behavior of these two species; (2) to describe qualitatively and quantitatively the compounds deposited during scent marking by males of each species; (3) to compare the deposited compounds, also qualitatively and quantitatively, with those found in the male labial gland; and (4) to detect the deposited substances in the air around marked sites.

METHODS AND MATERIAL

Study Site

The field work was conducted between July 7 and August 14, 1993, at the Vilanda field station, 100 km east of Göteborg in southwest Sweden. Patrol routes established in nature can be several hundred meters long (Bringer, 1973). It was therefore necessary to provide the bumblebees with a restricted area where their natural behavior could be observed continuously. For this purpose two greenhouses ($3.5 \times 2 \times 2$ m for each) were used, one for keeping bumblebee males and the other for the experiments.

Bumblebee males used in the study were collected in the wild, either close to the study area or in the Göteborg Botanical Garden. After three days of

acclimatization in the greenhouses, the bumblebee males exhibited their normal premating behavior. Fresh branches of birch (*Betula verrucosa* Ehrh., Betulaceae) were collected daily and placed in water bottles inside the greenhouses in order to provide the males with a natural substrate to scent mark. Because no preferences for certain tree species by the bumblebees are known, birch was chosen because it is abundant and available in the study area. Leaves and small branches were readily used as marking places by the bumblebee males. The bumblebees were provided diluted honey–water (50%) in small, blue and yellow pieces of plastic boxes (LEGO) at a specific site in the greenhouse.

Experimental Design

Usually, four to six acclimatized males were kept overnight in one of the greenhouses. At 7:30 hr next morning, before the males started their marking behavior, all eight birch branches were replaced by new ones. To reduce the risk of contamination from wild bumblebees outside the greenhouses, the new birch branches had been collected in a dense forest in which no bumblebee males were ever observed. When one male had started to scent mark, all other males were caught in a net and removed from the greenhouse. A piece of thread was placed on the petiole of every leaf the male scent marked. All leaves were collected with a pair of tweezers. As controls, unmarked leaves from the same branches were collected. When the male had marked 10 leaves, it was captured and put in liquid nitrogen (−196°C) for later analysis of the remaining contents of its labial glands; the glands were dissected from the heads and subjected to extraction. In this way it was possible to compare the chemicals deposited on the leaves with the chemicals found in the glands of individual males. A total of 22 *B. pratorum* and 10 *B. lapidarius* males were used as described above.

Ten marked and 10 unmarked leaves were placed separately in two glass containers for 9 hr and subjected to headspace adsorption as follows. The glass containers had a charcoal filter at one end and the exhaustion end was connected to a pump. Teflon tubes (4.5 cm long, 3 mm in diameter) packed with Porapak Q (mesh size 80/100, Supelco Inc.) were placed between the pump and the sample container. The total volume of each glass container was 250 cm^3 and the airflow was 125 cm^3/min. After 3 hr, the two tubes were replaced with new ones that were used for the last 6 hr of adsorption. Finally, after 9 hr, each leaf was washed separately with 1.0 ml of diethyl ether. This was done by holding the leaves over a glass funnel while the diethyl ether was applied with a pipet and allowed to drop down the funnel. The tubes were then stored in liquid nitrogen until analysis. In addition, 10 marked and 10 unmarked leaves were washed with 1.0 ml of diethyl ether each without being subjected to headspace collection. In total, 14 *B. pratorum* and 10 *B. lapidarius* males were used.

Sample Preparation

The experimental design outlined above gave four different types of samples: gland samples—labial glands from the bumblebee males; wash samples—solutions from the washed leaves after they had been subjected to 9-hr of headspace adsorption; instant wash samples—solutions from the instantly washed leaves; and headspace samples—tubes with volatiles adsorbed on Porapak.

Prior to the chemical analysis by GC-MS, the different samples were treated as follows:

Gland Samples. The labial glands in *Bombus* and *Psithyrus* consist of a thoracic and a cephalic part, and both parts have a left and a right portion. The thoracic part of the labial gland produces saliva and the cephalic part produces the chemicals used by the males in their scent-marking behavior (Ågren, 1979). The two portions of the cephalic labial gland each resemble a bunch of grapes and are most easily removed from the head when frozen. One of the two frozen cephalic labial portions was randomly chosen and dissected from the head of each male. Each gland was weighed and put into a 0.3 ml Reacti-Vial chilled with dry ice (−78°C). Thereafter, 150 μl diethyl ether containing 50 ng/μl pentadecanol as quantification standard was added to the Reacti-Vial, and the vials were put in an ultrasonic bath for 15 min at room temperature. After ultrasonic treatment, 100 μl of the total extract was transferred to a new vial and analyzed.

Wash and Instant Wash Samples. Both types of wash samples originally had a volume of about 8 ml, i.e., the remaining volume after 10 leaves had been washed with 1 ml each of diethyl ether. Pentadecanol was added as an internal standard (6.25 μg for *B. pratorum* and 12.5 μg for *B. lapidarius*), and the samples were then concentrated by evaporation at ambient temperature (about 20°C) until the volumes had been reduced to 250 μl.

Headspace Samples. Prior to use, all tubes containing the adsorbent medium were conditioned by washing with 2 ml methanol, 2 ml diethyl ether, and 2 ml pentane successively and then by drying with nitrogen gas for about 5 min. After conditioning, as well as after use for headspace adsorption, the tubes were wrapped in aluminum foil (max. for two weeks) and stored in a freezer at −20°C. The volatiles in the adsorbent medium were extracted with 600 μl of diethyl ether. Pentadecanol was added as a quantification standard (6.25 μg) to 250 μl of this extract, which then was concentrated to 100 μl by evaporation at ambient temperature (about 20°C).

Chemical Analysis

All chemical analyses were made by combined gas chromatography–mass spectrometry (GC-MS) on a HP 5890 gas chromatograph (GC) connected to a

HP mass selective detector (MS). The GC was equipped with a CP-Sil-5 column (Chrompack) or an OV-351 column (GeneTec), both 30 m × 0.25 mm. Helium was used as the carrier gas at a constant flow 0.7 ml/min (30 cm/sec) at 50°C. The injector temperature was 220°C. After sample injection, the GC oven was kept at 50°C for 2 min and the temperature was then increased 10°C/min to 240°C. The maximum temperature was kept for 40 min.

Volatile compounds were identified by comparing chromatographic retention values and mass spectral data with those of reference compounds.

Quantification and Statistical Analysis

Data in the form of proportions or percentages were transformed (arcsine transformations) in order to achieve a normal distribution from which means and standard errors (SE) were calculated (Zar, 1984). The percentage values were transformed before calculating the means and SE, and then transformed back to percentages. The absolute amounts of identified compounds were obtained by comparison to individual standard curves. Means were compared using the nonparametric Mann-Whitney *U* test.

RESULTS

Scent-Marking Behavior

Birch leaves were used as marking objects by males of both species. The scent was applied as the male alighted on the leaf and walked along the edge holding it between the mandibles. A prominent difference was found between males of the two species regarding scent-marking behavior. *Bombus pratorum* males scent marked leaves all over the birch branches, and it was not possible to predict the next site to be marked. Some individuals also flew at ground level and used grass for marking, while still others scent marked parts of the greenhouse itself. In contrast, *B. lapidarius* males showed high consistency in their selection of scent-marking sites. They only scent marked the uppermost leaves and twigs on the branches, which would correspond to the top shoots of trees used by this species in nature. Furthermore, the *B. pratorum* males were hasty in their marking behavior. They were more likely to mark only a part (a quarter or half) of a leaf edge before leaving it, either to choose another leaf nearby or to fly to one of the other birch branches. The opposite was the case in *B. lapidarius* males; when they started to scent mark the edge of one leaf, they most often continued to mark it all around, and by following the twig, also scent marked the neighboring leaves. While on the leaf, the actual scent marking was done in a slower manner compared to *B. pratorum* males.

Gland Samples

GC-MS analyses of the labial gland secretion of *B. pratorum* and *B. lapidarius* showed that the two species had compound mixtures that were qualitatively distinct from each other (Figure 1). All the compounds found with a mean percentage of $>0.1\%$ of the total were characterized (Table 1). The main compounds in *B. pratorum* were the three isoprenoids, farnesol, geranylgeranyl acetate, and farnesyl acetate, followed by three fatty acid derivatives, (*Z*)-11-octadecenol, hexadecanol, and a pentacosadiene. In *B. lapidarius* the two main

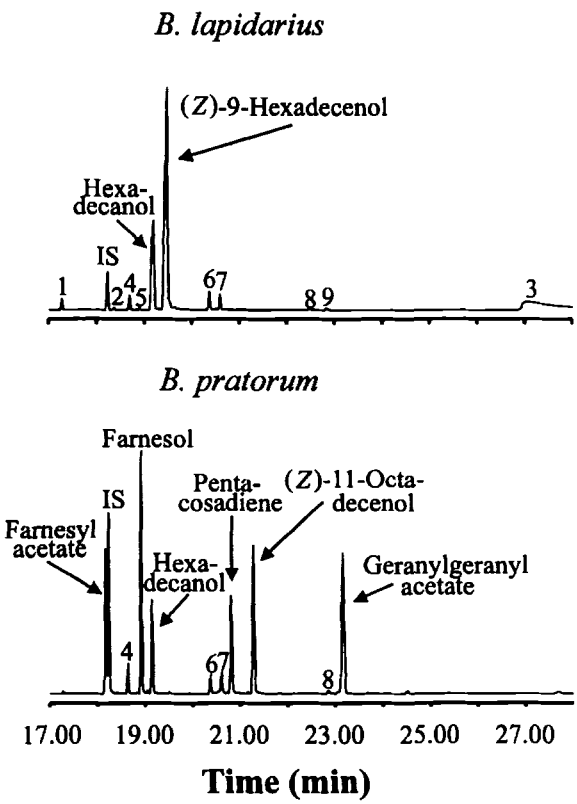


FIG. 1. Gas chromatograms of the cephalic labial gland from individual males of *B. lapidarius* and *B. pratorum*, respectively. Names of major compounds are given in the diagram while smaller peaks are identified by their peak numbers given in Table I. Same peak number in both species is applied to identical compounds. IS = internal standard, pentadecanol, added prior to analysis.

TABLE 1. AMOUNT AND INDIVIDUALS (N) WITH DETECTABLE AMOUNTS OF COMPOUNDS FOUND IN LABIAL GLAND SECRETION OF 12 *B. lapidarius* AND 22 *B. pratorum* MALES[a]

Compound	*B. lapidarius* Relative amount (%) Mean	Range[b]	N	*B. pratorum* Relative amount (%) Mean	Range[b]	N
Farnesol				22.3	15.6–26.2	22
Farnesyl acetate				10.8	4.2–16.9	22
Geranylgeranyl acetate				24.0	18.9–34.5	22
Tetradecanol (1)	0.8	0.5–1.2	12			
Hexadecanol	22.9	18.7–30.1	12	10.0	7.2–13.5	22
(Z)-9-Hexadecenol	52.7	43.0–66.8	12			
(Z)-11-Octadecenol				15.0	12.7–18.6	22
(Z)-9-Ethyl hexadecenoate (2)	0.4	0.1–1.3	10			
Hexadecenoic acid (3)	11.4	10.3–27.1	9			
Tricosane (4)	1.3	0.1–3.1	12	2.0	1.4–4.0	21
Tricosene (5)	0.1	0.1–0.5	8			
Pentacosane (6)	1.5	0.8–2.9	12	1.9	1.3–5.9	21
Pentacosene (7)	1.2	0.7–2.0	12	1.7	1.2–2.7	21
Pentacosadiene				8.4	4.8–14.7	22
Heptacosane (8)	0.1	0.1–0.3	6			
Heptacosene (9)	0.1	0.1–0.4	6	0.2	0.3–0.8	15

[a]The first three compounds are isoprenoids, while the other are all fatty acid derivatives. The number in parentheses after a compound name corresponds to the minor peak number in the chromatograms in Figure 1.

[b]The minimum amounts are from those males in which the compound was detected.

compounds, (Z)-9-hexadecenol and hexadecanol, are fatty acid derivatives. The most prominent difference between the two species was the presence of three isoprenoids in the labial gland secretion of *B. pratorum* males. The secretions in both species contained straight-chain saturated or monounsaturated hydrocarbons ranging from 23 to 27 carbon atoms. One exception was the large amount of the pentacosadiene present only in *B. pratorum*.

The absolute amount per milligram gland tissue for the main compounds in the two species are shown in Figure 2. Note that the labial gland in *B. lapidarius* males contain a much larger total amount of secretion, approximately 40 μg/mg gland compared to 0.2 μg/mg gland in *B. pratorum*. The average weight of the labial glands used were 1.3 ± 0.2 mg in *B. lapidarius* (mean ± SE, $N = 12$) and 1.7 ± 0.2 mg in *B. pratorum* ($N = 22$). The difference was not significant (Mann-Whitney U test, $P = 0.16$).

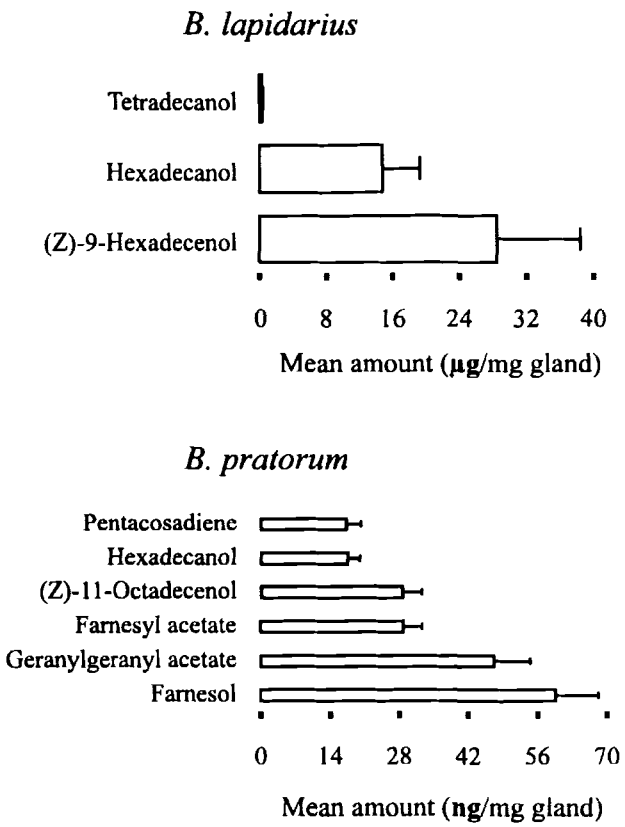


FIG. 2. Mean amount and upper standard error value for major compounds found in the labial gland secretions of *B. lapidarius* and *B. pratorum* males. Note that the amounts are in micrograms for *B. lapidarius* and nanograms for *B. pratorum*.

Wash and Instant Wash Samples

The birch leaves themselves gave a considerable background in the gas chromatograms from both kinds of wash samples (Figure 3). However, labial gland compounds from both bumblebee species were found in samples from marked leaves but not in samples with unmarked leaves. In *B. lapidarius* the two main compounds, (Z)-9-hexadecenol and hexadecanol, were found in amounts large enough to permit quantitative analysis (Figure 4). In both kinds of washes, the ratios between the compounds were approximately the same as the ratios found in the gland secretion; in the gland samples the mean ratio

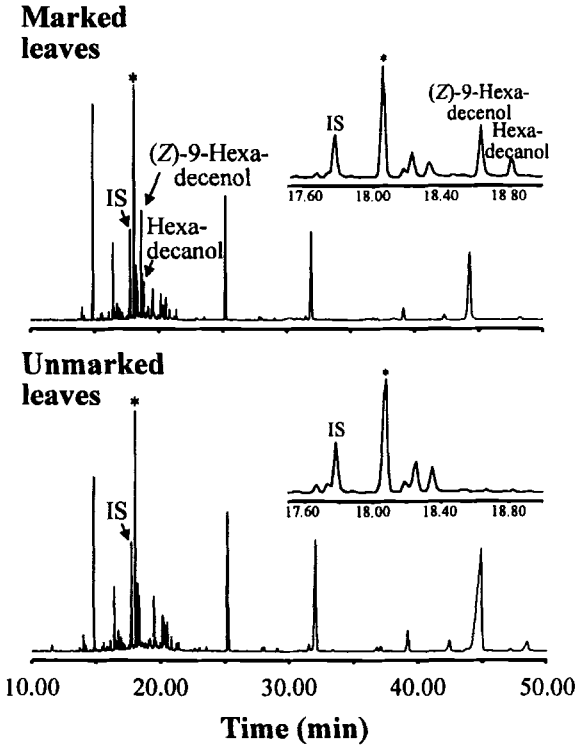


FIG. 3. Part of two chromatograms (ranging from 10 to 50 min) showing a sample of 10 birch leaves marked by a *B. lapidarius* male and a sample of 10 control leaves (unmarked) from the same birch. An inserted and enlarged part of each chromatogram shows the presence of two labial gland compounds, (Z)-9-hexadecenol and hexadecanol, in the sample with marked leaves. The internal standard is denoted IS and the asterisk denotes a compound from the birch to aid orientation in the insert.

between the amounts of hexadecanol and (Z)-9-hexadecenol was 0.53 (Figure 2), while the ratios were 0.58 and 0.56 for wash and instant wash samples, respectively (Figure 4). Furthermore, the mean amounts were larger for the two compounds in the instant wash samples compared to those in the wash made after 9 hr of headspace sampling. However, the differences were not statistically significant (Mann-Whitney U test, $P = 0.18$ and $P = 0.08$ for (Z)-9-hexadecenol and hexadecanol, respectively).

All the wash samples of both types from *B. pratorum* contained trace amounts of the following five labial gland compounds: farnesol, geranylgeranyl acetate, farnesyl acetate, (Z)-11-octadecenol, and hexadecanol. None of these

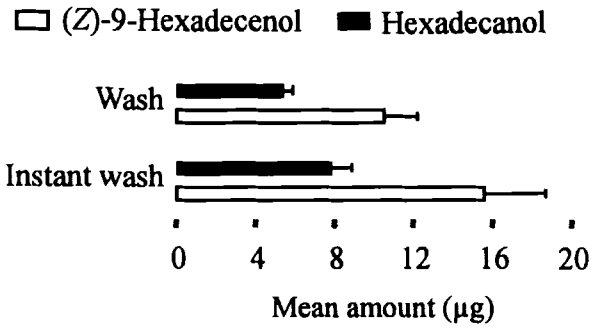


FIG. 4. Mean amount and upper standard error value for compounds deposited on leaves by *B. lapidarius* males. Mean ratio between the amounts of hexadecanol and (Z)-9-hexadecenol were 0.58 for wash and 0.56 for instant wash compared with 0.53 in the labial gland. Shaded bars correspond to hexadecanol and open bars to (Z)-9-hexadecenol.

five compounds were present in any of the control samples with unmarked leaves. As a consequence of the small amounts found and the fact that peaks representing labial gland compounds overlapped with background peaks from the birch leaves, no attempt was made to quantify these components.

Headspace Samples

Only trace amounts of compounds present in the labial gland were found in the air surrounding marked birch leaves. In *B. lapidarius* the main component of the labial gland, (Z)-9-hexadecenol, was detected in 17 of the 20 samples (85%). In the three cases where this compound was not detectable, all samples were from the first 3 hr of headspace sampling. In the 44 samples of *B. pratorum*, 34 (77%) contained farnesol and 32 (73%) contained farnesyl acetate. A few of the samples were strong enough to permit a relative comparison between the two compounds; approximately twice as much farnesyl acetate as farnesol was present. Thus, for *B. pratorum* its main labial gland component (farnesol) together with the compound present in third largest amount (farnesyl acetate) could be detected in these samples. In contrast, the compound in second largest amount, geranylgeranyl acetate, was not detected.

DISCUSSION

The labial gland secretions in the two species of bumblebees studied here consist of species-specific blends. This is a feature found in all the earlier analyses of 37 species and two forms of Scandinavian bumblebees (Bergström

and Svensson, 1973; Bergström et al., 1973; Svensson and Bergström, 1977, 1979; Cederberg et al., 1984; Applegren et al., 1991; Bergman et al., 1996) as well as in three North American species (Bergström et al., 1996). The most conspicuous difference between the two species is that *B. pratorum* uses two different biosynthetic pathways to produce the labial compounds and *B. lapidarius* uses only one of them (Figure 5). The three isoprenoids in *B. pratorum* originate from the mevalonic acid pathway, while fatty acid derivatives, such as the pentacosadiene (*B. pratorum*), hexadecanol (both species), and (*Z*)-9-hexadecenol (*B. lapidarius*), originate from the polyketide pathway (Torssell, 1983).

Several straight chain hydrocarbons containing an odd number of carbon atoms ranging from 23 to 27 were found in the two species currently investigated. Such hydrocarbons have been reported in all previous analyses of male labial secretion in bumblebees (Bergström et al., 1973; Ågren et al., 1979; Bergström et al., 1981; Svensson et al., 1984; Cederberg et al., 1984; Lanne et al., 1987; Applegren et al., 1991). In a comparative study of five bumblebee

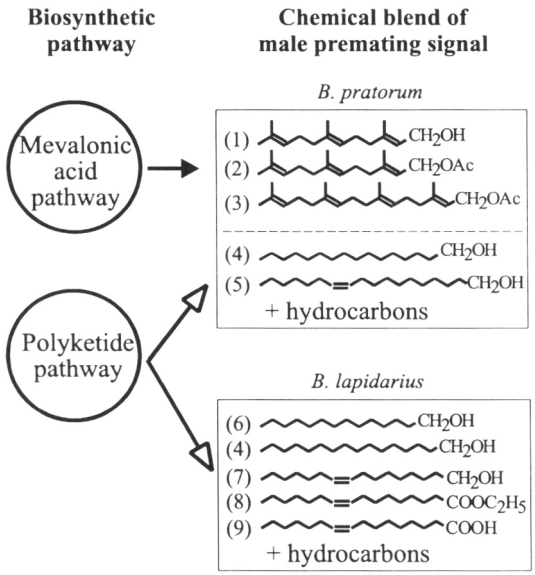


FIG. 5. Schematic representation of the two biosynthetic pathways leading to species-specific premating signals. Note that *B. pratorum* uses two different pathways to produce its labial gland blend while *B. lapidarius* uses only one. (1) Farnesol; (2) farnesyl acetate; (3) geranylgeranyl acetate; (4) hexadecanol; (5) (*Z*)-11-octadecenol; (6) tetradecanol; (7) (*Z*)-9-hexadecenol; (8) (*Z*)-9-ethyl hexadecenoate; (9) (*Z*)-9-hexadecenoic acid (tentative position and geometry of the unsaturation).

species including both *B. pratorum* and *B. lapidarius*, Oldham et al. (1994) demonstrated that the outer cuticular surface is covered by a mixture of compounds comprised mainly of the same straight-chain hydrocarbons as those found in the labial gland. The presence of hydrocarbons in the gland samples is most likely due to the fact that the labial gland is derived from epidermal tissue (Snodgrass, 1956). However, we believe that the straight-chain hydrocarbon pentacosadiene present in *B. pratorum* is produced in the labial gland for communication purposes in the scent marks. The reasons for this are that pentacosadiene is produced in larger amounts compared to the other hydrocarbons. Furthermore, pentacosadiene has not been found in any of the previous analyses mentioned above, nor was it found in the mixture covering the cuticle in five bumblebee species (Oldham et al., 1994).

The analyses of labial gland secretion in *B. pratorum* and *B. lapidarius* in this study agree well with findings from previous studies (Svensson and Bergström, 1977; Bergström et al., 1981). This is, however, the first time quantitative data are presented for these two species, revealing an approximate 200-fold difference in total amount of labial compounds between the two species. Although males of *B. lapidarius* are larger than *B. pratorum* males, this difference in body size cannot explain the difference in total amounts. In fact, the labial gland of *B. pratorum* weighed just as much as that of *B. lapidarius*. It is more likely that the difference reflects a disparity in the premating behavior of the two species. One such factor may be the aboveground height of objects that the males use for scent marking and subsequently patrol. While *B. lapidarius* is known to use the treetop level for patrolling, males of *B. pratorum* exhibit their premating behavior at ground to bush level (Haas, 1949a; Krüger, 1951; Bringer, 1973). The wind and solar radiation are usually stronger at treetop level, and this may call for larger amounts of scent compounds in order to keep the signaling effective and persistent throughout the day. Another factor could be that by depositing more, *B. lapidarius* compensates for the lower volatility of the main marking compound compared to the more volatile ones of *B. pratorum*. Further comparative studies of different bumblebee species are needed to assess the magnitude of variation in the amount of secretion and to properly evaluate the biological consequences of this variation.

In the past, two different glands have been proposed to be the origin of the compounds used by the bumblebee males for scent-marking: the mandibular gland (Haas, 1952) and the cephalic part of the labial gland (Kullenberg et al., 1973; Ågren et al., 1979). The present study strongly supports the cephalic portion of the labial gland as the source for the scent-marking compounds in bumblebee males. The main labial compounds in both species occur on the leaves scent marked by males and, at least in *B. lapidarius*, also in the same ratio as they do in the gland. Furthermore, although all wash samples were searched for compounds other than those known from the labial gland, no such

compounds were found. For instance, one might think that some other head gland, such as the mandibular glands, also would contribute to the scent markings. The specific case with the mandibular gland can be ruled out for *B. lapidarius* since the chemical content of the mandibular gland was studied by Cederberg (1977). He showed that the mandibular gland in all castes and sexes mainly contained butyric acid with the two ketones, 2-pentanone and 2-heptanone. Butyric acid was found to be the main component in the mandibular secretion of eight *Bombus* and two *Psithyrus* species investigated. Not even a trace of these compounds was found in any of the wash samples in the present study. There is, however, a small possibility that compounds originating from a gland other than the labial gland were present in minor amounts after all but were not noticed because they may have been masked by background compounds from the birch leaves themselves. In order to determine if this is the case or not, it would be interesting to investigate scent marks deposited by bumblebee males on some kind of inert substrate. A possible method could be to allow bumblebee male to scent mark artificial leaves made of glass.

Scent marks made by bumblebee males are thought to work as a pre-copulatory attracting signal to make contact with females (Svensson, 1980). A virgin queen ready for mating is guided by the scent to a marked place, where she alights and waits for the male to appear. In addition, bumblebee males only seem to respond to queens as long as they meet each other at a scent-marked site (Awram, 1970; Free, 1971). In both *B. pratorum* and *B. lapidarius* the compounds deposited on marked leaves were detectable from the surrounding air, thus lending support for the attract-from-a-distance hypothesis. It is interesting to compare the Scandinavian bumblebee premating system with that of *Xylocopa* species, carpenter bees in the family Anthophoridae. Male *Xylocopa* spp. exhibit a variety of premating behaviors (McAuslane et al., 1990), including a lek polygyny system in which scent marking at certain landmarks is an important behavioral component. Minkley et al. (1991) showed that the two main compounds in the thoracic gland secretion from males of *X. varipuncta* worked as long-range attractants on conspecific females. The two compounds are the isoprenoids, *trans*-geranylgeraniol and *trans*-farnesal, while *X. micheneri* only contained fatty acid derivatives (Andersen et al., 1988). Both classes of compounds that constitute the labial gland secretion in Scandinavian bumblebee species are present also in *Xylocopa* species. Furthermore, since these chemical signals can act as long-range attractants in the *Xylocopa* premating system it does not seem too farfetched that the same is possible in the *Bombus* system.

It is interesting to note that *B. pratorum* together with several other species of bumblebees also synthesize heavier diterpenes, such as geranylgeranyl acetate, apart from relatively volatile compounds. None of the headspace samples from this species contained geranylgeranyl acetate, while the two sesquiterpenes, farnesol and farnesyl acetate, were found on average in 75% of all samples.

This makes sense since the vapor pressure of geranylgeranyl acetate is about 20 times less than the two sesquiterpenes [approximated from Jordan (1954)]. One can speculate that the larger molecules, i.e., the less volatile compounds, such as the diterpenes and the larger fatty acid derivatives, could be important for close-range orientation and possibly for maintaining scented guideposts even in sunny and/or windy weather. It is likely that these less volatile compounds remain detectable at least until the next day. These compounds may then help the bumblebee male to reconstruct the previous day's flight path, which is then reinforced by new scents (Awram, 1970).

CONCLUSIONS

The bumblebee males of *B. pratorum* and *B. lapidarius* differ in their scent-marking behavior in at least two aspects: the height above ground of the objects marked and the thoroughness with which the markings are done. Due to the small amount of scent compounds in the labial glands of *B. pratorum*, about 200 times less than in *B. lapidarius*, together with their very hasty marking behavior, this species deposits far less than *B. lapidarius*. Compounds deposited during scent marking by males of each species differ qualitatively as well as quantitatively. In both species the compounds deposited are the same as those found in their cephalic labial glands. In *B. lapidarius* at least one and in *B. pratorum* a few of the marking compounds are detectable in the air around marking sites. There should no longer be any doubt that the compounds originating from the cephalic part of the male labial gland are those used for scent marking and most likely are important as signals in the premating behavior of Scandinavian bumblebees.

Acknowledgments—We are much indebted to Solveig Svensson for assistance in the field and Monica Appelgren for preparation of the samples prior to analysis. Thanks are also due to Hans Alborn, Susanna Andersson, Heather McAuslane, Mikael Stenström and Lennart Ågren for criticism of an earlier draft of the manuscript. Heather McAuslane kindly improved the language. The work was financially supported by the Swedish Natural Science Research Council, the Royal Society of Arts and Sciences (Göteborg) and the Wilhelm and Martina Lundgren Science Foundation.

REFERENCES

ÅGREN, L., CEDERBERG, B., and SVENSSON, B. G. 1979. Changes with age in ultrastructure and pheromone content of male labial glands in some bumblebee species (Hymenoptera, Apidae). *Zoon* 7:1-14.

ANDERSEN, J. F., BUCHMANN, S. L., WEISLEDER, D., PLATINER, R. D., and MINCKLEY, R. L. 1988. Identification of thoracic gland constituents from male *Xylocopa* spp. (Hymenoptera: Anthophoridae) from Arizona. *J. Chem. Ecol.* 14:1153-1162.

ANDERSSON, H. 1992. The cuckoo bumblee bee *Psithyrus versalis* (Geoffroy) (Hymenoptera,

Apidae) lives and flourishes in Sweden, Scania. *Entomol. Tidskr.* 113:42–43 (in Swedish with English summary).

APPELGREN M., BERGSTRÖM, G., SVENSSON, B. G., and CEDERBERG B. 1991. Marking pheromones of *Megabombus* bumble bee males. *Acta Chem. Scand.* 45:972–974.

AWRAM, W. J. 1970. Flight route behaviour of bumblebees. PhD dissertation. University of London, London.

BERGMAN, P., BERGSTRÖM, G., and APPELGREN, M. 1996. Labial gland marking secretion in males of two Scandinavian cuckoo bumblebee species. *Chemoecology* 7:140–145.

BERGSTRÖM, G., and SVENSSON, B. G. 1973. Studies on natural odoriferous compounds VIII. Characteristic marking secretions of the forms *lapponicus* and *scandinavicus* of *Bombus lapponicus* Fabr. (Hymenoptera, Apidae). *Chem. Scr.* 4:231–238.

BERGSTRÖM, G., KULLENBERG B., and STÄLLBERG-STENHAGEN, S. 1973. Studes on natural odoriferous compounds VII. Recognition of two forms of *Bombus lucorum* L. (Hymenoptera: Apidae) by analysis of the volatile marking secretion from individual males. *Chem. Scr.* 4:174–182.

BERGSTRÖM, G., SVENSSON, B. G., APPELGREN, M., and GROTH, I. 1981. Complexity of bumble bee marking pheromones: biochemical, ecological and systematical interpretations, pp. 175–183, *in* P. E. Howse and J.-L. Clément (eds.). Biosystematics of Social Insects. Academic Press, London.

BERGSTRÖM, G., BERGMAN, P., APPELGREN, M., and SMITH, J. O. 1996. Labial gland chemistry of three species of bumblebees (Hymenoptera, Apidae) from North America. *Bioorg. Med. Chem.* 4:515–519.

BRINGER, B. 1973. Territorial flight of bumble-bee males in coniferous forest on the northernmost part of the island of Öland. *Zoon Suppl.* 1:15–22.

CEDERBERG B. 1977. Chemical basis for defense in bumble bees. *Proc. 8th Int. Congr. I.U.S.S.I.* 1977:77.

CEDERBERG B., SVENSSON, B. G., BERGSTRÖM, G., APPELGREN M., and GROTH, I. 1984. Male marking pheromones in north European cuckoo bumble bees, *Psithyrus* (Hymenoptera, Apidae). *Nova Acta Regiae Soc. Sci. Ups., Ser. V:C* 3:161–166.

DARWIN, C. 1886. Über die Wege der Hummelmännchen, pp. 84–88, *in* E. Krause (ed.). Gesammelte Schriften von Charles Darwin, Vol. 2. Leipzig.

ESTOUP, A., SCHOLL, A., POUVREAU, A., and SOLIGNAC, M. 1995. Monoandry and polyandry in bumble bees (Hymenoptera; Bombinae) as evidenced by highly variable microsatellites. *Mol. Ecol.* 4:89–93.

FRANK, A. 1941. Eigenartige Flugbahnen bei Hummelmännchen. *Z. Vergl. Physiol.* 28:467–484.

FREE, J. B. 1971. Stimili eliciting mating behaviour of bumble-bee (*Bombus pratorum* L.) males. *Behavior* 40:55–61.

HAAS, A. 1946. Neue Beobachtungen zum Problem der Flugbahnen bei Hummelmännchen. *Z. Naturforsch.* 1:596–600.

HAAS, A. 1949a. Arttypische Flugbahnen von Hummelmännchen. *Z. Vergl. Physiol.* 31:281–307.

HAAS, 1949b. Gesetzmässiges Flugverhalten der Männchen von *Psithyrus silvestris* Lep. und einiger solitärer Apiden. *Z. Vergl. Physiol.* 31:671–683.

HAAS A. 1952. Die Mandibeldrüse als Duftorgan bei einigen Hymenopteren. *Naturwissenschaften* 39:484.

HAAS, A. 1967. Vergleichende Verhaltensstudien zum Paarungsschwarm der Hummeln (*Bombus*) und Schmarotzerhummeln (*Psithyrus*). *Z. Tierpsychol.* 24:257–277.

JORDAN, T. E. 1954. Vapour Pressure of Organic Compounds. Interscience Publishers, London.

KRÜGER, E. 1951. Über die Bahnflüge der Männchen der Gattungen *Bombus* und *Psithyrus* (Bombidae Hymenopt.). *Z. Tierpsychol.* 8:61–75.

KULLENBERG, B. 1956. Field experiments with chemical sexual attractants on aculeate Hymenoptera males. *I. Zool. Bidr. Upps.* 31:253–352.

KULLENBERG, B. 1973. Field experiments with chemical attractants on aculeate Hymenoptera males. II. *Zoon Suppl.* 1:31–42.

KULLENBERG, B., BERGSTRÖM, G., BRINGER, B., CARLBERG B., and CEDERBERG B. 1973. Observations on scent marking by *Bombus* Latr. and *Psithyrus* Lep. males (Hym., Apidae) and localization of site of production of the secretion. *Zoon Suppl.* 1:23–30.

LANNE, B. S., BERGSTRÖM, G., WASSGREN A.-B., and TÖRNBÄCK B. 1987. Biogenetic pattern of straight chain marking compounds in male bumble bees. *Comp. Biochem. Physiol. B. Biochem* 88:631–636.

LØKEN, A. 1973. Studies of Scandinavian bumblebees (Hymenoptera, Apidae). *Nor. Entomol. Tidsskr.* 20:1–218.

LØKEN, A. 1984. Scandinavian species of the genus *Psithyrus* Lepeletier (Hymenoptera: Apidae). *Entomol. Scand. Suppl.* 23:1–45.

MCAUSLANE, H. J., VINSON, S. B., and WILLIAMS H. J. 1990. Change in mandibular and mesosomal gland contents of male *Xylocopa micans* (Hymenoptera: Anthophoridae) associated with mating system. *J. Chem. Ecol.* 16:1877–1885.

MINCKLEY, R. L., BUCHMANN, S. L., and WCISLO, W. T. 1991. Bioassay evidence for a sex attractant pheromone in the large carpenter bee, *Xylocopa varipuncta* (Hymenoptera: Anthophoridae). *J. Zool. London* 224:285–291.

NEWMAN, H. W. 1851. Habits of the Bombinatrices. *Proc. Entomol. Soc. London* 1:86–92.

OLDHAM, N. J., BILLEN, J., and MORGAN E. D. 1994. On the similarity of the Dufour gland secretion and the cuticular hydrocarbons of some bumblebees. *Physiol. Entomol.* 19:115–123.

PRŶS-JONES, O. E., and CORBET, S. A. 1987. Bumblebees. Naturalists' Handbooks No. 6. Cambridge University Press, Cambridge.

RÖSELER, P.-F. 1973. Die Anzahl der Spermien im Receptaculum Seminis von Hummelköniginnen (Hym., Apoidea, Bombinae). *Apidologie* 4:267–274.

SLADEN, F. W. L. 1912. The Humble-bee. Macmillan, London.

SNODGRASS, R. E. 1956. Anatomy of the Honey Bee. Constaple and Company, London.

STEIN, G. 1956. Beiträge zur Biologie der Hummel (*B. terrestria* L., *B. lapidarius* L. u.a.). *Zool. Jahrb., Abt. System., Ökol. Geogr.* 84:439–462.

SVENSSON, B. G. 1979a. Patrolling behaviour of bumble bee males (Hymenoptera, Apidae) in a subalpine/alpine area, Swedish Lapland. *Zoon* 7:67–94.

SVENSSON, B. G. 1979b. *Pyrobombus lapponicus* auct., in Europe recognized as two species: *P. lapponicus* (Fabricius, 1793) and *P. monticola* (Smith, 1849) (Hymenoptera, Apoidea, Bombinae). *Entomol. Scand.* 10:274–296.

SVENSSON, B. G. 1980. Species-isolating mechanisms in male bumble bees (Hymenoptera, Apidae). PhD dissertation. University of Uppsala, Uppsala, Sweden.

SVENSSON, B. G., and BERGSTRÖM, G. 1977. Volatile marking secretions from the labial gland of north European *Pyrobombus* D. T. males (Hymenoptera, Apidae). *Insectes Soc.* 24:213–224.

SVENSSON B. G., and BERGSTRÖM, G. 1979. Marking pheromones of *Alpinobombus* males. *J. Chem. Ecol.* 5:603–615.

SVENSSON, B. G., APPELGREN, M., and BERGSTRÖM, G. 1984. Geranylgeranyl acetate and 2-heptadecanone as the dominant marking secretion components of the labial glands in the bumble bee *Alpigenobombus wurfleini. Nova Acta Regiae Soc. Sci. Ups., Ser. V:C* 3:145–147.

TORSSELL, K. B. G. 1983. Natural Product Chemistry. A Mechanistic and Biosynthetic Approach to Secondary Metabolism. John Wiley, New York.

ZAR, J. H. 1984. Biostatistical Analysis, 2nd ed. Prentice-Hall, Englewood Cliffs, New Jersey.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

DECREASED ATTRACTION OF *Anastrepha ludens* TO COMBINATIONS OF TWO TYPES OF SYNTHETIC LURES IN A CITRUS ORCHARD

D. C. ROBACKER[1,*] and R. R. HEATH[2]

[1]*Crop Quality and Fruit Insects Research, ARS, USDA*
2301 South International Blvd.
Weslaco, Texas 78596

[2]*Insect Attractants, Behavior, and Basic Biology Laboratory, ARS, USDA*
P.O. Box 14565
Gainesville, Florida 32604

(Received April 25, 1996; accepted December 17, 1996)

Abstract—Combinations of the previously developed attractants CEHO from host fruit aroma and AMPu from volatile metabolites of amino acids were evaluated for attractiveness to gamma-irradiated Mexican fruit flies, *Anastrepha ludens*, in a citrus orchard. In one experiment, McPhail traps with polyvinyl chloride (PVC) lures loaded with CEHO (10:1:1:50 mixture of 1,8-cineole, ethyl hexanoate, hexanol, and ethyl octanoate) were more attractive than blank traps. McPhail traps with AMPu (10:10:1 mixture of ammonium bicarbonate, methylamine HCl, and putrescine) were more attractive than blank and CEHO traps. Traps containing both AMPu and CEHO lures were less attractive than traps containing AMPu alone. In another experiment, sticky yellow panels and spheres were used to compare attractiveness of the same two attractants in different formulations. Sticky traps baited with membrane formulations of CEHO (10:1:1:10 mixture of the chemicals listed above) were not significantly attractive. Sticky traps with polypropylene tubes containing an agar formulation of AMPu (6:10:1 mixture of ammonium carbonate, methylamine HCl, and putrescine) were more attractive than blank and CEHO baited traps. As in the first experiment, traps with both AMPu and CEHO lures were less attractive than traps with AMPu alone. Results indicate that attractive host-odor volatiles and attractive amino-acid metabolites interact negatively with regard to attractiveness to the Mexican fruit fly.

Key Words—Attractants, Mexican fruit fly, Diptera, Tephritidae, *Anastrepha ludens*, host odors, ammonia, amines.

*To whom correspondence should be addressed.

0098-0331/97/0500-1253$12.50/0 © 1997 Plenum Publishing Corporation

INTRODUCTION

In the past five years, development and laboratory bioassay of two different types of attractants were reported for the Mexican fruit fly, *Anastrepha ludens* Loew. The first was a four-component attractant (CEHO) developed from yellow chapote fruit (*Sargentia greggii* S. Wats.), a natural host of the fly (Robacker et al., 1992). The second was a three-component attractant (AMPu) containing metabolites from biological degradation of amino acids (Robacker and Warfield, 1993). Both of these attractants were more attractive than *Torula* yeast, the most commonly used bait for Mexican fruit flies in flight chamber tests in a greenhouse. In tests in a citrus orchard both were significantly more attractive than blank traps, but AMPu was not more attractive than *Torula* yeast (Robacker, 1995), and CEHO was less attractive than *Torula* yeast (Robacker and Heath, 1996). The purpose of this work was to determine if combinations of CEHO and AMPu would be more attractive than either alone.

METHODS AND MATERIALS

Insects. Flies were from a culture that had been maintained on laboratory diet for about 100–120 generations with no wild-fly introductions. Flies were irradiated with 70–92 Gy (^{137}Cs source) one to two days before adult eclosion, to comply with quarantine laws for releasing *A. ludens*. Mixed-sex groups of 180–200 flies were kept in 473 ml cardboard cartons with screen tops until used in tests. Laboratory conditions for holding flies were 22 ± 2°C, 50 ± 20% relative humidity, and photophase from 06:30 to 19:30 hr provided by fluorescent lights. Flies were fed sucrose and water until the time of release. Flies were released into the orchard when 2–10 days old during the late afternoon of the day before a test. Approximately 2000 flies were distributed equally in the test area of the orchard.

Test Chemicals and Formulations. Two types of CEHO lures were used. One was a polyvinyl chloride (PVC) formulation prepared by Scentry, Inc. (now Ecogen, Inc., Langhorne, Pennsylvania). These lures contained 1,8-cineole, ethyl hexanoate, and ethyl octanoate obtained from Aldrich Chemical Co., Inc., (Milwaukee, Wisconsin), and hexanol obtained from Sigma Chemical Company (St. Louis, Missouri). All four chemicals were >99% pure. Each lure contained 120 mg of a 10:1:1:50 mixture of 1,8-cineole, ethyl hexanoate, hexanol, and ethyl octanoate, respectively. These PVC lures were more attractive than *Torula* yeast in flight-chamber experiments (Robacker et al., 1992).

The second CEHO lure was a membrane-based formulation like that described previously for synthetic pheromone of the papaya fruit fly (*Toxotrypana curvicauda* Gerstaecker) (Heath et al., 1996). Briefly, the lure was a

3- × 5-cm envelope made from impermeable polyethylene containing a high-density polyethylene membrane (Consep Inc., Bend, Oregon). Chemicals diffusing through the membrane were released from the lure through a 1.17-cm-diam. hole on one side of the envelope. Two loadings of the chemicals were used. The low loading was 25 μl of a 10:1:1:10 mixture of the chemicals; and the high loading was 100 μl of the same mixture. Chemicals were obtained from Aldrich and were >99% pure. McPhail traps containing these membrane formulations were significantly more attractive than water blanks and significantly less attractive than *Torula* yeast in citrus orchard experiments (Robacker and Heath, 1996).

AMPu also was tested in two formulations. One formulation was an aqueous solution of ammonium bicarbonate, methylamine HCl, and putrescine. The chemicals were obtained from Sigma and were at least 98% pure. Concentrations of the three chemicals in the formulation were 400:400:40 μg/ml, respectively, at a pH of 8.8. These concentrations were the most attractive in previous experiments (Robacker, 1995).

The second AMPu formulation was an agar gel for use in dry traps. Ammonium carbonate was substituted for ammonium bicarbonate because of its greater solubility in water, with the molar ratios of ammonia, methylamine, and putrescine the same as in the aqueous formulation. Ammonium carbonate was obtained from Aldrich and was at least 98% pure. The AMPu-agar formulation was prepared by mixing hot agar (Bacto Agar, Difco Laboratories, Detroit, Michigan) solution with aqueous AMPu in 1.9 ml polypropylene microcentrifuge tubes (A. Daigger & Company, Inc., Wheeling, Illinois). Final agar concentration was 1%. AMPu component concentrations in agar tubes were 60 mg/ml ammonium carbonate, 100 mg/ml methylamine HCl, and 10 mg/ml putrescine. Final pH of the AMPu tubes was 8.5–8.8. Sticky traps baited with AMPu-agar tubes prepared in this way were equal in attractiveness to *Torula* yeast in McPhail traps in earlier research (Robacker, 1995).

Experiment 1. Attractiveness of AMPu-CEHO Combinations in McPhail Traps. Two AMPu-CEHO combinations were evaluated against AMPu alone, CEHO alone, and a water blank in McPhail traps. PVC formulations of CEHO and aqueous formulations of AMPu were used in this experiment. PVC CEHO lures were used when 6–18 days old. Previous laboratory research indicated lures were most attractive between the ages of 10–15 days (Robacker et al., 1992) and preliminary field work indicated lures were actually repellent to flies before 6 days.

Traps with AMPu-CEHO combinations or AMPu alone contained 200 ml of the aqueous AMPu formulation in the trap reservoir. One AMPu-CEHO trap and the trap with CEHO alone contained a PVC CEHO lure suspended inside the trap 1–2 cm above the entrance hole on the underside of the trap. The trap with CEHO alone also contained 200 ml of water. The second AMPu-CEHO

combination used a PVC CEHO lure fastened to the top on the outside of the trap. CEHO lures used in the three CEHO treatments were always of the same age within each replication of the experiment. All traps contained amber coloring in the water from a combination of red, yellow, and green food colors (McCormick & Co., Inc., Baltimore, Maryland) and 0.01% Triton (Rohm and Haas Co., Philadelphia, Pennsylvania) as a wetting agent.

The experiment was conducted in a mixed citrus orchard located near the laboratory in Weslaco, Texas. The orchard contained several varieties of orange, lemon, grapefruit, and tangerine trees of varying ages. One row of Ruby Red grapefruit (*Citrus paradisi*) and one row of Dancy tangerine (*C. reticulata*) were chosen for tests since they contained relatively large (2–3 m height) fruit-bearing trees. Two linear blocks of five consecutive trees each were used in each row, for a total of four blocks in the orchard. One each of the two AMPu–CEHO combinations, an AMPu trap, a CEHO trap, and a water blank trap were tested in each block. Each test lasted one day. Traps were placed in the orchard during the morning and removed for fly counts and cleaning on the following morning. Traps were hung one to a tree, north of center, at 1–2 m height.

Two series of one-day tests using two different sets of PV CEHO lures were conducted. In each series, one-day tests were conducted every two to four days. CEHO lures were suspended in a fume hood in the laboratory at 22 ± 2°C on nontest days. Four one-day tests were conducted in one series and five in the second for a total of 36 replications of each treatment (9 days × 4 blocks per day). Positions of treatments within each block were randomized for the first one-day test of each series. Positions of treatments in consecutive one-day tests were not randomized but were moved sequentially within each block. One series of one-day tests was conducted from August 9 to 20 and the second from October 1 to 13, 1993.

Experiment 2. Attractiveness of AMPu–CEHO Combinations on Sticky Traps. Four AMPu–CEHO–trap-type combinations were evaluated against various AMPu alone, CEHO alone, and blank trap controls. Membrane formulations of CEHO and agar formulations of AMPu were used in this experiment. CEHO lures were used between the ages of 0 and 30 days because previous research indicated attractiveness of these lures did not change as they aged up to one month (Robacker and Heath, 1996). AMPu lures were used only during the first day after removal from the refrigerator. The lures are highly attractive during this time (Robacker, 1995). Two types of sticky traps were used. One was a yellow ball trap (13 cm diam.) and the second was a yellow panel trap (13 × 18 cm). Traps were coated with Tangle-Trap (Tanglefoot Company, Grand Rapids, Michigan). Both traps had been evaluated previously with and without lures (Robacker, 1992, 1995). CEHO and AMPu lures were attached to the tops of the traps, on opposite sides when both lures were used on the same trap. Lure–trap combinations were: AMPu–CEHO (low loading)–ball;

AMPu–CEHO (low)–panel; AMPu–CEHO (high loading)–ball; AMPu–CEHO (high)–panel; AMPu–ball; AMPu–panel; CEHO (low)–ball; CEHO (low)–panel; CEHO (high)–ball; CEHO (high)–panel; unbaited ball; and unbaited panel.

The experiment was conducted in the same orchard as experiment 1 except that the four blocks were enlarged to contain eight trees each instead of five. One block in each of the two rows was used to test the four lure–trap combinations containing the low loading of CEHO, plus the two AMPu alone combinations, and the two blank traps. The other two blocks were used to test the four combinations containing the high loading of CEHO, plus the AMPu alone combinations and the blank traps. Experimental procedures were similar to experiment 1. Ten one-day tests were conducted from May 26 to July 27, 1994. Because two of the four blocks were used to test the low loading of CEHO and two were used to test the high loading, in essence two experiments were conducted at the same time. In each, 20 replications of each treatment were conducted.

Statistical Analyses. The numbers of males and females captured by individuals traps were transformed to log $(X + 1)$ to stabilize variance. Two-way analysis of variance (ANOVA) was conducted for both males and females for each experiment (Abacus Concepts, 1989) to test effects of test day, lure–trap type, and interactions of day with lure–trap type. For experiment 2, separate ANOVAs were conducted for blocks containing the low and high loadings of CEHO. Single degree-of-freedom contrasts were conducted to test effects of trap type (ball versus panel), lure type (AMPu versus water or blank, CEHO versus water or blank), and interactions of AMPu with CEHO.

Experiment 2 was reanalyzed after combining data from balls and panels and from blocks containing low and high loadings of CEHO. Replications over space (four blocks) were treated equivalently to replications over time (one-day tests). Two-way ANOVAs were conducted for both males and females to test lure effects and to remove effect of test day from the residual. The test day × lure interaction was left out of the models. Experiment 1 was reanalyzed similarly. Fisher's protected least significant difference (LSD) method was used to compare lure means. Paired t tests were conducted to test attractiveness of CEHO lures relative to water blanks within the same block and one-day test.

RESULTS

Experiment 1. Attractiveness of AMPu–CEHO Combinations in McPhail Traps. Test day × lure interactions were not significant according to ANOVAs for both males and females. ANOVAs (without test day × lure interaction terms) showed that McPhail traps containing aqueous AMPu were significantly more attractive than traps containing water or CEHO alone to both male and female

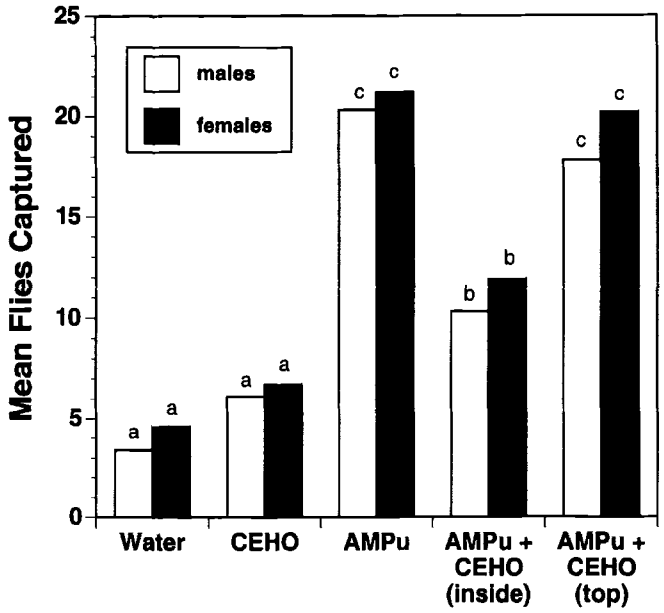


FIG. 1. Captures of *A. ludens* in McPhail traps baited with combinations of aqueous AMPu and PVC CEHO lures inside or on top of traps, compared with traps containing only water, a CEHO lure, or AMPu (experiment 1). Within each sex, bars with the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$; $N = 36$ for each trap/lure configuration).

flies (Figure 1) ($F = 46.9, 41.1$; $df = 4,167$; $P < 0.0001$ for males and females, respectively). Traps baited with CEHO alone were not significantly attractive by the LSD test. However, CEHO traps were more attractive than traps containing only water by a paired t test comparing traps within the same block, summed over males and females ($P < 0.05$, $t = 2.3$, $df = 34$). Traps baited with AMPu-CEHO combinations in which the CEHO PVC lure was located inside the trap were significantly less attractive than traps baited with AMPu alone to both males and females. Combinations with the CEHO lure on top of the trap were not significantly different from AMPu alone.

Experiment 2. Attractiveness of AMPu-CEHO Combination on Sticky Traps. Test day × lure interactions were not significant in any of the four two-way ANOVAs (highest $F = 1.1$; $df = 63,89$; $P = 0.29$ for males, high loading of CEHO). No significant differences were found between panel and ball traps (highest $F = 0.84$; $df = 1,64$; $P = 0.36$ for single degree-of-freedom contrast for males, low loading of CEHO). AMPu-baited traps were significantly more attractive than blanks in all four ANOVAs (lowest $F = 7.2$; $df = 1,64$; $P <$

0.01 for single degree-of-freedom contrast for males, low loading of CEHO). Neither loading of CEHO was significantly attractive to either males or females, although the numbers of flies captured by CEHO-baited traps were numerically higher than those captured by blank traps in each case (highest $F = 2.3$; $df = 1,64$; $P = 0.13$ for single degree-of-freedom contrast for females, low loading of CEHO). In the two ANOVAs for the high loading of CEHO, the AMPu × CEHO interaction was significant (lower $F = 4.0$; $df = 1,80$; $P < 0.05$ for single degree-of-freedom contrast for females). AMPu × CEHO interactions were not significant at the 5% level for either males or females for the low loading of CEHO, but the effect was similar to that of the high loading (lower $F = 1.9$; $df = 1,64$; $P = 0.17$ for single degree-of-freedom contrast for females). The interactions occurred because AMPu–CEHO combinations were less attractive than AMPu alone.

The statistical analyses presented above indicated no differences between ball traps and panel traps, or between low and high loadings of CEHO, with regard to captures of flies. Moreover, test day × lure interactions were not significant. Therefore, data were combined for ball and panel traps and for low and high loadings of CEHO. Test day × lure interactions were left out of the model. These simplified ANOVAs gave results that were essentially the same as in experiment 1. Traps baited with AMPu were significantly more attractive than blank traps or traps baited with CEHO to both male and female flies (Figure 2) ($F = 17.2, 41.2$; $df = 3,291$; $P < 0.0001$ for males and females, respectively). Traps baited with CEHO alone were not significantly more attractive than blank traps either by LSD or paired t tests. Traps baited with combinations of AMPu and CEHO were significantly less attractive than traps baited with AMPu alone to both male and female flies.

DISCUSSION

Combinations of AMPu with CEHO were either less attractive or no more attractive than AMPu alone in both experiments. This result cannot be attributed simply to the CEHO lures acting as repellents. Traps baited with membrane-based CEHO lures were not less attractive than blank traps, and PVC CEHO lures were more attractive than blank traps. Furthermore, in previous work, McPhail traps baited with membrane-based CEHO lures identical to those tested here were significantly more attractive than McPhail traps containing only water (Robacker and Heath, 1996).

Combinations of attractants that act on different appetitive behaviors, and perhaps on different central nervous system centers (Robacker, 1993), have usually proven less attractive than the more potent of the two attractants alone for *A. ludens*. The effect has now been demonstrated for combinations of pher-

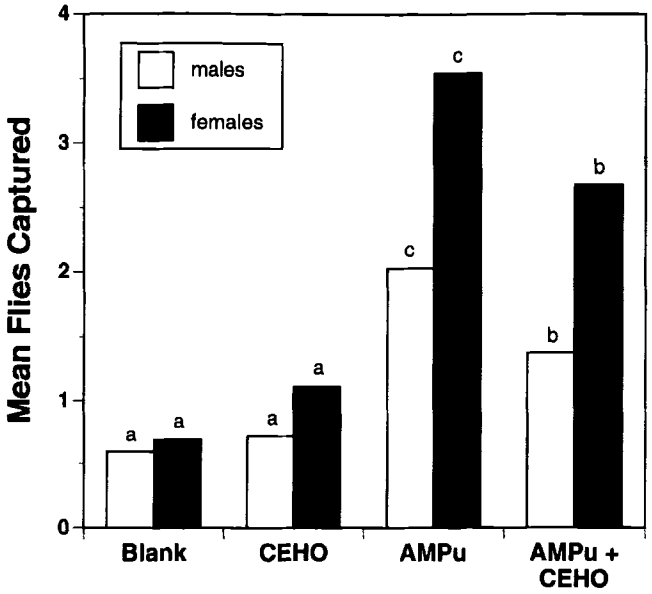


FIG. 2. Captures of *A. ludens* on sticky traps baited with a combination of an AMPu–agar lure and a membrane-based CEHO lure, compared with blank traps or traps baited with a CEHO or AMPu lure alone (experiment 2). Within each sex, bars with the same letter are not significantly different from each other by Fisher's protected LSD ($P < 0.05$; $N = 76$ for each trap/lure configuration).

omone with odor of fermented host fruit (Robacker and Garcia, 1990), chemicals from host fruit odor with bacterial odor (Robacker, 1991), and acetic acid with AMPu (Robacker et al., 1996). In each case, laboratory experiments indicated that the motivational state of the fly determined which of the two attractants of the combination elicited attraction and which inhibited the response to the combination. For example, sexually active female flies were strongly attracted to pheromone and less so to the combination of pheromone and fruit odor, whereas sexually immature, sugar-starved females were attracted more to fruit odor than to the combination (Robacker and Garcia, 1990). Similar decreased attraction to combinations of pheromone and protein baits was reported in *Bactrocera oleae* Gmelin (Haniotakis and Skyrianos, 1981; Haniotakis and Vassiliou-Waite, 1987; Zervas, 1989). However, several cases are known in Tephritidae in which combinations of attractants that apparently act on different appetitive behaviors were more attractive than either alone (Zervas, 1989; Landolt et al., 1992; MacCollom et al., 1992, 1994; Robacker et al., 1996). These findings indicate that attraction of fruit flies to semiochemicals is highly complex, involving

interactions of chemicals with each other and various effects of physiological states of the flies on responses to the chemicals.

Acknowledgments—I thank Pravait Kaochoung (Office of Atomic Energy for Peace, Bankhen, Bangkok, Thailand) and Maura Rodriguez, Marco Gomez, and Cyndi Rodriguez for technical assistance; Sammy Ingle for insects; and A. W. Guenthner (USDA-APHIS, Mission, Texas) for irradiation of pupae. Use of a product brand in this work does not constitute an endorsement by the USDA.

REFERENCES

ABACUS CONCEPTS. 1989. SuperANOVA. Abacus Concepts, Inc., Berkeley, California.

HANIOTAKIS, G. E., and SKYRIANOS, G. 1981. Attraction of the olive fruit fly to pheromone, McPhail, and color traps. *J. Econ. Entomol.* 74:58–60.

HANIOTAKIS, G. E., and VASSILIOU-WAITE, A. 1987. Effect of combining food and sex attractants on the capture of *Dacus oleae* flies. *Entomol. Hell.* 5:27–33.

HEATH, R. R., EPSKY, N. D., JIMENEZ, A., DUEBEN, B. D., LANDOLT, P. J., MEYER, W. L., ALUJA, M., RIZZO, J., CAMINO, M., JERONIMO, F., and BARANOWSKI, R. M. 1996. Improved pheromone-based trapping systems to monitor *Toxotrypana curvicauda* (Diptera: Tephritidae). *Fla. Entomol.* 79:37–48.

LANDOLT, P. J., REED, H. C., and HEATH, R. R. 1992. Attraction of female papaya fruit fly (Diptera: Tephritidae) to male pheromone and host fruit. *Environ. Entomol.* 21:1154–1159.

MACCOLLOM, G. B., LAUZON, C. R., WEIRES, R. W., JR., and RUTKOWSKI, A. A. 1992. Attraction of adult apple maggot (Diptera: Tephritidae) to microbial isolates. *J. Econ. Entomol.* 85:83–87.

MACCOLLOM, G. B., LAUZON, C. R., PAYNE, E. B., and CURRIER, W. W. 1994. Apple maggot (Diptera: Tephritidae) trap enhancement with washed bacterial cells. *Environ. Entomol.* 23:354–359.

ROBACKER, D. C. 1991. Specific hunger in *Anastrepha ludens* (Diptera: Tephritidae): Effects on attractiveness of proteinaceous and fruit-derived lures. *Environ. Entomol.* 20:1680–1686.

ROBACKER, D. C. 1992. Effects of shape and size of colored traps on attractiveness to irradiated, laboratory-strain Mexican fruit flies (Diptera: Tephritidae). *Fla. Entomol.* 75:230–241.

ROBACKER, D. C. 1993. Understanding olfactory attraction in *Anastrepha* using *A. ludens* as a model system, pp. 201–206, *in* A. Aluja and P. Liedo (eds.). Fruit Flies: Biology and Management. Springer-Verlag, New York.

ROBACKER, D. C. 1995. Attractiveness of a mixture of ammonia, methylamine and putrescine to Mexican fruit flies (Diptera: Tephritidae) in a citrus orchard. *Fla. Entomol.* 78:571–578.

ROBACKER, D. C., and GARCIA, J. A. 1990. Responses of laboratory-strain Mexican fruit flies, *Anastrepha ludens*, to combinations of fermenting fruit odor and male-produced pheromone in laboratory bioassays. *J. Chem. Ecol.* 16:2027–2038.

ROBACKER, D. C., and HEATH, R. R. 1996. Attraction of Mexican fruit fly (Diptera: Tephritidae) to lures emitting host-fruit volatiles in a citrus orchard. *Fla. Entomol.* 79:600–602.

ROBACKER, D. C., and WARFIELD, W. C. 1993. Attraction of both sexes of Mexican fruit fly, *Anastrepha ludens*, to a mixture of ammonia, methylamine, and putrescine. *J. Chem. Ecol.* 19:2999–3016.

ROBACKER, D. C., WARFIELD, W. C., and FLATH, R. A. 1992. A four-component attractant for the Mexican fruit fly, *Anastrepha ludens* (Diptera: Tephritidae), from host fruit. *J. Chem. Ecol.* 18:1239–1254.

ROBACKER, D. C., MORENO, D. S., and DEMILO, A. B. 1996. Attractiveness to Mexican fruit flies of combinations of acetic acid with ammonium/amino attractants with emphasis on effects of hunger. *J. Chem. Ecol.* 22:499–511.

ZERVAS, G. A. 1989. Significant increase of *Dacus oleae* trapping using sex and food attractants separately in the same tree, pp. 433–442, *in* R. Cavalloro (ed.). Fruit Flies of Economic Importance. Balkema, Rotterdam.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

MEXICAN FRUIT FLY ATTRACTANTS: EFFECTS OF 1-PYRROLINE AND OTHER AMINES ON ATTRACTIVENESS OF A MIXTURE OF AMMONIA, METHYLAMINE, AND PUTRESCINE

D. C. ROBACKER,[1,*] A. B. DEMILO,[2] and D. J. VOADEN[2]

[1]*Crop Quality and Fruit Insects Research, ARS, USDA*
2301 South International Blvd.
Weslaco, Texas 78596

[2]*Insect Chemical Ecology Laboratory, ARS, USDA*
Bldg. 007, Rm 301, BARC-West
Beltsville, Maryland 20705

(Received May 13, 1996; accepted December 17, 1996)

Abstract—Several amines were tested alone and in combination with AMPu, an attractant mixture containing ammonium bicarbonate or ammonium carbonate, methylamine hydrochloride, and putrescine, for attractiveness to Mexican fruit flies (*Anastrepha ludens* Loew). In laboratory bioassay, 1-pyrroline, 3-pyrroline, 2-(methylamino)ethanol, spermidine, spermine, and indole-3-acetic acid were significantly more attractive than solvent controls. In orchard tests, traps baited with combinations of AMPu with dimethylamine hydrochloride, ethylamine, 2,5-dimethylpyrazine, or pyrrolidine captured fewer flies than traps baited with AMPu alone. Traps containing AMPu plus additional ammonium bicarbonate were much less attractive than AMPu alone. Combinations of AMPu with 1-pyrroline were about 50% more attractive than AMPu alone to both males and females. Combinations of AMPu with 3-pyrroline were not significantly more attractive than AMPu alone.

Key Words—Attractants, Mexican fruit fly, Diptera, Tephritidae, *Anastrepha ludens*, ammonia, methylamine, putrescine, 1-pyrroline.

INTRODUCTION

Robacker and Warfield (1993) described a three-component attractant for *Anastrepha ludens* Loew consisting of ammonium bicarbonate, methylamine hydro-

*To whom correspondence should be addressed.

0098-0331/97/0500-1263$12.50/0 © 1997 Plenum Publishing Corporation

chloride, and putrescine (AMPu). Robacker (1995) showed that yellow sticky panel traps baited with AMPu formulated into agar were equal in attractiveness to McPhail traps baited with *Torula* yeast, a standard attractant for many Tephritidae. Robacker et al. (1993) and Robacker and Flath (1995) demonstrated that attractiveness of bacterial odor to the Mexican fruit fly is largely due to chemicals containing protonatable nitrogen, such as the components of AMPu. The purpose of the current research is to evaluate attractiveness to Mexican fruit flies of various nitrogen-containing chemicals alone and in combination with AMPu, as part of ongoing research to develop more powerful attractants for the Mexican fruit fly and other Tephritidae.

Test chemicals were selected based on several factors. Ammonium bicarbonate was selected, even though it is already a component of AMPu, primarily to determine if attractiveness of the mixture could be increased with a greater concentration of this important component. Ammonia, dimethylamine, ethylamine, isoamylamine, and 2,5-dimethylpyrazine were identified in volatiles above bacterial cultures attractive to the Mexican fruit fly (Robacker and Flath, 1995). Ethanolamine, 2-(methylamino)ethanol, isoamylamine, spermidine, and spermine were selected because they were reported as products of various other microorganisms (Miller, 1961). Most of the aforementioned chemicals as well as pyrrolidine, indole-3-acetic acid, and 1-pyrroline are also known amino acid metabolites (Lehninger, 1970; Amoore et al., 1975). Amino acid metabolites were considered important because hunger for protein has been hypothesized as the driving force in attraction of fruit flies to proteinaceous baits (Wakabayashi and Cunningham, 1991; Heath et al., 1995), to bacterial odor (Drew and Lloyd, 1989; Robacker and Moreno, 1995), and to AMPu (Robacker and Warfield, 1993). Furthermore, pyrrolidine is a known attractant for the melon fly, *Bactrocera cucurbitae* Coquillett (Wakabayashi and Cunningham, 1991), and both pyrrolidine and ethanolamine proved attractive to Mexican fruit flies in laboratory experiments (Robacker and Warfield, 1993). 1-Pyrroline has been identified as an attractive pheromone component produced by male Mediterranean fruit flies (*Ceratitis capitata* Wiedemann) (Baker et al., 1985; Flath et al., 1993; Jang et al., 1994). It has also been suggested that 1-pyrroline, a natural contaminant of putrescine formed by spontaneous oxidation (Amoore et al., 1975), may be responsible for the attractiveness of putrescine to fruit flies (Robacker and Warfield, 1993). 3-Pyrroline was selected because of its structural similarity to 1-pyrroline.

Two types of experiments were conducted. Laboratory bioassays were first conducted to assess the attractiveness of individual test chemicals. Chemicals that were most attractive in these tests were then tested in orchard experiments in combinations with AMPu.

METHODS AND MATERIALS

Insects. Flies were from two cultures. Flies used in orchard tests involving aqueous AMPu in McPhail traps were from a culture that originated from mangoes (*Mangifera indica* L.) collected in Morelos, Mexico, in 1953. Flies used in all other experiments were from a culture that originated from yellow chapote fruit (*Sargentia greggii* S. Wats.), a native host of the fly, collected in Nuevo Leon, Mexico, in 1987. Both cultures had been maintained on laboratory diet since establishment. Flies used in orchard tests were irradiated with 70–84.7 Gy (^{137}Cs source) one to two days before adult eclosion for release into the orchard, to comply with quarantine laws for releasing *A. ludens*. Flies used in laboratory bioassays were not irradiated. Mixed-sex groups of 180–200 flies were kept in 0.5-liter cardboard cartons with screen tops until used in tests. Flies were fed sucrose and water but not protein until the time of testing. This feeding regime yields maximum attraction to volatiles from bacterial cultures (Robacker and Garcia, 1993) that contain chemicals similar to those tested in this work. Laboratory conditions for holding flies were 20–25°C, 50–70% relative humidity and photophase from 06:30 to 19:30 hr provided by fluorescent lights.

Test Chemicals. Test chemicals (purity) obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin) were ammonium carbonate (ACS Reagent quality), 3-pyrroline (97% pure), 2-(methylamino)ethanol (99%), spermidine (99%), spermine (99%), putrescine (98%), indole 3-acetic acid (98%), isoamylamine (99%), 2,5-dimethylpyrazine (99%), and ethanolamine (99%). Test chemicals obtained from Sigma Chemical Co. (St. Louis, Missouri) were ammonium bicarbonate (99%), methylamine hydrochloride (99%), dimethylamine hydrochloride (95%), 70% ethylamine in water (99%), and pyrrolidine (99%).

1-Pyrroline was synthesized by acid hydrolysis of 4-aminobutyraldehyde diethyl acetal by a method modified from that of Schopf and Oechler (1936), as follows: A 50-ml conical flask containing a magnetic stirbar was cooled in an ice-water bath and charged with 0.36 g of 90% 4-aminobutyraldehyde diethyl acetal (Aldrich) and 4 ml of 2 N HCl (Fisher Scientific, Fair Lawn, New Jersey). The reaction mixture was stirred for 25 min before 6 ml of 1.2 M potassium carbonate (J. T. Baker Chemical Co., Phillipsburg, New Jersey) was stirred into the flask (resulting pH > 12). The synthesis of 1-pyrroline was confirmed by extracting (3× 5 ml) the aqueous solution with diethyl ether (E. M. Science, Gibbstown, New Jersey) and analyzing the combined and dried (anhydrous magnesium sulfate, Fisher) extract by GC-MS using a Hewlett Packard (Avondale, Pennsylvania) model 5890A equipped with a 5971A mass selective detector (MSD) and HP5 bonded-phase fused-silica capillary column (25 m, 0.2 mm ID, 0.11 μm film). GC conditions were: split injection (100:1) at 250°C, col-

umn oven 50°C isothermal, and helium carrier gas flow rate 1 ml/min. MSD (electron impact ionization) conditions were: ionization voltage 70 eV, and ion source temperature 180°C. Under these conditions, 1-pyrroline eluted at 2.02 min. Ion masses and relative abundances were: 69 (M^+, 100), 68 (61.7), 67 (2.2), 54 (6.0).

1-Pyrroline was used as an aqueous solution in behavioral bioassays within five days of its synthesis and was kept refrigerated until used. Gas chromatographic examination of the 1-pyrroline solution was conducted within a day of synthesis on three occasions to determine purity and concentration. GC analyses were conducted with a Shimadzu GC 17A (Shimadzu Scientific Instruments, Inc., Columbia, Maryland) equipped with a flame ionization detector. The primary GC column was a DB-1 column (60 m, 0.32 mm ID, 5 μm film) (J & W Scientific, Folsom, California). 1-Pyrroline was sampled by solid-phase microextraction (SPME) with a 100-μm polydimethylsiloxane-coated fiber (Supelco, Inc., Bellefonte, Pennsylvania) that was inserted for 10 min into 0.2 ml of a 1 : 100 dilution of the 1-pyrroline reaction mixture. On-column injection was accomplished by thermal desorption of the fiber at 200°C in the tip of a 16-cm length of 0.53-mm-ID deactivated fused silica capillary tubing (Supelco) positioned in the on-column injection port. The 0.53-mm desorption tube was connected to the primary column by a GlasSeal connector (Supelco, Inc.). Column-oven conditions were 140–200°C at 5°C/min. Carrier gas was helium at a linear velocity of 30 cm/sec. Purity of the 1-pyrroline product was approximately 80% by total FID peak area comparisons. Quantitation of 1-pyrroline was done using a calibration curve constructed with known amounts of pyrrolidine, which is similar in structure and retention time to 1-pyrroline. Calculated concentration of 1-pyrroline (mean $\pm$ SE) was 14.5 $\pm$ 3.8 mg/ml ($N = 3$) in the reaction mixture. This concentration indicates that conversion of 4-aminobutyraldehyde diethyl acetal to 1-pyrroline (and ethanol by-product) was approximately 100%.

Laboratory Bioassays. Experiments were conducted to assess attractiveness of individual test chemicals that had not been evaluated previously using cage-top bioassays as in Robacker and Warfield (1993). Attractiveness of ammonia, dimethylamine, ethylamine, isoamylamine, 2,5-dimethylpyrazine, pyrrolidine, and ethanolamine to Mexican fruit flies was established in earlier work (Robacker and Warfield, 1993; Robacker and Flath, 1995). Four solutions of each chemical that had not been tested in earlier research were prepared. 1-Pyrroline, 3-pyrroline, 2-(methylamino)ethanol, and spermidine were prepared at concentrations of 0.001, 0.01, 0.1, and 1 mg/ml in water. Spermine was prepared at 0.01, 0.1, 1, and 10 mg/ml in water. Indole 3-acetic acid was prepared at 0.01, 0.1, 1, and 10 mg/ml in methanol. Test concentrations of the latter two chemicals were 10 times higher than those of the other chemicals because of their lower

volatilities. Chemicals were tested in 10 μl aliquots of the solutions, resulting in test quantities ranging from 10 ng to 100 μg of the individual chemicals.

Bioassays were conducted using aluminum-screened cages (30 cm/side) each containing one carton of 180–200 flies. Flies were tested when 5–14 days old between 10:00 and 14:00 hr under a combination of fluorescent and natural light. Tests were conducted by placing four filter paper triangles (3 cm/side), two containing test chemicals and two containing 10 μl of either water or methanol (controls for the respective solvents of the test chemicals), near the corners on the top of a cage. Papers containing test chemicals were located diagonally from each other on the cage top, as were the papers containing solvent. The filter papers were raised 5 mm above the cage top using plastic rings to ensure that olfaction and not contact chemoreception was solely responsible for the response of the flies. The numbers of flies beneath each paper were counted once each minute for 10 min. Totals of the 10 counts at each treatment and at the controls were calculated, and these totals were used in paired *t* tests to determine if each chemical was more attractive than a solvent control. Ten replications were conducted for each test chemical.

AMPu Preparations. AMPu was used either as an aqueous solution or an agar mixture. Each was adjusted to pH 8.8 with saturated NaOH (Fisher). For most experiments, the aqueous solution contained ammonium bicarbonate, methylamine hydrochloride, and putrescine at concentrations of 0.4, 0.4, and 0.04 mg/ml, respectively. In the experiment to evaluate additional ammonium bicarbonate, AMPu component concentrations were 0.2, 0.2, and 0.02 mg/ml. Both sets of AMPu concentrations were highly attractive in previous research (Robacker, 1995). The agar mixture of AMPu contained ammonium carbonate (instead of ammonium bicarbonate), methylamine hydrochloride, and putrescine at 60, 100, and 10 mg/ml. Molar ratios of ammonia to the other two components were approximately equal in the two preparations. Agar tubes were prepared by mixing equal volumes of hot agar solution (Bacto Agar, Difco Laboratories, Detroit, Michigan) and aqueous AMPu (120, 200, and 20 mg/ml of the three chemicals) in 1.9 ml polypropylene microcentrifuge tubes (A. Daigger & Company, Inc., Wheeling, Illinois) to a volume of 1.7 ml. Final agar concentration was 1% in the tubes.

Orchard Tests. Experiments were conducted in a citrus orchard to assess the effects on attractiveness to Mexican fruit flies of combining test chemicals with AMPu. A mixed citrus orchard located near the laboratory in Weslaco, Texas, was used for all field experiments. The orchard contained several varieties of orange, lemon, grapefruit, and tangerine trees of varying ages. One row of Ruby Red grapefruit (*Citrus paradisi* MacFadyen) and one row of Dancy tangerine (*C. reticulata* Blanco) were chosen for tests. Two linear blocks of

eight consecutive trees each were used in each row, for a total of four blocks in the orchard.

In the first series of experiments, combinations of test chemicals with aqueous AMPu were tested in standard glass McPhail traps (Baker et al., 1944). Six experiments were conducted, each to test one chemical. In five experiments,

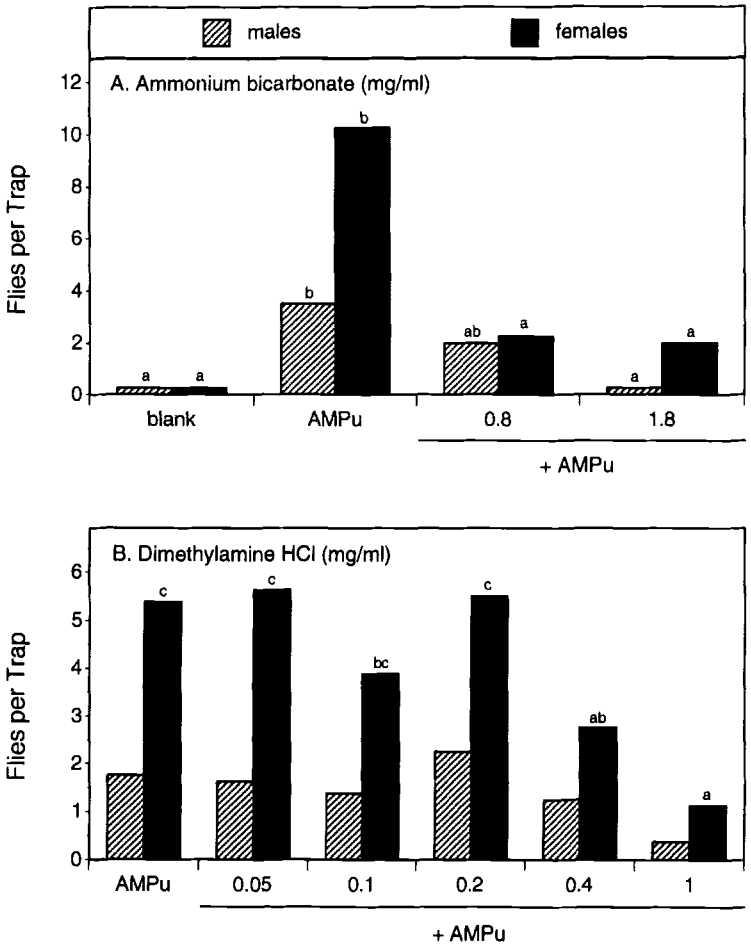


FIG. 1. Mean captures of *A. ludens* in McPhail traps containing AMPu or combinations of AMPu with different concentrations of test chemicals. (A) Ammonium bicarbonate ($N = 4$ replications of each treatment); (B) dimethylamine hydrochloride ($N = 8$). Bars with the same letter, within males or females, are not significantly different by Fisher's protected LSD ($P < 0.05$). Bars without letters indicate a nonsignificant F test.

five concentrations of the test chemical dissolved in 200 ml of AMPu solution were evaluated. Test chemicals were dimethylamine hydrochloride, ethylamine, isoamylamine, 2,5-dimethylpyrazine, and pyrrolidine. In the sixth experiment, only two concentrations of ammonium bicarbonate were evaluated. Concentrations of the six test chemicals are shown in Figure 1-3. Note that the concen-

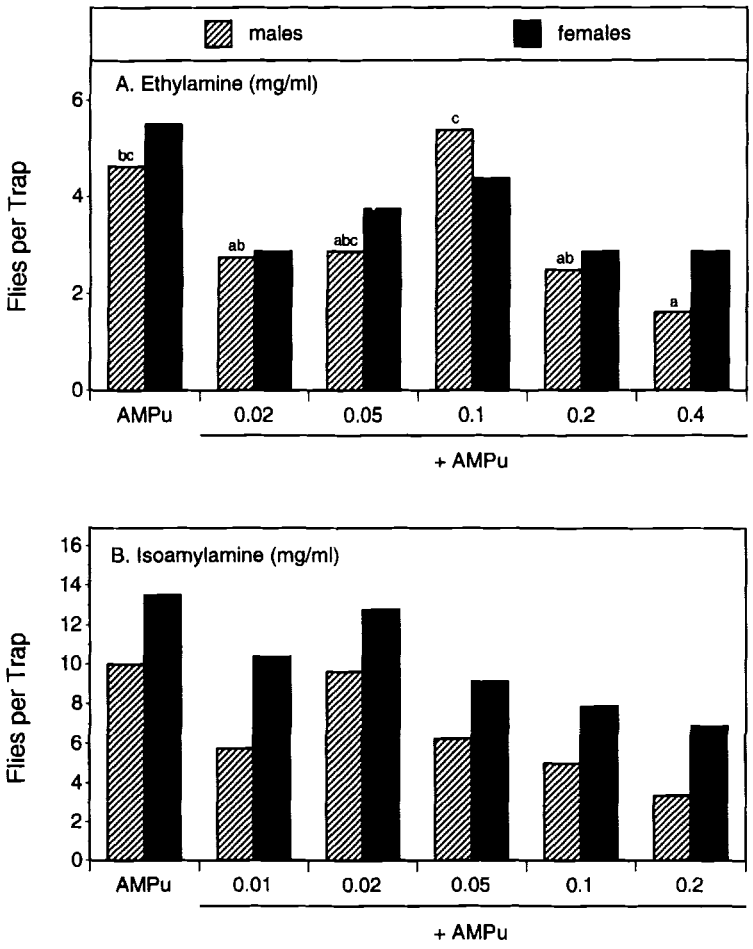


FIG. 2. Mean captures of *A. ludens* in McPhail traps containing AMPu or combinations of AMPu with five concentrations of test chemicals. (A) Ethylamine ($N = 8$ replications of each treatment); (B) isoamylamine ($N = 8$). Bars with the same letter, within males or females, are not significantly different by Fisher's protected LSD ($P < 0.05$). Bars without letters signify a nonsignificant F test.

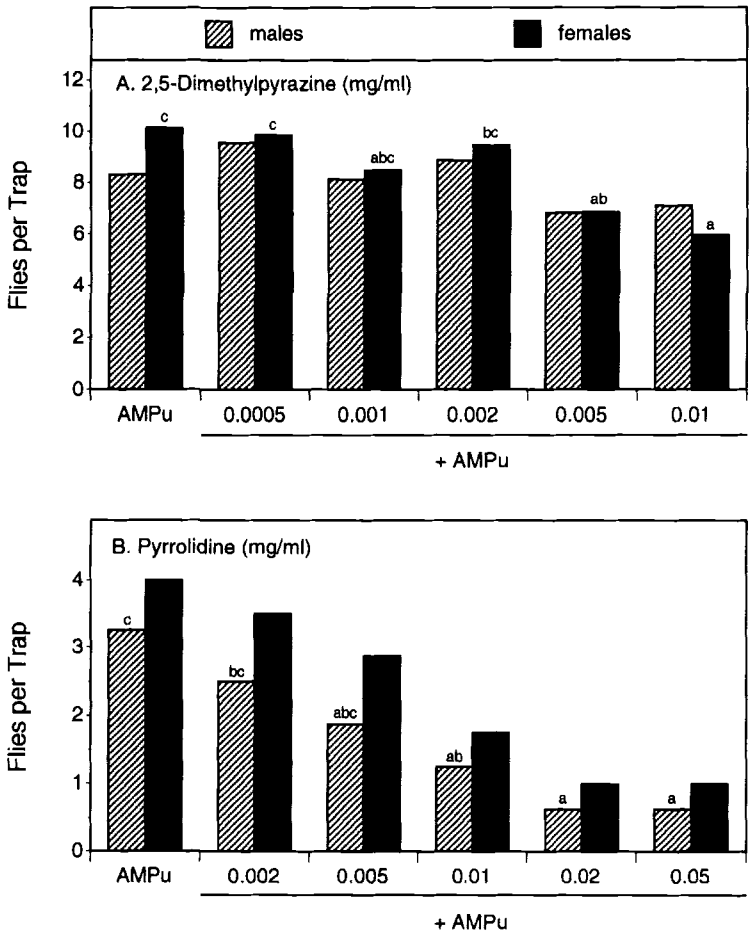


FIG. 3. Mean captures of *A. ludens* in McPhail traps containing AMPu or combinations of AMPu with five concentrations of test chemicals. (A) 2,5-Dimethylpyrazine ($N = 28$ replications of each treatment); (B) pyrrolidine ($N = 8$). Bars with the same letter, within males or females, are not significantly different by Fisher's protected LSD ($P < 0.05$). Bars without letters signify a nonsignificant F test.

trations of ammonium bicarbonate shown in Figure 1 (0.8 and 1.8 mg/ml) are the amounts that were added to that already in AMPu (0.2 mg/ml). A McPhail trap containing AMPu alone was included in each block in all experiments and a McPhail trap containing water alone was included in each block in the ammonium bicarbonate experiment. All traps contained 0.01% Triton X-100 (Rohm and Haas Co., Philadelphia, Pennsylvania) as a surfactant.

Concentrations chosen for testing were based primarily on previous research

that demonstrated the most attractive concentrations of the AMPu components in cage-top bioassays are about five times higher than the most attractive concentration in McPhail traps (Robacker and Warfield, 1993; Robacker, 1995). Therefore, test concentrations generally were centered at about 20% of the most attractive concentrations observed in cage-top bioassays. 2,5-Dimethylpyrazine was tested at lower concentrations than suggested by results of cage-top bioassays (Robacker and Flath, 1995) because preliminary bioassays in a flight chamber indicated these were the most attractive concentrations (D. C. Robacker, unpublished data).

Traps were hung one to a tree, 1–2 m above the ground, on the northwest sides of trees. Traps were placed in the orchard during the morning and removed for fly counts and cleaning on the following morning. Positions of treatments within each block were randomized for the first replication of each experiment. Positions of treatments in consecutive replications were not randomized but were moved sequentially within each block. Flies were released into the test orchard when 4–12 days old during the late afternoon of the day before a test. Approximately 2000 flies were distributed equally among the 32 test trees in the four blocks. The test of ammonium bicarbonate was not replicated over time (N = 4 replicates of each treatment over space); two replications each were conducted for dimethylamine hydrochloride, ethylamine, isoamylamine, and pyrrolidine (N = 8 replicates of each treatment); and seven replications were conducted for 2,5-dimethylpyrazine (N = 28).

In the second series of experiments, combinations of test chemicals with AMPu–agar tubes were tested on Pherocon AM traps (Trece, Inc., Salinas, California). Pherocon AM traps are yellow cardboard panels (14 × 23 cm) with a sticky coating. Four chemicals were evaluated over the course of five experiments. Test chemicals were dimethylamine hydrochloride, ethanolamine, 1-pyrroline, and 3-pyrroline. In most experiments, five concentrations of the test chemical were evaluated in combination with AMPu–agar tubes. All chemicals except 1-pyrroline were tested in this way. In each experiment, the test chemical was formulated into agar in a 1.9-ml microcentrifuge tube by the same method as for AMPu. Tubes containing AMPu and those containing the various concentrations of test chemicals were fastened to opposite sides of the traps. Except for tubes containing aqueous 1-pyrroline solutions, all tubes were fastened to traps with their caps open. The pH of the dimethylamine hydrochloride tubes was adjusted to 8.8 with NaOH. The pH was not changed in the other test chemical tubes. A Pherocon trap containing only an AMPu tube, one containing only a tube with the middle concentration of test chemical, and one containing no bait were included in each block in each of the five experiments described above for a total of eight traps per block.

Two experiments were conducted to evaluate 1-pyrroline. The first experiment was conducted in the same manner as for the other test chemicals except that the 1-pyrroline tubes contained aqueous 1-pyrroline instead of agar for-

mulations. These tubes were fastened to traps with their caps closed but with a pinhole in the middle of the cap. The pH of the 1-pyrroline solutions was not changed from the high pH of the reaction mixture (>12). The second experiment with 1-pyrroline differed from the first in two respects. First, only three concentrations of 1-pyrroline were evaluated and no tube containing 1-pyrroline alone was tested. Second, the 1-pyrroline was not put into separate tubes but

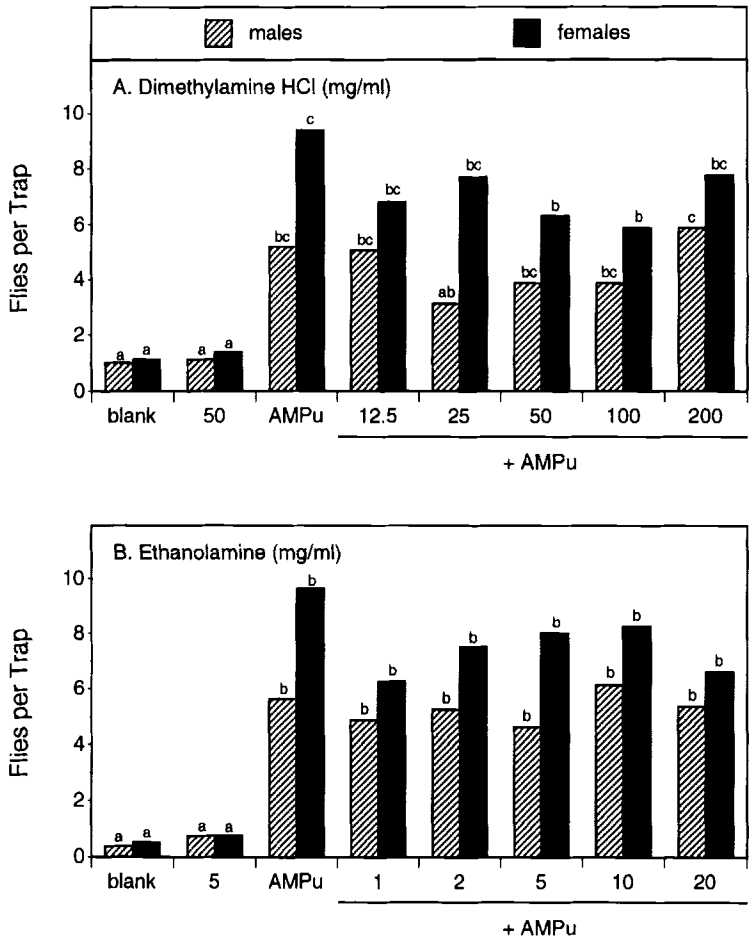


FIG. 4. Mean captures of *A. ludens* on Pherocon AM traps baited with a test chemical, AMPu, or combinations of AMPu with five concentrations of test chemicals. (A) Dimethylamine hydrochloride (N = 16 replications of each treatment); (B) ethanolamine (N = 8). Bars with the same letter, within males or females, are not significantly different by Fisher's protected LSD ($P < 0.05$).

was formulated directly into the AMPu–agar tubes. This was done by mixing 1-pyrroline solution with aqueous AMPu before the hot agar was added to the tubes. The pH of the AMPu–1-pyrroline–agar tubes was adjusted to 8.8 with NaOH. Thus, the pH of the AMPu–1-pyrroline–agar tubes and the AMPu–agar tubes was equal. Concentrations of the four chemicals tested in combination with AMPu/agar tubes are shown in Figures 4–6.

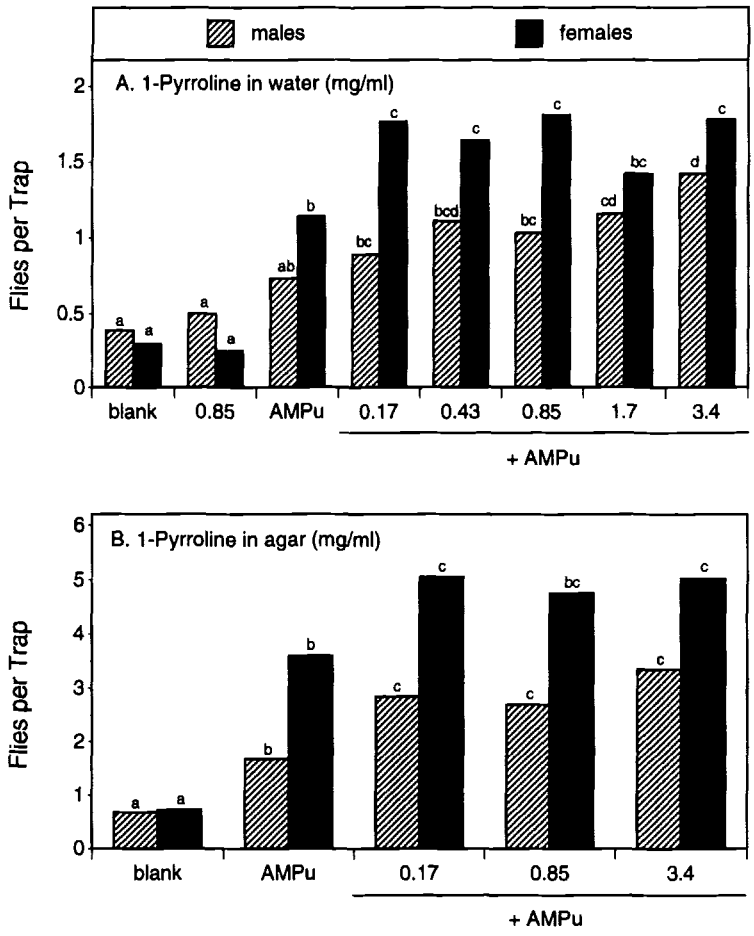


FIG. 5. Mean captures of *A. ludens* on Pherocon AM traps baited with 1-pyrroline, AMPu, or combinations of AMPu with three to five concentrations of 1-pyrroline. (A) 1-Pyrroline in water in one tube and AMPu in agar in a second tube ($N = 64$ replications of each treatment); (B) 1-Pyrroline and AMPu in agar in the same tube ($N = 40$). Bars with the same letter, within males or females, are not significantly different by Fisher's protected LSD ($P < 0.05$).

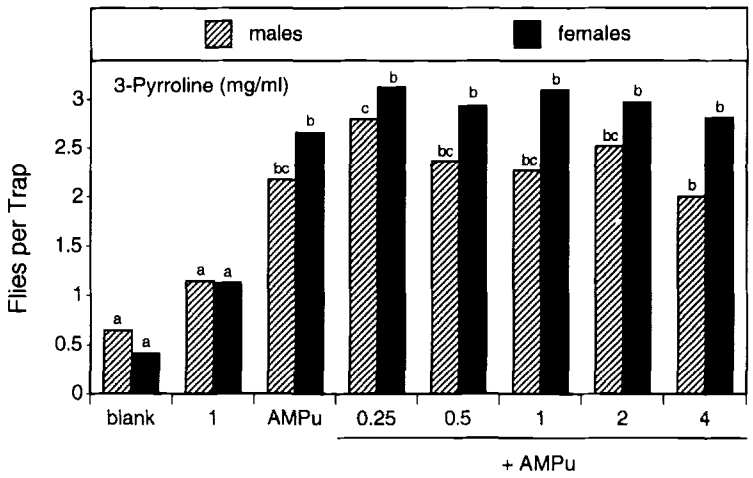


FIG. 6. Mean captures of *A. ludens* on Pherocon AM traps baited with 3-pyrroline, AMPu, or combinations of AMPu with five concentrations of 3-pyrroline (N = 64 replications of each treatment). Bars with the same letter, within males or females, are not significantly different by Fisher's protected LSD ($P < 0.05$).

The concentrations of dimethylamine hydrochloride and ethanolamine chosen for testing in agar formulations were based primarily on previous research that demonstrated that the most attractive concentrations of the AMPu components in agar are about 100 times higher than the most attractive concentrations in cage-top bioassays (Robacker and Warfield, 1993; Robacker, 1995). Therefore, test concentrations of these chemicals were centered at about 100 times the most attractive concentrations observed in cage-top bioassays. 1-Pyrroline was not very attractive at any concentration in cage-top bioassays. The concentrations of 1-pyrroline chosen for testing were based in part on literature that indicated it is a primary odor to human olfaction and thus could be detected at concentrations much lower than for similar chemicals (Amoore et al., 1975). Furthermore, Jang et al. (1994) showed that 1-pyrroline elicited attraction of Mediterranean fruit flies at concentrations much lower than those of other pheromone components. Test concentrations of 3-pyrroline were similar to those of 1-pyrroline.

Orchard testing procedures were the same as those used for chemicals tested in McPhail traps. Four replications over time were conducted for dimethylamine hydrochloride (N = 16 replicates of each treatment), 2 for ethanolamine (N = 8), 16 for 1-pyrroline in aqueous formulation (N = 64), 10 for 1-pyrroline in agar (N = 40), and 16 for 3-pyrroline (N = 64).

Two-way ANOVA testing effects of bait and test day (replication over

time) was conducted for each orchard experiment for captures of both males and females using SuperANOVA (Abacus Concepts, 1989). For the purpose of these analyses, the four blocks in the orchard were treated as trap-bait replications. Means separations were conducted by Fisher's protected least significant difference method. A two-way ANOVA was conducted for the test of 1-pyrroline–AMPu combinations in agar to determine if the combinations attracted males and females in different relative numbers than were attracted by AMPu alone. Data from blank traps were not used in this analysis of trap bait by sex interaction.

RESULTS

Laboratory Bioassays. All chemicals were significantly more attractive than solvent controls at one or more test quantities (Table 1). The most attractive chemicals were 3-pyrroline and spermidine, which were about twice as attractive as controls. 1-Pyrroline and indole 3-acetic acid were the least attractive chemicals.

Orchard Tests. Except for the experiments involving 1-pyrroline and 3-pyrroline, the general outcome of these experiments was that combinations of test chemicals with AMPu were less attractive than AMPu alone. Ammonium bicarbonate–AMPu combinations were much less attractive than AMPu alone (males: $F = 3.0$; $df = 5,18$; $P < 0.05$; and females: $F = 4.5$; $df = 5,18$; $P < 0.01$) (Figure 1A). Combinations of AMPu with the higher concentrations of dimethylamine hydrochloride, ethylamine, isoamylamine, 2,5-dimethylpyrazine, pyrrolidine, and ethanolamine generally captured fewer flies, especially

TABLE 1. MEAN COUNTS OF *A. ludens* AT SOLVENT BLANKS OR TEST CHEMICALS USING CAGE-TOP BIOASSAYS

	10 ng		100 ng		1 μg		10 μg		100 μg	
	Test	Solvent	Test	Solvent	Test	Solvent	Test	Solvent	Test	Solvent
1-Pyrroline	27.2	24.3	22.5	22.9	22.2	24.0	29.2[a]	26.1		
3-Pyrroline	17.3	18.0	16.3	13.6	19.0[a]	12.7	32.9[a]	16.5		
2-(Methylamino) ethanol	10.2	12.6	12.5	10.3	14.6	14.0	16.5[a]	10.8		
Spermidine	11.4	13.3	15.7	11.6	19.2[a]	11.9	20.7[a]	10.7		
Spermine			15.0	16.8	18.2	18.0	21.5	17.4	27.2[a]	16.7
Indole 3-acetic acid			13.4	14.6	18.2	17.1	17.9	16.8	17.5[a]	13.1

[a]The mean response to a chemical was significantly different from the response to a solvent control by paired t tests ($P < 0.05$).

females, than AMPu alone (Figures 1–4). ANOVAs were significant for captures of females in the test of dimethylamine hydrochloride in McPhail traps ($F = 4.7$; $df = 5,41$; $P < 0.01$), males in the ethylamine experiment ($F = 2.4$; $df = 5,41$; $P < 0.05$), females in the 2,5-dimethylpyrazine experiment ($F = 3.0$; $df = 5,156$; $P < 0.05$), males in the pyrrolidine experiment ($F = 3.7$; $df = 5,41$; $P < 0.05$), males and females in the test of dimethylamine hydrochloride in agar formulations (males: $F = 5.6$; $df = 7,117$; $P < 0.001$; and females: $F = 7.8$; $df = 7,117$; $P < 0.001$), and males and females in the ethanolamine experiment (males: $F = 5.6$; $df = 7,55$; $P < 0.001$; and females: $F = 7.1$; $df = 7,55$; $P < 0.001$). In some cases such as ethanolamine, no individual combinations were significantly less attractive than AMPu alone despite large ANOVA F values. Traps baited with dimethylamine hydrochloride alone or ethanolamine alone (Figure 4) were not significantly more attractive than blank traps. Ammonium bicarbonate, ethylamine, isoamylamine, 2,5-dimethylpyrazine, and pyrrolidine were not tested alone.

Combinations of 1-pyrroline with AMPu were significantly more attractive to flies than AMPu alone. This was true whether 1-pyrroline was tested in a separate tube from the AMPu (Figure 5A) or mixed into the AMPu/agar formulation (Figure 5B). Traps baited with combinations of AMPu and 1-pyrroline in separate tubes captured 53% more males and 48% more females than AMPu-alone traps, summed over all 1-pyrroline concentrations. Likewise, traps baited with combinations of AMPu and 1-pyrroline mixed in agar in the same tube captured 77% more males and 37% more females than AMPu-alone traps. The relative attractiveness of the combinations to males and females was not significantly different from the relative attractiveness of AMPu alone (bait × sex interaction from the experiment in which 1-pyrroline was mixed with AMPu in agar: $F = 0.004$; $df = 1,316$; $P = 0.95$). ANOVAs were highly significant for both males and females in the experiment with separate tubes (males: $F = 6.3$; $df = 7,489$; $P < 0.0001$; and females: $F = 13.2$; $df = 7,489$; $P < 0.0001$), and in the experiment with 1-pyrroline mixed into the agar (males: $F = 13.3$; $df = 4,186$; $P < 0.001$; and females: $F = 15.1$; $df = 4,186$; $P < 0.001$). Traps baited with 1-pyrroline alone were not significantly more attractive than blank traps.

Combinations of 3-pyrroline and AMPu were not significantly more attractive than AMPu alone (Figure 6), although these combinations captured 10% more males and 12% more females than AMPu alone. Traps baited with 3-pyrroline alone were not significantly more attractive than blank traps.

DISCUSSION

Previous research with *A. ludens* has demonstrated that combinations of attractants from different attractant systems generally are less attractive than the

more potent of the two attractants alone (Robacker, 1993). In this context, an attractant system is defined as a group of chemicals, receptors, and central nervous system centers that function together in producing behavior related to some biological function. Two examples were combinations of pheromones (sexual behavior system) with host-fruit odor (sugar-feeding system), in which combinations were less attractive than pheromone alone to sexually active female flies (Robacker and Garcia, 1990), and host-fruit odor with bacterial odor (protein-feeding system), in which combinations were less attractive than host-fruit odor to sugar-hungry flies (Robacker, 1991). However, combining attractive chemicals from the same system, such as hexanol and ethyl hexanoate identified from host-fruit volatiles, usually has additive effects on attractiveness (Robacker et al., 1990). The combinations used in this study would seem to be of the latter type because all the chemicals tested, including AMPu, are attractive to sugar-fed, protein-starved flies (Robacker and Warfield, 1993; Robacker and Flath, 1995). Nevertheless, many of the combinations of these chemicals with AMPu were less attractive than AMPu alone.

The explanation for the very low attractiveness of the AMPu/ammonium bicarbonate combinations may be as simple as irritation caused by high concentrations of ammonia on various receptor types. One possible explanation for the low attractiveness of the other combinations could be competition for the same olfactory receptor. This seems likely for dimethylamine and ethylamine, which may compete for the same receptor sites as methylamine. The same explanation was proposed for two pheromone components, (*Z*)-3-nonenol and (*Z*,*Z*)-3,6-nonadienol, each of which synergized the effect of a third pheromone component, but the first alcohol inhibited the effect of the latter when they were present together (Robacker and Hart, 1985). A similar hypothesis can be offered for some of the other chemicals, although their structures differ enough from the AMPu chemicals that other explanations may be more appropriate.

Combinations of 1-pyrroline with AMPu were more attractive than AMPu alone even though 1-pyrroline was nearly unattractive by itself. 1-Pyrroline is the only semiochemical studied to date that is almost unattractive by itself but that synergizes attractiveness of another set of chemicals that are attractive to Mexican fruit flies engaged in food-finding behavior.

1-Pyrroline appears to be a rather ubiquitous naturally occurring chemical as its odor is present in many products of plant and animal origin, probably as a result of enzymatic degradation of certain amino acids and spontaneous oxidation of various amino acid metabolites (Amoore et al., 1975). 1-Pyrroline is important to human olfaction as a primary odor (Amoore et al., 1975), and it has been identified as an attractive component of male-produced volatiles of the Mediterranean fruit fly (Baker et al., 1985). Interestingly, Jang et al. (1994) reported that the attractiveness to sexually active female Mediterranean fruit flies of 1-pyrroline by itself is small, but it synergizes the attractiveness of other pheromonal components.

Neither 3-pyrroline nor pyrrolidine performed the same way as 1-pyrroline despite structural similarities. Both were considerably more attractive than 1-pyrroline when tested alone, and neither significantly increased the attractiveness of AMPu in orchard tests. Pyrrolidine actually decreased the attractiveness of AMPu, a result similar to one observed when pyrrolidine decreased the attractiveness of a more complex mixture of amines and acids in laboratory experiments (Robacker and Warfield, 1993). The results suggest that the imine (−N=CH−) functionality of 1-pyrroline may be a critical molecular feature in olfactory reception. However, the low pK_a (6.7) of 1-pyrroline (Amoore et al., 1975) may also account for its greater attractiveness than the strongly basic 3-pyrroline (pK_a 10.5) (Amoore et al., 1975) because at pH 8.8 (pH at which 3-pyrroline was tested) it would exist mostly in the ionized, nonvolatile form. In contrast, 1-pyrroline (tested at pH 8.8 or higher) would exist almost entirely in the nonionized, volatile form. Thus, the concentration of 1-pyrroline evolving from the preparations should be greater than that of 3-pyrroline at a given concentration in solution. Pyrrolidine, like 3-pyrroline, is a strong base (pK_a 11.0) (The Merck Index, 1983) and would be mostly in the ionized, nonvolatile form at pH 8.8 at which it was tested. However, pyrrolidine was repellent in the largely ionized form and increasing its concentration in the air probably would only increase its repellency.

AMPu has already proven as attractive as *Torula* yeast to Mexican fruit flies in citrus orchard tests (Robacker, 1995). Results of this work suggest that attractiveness of AMPu could be enhanced by addition of 1-pyrroline. Although 1-pyrroline has been reported as unstable in solution (Nomura et al., 1977), 1-pyrroline trimer in neat form is stable and evolves 1-pyrroline monomer as an evaporation product (Baker et al., 1992). Consequently, it may be possible to develop a formulation of AMPu and 1-pyrroline trimer that would emit the four attractive components at stable rates for periods of perhaps a month or more.

Acknowledgments—We thank Maura Rodriguez and Cyndi Rodriguez for technical assistance; Sammy Ingle for insects; and USDA-APHIS (Mission, Texas) for irradiation of pupae. We also thank Ken Hagen (Professor of Entomology, Emeritis, University of California, Berkeley) for discussion during the developmental phase of the work, and Dr. Chang-Joo Lee (USDA-ARS, Insect Chemical Ecology Laboratory, Beltsville, Maryland) for useful criticism of the manuscript. This work was partially supported by a grant from the Citrus Research Board (Visalia, California, Project 58-6204-4-038). Use of a product brand in this work does not constitute an endorsement by the USDA.

REFERENCES

ABACUS CONCEPTS. 1989. SuperANOVA. Abacus Concepts, Berkeley, California.

AMOORE, J. E., FORRESTER, L. J., and BUTTERY, R. G. 1975. Specific anosmia to 1-pyrroline: The spermous primary odor. *J. Chem. Ecol.* 1:299–310.

BAKER, A. C., STONE, W. E., PLUMMER, C. C., and MCPHAIL, M. 1944. A review of studies on the Mexican fruitfly and related Mexican species. U.S. Department of Agriculture Misc. Publ. 531.

BAKER, J. D., HEATH, R. R., and MILLAR, J. G. 1992. An equilibrium and stability study of Δ^1-pyrroline. *J. Chem. Ecol.* 18:1595–1602.

BAKER, R., HERBERT, R. H., and GRANT, G. G. 1985. Isolation and identification of the sex pheromone of the Mediterranean fruit fly, *Ceratitis capitata* (Wied.). *J. Chem. Soc. Chem. Commun.* 1985:824–825.

DREW, R. A. I., and LLOYD, A. C. 1989. Bacteria associated with fruit flies and their host plants, pp. 131–140, *in* A. S. Robinson and G. Hooper (eds.). Fruit Flies, Their Biology, Natural Enemies and Control, Vol. 3A. Elsevier, New York.

FLATH, R. A., JANG, E. B., LIGHT, D. M., MON, T. R., CARVALHO, L., BINDER, R. G., and JOHN, J. O. 1993. Volatile pheromonal emissions from the male Mediterranean fruit fly: Effects of fly age and time of day. *J. Agric. Food Chem.* 41:830–837.

HEATH, R. R., EPSKY, N. D., GUZMAN, A., DUEBEN, B. D., MANUKIAN, A., and MEYER, W. L. 1995. Development of a dry plastic insect trap with food-based synthetic attractant for the Mediterranean and Mexican fruit flies (Diptera: Tephritidae). *J. Econ. Entomol.* 88:1307–1315.

JANG, E. B., LIGHT, D. M., BINDER, R. G., FLATH, R. A., and CARVALHO, L. A. 1994. Attraction of female Mediterranean fruit flies to the five major components of male-produced pheromone in a laboratory flight tunnel. *J. Chem. Ecol.* 20:9–20.

LEHNINGER, A. L. 1970. Biochemistry. Worth Publishers, New York, 833 pp.

MILLER, M. W. 1961. The Pfizer Handbook of Microbial Metabolites. McGraw-Hill, New York, 772 pp.

NOMURA, Y., OGAWA, K., TAKEUCHI, Y., and TOMODA, S. 1977. One-step synthesis and structural confirmation of 1-pyrroline trimer. *Chem. Lett.* 1977:693–696.

ROBACKER, D. C. 1991. Specific hunger in *Anastrepha ludens* (Diptera: Tephritidae): Effects on attractiveness of proteinaceous and fruit-derived lures. *Environ. Entomol.* 20:1680–1686.

ROBACKER, D. C. 1993. Understanding olfactory attraction in *Anastrepha* using *A. ludens* as a model system, pp. 201–206, *in* M. Aluja and P. Liedo (eds.). Fruit Flies: Biology and Management. Springer-Verlag, New York.

ROBACKER, D. C. 1995. Attractiveness of a mixture of ammonia, methylamine and putrescine to Mexican fruit flies (Diptera: Tephritidae) in a citrus orchard. *Fla. Entomol.* 78:571–578.

ROBACKER, D. C., and FLATH, R. A. 1995. Attractants from *Staphylococcus aureus* cultures for Mexican fruit fly, *Anastrepha ludens*. *J. Chem. Ecol.* 21:1861–1874.

ROBACKER, D. C., and GARCIA, J. A. 1990. Responses of laboratory-strain Mexican fruit flies, *Anastrepha ludens*, to combinations of fermenting fruit odor and male-produced pheromone in laboratory bioassays. *J. Chem. Ecol.* 16:2027–2038.

ROBACKER, D. C., and GARCIA, J. A. 1993. Effects of age, time of day, feeding history, and gamma irradiation on attraction of Mexican fruit flies (Diptera: Tephritidae), to bacterial odor in laboratory experiments. *Environ. Entomol.* 22:1367–1374.

ROBACKER, D. C., and HART, W. G. 1985. (*Z*)-3-Nonenol, (*Z*,*Z*)-3,6-nonadienol and (*S*,*S*)-(−)-epianastrephin: Male produced pheromones of the Mexican fruit fly. *Entomol. Exp. Appl.* 39:103–108.

ROBACKER, D. C., and MORENO, D. S. 1995. Protein feeding attenuates attraction of Mexican fruit flies (Diptera: Tephritidae) to volatile bacterial metabolites. *Fla. Entomol.* 78:62–69.

ROBACKER, D. C., and WARFIELD, W. C. 1993. Attraction of both sexes of Mexican fruit fly, *Anastrepha ludens*, to a mixture of ammonia, methylamine, and putrescine. *J. Chem. Ecol.* 19:2999–3016.

ROBACKER, D. C., TARSHIS MORENO, A. M., GARCIA, J. A., and FLATH, R. A. 1990. A novel attractant for Mexican fruit fly, *Anastrepha ludens*, from fermented host fruit. *J. Chem. Ecol.* 16:2799–2815.

ROBACKER, D. C., WARFIELD, W. C., and ALBACH, R. F. 1993. Partial characterization and HPLC isolation of bacteria-produced attractants for the Mexican fruit fly, *Anastrepha ludens*. *J. Chem. Ecol.* 19:543–557.

SCHOPF, C., and OECHLER, F. 1936. Die Synthese des desoxyvasicins unter physiologischen bedingungen. *Ann. Chem.* 523:1–29.

The Merck Index. 1983. Merck & Co., Inc., Rahway, New Jersey.

WAKABAYASHI, N., and CUNNINGHAM, R. T. 1991. Four-component synthetic food bait for attracting both sexes of the melon fly (Diptera: Tephritidae). *J. Econ. Entomol.* 84:1672–1676.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

ELECTROPHYSIOLOGICAL AND BEHAVIORAL EVIDENCE FOR A SEX PHEROMONE IN THE WASP *Bephratelloides pomorum*

WALTER SOARES LEAL,[1,*] JOSE INACIO LACERDA MOURA,[2] JOSE MAURICIO S. BENTO,[3] EVALDO FERREIRA VILELA,[3] and PIERRE BARBOSA PEREIRA[2]

[1]*Laboratory of Chemical Prospecting*
National Institute of Sericultural and Entomological Science (NISES)
1-2 Ohwashi, Tsukuba 305 Japan

[2]*CEPLAC/CEPEC, Km 22 Rodovia Ilheus-Itabuna*
CP 7, Itabuna-BA, Brazil

[3]*Departamento de Biologia Animal*
Universidade Federal de Viçosa
36570-000, Viçosa-MG, Brazil

(Received August 7, 1996; accepted December 17, 1996)

Abstract—Field experiments showed that males of the seed-feeding, Neotropical wasp *Bephratelloides pomorum* are strongly attracted to females. That this attraction was semiochemically mediated was demonstrated by both the response of male antennae to the whole-body extract of females and by behavioral bioassays. In the field, males were captured in traps baited with female thoraces, but not with other body parts (abdomen and head). Volatiles collected from the headspace of female wasps or whole-body extracts, concentrated and transferred to cotton plugs, elicited a full sequence of sexual behavior in male wasps. The attraction of males to female-baited traps showed a peak of activity at noon, but in indoor bioassays male responded to female extracts even at night.

Key Words—*Bephratelloides pomorum*, *B. cubensis*, *Annona muricata*, portable EAG, sex pheromone, Hymenoptera, Eurytomidae.

INTRODUCTION

Since the milestone identification of bombykol as a sex pheromone for the silk moth, *Bombyx mori* (Butenandt et al., 1961), well over 500 sex pheromones

*To whom correspondence should be addressed.

0098-0331/97/0500-1281$12.50/0 © 1997 Plenum Publishing Corporation

have been identified (Mayer and McLaughlin, 1991) and evidence for their occurrence in various species has been well documented. While members of the Lepidoptera are by far the most thoroughly studied, there are other groups for which our knowledge of chemical communication is extremely scanty. This discrepancy, partially dictated by the economic importance of the insects studied, is observed even within certain orders. In the Hymenoptera, for example, pheromone research has been focused mainly on social insects (honeybees and ants), whereas chemical communication is terra incognita for other families. The family Eurytomidae (Hymenoptera) presents some interesting cases because the occurrence of sex pheromones is not obvious in some species. For example, one representative of the group, *Bephratelloides cubensis*, is thelytokous; males are extremely rare and daughters are produced parthenogenetically (Nadel and Peña, 1991).

Another representative of the family, the seed-feeding Neotropical wasp *Bephratelloides pomorum* (Fabricius) reproduces sexually and is an economically important pest in Honduras, Costa Rica, Panama, Trindad Tobago, Colombia, Venezuela, Ecuador, Peru, Bolivia, Belize, Surinam, French Guiana, and Brazil (Griessell and Schauff, 1990). It is a small wasp (body length 5–10 mm) that causes serious losses to commercial production of atemoyas (*Annona* species). In Brazil, this pest is so devastating on *A. muricata* that the commercial production of this plant is extremely dependent on a new strategy for control. *B. pomorum* lays eggs singly in seeds; larvae feed on the endosperm and accelerate the plants' metabolism. Pupation occurs in the seed, and adults create a tunnel from the seed through the fruit tissues. Infested fruits are 100% lost to the fresh fruit market and can be commercialized only as pulp, but even so the losses are as high as 80% (Moura and Pereira, unpublished data).

Because the wasp is well protected inside the seeds, it cannot be controlled with conventional insecticides. This scenario prompted us to investigate the feasibility of using semiochemicals for the control of *B. pomorum* in order to save the commercial production of *A. muricata* in Brazil.

We describe here electrophysiological and behavioral evidence that *B. pomorum* reproduces sexually and male attraction to females is semiochemically mediated.

METHODS AND MATERIALS

Insects. Virgin females and unmated males of *B. pomorum* were collected from seeds of *A. muricata* obtained from a farm, Fazenda Esperança, in Eunápolis-BA, Brazil. The wasps were segregated by sex, placed inside plastic bottles (1.5 liters, 30 wasps per bottle) with wet pieces of cotton. They were

kept at room temperature and under field light conditions and were used within three days.

Electroantennogram (EAG). A new portable battery-operated EAG system was assembled (Figure 1). Excised wasp antennae were placed on a previously described acrylic stage (Leal et al., 1992). Electrodes were connected to the amplifier, and the stage was placed inside a glass tube 2 cm away from the source of the stimuli. Air (300 ml/min) was pumped through with a battery-operated mini pump (MP-05CF, Shibata, Tokyo, Japan). The output of the amplifier was connected to a multivoltimeter equipped with minimal/maximal

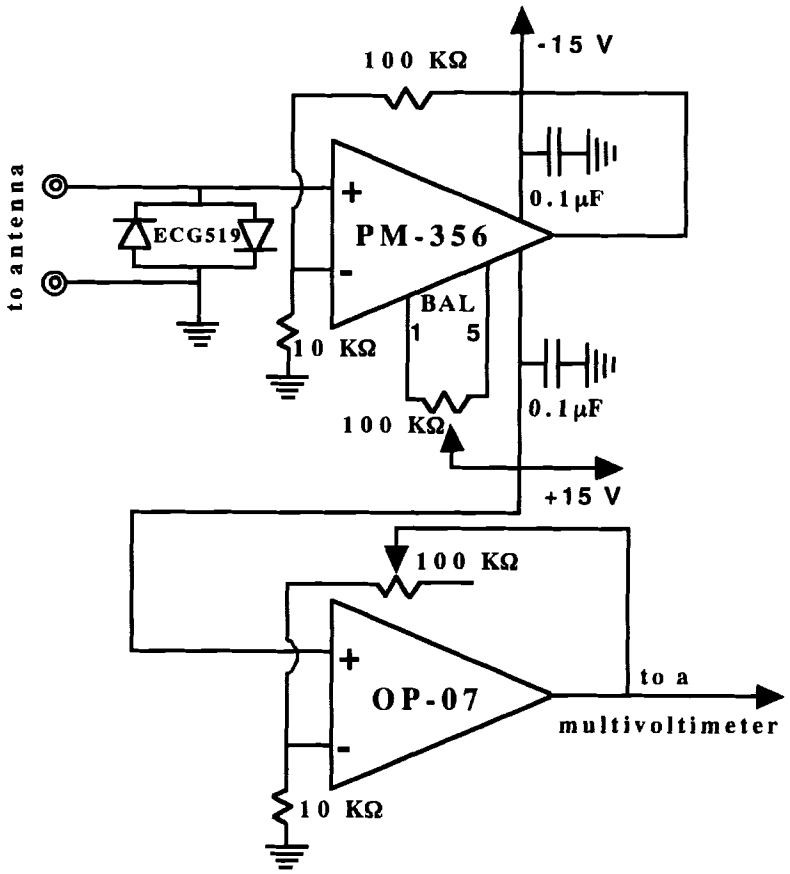


FIG. 1. Circuit diagram of a battery-operated EAG system used for measurements in the field.

hold function (BK Test Branch 389; Chicago, Illinois). Before each stimulus was applied, the multivoltimeter was reset and the maximal voltage was recorded. The test samples were loaded in disposable Pasteur pipets and introduced into the airflow with a silicone bulb (3 ml). Antennal responses to different stimuli were recorded with the same antenna (10 repetitions) and replicated with different antennae ($N = 3$–4).

Pheromone Collections. Whole-body extracts were obtained by washing the wasps with hexane for 3–5 min followed by filtration and concentration. Airborne volatiles were collected in the field in an all-glass aeration apparatus. Airflow (1 l/min) was obtained with a water aspirator. Air was passed through an activated charcoal column and humidified before entering the chamber. Volatiles trapped on Super Q (Alltech, Deerfield, Illinois) were extracted with hexane (10 ml) and concentrated to 0.1 insect-equivalent per day per microliter.

Indoor Bioassays. Responses of *B. pomorum* males to extracts were observed inside cylindrical plastic tubes (12 cm long, 2.5 cm ID), containing lateral openings (about 1 mm ID). Five to 10 males were placed inside each arena and their responses ($N = 3$) to cotton plugs impregnated with extracts or hexane only were observed for 15 min under field conditions and still air.

Field Experiments. Field tests were conducted at the Fazenda Esperança, Eunápolis-BA, Brazil, in a 12-ha plantation of *A. muricata.* Traps were made of waterproof paper (20 × 20 cm) coated on both sides with adhesive polybutadiene. An insect cage made of a plastic tube (2.5 × 5 cm) and closed at both sides with cheese cloth was placed in the center of the trap. Attraction to traps was observed with three repetitions for each of the following treatments: caged males, caged females, and unbaited traps. Male and female traps were baited with three, six, or nine individuals per cage. Daily activity of wasp sex attraction in the field was quantified with three traps, each containing six caged females placed in the field at 7:00 AM and observed every hour until 7:00 PM (February 1995). Field temperature and relative humidity were also recorded. For the identification of the body part involved in the sex pheromone release, 10 females were sectioned into head, thorax, and abdomen and these parts were placed directly on one surface of the sticky traps. The experiments were carried out with one trap per treatment per day for four consecutive days, with the treatment being renewed every day. Traps were placed at 1.5 m above the ground with an intertrap distance of 7 m. Capture data were transformed to log $(x + 1)$ before differences among means were tested for significance by ANOVA with the JMP Software, Version 2 (Anonymous, 1989). Treatments followed by the same letters are not significantly different at the 5% level in the Tukey-Kramer honestly significant difference test.

Analytical Procedures. Gas chromatography (GC) was carried out on a Hewlett-Packard 5890 II Plus instrument equipped with a split/splitless injector, electronic pressure control, a flame ionization detector, and an HP 3365 Series

II Chemstation. High-resolution GC analyses were performed with polar and nonpolar capillary columns: HP-Innowax and HP-5MS (30 m × 0.25 mm × 0.25 μm; Hewlett-Packard), respectively. These columns were operated at 70°C for 1 min, increased to 230°C at a rate of 10°C/min, and held at this temperature for 10 min. Low-resolution mass spectrometry (MS) was carried out with an HP 5890 II Plus gas chromatograph linked to a mass selective detector MSD 5972 (Hewlett-Packard). Chromatographic resolution was achieved on a HP-Innowax column operated under the same conditions as described above. Mass spectrometry was also performed using a Hewlett-Packard gas chromatograph electron ionization detector GCD Series, equipped with a HP-5MS capillary column that was operated at 50°C for 1 min, increased to 150°C at a rate of 5°C/min, held at this temperature for 1 min, increased again to 230°C at a rate of 10°C/min, and finally held at this temperature for 10 min.

RESULTS AND DISCUSSION

Field experiments with wasp-baited traps showed that males were strongly attracted to virgin females (Figure 2). Under the same conditions, traps baited with males captured neither females nor males. Throughout the experiments, not a single wasp was captured in either male-baited or control traps.

Two lines of evidence demonstrated that the female attraction was chemically mediated. First, electrophysiological experiments conducted in the field

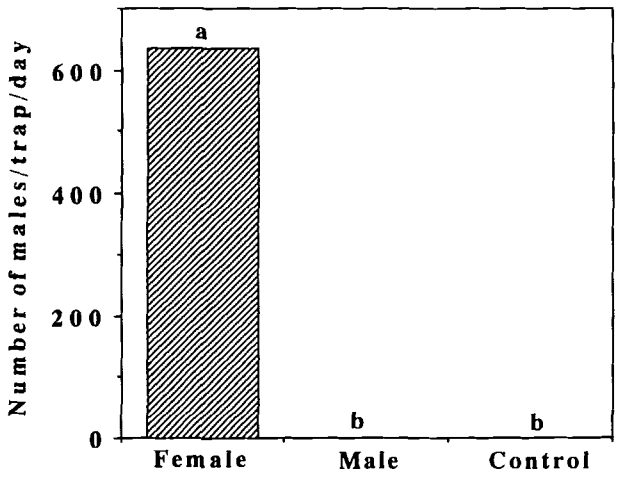


FIG. 2. Captures of male *B. pomorum* in traps baited with female or male wasps and in control traps.

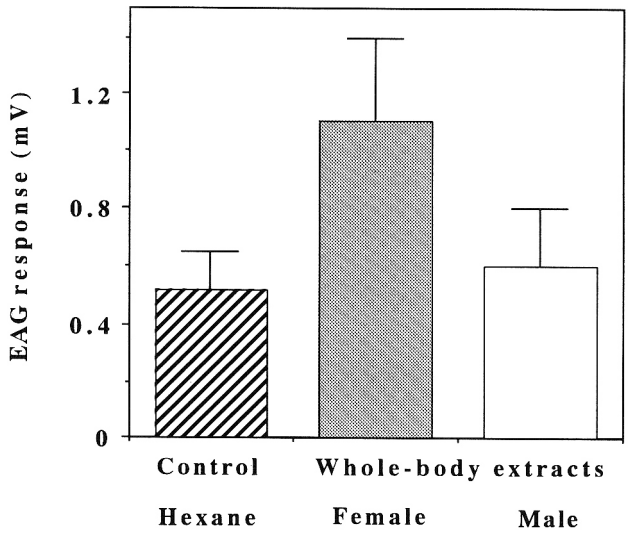


FIG. 3. Electroantennogram responses of antennae of male *B. pomorum* to whole-body extracts of female and male wasps. The response to male extract did not differ from that to control (hexane only). Bars represent SEM.

showed that male antennae gave significant response to female whole-body extract, but the response to male whole-body extract was not significantly different from the control (Figure 3). Male antennae responded also to the airborne volatiles from females, but not to the volatiles collected from males. Secondly, males responded behaviorally to the extracts from females both in a preliminary field test as well as in indoor bioassays. When a piece of filter paper (2 × 2 cm) was impregnated with 10 insect-equivalents of the female whole-body extract and placed on an atemoya leaf, males resting on the fruits in the vicinity started to search for females and were attracted to the source. Likewise, males displayed a clear sexual behavior in an indoor bioassay. They were not only attracted to cotton balls impregnated with 0.1 female-equivalent of the airborne volatiles or whole-body extracts, but also tried to copulate with the cotton plugs (Figure 4).

In order to identify the body part(s) involved in the sex pheromone release, male attraction to traps baited with different body parts was investigated in the field. Male response to thorax-baited traps was clear-cut, but traps baited with heads or abdomens did not capture any wasps (Figure 5). Interestingly, males tried to copulate with thoraces even a few hours after they were excised.

Airborne volatiles and whole-body extracts of both males and females were prepared in Brazil and brought to Japan for chemical analyses. Gas chromatog-

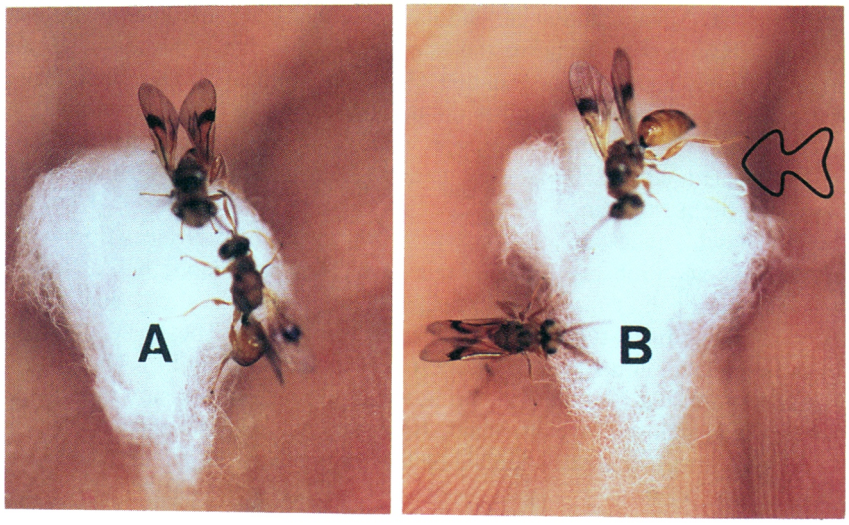


FIG. 4. Response of *B. pomorum* males to a piece of cotton impregnated with female extract. Males were attracted, searched the surface of the bait (A), and raised their wings, and bent their abdomen (B, arrow) in attempt to copulate with the source.

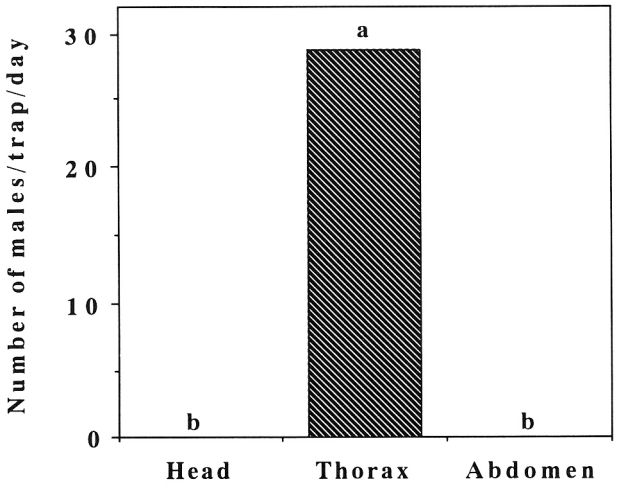


FIG. 5. Catches of male *B. pomorum* in traps baited with parts of the female body.

raphy and GC-MS measurements did not show any particular chemical dimorphism. This is probably due to the occurrence of active semiochemical(s) at very low levels and/or to degradation of the pheromone(s). The identification of the active peak(s) would require the utilization of a sensitive technique such as GC-EAD. First attempts to import male wasps into Japan for GC-EAD measurements (and other bioassays) were unsuccessful because all insects died during the flight to Japan.

In the field, males are frequently found resting on fruit surfaces from which females emerge. Probably, females release the sex pheromone while chewing their way out of the fruit. Upon emergence, the female is highly attractive to males, and one male succeeds in mounting the female. They take flight and when the female returns to oviposit, no males are attracted to her. The fact that males are "arrested" on the fruit surface may give the impression that the sex pheromone is a close-range attractant. However, field experiments showed that males were also captured in female-baited traps placed as far as 25 m away from the host plant.

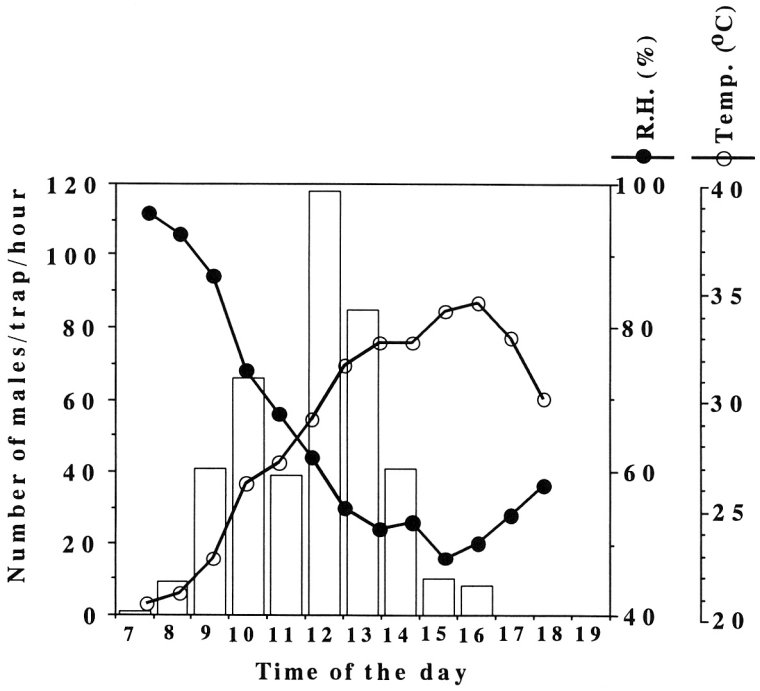


FIG. 6. Hourly fluctuation patterns of *B. pomorum* male captures in the field in traps baited with live females. Relative humidity (R.H.) and relative temperature are also displayed.

The hourly fluctuation pattern of *B. pomorum* in the field was studied with female-baited traps. Activity was observed from 8:00 AM to 4:00 PM with a peak at noon (Figure 6), but in indoor bioassays, males responded to female-extracts even at 7:00 PM. Under natural conditions, male activity is probably controlled by female release of the sex pheromone, and the male ability to respond to the stimulus does not seem to be limited to a narrow time window.

Although the sex ratio of the wasp in the field throughout the year is yet to be investigated, a random collection of wasps obtained on December 19–22, 1995, by opening seeds of the host plant gave a higher number of females. It is worth noting, however, that some fruits house only male wasps, whereas in other fruit, females are predominant (80% or more). Therefore, it is reasonable to assume that females need a long-range pheromone to attract males.

In conclusion, electrophysiological and behavioral evidence supports the occurrence of a long-range, female-released sex pheromone in *B. pomorum*. Work is now in progress to chemically characterize the semiochemical(s).

Acknowledgments—This research was supported in part by a special coordination fund for promoting science and technology by the Science and Technology Agency, Japan granted to W.S.L. We are indebted to Kazuaki Kamijo and Nelson W. Perioto (Instituto Biológico, Ribeirão Preto-São Paulo) for their independent taxonomic identification of the wasp in Japan and Brazil, respectively. We thank also Kenjiro Kawazaki, Hubert Wojtasek, and Daniel Ibarra for critical review of the manuscript.

REFERENCES

ANONYMOUS. 1989. Software for Statistical Visualization on the Apple Macintosh. JMP Introductory Guide. SAS Institute, Inc., Cary, North Carolina.

BUTENANDT, A., BECKMANN, R., and STAMM, D. 1961. Über den Sexuallockstoff des Seidenspinners, II. Konstitution und konfiguration des Bombykols. *Hopper-Seyler's Z. Physiol. Chem.* 324:84–87.

GRIESSEL, E. E., and SCHAUFF, M. E. 1990. A synopsis of the seed-feeding genus *Bephratelloides* (Chalcidoidea: Eurytomidae). *Proc. Entomol. Soc. Wash.* 92:177–187.

LEAL, W. S., MOCHIZUKI, F., WAKAMURA, S., and YASUDA, T. 1992. Electroantennographic detection of *Anomala cuprea* Hope (Coleoptera: Scarabaeidae) sex pheromone. *Appl. Entomol. Zool.* 27:289–291.

MAYER, M. S., and MCLAUGHLIN, J. R. 1991. Handbook of Insect Pheromones and Sex Attractants, CRC, Boca Raton, Florida.

NADEL, H., and PEÑA, J. E. 1991. Seasonal oviposition and emergence activity of *Bephratelloides cubensis* (Hymenoptera: Eurytomidae), a pest of *Annona* species in Florida. *Environ. Entomol.* 20:1053–1057.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

CUTICULAR HYDROCARBONS OF *Glossina*, III: SUBGENERA *Glossina* AND *Nemorhina*

BRUCE D. SUTTON[1] and DAVID A. CARLSON[2,*]

[1]*Division of Plant Industry, Florida Department of Agriculture & Consumer Services Gainesville, Florida 32614*

[2]*USDA-ARS, Center for Medical, Agricultural and Veterinary Entomology Gainesville, Florida 32604*

(Received June 20, 1995; accepted December 19, 1996)

Abstract—The cuticular methyl-branched alkanes of tsetse flies of the subgenera *Glossina* (sensu stricto, formerly *morsitans*) and *Nemorhina* (formerly *palpalis*) were identified and quantified by capillary gas–liquid chromatography (GC) and gas chromatography–mass spectrometry (GC-MS). Males of *Glossina* (*Nemorhina*) are differentiated from *G.* (*Glossina*) by dominant 27-, 28-, and/or 29-carbon backbone trimethylalkanes with the methyl positions at 3,7,11-, 4,8,12-, and 3,7,11-, respectively. All females contain major quantities of long-chain internally branched di- and/or trimethylalkanes that were previously implicated as mediators of sexual behavior in males. Taxa within these two subgroups that are closely related and/or conspecific, based upon conventional morphological and ecological criteria, exhibit similar GC patterns and similar internally branched di- and trimethylalkane isomers in females. Examination of these potentially stimulatory methylalkanes may provide reasons for the reproductive isolation of closely related species from each other.

Key Words—Tsetse fly, sex pheromone, gas–liquid chromatography, mass spectrometry, methylalkanes, patterns, Diptera, hydrocarbons.

INTRODUCTION

The cuticular lipids of the tsetse fly (*Glossina* Wiedemann) (Diptera: Glossinidae) have come under scrutiny as a result of the identification of hydrocarbon constituents having biological activity. The existence of a female-produced compound in the epicuticular waxes of the tsetse fly capable of stimulating male sexual behavior was first demonstrated for *Glossina morsitans morsitans* West-

*To whom correspondence should be addressed.

0098-0331/97/0500-1291$12.50/0 © 1997 Plenum Publishing Corporation

wood (Langley et al., 1975). The compound found to be most active in initiating male copulatory activity in the lab was isolated and identified as a trimethylalkane, 15,19,23-trimethylheptatriacontane (Carlson et al., 1978). Lesser activity was reported for two dimethyl-branched compounds, 15,19- and 17,21-dimethylheptatriacontane.

A sex stimulant pheromone was also demonstrated in adult female *G. pallidipes* Austen (McDowell et al., 1981, Langley et al., 1982), and the compound eliciting the maximal response in males of this species was identified as 13,23-dimethylpentatriacontane (Carlson et al., 1984). Males of *G. austeni* Newstead were reported to exhibit a copulatory response to synthetic 15,19-dimethyltritriacontane (Huyton et al., 1980), although the identity of a sex pheromone in this species remains ambiguous. In addition, epicuticular constituents have been suggested as promoting sexual activity in *G. palpalis palpalis* Robineau-Desvoidy (Offor et al., 1981). Two unusual dimethylalkanes, 11,23- and 13,25-dimethylheptatriacontane, that have 11 methylene units between the methyl branches were found in *G. tachinoides* Westwood females as an inseparable mixture (Nelson et al., 1988). The natural hydrocarbons extracted from female *G. tachinoides* were stimulatory to males (Messoussi et al., 1994), but only recently have these compounds been synthesized (Matsuyama and Mori, 1994) and available for bioassay. Initial results (Carlson, unpublished data) indicate that synthetic 11,23- and 13,25-dimethylheptatriacontane release sexual responses in male *G. tachinoides*.

The identification of long-chain methylalkanes in the hydrocarbon constituent of epicuticular waxes in tsetse flies that exhibited highly specific behavioral activity and the likelihood that such compounds would be species specific stimulated the initial study of the cuticular hydrocarbon patterns and composition of a number of *Glossina* taxa. Species of the subgenus *Glossina* Wiedemann sensu stricto (the *morsitans* group; *G. m. morsitans*, *G. austeni* and *G. pallidipes*) have been reported to have cuticular methylalkane compositions comprised of homologous series of externally branched monomethylalkanes (2- and 3-methylalkanes), internally branched monomethylalkanes, dimethylalkanes, and trimethylalkanes (Nelson and Carlson, 1986). There were only low levels of *n*-alkanes detected. The 2-methylalkanes were predominant in males, while the females were found to possess, in addition to the 2-methylalkanes, substantial quantities of internally branched dimethyl- or trimethylalkanes corresponding to the putative sex pheromones.

Glossina fuscipes fuscipes Newstead, *G. palpalis palpalis*, *G. p. gambiensis* Vanderplank, and *G. tachinoides*, of the subgenus *Nemorhina* Robineau-Desvoidy (the *palpalis* group), have also been investigated (Nelson et al., 1988). The females of these taxa were similar to those of *G.* (*Glossina*) in that the patterns were dominated by homologous series of 2-methylalkanes together with series of internally branched di- or trimethylalkanes. Males were characterized

by patterns having substantial amounts of 27- or 29-carbon backbone 3,7,11-trimethylalkanes and a 28-carbon backbone, 4,8,12-trimethylalkane in addition to the 2-methylalkane series. The single species of the subgenus *Austenina* Townsend (the *fusca* group), *G. brevipalpis* Newstead, for which the cuticular methylalkanes have been determined, exhibits a pattern quite distinct from the species of *G.* (*Glossina*) and *G.* (*Nemorhina*) (Nelson et al., 1988). In *G. brevipalpalis*, tetramethylalkanes are a significant component of the methylalkanes, with 3,7,11,15-tetramethylhentriacontane the predominant isomer, and the internally branched compounds in minor quantities. In recent trials of the hydrocarbon fraction from females of this species, conspecific colony males were strongly sexually stimulated, implicating 3,7,11,15-tetramethylalkane homologs in modifying sexual behavior in this species (D.A.C., unpublished data).

It is possible to elucidate the possible taxonomic correlations in methylalkane patterns within the genus *Glossina* and to identify female-specific methylalkanes having potential for biological activity in taxa not yet investigated. Examination of these potentially stimulatory methylalkanes may provide reasons for the reproductive isolation of closely related species from each other, by the presence or absence of essential components. Thus we have analyzed the hydrocarbons of all but one of the remaining taxa of the *Glossina* and *Nemorhina* subgenera.

METHODS AND MATERIALS

The methylalkane composition was determined for adult males and females of the following species: *G. morsitans centralis* Machado, *G. m. submorsitans* Newstead sensu Machado, *G. longipalpis* Wiedemann and *G. swynnertoni* Austen of *G.* (*Glossina*) s. str. together with *G. caliginea* Austen, *G. fuscipes martinii* Zumpt, *G. pallicera pallicera* Bigot, and *G. p. newsteadi* Austen of *G.* (*Nemorhina*). Specimens of the sole remaining member of *G.* (*Nemorhina*), *G. fuscipes quanzensis* Pires, were not available for analysis.

Each dried specimen was extracted in hexane and the hydrocarbons isolated from other lipid components by elution with hexane (3 ml) using silica gel chromatographic columns packed in Pasteur pipets (50 mm × 6 mm ID) as previously described (Carlson et al., 1984). The alkanes were obtained by elution with hexane (3 ml) from 5% silver nitrate-impregnated silica gel columns (50 mm × 6 mm ID). Analysis of cuticular components involved quantification of eluents by gas–liquid chromatography (GC) utilizing a 50-m × 0.15-mm-ID fused-silica capillary column having a 0.25-μm dimethyl siloxane stationary phase (BP-1, Scientific Glass Engineering Inc., Austin, Texas) fitted to a Tracor 540 gas chromatograph (Tracor, Austin, Texas) with a cool on-column injector

(OCI-3, Scientific Glass Engineering) and flame-ionization detector. H_2 carrier was used with a linear flow velocity of 35 to 45 cm/sec. Each sample was reconstituted in 10–70 μl of hexane as necessary to provide optimal and consistent stationary phase loading, and 1–2 μl was injected. Each GC run was temperature programmed as follows: initial hold at 60°C (2 min), ramp 20°/min to 230°C, then 1°/min to 350°C, final hold (20 min). Electron ionization (EI) and chemical ionization (CI) mass spectra were obtained using Hewlett-packard 5988A MS interfaced to a HP 5890 gas chromatograph (GC) fitted with an OCI-3 injector. The column utilized was 30 m × 0.32 mm ID (DB-1, J&W Scientific, Folsom, California) having a 0.25-μm polymethylsiloxane stationary phase. He carrier was used at a linear velocity of 40 cm/sec. The MS interface was maintained at 315°C, electron voltage at 70 eV, and the system parameters manually optimized in order to enhance the high-mass spectra. The mass spectral scan range extended from *m/z* 70 to *m/z* 750 at 1 scan/sec. MS scans were manually background subtracted prior to interpretation. Methane (700 torr) was used as the reagent gas for CI (electron voltage at 30 eV, ion source temperature at 100°C).

The identification of cuticular methylalkane components based upon EI mass spectra followed the conventions for their interpretation, including the following for types and partial locations of branches; A = internal monomethyl, B = internal dimethyl, C = internal trimethyl, D = internal tetramethyl, A′ = 2- (3- or 4-) methyl, B′ = 3,7-dimethyl, and C′ = 3,7,11- or 4,8,12-trimethyl. (Carlson et al., 1984; Nelson and Sukkestad, 1970; Nelson and Carlson, 1986; Nelson et al., 1980, 1988). The CI mass spectra were used to assign molecular weights to eluents.

Label data for all specimens are given in Appendix 1. Specimens are vouchered at the The Natural History Museum, London, U.K. [NHM, formerly British Museum (Natural History), or the Florida State Collection of Arthropods, FDACS, Gainesville, Florida, (FSCA)].

RESULTS

The *n*-alkanes and methylalkanes identified by GC-MS from *G. morsitans centralis*, *G. m. submorsitans*, *G. longipalpis*, and *G. swynnertoni* of the subgenus *Glossina* and *G. caliginea*, *G. fuscipes martinii*, *G. pallicera pallicera*, and *G. p. newsteadi* of the subgenus *Nemorhina* are presented in Tables 1 and 2 for females and males, respectively. The GC profiles for males and females of each of the taxa are shown in Figures 1–4 and the major peak areas tabulated in Tables 3 and 4. The surface alkanes of these species of tsetse flies were comprised of long-chain methyl-branched alkanes with *n*-alkanes present as minor components. The dominant methylalkanes common to males and

TABLE 1. NORMAL AND METHYL ALKANES OF *Glossina* (*Glossina*)[a]

KI	Methyl-branching	*G. m. centralis*		*G. m. submorsitans*		*G. longipalpis*		*G. swynnertoni*	
		M	F	M	F	M	F	M	F
2463	2-	–	–	+	+	–	–	+	+
2500	*n*-C_{25}	+	+	+	+	–	–	+	+
2533	11-; 13-	++	++	++	++	–	–	++	++
2563	11, *x*-	–	–	*I* = 3	*I* = 3	–	–	–	–
		2-	–	+	+	+	–	–	+
2572	7, *x*-	–	–	–	*I* = 3	–	–	–	–
2583	5, *x*-	–	–	*I* = 3/5	*I* = 3/5	*I* = 3/5	*I* = 3/5	–	–
2600	*n*-C_{26}	+	+	+	+	t	+	+	+
2610	5, *x*, *y*-	–	–	–	–	–	–	*I* = 3/3	*I* = 3, 3/3, 5?
2633	9-; 11-;	–+	–+	?+	–+	––	––	–+	–+
	12-; 13-;	–+	–+	––	++	––	––	––	––
	14-; 15-	––	––	––	++	––	––	––	––
2640	3, *x*, *y*-	t	–	–	t	–	–	–	–
2655	11, *x*-;	–	–	–	–	–	–	–	–
	9, *x*-	–	–	–	*I* = 3	–	–	–	–
2665	2-	+	+	+	+	+	+	+	+
2672	7, *x*-	–	–	–	*I* = 3	–	–	–	–
2683	5, *x*-	–	–	t	*I* = 3?/5/7	–	*I* = 3/5/7?	–	–
2700	*n*-C_{27}	+	+	+	+	+	+	+	+
2720	4, *x*, *y*-	–	–	t	t	t	t	t	t
2733	9-; 11-;	–+	–+	–+	++	–+	–+	–+	–+
	13-	+	+	+	+	+	+	+	+
2739	7-	–	–	–	+	–	–	–	–
2755	11, *x*-;	–	–	–	*I* = 3	–	–	–	–
	9, *x*-	–	–	–	*I* = 3	–	–	–	*I* = 3
2763	2-	+	+	+	+	+	+	+	+
2771	7, *x*-	–	–	t	*I* = 3/5	–	–	–	–

TABLE 1. CONTINUED

KI	Methyl-branching	*G. m. centralis* M	*G. m. centralis* F	*G. m. submorsitans* M	*G. m. submorsitans* F	*G. longipalpis* M	*G. longipalpis* F	*G. swynnertoni* M	*G. swynnertoni* F
2781	5, *x*-	−	−	t	*I* = 5/7	−	*I* = 3	t	*I* = 3/5/7
2800	*n*-C_{28}	t	t	+	+	−	−	−	t
2833	9-; 10-;	−+	−−	−−	++	−−	−−	−−	−+
	11-; 12-;	+−	+−	+−	++	−+	+−	−−	−−
	13-; 14-	+−	+−	+−	++	+−	−−	+−	+−
2840	3, *x*, *y*-	t	t	t	*I* = 3, 3	t	t	t	*I* = 3, 3/3, 5
2863	2-	+	+	+	+	+	+	+	+
2873	7, *x*-	−	−	−	*I* = 3/5	−	−	−	+
2882	5, *x*-	−	−	−	*I* = 9?	−	−	−	−
2900	*n*-C_{29}	+	+	+	+	+	t	+	+
2920	4, *x*, *y*-	−	−	−	−	−	−	−	−
2933	9-; 11-;	−+	−+	−+	−+	−+	−+	−+	−+
	13-; 15-	−+	−+	++	++	++	+−	++	+−
2955	9, *x*-;	−	−	−	−	*I* = 3	−	−	−
	11, *x*-	−	−	−	−	−	−	−	−
2963	2-	+	+	+	+	+	+	+	+
2975	7, *x*-	−	−	−	*I* = 5	−	−	−	−
3000	*n*-C_{30}	t	t	+	+	−	−	−	−
3033	11-; 12-;	+−	−−	++	++	++	−−	++	−+
	13-; 14-;	+−	−−	−+	++	+−	+−	−+	−+
	15-	−	−	−	−	−	−	+	+
3040	3, *x*, *y*-	−	−	−	−	−	−	−	−
3063	2-	+	+	+	+	+	+	+	+
3100	*n*-C_{31}	t	t	+	+	+	t	−	−
3133	9-; 11-;	−+	−+	−+	−+	−+	−+	−+	−−
	13-, 15-	+−	+−	++	++	++	−−	++	++

3163	2-	+	+	+	+	–	–	+	+
3155-	9, *x*-;	*I* = 3	–	–	–	–	–	–	–
3163	11, *x*-;	*I* = 3	–	*I* = 3	*I* = 3	*I* = 3	*I* = 3/9	*I* = 3	*I* = 3/9
	13, *x*-	–	–	–	–	–	–	–	*I* = 3
3172	7, *x*-	–	–	t	–	–	–	–	–
3180	5, *x*-	–	–	*I* = 7	–	–	–	–	–
3170	11, *x*, *y*-	t	t	t	t	t	t	t	t
3180	7, *x*, *y*-	–	–	*I* = 3,3	–	–	–	–	–
3200	*n*-C_{32}	–	–	–	+	–	–	–	–
3233	11-; 13-;	++	+–	++	+–	+–	+–	+–	++
	15-	–	–	+	–	–	–	–	–
3210	5, *x*, *y*-	*I* = 3, 3	–	*I* = 3, 3	–	–	–	*I* = 3, 3	*I* = 3, 3
3255	10, *x*-;	–	–	*I* = 3	–	*I* = 3	–	–	–
	11, *x*-;	*I* = 3	?	*I* = 3	?	?	?	*I* = 3	*I* = 3
	12, *x*-	–	–	–	–	*I* = 3	–	–	–
3263	2-	+	+	+	+	+	+	–	–
3270	10, *x*, *y*-	–	–	t	t	–	–	t	–
3280	6, *x y*-			*I* = 3, 3	–	–	–	–	–
3333	11-; 13-;	+–	?–	–+	+–	?–	?–	?–	?–
	15-	–	–	–	–	–	–	–	–
3355-	11, *x*-;	*I* = 3	*I* = 3	*I* = 3	*I* = 3	*I* = 3	*I* = 3/9	*I* = 3	*I* = 3/9
3363	13, *x*-;	–	–	–	–	–	–	–	–
	15, *x*-	–	–	–	–	–	–	–	–
3363	2-	–	–	–	+	–	–	–	–
3370	9, *x*, *y*-;	–	–	–	–	–	–	–	–
	11, *x*, *y*-;	?	?	?	?	?	?	t	–
	13, *x*, *y*	–	–	–	–	?	–	–	–
3380	7, *x y*-	*I* = 3, 3	–	*I* = 3, 3	–	–	–	–	*I* = 3,3
3410	5, *x y*-	–	–	*I* = 5, 3	t	–	–	–	–
3433	12-; 13-;	++	–+	++	?–	–?	+–	??	–?
	14-	–	–	+	–	–	–	–	–
3455	12, *x*-;	–	?	*I* = 3	*I* = 3	*I* = 3	*I* = 9	*I* = 3	*I* = 3
	11, *x*-	*I* = 3	–	*I* = 3	?	–	–	–	–

TABLE 1. CONTINUED

KI	Methyl-branching	*G. m. centralis*		*G. m. submorsitans*		*G. longipalpis*		*G. swynnertoni*	
		M	F	M	F	M	F	M	F
3463	2-	+	+	–	+	–	+	–	–
3470	8, *x y*-	–	?	–	t	t	t	–	–
3480	6, *x y*-	–	–	t	–	–	–	*I* = 3/?	t
3533	13-; 15-	+–	––	?–	?–	––	++	+–	+–
3555-	11, *x*-;	*I* = 3	–	*I* = 3	–	*I* = 3	–	*I* = 3	*I* = 3/9
3563	13, *x*-;	*I* = 3	–	*I* = 3	*I* = 9	*I* = 3	*I* = 3	?	*I* = 9
	15, *x*-	–	*I* = 3	–	*I* = 3	–	–	–	*I* = 3
3570	11, *x y*-;	–	*I* = 3, ?	–	–	*I* = 3, ?	–	–	–
	13, *x*, *y*-	–	*I* = 3, 3	–	*I* = 3, 3	*I* = 3, ?	–	–	*I* = 3, 3?
3582	7, *x*, *y*-	–	–	*I* = 3, 3	–	–	–	–	–
3610	5, *x*, *y*	–	–	*I* = 5, 3	–	–	–	–	–
3655-	12, *x*-;	*I* = 3	–	*I* = 3	–	*I* = 3	–	*I* = 9	*I* = 9
3663	13, *x*-;	–	–	–	–	–	–	–	–
	14, *x*-;	–	–	–	*I* = 3	*I* = 3	–	–	–
	15, *x*-;	*I* = 3	*I* = 3	–	*I* – 3	–	–	*I* = 3	*I* = 3
	16, *x*-	–	*I* = 3	–	*I* = 3	–	–	–	*I* = 3
3670	13, *x*, *y*-;	–	–	–	–	?	–	–	–
	14, *x y*-	–	*I* = 3, 3	?	*I* = 3, 3	–	–	–	*I* = 3, 3?
3733	11-; 13-;	––	––	––	––	––	–+	––	––
	15-; 17-	––	––	––	––	––	++	––	––
3755-	11, *x*-;	–	–	*I* = 3	–	–	–	–	–
3763	13, *x*-;	–	–	*I* = 3	*I* = 3	*I* = 3	*I* = 3	–	*I* = 9
	15, *x*;	*I* = 3	*I* = 3	*I* = 3	*I* = 3	–	–	–	–
	17, *x*-	*I* = 3	*I* = 3	–	–	–	–	–	–3770
	11, *x*, *y*-;	–	–	–	–	–	*I* = 3, ?	–	–

	13, *x*, *y*-;	–	–	–	–	–	?	–	–
	15, *x*, *y*-	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	–	*I* = 3, 3	–	–
3790	11, *x*, *y* *z*-;	–	–	–	*I* = 3, 3, 3	–	–	–	*I* = 3, 3, 3
3855-	12, *x*-;	–	–	–	–	–	–	–	*I* = 9
3863	15, *x*-;	–	*I* = 3	–	*I* = 3				*I* = 3
	16, *x*-;	–	*I* = 3	–	*I* = 3	–	–	–	*I* = 3
	17, *x*-	–	–	–	–	–	–	–	*I* = 3
3870	15, *x*, *y*-;	–	*I* = 3, 3	?	*I* = 3, 3	–	*I* = 3, 3	–	–
	16, *x*, *y*-	–	*I* = 3, 3	–	–	–	–	–	*I* = 3, 3
3955-	13, *x*-;	–	–	–	–	–	–	–	*I* = 9/11?
3963	15, *x*-;	–	*I* = 3	–	–	–	–	–	–
3970	15, *x*, *y*-	–	*I* = 3, 3	–	*I* = 3, 3	–	t	–	*I* = 3, 3

[a] +, isomer present; –, isomer not present at detectable levels; t, peak present but identification of isomers ambiguous; ?, methyl-branching ambiguous. Each mark represents one compound or one isomer.

[b] *x*, position of the second methyl branch; *y*, position of the third methyl branch. **I**, = number of methylene carbons between branches.

TABLE 2. NORMAL AND METHYL ALKANES OF *Glossina (Nemorhina)*[a]

KI	Methyl branching	*G. f. martinii*		*G. caliginea*		*G. p. pallicera*		*G. p. newsteadi*	
		M	F	M	F	M	F	M	F
2500	*n*-C_{25}	+	+	+	+	+	+	+	+
2563	2-	+	+	+	+	+	+	+	+
2600	*n*-C_{26}	+	+	+	+	+	+	+	+
2640	3, *x*, *y*-	*I* = 3, 3	*I* = 3, 3	–	–	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3
2663	2-	+	+	+	+	+	+	+	+
2700	*n*-C_{27}	+	+	+	+	+	+	+	+
2720	4, *x*, *y*-	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3
2763	2-	+	+	+	+	+	+	+	+
2800	*n*-C_{28}	t	t	+	t	+	+	t	t
2810	5, *x*, *y*-	–	–	–	–	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3
2840	3, *x*, *y*-	*I* = 3, 3 3, 5	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3
2863	2-	+	+	+	+	+	+	+	+
2900	*n*-C_{29}	–	–	+	t	+	+	+	+
2920	4, *x*, y-	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3
2963	2-	+	+	+	+	+	+	+	+
3000	*n*-C_{30}	–	–	+	–	–	–	–	–
3010	5, *x*, *y*	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3 3, 5	–	–	–	–	–
3040	3, *x*, *y*-	*I* = 3, 3	*I* = 3, 3	*I* = 3, 3 3, 5	–	–	–	–	–

3063	2-	+	+	+	+	+	+	+	+
3100	n-C_{31}	–	–	+	–	–	–	–	–
3155	13, x-;	–	–	–	I = 3	–	I = 3	–	–
	11, x-;	–	–	–	–	–	I = 3	–	–
3163	2-	+	+	+	+	+	?	+	+
3170	11, x, y-	–	–	–	I = 3, 3	–	I = 3, 3	–	–
3263	2-	+	+	?	?	–	–	–	–
3255	10, x-;	–	–	–	–	–	I = 3	I = ?	I = 3
	12, x-	–	–	–	?	–	I = 3	I = ?	I = 3
3271	10, x, y-;	–	–	–	–	–	I = 3, 3	–	–
	12, x, y-	–	I = 3, 3?	–	–	–	I = 3, 3	–	–
3355	11, x-;	–	I = 3	–	?	–	I = 3	I = 3	I = 3
	13, x;	–	I = 3	I = 3	I = 3	–	I = 3	I = 3	I = 3
	15, x-	–	–	–	I = 3	–	–	–	–
3363	2-	+	+	?	?	–	–	–	–
3370	11, x, y-	–	I = 3, 3	–	I = 3, 3	–	I = 3, 3	I = 3, 3	I = 3, 3
3455	12, x-;	–	I = 3	–	?	–	t	–	–
	10, x-	–	–	–	–	–	t	–	–
3470	12, x, y-	–	I = 3, 3?	–	–	–	–	I = 3, 3	?
3555	11, x-;	–	I = 3	–	–	–	t	I = 3	I = 3
	13, x-	–	I = 3	–	–	–	t	I = 3	I = 3
3570	11, x, y;	–	I = 3, 3?	–	–	–	t	I = 3, 3	I = 3, 3
	13, x, y-	–	I = 3, 3?	–	I = 3, 3	–	–	–	–

[a] +, isomer present; –, isomer not present at detectable levels; t, peak present at trace levels but identification of isomers ambiguous; ?, methyl-branching ambiguous; x, y, and I as in Table I.

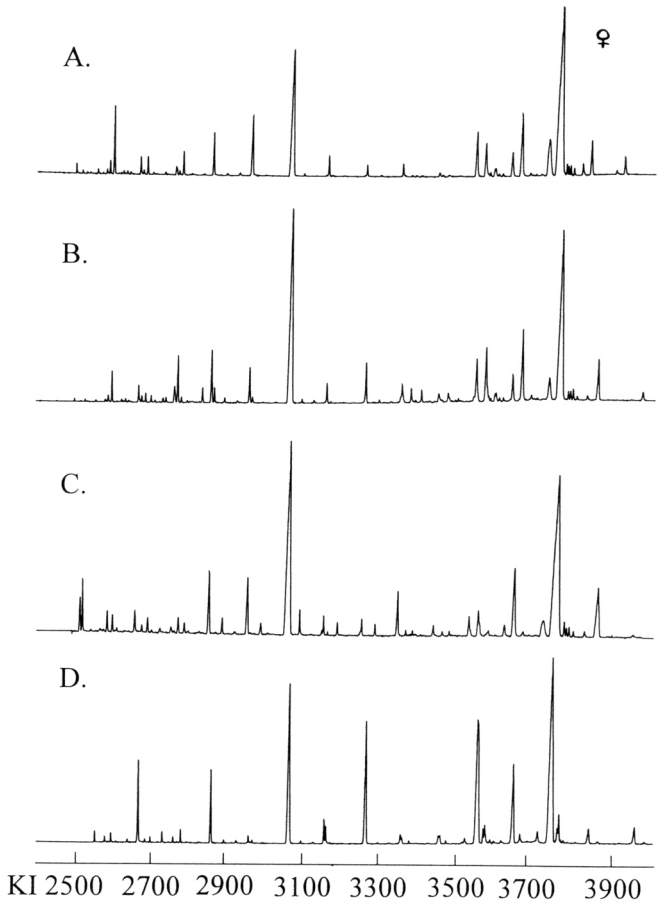


FIG. 1. GC of females *Glossina* (*Glossina*) cuticular alkanes: (A) *G. morsitans centralis*; (B) *G. m. submorsitans*; (C) *G. swynnertoni*; (D) *G. longipalpalis*.

females in both subgenera consisted of an odd-carbon-number backbone series of 2-methylalkanes having modes at 29 or 31 carbons, and a homologous even-carbon-number backbone series with a mode at 30 carbons. Internally branched monomethylalkanes were present at relatively low levels with the 15-, 13-, and 11- isomers coeluting at Kovats index (Kovats, 1965) KI *XX*28–*XX*33, where *XX* refers to the first two digits of the KI number, and internal branching refers to branch positions near the center of the carbon chain. There was no unambiguous evidence for the presence of 3- or 4-methylalkanes in any of the taxa

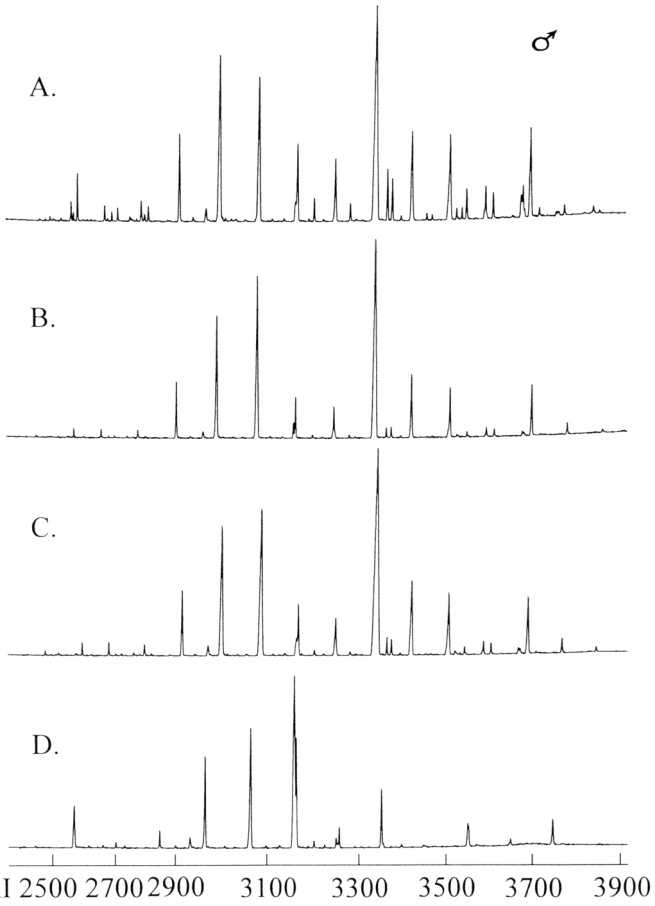


FIG. 2. GC of male *Glossina* (*Glossina*) cuticular alkanes: (A) *G. morsitans centralis*; (B) *G. m. submorsitans*; (C) *G. swynnertoni*; (D) *G. longipalpalis*.

using either the 30-m or 50-m column, the latter producing about 300,000 theoretical plates.

Adult females were characterized by the presence of relatively large quantities of internally branched di- and/or trimethylalkanes in addition to the 2-methylalkanes. These compounds were present in the males, if detected, in significantly smaller quantities. Internally branched tetramethylalkanes were also present albeit at relatively low levels.

Subgenus Glossina. The methylalkanes of *G. morsitans centralis* and

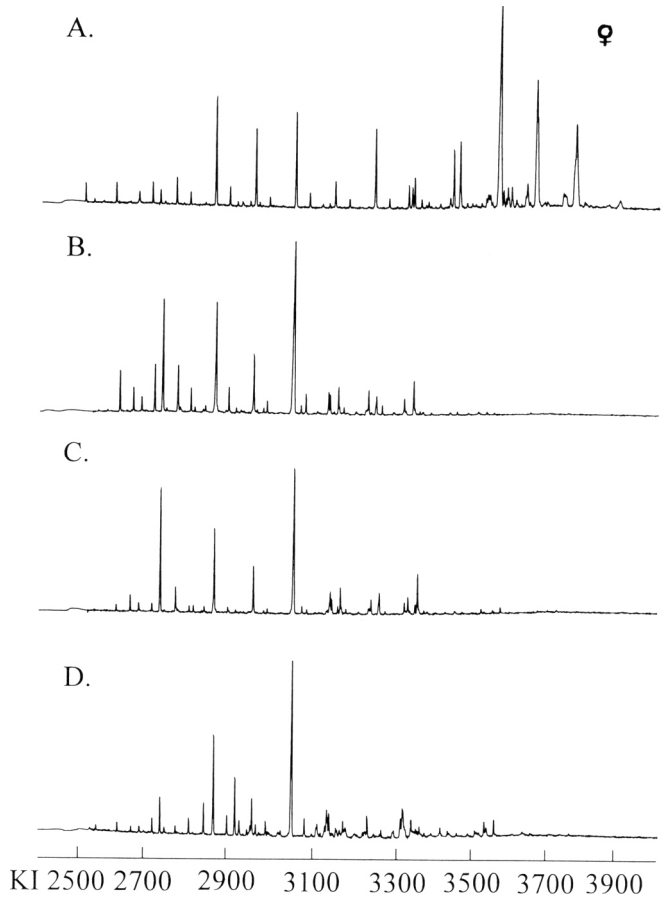


FIG. 3. GC of female *Glossina* (*Nemorhina*) cuticular alkanes: (A) *G. fuscipes martinii*; (B) *G. pallicera pallicera*; (C) *G. p. newsteadi*; (D) *G. caliginea.*

G. m. submorsitans were essentially identical (Figure 1), being dominated by a peak at KI 3770 (37.6% and 33.7% of total, respectively). EI mass spectra of this peak showed major fragments at *m/z* 224/225, 295, and 365. The 224/225 doublet with *m/z* 224 > 225 indicated cleavage internal to the methyl branch that is closest to the end of the chain, and the lack of a second even/odd doublet showed that the compound is symmetric. Minor fragments at *m/z* 196/197 (in part), 266/267, and 336/337 were of a magnitude consistent with primary carbonium ions of the *m/z* 224/225, 295, and 365 secondary ions, respectively,

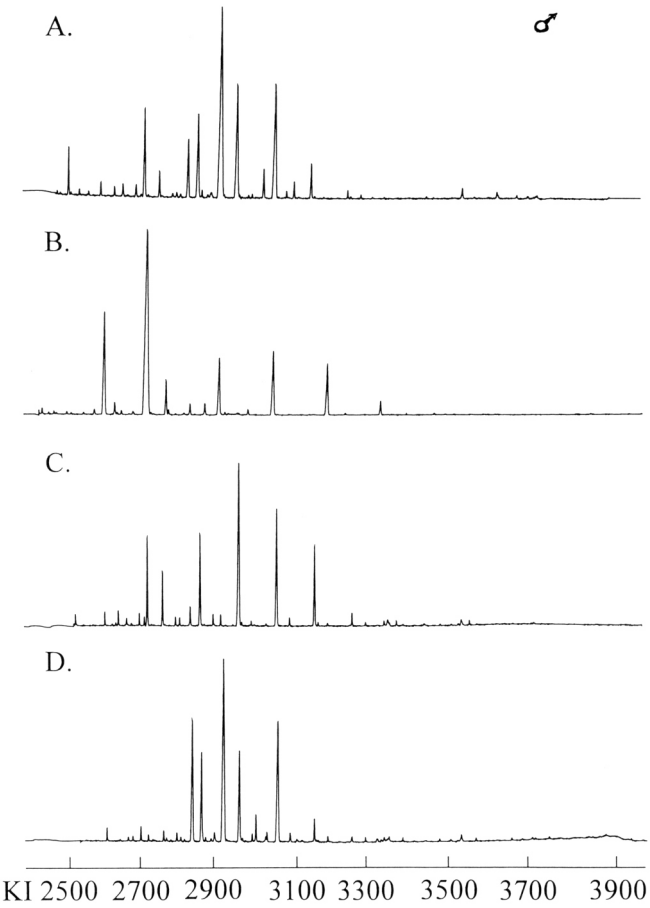


FIG. 4. GC of male *Glossina* (*Nemorhina*) cuticular alkanes: (A) *G. fuscipes martinii*; (B) *G. pallicera pallicera*; (C) *G. p. newsteadi*; (D) *G. caliginea*.

and did not necessarily indicate the presence of additional isomers. This fragmentation pattern was consistent with 15,19,23-trimethylheptatriacontane, having a pattern of methylene interruptions (*I*) between the branch points of the form $I = 3,3$. Both EI mass spectra and KI were found to be identical with both the synthetic and the natural compound originally identified from *G. morsitans morsitans* (Carlson et al., 1978). The $[M-15]^+$ fragment at *m/z* 547 was not identifiable in the EI spectra but CI-MS confirmed the presence of 40 carbons. Homologs of 15,19,23-trimethylheptatriacontane were also present with

TABLE 3. ALKANES OF *G. (Glossina)* AND *G. (Nemorhina)* FEMALES

Type[a]	KI	Percent of total peak area (mean ± SD)[b]							
		G.m.c.	G.m.s.	G.s.	G.l.	G.f.m.	G.p.p.	G.p.n.	G.c.
26	2600	0.1 ± 0.0	0.2 ± 0.2	1.2 ± 0.7	0.4 ± 0.4	0.4 ± 0.3	1.6 ± 1.2	0.6 ± 0.1	0.9 ± 0.5
26A	2633	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0		0.2 ± 0.1	0.5 ± 0.6	0.1 ± 0.1
25C′	2640		0.1 ± 0.1			0.7 ± 0.2	1.6 ± 0.1	1.8 ± 0.5	0.3 ± 0.1
26A′	2663	1.1 ± 0.4	0.8 ± 0.2	0.8 ± 0.3	1.7 ± 1.4	0.5 ± 0.1	1.2 ± 0.3	0.6 ± 0.1	0.8 ± 0.4
27	2700	0.1 ± 0.0	0.3 ± 0.2	0.4 ± 0.4	0.5 ± 0.6	1.0 ± 0.4	2.2 ± 1.5	0.7 ± 0.0	1.1 ± 0.2
26C′	2720		0.1 ± 0.0		0.1 ± 0.1		10.3 ± 0.9	12.2 ± 2.7	1.7 ± 1.2
27A	2733	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.2	0.3 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.4 ± 0.3	0.4 ± 0.2
27A′	2763	1.3 ± 0.5	0.9 + 0.4	0.3 ± 0.1	0.6 ± 0.3	0.5 ± 0.3	3.2 ± 0.9	2.3 ± 1.2	0.6 ± 0.2
28A	2833	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
27C′	2840	0.0 ± 0.1	0.4 ± 0.3	0.1 ± 0.0		0.1 ± 0.1	0.4 ± 0.1	1.1 ± 0.5	3.4 ± 1.8
28A′	2863	1.9 ± 0.8	2.0 ± 0.9	2.7 ± 1.3	2.1 ± 0.9	5.0 ± 2.3	14.3 ± 4.3	7.1 ± 5.7	10.4 ± 3.5
28C′	2920					0.1 ± 0.1	0.5 + 0.4	0.6 ± 0.5	5.7 ± 1.7
29A	2933	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.1	0.4 ± 0.2	0.3 ± 0.1	0.6 ± 0.7	0.2 ± 0.0	1.1 ± 0.5
29A′	2963	3.7 ± 0.9	1.9 ± 0.6	4.5 ± 1.3	1.0 ± 0.6	3.5 ± 0.9	4.9 ± 1.0	5.3 ± 1.0	4.1 ± 0.3
30A	3033		0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.4 ± 0.4	0.1 ± 0.0	1.1 ± 0.4
29C′	3040					0.2 ± 0.1	0.9 ± 1.2	0.3 ± 0.1	0.2 ± 0.2
30A′	3063	19.6 ± 2.2	23.1 ± 5.9	26.9 ± 0.4	10.4 ± 2.7	4.7 ± 3.5	26.1 ± 6.5	18.3 ± 9.3	21.3 ± 0.4
31A	3133		0.1 ± 0.1		0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.2	2.2 ± 0.1
31B	3155	1.5 ± 0.5	1.4 ± 0.7	2.2 ± 1.7	4.5 ± 3.7	1.1 ± 0.5	5.0 ± 3.3	5.5 ± 1.2	7.8 ± 0.4
31C	3170	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0		6.3 ± 3.3	2.4 ± 3.2	5.8 ± 0.8
32A	3233		0.1 ± 0.0		0.1 ± 0.1	0.1 ± 0.1	0.6 ± 0.3	0.3 ± 0.2	1.0 ± 0.6
32B	3255	1.3 ± 0.8	3.1 ± 0.5	1.5 ± 0.8	8.5 ± 0.8	3.9 ± 1.1	2.5 ± 0.8	5.8 ± 3.6	3.8 ± 0.2
32C	3270		0.1 ± 0.0				3.1 ± 0.8	2.0 ± 2.6	2.2 ± 0.3
33A	3333		0.1 ± 0.1		0.2 ± 0.1	0.1 ± 0.2	0.4 ± 0.1	0.3 ± 0.1	1.0 ± 0.4
33B	3355	0.8 ± 0.1	1.7 ± 1.1	7.4 ± 6.5	1.6 ± 0.7	3.5 ± 1.9	3.4 ± 1.2	12.8 ± 11.5	8.9 ± 0.4
33C	3570		0.6 ± 0.4		0.1 ± 0.1	0.5 ± 0.2	6.3 ± 7.1	6.6 ± 0.5	5.0 ± 0.6

34A	3433		0.1 ± 0.0		0.2 ± 0.0	0.1 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.2
34B	3455	0.8 ± 0.4	0.9 ± 0.1	1.6 ± 1.2	1.4 ± 0.3	3.9 ± 1.3	0.5 ± 0.2	2.0 ± 1.9	0.8 ± 0.1
34C	3470	0.2 ± 0.0	0.7 ± 0.4	0.2 ± 0.2	0.3 ± 0.1	4.1 ± 1.1	0.3 ± 0.1	0.7 ± 0.0	0.9 ± 0.0
35A	3533		0.1 ± 0.1		0.5 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.1
35B	3555	4.1 ± 0.1	3.4 ± 0.8	2.6 ± 1.6	19.0 ± 1.7	2.7 ± 0.9	0.4 ± 0.3	4.4 ± 4.9	1.5 ± 0.5
35C	3570	3.2 ± 0.0	4.5 ± 2.0	1.8 ± 0.7	2.8 ± 0.7	24.4 ± 8.7	0.6 ± 0.4	2.8 ± 3.3	2.4 ± 0.4
35D	3590	0.2 ± 0.0	1.1 ± 0.5		0.3 ± 0.0	2.0 ± 1.1	0.0 ± 0.1	0.1 ± 0.0	0.7 ± 0.9
36A	3633		0.1 ± 0.1		0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.2
36B	3655	2.2 ± 0.0	1.8 ± 0.2	1.1 ± 0.3	6.6 ± 1.0	2.6 ± 0.9	0.1 ± 0.1	0.4 ± 0.3	0.1 ± 0.1
36C	3670	6.3 ± 0.3	5.7 ± 0.2	3.9 ± 2.9	0.7 ± 0.1	14.3 ± 3.1	0.1 ± 0.1	0.2 ± 0.1	0.4 ± 0.6
36D	3690	0.1 ± 0.0	0.4 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	0.7 ± 0.2		0.1 ± 0.1	0.1 ± 0.2
37A	3733	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.2	0.1 ± 0.0		0.1 ± 0.1	0.1 ± 0.2
37B	3755	6.6 ± 0.1	3.0 ± 0.8	3.4 ± 0.9	27.5 ± 4.1	2.3 ± 0.8	0.1 ± 0.1	0.4 ± 0.3	0.3 ± 0.4
37C	3770	37.6 ± 0.3	33.7 ± 4.4	27.4 ± 9.9	2.6 ± 0.3	12.3 ± 3.5		0.2 ± 0.2	0.2 ± 0.3
37D	3790	0.9 ± 0.1	0.8 ± 0.4	0.7 ± 0.4	0.2 ± 0.0	0.6 ± 0.1		0.1 ± 0.1	0.1 ± 0.1
38A	3833		0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1			
38B	3855	0.9 ± 0.0	0.5 ± 0.3	0.4 ± 0.0	1.3 ± 0.3	0.7 ± 0.5			0.1 ± 0.2
38C	3870	2.7 ± 0.3	4.2 ± 1.2	5.4 ± 1.0	0.2 ± 0.0	1.2 ± 0.1			
38D	3890	0.0 ± 0.1	0.1 ± 0.1			0.1 ± 0.1			
39A	3933				0.1 ± 0.0				0.1 ± 0.1
39B	3955	0.3 ± 0.0	0.3 ± 0.2	0.3 ± 0.2	1.7 ± 0.4				0.1 ± 0.2
39C	3970	1.2 ± 0.2	0.9 ± 0.6	1.7 ± 2.1	0.2 ± 0.0	0.2 ± 0.1			0.2 ± 0.2
39D	3990					0.5 ± 0.3			

[a] A = internal methyl, B = internal dimethyl, C = internal trimethyl, D = internal tetramethyl, A′ = 2-methyl, C′ = 3, 7, 11- or 4, 8, 12-trimethyl alkanes.

[b] *G. m. centralis* (G.m.c.). *G. m. submorsitans* (G.m.s.), *G. swynnertoni* (G.s.), *G. longipalpis* (G.l.), *G. f. martinii* (G.f.m.), *G. p. pallicera* (G.p.p.), *G. p. newsteadi* (G.p.n.), *G. caliginea* (G.c.).

TABLE 4. ALKANES OF *G. (Glossina)* AND *G. (Nemorhina)* MALES

Type[a]	KI	Percent of total peak area (mean ± SD)[b]							
		G.m.c.	G.m.s.	G.s.	G.l.	G.f.m.	G.p.p.	G.p.n.	G.c.
26	2600	0.1 ± 0.0	0.3 ± 0.2		0.3 ± 0.2	0.7 ± 0.1	0.8 ± 0.3	0.9 ± 0.5	0.4 ± 0.3
26A	2633	0.1 ± 0.0	0.1 ± 0.0			0.1 ± 0.1	0.1 ± 0.0	1.0 ± 1.4	
25C′	2640	0.1 ± 0.0				1.6 ± 1.9	7.6 ± 6.7	1.2 ± 0.9	0.1 ± 0.1
26A′	2663	0.5 ± 0.0	0.5 ± 0.3	0.5 ± 0.0	2.4 ± 2.2	0.7 ± 0.1	1.2 ± 0.6	0.4 ± 0.3	0.5 ± 0.4
27	2700	0.3 ± 0.2	0.4 ± 0.1	0.1 ± 0.0	0.4 ± 0.2	0.8 ± 0.0	0.9 ± 0.5	0.8 ± 0.4	1.0 ± 0.6
26C′	2720			0.1 ± 0.1	0.1 ± 0.0	14.5 ± 12.7	36.8 ± 11.5	10.6 ± 2.1	0.9 ± 0.6
27A	2733	0.3 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1
27A′	2763	0.8 ± 0.1	1.0 + 0.5	0.5 ± 0.0	0.5 ± 0.3	1.8 ± 0.5	4.4 ± 1.7	4.3 ± 1.3	0.7 ± 0.1
28A	2833	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0				
27C′	2840		0.2 ± 0.2			5.2 ± 0.3	0.9 ± 0.3	1.7 ± 0.3	17.5 ± 3.5
28A′	2863	3.9 ± 0.3	1.8 ± 0.3	3.7 ± 0.2	1.4 ± 0.5	7.2 ± 1.9	10.1 ± 3.1	7.7 ± 5.0	10.0 ± 1.1
28C′	2920					22.0 ± 12.4	0.6 + 0.3	0.9 ± 0.3	33.6 ± 3.9
29A	2933	0.6 ± 0.5	0.3 ± 0.2	0.8 ± 0.1	1.4 ± 0.4		0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
29A′	2963	14.0 ± 0.1	12.8 ± 8.8	12.2 ± 0.2	6.7 ± 2.8	13.5 ± 3.2	17.7 ± 6.6	31.2 ± 0.7	6.9 ± 3.7
30A	3033	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1		0.1 ± 0.1	0.1 ± 0.1	0.4 ± 0.2
29C′	3040					2.9 ± 2.1	0.2 ± 0.2	0.3 ± 0.1	0.6 ± 0.2
30A′	3063	14.1 ± 3.3	21.7 ± 9.0	18.5 ± 1.8	19.1 ± 1.3	16.8 ± 6.6	10.3 ± 2.7	14.6 ± 4.4	4.7 ± 3.5
31A	3033	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.3		0.2 ± 0.1	0.1 ± 0.2	0.4 ± 0.2
31B	3055	6.4 ± 0.8	8.0 ± 4.1	4.1 ± 0.1	29.6 ± 12.1	3.3 ± 0.8	1.9 ± 0.4	12.0 ± 2.7	1.6 ± 0.4
31C	3070	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.1 ± 0.1
32A	3233	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
32B	3255	4.3 ± 0.6	4.9 ± 1.7	2.8 ± 0.1	5.5 ± 1.7	0.5 ± 0.2	0.4 ± 0.2	1.1 ± 0.1	0.6 ± 0.2
32C	3270		0.5 ± 0.7		0.0 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.7

33A	3333	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.2		0.2 ± 0.2	0.2 ± 0.1	0.6 ± 0.4
33B	3355	29.7 ± 4.1	29.3 ± 1.3	35.6 ± 1.1	19.2 ± 11.4	0.4 ± 0.3	0.5 ± 0.4	1.7 ± 0.6	1.3 ± 1.0
33C	3370	0.1 ± 0.0	1.3 ± 1.6		0.1 ± 0.0	0.2 ± 0.3	0.4 ± 0.3	1.5 ± 1.1	0.9 ± 0.4
34A	3433	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.1		0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
34B	3455	6.7 ± 0.4	4.4 ± 0.8	6.5 ± 0.4	1.2 ± 0.6	0.5 ± 0.5	0.2 ± 0.2	0.5 ± 0.3	0.3 ± 0.2
34C	3470		1.5 ± 2.1	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.2	0.1 ± 0.1	0.6 ± 0.7	0.7 ± 0.4
35A	3533	0.1 ± 0.0	0.2 ± 0.1		0.2 ± 0.0	0.1 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.3
35B	3555	6.0 ± 1.5	3.2 ± 0.3	5.0 ± 0.3	4.7 ± 1.4	0.3 ± 0.1	0.5 ± 0.5	1.3 ± 0.1	1.1 ± 0.3
35C	3570	0.3 ± 0.1	0.4 ± 0.3	0.4 ± 0.0	0.3 ± 0.1	1.7 ± 1.1	0.4 ± 0.4	1.7 ± 1.5	0.7 ± 1.0
35D	3590	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.6 ± 1.1	0.1 ± 0.2	0.1 ± 0.0		
36A	3633	0.1 ± 0.0	0.0 ± 0.1		0.3 ± 0.5		0.2 ± 0.1		0.1 ± 0.1
36B	3655	1.5 ± 0.5	0.4 ± 0.0	0.8 ± 0.0	0.8 ± 0.3	0.3 ± 0.2	0.2 ± 0.2	0.2 ± 0.0	0.2 ± 0.2
36C	3670	0.7 ± 0.5	0.6 ± 0.4	0.6 ± 0.0	0.1 ± 0.1	1.0 ± 0.4	0.1 ± 0.1	0.4 ± 0.3	0.2 ± 0.3
36D	3690		0.1 ± 0.1			0.1 ± 0.0	0.1 ± 0.1		
37A	3733	0.2 ± 0.0	0.1 ± 0.1		0.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.4
37B	3755	2.5 ± 1.2	0.8 ± 0.4	0.8 ± 0.0	2.8 ± 1.4	0.5 ± 0.2	0.4 ± 0.3	0.4 ± 0.1	0.7 ± 0.6
37C	3770	4.0 ± 3.3	2.7 ± 0.2	4.5 ± 0.0	0.2 ± 0.1	0.9 ± 0.5	0.5 ± 0.4	0.6 ± 0.8	0.6 ± 0.6
37D	3790					0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.0	
38A	3833	0.1 ± 0.0	0.1 ± 0.1			0.2 ± 0.4	0.1 ± 0.1	0.1 ± 0.0	
38B	3855	0.4 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
38C	3870	0.3 ± 0.3	0.3 ± 0.1	0.8 ± 0.1		0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
38D	3890					0.1 ± 0.0	0.0 ± 0.1		
39A	3933		0.1 ± 0.1			0.1 ± 0.1			
39B	3955	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0		0.2 ± 0.1		0.1 ± 0.1	0.1 ± 0.2
39C	3970	0.4 ± 0.2	0.2 ± 0.0	0.3 ± 0.0		0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.2
39D	3990	0.1 ± 0.1				0.1 ± 0.1		0.1 ± 0.1	

[a] A = internal methyl, B = internal dimethyl, C = internal trimethyl, D = internal tetramethyl, A′ = 2-methyl, C′ = 3, 7, 11- or 4, 8, 12-trimethyl alkanes.
[b] *G. m. centralis* (G.m.c.). *G. m. submorsitans* (G.m.s.), *G. swynnertoni* (G.s.), *G. longipalpis* (G.l.), *G. f. martinii* (G.f.m.), *G. p. pallicera* (G.p.p.), *G. p. newsteadi* (G.p.n.), *G. caliginea* (G.c.).

backbone carbon numbers of 38 and 39. In addition, smaller quantities of other trimethylalkane isomers were present with methyl branching ranging from 3,*x*,*y*- to 9,*x*,*y*- and 13,*x*,*y*- to 14,*x*,*y*-, where *x* refers to the position of the second methyl group, and *y* refers to the position of the third methyl group. The 3,*x*,*y*- the 7,*x*,*y*- isomers were the predominant trimethylalkanes at backbone carbon numbers below 35, with much less of the even backbone homologs. Dimethylalkanes were present at relatively low levels with methyl branching from 5,*x*- to 17,*x*-. The internally branched isomers, 11,*x*- to 17,*x*-, coeluted, forming a homologous series of peaks at KI *XX*55 and were quantitatively correlated with the 15,19,23- series. The CI-MS confirmed the presence of small quantities of tetramethylalkanes at KI *XX*90 with the mode at KI 3790, and the EI spectra for the compound at KI 3790 was consistent with a single isomer, 11,15,19,23-tetramethylheptatriacontane.

In adult males of *G. m. centralis* and *G. m. submorsitans*, 15,19,23-trimethylheptatriacontane was present only as a minor component (4 and 2.7%, respectively), whereas the major peak (29 and 29%) was identified as 11,15-dimethyltritriacontane (KI 3355). This compound was also present in adult females (range 0.8–1.7%).

Both males and females of *G. swynnertoni* also exhibited methylalkane patterns within the range of variation seen in the *G. morsitans* subspecies (Figures 1 and 2). EI mass spectra of the predominant peak in adult females (27.4%) at KI 3770 showed major fragments as above for 15,19,23-trimethylheptatriacontane. Hydrocarbon patterns and composition of adult males of *G. sywnnertoni* were essentially identical with *G. morsitans* males.

Female *G. longipalpalis* exhibited a pattern in which trimethylalkanes, although present, were not dominant. In this species the predominant peak was found to vary between KI 3563 and KI 3763, depending on the individual (19.0 and 27.5%, respectively). EI mass spectra of the peak at KI 3563 (Figure 5a) showed major fragments *m/z* 168/169, 196/197, 224/225, 323, 351, 379, and 505 $[M-15]^+$. Examination of successive scans across the KI 3563 peak for each of the *G. longipalpis* females indicated that the doublet at *m/z* 196/197 was strongly correlated with the fragment at 351 and that the remaining fragments were associated with another dimethyl isomer. The *m/z* 196/197 and 351 fragments were consistent with 13,23-dimethylpentatriacontane, which has an interruption of nine methylenes between methyl branches ($I = 9$). The remaining fragments were consistent with the 11,21- homolog. The EI spectra and KI of this peak were consistent with that reported for natural and synthetic 11,21- and 13,23-dimethylpentatriacontane in *G. pallidipes* (Carlson et al., 1984). In all specimens examined, 13,23-dimethylpentatriacontane was the dominant isomer at KI 3563 with the ratio of the abundances of the *m/z* 351 and 379 fragments greater than 2 : 1. The EI mass spectra of the apex of the peak at KI 3763 (Figure 5b) showed major fragments at *m/z* 196/197, 224/225, 351, 379, and 533

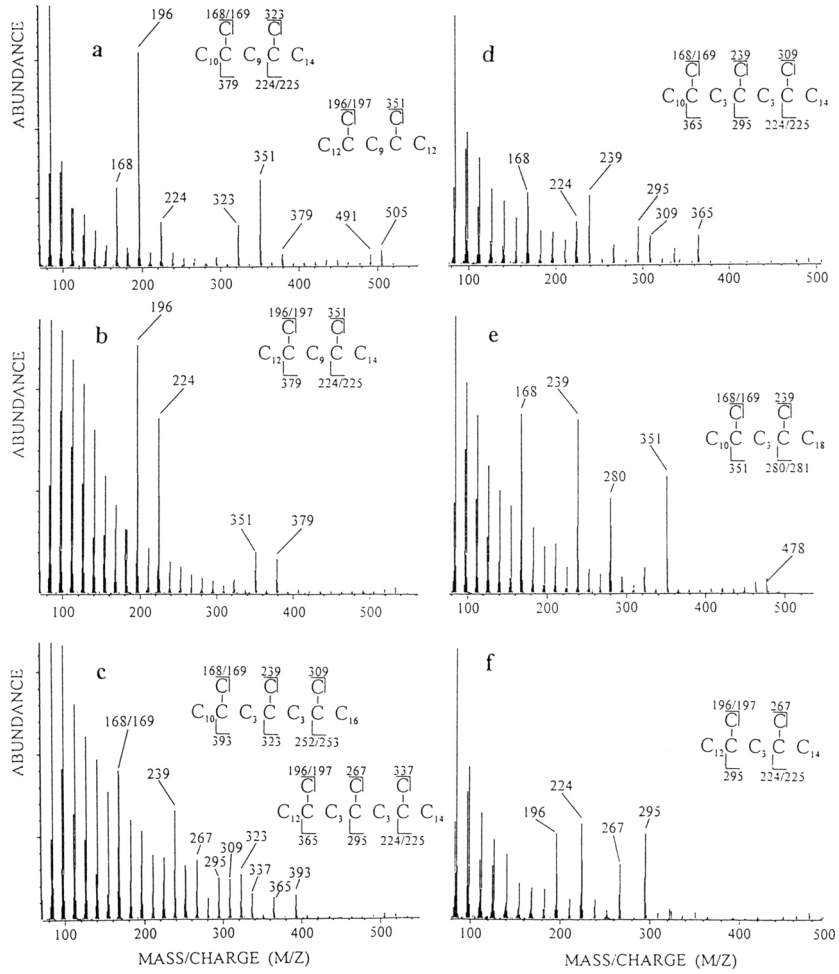


FIG. 5. GC-MS spectra of apex of GC-MS peaks at: (a) KI 3563, *G. longipalpis* female; (b) KI 3763, *G. longipalpis* female; (c) KI 3570, *G. f. martinii* female; (d) KI 3370, *G. p. pallicera* female; (e) KI 3355, *G. p. pallicera* female (*m/z* 477 ion recorded as 478 due to mass defect); (f) KI 3153, *G. caliginea* female.

$[M-15]^+$, consistent with 13,23-dimethylheptatriacontane ($I = 9$); this molecular weight was consequently confirmed by CI, and no other isomers were detected. The EI scans at the front of the peak at KI 3563 suggested the presence of additional isomers of 11,*x*-, 13,*x*-, and/or 15,*x*-dimethylalkanes with $I = 3$. Dimethylalkane isomers with three methylene units between methyl positions

were identified ranging in backbone carbon number from 27 to 38, with the series mode at 31 carbons. The KI 3155 peak consisted of 11,*x*- and 13,*x*-dimethylalkanes having $I = 3$ and was partially resolved from the corresponding isomers having $I = 9$ eluting at KI 3163. Dimethylalkanes having intermediate numbers of methylene units (5- or 7-) were not identified. Internally branched trimethylalkanes of the form 11,15,*y*-, 13,17,*y*-, and/or 15,19,*y*- were also present at KI *XX*70 with the largest peaks at KI 3570 and KI 3770. Given the complexity of the EI spectra as a result of the presence of multiple trimethylalkane isomers and the paucity of fragments, it was not possible to reliably reconstruct the more internal methyl positions; however, the fragments suggest that more than three methylene units are present between the two most interior methyl branches in at least some isomers. Other trimethylalkanes were present with backbone carbon numbers below 33. Tetramethylalkanes having a 37-carbon backbone were present at KI 3790.

Males of *G. longipalpis* were found to have negligible quantities of dimethylalkanes having nine methylene units between methyl branches. The predominant internally branched methyl-branched alkane in males was identified as 11,15-dimethyltritriacontane (29.6%).

Subgenus Nemorhina. Females of *G. f. martinii* were found to possess, in addition to the 2-methylalkane series, a major peak eluting at KI 3570 (Figure 3). The CI mass spectra indicated the presence of 38 carbons, and the EI mass spectra (Figure 5c) showed a major doublet at *m/z* 168/169 with 168 > 169 and the next significant fragment at *m/z* 239. This was consistent with one methyl on position 11 and a second methyl at position 15. The other significant ions that could result from fragmentation of a 11,15,*y*-trimethylalkane were at *m/z* 309, 337, 365, and 393. For $I = 3,3$, the *m/z* 309 fragment would result from the third methyl at position 19. The remaining fragments at *m/z* 393, 323, and 252/253 were consistent with a 11,15,19-trimethylalkane having a 35-carbon backbone as identified as the minor isomer in *G. p. palpalis* (Nelson et al., 1988). The 252/253 doublet did show 252 > 253 as consistent with a terminal fragment (one that had contained only one methyl branch); however the abundance of this ion relative to *m/z* 323 is low. This could be an artifact resulting from background subtraction. The remaining fragments were consistent with the 13,17,21- isomer, although the 196/197 doublet is not evident. Alternatively, isomers having five or more methyl units between the more interior methyl branches may be present. The strong ions at *m/z* 168/169, 239, 323, and 393 suggest that the dominant isomer is 11,15,*x*-trimethylpentatriacontane; however, the exact branching sequence remains questionable. Dimethylalkanes, predominantly 11,15- and 13,17-isomers, were also present at low levels in females of *G. f. martinii*.

The methylalkane patterns of adult *G. fuscipes martinii* males (Figure 4) were dominated by peaks at KI 2720 or KI 2920 (30–60% of total), depending

upon the individual, in addition to the homologous series of 2-methylalkanes. The EI mass spectra of the peak at KI 2920, the predominant peak in most individuals, showed major fragments at *m/z* 141, 211, 252/253, 323, and 393. Although the doublet at *m/z* 70/71 expected with EI fragmentation of 4,8,12-trimethyloctacosane was not distinguishable from the alkyl background, the remaining fragments and the CI mass spectra were consistent with this structure. There was no evidence to support the presence of other isomers in this peak. Similarly, EI mass spectra of the peak at KI 2720 showed major ions at *m/z* 141, 211, 224/225, 295, and 365 consistent with 4,8,12-trimethylhexacosane, the KI 2920 homolog. The homologous 3,7,11-trimethylpentacosane (KI 2640), heptacosane (KI 2840), and nonacosane (KI 3040) were present in smaller quantities. These trimethylalkanes were only found as minor components in the adult females of *G. f. martinii.*

The females of both subspecies of *G. pallicera* exhibited variability in the relative abundances of long-chain internally branched methylalkanes (<5% to 15%). EI scans of the predominant peak (KI 3370) in most individuals examined (Figure 5d) showed major fragments at *m/z* 168/169, 224/225, 239, 295, 309, and 365 consistent with a 11,15,19-trimethylalkane having a 33-carbon backbone. Homologs were present at KI 3170 and KI 3570. In one adult female of *G. p. newsteadi* the corresponding dimethylalkane series was predominant. EI scans of the mode of this series at KI 3355 (Figure 5e) showed major fragments *m/z* 168/169, 239, 280/281, 351, and 477 $[M-15]^+$. This was consistent with 11,15-dimethyltritriacontane, the dimethyl analog of the predominant trimethylalkane isomer.

G. pallicera males of both subspecies had methylalkane compositions similar to that of *G. f. martinii* (Table 1). The 4,8,12-trimethylalkane and 2-methylalkanes series had modes at 27 and 29/30 carbons, respectively.

Internally branched alkanes were present as minor constituents in the two adult females of *G. caliginea* analyzed (Figure 3). In both sexes the methylalkanes were dominated by 3,7,11-/4,8,12-trimethylalkanes and 2-methylalkanes, with the former predominant in the males. EI mass spectra of the peak at KI 3153 in the females (Figure 5f) showed major fragments at *m/z* 196/197, 224/225, 267, and 295, consistent with a 13,17-dimethylalkane having a backbone carbon number of 31. The corresponding peak at KI 3355 showed major fragments at *m/z* 224/225 and 295 together with less abundant fragments at 196/197, 252/253, 267, and 323. A series of scans across this peak indicated that the former fragments were strongly correlated. This is consistent with a symmetric 15,19-dimethylalkane having a 33-carbon backbone as identified in males of *G. m. morsitans* (Nelson and Carlson, 1986). The remaining fragments were also correlated and represent an isomer eluting slightly later than the 15,19-dimethylalkane. That fragmentation was interpreted as the 33-carbon backbone homolog of the 13,17-dimethylalkane at KI 3155. A second homologous series

of internally branched alkanes (KI 3170 to KI 3570) was also present in the cuticular extracts of adult females of *G. caliginea*. EI mass spectra of the series mode at KI 3370 showed major fragments at 168/169, 224/225, 239, 295, 309, and 365, and the CI mass spectra indicated the presence of 36 carbons. This was consistent with a 33-carbon backbone trimethylalkane having an 11,15,19-structure. The corresponding 31-carbon backbone homolog was identified at KI 3155. EI mass spectra of the remaining significant peak in this series at KI 3570 showed major fragments at *m/z* 196/197, 224/225, 267, 295, 337, and 365 and was identified as 13,17,21-trimethylpentatriacontane. Dimethylalkanes and trimethylalkanes were also present in adult males of *G. caliginea* albeit at low levels. The mode for the 4,8,12-trimethylalkane series in *G. caliginea* males was at 29 carbons in the two individuals analyzed and the mode for the 2-methylalkane series at 30 carbons.

DISCUSSION

Tsetse flies of the subgenera *Glossina* and *Nemorhina* exhibit common patterns of cuticular alkanes consisting of even- and odd-carbon-number backbone homologous series of 2-methylalkanes, low levels of *n*-alkanes and, especially in females, long-chain internally branched di- and trimethylalkanes having backbone carbon numbers in the range of 31–39 carbons. Adults of both subgenera exhibit sexual dimorphism in the relative proportion of the internally branched methylalkanes, with the longer di- and/or trimethyl isomers often being the predominant saturated hydrocarbon compounds in females, and are implicated as mediators of sexual response in males of several species.

The subgenus *Nemorhina* shows a homologous series of 3,7,11- (KI *XX*40, exclusively odd-carbon-number backbone) and 4,8,12-trimethylalkanes (KI *XX*20, exclusively even-carbon-number backbone). The relative proportion of these compounds is sexually dimorphic in adults, with the 4,8,12- series of isomers the predominant methylalkanes in most males. Mass spectra for both series of trimethylalkanes were described from *G. palpalis palpalis* males (Nelson et al., 1988). The biosynthetic pathway is probably similar for this series, as they often appear together, and the remainder of the molecule is the same after the first branch point in an odd-/even-carbon-number backbone pair. The first published examples of trimethyls with both branching patterns were larger C_{34}–C_{39} alkanes found in ants (Martin and MacConnell, 1970), whereas only 3,7,11-trimethylheptacosane was reported in fire ants (Nelson et al., 1980). These trimethylalkanes are present at trace levels in the *G.* (*Glossina*) taxa. In addition, internally branched monomethylalkanes are relatively less common in taxa of *G.* (*Nemorhina*) than in *G.* (*Glossina*).

The predominant internally branched di- and trimethylalkanes in adult males

and females are not correlated with putative subgeneric status. Adult males in both subgenera are characterized by 11,15- and/or 13,17-dimethylalkanes having 31- or 33-carbon backbones as the most abundant internally branched alkane(s). The predominant internally branched methylalkanes in adult females with *G. (Glossina)* or *G. (Nemorhina)* varies in the number and position of methyl branches present, the number of methylene units between methyl branches, and the backbone carbon number; however, closely related taxa have similar cuticular methylalkane compositions and patterns. It should be noted that complications are possible in that some compounds may be transferred between the sexes by contact.

Within *G. (Glossina)*, taxa can be divided into three groups delineated by the predominant internally branched alkanes in adult females. The dominant isomer in female *G. m. morsitans*, *G. m. centralis*, *G. m. submorsitans*, and *G. swynnertoni* is 15,19,23-trimethylheptatriacontane (Carlson et al., 1993), although the mass spectra for the last three taxa were not reported until this work. The presence of similar methylalkane compositions and relative abundances in these closely related taxa suggests that this compound may be involved in mediating sexual response in males of all members of this complex, although this has been shown experimentally only in *G. m. morsitans* (Huyton et al., 1980), as colonies of the related species are rare or unavailable. The major internally branched methylalkanes in *G. longipalpis* are the homologs 13,23-dimethylpentatriacontane and 13,23-dimethylheptatriacontane. The cuticular methylalkane composition of adult females of the morphologically and ecologically similar species, *G. pallidipes*, is dominated by 13,23-dimethylpentatriacontane, which has been implicated as a contact sex pheromone (Carlson et al., 1984). The remaining species within *G. (Glossina)*, *G. austeni*, does not fit into either of these two classes. The predominant internally branched alkanes in adult females of *G. austeni* were reported as mixtures of 11,*x*-, 13,*x*-, and 15,*x*-dimethylalkanes having three or five methylene units between branch points (Nelson and Carlson, 1986); however, the relative abundances of these compounds were low and sexual dimorphism poorly defined. In addition, there appears to be considerable intraspecific variation in the predominant isomer in females (Sutton, unpublished data). While males of *G. austeni* were reported to show a positive response to synthetic 15,19-dimethyltritriacontane (Huyton et al., 1980), repeated bioassays of the natural methylalkanes were inconclusive (P. A. Langley, personal communication). Recent bioassays of the alkene fraction of cuticular extracts (Carlson, unpublished data) have shown activity comparable to that of the sexually mature unmated female. This suggests that the cuticular mediators for male sexual response have shifted in *G. austeni* to the unsaturated analogs of the internally branched methylalkanes.

In *G. p. palpalis* females, 13,17,21-trimethylheptatriacontane is the most abundant isomer present, making up almost one third of the total methylalkane

composition (Nelson et al., 1988), and has been implicated in mediating sexual behavior (Offor et al., 1981), although bioassays of this compound have not shown full activity in comparison to total hydrocarbon extracts. It is possible that polar constituents of the cuticle in this species are also involved in promoting sexual response in males. The cuticular alkane composition of females of *G. f. martinii* is essentially identical with that reported for *G. f. fuscipes* (Nelson et al., 1988) in that 13,17,21?-trimethylheptatriacontane was predominant, although the 11,15,19?- isomer was also present at significant levels, where the position of the third methyl branch was ambiguous. It is possible that the 11,15,19- and/or 13,17,21-trimethylheptatriacontanes have similar bioactivity in all *G. palpalis* and *G. fuscipes*, perhaps in combination with yet to be determined polar compounds.

In *G. pallicera* females, in contrast, the relative proportion of the 11,15,19-, and 13,17,21-trimethylalkanes in the individuals examined was far lower, less than 5–15% of the total alkanes and the 11,15,19- isomer was predominant. In addition, the 11,15- and 13,17-dimethyl homologs approached the trimethylalkanes in abundance.

The two females of *G. caliginea* examined did not exhibit clearly defined sexual dimorphism in the amounts of internally branched alkanes present or a dominant isomer. The methylalkane isomers present were consistent with those seen in *G. pallicera* and *G. palpalis* (Nelson et al., 1988).

The remaining species, *G. tachinoides*, is unique within the *G.* (*Nemorhina*) in that the dominant methylalkanes in adult females are 37-carbon backbone dimethylalkanes of the form 11,*x*- and 13,*x*- with 9 and 11 methylene units between the branch points.

It is not clear whether the differences observed in the relative abundances of internally branched trimethylalkanes in adult females of *G. palpalis/G. fuscipes* and *G. pallicera/G. caliginea* represent sampling bias, such as age variation, or a fundamental shift in the pheromone system, as appears to have occurred in *G. austeni*. Marked sexual dimorphism in the predominant internally branched alkanes in *G. tachinoides*, 11,23- and 13,25-dimethylheptatriacontane, began three days before eclosion, but did not reach its maximum extent until three days or more after emergence (Messoussi et al., 1994). The small degree of differentiation in the relative abundances of di- and trimethylalkanes between male and female *G. pallicera* and *G. caliginea* in comparison to *G. palpalis* and *G. fuscipes* may reflect the degree of sexual maturation of the females available for analysis. Further work is needed to establish the presence or absence of a common pheromone system between these four species.

Within *G.* (*Glossina*) and *G.* (*Nemorhina*), the interspecific variation in the internally branched alkanes in adult females implicated or suspected as mediating sexual response in males appears to represent a simple shift in isomer abundance rather than separate, de novo biosynthesis of each individual compound. In all

females, common patterns are observed that include several homologous series of internally branched di- and/or trimethylalkanes having backbone carbon numbers of 31–39. The predominant isomers are of the form 11,*x*-, 13,*x*-, 15,*x*-, 11,*x*,*y*-, 13,*x*,*y*-, and 15,*x*,*y*-, although it appears likely that complete di- and trimethylalkane series from 3,*x*- to 15,*x*- or 17,*x*- and 3,*x*,*y*-/4,*x*,*y*- to 15,*x*,*y*- or 17,*x*,*y*- exist in the cuticular lipids of all adults. In general, isomers having methyl branches close to an end position are most abundant at backbone carbon numbers below 31. The internally branched dimethylalkanes form two homologous series having 3 or 9 and/or 11 methylene units between the branch points. The former series is predominant in the males and has the mode at 31 or 33 carbons. The dimethylalkane series having 9 and/or 11 methylene units between branch points has the mode at 35 or 37 carbons and overlaps the former series in the region of 31–35 carbons. On high-resolution chromatographic columns such as the 50-m column used here with $>100{,}000$ theoretical plates, these two series partially resolve at each carbon number, forming a bimodal peak. If dimethylalkane isomers having intermediate numbers of methylene units between branch points exist, i.e., $I = 5$ and/or 7, they are usually below detection threshold, except in *G. austeni*. The internally branched trimethylalkanes, especially those isomers involved in behavioral modification, are predominantly of an $I = 3,3$ pattern, although other isomers may be present. There is a general trend in that the internally branched di- and trimethylalkanes having biological activity in the tsetse flies are symmetrical (or nearly so) in terms of the methyl branch positions on the alkane backbone.

The differences in the major internally branched alkanes in adult females between species, or groups of species, in *G.* (*Glossina*) and *G.* (*Nemorhina*) represent shifts in the relative abundances of the two dimethylalkane series and the trimethylalkane series. In addition, quantitative shifts in isomer composition within a series and at a given backbone carbon number may occur and exhibit geographical trends as reported for *G. pallidipes* (Carlson et al., 1984). The aberrant *G. austeni* represents the greatest extreme in this pattern.

Sexual dimorphism in tsetse may also result from relative shifts in methylalkane abundance rather than separate, specialized biosynthesis of each internally branched di- and trimethylalkane in females. Quantification of the methylalkane composition in tsetse males is complicated by the potential transfer of cuticular components during mating from females. This phenomenon is known in laboratory colonies since unmated males possessed very small quantities of trimethylalkanes normally produced by females (Nelson and Carlson, 1986). Conversely, the transfer of species-specific cuticular alkenes from male to female during mounting was observed by GC in seven members of *G.* (*Glossina*) and *G.* (*Nemorhina*) (Carlson and Langley, 1986), and the absence of compounds produced by the opposite sex was noted in conspecific virgin flies. We utilized wild males that would be expected to contain no conspecific female-produced

compounds if naive but may have acquired materials during mating, although this appears unlikely because of low populations. Thus it is not possible a posteriori to determine whether the low levels of the long-chain internally branched alkanes found in situ are male-produced, since males that have never had contact with females after eclosion are not readily available except in the laboratory. In addition, it was suggested that unmated blood-fed female tsetse cannot be found in the field (P. A. Langley, personal communication), implying that males continue to attempt copulation with all females, regardless of their mated status, and thereby accumulate more than trace amounts of conspecific female-produced trimethylalkanes.

The methylalkanes described here have chiral potential, but are of unknown stereochemistry. Although this is a subject beyond the scope of this paper, chirality may contribute to the activity or inactivity of synthetic hydrocarbons made for biological studies. Resolution of natural enantiomers is likely to be difficult because of limited optical rotation, as well as their lack of chromophores or other "handles."

The cuticular alkane patterns and composition of the remaining taxa of the subgenus *Austenina* (the *fusca* group) will be reported in a subsequent paper.

Acknowledgments—We especially thank Adrian Pont, formerly of the British Museum (Natural History), London, for his expert and patient cooperation in providing identified specimens. We thank Julio Hector for sample preparation and conducting the GC-MS analysis. We thank D. R. Nelson, M. Haverty, and four anonymous reviewers for their comments on this manuscript.

APPENDIX 1: SPECIMENS EXAMINED

Glossina (Glossina)

G. morsitans centralis: males—West Tanzania, Tabora Region, Tabora District, Kubunde village (15 km NW Tabora town), alt. 1200 m, Igombe R., 8 April 1982 (N = 2) (FSCA); N. Rhodesia, Nawadia District, Kazemba's village, 21-IV-1904, W. P. Kennelly, 1905-1979. (NHM); Zambia, Central Province, E edge of Kafue fly belt on main road, 14°69′S 26°40′E, Davies, 16/7/82 (N = 2) (FSCA); females—West Tanzania, Tabora Region, Tabora District, Kubunde village (15 km NW Tabora town), alt. 1200 m, Igombe R., 8 April 1982 (N = 2) (FSCA); Egyptian Sudan, Mengalla District, 16-IX-1921, Capt. Neil Cantlie M.C., BM 1922-98 (NHM); Zambia, Central Province, E edge of Kafue fly belt on main road, 14°69′S 26°40′E, Davies, 16/7/82 (N = 2) (FSCA). *G. m. submorsitans:* males—Upper Volta (FSCA); Uganda, Gondokoro, 1913 (BM); Ranch de Madina, Reg: Sikasso, Mali, 22 September 1979, A. Cisse (N = 2) (FSCA); females—Upper Volta (FSCA); Nigeria (FSCA); Uganda, 2 miles north of Lake Nwamia, 1913 (NHM); Ranch de Madina, Reg:

Sikasso, Mali, 22 September 1979, A. Cisse (N = 2) (FSCA). *G. swynnertoni:* males—Tanganyika, Shinyanga, 1954 (N = 4) (FSCA); females—Tanganyika, Atasha, 13-VIII-1950, E. Burtt, BM 1959-638 (NHM); Tanzania, Lake Manyara, October 22, 1981, D. Carlson (FSCA); Tanganyika, Shinyanga, 1954 (N = 3) (FSCA): *G. longipalpis:* males—Ivory Coast, February 1987, T. Jordan (FSCA); N. Nigeria, R. Niger, Horin Province, nr. Jebba, October 1909, Dr. C. W. McLeay W.A.M.S. (NHM); Gold Coast, Volta River, March 1908, G. C. Dudgeon, 1908-201 (NHM); W. Ashanti (grass country), F. E. Fell, 13-VII-1911, 1911-220 (NHM); females—Ivory Coast, February 1987, T. Jordan (N = 3) (FSCA); Sebe, S. Nigeria, May 1908, G. C. Dudgeon, 1908-201 (NHM); Gwatehipi, Abuja, Niger Province, May 1961, Brit. Mus. 1962-678 (NHM); S. Nigeria, Lagos, G. C. Dudgeon, April 1908, 1908-201 (NHM)

Glossina (Nemorhina)

G. fuscipes martinii: males—L. Tanganyika, Mpulungu, 31-V-1948, E. Burtt, B.M. 1959-638 (NHM); Tanganyika, 1948, Dr. E. Burtt (NHM); Tanganyika, 1948, E. Burtt (unfed) (NHM); females—L. Tanganyika, Mpulungu, 12-VIII-1948, E. Burtt, B.M. 1959-638 (NHM); L. Tanganyika, 21.5.48 (NHM); Tanganyika, 1948, E. Burtt (unfed) (NHM): *G. pallicera pallicera:* males—Ashanti, Western Prov., on road between Lechiri and Lanuso, 25-XII-1911, M. Wade (NHM); Gold Coast, Ashanti Sunyani, 7.7.1914, A. Ingram (NHM); Ashanti, West Africa, October 27, 1907, W. M. Graham, 1908-245 (NHM); females—Ashanti, 1912, Watson (NHM); Ashanti, April 1912, J. E. Zell (NHM); Suji, Liberia, W. Africa, 21-V-1909, Major A. Pearse, R.A.M.C., 1909-184 (NHM): *G. p. newsteadi:* Males—Belgian Congo, Lower Somami R., R. Elipa, X-1929, J. Schwetz (NHM); NE Belgian Congo, R. Ubele, Monja Terr., May 1929, J. Schwetz (NHM); females—Belgian Congo, X-1929, J. Schwetz (NHM); NW Ashanti: Chirat to Sunyani, 9-XII-1911, J. E. Fell, Presented by Ent. Res. Committee (NHM): *G. caliginea:* Males—S. Nigeria, Ori R., 10-III-1911, W. A. Lamborn, 1911-462 (NHM) Douala, French Cameroons, J. Rageau, 11-IV-1950, Presented by Com. Inst. Ent. BM 1950-16 (NHM); Belgian Congo, 1959 (NHM); females—Missellele, nr. Tiko, B.C., November 1959, Brit. Mus. 1962-678 (NHM); French Cameroon, 1937 (NHM)

REFERENCES

CARLSON, D. A., and LANGLEY, P. A. 1986. Tsetse alkenes: Appearance of novel sex-specific compounds as an effect of mating. *J. Insect Physiol.* 32:781–790.

CARLSON, D. A., LANGLEY, P. A., and HUYTON, P. A. 1978. Sex pheromone of the tsetse fly: Isolation, identification and synthesis of contact aphrodisiacs. *Science* 201:750–753.

CARLSON, D. A., NELSON, D. R., LANGLEY, P. A., COATES, T. W., DAVIS, T. L., and LEEGWATER-

VANDER LINDEN, M. 1984. Contact sex pheromone in the tsetse fly *Glossina pallidipes* Austen: Identification and synthesis. *J. Chem. Ecol.* 10:429–450.

CARLSON, D. A., MILSTREY, S. K., and NARANG, S. K. 1993. Genetic classification of the tsetse flies using cuticular hydrocarbons. *Bull. Entomol. Soc.* 83:507–515.

HUYTON, P. M., LANGLEY, P. A., CARLSON, D. A., and SCHWARZ, M. 1980. Specificity of contact sex pheromones in tsetse flies, *Glossina* spp. *Physiol. Entomol.* 5:253–264.

KOVATS, E. 1965. Gas chromatographic characterization of organic substances in the retention index system. *Adv. Chromatogr.* 1:229–235.

LANGLEY, P. A., PIMLEY, R. W., and CARLSON, D. A. 1975. Sex recognition pheromone of the tsetse fly. *Nature* 254:51–53.

LANGLEY, P. A., COATES, T. W., and CARLSON, D. A. 1982. A sex recognition pheromone in the tsetse fly, *Glossina pallidipes* Austen. *Experientia* 38:473–475.

MARTIN, M. M., and MACCONNELL, J. G. 1970. The alkanes of the ant, *Atta colombica*, *Tetrahedron* 26:307–319.

MATSUYAMA, K., and MORI, K. 1994. Synthesis of a stereoisomeric mixture of 13,25-, 11,21- and 11,23-dimethylheptatriacontane, the contact sex pheromone of the tsetse fly, *Glossina tachinoides*. *Biosci. Biotech. Biochem.* 58:539–543.

MCDOWELL, P. G., WHITEHEAD, D. L., and CHAUDHURY, F. B. 1981. The isolation and identification of the cuticular sex-stimulant pheromone of the tsetse *Glossina pallidipes* Austen (Diptera: Glossinidae). *Insect Sci. Appl.* 2:181–187.

MESSOUSSI, S., WICKER, C., ARIENTI, M., CARLSON, D. A., and JALLON, J. M. 1994. Hydrocarbons in species recognition in insects, *in* D. L. Hawksworth (ed.). The Identification and Characterization of Pest Organisms. CAB International. Wallingford.

NELSON, D. R., and CARLSON, D. A. 1986. Cuticular hydrocarbons of the tsetse flies *Glossina morsitans*, *G. austeni* and *G. pallidipes*. *Insect Biochem.* 16:403–416.

NELSON, D. R., and SUKKESTAD, D. R. 1970. Normal and branched aliphatic hydrocarbons from eggs of the tobacco hornworm. *Biochemistry* 9:4601–4611.

NELSON, D. R., FATLAND, C. L., HOWARD, R. W., MCDANIEL, C. A., and BLOMQUIST, G. J. 1980. Re-analysis of the cuticular methylalkanes of *Solenopsis invicta* and *S. richteri*. *Insect Biochem.* 10:163–168.

NELSON, D. R., CARLSON, D. A., and FATLAND, C. L. 1988. Cuticular hydrocarbons of the tsetse flies, II: *G. p. palpalis*, *G. p. gambiensis*, *G. fuscipes*, *G. tachinoides*, and *G. brevipalpis*. *J. Chem. Ecol.* 14:963–987.

OFFOR, I. I., CARLSON, D. A., GADZAMA, N. N., and BOZIMO, H. T. 1981. Sex-recognition pheromone in the West African tsetse fly, *Glossina palpalis palpalis* (Robineau-Desvoidy). *Insect Sci. Appl.* 1:417–420.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

TOXICOLOGICAL EFFECTS OF *Lycopersicon hirsutum* f. *glabratum* AND BEHAVIORAL RESPONSE OF *Macrosiphum euphorbiae*

LUCIANA MUSETTI and JONATHAN J. NEAL*

Department of Entomology, Purdue University, 1158 Entomology Hall, West Lafayette, Indiana 47907-1158

(Received August 12, 1996; accepted December 20, 1996)

Abstract—Volatiles in leaf extracts of *Lycopersicon hirsutum* f. *glabratum* disrupted settled *Macrosiphum euphorbiae* and caused 100% mortality over 24 hr. There was a single peak of activity among fractions separated by silica gel column chromatography. 2-Tridecanone was the major component of all active fractions, and responses to 2-tridecanone alone were similar to the initial extract. Another major component, 2-undecanone had no effect on *M. euphorbiae* either alone or as a synergist of 2-tridecanone. Trichome exudates and 2-tridecanone applied to artificial membranes inhibited feeding and caused mortality over 24 hr. Topical LD_{50} for 2-tridecanone was 1.0 μg/individual. The lab colony displayed little variation in susceptibility to 2-tridecanone. The effectiveness of 2-tridecanone in reducing infestation by *M. euphorbiae* and evidence of behavioral resistance in *M. euphorbiae* to 2-tridecanone are discussed.

Key Words—*Lycopersicon hirsutum*, *Macrosiphum euphorbiae*, pink potato aphid, glandular trichomes, 2-tridecanone, 2-undecanone, resistance, toxicity, feeding disruption.

INTRODUCTION

Resistance of *Lycopersicon hirsutum* f. *glabratum* C. H. Mull, accessions PI 126449 and PI 134417 to the potato aphid, *Macrosiphum euphorbiae* (Thomas), is associated with the glandular trichomes present on the leaf surface (Musetti, 1996). Intoxification and decreased probing and settling were noted in these aphids on this plant. Extracted volatiles of PI 134417 plants are toxic to other aphid species (Kennedy and Yamamoto, 1979). 2-Tridecanone has been iden-

*To whom correspondence should be addressed.

0098-0331/97/0500-1321$12.50/0 © 1997 Plenum Publishing Corporation

tified as an abundant constituent of type VI glandular trichomes and the major toxic component of PI 134417 and other accessions of *L. hirsutum* f. *glabratum* (Williams et al., 1980; Dimock and Kennedy, 1983; Lundgren et al., 1985; Lin et al., 1987). 2-Undecanone also occurs in trichomes of *L. hirsutum* f. *glabratum* (Soost et al., 1968; Lundgren et al., 1985) and has been associated with insect resistance (Farrar and Kennedy, 1987; Lin et al., 1987).

The objective of this study was to identify the allelochemicals from *L. hirsutum* f. *glabratum* accessions PI 126449 and PI 134417 that are responsible for toxicity to and behavioral changes in *M. euphorbiae*. Two behavioral assays, disruption of settling and feeding inhibition, were used to test plant extracts, separated fractions, and the methyl ketones 2-tridecanone and 2-undecanone. Vapor toxicity of plant extracts and vapor and topical toxicity of 2-tridecanone and 2-undecanone were measured. The amounts of 2-tridecanone in extracts and trichome heads were measured to determine whether the effects of the plant extracts on *M. euphorbiae* could be due to 2-tridecanone alone.

METHODS AND MATERIALS

Insects. A colony was established from a single apterous *M. euphorbiae* collected from a tomato plant in West Lafayette, Indiana, in August 1993. The colony was maintained on *L. esculentum* at 23°C, 65% relative humdity and 16:8 photophase.

Plants and Extracts. Seeds of *Lycopersicon hirsutum* f. *glabratum* accessions PI 126449 and PI 1324417 and *L. esculentum* cv. Ohio were obtained from Dr. Edward Tigchelaar, Department of Horticulture, Purdue University. Plants were grown in a greenhouse supplemented with metal halide illumination in a 16:8 photophase during summer 1994. Extracts were obtained from fully expanded, healthy leaves from 5- to 6-week-old plants by steeping in hexane for 1 hr. The extracts were filtered through Whatman No. 1 filter paper and the excess solvent partially removed by evaporation. Extracts were stored in glass vials at −20°C. Type VI glandular trichome heads were collected with a glass needle from 9 cm^2 of upper surface of fully developed leaves from 5- to 6-week-old PI 134417 and PI 126449 greenhouse plants. Trichome heads were counted and rinsed in 10 μl dichloromethane.

Column Chromatography. Extracts of *L. hirsutum* plants were fractioned by flash column chromatography (20-mm × 100-mm silica gel) using a hexane, dichloromethane, methanol series. Fractions (50 ml) were reduced to a ml for bioassays.

Gas Chromatography. Extracts and fractions from flash chromatography and type VI trichome heads were analyzed for 2-tridecanone and 2-undecanone content using a Varian 3400 gas chromatograph, 30-m × 0.32-mm DB1 fused

silica column (J&W Scientific), coupled to a flame ionization detector. Temperature program was: 100°C 4 min; 100–146°C at 4°C/min; 146–250°C at 30°C/min. 2-Tridecanone and 2-undecanone standards and 8-pentadecanone, internal standard, were purchased from Fluka AG. 2-Undecanone and 2-tridecanone retention times were 7.9 and 14.0 min, respectively.

Settling Disruption Assay. Ten aphids were allowed to settle on a fresh *L. esculentum* var. Ohio leaflet in a clear plastic cup (38 mm diameter × 40 mm deep with a snap lid). Filter paper discs (25 mm) treated with 20 μl of test solution or solvent (control) were allowed to evaporate for 30 min. Treatment discs were placed in the cup separated from the aphids by a perforated acrylic sheath. Settling disruption (number of aphids abandoning the feeding site), restlessness (number of aphids walking continuously) and intoxication (uncoordinated, slow movements or knock-down) were assessed. Ten cages were tested per treatment.

Topical Toxicity of 2-Tridecanone. To determine the LD_{50} of 2-tridecanone to adult *M. euphorbiae*, a series of eight 2-tridecanone doses (from 0.2 to 2.4 μg) and an acetone control were applied on the dorsal surface of apterous adult aphids (100/concentration) in 10 nl of acetone. Droplets were applied with an ISCO motorized syringe drive coupled to a 10-μl Hamilton syringe fitted with a finely drawn glass needle. Treated aphids were placed on fresh *L. esculentum* leaflets (20/leaflet) and kept in Petri dishes lined with moist filter paper. Mortality was recorded after 24 hr.

Feeding Cage Assay. Feeding solution, 15 ml of 20% sucrose, was placed in plastic cups (as described above) and sealed with Parafilm. Trichome exudates of Ohio, PI 126449, and PI 134417 were applied by pressing the upper surface of two fresh leaflets against the Parafilm membrane. Leaflets were from third and fourth fully developed leaves of 5- to 6-week-old greenhouse plants. Acetone (100 μl) was applied to the membranes to distribute the exudates evently. Membranes treated with acetone alone served as controls. Treated membranes were dried for 30 min. Plastic cups (as described above) were divided into two aphid test chambers with thin foam (0.5 mm). Ten aphids were placed in each chamber. The cup containing feeding solution was inverted so that the treated membrane formed the top of the chambers.

2-Tridecanone and 2-undecanone were tested alone and in mixtures on adult aphids in choice and no-choice situations. Test chemicals were applied to feeding cage membranes in 50 μl acetone and allowed to dry for 30 min. In no-choice tests, the entire membrane was treated. In choice tests, one half of the membrane was treated. Because the total treated membrane area in the no-choice test is twice that of the choice test, the amount of tridecanone per cage at a given membrane concentration is twice as great in the no-choice test as in the choice tests. A total of 12 replicates, with 10 aphids per replicate, was tested for each treatment. Mortality and the number of aphids settled and moving were

evaluated at 2, 4, 6, and 24 hr. LC_{50} values at 24 hr were estimated. Stylet sheaths were stained with safranin, the membranes inverted onto a grid, and stylet sheaths within a 147-mm^2 area counted under a stereoscopic microscope (Neal et al., 1990). Feeding inhibition was calculated as follows:

$$\text{Feeding inhibition} = 100\% - \frac{\text{stylet sheaths density (treated)}}{\text{stylet sheaths density (control)}}$$

EC_{50} values for feeding inhibition were estimated.

Statistical Analysis. LC_{50} and feeding inhibition EC_{50} were determined by probit analysis using Microprobit 3.0 (Dr. Thomas C. and Alexandra Sparks, unpublished). This program uses Abbott's transformation for control mortality and calculates log dose probit lines according to the iterative process described by Finney (1971). Aphid response to extract volatiles, feeding inhibition, and

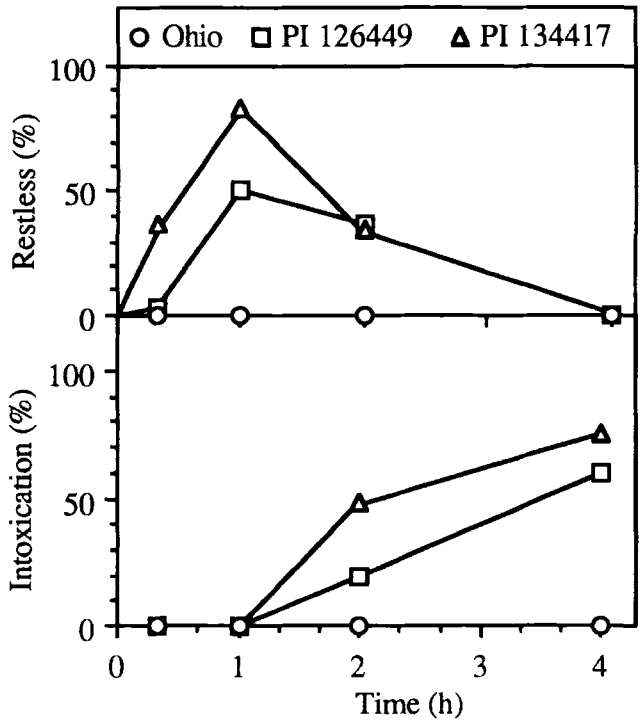


FIG. 1. Behavioral responses of settled *Macrosiphum euphorbiae* adults to *Lycopersicon esculentum* and *L. hirsutum* f. *glabratum* extract volatiles. Restless behavior describes aphids that have abandoned the feeding site and are wandering. With time, restless aphids become intoxicated as evidenced by a staggering gait or knockdown.

feeding cage mortality were compared by contingency analyses (G test). Concentrations of 2-tridecanone and 2-undecanone in *L. hirsutum* f. *glabratum* accessions were compared using Student's *t* test.

RESULTS

Effect of Extracts on Aphid Settling Behavior. Both PI 134417 and PI 126449 extracts disrupted settled aphids. No behavioral response or intoxication was observed in cages with Ohio extract (Figure 1). The first signs of settling disruption, characterized by intense antennation, were observed by 0.2 hr after introduction of PI 134417 extracts. At 0.3 hr, a significant number of aphids exposed to PI 134417 extracts had abandoned feeding and were walking, compared to none in the PI 126449 or Ohio treatments ($P < 0.001$, G test). Moving aphids did not settle again during the test period. At 0.3 and 1 hr, the percentage of restless aphids was significantly higher in the PI 134417 than the PI 126449 treatment and significantly higher in both *L. hirsutum* treatments than the Ohio treatment ($P < 0.001$, G test). Decrease in restlessness after 1 hr was due to an increase in intoxication. Percentage intoxication was significantly greater in the PI 134417 than the PI 126449 treatment only at 2 hr ($P < 0.05$, G test). PI 134417 extract volatiles were more effective (restlessness + intoxication) than PI 126449 against the aphids at 0.3, 1 and 2 hr, but not at 4 hr ($P = 0.05$, G test). No mortality was observed at 4 hr.

Determination of Active Fractions. After separation of extract components by silica gel chromatography, all activity (as measured by feeding disruption and restlessness activity at 1 hr and mortality at 24 hr) resided in a single peak of dichloromethane fractions (Figure 2). Activity was not present in the hexane eluant and was completely eluted by dichloromethane. Fractions with the highest biological activity caused 100% mortality after 24 hr. Active fractions had a strong 2-undecanone odor.

Gas Chromatographic Analyses. The predominant chemical in all active fractions of both *L. hirsutum* accessions, 2-tridecanone, was present at 10.7 mg/ml or greater (equivalent to 214 μg of 2-tridecanone per test cage), while no inactive fraction contained more than 4.8 mg/ml. For the most active fractions of both *L. hirsutum* accessions, 2-tridecanone and 2-undecanone contributed 89% of the area counts (GC-FID). Ratios of 2-tridecanone to 2-undecanone were about 4:1 in both PI 126449 and PI 134417 extracts and concentration was not significantly different (Table 1; $P > 0.05$, Student's *t*). 2-Tridecanone and 2-undecanone were not detected in extracts of Ohio. Type VI trichome heads from PI 126449 and PI 134417 averaged 9.9 and 14.6 ng of 2-tridecanone, respectively. These values were not significantly different ($P > 0.05$, Student's *t*).

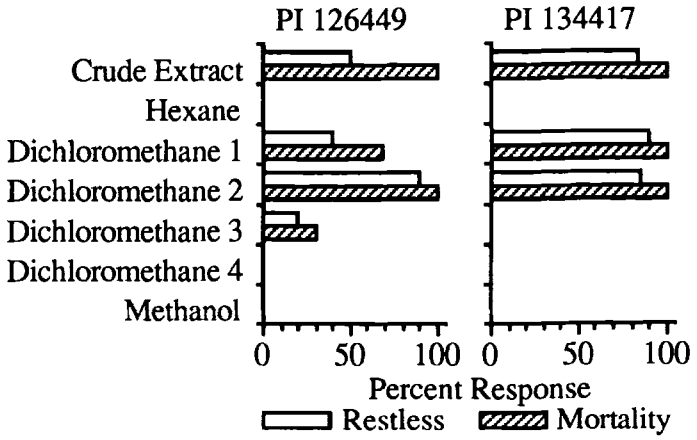


FIG. 2. Restless activity at 1 hr and 24-hr mortality of settled *Macrosiphum euphorbiae* adults exposed to *L. hirsutum* f. *glabratum* volatiles present in extracts and extract fractions.

Effect of 2-Tridecanone and 2-Undecanone Volatiles on Aphid Settling Behavior. Aphid settling disruption response at 1 hr to 2-tridecanone was similar to whole leaf extracts and active fractions from PI 126449 and PI 134417 (Figure 3). The EC_{50} for restlessness at 1 hr for 2-tridecanone, was 220 μg/cage (195–250 μg, 95% CI). Intoxication at 1 hr was only observed at the highest 2-tridecanone concentration (680 μg/cage). The highest concentration of 2-undecanone (680 μg/cage) had no effect.

Toxicity of 2-Tridecanone. The topical LD_{50} of 2-tridecanone to *M. euphorbiae* adults was 1.0 μg per aphid (Figure 4). Control mortality was less than

TABLE 1. 2-TRIDECANONE AND 2-UNDECANONE CONTENT PER LEAF AREA (MEAN ± SD) AND PERCENT FOLIAGE FRESH WEIGHT FFW, (RANGE) IN *L. hirsutum* f. *glabratum* LEAF EXTRACTS

	2-Tridecanone		2-Undecanone	
	μg/cm²	% FFW	μg/cm²	% FFW
PI 126449	19.5 ± 6.6	0.08–0.10	5.2 ± 1.7	0.02–0.03
PI 134417	28.7 ± 7.5	0.12–0.17	7.1 ± 1.8	0.03–0.04

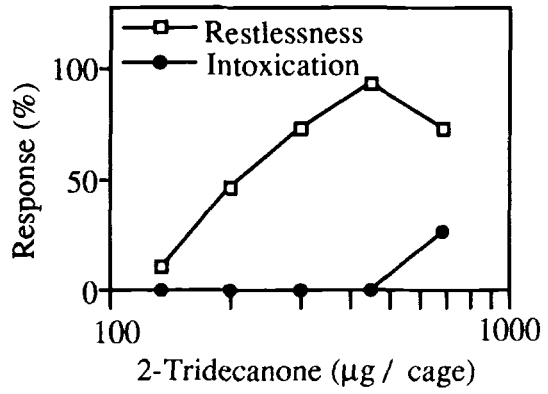


FIG. 3. Behavioral responses of settled *Macrosiphum euphorbiae* adults to 2-tridecanone volatiles at 1 hr.

5%. The slope, 4.5 probits/log, indicates a relatively homogeneous population with little variation in susceptibility to 2-tridecanone within the lab colony.

Effect of Exudates on Aphid Mortality and Feeding Behavior. In applications to feeding cage membranes, exudates of both PI 134417 and PI 126449 caused significant mortality compared to Ohio exudates and the control (Figure 5; $P < 0.05$, G test). Mortality was significantly higher from PI 134417 than PI 126449 exudate ($P = 0.03$, G test). Control mortality was less than 5%.

2-Tridecanone caused mortality, but 2-undecanone had no effect in choice and no-choice tests (Figure 6). In no-choice tests, the 24-hr LC_{50} for 2-tridecanone alone (64 $\mu g/cm^2$) was not significantly different from the LC_{50} cal-

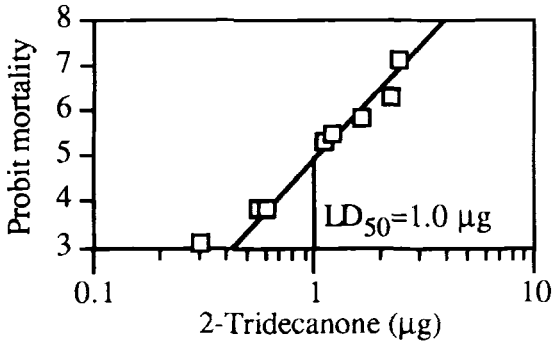


FIG. 4. Topical toxicity of 2-tridecanone to adult *Macrosiphum euphorbiae*.

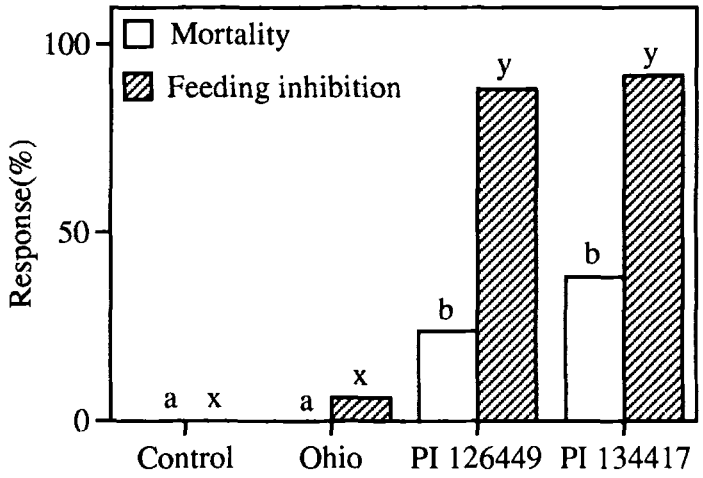


FIG. 5. Mortality and feeding inhibition of *Macrosiphum euphorbiae* adults feeding through artificial membrane treated with glandular trichome exudates. Feeding inhibition was calculated as 100% minus the stylet sheath density of the treatment as a percent of the control cage average. Columns labeled by the same letter (a, b, x, y) are not significantly different (G test; $P = 0.05$).

culated from the 2-tridecanone component of the tridecanone–undecanone mixture (56 $\mu g/cm^2$; Table 2). In choice tests, only the highest concentration of 2-tridecanone (132 $\mu g/cm^2$) produced greater than 50% mortality. Control mortality was always lower than 5%.

Exudates of both PI 134417 and PI 126449 inhibited feeding compared to Ohio exudate and controls (Figure 5, $P = 0.05$; G test). Ohio exudate had no significant effect on feeding.

2-Tridecanone reduced the number of stylet sheaths, but 2-undecanone had no effect. Feeding inhibition EC_{50} for the no-choice test and the treated area of the choice test were significantly lower than for the untreated area in the choice test for both 2-tridecanone and the 2-tridecanone–2-undecanone mixture (Table 2). 2-Tridecanone feeding inhibition EC_{50} for the no-choice test was significantly lower than for the choice-treated area as determined from 95% confidence intervals.

DISCUSSION

2-Tridecanone alone could be responsible for the behavioral and toxic effects of accessions PI 126449 and PI 134417 on *M. euphorbiae*. Extract fractions

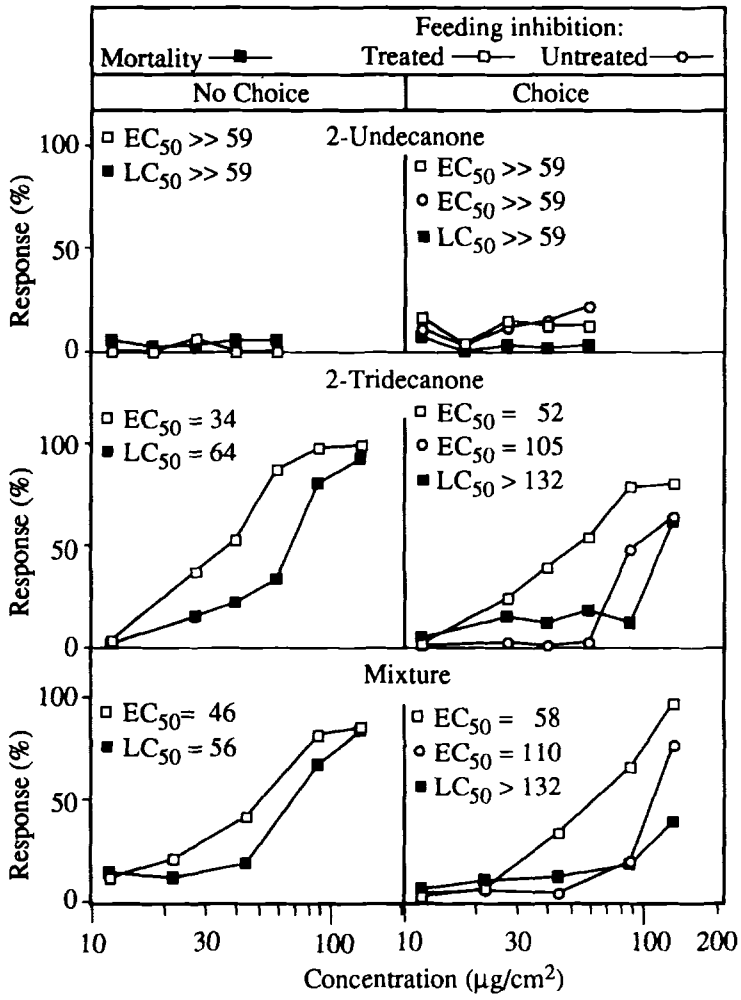


FIG. 6. Mortality and feeding inhibition (24 hr) of *Macrosiphum euphorbiae* adults feeding through artificial membrane treated with 2-tridecanone and 2-undecanone, alone and in mixtures. Treated membrane area was 11.3 cm^2 and 5.7 cm^2 in the no-choice and choice treatments, respectively.

tested at 214 μg 2-tridecanone per cage or higher disrupted settling while those with less than 96 μg/cage were inactive. 2-Tridecanone alone disrupted settling at 200 μg/cage and was not active at 90 μg/cage. Thus, the active range is identical. Exudate effects on feeding and mortality in feeding cages (transferring all the trichomes from two PI 134417 leaves would produce a 2-tridecanone

TABLE 2. TOXICITY (LC_{50} AND 95% CI) AND FEEDING INHIBITION (EC_{50} AND 95% CI) OF 2-UNDECANONE AND 2-TRIDECANONE, ALONE AND IN MIXTURES, TO ADULT *Macrospihum euphorbiae* IN CAGES WITH FEEDING MEMBRANES TREATED IN CHOICE AND NO-CHOICE PRESENTATIONS

	LC_{50} ($\mu g/cm^2$)[a]		EC_{50} ($\mu g/cm^2$)[a]		
Treatment	No-Choice	Choice	No-Choice	Choice treated	Choice untreated
2-Undecanone	$\gg 59$	$\gg 59$	$\gg 59$	$\gg 59$	$\gg 59$
2-Tridecanone	63.6 (58.0–69.3)	> 132	34.2 (29.6–38.4)	52.3 (44.6–61.8)	105.5 (87.8–149.6)
Mixtures	56.2 (39.0–80.7)	> 132	45.8 (35.7–58.5)	58.1 (41.0–76.5)	109.7 (86.8–142.7)

[a]Treated membrane area was 11.3 cm^2 and 5.7 cm^2 in the no-choice and choice treatments, respectively.

density of 29 $\mu g/cm$) were similar to 30 $\mu g/cm$ 2-tridecanone. 2-Tridecanone has similar toxic effects on other arthropods including other species of aphids (Williams et al., 1980; Lin et al., 1987).

2-Undecanone, the second major constituent of *L. hirsutum* trichome exudates, affects some insects but not others. Lin et al. (1987) have shown that 2-undecanone has a synergistic effect on 2-tridecanone toxicity to neonate larvae of *Keiferia lycopersicella* and *Spodoptera exigua*. Farrar and Kennedy (1987) demonstrated undecanone effects on *Helicoverpa zea* but found no effect on *Manduca sexta*. However, 2-undecanone is not deterrent, repellent, or otherwise effective either alone or as a synergist of 2-tridecanone activity against *M. euphorbiae*. Lin et al. (1987) speculated that 2-undecanone could indirectly enhance resistance to aphids through effects on physical characteristics of the trichome exudates, such as viscosity.

The repellent and deterrent effects of 2-tridecanone may be more important in resistance to *M. euphorbiae* than toxicity. In choice tests, feeding was inhibited at 2-tridecanone concentrations that cause little mortality. *M. euphorbiae* will quickly abandon leaves of PI 126449 and PI 134417 without probing (Musetti, 1996). Unless confined, few dead *M. euphorbiae* are found on *L. hirsutum* plants (Musetti, 1996). Feeding is equally inhibited by 2-tridecanone on no-choice and choice-treated membranes, indicating that the mechanism of resistance is not nonpreference (Painter, 1951). 2-Tridecanone has an antibiotic effect on aphids in a confined space, but its action in the field is probably primarily due to antixenosis.

There is evidence of behavioral adaptation to 2-tridecanone. *M. euphorbiae*

can establish on *L. hirsutum* in the field or greenhouse (Quiros et al., 1977; Musetti, 1996), although infestations are reduced compared to *L. esculentum.* On *L. hirsutum* leaflets, *M. euphorbiae* elevate their bodies by extending their legs to avoid contact with trichomes while walking and feeding (Musetti, 1996). This posture may be easier to maintain on the abaxial surface of the leaf than on the adaxial surface. *M. euphorbiae* also avoid the central portion of the adaxial surface of *L. hirsutum* leaflets, confining movement to the abaxial surface and leaflet edge (Musetti, 1996). *M. euphorbiae* that settle on *L. hirsutum* are exclusively on the abaxial surface. This contrasts with *L. esculentum*, on which some aphids settle on the adaxial surface. At the PI 134417 average of 14.6 ng 2-tridecanone in each trichome head, an aphid would only have to accumulate the contents of 69 trichomes to receive the LD_{50} (1 μg). At 9.8 trichomes/mm^2 (Musetti, 1996), the average distance between trichomes is 0.32 mm, less than half the average width (0.75 mm) of an adult *M. euphorbiae.* Moving only 11 mm could bring an aphid in contact with 69 trichomes. Thus, behavioral avoidance is important for *M. euphorbiae* that establish on *L. hirsutum.*

There has been considerable interest in incorporating 2-tridecanone into commercial tomato varieties as a resistance factor. Based on field observations (Musetti, 1996) and laboratory evaluations, 2-tridecanone would likely be effective in reducing infestation by *M. euphorbiae* if incorporated into commercial varieties. However, populations of *M. euphorbiae* do establish on 2-tridecanone-containing plants and the potential for development of an *M. euphorbiae* biotype resistant to 2-tridecanone needs to be evaluated. Development of biotypes resistant to 2-tridecanone could be exacerbated because of adverse effects of 2-tridecanone on important natural enemies of pest species (Kennedy, 1984; Kashyap et al., 1991; Farrar and Kennedy, 1993). Both as a resistance management strategy and to provide more complete control, combining of 2-tridecanone with other chemical and physical resistance factors could prove more useful.

Acknowledgments—We thank the late Dr. Edward C. Tigchelaar for his contributions to this project, Dr. Thomas C. and Alexandra Sparks for the "Microprobit 3.0" computer program used for probit analysis, Jerry Blosser and Dr. Paul Grover for technical assistance, and Dr. Linda Mason and Richard Shade for critical review of this manuscript. This project was supported by Hatch Project IND58058 and a scholarship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior)-Brazil, to L.M. This is paper number 15144 of the Purdue University Agricultural Experiment Station.

REFERENCES

DIMOCK, M. B., and KENNEDY, G. G. 1983. The role of glandular trichomes in the resistance of *Lycopersicon hirsutum* f. *glabratum* to *Heliothis zea. Entomol. Exp. Appl.* 33:263–268.

FARRAR, R. R., and KENNEDY, G. G. 1987. 2-Undecanone, a constituent of the glandular trichomes of *Lycopersicon hirsutum* f. *glabratum:* Effects on *Heliothis zea* and *Manduca sexta* growth and survival. *Entomol. Exp. Appl.* 43:17–23.

FARRAR, R. R., and KENNEDY, G. G. 1993. Field cage performance of 2 tachinid parasitoids of the tomato fruitworm on insect resistant and susceptible tomato lines. *Entomol. Exp. Appl.* 67:73–78.

FINNEY, D. J. 1971. Probit Analysis, 3rd ed. Cambridge University Press, Cambridge.

KASHYAP, R. K., KENNEDY, G. G., and FARRAR, R. R. 1991. Mortality and inhibition of *Helicoverpa zea* egg parasitism rates by *Trichogramma* in relation to trichome methyl ketone-mediated insect resistance of *Lycopersicon hirsutum* f. *glabratum*, accession PI 134417. *J. Chem. Ecol.* 17:2381–2395.

KENNEDY, G. G. 1984. 2-Tridecanone, tomatoes and *Heliothis zea:* Potential incompatibility of plant antibiosis with insecticidal control. *Entomol. Exp. Appl.* 35:305–311.

KENNEDY, G. G., and YAMAMOTO, R. T. 1979. A toxic factor causing resistance in a wild tomato to the tobacco hornworm and some other insects. *Entomol. Exp. Appl.* 26:121–126.

LIN, S. Y., TRUMBLE, J. T., and KUMAMOTO, J. 1987. Activity of volatile compounds in glandular trichomes of *Lycopersicon* species against two insect herbivores. *J. Chem. Ecol.* 13:837–850.

LUNDGREN, L., NORELIUS, G., and STENHAGEN, G. 1985. Leaf volatiles from some wild tomato species. *Nord. J. Bot.* 5:315–320.

MUSETTI, L. 1996. Resistance of two accessions of *Lycopersicon hirsutum* f. *glabratum* C.H. Mull to apterous forms of the pink potato aphid, *Macrosiphum euphorbiae* (Thomas) (Homoptera: Aphididae). Doctoral dissertation. Purdue University, West Lafayette, Indiana.

NEAL, J. J., TINGEY, W. M., and STEFFENS, J. C. 1990. Sucrose esters of carboxylic acids in glandular trichomes of *Solanum berthaultii* deter settling and probing by green peach aphid. *J. Chem. Ecol.* 16:487–497.

PAINTER, R. H. 1951. Insect Resistance in Crop Plants. Macmillan, New York, 520 pp.

QUIROS, C. F., STEVENS, M. A., RICK, C. M., and KOK-YOKOMI, M. L. 1977. Resistance in tomato to the pink form of the potato aphid (Macrosiphum euphorbiae Thomas): The role of anatomy, epidermal hairs and foliage composition. *J. Am. Soc. Hortic. Sci.* 102:166–171.

SOOST, R. K., SCORA, R. W., and SIMS, J. J. 1968. Contributions to the chromatographic analysis of the leaf oils in the genus *Lycopersicon. Proc. Am. Soc. Hortic. Sci.* 92:568–571.

WILLIAMS, W. G., KENNEDY, G. G., YAMAMOTO, R. T., THACKER, J. D., and BORDNER, J. 1980. 2-Tridecanone: Naturally-occurring insecticide from the wild tomato species *Lycopersicon hirsutum* f. *glabratum. Science* 207:888–889.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

AGGREGATION PHEROMONE OF *Pityokteines elegans*

J. E. MACÍAS-SÁMANO,[1] J. H. BORDEN,[1,*] H. D. PIERCE, JR.,[2] R. GRIES,[1] and G. GRIES[1]

[1]*Centre for Pest Management, Department of Biological Sciences*
[2]*Department of Chemistry*
Simon Fraser University
Burnaby, British Columbia V5A 1S6, Canada

(Received September 19, 1996; accepted December 20, 1996)

Abstract—In laboratory bioassay experiments, the beetles *Pityokteines elegans* were attracted to volatiles captured from bolts of grand fir, *Abies grandis*, colonized by *P. elegans* males. Male-specific volatiles detected by coupled gas chromatographic–electroantennographic detection (GC-EAD) analysis and by GC–mass spectrometry employing a chiral column were: (*S*)-(−)-ipsenol, (+)- and (−)-ipsdienol, and ipsenone. Field experiments demonstrated that 1 : 1 combinations of (−)-ipsenol and (±)-ipsdienol strongly attracted insects of both sexes to multiple-funnel traps. No beetles were attracted to any of these compounds alone, and both enantiomers of ipsdienol were required with (−)-ipsenol to induce attraction. Ipsenol and ipsdienol are now reported as pheromones of five *Pityokteines* species. Specificity of semiochemical-based communication between sympatric *P. elegans* and *P. minutus* appears to be based on host preference and on the composition and chirality of the pheromone blend.

Key Words—Semiochemical, secondary attraction, pheromone, *Pityokteines elegans*, enantiomer, ipsenol, ipsdienol, ipsenone.

INTRODUCTION

The bark beetle, *Pityokteines elegans* (Swaine) (Coleoptera: Scolytidae), colonizes grand fir, *Abies grandis* (Doug.) Lindl.; red fir, *A. magnifica* A. Murr.; white fir, *A. concolor* (Gord. & Glend.) Hildebr.; and possibly Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco (Bright and Stark, 1973; Bright, 1976; S. L. Wood, 1982). The biology of *P. elegans* is unknown, but it is suspected

*To whom correspondence should be addressed.

0098-0331/97/0500-1333$12.50/0 © 1997 Plenum Publishing Corporation

to be similar to that of related species such as *P. sparsus* LeConte in balsam fir, *A. balsamea* (L.) Mill., and *P. minutus* Swaine in subalpine fir, *A. lasiocarpa* (Hook) Nutt. (Bright, 1976; S. L. Wood, 1982).

All *Pityokteines* spp. are polygamous (S. L. Wood, 1982). Males are the pioneer sex (Chararas, 1962; Dajoz, 1980; D. L. Wood, 1982) and build a star-shaped nuptial chamber in the phloem of cut, fallen, or dying trees (Belyea, 1952; Chararas, 1962; Bright, 1976; Hosking and Knight, 1976; S. L. Wood, 1982). *P. elegans*, *P. sparsus*, *P. minutus*, and *P. lasiocarpi* (Swaine) are reportedly never found in true firs or in the absence of other bark beetles, suggesting that they are secondary colonizers (Belyea, 1952; Hertert et al., 1975; Hosking and Knight, 1976; Basham, 1986; Furniss and Carolin, 1986; R. L. Livingston, personal communication, Insects and Diseases Section, Idaho Department of Lands, Coeur d'Alene, Idaho). *Pityokteines elegans* usually inhabits trees weakened by root diseases and other insects, notably the fir engraver, *Scolytus ventralis* LeConte (Hertert et al., 1975; S. L. Wood, 1982).

It is generally accepted that scolytid beetles search for and detect suitable hosts by using visual and chemical cues (D. L. Wood, 1982; Borden, 1982). Most scolytids achieve mass attack of a scarce host resource (Atkins, 1966) through a secondary attraction response to a blend of host kairomones and beetle-produced aggregation pheromones (D. L. Wood, 1982; Birch, 1984). This phenomenon benefits the beetles by signaling both the location of a suitable host and the presence of potential mates (Borden, 1985).

Secondary attraction has been well demonstrated in several *Pityokteines* spp. Ipsenol (2-methyl-6-methylene-7-octen-4-ol) and ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) are reported as pheromones for the sympatric species, *P. curvidens* Ger. and *P. vorontzovi* Jacob, both of which infest silver fir, *A. alba* Mill, in Europe (Harring et al., 1975; Harring and Mori, 1977; Harring, 1978). *Pityokteines vorontzovi* males produce ipsenol, ipsdienol, and (*Z*)-verbenol, (*Z*)-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol (Harring, 1978). In the field, *P. vorontzovi* males were more attracted than females to mixtures of ipsenol plus (*R*)-(−)-ipsdienol. Male *P. curvidens* produce ipsenol and (*Z*)-verbenol (Harring et al., 1975; Harring and Mori, 1977; Harring, 1978). In field tests, both sexes were attracted to (*S*)-(−)-ipsenol; the antipode was biologically inactive and (±)-ipsenol attracted fewer beetles than (*S*)-(−)-ipsenol (Harring and Mori, 1977). Neither species responded to (*Z*)-verbenol. Males of *P. spinidens* Reitt. produce both ipsenol and ipsdienol, but neither was bioactive in field tests (Harring, 1978).

North American *P. minutus* produce (*S*)-(−)-ipsenol and (*S*)-(+)-ipsdienol in a 1.3:1 ratio. (Camacho-Vera, 1993). In laboratory bioassays, males were equally attracted to both components alone and combined, but females were more strongly attracted to the combination. In the field, only (−)-ipsenol was attractive.

Our objectives were to: (1) identify candidate pheromones produced by *P. elegans*; (2) determine the bioactivity of these compounds in the laboratory and field; and (3) investigate which optical isomers mediate attraction of *P. elegans* in the field.

METHODS AND MATERIALS

Collection of Insects and Host Materials. Bolts of grand fir, both uninfested and infested with *S. ventralis* and *P. elegans*, were collected in August and September 1993–1995, from felled trees near Coeur d'Alene, Idaho. They were kept at 2°C until used. To obtain beetles, infested logs were transferred into mesh screen cages held at 24–30°C; water was sprayed on them every five to six days to prevent dessication. Emerged beetles were collected daily, sexed by the presence (females) or absence (males) of a setal brush on their frons (Bright, 1976), and held at 4°C in jars with moistened paper.

Collection and Analysis of Volatiles. Preformed entrance holes (1.5 mm diam.), approximately 3 cm apart, were drilled through the bark of fresh grand fir bolts ca. 21 cm long and 12 cm diam. These bolts were set inside separate glass aeration chambers (28 cm long and 15 cm diam.); 200 males, 200 females, or 200 males plus 200 females were added, and allowed to bore into the bark. Uninfested bolts were set in similar devices and used as controls. Air was drawn through the chamber at 1.7 liter/min and then through glass tubing (14 mm OD, 20 cm long) containing Porapak-Q (Byrne et al., 1975). Volatiles were eluted from the trap with 150 ml of distilled pentane and the eluent concentrated to 5 ml by distillation in a 30-cm Dufton column.

Differential diagnosis (Vité and Renwick, 1970) of male- and female-produced volatiles allowed identification of sex-specific compounds. GC analyses employed Hewlett Packard 5830A, 5880A, and 5890A instruments equipped with capillary inlet systems and flame-ionization detectors. Capillary columns (30 m × 0.25 or 0.32 mm ID) coated with SP-1000 (Supelco, Bellefonte, Pennsylvania) or DB-1 (J&W Scientific, Inc., Folsom, California) were used. A Hewlett Packard 5895B fitted with the above columns was employed for coupled GC–mass spectrometry (MS). The injection port, transfer line, and ion source were 260°, 250°, and 200°C, respectively. Helium was the carrier gas for the GC and GC-MS. Compound identities were confirmed by comparison of their mass spectra with those of authentic samples. Enantiomeric compositions were determined by GC analyses using Chirasil-Dex (8) (30 m × 0.25 mm ID, V. Schurig, Tübingen, Germany) and Cyclodex-B columns (30 m × 0.25 mm ID, J&W).

Captured volatiles were also subjected to coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses (Arn et al., 1975)

adapted for intact bark beetles (Gries, 1995). Antennal responses were amplified utilizing a custom-built amplifier with a passive low-pass filter and a cutoff frequency of 10 kHz.

Laboratory Experiments. Behavioral activity of captured volatiles was tested in laboratory experiments employing a single-choice, open-arena olfactometer (Stock and Borden, 1983). Prior to bioassays, beetles were placed in groups of 10 (sexes kept separately) into Petri dishes with moistened paper and held at 21°C and 69 lux for 2 hr. A group of 10 beetles was released in the center of a filter paper arena (18.5 cm diam.) and the beetles were allowed 3 min to walk upwind against a gentle airstream (700 ml/min) of medical air, released through a filter-paper-lined tube (10 cm diam.) impregnated with volatile extract in pentane or a pentane control stimulus (Table 1). A beetle that entered an arena 2.0 × 2.0 × 3.0 cm at the outlet of the glass tube was classed a responder.

(*S*)-(−)-Ipsenol (97.1% optically pure), (±)-ipsdienol (96.9% pure), (*S*)-(+)-ipsdienol (98.3% optically pure) and (*R*)-(−)-ipsdienol (98.4% optically pure) were purchased from Phero Tech Inc., Delta, British Columbia. Except for results of chemical analyses, enantiomers are referred to hereafter as either (−) or (+). Ipsenone was synthesized by chronic acid oxidation of racemic

TABLE 1. RESPONSES OF WALKING MALE OR FEMALE *P. elegans* IN LABORATORY BIOASSAY EXPERIMENTS 1–3 (5 GROUPS OF 10 INSECTS PER TREATMENT)

Experiment	Treatment	Dose	Percent response ($\bar{X}$ ± SE)[a] Males	Females
1	Pentane control	1 μl	26 ± 4.0a	36 ± 5b
	Host volatiles	5 gram-hours	12 ± 3.7a	18 ± 5.5c
	Host + female volatiles	5 beetle-hours	24 ± 6.0a	40 ± 7.5b
	Host + male + female volatiles	5 beetle-hours	26 ± 10.0a	55 ± 8ab
	Host + male volatiles	5 beetle-hours	42 ± 7.3a	65 ± 5.5a
2	Pentane control	1 μl	38 ± 8.0ab	38 ± 3.7a
	Ipsenone	1 μg	18 ± 5.6b	26 ± 6.5a
	(−)-Ipsenol	1 μg	40 ± 3.1a	30 ± 4.4a
	(±)-Ipsdienol	1 μg	44 ± 9.2a	22 ± 5.5a
3	Pentane control	1 μl	24 ± 11.5a	10 ± 4.4b
	(−)-Ipsenol + (±)-ipsdienol	1:1 ng	36 ± 9.7a	42 ± 5.8a
		1:0.5 ng	44 ± 10.2a	38 ± 8.6a
	(−)-Ipsenol + (±)-ipsdienol + ipsenone	1:1:1 ng	36 ± 4.0a	48 ± 7.3a
		1:0.5:0.1 ng	36 ± 4.0a	42 ± 3.7a

[a]Means within an experiment and column followed by the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh multiple range test, $P > 0.05$.

ipsenol (Brown et al., 1971) and was 97.2% pure after purification by preparative thin-layer chromatography on silica gel (pentane–ether, 4:1, v/v). The NMR spectral data were in agreement with those reported by Fish et al. (1984).

Attractiveness of test stimuli to walking beetles was tested in three laboratory bioassay experiments (experiments 1–3, Table 1). Experiment 1 tested volatiles from male-, female-, and male- plus female-infested bolts, using pentane or medical air as control stimuli. Experiment 2 tested synthetic (*S*)-(−)-ipsenol, (±)-ipsdienol, or ipsenone, employing 1 μl of pentane as a control stimulus. Experiment 3 tested (−)-ipsenol in combination with (±)-ipsdienol alone or with ipsenone. Doses were much lower than in experiments 1 and 2 and were adjusted to approach the level of ipsenone (4 ng) in 1 beetle-hour (bh) of aeration (experiment 1).

Field Experiments. Compounds that were antennally active or attractive in the laboratory were field tested in a mature *Abies grandis/Acer rubrum* forest with Douglas fir well represented (Steel and Gier-Hayes, 1992), located 10 km north of Coeur d'Alene, Idaho. Twelve-unit, multiple-funnel traps (Lindgren, 1983) (Phero Tech Inc.) baited with candidate pheromones were deployed in 10 randomized complete blocks, with ≥15 m between traps and 15 m between lines.

All compounds were released from bubble caps (Phero Tech Inc.) with butanediol added as stabilizer for ipsenol and ipsdienol. Release rates of ipsenol, ipsdienol, and ipsenone, determined at 22°, 22°, and 27°C (Phero Tech Inc.), were 0.15, 0.15, and 0.5 mg/24 hr, respectively.

All field experiments (Table 2; Figure 4 below) tested potential synergism between candidate pheromones. Captured beetles were bagged and frozen until they could be counted and sexed. Experiment 4 tested (−)-ipsenol and (±)-ipsdienol singly and in combination. Experiment 5 tested (−)- or (±)-ipsenol plus (±)-ipsdienol. Experiment 6 tested (−)-ipsenol in combination with (+)-ipsdienol, (−)-ipsdienol, or (±)-ipsdienol. Experiment 7 tested (±)-ipsenol and (±)-ipsdienol at 2:1 or 1:1 ratios. The final experiment (experiment 8) tested (±)-ipsenol plus (±)-ipsdienol alone and in combination with ipsenone.

Statistical Analysis. Numbers of captured male and female beetles were analyzed separately using SAS computer software (SAS Institute, 1990). Percentages of beetles responding in laboratory bioassays were transformed by $x' = \arcsin\sqrt{x}$ (Zar, 1984) and analyzed with the Ryan-Einot-Gabriel-Welsh multiple range test (Day and Quinn, 1989). The same test was used for data from field experiments, but with a $x' = \log_{10}(x + 1)$ transformation (Zar, 1984).

RESULTS

In experiment 1, female *P. elegans* were more strongly attracted to volatiles from male- than from female-infested or uninfested bolts (Table 1). Volatiles

TABLE 2. NUMBERS OF *P. elegans* CAPTURED IN MULTIPLE FUNNEL TRAPS IN EXPERIMENTS 7 AND 8[a]

Experiment	Treatment	Respective release rates (mg/24 hr)	Beetles captured ($\bar{X} \pm$ SE)[b]	
			Males	Females
7	Unbaited trap (control)		0.0b	0.3 ± 0.6b
	(±)-Ipsenol + (±)-ipsdienol	0.15 and 0.07	1.5 ± 0.3a	8.3 + 1.6a
	(±)-Ipsenol + (±)-ipsdienol	0.3 and 0.07	1.0 ± 0.4a	8.1 ± 2.6a
8	Unbaited trap (control)		0.0b	0.2 ± 0.1b
	(±)-Ipsenol + (±)-Ipsdienol	0.3 and 0.07	4.4 ± 0.7a	22.5 ± 6.0a
	(±)-Ipsenol + (±)-ipsdienol + ipsenone	0.3, 0.07 and 0.26	5.2 ± 0.9a	25.9 ± 6.1a

[a]Experiment 7 ($N = 10$) tested two different doses of (±)-ipsenol in combination with (±)-ipsdienol, and experiment 8 ($N = 10$) tested the effect of adding ipsenone to the blend with the lowest dose of (±)-ipsenol. Experiments were conducted 10 km north of Coeur d'Alene, Idaho, on the following dates: experiment 7, Jun 28–July 20; experiment 8, June 28–August 15, 1995.

[b]Means within an experiment and column followed by the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh multiple range test, $P > 0.05$.

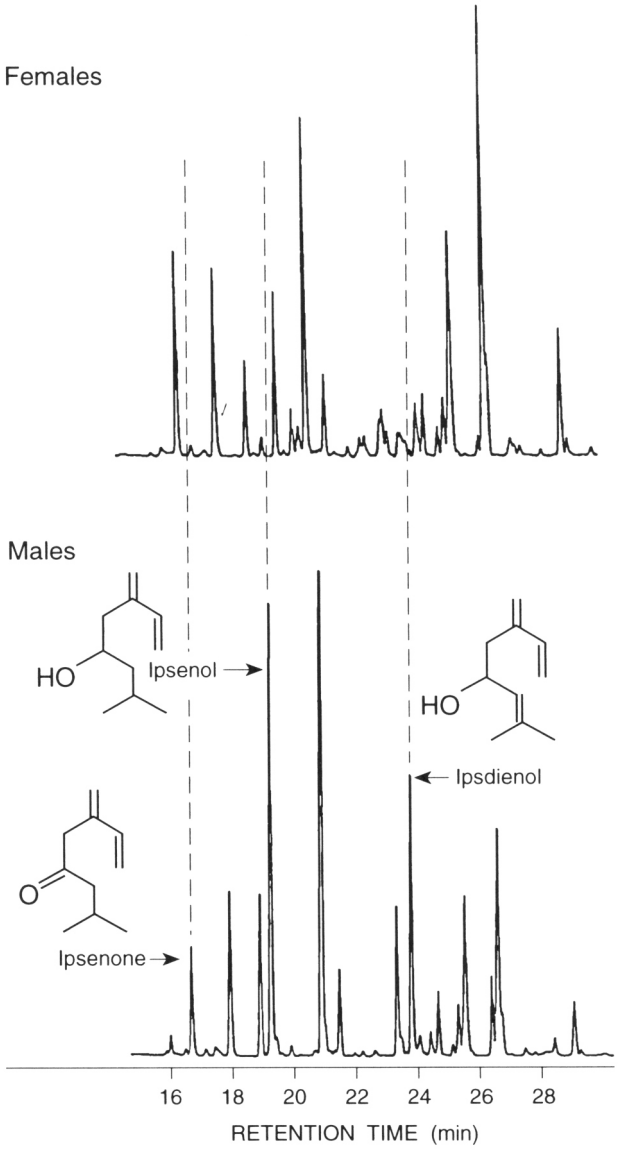


FIG. 1. Comparative chromatograms of volatiles produced by male and female *P. elegans*, revealing male-specific production of ipsenone, ipsenol, and ipsdienol.

from male-infested bolts were not more attractive than volatiles from the male-plus female-infested bolts. Responses by males did not differ among treatments.

Based on differential diagnosis, ipsenone, ipsenol, and ipsdienol were produced only by male beetles (Figure 1). All three compounds consistently elicited antennal responses in GC-EAD analyses (Figure 2). Chromatography of male-produced volatiles on a chiral column, which separates optical isomers of ipsenol and ipsdienol, revealed that *P. elegans* males produced (−)-ipsenol and (−)- and (+)-ipsdienol in a 2 : 1 ratio (Figure 3).

In laboratory bioassays, none of the stimuli, (−)-ipsenol, (±)-ipsdienol, or ipsenone, induced a response that was significantly different from that to the control stimulus (Table 1, experiment 2). The binary blend of ipsenol plus (±)-ipsdienol and the ternary blend with ipsenone added were equally attractive to females and more attractive than the pentane control stimulus (Table 1, experiment 3).

In the field, neither (−)-ipsenol nor (±)-ipsdienol alone attracted beetles

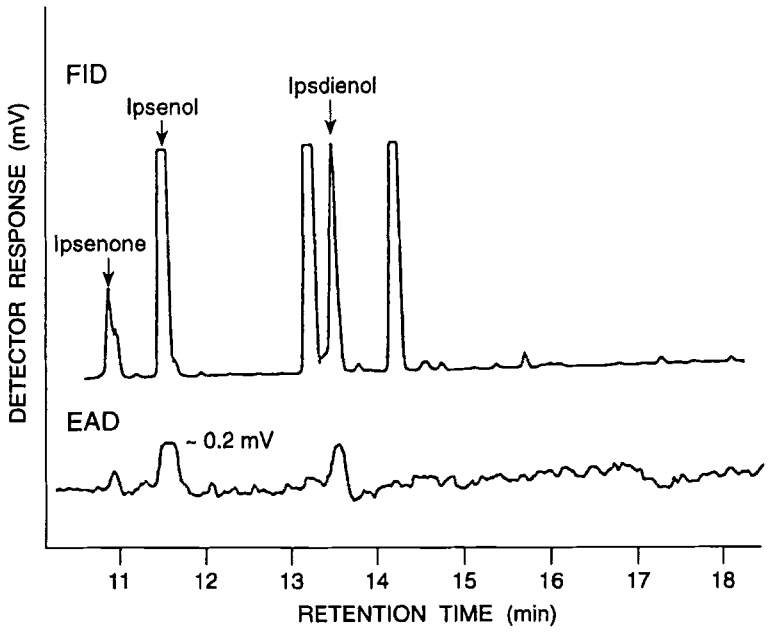


FIG. 2. Representative recording of flame ionization detector (FID) and electroantennographic detector (EAD: female *P. elegans* antenna) responses to 5 beetle-hour equivalents of volatiles released from a grand fir bolt infested with *P. elegans* males. Chromatography: DB-5 column; temperature program: 50°C (1 min), 3°C/min to 100°C then 20°C/min to 200°C.

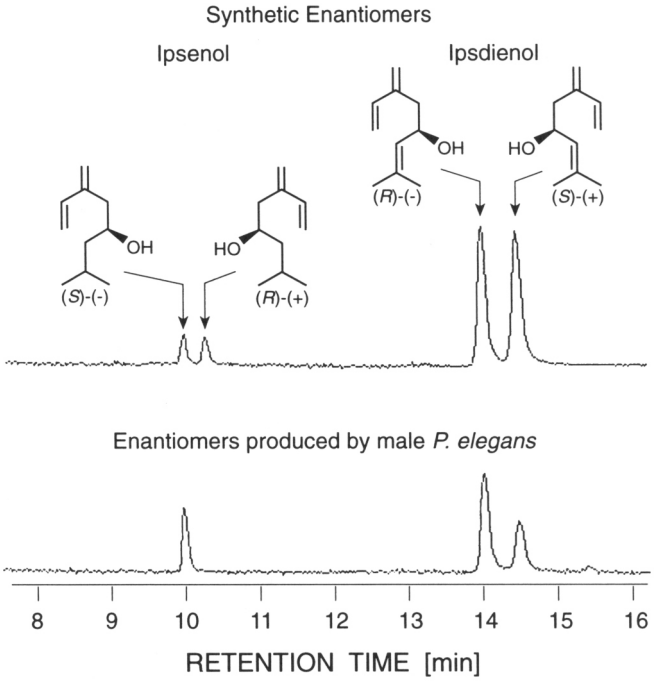


FIG. 3. Selected ion *m/z* 137 (ipsenol) and *m/z* 135 (ipsdienol) chromatograms (Hewlett-Packard 5985B) of enantiomeric and male *P. elegans*-produced ipsenol and ipsdienol; *m/z* 137 and *m/z* 135 were the parent ions of full-scan mass spectra in chemical ionization (isobutane) mode. Chromatography: split injection; Cyclodex-B column; 110°C isothermal; linear flow velocity of carrier gas: 35 cm/sec; injector temperature: 220°C.

(Figure 4, experiment 4). In contrast, (−)- or (±)-ipsenol plus (±)-ipsdienol attracted females and attracted males in low but significant numbers (Figure 4; experiments 4–6). Both enantiomers of ipsdienol were required to induce attraction (Figure 4, experiment 6). (±)-Ipsenol plus (±)-ipsdienol at 1:1 or 2:1 ratios were equally attractive (Table 2, experiment 7). Addition of ipsenone to the 2:1 blend of (±)-ipsenol and (±)-ipsdienol had no effect on the number of beetles captured (Table 2, experiment 8).

DISCUSSION

Enantioselective production of, and response to, pheromones has been demonstrated in many species of scolytids (Birch, 1984; Borden, 1985; Byers, 1989).

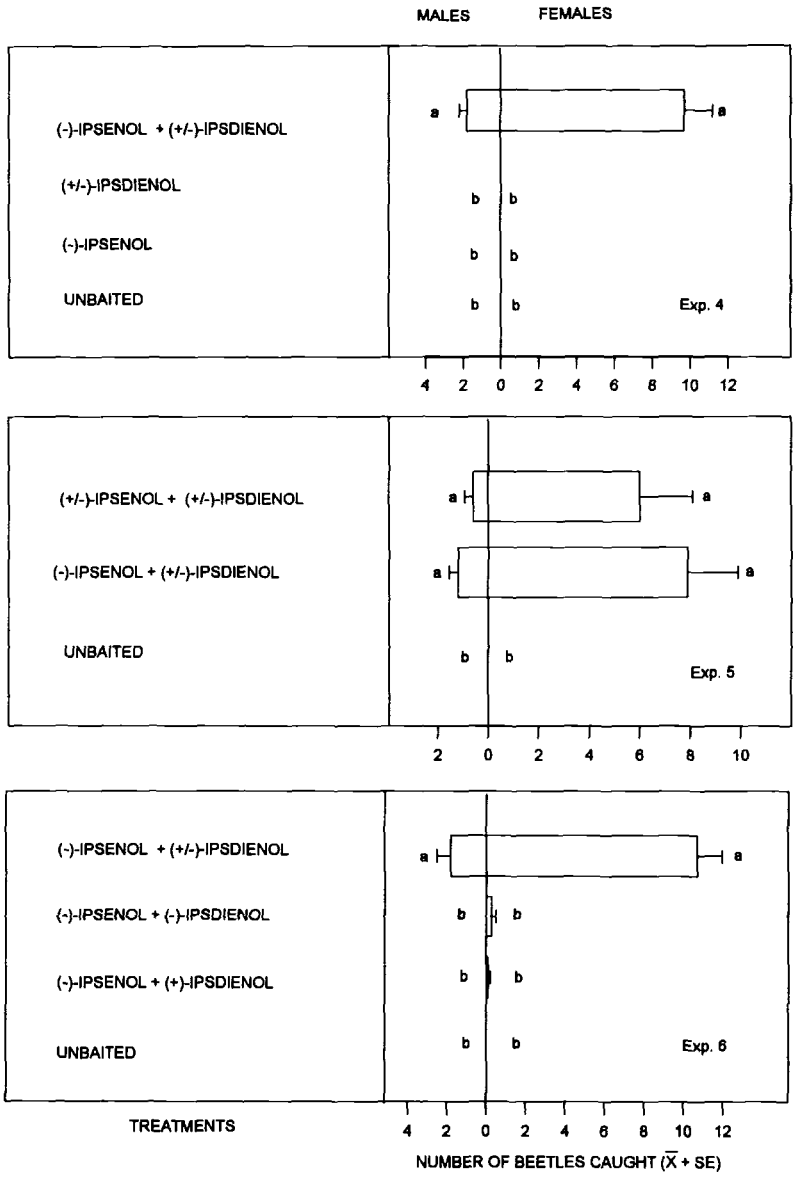


FIG. 4. Numbers of male and female *P. elegans* caught in multiple-funnel traps in experiments 4 and 5 (June 20–July 6, 1994) and experiment 6 (July 21–August 11, 1995) Coeur d'Alene, Idaho; $N = 10$ for each experiment. Release rate for (±)-, (+)-, and (−)-ipsdienol was 0.15 mg/24 hr. Release rate for (−)-ipsenol in combination with (±)-ipsdienol was 0.15 mg/24 hr and in combination with (+)- or (−)-ipsdienol was 0.30 mg/24 hr. Release rate for (±)-ipsenol in combination with (±)-ipsdienol was 0.3 mg/24 hr. Bars for each sex within each experiment associated with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh test, $P > 0.05$.

To our knowledge the *P. elegans* communication system is the first example in which three chiral compounds are required to mediate any attraction. Male *P. elegans* produce (−)-ipsenol, (−)-ipsdienol, and (+)-ipsdienol (Figure 3). Lack of attractiveness of these compounds singly or in binary combinations (Figure 4, experiments 4 and 6) and attraction only when all three are present (Tables 1 and 2; Figure 4) provides compelling evidence that each of the three chiral terpene alcohols is an essential component of the aggregation pheromone. The relatively low catches to the ternary blend (Figure 4) is probably not due to a missing component. Rather, as we have later determined, the experiments were conducted between the two major seasonal flight periods of the insect. *Pityokteines elegans* produces ipsenone, and its antennae can perceive it; however, it was not behaviorally active and is of unknown function.

Electrophysiological studies have revealed that species such as *Ips pini* Say, *I. typographus* L., *I. paraconfusus* Lanier, *Scolytus multistriatus* (Marsham), *S. scolytus* (F.), and *Trypodendron lineatum* (Olivier) have olfactory receptor cells specific to optical isomers of aggregation pheromones (Mustaparta et al., 1980, 1984; Angst, 1981; Wadhams et al., 1982; Tømeras et al., 1984). Based on the specificity of receptor cells in the above species and the absolute requirement of *P. elegans* for the specific optical isomers of ipsenol and ipsdienol, we suggest that each of the three components is perceived by specific olfactory receptor cells in *P. elegans* antennae. Because (+)-ipsenol is not produced by male *P. elegans* (Figure 3), and neither enhances nor reduces attractiveness of the ternary pheromone blend (Figure 4, experiment 5), *P. elegans* may lack receptor sites for (+)-ipsenol.

The 2:1 ratio of the (*R*)-(−)- and (*S*)-(+)-ipsdienol produced by male *P. elegans* is identical but in reverse order to that reported for the pine engraver, *Ips pini* (Say), from California (Fish et al., 1984). However, as for *I. pini* (Miller et al., 1989), individual male *P. elegans* may produce ipsdienol of variable enantiomeric composition.

Ipsenol and ipsdienol are the only pheromones identified in the genus *Pityokteines* (Table 3), suggesting that pheromonal communication by sympatric congeners requires specificity in the enantiomeric composition of pheromones, as revealed in the genera *Ips* (Vanderwel and Oehlschlager, 1987) and *Dryocoetes* (Camacho et al., 1993). *Pityokteines elegans* and *P. minutus* are partially sympatric in the Pacific Northwest. *Pityokteines minutus* is found primarily in subalpine fir, which is not a host for *P. elegans* (Bright and Stark, 1973; Bright, 1976; S. L. Wood, 1982). The ranges of grand fir, the principal host of *P. elegans*, and subalpine fir overlap substantially (Hitchcock et al., 1959). In addition to host preference, differential pheromone blends obviously contribute to reproductive isolation of the two sympatric congeners (Table 3). *Pityokteines elegans* would not be cross-attracted to *P. minutus*, which does not produce the critical (−)-ipsdienol. In turn, *P. minutus* is attracted in the field only to (−)-ipsenol (Camacho-Vera, 1993). We suggest that (−)-ipsdienol may deter

TABLE 3. MALE-PRODUCED PHEROMONES IN *Pityokteines* spp.

Species	Pheromone produced	Attractive enantiomer (location of bioassay)	References
P. curvidens	Ipsenol	(−)-Ipsenol (field)	Harring et al. (1975), Harring and Mori (1977), Harring (1978)
P. spinidens	Ipsenol,[a] ipsdienol[a]	Inconclusive results	Harring (1976)
P. vorontzovi	Ipsenol,[a] ipsdienol[a]	Ipsenol[b] + (−)-ipsdienol (field)	Harring (1978)
P. minutus	(−)-Ipsenol, (+)-Ipsdienol	(−)-Ipsenol (field), 1:1 blend of both enantiomers attractive to females (laboratory)	Camacho-Vera (1993)
P. elegans	(−)-Ipsenol, (−)-Ipsdienol (+)-ipsdienol	(−)-Ipsenol + (−)-ipsdienol and (+)-ipsdienol (laboratory and field	Figures 1–4

[a]Chirality not yet determined.
[b]Bioactive enantiomers not yet determined.

cross attraction to *P. minutus* to *P. elegans*, since this component is not present in the former species (Camacho-Vera, 1993).

One potential application for the pheromones of secondary bark beetles is to use them for induction of competitive displacement or exclusion (Payne and Richerson, 1985; Borden, 1992) of primary, tree-killing species. Discovery of the aggregation pheromones of *P. elegans* will allow induced competition to be tested as a potential management tactic for *S. ventralis*.

Acknowledgments—We thank L. J. Chong, B. Cinel, S. L. Van Learhoven, M. E. Greenwood, and G. A. Yakel for assistance and S. J. Gast, and J. L. Wallace, USDA, Forest Service, Idaho Panhandle National Forest, and R. L. Livingston, Idaho Department of Lands, for advice and support for field experiments. The research was supported by the Natural Sciences and Engineering Council of Canada, the Canadian Forest Service, the Science Council of British Columbia, the Coast Forest and Lumber Sector, the Interior Lumber Manufacturers' Association, the Cariboo Lumber Manufacturers' Association, seven forest industries and by scholarships to J.E.M.-S. from the Comisión Nacional para el Desarrollo de la Ciencia y la Tecnología (CONACyT), México and the Organization of American States, Washington, D.C.

REFERENCES

ANGST, M. 1981. Sinnesphysiologische Untersuchungen über die Pheromon-perzeption von *Scolytus*-Arten, besonders *Scolytus multistriatus:* Feldversuche, Electroantennogramme und Eizelzellableitungen von den Antennen. Thesis. ETH, Zurich.

ARN, H., STÄDLER, E., and RAUSCHER, S. 1975. The electroantennographic detector—selective and sensitive tool in the gas chromatographic analysis of insects pheromones. *Z. Naturforsch.* 30c:722–725.

ATKINS, M. D. 1966. Behavioral variations among scolytids in relation to their habitat. *Can. Entomol.* 98:285–288.

BASHAM, J. T. 1986. Biological factors influencing stem deterioration rates and salvage planning in balsam fir killed after defoliation by spruce budworm. *Can. J. For. Res.* 16:1217–1229.

BELYEA, R. M. 1952. Death and deterioration of balsam fir weakened by spruce budworm defoliation in Ontario. II. An assessment of the role of associated insect species in the death of severely weakened trees. *J. For.* 50:729–738.

BIRCH, M. C. 1984. Aggregation in bark beetles, pp. 331–353, *in* W. J. Bell and R. T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, New York.

BORDEN, J. H. 1982. Aggregation pheromones, pp. 74–139, *in* J. B. Mitton, and K. B. Sturgeon (eds.). Bark Beetles in North American Conifers. University of Texas Press, Austin, Texas.

BORDEN, J. H. 1985. Aggregation pheromones, pp. 257–285, *in* G. A. Kerkurt and L. I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry, and Pharmacology, Vol. 9. Pergamon Press, Oxford.

BORDEN, J. H. 1992. Two tree baiting tactics for management of bark beetles with semiochemicals. *J. App.. Entomol.* 114:201–207.

BRIGHT, D. E., JR. 1976. The insects and arachnids of Canada and Alaska. 2. Coleoptera: Scolytidae. Canada Department of Agriculture, Ottawa, Publ. 1576.

BRIGHT, D. E., and STARK, R. W. 1973. The bark beetles and ambrosia beetles of California. Coleoptera: Scolytidae and Platypodidae. Bulletin, California Insect Survey, Vol. 16. University of California Press, Berkeley.

BROWN, H. C., GARG, C. P., and KWANG-TING, L. 1971. The oxidation of secondary alcohols in diethyl ether with aqueous chromic acid. A convenient procedure for the preparation of ketones in high epimeric purity. *J. Org. Chem.* 36:390–397.

BYERS, J. A. 1989. Chemical ecology of bark beetles. *Experientia* 45:271–283.

BYRNE, K. J., GORE, W. E., PEARCE, G. T., and SILVERSTEIN, R. M. 1975. Porapak-Q collection of airborne organic compounds serving as models for insect pheromones. *J. Chem. Ecol.* 1:1–7.

CAMACHO-VERA, A. D. 1993. Semiochemical-based communication in three species of bark beetles in subalpine forest of British Columbia. PhD thesis. Simon Fraser University, Burnaby British Columbia.

CAMACHO, A. D., PIERCE, H. D., JR., and BORDEN, J. H. 1993. Geometrical and optical isomerism of pheromones in two sympatric *Dryocoetes* species (Coleoptera: Scolytidea), mediates species specificity and response level. *J. Chem. Ecol.* 19:2169–2182.

CHARARAS, C. 1962. Étude biologique des scolytides de conifères. Editions Paul Lechevalier, Paris, 556 pp.

DAJOZ, R. 1980. Ecologie des insectes forestiers. Gauthier-Vilars, Bordas, Paris.

DAY, R. W., and QUINN, G. P. 1989. Comparisons of treatments after an analysis of variance in ecology. *Ecol. Monogr.* 59:433–463.

FISH, R. H., BROWN, L. E., and BERGOT, B. J. 1984. Pheromone biosynthetic pathways: Conversion of ipsdienone to (−)-ipsdienol, a mechanism for enantioselectivity reduction in the male bark beetle, *Ips paraconfusus*. *J. Chem. Ecol.* 10:1057–1064.

FURNISS, R. L., and CAROLIN, V. M. 1986. Western forest insects. USDA Forest Service Miscellaneous Publication No. 1339.

GRIES, G. 1995. Prospects of new semiochemicals and technologies, pp. 44–47, *in* S. Salom and K. Hobson (eds.). Applications of Semiochemicals for Management of Bark Beetle Infestations—Proceedings of an Informal Meeting. USDA Forest Service, General Technical Report INT-GTR-318.

HARRING, C. M. 1978. Aggregation pheromones of the European fir engraver beetles *Pityokteines curvidens*, *P. spinidens*, and *P. vorontzovi* and the role of juvenile hormone in pheromone biosynthesis. *Z. Angew. Entomol.* 85:281–317.

HARRING, C. M., and MORI, K. 1977. *Pityokteines curvidens* Germ. (Coleoptera: Scolytidae): Aggregation response to optically pure ipsenol. *Z. Angew. Entomol.* 82:321–329.

HARRING, C. M., VITÉ, J. P., and HUGHES, P. R. 1975. Ipsenol, der populätionslockstoff des krummzahnigen Tannenborken-käfers. *Naturwissenschaften* 62:488.

HERTERT, H. D., MILLER, D. L., and PARTRIDGE, A. D. 1975. Interaction of bark beetles (Coleoptera: Scolytidae) and root-rot pathogens in grand fir in northern Idaho. *Can. Entomol.* 107:829–904.

HITCHCOCK, C. L., CRONQUIST, A., and OWNBERG, M. 1959. Vascular cryptograms, gymnosperms, and monocotyledons. Part 1, *in* C. L. Hitchcock, A. Cronquist, M. Ownberg, and J. W. Thompson (eds.). Vascular plants of the Pacific Northwest. University of Washington Press, Seattle.

HOSKING, G. P., and KNIGHT, F. B. 1976. Investigations on the life history and habits of *Pityokteines sparsus* (Coleoptera: Scolytidae). Life Sciences and Agriculture Experiment Station, University of Maine, Orono, Technical Bulletin 8.

LINDGREN, B. S. 1983. A multiple funnel trap for scolytid bark beetles. *Can. Entomol.* 115:299–302.

MILLER, D. R., BORDEN, J. H., and SLESSOR, K. N. 1989. Inter- and intrapopulation variation of the pheromone, ipsdienol produced by male pine engravers, *Ips pini* (Say) (Coleoptera: Scolytidae). *J. Chem. Ecol.* 15:233–247.

MUSTAPARTA, H., ANGST, M. E., and LANIER, G. N. 1980. Receptor discrimination of enantiomers of the aggregation pheromone ipsdienol, in two species of *Ips*. *J. Chem. Ecol.* 6:689–701.

MUSTAPARTA, H., TØMERAS, B. A., BAECKSTRÖM, P., BAKKE, J. M., and OHLOFF, G. 1984.

Ipsdienol-specific receptor cells in bark beetles: Structure activity relationships of various analogs and the deuterium-labeled ipsdienol. *J. Comp. Physiol. A.* 154:591–596.

PAYNE, T. L., and RICHERSON, J. V. 1985. Pheromone-mediated competitive replacement between two bark beetle populations: Influence on infestation suppression. *Z. Angew. Entomol.* 99:131–138.

SAS INSTITUTE. 1990. SAS System for Personal Computers, Release 6.04. SAS Institute Inc., Cary, North Carolina.

STEEL, R., and GIER-HAYES, K. 1992. The grand fir/mountain maple habitat type in central Idaho: Succession and management. USDA Forest Service General Technical Report INT-284.

STOCK, A. J., and BORDEN, J. H. 1983. Secondary attraction in the western balsam bark beetle, *Dryocoetes confusus* (Coleoptera: Scolytidae). *Can. Entomol.* 115:539–550.

TØMERAS, B. A., MUSTAPARTA, H., and GREGOIRE, J. C. 1984. Receptor cells in *Ips typographus* and *Dendroctonus micans* specific to pheromones of the reciprocal genus. *J. Chem. Ecol.* 10:759–769.

VANDERWEL, D., and OEHLSCHLAGER, A. C. 1987. Biosynthesis of pheromones and endocrine regulation of pheromone production in Coleoptera, pp. 175–215, *in* G. D. Prestwich and G. J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, New York.

VITÉ, J. P., and RENWICK, J. A. A. 1970. Differential diagnosis and isolation of population attractants. *Contrib. Boyce Thompson Inst.* 24:323–328.

WADHAMS, L. J., ANGST, M. E., and BLIGHT, M. M. 1982. Responses of the olfactory receptors of *Scolytus scolytus* (F.) (Coleoptera: Scolytidae) to the stereoisomers of 4-methyl-3-heptanol. *J. Chem. Ecol.* 8:477–492.

WOOD, D. L. 1982. The role of pheromones, kairomones and allomones in the host selection and colonization behavior of bark beetles. *Annu. Rev. Entomol.* 27:411–446.

WOOD, S. L. 1982. The bark and ambrosia beetles of North and Central America (Coleoptera: Scolytidae), a taxonomic monograph. *Great Basin Nat. Mem.* 6.

ZAR, J. H. 1984. Biostatistical Analysis, 2nd ed. Prentice Hall, Englewood Cliffs, New Jersey.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

EVIDENCE FOR A SUBSTRATE-BORNE SEX PHEROMONE IN THE PARASITOID WASP *Trichogramma brassicae*

FRANCOIS POMPANON,* BENOIT DE SCHEPPER, YVAN MOURER, PIERRE FOUILLET, and MICHEL BOULETREAU

Laboratoire de Biométrie, Génétique et Biologie des Populations
UMR CNRS 5558, Université Claude Bernard-Lyon I
43 Bd du 11 Novembre 1918, 69622 Villeurbanne cedex, France

(Received July 1, 1996; accepted January 5, 1997)

Abstract—While the use of chemical cues in host location is well known in parasitoid insects, little is known about their role in sexual communication. Sex pheromones were investigated in the egg parasitoid *Trichogramma brassicae*, and the evidence for a substrate-borne pheromone was demonstrated. Mated and unmated males concentrated their walking in an area where virgin females (< 1 or 5 days old) had walked. Males were tested 15 min after females left the area. Male response decreased over time but was always significant during the 8 min of tracking. This suggests that females deposit chemicals on the substrate for mate finding. The pheromone is soluble in hexane, as shown by two kinds of extracts: female body rinsing and rinsing of the flask where females had walked. Hexane extracts resulted in a positive response by males. The response to different doses showed a saturation above a threshold of 4–8 female equivalents. The results do not exclude the possibility that a volatile part of the pheromone attracts males over long distances. Substrate-borne pheromones may increase mating chances for virgin females searching for oviposition sites, and may also increase mating chances on emergence sites by reducing male dispersion.

Key Words—Sex pheromone, substrate-borne chemical, egg parasitoid, Hymenoptera, Trichogrammatidae, *Trichogramma brassicae*, mating structure, videotracking.

INTRODUCTION

Insect communication mainly involves hearing, vision, and olfaction, the dominant sense depending on the habitat and the ecology of the species (Lewis,

*To whom correspondence should be addressed.

0098-0331/97/0500-1349$12.50/0 © 1997 Plenum Publishing Corporation

1984). While vision (Pak et al., 1991; Wäckers, 1994) or mechanoreception (Vet and van Alphen, 1985) are used by parasitoid insects, chemicals seem to constitute the main means of communication. Chemical cues are widely used by parasitoid insects for host habitat location (Vinson, 1984), host location (Weseloh, 1981), and host selection (Arthur, 1981). Chemicals are also involved in the intraspecific communication of parasitoids, such as sexual communication (see Discussion), as well as in intra- and interspecific competitive interactions (Janssen et al., 1995; Bernstein and Driessen, 1996). Thus, chemical communication is involved in parasitic strategies that involve the expression of complex behaviors in response to various stimuli, resulting in optimized reproduction and host exploitation.

The genetic structure of a population depends on its mating structure, and more and more approaches are being used to analyze such structures in parasitoid populations. For instance, authors look at the temporal distribution of adult emergence (Pompanon et al., 1995), the occurrence of dispersion before or after mating (Antolin and Strand, 1992; Nadle and Luck, 1992), and the genetic structure of the population (Kazmer and Luck, 1991) to appreciate the relative importance of outcrossing and intrapatch mating. Until now, however, little attention has been paid to the role of chemical communication in mate finding in parasitoid Hymenoptera or to its consequences on the mating structure. While volatile and contact sex pheromones have been found in several parasitoid species, little is known about the role of substrate-borne chemicals in sexual communication. Here we report the existence of a substrate-borne sex pheromone deposited by female *Trichogramma brassicae*, an egg parasitoid of various lepidopteran pests. The effects of female deposits and of hexane extracts from virgin and mated females were tested and male responses were analyzed.

METHODS AND MATERIALS

Biological Material. The strain of *Trichogramma brassicae* Bezdenko (Hymenoptera, Trichogrammatidae) used for experiments originates from Moldavia and was reared using *Ephestia kuehniella* (Lepidoptera, Pyralidae) eggs killed by UV radiation. Rearing and experiments were performed under 12L : 12D photoperiod (light phase from 08:00–20:00 hr) at 25°C and 70% relative humidity. Parasitized eggs of *Ephestia* were isolated before wasp emergence to obtain virgin adults for experiments. Mated adults were obtained by keeping newly emerged males and females together for 1 hr. Wasps were studied on the day of emergence (age $<$ 10 hr of adult life).

Experimental Design. Experiments were performed in cylindrical arenas (height 1 mm, diameter 3 cm) made of a punched Plexiglas plate sandwiched between two glass plates. Each arena was divided into two equal parts by a

(chemically inert) silicone bar. A *Trichogramma* female was allowed to walk in one zone for 30 min (marking phase, Figure 1A), which resulted in a homogenous exploration of this half of the arena. Then, the silicone bar was removed and, after 60 sec, a male was placed in the arena (test phase, Figure 1B and C). The male's path was tracked for 8 min with an automatic video tracking device. The arena was placed under a circular fluorescent tube (daylight, 300 lux) and was filmed with a video camera (Canon CI-20PR) equipped with a 90-mm lens (Tamron). Each picture (25/sec) was digitized (video card, Secad), and the insect coordinates recorded (software, Cible1, Secad).

Positive evidence of female marking was measured by a choice index reflecting the time that the male spent in the marked half of the arena: $I = (2\ T_m - T_t)/T_t$, where T_t is the total time of tracking and T_m the time spent in the marked zone. This index ranges from -1 (all time spent in the non marked zone) to $+1$ (all time spent in the marked zone), and is null when time is equally shared between the two zones. The index was calculated only when males actually moved. However, in a preliminary experiment, insects were found active during the entire 8 min of recording, whatever their group: the mean time spent moving by virgin males was 7 min 38 sec $\pm$ 6 sec when there was virgin female marking ($N = 18$) and 7 min 19 sec $\pm$ 10 sec for controls without marking ($N = 16$) (95.4 $\pm$ 1.4% and 91.5 $\pm$ 1.9%, respectively). This differ-

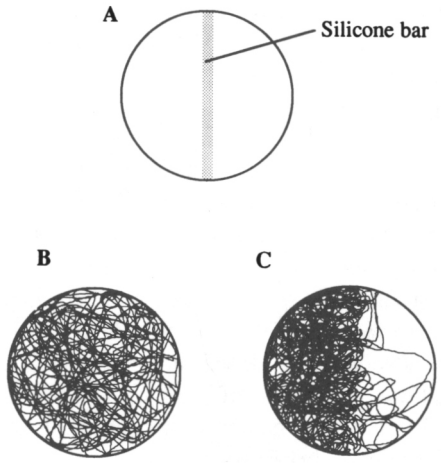


FIG. 1. Experimental procedure and examples of male trails. (A) Marking phase: cylindrical arena is divided into two equal parts with a silicone bar. A female is allowed to walk for 30 min in one half of the arena. (B) Test phase for control: path of a male (recorded for 8 min) when no female was present during the marking phase. (C) Test phase: path of a male (recorded for 8 min) after a female had walked in the left test.

ence was not statistically significant (Student's t-test after angular transformation arcsin $\sqrt{x}$, $t = 1.78$, $P > 0.05$).

Response to Female Trails. For each replicate, one virgin female was allowed to walk in the half arena for 30 min. As a control, a silicone bar was placed in an arena without any female for the same duration. After removing the female and/or the silicone bar, a male was placed at the center of the arena and tracked. This experiment was performed on four consecutive days from 10:00 to 18:00 hr. Forty measurements were carried out each day (8 controls and 32 experimentals), each using new individuals. Variations of the index over time of day and day of experiment were tested. For each replicate, the index was calculated every 30 sec to follow its evolution during the measurement. The mean index of control individuals was used as an indifference range, and is noted on the figures.

To test the sexual nature of the chemical, three treatments (16 replicates each) were performed: (1) marking by a female and then tracking a male, (2) marking by a female and then tracking another female, (3) marking by a male and then tracking another male. All individuals were virgin.

The effect of mating on female emission was tested by using either virgin females (16 replicates) or mated ones (16 replicates) for the marking phase. Virgin males were tracked. Mating effect was also checked on male perception by comparing the response of virgin and mated males (16 replicates each) after marking by a virgin female. To test the persistence and stability of female marking, one male was deposited in the arena 1 or 15 min after female marking, tracked for 8 min, and then removed. The test was repeated 16 times. The choice index was calculated every 30 sec.

Pheromone Extracts. Two kinds of extractions were performed using hexane as a solvent, by rinsing either female deposits, or female bodies. Two extracts were done using either virgin or mated females. (1) For flask rinsing, 40 females were allowed to walk in a glass tube (5 mm diameter, 35 mm length) for 1 hr at 22°C. After removing females, the tube was rinsed with 40 μl of pure hexane for 5 min. This extract had a concentration of 1 "flask-rinsing" equivalent female/μl (frEF/μl). (2) For female body rinsing, 150 females were soaked in a tube and stirred with 50 μl of pure hexane for 5 min. Because a part of the solvent was retained by insect bodies, a second rinsing was done under the same conditions with 20 μl of hexane. The two extracts were mixed for a final volume of 50 μl. Thus, the concentration of this extract was 3 "body-rinsing" equivalent female/μl (brEF/μl).

Extracts were tested by covering the bottom of the arena with filter paper. Then 1–5 μl of pure or diluted (for doses <3 brEF) extract were deposited on one half of the arena, while the same volume of pure hexane was deposited on the other half. After evaporation of the solvent (5 min under ambient air), adult males were placed in the arena and tracked for 3 min.

RESULTS

Response to Female Trails. Males strongly concentrated their walking in the areas where virgin females had walked for 30 min. They frequently exhibited courtship behavior in the marked zones (lateral swing of the body). Whatever the time of day, the hourly mean of the choice index ranged from +0.4 to +0.8, compared to −0.2 to +0.2 in the control group. A two-way ANOVA showed there was no effect of day ($F_{3,7} = 1.6$, $P > 0.2$) or hour ($F_{7,21} = 0.9$, $P > 0.5$) on the mean index. The data were grouped to test the evolution of the male response during 8 min of tracking. Within each of the 30-sec intervals, the time spent moving in marked areas by males exposed to female marking was greater than the time spent by males in arenas when there was no marking (Mann-Whitney U tests, $P < 0.001$ except for the last interval, $P < 0.03$). Thus, the response was always significant despite its linear decrease with time (Figure 2).

While virgin males concentrated their searches on areas marked by virgin females, they did not spend more time where other virgin males had walked for 30 min. Moreover, virgin females did not respond to the marking of other females (Figure 3A). There was an effect of mating. Naive males were indifferent to areas where mated females had walked (Figure 3B). Results were the same with newly emerged females (age < 10 hr) and 5-day-old ones (Figure 3 B and C). The response of mated males to virgin female markings appeared slightly reduced compared to naive males (Figure 3B), but the difference was not significant (Student's t test $P > 0.5$). The decrease in male response was not affected by a 15-min delay between the end of the marking phase and the

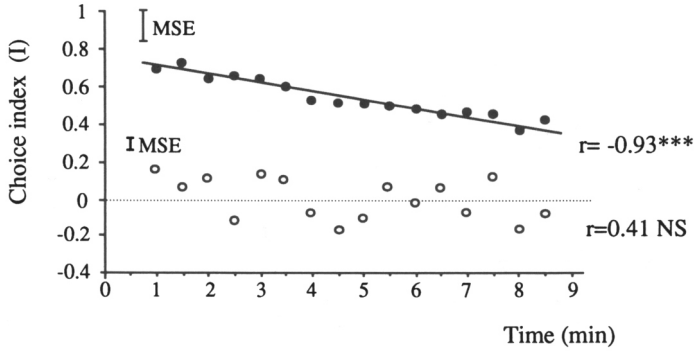


FIG. 2. Response of virgin males to virgin female marking. Male trail is recorded for 8 min and the choice index (reflecting the time spent moving in the marked area) is calculated every 30 sec. Black circles: presence of marking ($N = 128$); open circles: no marking ($N = 32$). MSE: mean standard error; r: coefficient of correlation, ***: $P < 0.001$; NS: not significant.

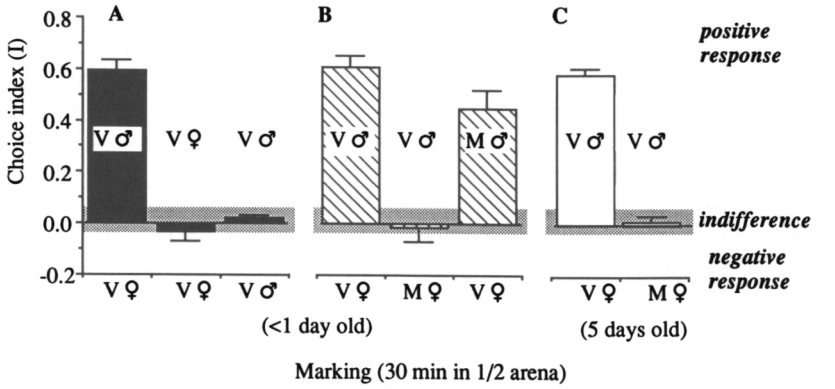


FIG. 3. Sex specificity of the pheromone. Symbols on the horizontal axis show the insect used for marking, symbols on the bar chart represent the insect tested. V: virgin; M: mated. Bars represent the mean choice index ± SE (16 replicates for each modality). The grey zone represents the indifference area (mean value of control males ± SE). (A) Emission and perception of the marking chemical; (B) effect of mating on female emission and on male perception; (C) effect of female age on marking.

introduction of the male. There was a linear decrease with time (Figure 4) that was similar whatever the delay. The slopes did not differ statistically (ANOVA, $P > 0.5$).

Response to Extracts. The response of males to hexane extracts was similar to that of female marking. Whatever the nature of the extract (flask or body

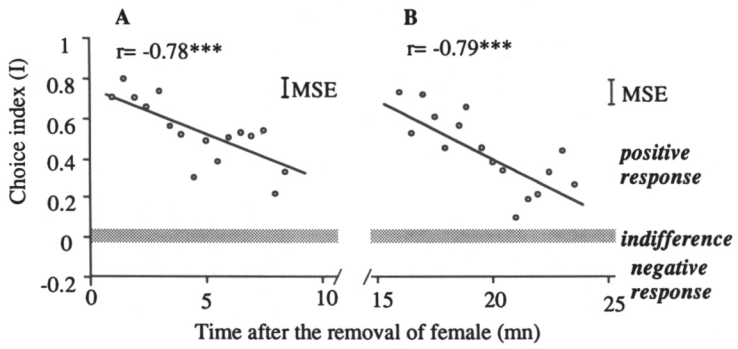


FIG. 4. Persistence of the marking by virgin females. (A) Response of males 1 min after female marking; (B) response of males 15 min after female marking. Sixteen replicates were performed. The two groups show a linear decrease of response ($y_1 = -0.047x + 0.759$; $y_2 = -0.061 + 0.708$). The regression lines are statistically parallel (ANOVA, $P > 0.5$). r: Coefficient of correlation. ***; $P < 0.001$. MSE: mean standard error.

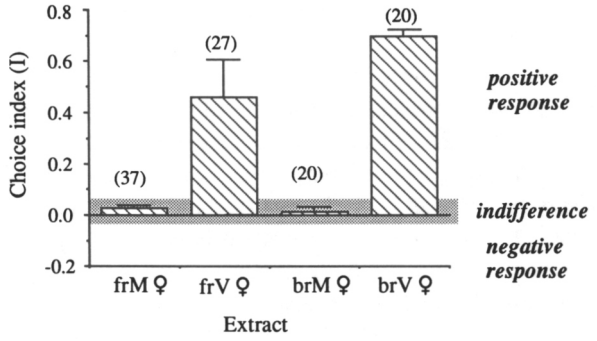


FIG. 5. Response of virgin males to hexane extracts. Bars represent the mean choice index (± SE) for a 1 μl-deposit of extract. This represents a dose of 1 frEF for flask-rinsing extract and 3 brEF for body-rinsing extract. The grey zone corresponds to indifference (mean value ± SE of control males). For each treatment the number of replicates is given in parentheses. fr: flask rinsing extracts, br: body rinsing extracts; M: mated female; V: virgin female.

rinsing), virgin males concentrated their walking in the areas where virgin female extracts were deposited. They did not respond to mated female extracts (Figure 5). For body rinse extracts, the male response increased with dose, but never exceeded a mean of 85% of active time spent on the marked area (Figure 6). Such a maximum corresponds to the classical shape of the dose–response curves obtained for other insects.

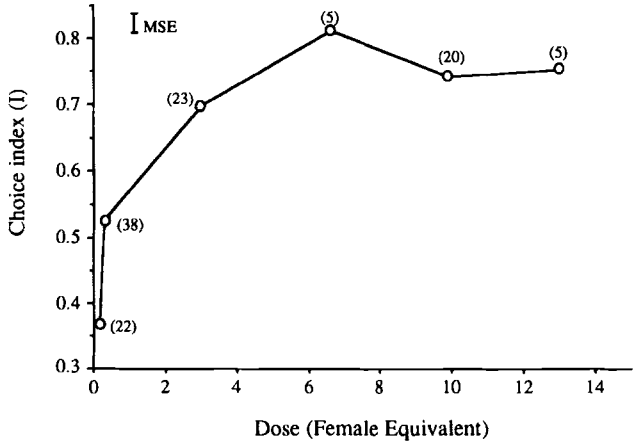


FIG. 6. Response of virgin males to various doses of body rinse extract. MSE: mean standard error. For each dose the number of replicates is given in parentheses.

DISCUSSION

Characteristics of Substrate-Borne Sex Pheromone (SSP). In insects, chemical cues deposited on substrates are involved in several inter- and intraspecific relationships. For instance, contact kairomones are involved in host location by parasitoid insects (Waage, 1978). Many cases have been studied in Hymenoptera, such as ants, which use trail pheromones for worker recruitment (Blum, 1974; Hölldobler, 1984). Contact chemicals are also involved in sexual interactions, e.g., sex pheromones eliciting male courtship behavior have been observed in several hymenopteran parasitoids (Assem et al., 1980; Vinson, 1972, 1978; Simser and Coppel, 1980; Mohamed and Coppel, 1987). Such pheromones have been demonstrated to be active at short distances in two *Brachymeria* species (Simser and Coppel, 1980), in *Apanteles glomeratus* (Kitano, 1975), and *Anisopteromalus calandrae* (Yoshida, 1978) where a "pheromone sphere" of less than 3 cm in diameter has been described.

Our results indicate that a sexual pheromone is responsible for mate finding in *Trichogramma brassicae.* Chemicals are emitted exclusively by virgin females and are perceived exclusively by males whatever their mating status. As in other parasitoid species (Mohamed and Coppel, 1980; Shu and Jones, 1993), pheromone extracts elicit male courtship behavior, proving a role for these chemicals in sexual interactions. Several pieces of evidence demonstrate that the pheromone is deposited by virgin females on the substrate. First, there is concentrated male searching within the area previously explored by the female, and the signal remains for at least 15 min, indicating a low volatility or a strong adsorption. Second, the strongly positive action of hexane extracts proves the efficiency of the chemicals when deposited on the substrate. Third, active extracts may be obtained by rinsing a glass flask where virgin females have walked. Thus, a substrate-borne sex pheromone induces concentrated male searching.

The role of substrate-borne chemicals in sexual interactions was previously suggested in two parasitoid species, *Ascogater reticulatus* (Kamano et al., 1989) and *Aphelinus asychis* (Fauvergue et al., 1995). In those species, males more frequently visited leaves previously exposed to virgin females, and in *Ascogaster reticulatus* hexane extracts of female bodies elicited male antennal searching (Kamano et al., 1989). In *Trichogramma brassicae*, although the role of chemical cues deposited by females on the substrate was demonstrated, we cannot exclude the possibility that sex pheromones act at a distance in nature, especially if other volatile components are also emitted. Many sexual pheromones used by parasitoids are volatile (Lewis et al., 1971; Eller et al., 1984; Mohamed and Coppel, 1987) and may also be strongly adsorbed onto glass (Shu and Jones, 1993). In the parasitoid *Brachymeria intermedia*, the efficiency of pheromone traps in the field suggests a volatile activity of the pheromone, which elicits male courtship behavior at short distances (Mohamed and Coppel, 1987). In

Trichogramma maidis (= *Trichogramma brassicae*), a volatile sex pheromone has been reported (Pintureau and Toonders, 1983). The presence of volatile sex pheromone components in *Trichogramma* species is not excluded, but it is clear that the diffusion of the signal from the marked to the unmarked area is weak or absent, because the male response remains unchanged 15 min after marking. Substrate-borne chemicals clearly are responsible for the concentrated male searching in areas previously explored by virgin females.

We did not observe in *Trichogramma* any particular female walking behavior, and thus the deposition of the chemicals remains open to question. In some parasitoids, sex pheromones originate from the female abdomen (Assem et al., 1980), more precisely from Dufour's gland (Vinson, 1978), or a pheromone-producing gland at the base of the second valvifer of the female genitalia (Tagawa, 1977). In other Hymenoptera, however, substrate-borne pheromones are deposited in various ways. In some ants, bees, and wasps, trails are laid as "footprints" (Blum, 1974). In the parasitoid *Ascogaster reticulatus*, the presence of tibial sex pheromone glands was suggested by the response of males to hexane extracts of female tibia (Kainoh and Oishi, 1993).

Study of Male Response. Several questions arise concerning the male response to female marking and hexane extracts. The variability in the response index results from the variability in both the female marking and the male response. In the bioassays, a long period of marking (30 min) was chosen to reduce the variability of marking (= between-female variability). However, the use of female extracts is a better way of reducing marking variability; the great number of females used (40–150) ensures the homogeneity of different extracts. Moreover, the use of the same extract for several tests leads to a repeatable stimulus. Thus, the variability in the response is due mainly to males.

Another point is the linear decrease in male response over the 8-min tracking period. It could be due to a low stability of the SSP, leading to decreased stimulation with time. This is unlikely, however, because male response is the same 1 or 15 min after the end of marking. Furthermore, separate tests have previously demonstrated that the efficiency of marking on glass remains unaffected after 12 hr in the open air. The possible low volatility of the SSP invading the arena also may lead to a decrease in male response. Alternatively, the decrease in male response can result from a saturation of male chemoreceptors. After an intensive unsuccessful search in a restricted area, males often give up in order to explore elsewhere. Further experiments are needed to investigate these nonexclusive explanations. Saturation of male receptors could explain why the male response does not increase linearly with the intensity of the stimulus above a certain dose, a well-known phenomenon in insects (Boeckh, 1984) and other animals.

Adaptive Value of SSP in Mate Finding. Volatile chemicals lead to long-distance location of hosts or mates and are involved on a large scale in the

spatial structuration of insect populations. In contrast, contact and short-distance active chemicals act as attractants, arrestants, or stimulants for a space-limited search and are responsible for the spatial structuring of the population on a smaller scale (Roland, 1990). Thus, volatile and contact chemicals are clearly related to different searching activities. Volatile sex pheromones are efficient when flight orientation of males towards females is possible. In such cases, emitting females remain immobile (creating a pheromone gradient) and do not search for hosts. In contrast, substrate-borne pheromones lead to short-distance location of virgin females, restricting male searching to a limited area, and efficiently orienting their search while walking. This is the case for *Trichogramma*, which fly passively in an airstream, but search actively by walking (Noldus et al., 1991).

Trichogramma species are polyphagous parasitoids exploiting isolated or patchily distributed hosts, and they emerge isolated or in a group depending on the species or the population within a species. SSP laid on substrate by virgin females searching for oviposition sites by walking increases their mating chances. Such a mechanism would be adaptive for females that disperse from an emergence site before mating, particularly parasitoids developing in isolated hosts, where dispersion before mating is the rule. If mating does not occur rapidly, in such arrhenotokous species, it would be adaptive to search for hosts and lay male offspring, while mating chances have not decreased, thus giving hope for further production of female offspring.

In parasitoids exploiting patchily distributed hosts, SSP would favor mating on the emergence site, thus preventing females from dispersing before mating. In various *Trichogramma* species, males emerge before females of the same patch and wait to mate with them (Waage and Ng, 1984; Pompanon et al., 1995). As soon as a few females have emerged, SSP would facilitate mating. After the emergence of several females, the emergence site would be covered by SSP and would limit male dispersion, keeping them there. This would increase the mating chances of later-emerging females. The actual duration of the effectiveness of the pheromone is still unknown. It depends on the stability of the chemicals, on the amount of SSP, and on the saturation of male perception. Because airborne pheromones are also emitted by virgin females (Pintureau and Toonders, 1983), the attraction of males from other patches may not be excluded, leading to outcrossing. Regardless, the SSP would be useful in the final step of mate location.

While the use of airborne cues is widespread in insects, the use of substrate-borne cues is less documented in sexual interactions. However, such substrate-borne chemicals can play a major role in mate location by insects orienting their search while walking. The adaptive value of such mechanisms may depend on the mating structure of the species (solitary or gregarious). Thus SSP would be an important element of population structure by conditioning mate encounters in both solitary and gregarious parasitoids.

Acknowledgments—Research was supported by the C.N.R.S. and C.E.E. Grant A.I.R. 1433. We thank Dr. X. Fauvergue for fruitful discussions.

REFERENCES

ANTOLIN, M. F., and STRAND, M. R. 1992. The mating system of *Bracon* (Hymenoptera: Braconidae). *Ecol. Entomol.* 17:1–17.

ARTHUR, A. P. 1981. Host acceptance by parasitoids, pp. 97–120, *in* D. A. Nordlund, R. L. Jones and W. J. Lewis (eds.). Semiochemicals, Their Role in Pest Control. John Wiley, New York.

ASSEM, J. VAN DEN, JACHMANN F., and SIMBOLOTTI, P. 1980. Courtship behaviour of *Nasonia vitripennis* (Hym., Pteromalidae): Some qualitative, experimental evidence for the role of pheromones. *Behavior* 75:301–307.

BERNSTEIN, C., and DRIESSEN, G. 1996. Patch-marking and optimal search patterns in the parasitoid *Venturia canescens*. *J. Anim. Ecol.* 65:211–219.

BLUM, M. S. 1974. Pheromonal sociality in the Hymenoptera, pp. 222–249, *in* M. C. Birch (ed.). Pheromones. North-Holland Publishing, Amsterdam.

BOECKH, J. 1984. Neurophysiological aspects of insect olfaction, pp. 83–104, *in* T. Lewis (ed.). Insect Communication, 12th Symposium of the Royal Entomological Society of London. Academic Press, London.

ELLER, F. J., BARTLET, R. J., JONES, R. L., and KULMAN, H. M. 1984. Ethyl (*Z*)-9-hexadecenoate a sex pheromone of *Syndipnus rubiginosus*, a sawfly parasitoid. *J. Chem. Ecol.* 10:291–300.

FAUVERGUE X., HOPPER, K. H., and ANTOLIN, M. F. 1995. Mate finding via a trail sex pheromone by a parasitoid wasp. *Proc. Natl. Acad. Sci. USA* 92:900–904.

HÖLLDOBLER B. 1984. Evolution of insect communication, pp. 349–377, *in* T. Lewis (ed.). Insect Communication, 12th Symposium of the Royal Entomological Society of London. Academic Press, London.

JANSSEN, A., VAN ALPHEN, J., SABELIS, M., and BAKKER, K. 1995. Specificity of odour-mediated avoidance of competition in *Drosophila* parasitoids. *Behav. Ecol. Sociobiol.* 36:229–235.

KAINOH, Y., and OISHI Y. 1993. Source of a sex pheromone of the egg-larval parasitoid, *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae). *J. Chem. Ecol.* 19:963–969.

KAMANO, Y., SHIMIZU, K., KAINOH, Y., and TATSUKI, S. 1989. Mating behavior of *Ascogaster reticulatus* Watanabe (Hymenoptera: Braconidae), an egg-larval parasitoid of the smaller tea tortrix, *Adoxophyes* sp. (Lepidoptera: Tortricidae). II. Behavioral sequence and a role of sex pheromone. *Appl. Entomol. Zool.* 24:372–378.

KAZMER, D. J., and LUCK, R. F. 1991. The genetic-mating structure of natural and agricultural populations of *Trichogramma*, pp. 107–110, *in* E. Wajnberg and S. B. Vinson (eds.). *Trichogramma* and Other Egg Parasitoids. Les Colloques de l'INRA No. 56.

KITANO, H. 1975. Studies on the courtship behavior of *Apanteles glomeratus* L. 2- Role of the male wing during courtship and the releaser of mounting and copulatory behaviour in the male. *Kontyû* 43:513–521.

LEWIS, T. 1984. The element and frontiers of insect communication, pp. 1–27, *in* T. Lewis (ed.). Insect Communication, 12th Symposium of the Royal Entomological Society of London. Academic Press, London.

LEWIS, W. J., SNOW, J. W., and JONES, R. L. 1971. A pheromone trap for studying populations of *Cardiochiles nigriceps*, a parasite of *Heliothis virescens*. *J. Econ. Entomol.* 64:1417–1421.

MOHAMED, M. A., and COPPEL, H. C. 1987. Pheromonal basis of courtship behavior in two gypsy moth parasitoids: *Brachymeria intermedia* (Nees) and *Brachymeria lasus* (Walker) (Hymenoptera: Chalcididae). *J. Chem. Ecol.* 13:1099–1113.

NADLE, H., and LUCK, R. F. 1992. Dispersal and mating structure of a parasitoid with a female-biased sex ratio: Implications for theory. *Evol. Ecol.* 6:270–278.

NOLDUS, L. P. J. J., VAN LENTEREN, J. C., and LEWIS, W. J. 1991. How *Trichogramma* parasitoids use sex pheromones as kairomones: Orientation behaviour in a wind tunnel. *Physiol. Entomol.* 16:313-327.

PAK, G. A., BERKHOUT, H., and KLAPWIJK, J. 1991. Do *Trichogramma* look for hosts?, pp. 77-80, *in* E. Wajnberg and S. B. Vinson (eds.). *Trichogramma* and Other Egg Parasitoids. Les Colloques de l'INRA No. 56.

PINTUREAU, B., and TOONDERS, F. B. 1983. Quelques résultats concernant l'étude de l'attraction des mâles par les femelles vierges chez *Trichogramma maidis* (Hym., Trichogrammatidae). *Bull. Mens. Soc. Linn. Lyon* 52:81-87.

POMPANON, F., FOUILLET, P., and BOULÉTREAU, M. 1995. Emergence rhythms and protandry in relation to daily patterns of locomotor activity in *Trichogramma* species. *Evol. Ecol.* 9:467-477.

ROLAND, J. 1990. Parasitoid aggregation: Chemical ecology and population dynamics, pp. 185-211, *in* M. Mackauer, L. E. Ehler, and J. Roland (eds.). Critical Issues in Biological Control. Intercept, Andover.

SHU, S., and JONES, R. L. 1993. Evidence for a multicomponent sex pheromone in *Eriborus terebrans* (Gravenhorst) (Hym.: Ichneumonidae), a larval parasitoid of the european corn borer. *J. Chem. Ecol.* 19:2563-2576.

SIMSER, D. H., and COPPEL, H. C. 1980. Female-produced sex pheromone in *Brachymeria lasus* and *B. intermedia* (Hym.: Chalcididae). *Entomophaga* 25:373-380.

TAGAWA, J. 1977. Localization and histology of the female sex pheromone-producing gland in the parasitic wasp, *Apanteles glomeratus*. *J. Insect Physiol.* 23:49-56.

VET , L. E. M., and VAN ALPHEN, J. J. M. 1985. A comparative functional approach to the host detection behaviour of parasitic wasps. I. A qualitative study on Eucoilidae and Alysinnae. *Oikos* 44:478-486.

VINSON, S. B. 1972. Courtship behavior and evidence for a sex pheromone in the parasitoid *Campoletis sonorensis* (Hymenoptera: Ichneumonidae). *Environ. Entomol.* 1:409-414.

VINSON, S. B. 1978. Courtship behavior and source of a sexual pheromone from *Cardiochiles nigriceps*. *Ann. Entomol. Soc. Am.* 71: 832-837.

VINSON, S. B. 1984. How parasitoids locate their hosts: A case of insect espionage, pp. 325-348, *in* T. Lewis (ed.). Insect Communication, 12th Symposium of the Royal Entomological Society of London. Academic Press, London.

WAAGE, J. K. 1978. Arrestment responses of the parasitoid, *Nemeritis canescens* to a contact chemical produced by its host, *Plodia interpunctella*. *Physiol. Entomol.* 3:135-146.

WAAGE, J. K., and NG, S. M. 1984. The reproductive strategy of a parasitic wasp. I. Optimal progeny and sex allocation in *Trichogramma evanescens*. *J. Anim. Ecol.* 53:401-415.

WÄCKERS, F. L. 1994. Visual cues in food and host foraging by hymenopterous parasitoids. *Norw. J. Agric. Sci.* 16:347-352.

WESELOH, R. M. 1981. Host location by parasitoids, pp. 79-93, *in* D. A. Nordlund, R. L. Jones and W. J. Lewis (eds.). Semiochemicals, Their Role in Pest Control. John Wiley, New York.

YOSHIDA, S. 1978. Behaviour of males in relation to the female sex pheromone in the parasitoid wasp, *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae). *Entomol. Exp. Appl.* 23:152-162.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

SEQUESTRATION AND METABOLISM OF HOST-PLANT FLAVONOIDS BY THE LYCAENID BUTTERFLY *Polyommatus bellargus*

MARTINA GEUDER,[1] VICTOR WRAY,[2] KONRAD FIEDLER,[3] and PETER PROKSCH[1,*]

[1]*Julius-von-Sachs-Institut für Biowissenschaften*
Universität Würzburg
Mittlerer Dallenbergweg 64, D-97082 Würzburg, Germany

[2]*Gesellschaft für Biotechnologische Forschung mbH*
Mascheroder Weg 1, D-38124 Braunschweig, Germany

[3]*Theodor-Boveri-Institut für Biowissenschaften*
Universität Würzburg
Am Hubland, D-97074 Würzburg, Germany

(Received July 23, 1996; accepted January 5, 1997)

Abstract—Larvae of the lycaenid butterfly *Polyommatus bellargus* were reared on leaves of *Coronilla varia* that are rich in flavone C-glycosides. Six flavonoids including isovitexin and isoorientin, as well as several of their congeners were isolated and identified by spectroscopic means. Comparative HPLC analysis of the host plant and of larvae, pupae, and imagines of *P. bellargus* indicated selective uptake of isovitexin versus isoorientin derivatives. Isovitexin-2″-*O*-xyloside was the major flavonoid detected in pupae and in imagines of *P. bellargus*. Several minor components were tentatively identified as quercetin- and kaempferol-*O*-glycosides based on their on line-UV spectra and by comparison with known standards. Since leaves of the host plant accumulate exclusively flavones, the flavonol glycosides are considered to be biotransformation products that are formed by the insects and/or by symbiotic bacteria. Imagines of *P. bellargus* caught in the wild exhibited similar flavonoid patterns compared to imagines reared in the laboratory. Within the imagines, approximately 80% of all flavonoids are stored in the wings (especially in the orange submarginal lunules), whereas the remaining 20% resides in the bodies. Female butterflies show a significantly higher flavonoid concentration than males. It is suggested that the sequestered flavonoids are involved in visual mate recognition.

Key Words—*Polyommatus bellargus*, Lepidoptera, Lycaenidae, *Coronilla varia*, Fabaceae, flavonoids, sequestration, insect–plant interactions.

*To whom correspondence should be addressed.

0098-0331/97/0500-1361$12.50/0 © 1997 Plenum Publishing Corporation

INTRODUCTION

Since flavonoids are ubiquitous secondary compounds found in every higher plant, as well as in many lower plants, most herbivorous insects can be expected to come into contact with them during their feeding stages. Butterflies from the families Lycaenidae, Papilionidae, and Nymphalidae are exceptional in this context as they frequently sequester flavonoids from their host plants (Morris and Thomson, 1963; Wilson, 1985, 1987; Harborne, 1991; Wiesen et al., 1994). It has been suggested that the accumulated flavonoids contribute to the UV-absorbing wing patterns of these daytime flying insects and hence may be involved in intra- or interspecific visual communication (Ford, 1941). In a number of butterfly species including members of the family Lycaenidae (Douwes, 1976; Bernard and Remington, 1991), patterns of UV reflectance and/or absorbance are indeed important for the location of conspecific males or females, corroborating the hypothesis that flavonoids are involved in visual mate recognition in butterflies.

Studies on flavonoid sequestration by butterflies, however, have been conducted mainly with wild-caught imagines with uncertain feeding histories (Wilson, 1985, 1986, 1987), which makes any comparison of flavonoid patterns found in butterflies with those from their putative host plants difficult. Recently, we studied the uptake and sequestration of flavonoids from two different host plants (*Coronilla varia* and *Medicago sativa*) by a Palearctic lycaenid butterfly, *Polyommatus icarus* Rott., under controlled laboratory conditions (Wiesen et al., 1994). We proved a selective uptake of flavonoids from the food sources as well as a bioconversion of host-plant-derived flavonols in the butterflies.

In the present paper, we have extended our studies of flavonoid metabolism to another lycaenid species, *Polyommatus bellargus* Rott. In contrast to the relatively polyphagous *P. icarus*, larvae of *P. bellargus* feed exclusively in nature on the leaves of two host plants, *Coronilla varia* and *Hippocrepis comosa* (both Fabaceae) (Thomas, 1983; Schurian, 1989). As with *P. icarus*, the adult butterflies of *P. bellargus* show a striking sexual dimorphism with the dorsal wing surfaces of the males a bright blue color while those of females are cryptically brownish. On the ventral wing surfaces, however, females have more prominent spot patterns than males. In our experiments with *P. bellargus*, we set out to investigate how these sexually dimorphic, relatively stenophagous insects metabolize the flavonoids encountered in their host plants. Similar to *P. icarus* we proved a selective uptake of flavonoids, and we obtained evidence for biotransformation reactions of dietary flavonoids occurring in the insects. The allocation of most of the sequestered flavonoids into distinct parts of the wings, as well as the observed strong sexual dimorphism with regard to flavonoid accumulation, suggest a role for these compounds in mate recognition.

METHODS AND MATERIALS

Insects. Butterflies were reared from eggs laid by field-caught females of *P. bellargus* (origin: Retzbach near Würzburg, northern Bavaria). Eggs, larvae, and pupae were kept in an environmental chamber (22.5°C, 15L:9D). After hatching from eggs, the caterpillars were placed in closed transparent plastic vials (250 ml) lined with moist filter paper. Cut foilage of *Coronilla varia* was provided in excess, and special care was taken to remove all inflorescences or flower buds from the food. Each day caterpillars were transferred to new clean rearing vials containing fresh food. After pupation, insects were kept without food in plastic vials of the same size until the butterflies eclosed. Under this regime, the individual development from egg to adult stage took 45–55 days (see Fiedler and Saam, 1994, for further data on rearing and development).

For chemical analysis, mature larvae, pupae, and adult butterflies were killed by deep-freezing (−20°C) and later freeze-dried. Larvae were kept without food for 2–4 hr prior to deep-freezing to ensure that the gut was empty. Adults were taken for analysis only after they had discharged their meconium. In addition, samples of larval frass were collected and stored.

In order to compare flavonoid patterns of laboratory-reared butterflies with naturally grown specimens, *P. bellargus* males from two localities in southern Turkey were analyzed. Turkish *P. bellargus* populations feed exclusively on *Coronilla varia* (Schurian, 1989; Hesselbarth et al., 1995), whereas populations in southern Germany utilize *Hippocrepis comosa* as host plant (Ebert and Rennwald, 1991).

Food Plant. Leaves of *C. varia* were collected around the campus of Würzburg University and fed to caterpillars as necessary. All plant material originated from the same plant population and was collected between May and July. Samples of leaves were stored at −20°C and freeze-dried for analysis.

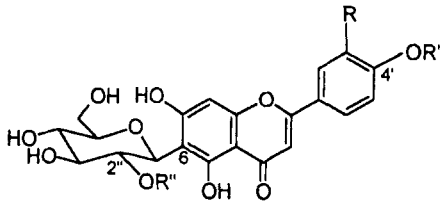
Flavonoid Analysis. For preparative isolation of flavonoids, freeze-dried leaves of *C. varia* were ground and extracted with 90% aqueous MeOH. The extract was concentrated and partitioned between H_2O/ethyl acetate and H_2O/*n*-butanol. All fractions were monitored for flavonoids by HPLC. The flavonoid-bearing *n*-butanol fraction was subjected to column chromatography on Polyamide SC-6 (Macherey & Nagel, Düren, Germany) using H_2O, H_2O/MeOH (50:50) or MeOH as eluents followed by column chromatography on Sephadex LH-20 (Sigma, Deisenhofen, Germany) with MeOH as eluent. Where necessary, final purification was achieved by column chromatography on an RP-18 lobar column (Merck, Darmstadt, Germany) with mixtures of H_2O and MeOH as eluent. Isolated flavonoids were identified based on their UV, mass, and ^1H and ^{13}C NMR spectra. ^1H and ^{13}C NMR spectra were recorded in DMSO on Bruker AM 300 or Bruker ARX 400 NMR spectrometers. FAB-MS spectra

were recorded on a Finnigan MAT 8430 mass spectrometer using glycerol as matrix. Structures of **1–6** (Figure 1, below) were determined from 1D (^1H and ^{13}C) and 2D (COSY, ^1H-detected direct, and long-range ^{13}C–^1H) correlations as well as by nOe experiments.

The HPLC system was from Pharmacia (Uppsala, Sweden) equipped with a photodiode array detector (Waters, Milford, Massachusetts). Samples were injected on a Nova-Pak C_{18} column (Waters) (150 × 3.9 mm, 4-μm pore size), and separated using a linear gradient from 100% A (10% MeOH, 90% H_2O adjusted to pH 2 with orthophosphoric acid) to 100% B (MeOH) in 50 min. Flavonoids were quantified by the internal or external standard methods using kaempferol (Roth, Karlsruhe, Germany) as standard.

RESULTS

Flavonoid Analysis of Leaves of Coronilla varia. Freeze-dried leaves of *C. varia* yielded six major flavone derivatives including isovitexin (**1**), isovitexin-4′-*O*-glucoside (**2**), isovitexin-2″-*O*-xyloside (**3**), isoorientin (**4**), isoorientin-4′-*O*-glucoside (**5**), and isoorientin-2″-*O*-xyloside (**6**) (Figure 1). All compounds were readily identified from their spectral data (UV, NMR, and MS), as well as from a comparison with published data (Sherwood et al., 1973).



	R	R'	R"
[1] Isovitexin	H	H	H
[2] Isovitexin-4'-O-glucoside	H	β-D-glucose	H
[3] Isovitexin-2"-O-xyloside	H	H	β-D-xylose
[4] Isoorientin	OH	H	H
[5] Isoorientin-4'-O-glucoside	OH	β-D-glucose	H
[6] Isoorientin-2"-O-xyloside	OH	H	β-D-xylose

FIG. 1. Structures of flavonoids identified in this study.

TABLE 1. DISTRIBUTION (%), CONCENTRATIONS, AND ABSOLUTE AMOUNTS OF FLAVOVOIDS IN *P. bellargus* AND IN LARVAL HOST PLANT *C. varia*

Compound	Leaves of *C. varia*	Larval feces	Larvae (N = 11)	Pupae (N = 7)	Female (N = 8)	Male (N = 13)
1[a]	2.0					
2	47.0	14.6				
3	4.0	27.0	37.4	67.0	60.2	42.3
4	2.0	47.2	21.7	7.0		
5	19.0					
6	26.0		9.0			
Unknowns		11.2	31.9	26.0	39.8	57.7
Conc. (mg/g dry wt)	13.7	7.4	7.0 ± 3.0	3.5 ± 2.0	6.5 ± 2.1	2.6 ± 0.5
Absolute amounts (μg/individual)			121.7 ± 35.0	62.5 ± 22.6	97.0 ± 25.8	34.2 ± 10.2

[a]Numbers of compounds refer to Figure 1.

Compound **2** was present as the major flavonoid, accounting for 47% of all leaf flavonoids (total concentration ca. 13.7 mg/g dry wt; Table 1), as indicated by HPLC analysis (Figure 2).

Flavonoid Analysis of Insects Feeding on Leaves of C. varia. HPLC analysis of feces from larvae of *P. bellargus* (Figure 2) revealed a different flavonoid pattern compared to that from leaves of *C. varia*. In feces, isorientin (**4**) followed by isovitexin-2″-*O*-xyloside (**3**) were the major phenolic compounds accounting

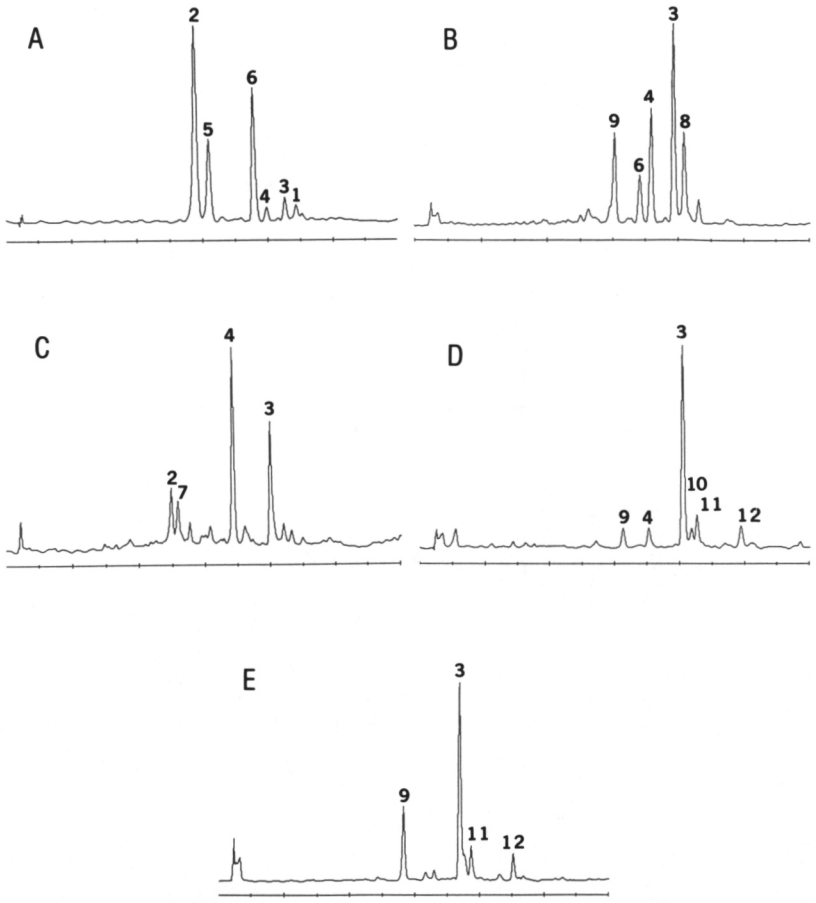


FIG. 2. Comparative HPLC analysis of flavonoids from leaves of *C. varia* (A) and from larvae (B), larval feces (C), pupae (D), and imagines (E) of *P. bellargus*. Numbers of compounds (**1–6**) follow Figure 1. Peaks **7–12** represent partially identified flavonoid metabolites originating through biotransformation in the insects.

for more than 74% of all flavonoids (total concentration ca. 7.4 mg/g dry wt; Table 1). In addition to the host-plant-derived flavone derivatives, an unknown compound (**7**) was detected in the feces that was not present in the leaves. Based on its UV spectrum (UV λ_{max}^{MeOH} nm: 272, 340), the unknown was tentatively identified as an additional flavone originating from larval biotransformation.

Larvae of *P. bellargus* contained isovitexin-2″-*O*-xyloside (**3**) and isoorientin (**4**) as major flavonoids (Figure 2, Table 1). In addition to these, two unknown flavone derivatives, **8** (UV λ_{max}^{MeOH} nm: 272, 340), and **9** (UV λ_{max}^{MeOH} nm: 272, 340), were present in the larvae and were tentatively identified as additional flavones formed in the larvae through metabolism of host-plant flavones. Flavonoid content of fourth-instar larvae was strongly correlated with larval dry weight ($rS = 0.82$, $P < 0.01$, $N = 11$), suggesting that flavonoid accumulation occurs steadily throughout that instar.

HPLC analysis of pupae of *P. bellargus* revealed a simplified flavone pattern compared to those of the larvae or leaves of *C. varia* (Figure 2). Isovitexin-2″-*O*-xyloside (**3**) was clearly the dominating flavone accounting for 67% of all flavonoids detected. Of the other compounds present in the host plant, only isoorientin (**4**) was detected. The biotransformation product **9** that had already been found in larvae was also present in the pupae. Further unknown biotransformation products included compounds **10–12**, which were detected only in pupae and imagines (Figure 2). Surprisingly, the UV spectra obtained for these latter compounds indicated that **10** (UV λ_{max}^{MeOH} nm: 255, 268 (sh), 355) and **11** (UV λ_{max}^{MeOH} nm: 255, 268 (sh), 355) were flavonol derivatives of the quercetin type, whereas **12** exhibited a UV spectrum (UV λ_{max}^{MeOH} nm: 268, 352) typical of a kaempferol derivative. Due to their minute concentrations compounds **10–12** could not be isolated in amounts sufficient for rigorous spectroscopic analysis. Their nature as flavonol rather than flavone derivatives, however, is beyond doubt as flavonols of the kaempferol or quercetin type can be easily differentiated from the corresponding flavones by UV spectroscopy (Mabry et al., 1970). Furthermore, compound **12** was found to coelute with the known kaempferol-3-*O*-glucoside when coinjected into the HPLC.

Male and female imagines of *P. bellargus* were similar to pupae with regard to their flavonoid patterns (Figure 2). Isovitexin-2″-*O*-xylosid (**3**) was again the major flavonoid detected, followed by the biotransformation products **9** as well as **11** (unknown quercetin derivative) and **12** (unknown kaempferol derivative, possibly kaempferol-3-*O*-glucoside). Male imagines that had been caught in the wild were similar to laboratory-grown imagines with regard to their flavonoid patterns. The major difference between males from both groups was in regard to a further biotransformation product that was detected only in wild-caught imagines and lacking in specimens that had been cultivated in the laboratory (data not shown). Based on its on-line-recorded UV spectrum, the compound was identified as another unknown quercetin derivative.

Since total flavonoid concentrations were similar for wild males and laboratory-reared ones, quantitative data from both groups were pooled for statistical evaluation (Table 1). Males (2.6 ± 0.5 mg/g dry wt, $N = 13$) contained significantly lower flavonoid concentrations than females (6.5 ± 2.1 mg/g dry wt, $N = 8$, U test: $P < 0.01$). Similarly, the absolute amounts of accumulated flavonoids differed greatly between the sexes (males: 34.2 ± 10.2 μg/individual, females: 97.9 ± 25.88 μg/individual, U test: $P < 0.01$), although the dry weights of the sexes showed less pronounced differences (males: 12.9 ± 1.8 mg; females; 15.4 ± 2.4 mg) (Table 1).

Localization of Flavonoids in Imagines of P. bellargus. When imagines of *P. bellargus* were dissected into wings and bodies and analyzed for flavonoids by HPLC, no differences were observed. Isovitexin-2″-*O*-xyloside (**3**) was always the major flavonoid in both body parts. Quantitative analysis, however, revealed that more than 80% of all flavonoids accumulated in the wings of males and females, whereas the remaining amounts resided in the bodies (Table 2). Within the wings, the highest concentrations of flavonoids (up to 0.4 μg/mm^2 for females) were detected in the distinct orange submarginal lunules that account for roughly 2.5% of the total wing area (Table 3). In the orange submarginal lunules of male butterflies, flavonoid concentrations were only up to 0.2 μg/mm^2 and were thus considerably lower than those in lunules of females (Table 3). The black and white colored discal, postdiscal, and submarginal ocelli of males and females, which account for approximately 45% or 38% of the total wing area, contained approximately 0.1 μg of flavonoids/mm^2. In particular, the orange lunules, as well as the ocelli that are present on the undersides of wings, are strongly UV-absorbing when viewed under UV_{365} nm (data not shown).

TABLE 2. QUANTITATIVE ANALYSIS OF FLAVONOIDS FROM BODIES AND WINGS OF MALE AND FEMALE IMAGINES OF *P. bellargus*[a]

	Female		Male	
	Body	Wings	Body	Wings
Dry wt (mg)	11.5	2.6	9.7	2.9
Absolute amounts of flavonoids (μg)	21.6	91.3	7.6	40.2
Conc. of flavonoids (μg/mg dry wt)	1.9	35.8	0.8	13.8

[a]Imagines used for the experiment were reared on leaves of *C. varia*. Data reported are means of three male and female imagines that were pooled for flavonoid analysis. No qualitative differences with regard to flavonoid patterns were observed for bodies and wings.

TABLE 3. DISTRIBUTION OF FLAVONOIDS IN DIFFERENT WING AREAS OF MALE AND FEMALE IMAGINES OF *P. bellargus*[a]

	Wing areas (fore- and hindwings combined)					
	Orange submarginal lunules		Black/white colored ocelli		Brown-colored area	
	Female	Male	Female	Male	Female	Male
Area of segment (%) compared to total wing area	2.5	2.2	37.9	44.8	59.7	53.0
Absolute amounts of flavonoids in different wing segments (ng/mm^2)	0.4	0.2	0.1	0.1	0.1	0.1

[a]Wings of three male and female imagines from a culture of *P. bellargus* reared on leaves of *C. varia* were pooled for the analysis. Data reported are means from these individuals.

DISCUSSION

Comparison of the flavonoid patterns from leaves of *C. varia* with those of pupae and imagines of *P. bellargus* indicates a selective sequestration of the host-plant flavone isovitexin-2″-*O*-xyloside (**3**) in reference to other isovitexin or isoorientin derivatives (Figures 1 and 2). Isoorientin derivatives (**4**–**6**, Figure 1), which are also present in the host plant appear to be mostly lost from the larvae by excretion (Figure 2). The observed sequestration of isovitexin-2″-*O*-xyloside in pupae and imagines of *P. bellargus* may be due to selective accumulation of this particular flavone derivative in reference to other isovitexin derivatives such as **1** or **2.** Alternatively, it is possible that isovitexin (**1**) or isovitexin-4′-*O*-glucoside (**2**) is converted to the xyloside (**3**) following uptake by the insects. Analysis of wild-caught imagines of *P. bellargus* yielded a similar flavonoid pattern to that found in laboratory-grown specimens. Thus, the results obtained in this study are not caused by laboratory conditions but may be extrapolated to specimens of *P. bellargus* in the field.

Surprisingly, pupae as well as imagines of *P. bellargus* were found to contain flavonol derivatives of the quercetin and kaempferol type (**10**–**12**, Figure 2). Flavonols are not present in leaves of *C. varia*, whereas they accumulate in flowers of the same species (Wiesen et al., 1994). Contamination of the larval food by flowers of *C. varia* could thus be a possible source of the flavonols detected in pupae and imagines. However, care was taken to avoid the presence of flowers in the larval food. Furthermore, larvae of *P. bellargus* are known to

reject flowers of *C. varia* when offered as food source. In addition, flavonol derivatives were detected only in pupae and imagines of *P. bellargus*, whereas they were lacking in the feeding stages as well as in the larval feces (Figure 2). Dietary flavone C-glycosides of the isovitexin and isoorientin type thus remain the only logical source of the flavonol derivatives detected in pupae and imagines.

Insect metabolism of dietary flavonoids has been demonstrated previously. For example, larvae of the lycaenid butterfly *Polyommatus icarus* are able to glucosylate kaempferol that is incorporated into artificial diet (Wiesen et al., 1994). Glycosylation of dietary flavonols (e.g., quercetin) has also recently been proven for the band-winged grasshopper, *Dissoteira carolina* (Hopkins and Ahmad, 1991). Cleavage of flavone C-glycosides, however, which is apparently found in *P. bellargus*, has not yet been described for insects. It has been reported, however, for symbiotic intestinal bacteria from humans. When isoorientin (**4**) was offered to intestinal bacteria of humans, the corresponding aglycone luteolin was recovered from the assays (Hattori et al., 1988). Thus, it is possible that symbiotic bacteria of *P. bellargus* are involved in the proposed biotransformation of flavone C-glycosides into their corresponding flavonol-*O*-glycosides.

Regardless of their origin, the flavonoids present in adult *P. bellargus* show a distinct sexual dimorphism with respect to their amounts and concentrations in wings of males and females. Females not only had consistently higher flavonoid loads and concentrations than males, but also enriched these pigments twice as strongly in those parts of the wings that contain the orange submarginal lunules. These sex differences, with a two-fold difference between males and females, were more pronounced in *P. bellargus* than in the related *P. icarus*, where females contain only 25–83% more flavonoids than males, depending on their larval diet (Wiesen et al., 1994; Burghardt et al., 1995). In the sexually monomorphic species *Aricia agestis*, flavonoid contents and concentrations showed no such sex differences (Ziegler et al., unpublished results). We also observed some variation between the Polyommatini species investigated as to exactly where in the wings the flavonoids are most concentrated.

These findings suggest that flavonoid accumulation in the wings may play a role in behavioral contexts where wing patterns could be important, such as species or mate recognition. As flavonoids accumulated by the lycaenids absorb UV light, they contribute to the complex color patterns of both the dorsal and ventral wing surfaces, which in addition contain UV-reflecting elements as well as patterns in the spectrum visible to humans. In butterflies, visual perception of wing patterns, particularly those in UV light, play a prominent role in mate location and mate selection (Silberglied, 1979, 1984). Physiological, behavioral, and morphological studies on Lycaenidae species also demonstrate the ability of these butterflies to extract information from UV patterns, which are often strictly species- or even sex-specific (Douwes, 1976; Bernard and Remington,

1991; Meyer-Rochow, 1991). Preliminary experiments on mate choice behavior of *P. icarus* further corroborate the hypothesis that flavonoid pigments are involved in mate selection. When given the choice between dummies made from flavonoid-rich (reared on flavonoid-rich inflorescences) or flavonoid-free females (reared on diets where females fail to sequester flavonoids), the males were significantly more attracted to those females that contained substantial amounts of flavonoid pigments (Otterbeck et al., unpublished results). Hence, the sequestration of flavonoids is not only another interesting example of physiological and chemical interactions between insects and their host plants, but also opens up a whole area of inquiry related to mate recognition and sexual selection fostered by host-plant-derived visual stimuli.

Acknowledgments—Financial support by a grant of the DFG (to K. F. and P. P.) and by the Fonds der Chemischen Industrie (to P. P.) is gratefully acknowledged.

REFERENCES

BERNARD, G. D., and REMINGTON, C. L. 1991. Color vision in *Lycaena* butterflies: Spectral tuning of receptor arrays in relation to behavioral ecology. *Proc. Natl. Acad. Sci. U.S.A.* 88:2783–2787.

BURGHARDT, F., FIEDLER, K., and PROKSCH, P. 1995. Wirtspflanzenabhängige Flavonoidmuster im Bläuling *Polyommatus icarus* (Lycaenidae). *Verh. Dtsch. Zool. Ges.* 88:256.

DOUWES, P. 1976. Mating behaviour in *Heodes virgaureae* with particular references to the stimuli from the female (Lepidoptera: Lycaenidae). *Entomol. Gen.* 2:232–241.

EBERT, G., and RENNWALD, E. (eds.) 1991. Die Schmetterlinge Baden-Württembergs. Band 1, Tagfalter 2. E. Ulmer, Stuttgart, 535 pp.

FIEDLER, K., and SAAM, C. 1994. Does ant-attendance influence development in 5 European Lycaenidae butterfly species? *Nota Lepid.* 17:5–24.

FORD, E. B. 1941. Studies on the chemistry of pigments in the Lepidoptera with reference to their bearing on systematics. 1. The anthoxanthins. *Proc. R. Entomol. Soc. London Ser. A.* 16:65–90.

HARBORNE, J. B. 1991. Flavonoid pigments, pp. 389–429, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites, 2nd. ed., Vol. 1. Academic Press, San Diego.

HATTORI, M., SHU, Y.-Z., EL-SEDAWY, A. I., NAMBA, T., KOBASHI, K., and TOMIMORI, T. 1988. Metabolism of homoorientin by human intestinal bacteria. *J. Nat. Prod.* 51:874–878.

HESSELBARTH, G., VAN OORSCHOT, H., and WAGENER, P. 1995. Die Tagfalter der Türkei unter Berücksichtigung der angrenzenden Länder, 3 vols. S. Wagener Verlag, Bocholt.

HOPKINS, T. L., and AHMAD, S. A. 1991. Flavonoid wing pigments in grasshoppers. *Experientia* 47:1089–1091.

MABRY, T. J., MARKHAM, K. R., and THOMAS, M. B. 1970. The Systematic Identification of Flavonoids. Springer, New York.

MEYER-ROCHOW, V. B. 1991. Differences in ultraviolet wing patterns in the New Zealand lycaenid butterflies *Lycaena salustius, L. rauparaha* and *L. feredayi* as a likely isolating mechanism. *J. R. Soc. N.Z.* 21:169–177.

MORRIS, S. H., and THOMSON, R. H. 1963. The flavonoid pigments of the marbled white butterfly (*Melanargia galathea* Seltz). *J. Insect Physiol.* 9:391–399.

SCHURIAN, K. G. 1989. Revision der Lysandra-Gruppe des Genus Polyommatus Latr. (Lepidoptera: Lycaenidae). *Neue Entomol. Nachr.* 24:1–181.

SHERWOOD, R. T., SHAMMA, M., MONIOT, J. L., and KROSCHEWSKY, J. R. 1973. Flavone C-glycosides from *Coronilla varia*. *Phytochemistry* 12:2275–2278.

SILBERGLIED, R. E. 1979. Communication in the ultraviolet. *Annu. Rev. Ecol. Syst.* 10:373–398.

SILBERGLIED, R. E. 1984. Visual communication and sexual selection among butterflies, pp. 207–223, *in* R. I. Vane-Wright and P. R. Ackery (eds.). The Biology of Butterflies. Academic Press, London.

THOMAS, J. A. 1983. The ecology and conservation of *Lysandra bellargus* (Lepidoptera: Lycaenidae) in Britain. *J. Appl. Ecol.* 20:59–83.

WIESEN, B., KRUG, E., FIEDLER, K., WRAY, V., and PROKSCH, P. 1994. Sequestration of host-plant-derived flavonoids by lycaenid butterfly *Polyommatus icarus*. *J. Chem. Ecol.* 20:2523–2538.

WILSON, A. 1985. Flavonoid pigments of butterflies in the genus *Melanargia*. *Phytochemistry* 24:1685–1691.

WILSON, A. 1986. Flavonoid pigments in swallowtail butterflies. *Phytochemistry* 25:1309–1313.

WILSON, A. 1987. Flavonoid pigments in chalkhill blue (*Lysandra coridon* Poda) and other lycaenid butterflies. *J. Chem. Ecol.* 13:473–493.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

EFFECTS OF FIFTH-INSTAR VOLATILES ON SEXUAL MATURATION OF ADULT DESERT LOCUST *Schistocerca gregaria*

YOUSIF O. H. ASSAD,[1] AHMED HASSANALI,[1,*] BALDWYN TORTO,[1] HASSANE MAHAMAT,[1] NABIL H. H. BASHIR,[2] and SAYED EL BASHIR[1]

[1]*International Center of Insect Physiology and Ecology, (ICIPE) P.O. Box 30772, Nairobi, Kenya*

[2]*Environment and Natural Resources Research Institute, (ENRRI) The National Center for Research P.O. Box 6090, Khartoum, Sudan*

(Received May 14, 1996; accepted January 6, 1997)

Abstract—The effect of the presence of gregarious fifth-instar nymphs on the sexual maturation of newly molted gregarious immature adult males and females of the desert locust *S. gregaria* was investigated by monitoring color changes (yellowing of body), sexual activity, aggregation-maturation pheromone titers (as measured by phenylacetonitrile levels in males), oocyte length, and oviposition time. Maturation was significantly delayed in adults that were reared together with fifth-instar nymphs (visual, tactile, and chemical signals present) with respect to all parameters measured. Male and female nymphs were equally effective in inducing this delay. Nymphs kept in an upper compartment of two-chamber cages (no visual or tactile contact possible) were similarly effective, but their feces were ineffective, suggesting the mediation of a volatile signal from the nymphs themselves. This was confirmed by examining the effect of volatiles trapped from nymphs and testing synthetic blends of previously identified nymphal aggregation pheromone components (C_6, C_8–C_{10} aliphatic aldehydes and acids, guaiacol, and phenol) on the sexual maturation of adults. These and previous studies suggest a dual role for nymphal volatiles as nymphal aggregants and adult maturation retardants, similar to the adult volatiles that are known as adult aggregants and maturation accelerants. The results are discussed in terms of the role of the two pheromone systems in synchronizing maturation of the gregarious adults.

*To whom correspondence should be addressed.

0098-0331/97/0500-1373$12.50/0 © 1997 Plenum Publishing Corporation

Key Words—*Schistocerca gregaria*, sexual maturation, hexanal, octanal, nonanal, decanal, hexanoic acid, octanoic acid, nonanoic acid, decanoic acid, guaiacol, phenol, pheromone.

INTRODUCTION

Two primer pheromone systems have been implicated in the regulation of maturation in the gregarious desert locust, *S. gregaria:* a maturation-accelerating pheromone associated with mature male adults (Norris, 1954; Loher, 1960; Amersinghe, 1978; Mahamat et al., 1993) and a maturation-retarding pheromone associated with young nymphal stages (Norris, 1964; Norris and Pener, 1965; Richards and El Mangoury, 1968). It has been suggested that the two effects operating sequentially facilitate synchronous maturation of the entire fledgling population (Popov, 1958; Norris, 1962, 1964; Uvarov, 1966; Richards and El Mangoury, 1968).

Recently, we traced the maturation-accelerating effect of mature males to volatiles from the males (Mahamat et al., 1993). The major components were anisole, veratrole, phenylacetonitrile, 4-vinyl veratrole, and benzaldehyde, with phenylacetonitrile comprising about 75–85% of the blend. Mature females did not produce significant amounts of these compounds. The emission of the blend precedes maturation, and also plays an aggregating role in the adult stage (Mahamat et al., 1993; Torto et al., 1994). Accelerated maturation in young males exposed to mature males was associated with earlier production of these compounds by the maturing males (Mahamat et al., 1993).

There are separate pheromone systems for the nymphal (second to fifth) and adult stages (Obeng-Ofori et al., 1993, 1994a). In view of the role of the adult pheromone as an adult aggregant and maturation accelerant, we wondered if the nymphal pheromone system (Torto et al., 1996) would also demonstrate a similar parsimony, that is if the maturation retarding effect of nymphs reported earlier are due to the pheromone responsible for nymphal aggregation. We reinvestigated the effects of the presence of fifth instars on the maturation of immature adults, and we compared the presence of live fifth instars with exposure to only volatiles from fifth instars, their feces, and nymphal synthetic aggregation pheromone comprising (C_6, C_8–C_{10} aliphatic aldehydes and acids, guaiacol, and phenol) (Torto et al., 1996). We monitored maturation as the time taken by immature adults to (1) change body color (yellowing), (2) copulate, (3) produce maturation–aggregation pheromone (as measured by phenylaceto-nitrile titre in males), (4) ovulate (as measured by oocytes length in females), and (5) oviposit. Herein we describe the results of this study.

METHODS AND MATERIALS

Insects

Experimental animals, i.e., crowded stock of the desert locust *S. gregaria*, were reared at the Insect and Animal Breeding Unit (IABU) of ICIPE. The colony was obtained from The Desert Locust Control Organization for Eastern Africa (DLCO-EA) in Addis Ababa, Ethiopia. Insects (300–400) of both sexes were reared under crowded conditions in aluminum cages (50 × 50 × 50 cm) in a well-aerated room (4.5 × 4.5 m) with a duct system that maintained a negative pressure. Temperature was 33 ± 2°C, relative humidity was 60%, and the photoperiod was 12L:12D. Fledgling males and females from the same batch (1–2 days old) were used to eliminate variability in physiological status. Fifth instars (2–3 days old) were replaced every three days to avoid the introduction of volatiles from insects molting into adults.

Experimental Cages

Two types of cages were used. The effect of live fifth instars (experiment I) was studied in standard aluminum cages (20 × 20 × 20 cm) with a sliding glass front and wire gauze on the top, bottom and two of the sides, and with the back made of aluminum sheath. In the other experiments (II, III, and IV) two-chamber cages were used. Each consisted of two stacked chambers of identical size (15 × 15 × 15 cm) with sliding glass doors at the front and wire gauze on the other three sides, top, and between the two chambers to allow test insects in the bottom chamber to perceive the volatiles emitted from the pheromone source from the upper chamber (Mahamat et al., 1993).

Collection of Volatiles

Monitoring Adult Emission. Volatiles were collected by aeration as described by Torto et al. (1994). Briefly, air from a compressed air cylinder was passed through a charcoal filter over locusts contained in a trapping chamber (10 cm long × 3.5 cm ID), and through 60 mg of charcoal packed between two glass wool plugs in 6-cm-long × 8-mm-ID glass tubes. A flow rate of 106 ml/min was maintained for 16 hr at 30 ± 1°C. Collections were done from three groups of three adult males exposed to: (1) fifth-instar nymphs (experiment I); (2) volatiles trapped from fifth nymphal instars (experiment II); (3) fifth-instar nymphal fecal volatiles (experiment III); and (4) no volatiles (control). For each treatment, collections were done on insects 12–16, 16–20, 20–24, 24–28, 28–32, 32–36, and 36–40 days from molt.

Collection of Nymphal Volatiles. Volatiles emitted by fifth instars

(50 insects, 3–5 days old, mixed sex) were collected as described above for 24 hr on 60 mg charcoal held in an ice bath (Torto et al., 1996), with the insects contained in a 1-liter three-necked flask. Trapped volatiles were eluted with 5 ml HPLC grade dichloromethane (DCM) (Aldrich Ltd.), concentrated under a stream of nitrogen at 0°C to 1000 μl, and stored at −15°C until used.

Analysis of Volatiles

Volatiles were analyzed as described by Torto et al. (1994). Extracts were concentrated to 100 μl and 250 ng of *o*-methyl acetophenone (internal standard) was added. An aliquot of 2.5 μl was analyzed by capillary gas chromatography (GC). Analyses were performed on a Hewlett-Packard (HP) 5890 series II gas chromatograph equipped with a flame ionization detector (FID) and a HP capillary column (Carbowax 20 M, 50 m × 0.32 mm ID × 0.3-μm film thicknesses) with nitrogen as the carrier gas at a flow rate of 0.35 ml/min. The oven temperature was initially isothermal at 60°C for 10 min, then programmed at 5°C/min to 180°C, held for 5 min, and raised at a rate of 10°C/min to 220°C, and held for 15 min. Chromatographic peaks were integrated using a HP 3393 integrator.

Experiment I: Effect of Fifth Instars on Maturation of Immature Adult Males and Females

Newly molted immature adult (1 to 2-day-old) males and females (referred to as recipients) from the gregarious colony were placed with (1 to 2-day-old) male or female fifth instars, sexually mature males, immature males, or immature females (referred to as signal sources) in aluminum cages described above. Recipient insects had visual, tactile, and olfactory contact with signal sources. Treatments are shown in Table 1. Each treatment was replicated five times. For treatments H–L (Table 1) an additional five replicates were set up to allow measurements of oocyte length. Insects were supplied with fresh wheat seedlings and wheat bran daily.

Recipient insects were checked daily for signs of maturation as assessed by: (1) changes in color (yellowing) in accordance with the stage III classification developed by Norris (1954) and recently used by Mahamat et al. (1993); (2) copulation time carried out with young males that had begun to develop coloration on the abdomen (young males were paired with fully mature females for about 6 hr daily and copulation activity noted; similarly test females were paired with mature males); (3) GC titers of trapped phenylacetonitrile from males at different ages (12–16, 16–20, 20–24, 24–28, 28–32, 32–36, and 36–40 days); and (4) age at which females begin ovipositing. Each female was marked and the tubes used for egg laying were changed every day. The number of eggs/pods oviposited was noted.

TABLE 1. TREATMENTS USED IN STUDYING MATURATION RESPONSE OF IMMATURE ADULT DESERT LOCUST TO PRESENCE OF FIFTH INSTARS (EXPERIMENT I)

Recipients (immature adult)	Signal sources	No. of insects recipient : signal
(A) males	immature adult males[a]	4:4
(B) males	immature adult females[a]	4:4
(C) males	fifth-instar females	4:4
(D) males	fifth-instar males	4:4
(E) males	fifth-instar male or female	4:1
(F) males	fifth-instar mixed sexes	4:2
(G) males	mature adult males	4:4
(H) females	immature adult males[a]	4:4
(I) females	immature adult females[a]	4:4
(J) females	fifth-instar females	4:4
(K) females	fifth-instar males	4:4
(L) females	mature adult males	4:4

[a] Control treatments.

Maturation in females was monitored by the length of oocytes. Terminal oocytes in females are about 0.7 mm in newly molted adult females and increase in length to about 7.0–8.0 mm when fully developed (Highnam and Lusis, 1962). Abdomens of adult females were cut open longitudinally and ovaries examined under a binocular microscope; the lengths of six developing terminal oocytes in each individual were measured with a micrometer. Measurements were made every second day from day 12 to 40.

Experiment II: Comparison of Effect of Volatiles of Fifth-Instar Nymphs and Their Feces

Groups of male and female immature adults were exposed to 1, 3, 6, or 9 g of feces from fifth-instar nymphs. In our colony, 0.75 g of feces is produced per day by one fifth instar. Experimental fecal doses, therefore, represent varying amounts of droppings produced by four fifth-instar nymphs. Test insects (newly molted fledgling adult males and females) were placed in the lower compartment of the two-chamber cage, and the feces, which were replaced daily, in the upper compartment. In controls, recipient immature adults were placed in the lower compartment of the cage and the signal sources (fifth-instar nymphs, immature adult males or females) in the upper chamber. The combinations shown in Table 2A were tested. Each treatment was replicated five times. For G–K treatments, an additional five replicates were performed. Maturation was monitored as described in experiment I.

TABLE 2. TREATMENTS USED IN STUDYING MATURATION RESPONSE OF IMMATURE ADULT DESERT LOCUST TO FIFTH-INSTAR VOLATILES

Recipient (4 immature adults)	Signal sources
A. Fifth instar or their fecal volatiles (experiment II)	
(A) males	immature adult males[a]
(B) males	fifth-instars mixed sexes
(C) males	fifth-instar feces (1g)
(D) males	fifth-instar feces (3g)
(E) males	fifth-instar feces (6g)
(F) males	fifth-instar feces (9g)
(G) females	immature adult females[a]
(H) females	fifth instars mixed sexes
(I) females	fifth-instar feces (1g)
(J) females	fifth-instar feces (3g)
(K) females	fifth-instar feces (6g)

Recipient (5 immature adults)	Signal sources
B. Extracts of fifth-instar volatiles (experiment III)	
(A) males	immature adult males[a]
(B) males	100 μl DCM[b] in light paraffin oil[a]
(C) males	fifth instars mixed sexes
(D) males	nymphal volatile extract (100 μl)
(E) females	immature adult females[a]
(F) females	100 μl DCM in light paraffin oil[a]
(G) females	fifth instars mixed sexes
(H) females	nymphal volatile extract (100 μl)

[a]Control treatments.
[b]DCM = dichloromethane.

Experiment III: Effect of Volatile Extracts from Fifth Instars

Volatiles collected from 50 fifth instars were concentrated to 1 ml before use. As concentrated, 20 μl of the sample represented the equivalent of 24 hr of emission from one nymph. One hundred microliters (representing 24 hr of emission of five nymphs) was prepared in light paraffin oil (Merck) (1000 μl) contained in 4-ml vials (Jencons, Bedfordshire, UK) with plastic snap caps with a 2-mm-diameter vent to moderate the release of the volatiles. The control consisted of dichloromethane (100 μl) in light paraffin oil (1000 μl). The combinations shown in Table 2B were tested. Recipient insects were placed in the lower compartment of the two-chamber cage and signal sources on the upper

compartment. Signal sources were changed daily. Each set was replicated five times with five additional replicates for female oocyte mesurement. Maturation was monitored as described in experiment I.

Experiment IV: Effect of Fifth Instar Aggregation Pheromone

Immature adults were exposed to different synthetic nymphal pheromone blends prepared in paraffin oil (Torto et al., 1996). A blend of synthetic aldehydes and acids (see (b) below) at a combined concentration of 1 $\mu g/\mu l$ was prepared. Three doses—5, 10, and 20 μg—were initially tested to determine the appropriate dose, and the dose of 10 μg was selected for use in subsequent assays. The following combinations of the nymphal pheromone components (see Table 3) were tested: (a) hexanal, octanal, nonanal, decanal, hexanoic acid, octanoic acid, nonanoic acid, decanoic acid, guaiacol, and phenol (4:9:20:20:14:50:100:16:0.0002:0.0002) (10 μg), (b) hexanal, octanal, nonanal, decanal, hexanoic acid, octanoic acid, nonanoic acid, and decanoic acid (4:9:20:20:14:50:100:16) (10 μg), (c) hexanal, octanal, nonanal, decanal, hexanoic acid, octanoic acid, nonanoic acid, and decanoic acid at the same ratio as in (b) (20 μg), (d) hexanal, octanal, nonanal, decanal, hexanoic acid, octanoic acid, nonanoic acid, and decanoic acid at the same ratio as in (b) (5 μg), (e) aldehydes only in the above ratio (10 μg), and (f) acids only in the above ratio (10 μg).

Recipient and signal sources were placed in the two-chamber cage. Signal

TABLE 3. COMBINATIONS OF NYMPHAL PHEROMONE CONSTITUENTS USED TO STUDY MATURATION RESPONSE OF IMMATURE ADULT MALE DESERT LOCUST (EXPERIMENT IV)

Recipient (5 immature male adults)
Signal sources
(1) fifth instars
(2) 10 μg (acids + aldehydes + guaiacol + phenol)
(3) 10 μg (acids + aldehydes)
(4) 20 μg (acids + aldehydes)
(5) 10 μg acids only
(6) DCM[b] + paraffin oil[a]
(7) 5 μg (acids + aldehydes)
(8) 10 μg aldehydes only
(9) immature adult males[a]

[a]Control treatments.
[b]DCM = dichloromethane.

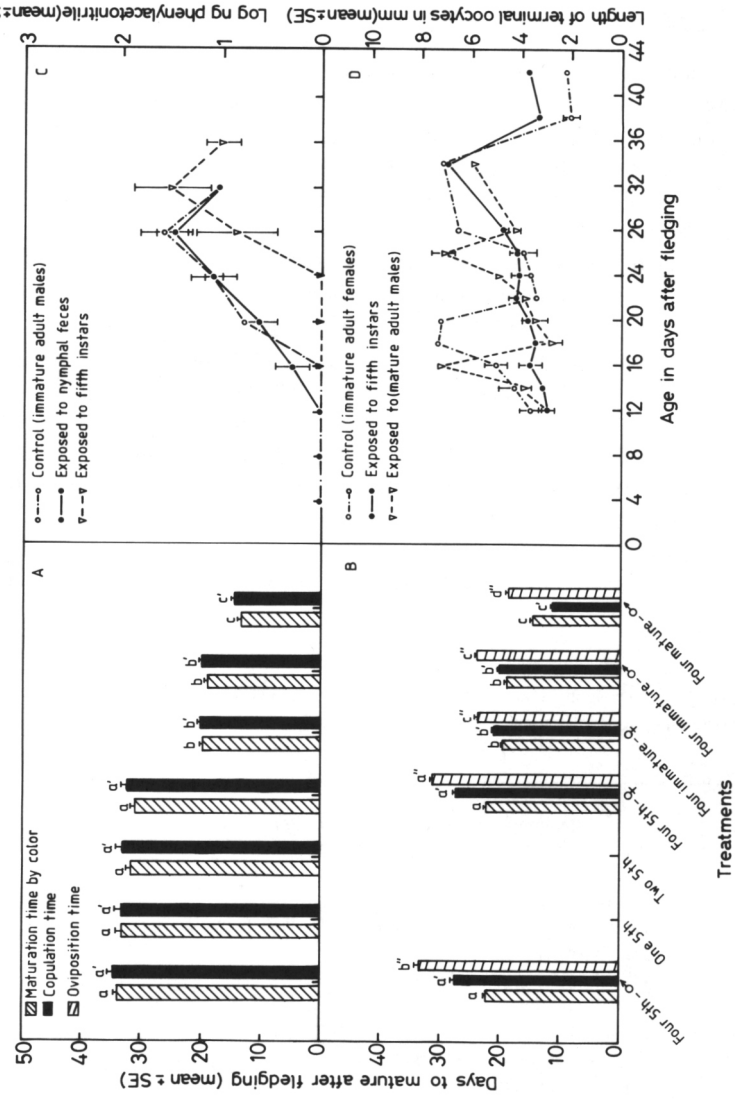


FIG. 1. Maturation time of immature adult (A) males and (B) females as assessed by color, copulation, and oviposition exposed to different treatments (Means with the same letter are not significantly different ($P < 0.05$). (C) Phenylacetonitrile produced by immature adult males exposed to fifth instars, fifth-instar feces, and control (immature adult males). (D) Oocyte length of immature adult females in the presence of fifth instars, mature males, and control (immature adult females).

sources were changed every five days. Each set was replicated four times. Maturation was monitored as described in experiment I.

Statistical Analysis

All data collected were subjected to analysis of variance (ANOVA) using SAS (SAS Institute Inc., Cary. North Carolina 27512-8000, 1987). Means were compared by the least significance difference (LSD) test.

RESULTS

Experiment I: Effect of Fifth Instars on Immature Adult Males and Females.

The effects of fifth instars (in the presence of visual, tactile and olfactory cues) on the maturation time of immature adult males and females are summarized in Figure 1A–D. A significant delay in maturation time compared to the controls (immature adult males and females) was obtained with respect to each parameter, and this was not affected by the number of nymphs placed with the immature adults as evidenced from tests with one, two, or four nymphs (Figure 1A). Both nymph sexes were equally effective in delaying maturation (Figures 1A and B). In the presence of mature adult males, there was significantly accelerated maturation compared to controls. These results confirm the maturation-retarding effect of nymphs on immature adults. There was no significant difference between sexes.

Experiment II: Effects of Volatiles and Feces from Fifth Instars on Adult Males and Females

Feces had no effect on the maturation of immature adults of both sexes (Figure 2A–C). There was no delay even though the dose range employed in the experiment included doses two- or threefold higher than that from average fecal droppings.

Experiment III: Effect of Nymph Trapped Volatiles on Immature Adult Males and Females

Significant delay in the maturation of immature males and females was obtained, demonstrating the volatile nature of the pheromone and its effectiveness in the absence of visual and tactile cues from the signal source (Figure 3A–D).

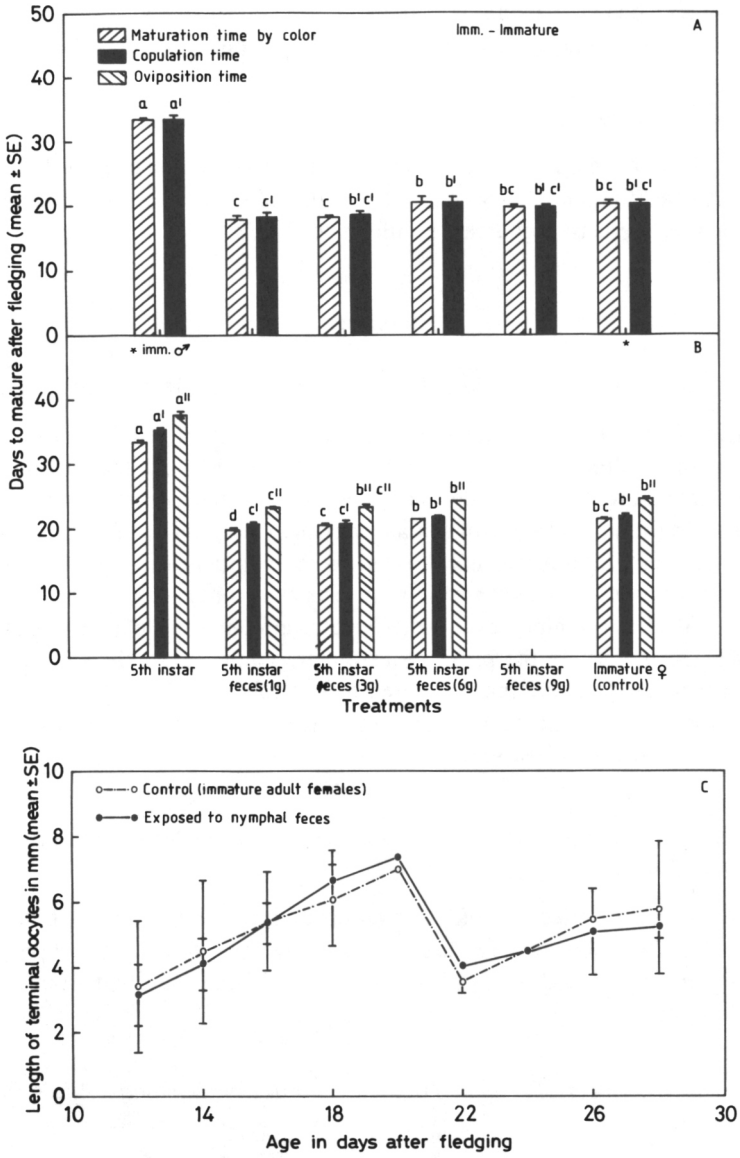


FIG. 2. Maturation time of immature adult (A) males and (B) females as assessed by color, copulation, and oviposition exposed to different treatments (Means with the same letter are not significantly different ($P < 0.05$). (C) Oocyte length of immature adult females exposed to fifth-instar feces and control (immature adult females).

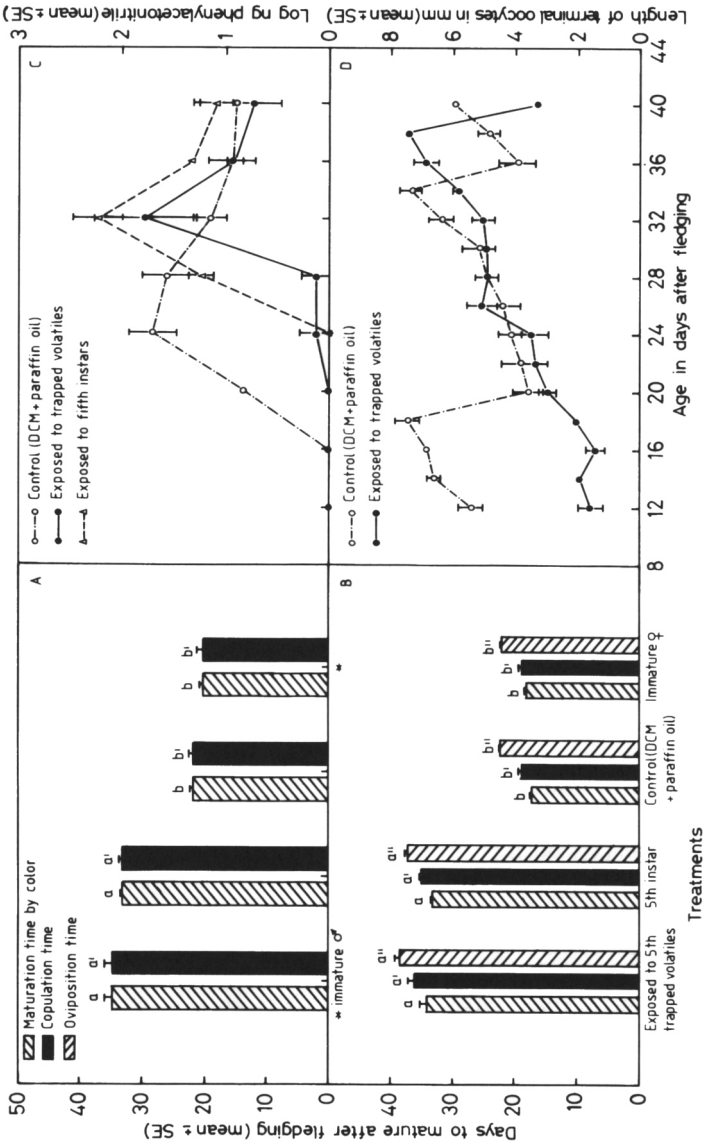


FIG. 3. Maturation time of immature adult (A) males and (B) females as assessed by color, copulation, and oviposition exposed to different treatments (Means with the same letter are not significantly different ($P < 0.05$). (C) Phenylacetonitrile produced by immature adult males exposed to trapped volatiles from fifth instars, live fifth instars, and control (paraffin oil + DCM). (D) Oocyte length of immature adult females exposed to trapped volatiles from fifth instars and control (paraffin oil + DCM).

Experiment IV: Effect of Fifth-Instar Aggregation Pheromone on Immature Adult Males

The blend of the acids and aldehydes at the same dose as that of the full blend (10 μg) was moderately active, but blends containing acids or aldehydes alone were not significantly different from controls (paraffin oil) in influencing maturation in immature adults (Figure 4A, C). The full synthetic blend of 10 compounds at 10 μg was as active as the volatiles from live nymphs in delaying maturation (Figure 4A and D). The difference in maturation time of immature males (exposed or unexposed to nymphal volatiles) between experiments III and IV (Figures 2A and 3A) is attributed to differences in batches of insects. Over a period of time, insects in an adult gregarious colony have been found to mature gradually earlier, probably as a result of accumulation of adult pheromone in the rearing room. Periodic cleaning of the rooms normally restores the duration of the maturation time.

DISCUSSION

The present study confirms the maturation-retarding effect of nymphs previously observed by Norris (1962, 1964; see also Richards and El Mangoury, 1968) and demonstrates conclusively that it is associated with volatiles emitted by fifth-instar nymphs. Both sexes are equally effective in delaying maturation of immature adults, which is consistent with our previous finding that there is no sex differentiation in the production of the nymphal pheromone (Obeng-Ofori et al., 1994b). This is different from the situation for adults, where only the males produce the pheromone (Mahamat et al., 1993; Torto et al., 1994; Obeng-Ofori et al., 1994b). In view of previous observations on the similarity in volatiles of second and fifth instars, a similar effect may be expected from other instars (Obeng-Ofori et al., 1993). The signal involved appears to be quite potent, and no significant reduction in its effect occurred even when the proportion of nymphs to young adults was reduced from 1:1 to 1:4 in our bioassays.

It has been suggested that in nature the retarding influence of hoppers tends to delay the maturation of early fledgers until the majority of nymphs have molted into adults (Norris, 1962, 1964; Richards and El Mangoury, 1968), In time, an increasing number of male adults will start to produce adult pheromone, which will accelerate maturation of other adults and result in maturation synchrony of the entire population (Norris, 1962; Norris and Pener, 1965; Mahamat et al., 1993). This could lead to simultaneous mating and facilitate communal oviposition critical for spatial and temporal cohesiveness of the progeny (Popov, 1958; Norris, 1963; Mahamat et al., 1993).

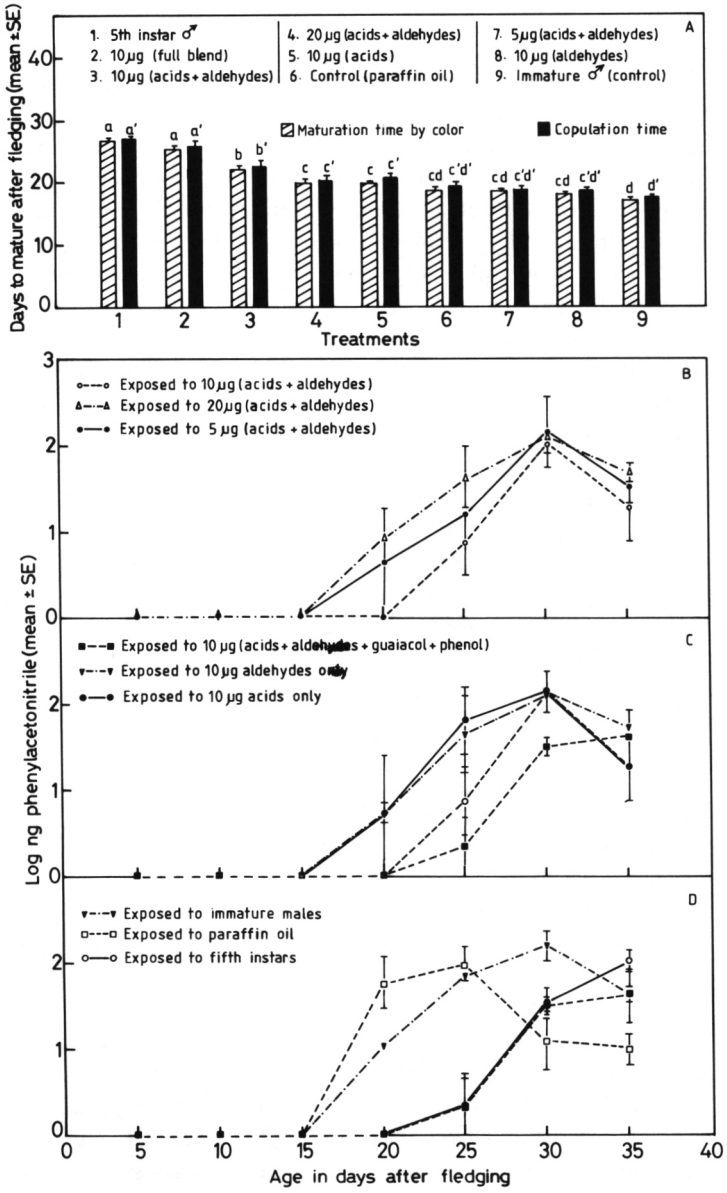


FIG. 4. Maturation time of immature adult (A) males as assessed by color and copulation exposed to a synthetic blend identified in volatiles from fifth instars (Means with the same letter are not significantly different ($P < 0.05$). Phenylacetonitrile produced by immature adult males exposed to a synthetic blend identified in volatiles from fifth instars, (B) acids + aldehydes at different doses; (C) full synthetic blend of 10 compounds compared to blend of acids + aldehydes, acids alone, or aldehydes alone; (D) full synthetic blend of 10 compounds compared to live fifth-instar volatiles, immature adult males, and paraffin oil.

The results of this study parallel those we reported earlier, which demonstrated a parsimonious role for the adult pheromone that serves as an adult aggregant (a releaser effect) and as a maturation accelerant (a primer effect) for young males and females (Mahamat et al., 1993; Torto et al., 1994). In the present study, the results clearly show that the nymphal pheromone blend composed of C_6 and C_8–C_{10} aldehydes and acids, phenol, and guaiacol, shown earlier to act as a nymphal aggregant (Torto et al., 1996), is a maturation retardant for the young adults.

Since immature and mature adult stages are behaviorally indifferent to nymphal pheromone (Obeng-Ofori et al., 1993), the manner in which this pheromone exerts its influence on young adults merits special comment. In previous studies, we showed that although young adults do not emit significant aggregation signals of their own, they nevertheless aggregate strongly in response to volatiles from nymphal feces and from their own feces (Obeng-Ofori et al., 1994a). In field observations, we found invariably large amounts of fecal droppings on areas where nymphs and fledglings co-occur. Volatiles derived from these two sources, unlike those from older and mature adults, are compositionally identical and consist of guaiacol and phenol, which account largely for the activity of the feces (Obeng-Ofori et al., 1994a). Response to the fecal volatiles explains the close association of nymphs and young adults in the field when the former are fledging (Uvarov, 1977). In the present study, we found that nymphal fecal volatiles alone have no measurable influence on the maturation of young adults, but the activity of the full synthetic pheromone blend (nymphal + fecal compounds) suggests that they augment nymphal volatiles in delaying maturation. Thus, the maturation-retarding influence would end once most nymphs have molted into adults.

The results provide further insight into the way maturation synchrony may be achieved in a gregarizing population. Associated with the delay in maturation in young males continuously exposed to nymphal volatiles is a delay in the production of the adult pheromone as measured by phenylacetonitrile titers. Since the adult pheromone is both an aggregating signal in the adult and a maturation accelerant for young males and females (Mahamat et al., 1993; Torto et al., 1994; Obeng-Ofori et al., 1994b), a delay in its production means a further delay in the maturation of early fledgers. In earlier stages of gregarization, when locust populations may not have attained a high degree of developmental synchrony, early fledgers may coexist with the final instar in different stages of development and also with earlier instars. Consequently, sexual maturation of early fledgers would be slowed down not only by their prolonged contact with nymphs but also by the delay in the production of the adult pheromone. However, as the population develops greater synchrony, contact with nymphs would be reduced and the adult pheromone could be produced earlier. The two pheromone systems would then increasingly fine-tune the synchronization process.

Delay in maturation of females is reflected in delay in color changes, mating, and oviposition. Mating of females exposed to pheromone occurred somewhat earlier than males (~27 days compared to ~34 days in males), but in the control insects there was no such difference. The significance of this is unclear. The delayed maturation effect is also reflected in delayed ovarial development. Growth of oocytes in females reared in the presence of nymphs was slower, with the first mature oocytes occurring 38 days after fledging. Development of mature oocytes in control females required only 22 days, and those exposed to mature males exhibited an even shorter developmental cycle of 16 days.

In summary, our studies confirm those by Norris (1954, 1964) and coworkers (Norris and Pener, 1965; Richards and El Mangoury, 1968) on the maturation-retarding effect of nymphs and the maturation-accelerating effect of older adults on young adults. Our results support the hypothesis that the two effects may function to synchronize the sexual maturation of young adults. We have identified the signals involved as the nymphal aggregation pheromone and adult aggregation pheromone, respectively, which, in addition to their releaser effects, act as primer inhibitory and acceleratory pheromones, respectively.

Acknowledgments—This work was partly supported by funds from IFAD, UNDP, SAREC, and AFESD coordinated by IFAD through the Consultative Group on Locust Research (CGLR) and its Scientific Advisory Committee (SAC), to whom we are most grateful. We are also grateful to the ICIPE ARPPIS program for a scholarship to Y.O.H.A. We thank the staff of the Chemical Ecology Department and Locust Breeding Unit (ICIPE) for their help in various ways.

REFERENCES

AMERASINGHE, F. P. 1978. Pheromonal effect on sexual maturation, yellowing, and the vibration reaction in immature male desert locust *Schistocerca gregaria*. *J. Insect Physiol.* 24:309–314.

HIGHNAM, K. C., and LUSIS, O. 1962. The influence of mature males of the neurosecretory control of ovarian development in the desert locust. *Qt. J. Microsc. Sci.* 103:73–83.

LOHER, W. J. 1960. The chemical acceleration of the maturation process and its hormonal control in the male of the desert locust. *Proc. R. Soc. B* 153:381–397.

MAHAMAT H., HASSANALI, A., ODONGO, H., TORTO, B., and EL BASHIR, S. 1993. Studies on the maturation-accelerating pheromone of the desert locust *Schistocerca gregaria* (Orthoptera: Acrididae). *Chemoecology* 4:159–164.

NORRIS, M. J. 1954. Sexual maturation in the desert locust *Schistocerca gregaria* (Forskål) with special reference to the effect of grouping. *Anti-Locust Bull.* 18:1–14.

NORRIS, M. J. 1962. Group effects on the activity and behavior of adult males on the desert locust, *Schistocerca gregaria* (Forskål) in relation to sexual maturation. *Anim. Behav.* 10:275–291.

NORRIS, M. J. 1963. Laboratory experiments on gregarious behavior in ovipositing females of the desert locust, *Schistocerca gregaria* (Forskål) in relation to sexual maturation. *Entomol. Exp. Appl.* 6:269–363.

NORRIS, M. J. 1964. Accelerating and inhibiting effects of crowding on sexual maturation in two species of locusts. *Nature* 203:784–785.

NORRIS, M. J., and PENER, M. P. 1965. An inhibitory effect of allatectomized males and females on the sexual maturation of young male adults of *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae). *Nature* 208:1122.

OBENG-OFORI, D., TORTO, B., and HASSANALI, A. 1993. Evidence for mediation of two releaser pheromones in the aggregation behavior of the gregarious desert locust, *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae). *J. Chem. Ecol.* 19:1665–1670.

OBENG-OFORI, D., TORTO, B., NJAGI, P. G. N., HASSANALI, A., and AMIANI, H. 1994a. Fecal volatiles as part of the aggregation pheromone complex of the desert locust, *Schistocerca gregaria* (Forskål) (Orthoptera:Acrididae). *J. Chem. Ecol.* 20:2077–2087.

OBENG-OFORI, D., NJAGI, P. G. N., TORTO, B., HASSANALI, A., and AMIANI, H. 1994b. Sex differentiation studies relating to releaser aggregation pheromone of the desert locust, *Schistocerca gregaria. Entomol. Exp. Appl.* 73:85–91.

POPOV, G. B. 1958. Ecological studies on oviposition by swarms of the desert locust *Schistocerca gregaria* (Forskål). *Entomol. Exp. Appl.* 6:279–303.

RICHARDS, M. J., and EL MANGOURY, M. A. 1968. Further experiments on the effects of social factor on the rate of sexual maturation in the desert locust. *Nature* 219:865–866.

TORTO, B., OBENG-OFORI, D., NJAGI, P. G. N., HASSANALI, A., and AMIANI, H. 1994. Aggregation pheromone system of the adult gregarious desert locust *Schistocerca gregaria* (Forskål). *J. Chem. Ecol.* 20:1749–1762.

TORTO, B., NJAGI, P. G. N., HASSANALI, A., and AMIANI, H. 1996. Aggregation pheromone system of nymphal gregarious desert locust *Schistocerca gregaria* (Forskål). *J. Chem. Ecol.* 22:2273–2281.

UVAROV, B. P. 1966. Grasshopper and Locusts, Vol. I. Cambridge University Press, Cambridge, 496 pp.

UVAROV, B. P. 1977. Grasshopper and Locusts, Vol. II. Center for Overseas Pest Research, London, 613 pp.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

ATTRACTION OF FEMALE ORIENTAL FRUIT FLY, *Bactrocera dorsalis*, TO VOLATILE SEMIOCHEMICALS FROM LEAVES AND EXTRACTS OF A NONHOST PLANT, PANAX (*Polyscias guilfoylei*) IN LABORATORY AND OLFACTOMETER ASSAYS

ERIC B. JANG,[1,*] LORI A. CARVALHO,[1] and JOHN D. STARK[2]

[1]*Tropical Fruit & Vegetable Research Laboratory*
Agricultural Research Service, US Department of Agriculture
P.O. Box 4459, Hilo, Hawaii 96720

[2]*Puyallup Research and Extension Center*
Washington State University
Puyallup, Washington 98371

(Received July 16, 1996; accepted January 7, 1997)

Abstract—Fresh whole leaves and solvent–water leaf extracts of the hedgerow plant panax, *Polyscias guilfoylei* (Bull), were tested for their attractiveness to male and female Oriental fruit flies, *Bactrocera dorsalis*, in laboratory flight tunnel and cage olfactometer bioassays. Fresh mature whole panax leaves were found to be attractive to mated female oriental fruit flies in the flight tunnel. Response of males and virgin females was low and in most instances not significantly different from controls. Attraction of mated female flies to the layers resulting from a methylene chloride–water partition or a hexane–water partition of freshly ground leaves using small McPhail traps was greatest in the methylene chloride fraction. When methylene chloride and water layers were tested competitively in a multiple-choice rotating olfactometer, the methylene chloride fraction was more attractive. Tests involving the methylene chloride–water interface (an emulsion of the two partitioned layers) with and without a standard attractant NuLure, showed the emulsion layer to be significantly more attractive than the other fractions or NuLure. In outdoor cage olfactometer assays of methylene chloride and water fractions, activity was greatest in the methylene chloride fraction. The results suggests that volatile semiochemicals from this nonhost plant are attractive to mated female Oriental fruit flies. The results are discussed in relation to the chemical ecology of *B. dorsalis* and the potential use of this nonhost plant for detection and control of female Oriental fruit flies in the field.

*To whom correspondence should be addressed.

0098-0331/97/0500-1389$12.50/0 © 1997 Plenum Publishing Corporation

Key Words—Oriental fruit fly, *Bactrocera dorsalis*, female attractant, panax, fruit fly control, semiochemicals, kairomones, Tephritidae.

INTRODUCTION

Dacine fruit flies from the family Tephritidae are a diverse group of more than 700 described species whose larvae infest many types of fleshy fruits found in tropical and subtropical regions (Metcalf, 1990). One polyphagous species, the Oriental fruit fly, *Bactrocera dorsalis* Hendel, is a serious pest of fruits and vegetables throughout the Asian Subcontinent and Pacific Rim countries including Hawaii, where the fly is established. The pest has been reported to attack over 173 different varieties of fruit and vegetables (Metcalf and Metcalf, 1992) and is considered a serious quarantine pest. Semiochemicals are involved in most current detection, control, and eradication technologies developed for tephritid fruit fly pests throughout the world (Jang and Light, 1996). They remain an important component of control programs, where the fly exists, and of detection programs, in areas where the fly does not exist but may become established.

The chemical ecology of these flies is complex, with flies responding behaviorally to both sex pheromones and various host and nonhost plant kairomones (for many monophagous as well as polyphagous species). The response of flies to semiochemicals has fascinated researchers since the observations of Howlett (1912) that oil of citronella (a nonhost) was attractive to males of two *Dacus* (*Bactrocera*) species. Currently, methyl eugenol, a potent male attractant first discovered by Howlett (1915) in oil of citronella, and various hydrolyzed protein products (e.g., NuLure), thought to be general food-type attractants for males and females of many tephritid species (Jang and Light, 1996), are used routinely for detection and/or control of Oriental fruit fly. While the potency of methyl eugenol as a male lure is well demonstrated, no comparable lure for females has yet been developed. Semiochemical attractants derived from nonhost plants have been reported for male *B. dorsalis* (primarily methyl eugenol) (Mitchell, 1965; Kawano et al., 1968; Fletcher et al., 1975; Tan, 1983). Jacobson et al. (1976) reported that asarylaldehyde, a distillate component from Indian calamus root oil, was attractive to female Oriental fruit flies. The need for a female-specific attractant for Oriental fruit fly as well as many other tephritid species has been regarded as a high priority in many fruit fly control programs for years (Jang and Light, 1996). Most studies looking for female attractants have focused primarily on host plants as a source of attractants (Chiu, 1990; Jang and Light, 1991; Light and Jang, 1996).

Stark (1995), studying the nocturnal behavior of tephritid fruit flies and their parasitoids in papaya-growing regions on the island of Kauai, reported a unique behavioral observation of Oriental fruit fly females in and around the

hedgerow plant panax, *Polyscias guifoylei* (Bull), bordering papaya fields in a commercial growing area of Moloa'a, Kauai. Female flies were observed leaving host plants (papaya) as dusk approached and landing on the undersides of panax leaves in the upper canopy, where they remained until dawn. Males followed the females, and, in some instances, were observed mating in the panax hedge. These observations were contrary to an earlier study by Stark et al. (1994) in commercial guava-growing regions on Kauai where Oriental fruit fly males and females were observed remaining to roost and mate in guava foliage throughout the night.

Based on the unique responses of females and males to the panax leaves reported by Stark (1995), we were interested in determining if volatile semiochemicals attractive to female Oriental fruit flies might exist in leaves of panax. This study reports on laboratory and outdoor olfactometer results showing the attraction of females to whole leaves and panax extracts. We discuss these results in regards to the usefulness of controlling fruit flies and the importance of studying nonhost as well as host plants in the chemical ecology of tephritid species.

METHODS AND MATERIALS

Insects. Adult flies used in this study were obtained as pupae from the mass-rearing unit at the USDA-ARS, Tropical Fruit & Vegetable Research Laboratory in Honolulu, Hawaii. Larvae were reared on a standard wheat, sugar, yeast diet (Tanaka et al., 1969). Pupae were shipped by air to Hilo where they were placed into 30-cm × 30-cm × 30-cm cubical aluminum screen cages containing sugar, water, and hydrolyzed protein. Flies were held at 24°C, 60–80% relative humidity, 12L:12D photoperiod until use. At least 24 hr prior to testing, cages of flies were placed in a 5°C walk-in refrigerator to immobilize them. Flies were separated by sex and placed into groups of either 25 or 50 males or females and placed in plastic containers (11.5 cm diameter × 7.5 cm deep) with nylon mesh covers containing sugar, water, and hydrolyzed protein. They were returned to the warmer temperature room (24°C) and held until use. Flies were approximately 9–11 days old when tested and presumed to be mated (ca. >95% of females from mixed cages are mated by day 7). For studies involving virgin females, flies were sexed within two days of emergence from pupae and held in groups of 50 females as above.

Leaves and Leaf Extracts. Mature undamaged leaves from panax plants, *Polyscias guilfoylei* (Bull) [L. H. Bailey], were taken from hedgerows bordering commercial papaya fields in the area of Moloa'a, Kauai, as described by Stark (1995). Leaves were placed in plastic bags and immediately shipped or hand-carried by air to Hilo. Bags of unused leaves were held in a refrigerator until

used. Prior to use, they were gently washed of all external debris with water and allowed to air dry at room temperature.

Extracts of panax were made by blending leaves in a methylene chloride–water (1 : 1) emulsion at a ratio of one part panax to two parts of the methylene chloride–water mixture (w/w) in an explosion-proof laboratory blender (Waring). A similar extraction was carried out using a hexane–water (1 : 1) partition. The resulting homogenates were strained through several layers of cheesecloth to remove particulate matter. The strained liquid was placed into several large glass separatory funnels (500 ml) and allowed to partition overnight (12–18 hr) under refrigeration. The following morning, the solvent layer (methylene chloride or hexane), a solvent–water interface layer (methylene chloride extract only), and the water layer were decanted off and placed in glass bottles. The five layers from the two extractions [CH_2Cl_2 layer, water layer from CH_2Cl_2–water partition, CH_2Cl_2–water emulsion (interface), hexane layer, and water layer from hexane–water partition] were kept refrigerated until used.

Laboratory and Outdoor Cage Bioassays. Tests of attractancy of panax leaves and extracts to flies were carried out in three different behavioral arenas. The first, a glass/metal laboratory flight tunnel consisted of a 0.9-m × 0.9-m × 2.8-m rectangular glass arena containing inlet and exit fans at the ends of the chamber, which produced a laminar flow of air (Jang and Light, 1991; Jang et al., 1994). Whole leaves (ca. 400 g) were placed in all-glass containers containing inlet and outlet ports (Jang and Light, 1991). The inlet port was connected to a tank of clean, breathing-quality compressed air, and the outlet port connected via Teflon tubing to the flight tunnel. Headspace odors from the leaves were swept from the container via the tubing into yellow plastic spheres hung inside the flight tunnel. Odors exited from small holes in the yellow plastic spheres. Exit flow rate into the spheres was 100 ml/min. Spheres were coated with Tanglefoot to trap alighting flies. A second sphere emitting only clean air served as a control.

Flight-tunnel tests of the various fractions were carried out using small invaginated glass McPhail traps in to which solvent or water fractions (or solvent or water controls) were placed. Approximately 5 ml of the fraction to be tested was added to 10 ml of water with two drops of Tween 20 added as a surfactant. Each test of whole leaves or extracts consisted of two-choice preference assays (two yellow balls or two McPhail traps) comparing an odor/fraction vs. a control or head-to-head comparisons of fractions. For each assay, 50 males and 50 females were released from the downwind end of the flight tunnel and allowed to respond freely for 2 hr. At the end of each test, trapped flies were counted, remaining flies removed, and the flight tunnel cleaned. All assays were run between 13:00 and 16:00 hr at a temperature of 26–28°C, under fluorescent lights (2000 lux) (Jang et al., 1994).

A laboratory multiple-trap rotating cage olfactometer described by Jang and Nishijima (1990) was used to compare different extracts in competitive tests. The olfactometer consisted of a 75-cm × 75-cm × 80-cm rectangular screened enclosure containing a 6-arm rotating hub. Glass McPhail traps or triangular Jackson traps containing individual fractions were hung on opposite arms of the rotating hub. Solvent or water layers of extract from ground panax leaves, solvent, or water controls, and, in some tests, NuLure (9% NuLure + 5% borax) (Miller Chemical Co., Hanover, Pennsylvania) were placed either in the water of the McPhail traps containing surfactant or directly onto a cotton wick suspended inside the Jackson traps. The hub was electrically rotated during a 2-hr assay period at a speed of 120 revolutions/hr. Fifty flies of each sex were released inside the cage and allowed to choose freely among the different extracts. Tests were run between 13:00 and 16:00 hr at 26–28°C under fluorescent lights (ca. 2000 lux). At the end of the test period, entrapped flies were removed and counted.

A larger-scale outdoor covered cage olfactometer employing the same principles as the indoor unit was used to confirm the laboratory findings. The outdoor olfactometer consisted of a 3-m × 3-m × 2.5-m rectangular wood-framed screen cage (screen on all four sides and the top) with a corrugated metal A-frame roof and wood floor. A rotating hub with arms approximately 62 cm long was hung from the center of the cage approximately 1.8 m from the floor. The outer arms of the motorized hub unit rotated at a rate of 90 rotations/hr. Extracts were placed in small glass McPhail traps as previously described. Approximately 900 flies of each sex were placed in the outdoor cage. Tests were conducted between 13:00 and 16:00 hr under ambient outside conditions (usually 23–28°C) and only natural (indirect) light was used. At the end of the test, flies were removed and counted.

Data Analysis. Results of the two-choice flight tunnel tests were compared using Student's *t*-test on SAS version 6.04 (Proc TTEST) (SAS Institute, 1990). Multiple-choice test results were analyzed using ANOVA, and means compared using the Tukey's studentized range (HSD) test (Proc GLM, SAS version 6.04) (SAS Institute, 1990). All analyses of significance were made at the $P < 0.05$ level of significance or lower.

RESULTS

Flight-Tunnel Assays. Female Oriental fruit flies were attracted to the odor of freshly picked panax leaves in laboratory flight-tunnel assays. Initial tests using 50 of each sex resulted in ca. 24% of released flies eventually being trapped (24 of 100) (Table 1). In these tests, 75% (18 of 24) of the total flies

TABLE 1. ATTRACTION OF ORIENTAL FRUIT FLY TO YELLOW SPHERES EMITTING HEADSPACE VOLATILES OF WHOLE PANAX LEAVES IN LABORATORY FLIGHT TUNNEL[a]

Treatment (50 males + 50 females)	N	Female capture (mean ± SEM)	Male capture (mean ± SEM)	Total capture (mean ± SEM)
Whole panax leaves vs. air	3	15 ± 2.6 a	4.3 ± 0.9 a	19.3 ± 3.2 a
	3	3.3 ± 0.3 b	1.7 ± 1.7 a	5 ± 2 b
Total		18.3	6.0	24.3

[a]Means followed by different letters within a column are significantly different at $P < 0.05$; t test (SAS, 1990).

captured were females, and 79% (15 of 19) of the flies attracted to panax odors were females.

When the water layer of the methylene chloride–water partition was compared with a water only control, the water layer captured significantly more females ($P < 0.01$) than water only (Table 2). Ninety-three percent of the mean number of flies (22 of 23.7) captured in McPhail traps containing the water layer were females. Tests of the methylene chloride-soluble layer against methylene chloride controls (both having a layer of water/surfactant above the fraction in the McPhail trap, see Methods and Materials) in the flight tunnel also showed a significantly greater ($P < 0.01$) capture with the methylene chloride layer than with the methylene chloride control (Table 2). In these tests, greater than 99% of the mean of total flies (22.4 of 22.5) captured in the methylene chloride layer were females. Ninety-nine percent of the mean of total flies captured in the test (24.5 of 24.7) were females. When the methylene chloride soluble layer was compared directly with the water layer, significantly more flies (80% or mean 29 of a mean total of 36.1) were attracted to the trap containing the methylene chloride layer. Ninety-three percent of the mean number of flies (27 of 29) captured in the methylene chloride layer were females (Table 2).

Virgin females with fully developed ovaries were tested to determine if the attraction of females was specific for mated females. Response of virgin females to the methylene chloride fraction (mean of 4.5 flies) was not significantly different from the corresponding methylene chloride control (mean of 1.67 flies) when tested in McPhail traps containing water and surfactant in the flight tunnel. Mean response was significantly lower ($P < 0.05$) compared to mated females of the same age.

Assays of the two layers from a hexane–water partition of ground leaves

TABLE 2. ATTRACTION OF ORIENTAL FRUIT FLIES TO FRACTIONS FROM CH_2Cl_2–WATER PARTITION OF PANAX LEAVES IN LABORATORY FLIGHT TUNNEL[a]

Treatment	N	Female capture (mean ± SEM)	Male capture (mean ± SEM)	Total capture (mean ± SEM)
Water layer of CH_2Cl_2:H_2O partition	5	22 ± 2.1 a	1.7 ± 0.8 a	23.7 ± 2.8
vs.				
Water control	5	0.1 ± 0.1b	0 ± 0 b	0.1 ± 0.1 b
Total		22.1	1.7	23.8
Methylene chloride layer of CH_2Cl_2:H_2O partition	9	22.4 ± 1.1 a	0.1 ± 0.1 a	22.5 ± 1.1 a
vs.				
Methylene chloride control	9	2.1 ± 0.4 b	0.1 ± 0.1 a	2.2 ± 0.4 b
Total		24.5	0.2	24.7
Methylene chloride layer of CH_2Cl_2:H_2O partition	7	27 ± 2.2 a	2 ± 1.8 a	29 ± 3.7 a
vs.				
Water layer of CH_2Cl_2:H_2O partition	7	4.8 ± 1.2 b	2.3 ± 1.5 a	7.1 ± 1.9 b
Total		31.8	4.3	36.1

[a]Layers (and their controls) were tested in McPhail-type traps; 50 males and 50 females released for each replication. Means followed by different letters within a column for each test group are significantly different at $P < 0.05$; t test (SAS, 1990).

showed no significant differences in attraction of females to either fraction (Table 3). This was in contrast to assays of the two layers from the methylene chloride–water partition. When the methylene chloride layer was tested against the hexane layer, flies (primarily females) were significantly more attracted to the methylene chloride layer (Table 3). Neither the hexane nor the methylene chloride solvent alone was attractive to Oriental fruit flies.

Olfactometer Assays. Competitive tests of both methylene chloride and water layers and their corresponding controls were conducted in an indoor rotating cage olfactometer. We also tested the methylene chloride–water interface-layer, an amorphous emulsion layer containing a mixture of the two separate layers as well as a small amount of particulate matter (Table 4, A). Interestingly, the interface layer caught significantly more ($P < 0.05$) flies than either the methylene chloride layer or the water layer. Both layers, however, caught a greater number of flies than controls. Ninety-six percent of the mean number

TABLE 3. RESPONSE OF ORIENTAL FRUIT FLIES TO FRACTIONS FROM $CH_2Cl_2 : H_2O$ PARTITION OR Hexane : H_2O PARTITION OF PANAX LEAVES IN LABORATORY FLIGHT TUNNEL[a]

Treatment	N	Female capture (mean ± SEM)	Male capture (mean ± SEM)	Total capture (mean ± SEM)
Hexane layer of $C_6 : H_2O$ partition	4	8.8 ± 2.5 a	1.7 ± 1.7 a	10.5 ± 4.1 a
vs.				
Water layer of $C_6 : H_2O$ partition	4	9.5 ± 2.1a	5.2 ± 4.9 a	14.7 ± 6.9 a
Total		18.3	6.9	25.2
Methylene chloride layer of $CH_2Cl_2 : H_2O$ partition	5	18.8 ± 2.6 a	0.2 ± 0.2 a	19 ± 2.8 a
vs.				
Hexane layer of $C_6 : H_2O$ partition	5	5.8 ± 1.7 b	0.2 ± 0.2 a	6 ± 1.7 b
Total		24.6	0.4	25

[a]Layers were tested in McPhail-type traps; 50 males and 50 females released for each replication. Means followed by different letters within a column for each test group are significantly different at $P < 0.05$; t test (SAS, 1990).

of flies captured (17 of 17.7) in the methylene chloride layer were females. Eighty-eight percent of the mean total flies captured (28.1 of 31.6) in all traps were females. In additional tests, which included the protein hydrolysate NuLure, the interface layer was significantly more attractive to females than any of the other fractions tested (Table 4, B). Both NuLure and the methylene chloride layer were not significantly different from water only (control). The interface layer caught 100% females. Males were significantly more attracted to NuLure than to any of the other treatments. Total fly capture was over 46% of the total flies released.

Flies tested in the outdoor covered olfactometer with methylene chloride and water-soluble fractions confirmed laboratory studies (Table 5). Significantly more flies were caught in the traps containing the methylene chloride layer than in the water layer. All flies captured in the methylene chloride layer were females.

DISCUSSION

The results of the study, confirmed by both laboratory and outdoor assays, suggest that volatile compounds from leaves of the hedgerow plant panax are

TABLE 4. COMPARATIVE ATTRACTION OF ORIENTAL FRUIT FLY TO LAYERS OF METHYLENE CHLORIDE–WATER PARTITION OF PANAX LEAVES IN INDOOR ROTATING OLFACTOMETER[a]

Treatment	N	Female capture (mean ± SEM)	Male capture (mean ± SEM)	Total capture (mean ± SEM)
Part A				
Water layer of $CH_2Cl_2:H_2O$ partition	8	2.7 ± 0.9 bc	1.8 ± 0.7 a	4.5 ± 1.1 b
Methylene chloride control	8	1.6 ± 0.9 c	0.2 ± 0.2 a	1.8 ± 0.9 c
Methylene chloride layer of $CH_2Cl_2:H_2O$ partition	8	6.6 ± 1.5 b	0.5 ± 0.3 a	7.1 ± 1.5 b
Water control	8	0.2 ± 0.2 c	0.3 ± 0.2 a	0.5 ± 0.3 c
Middle interface layer of $CH_2Cl_2:H_2O$ partition	8	17 ± 1.5 a	0.7 ± 0.4 a	17.7 ± 1.3 a
Total		28.1	3.3	31.6
Part B				
Middle interface layer of $CH_2Cl_2:H_2O$ partition	3	27.7 ± 3.1 a	0 ± 0 b	27.7 ± 3.1 a
NuLure	3	4.2 ± 0.7 b	6.3 ± 0.9 a	10.5 ± 1.5 b
Water control	3	2.0 ± 1.1 b	2.7 ± 1.3 b	4.7 ± 2.5 b
Methylene chloride control	3	3.7 ± 2.2 b	0 ± 0 b	3.7 ± 2.2 b
Total		37.6	9.0	46.6

[a] Layers were tested in McPhail-type traps; 50 males and 50 females per replication. Means followed by different letters within a column for each test group are significantly different at $P < 0.05$; Tukey's studentized range test (SAS, 1990).

attractive to mated female Oriental fruit flies. Tests of the layers of a methylene chloride–water partition confirmed that the active principle most likely resides in the methylene chloride fraction. We believe that the initial partition assays in the flight tunnel, which showed some attraction of females to the water-soluble portion of the extract (Table 2), can be explained by the intermediate polarity of methylene chloride (e.g., the water layer likely contains a small amount of methylene chloride). Head-to-head tests clearly show the methylene

TABLE 5. COMPARATIVE ATTRACTANCY OF ORIENTAL FRUIT FLY TO METHYLENE CHLORIDE–WATER PARTITION OF PANAX LEAVES IN OUTDOOR ROTATING OLFACTOMETER[a]

Treatment	N	Female capture (mean ± SEM)	Male capture (mean ± SEM)	Total capture (mean ± SEM)
Methylene chloride layer of $CH_2Cl_2:H_2O$ partition	5	67.2 ± 1.9 a	0 ± 0 b	67.2 ± 19.1 a
Methylene chloride control	5	0.6 ± 0.4 b	0.2 ± 0.2 ab	0.8 ± 0.4 b
Water control	5	5.2 ± 2.7 b	1.0 ± 0.8 ab	6.2 ± 3.3 b
Water layer of $CH_2Cl_2:H_2O$ partition	5	25.6 ± 9.4 ab	2.6 ± 0.9 a	28.2 ± 9.2 ab
Total		98.6	3.8	102.4

[a]Layers were tested in McPhail-type traps. Means followed by different letters within a column are significantly different at $P < 0.05$; Tukey's studentized range test (SAS, 1990).

chloride layer to be more attractive than the water layer. We believe that the strong attraction of the methylene chloride–water interface layer to flies can be explained in a similar way. The extract was partitioned for 24 hr or less before the extracts were separated. Allowing the emulsion to separate for several days largely reduces the volume (and activity) of the interface layer. An alternative explanation is that attractive components present in both the water layer and the methylene chloride layer are present in the emulsion.

We were perplexed as to how the methylene chloride-soluble fraction could be so attractive to females in the glass McPhail traps where the methylene chloride layer actually lay below the water/surfactant added to each trap to capture the flies. We believe that the water/surfactant emulsion actually helps in the controlled release of the active constituents from the methylene chloride solvent. Nigg et al. (1994) reported a similar phenomena in studying components from host plants attractive to Caribbean fruit fly, *Anastrepha suspensa*. They hypothesized that attractive components from leaf surfaces wet with dew may actually improve the attraction of Caribbean fruit fly to leaf volatiles over dry leaf surfaces. Response of flies to the methylene chloride layer without water in McPhail traps was low. We ascribe this to the repellent effect of many solvents in tephritid fruit fly olfactory assays. Results of assays in which solvent extract was placed directly on cotton wicks indicated that females were more attracted to the solvent–water–surfactant in the McPhail traps.

Although the precise role of panax in the chemical ecology of the Oriental fruit fly is not known, the study by Stark et al. (1994) and Stark (1995) and the results of this one demonstrate the need to study carefully the chemical ecology of polyphagus tephritid species, especially in the search for new and improved attractants. Metcalf (1990) cites the paper by Howlett (1912), who reported that oil of citronella was attractive to males of two species of *Dacus*, as a landmark observation in the chemical ecology of insects and, in particular, the dacine fruit flies. Since that time, the active ingredient from oil of citronella, methyl eugenol (Howlett, 1915), has been found in other nonhost plants as well (Mitchell, 1965; Kawano et al., 1968; Fletcher et al., 1975; Tan, 1983). Thus, the discovery of semiochemicals from nonhost plants attractive to females although surprising, should not be unexpected. Many plant volatiles are more or less ubiquitous in various host as well as nonhost plants and provide insects with a plethora of semiochemicals in their environment. Plant odors such as common "green leaf" volatiles (GLVs) present in leaves have been shown to modulate or enhance tephritid behavior, while other semiochemicals, such as the odor of ripening fruit, serve as primary kairomones (Light and Jang, 1996).

This paper is one of the few reports of a female-specific attractant derived from a nonhost plant for this species. Jacobson et al. (1976) reported that asarylaldehyde, a component of Indian calamus rootoil, was attractive to male and female oriental fruit flies in outdoor cage bioassays. The components in panax, however, are responsible for a female-specific attraction. The apparent preference of mated females for panax over other vegetation in the area is particularly interesting. Response of virgin females to leaf extracts was low.

Of particular interest for this species is the apparent plasticity in behavioral repertoires exhibited by flies inhabiting different ecological niches in or around host material. The Oriental fruit fly does not appear to migrate from commercial guava orchards (Stark et al., 1994), roosting (and mating) instead among the dense foliage of the guava trees throughout the night. In contrast, the vegetation in commercial papaya is less dense, and Stark (1995) initially hypothesized that movement from the commercial papaya to the panax was in response to the lack of roosting sites in the papaya. Stark (1995) reported observing females leaving the papaya fields and finding females in panax, apparently preferring the panax to other hedgerow plants in the area such as wiliwili, *Erythrina tahitensis*. Males followed females and were sometimes observed mating in the panax.

The attraction of mated females over virgin females to panax is a mystery. Stark (1995) did not identify the mating status of observed flies, but observed mating in the panax hedge in some instances. Host as well as nonhost kairomones from leaves, bark, and stems may act as "co-active attractants" (Light and Jang, 1996), which may increase the attractancy of a primary semiochemical (e.g., pheromone) and/or serve as "alightment-landing stimuli" for rendezvous sites and roosting. Thus, volatile semiochemicals from panax may elicit some

yet unidentified behavior specific to mated females. The precise role is at present unclear.

The potential of a female-specific attractant for the Oriental fruit fly is an exciting discovery and suggests the potential for the development of female lures. Extracts from panax may also selectively be attractive to other fruit fly species, and these are currently being tested. Further behavioral observations on the chemical ecology of polyphagus tephritid species and their parasitoids will likely bring new insight to the role of semiochemicals in their behavior and possibly lead to discoveries useful in detection, control, and eradication of these pests.

REFERENCES

CHIU, H. T. 1990. Bioassay of ovipositional attraction for *Dacus dorsalis* Hendel. *Chin. J. Entomol.* 10:343–352.

FLETCHER, B. S., BATEMAN, M. A., HART, N. K., and LAMBERTON, J. A. 1975. Identification of a fruit fly attractant in the Australian plant *Zieria smithii* as *O*-methyl eugenol. *J. Econ. Entomol.* 68:815–816.

HOWLETT, F. M. 1912. The effects of oil of citronella on two species of *Dacus*. *Trans. R. Entomol. Soc. London* 60:412–418.

HOWLETT, F. M. 1915. Chemical reactions of fruit flies. *Bull. Entomol. Res.* 6:297–305.

JACOBSON, M., KEISER, I., MIYASHITA, D. H., and HARRIS, E. J. 1976. Indian calamus root oil: Attractiveness of the constituents to Oriental fruit flies, melon flies and Mediterranean fruit flies. *Lloydia* 39:412–415.

JANG, E. B., and LIGHT, D. M. 1991. Behavioral responses of female Oriental fruit flies to the odor of papayas at three ripeness stages in a laboratory flight tunnel (Diptera: Tephritidae). *J. Insect Behav.* 4:751–762.

JANG, E. B., and LIGHT, D. M. 1996. Olfactory semiochemicals of tephritids, pp. 73–90, *in* B. A. McPheron and G. J. Steck (eds.). Fruit Fly Pests: A World Assessment of Their Biology and Management. St. Lucie Press, Delray Beach, Florida.

JANG, E. B. and NISHIJIMA, K. A. 1990. Identification and attractancy of bacteria associated with *Dacus dorsalis* (Diptera: Tephritidae). *Environ. Entomol.* 19:1726–1731.

JANG, E. B., LIGHT, D. M., BINDER, R. G., FLATH, R. A., and CARVALHO, L. A. 1994. Attraction of female Mediterranean fruit flies to the five major components of the male-produced pheromone in a laboratory flight tunnel. *J. Chem. Ecol.* 20:9–20.

KAWANO, Y., MITCHELL, W. C., and MATSUMOTO, H. 1968. Identification of the male Oriental fruit fly attractant in the golden shower blossom. *J. Econ. Entomol.* 61:986–988.

LIGHT, D. M., and JANG, E. B. 1996. Plant volatiles evoke and modulate tephritid behavior, pp. 123–133, *in* B. A. McPheron and G. J. Steck (eds.). Fruit Fly Pests: A World Assessment of Their Biology and Management. St. Lucie Press, Delray Beach, Florida.

METCALF, R. L. 1990. Chemical ecology of dacine fruit flies (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.* 83:1017–1030.

METCALF, R. L., and METCALF, E. R. 1992. Fruit flies of the family Tephritidae, pp. 109–152, *in* R. L. Metcalf, and E. R. Metcalf, (eds.). Plant Kairomones in Insect Ecology and Control. Chapman Hall, New York.

MITCHELL, W. C. 1965. Notes and exhibitions. *Proc. Hawaii. Entomol. Soc.* 19:25.

NIGG, H. N., MALLORY, L. L., SIMPSON, S. E., CALLAHAN, S. B., TOTH, J. P., FRASER, S., KLIM, M., NAGY, S., NATION, J. L., and ATAWAY, J. A. 1994. Caribbean fruit fly, *Anastrepha suspensa* (Loew), attraction to host fruit and host kairomones. *J. Chem. Ecol.* 20:727–743.

STARK, J. 1995. Nocturnal behavior of oriental fruit flies and melon flies (Diptera: Tephritidae) and associated parasitoids in a commercial papaya growing region on Kauai, Hawaii. *Proc. Hawaii. Entomol. Soc.* 32:149–151.

STARK, J. D., VARGAS, R. I., and WALSH, W. A. 1994. Temporal synchrony and patterns in an exotic-host-parasitoid community. *Oecologia* 100:196–199.

SAS Institute. 1990. SAS/STAT User's Guide. Release 6.04. SAS Institute, Cary, North Carolina.

TAN, K. H. 1983. Responses of *Dacus* (Diptera: Tephritidae) to *Ocimum sanctum* (Linn.) extracts and different synthetic attractants in Penang, Malaysia, pp. 513–521, *in* R. Cavalloro (eds.). Fruit Flies of Economic Importance. Balkema, Rotterdam.

TANAKA, N., OKUMOTO, R., and CHAMBERS, D. L. 1969. Low cost larval rearing medium for mass production of oriental and Mediterranean fruit flies. *J. Econ. Entomol.* 62:967–968.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

RESOLUTION OF PHEROMONAL EPOXYDIENES BY CHIRAL HPLC, STEREOCHEMISTRY OF SEPARATED ENANTIOMERS, AND THEIR FIELD EVALUATION

XU-RONG QIN, TETSU ANDO,* MASANOBU YAMAMOTO, MANABU YAMASHITA, KAZUTAKA KUSANO, and HIROSHI ABE

Graduate School of Bio-Applications and Systems Engineering
Tokyo University of Agriculture and Technology
Koganei, Tokyo 184, Japan

(Received August 23, 1996; accepted January 7, 1997)

Abstract—Resolution of insect pheromonal *cis*-epoxydiene racemates derived from (*Z*,*Z*,*Z*)-3,6,9-trienes with a C_{18}–C_{23} chain was examined utilizing chiral HPLC columns, and the result showed that a Chiralpak AS column was suitable to separate enantiomers of the 3,4-epoxides, and a Chiralpak AD column was indispensable for the resolution of the racemic 6,7- and 9,10-epoxides. The absolute configuration of the enantiomers of the 3,4- and 9,10-epoxides separated by HPLC was studied after methanolysis of their epoxy rings. Examination of the ^{1}H NMR data from esters of the methoxyalcohols produced by a modified Mosher's method with (*S*)- and (*R*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid indicated that the dextrorotatory parent epoxides with a shorter R_t were 3*S*,4*R* and 9*S*,10*R* isomers and the levorotatory enantiomers having a longer R_t possessed 3*R*,4*S* and 9*R*,10*S* configuration. Field tests with both enantiomers of (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene separated by HPLC with the chiral column revealed new specific attraction of geometrid forest defoliators, *Pachyerannis obliquaria*, to the 3*R*,4*S* isomer and *Zethenia albonotaria nesiotis* to the 3*S*,4*R* isomer.

Key Words—Chiral HPLC, sex pheromone, Lepidoptera, Geometridae, epoxydiene, stereochemistry, Mosher's method.

INTRODUCTION

There have been a number of reports of (*Z*,*Z*,*Z*)-3,6,9-trienes and the related *cis*-3,4-, 6,7-, and 9,10-epoxydienes as lepidopteran sex pheromone components and attractants (Arn et al., 1992; Ando et al., 1995, and references therein).

*To whom correspondence should be addressed.

0098-0331/97/0500-1403$12.50/0 © 1997 Plenum Publishing Corporation

The *cis*-epoxides include chiral centers, and many efforts have been made to synthesize their enantiomers for deeper understanding of the relationships between the stereochemistry and biological activity. Each chiral epoxydiene has been successfully synthesized stereoselectively by applying Sharpless epoxidation of an allylic alcohol (Cossé et al., 1992; Millar et al., 1990; Millar and Underhill, 1986; Mori and Brevet, 1991; Mori and Ebata, 1986; Szöcs et al., 1993; Wong et al., 1985). However, cooling under −20°C for a long time (more than two days) is necessary for the asymmetric epoxidation, and the optical purity is not perfect even under the optimized condition. Preparation of the optically pure sample requires recrystallization of the 3,5-dinitrobenzoate of the epoxyalcohol produced by asymmetric epoxidation. Furthermore, synthesis of the allylic alcohol intermediate for 3,4-epoxydienes is not simple, and many steps are necessary to accomplish the syntheses of 6,7- and 9,10-epoxydienes after asymmetric epoxidation. Recently, Mori used pig pancreatic lipase-catalyzed asymmetric hydrolysis of a *meso*-diacetoxyepoxybutane to produce optically active monoacetoxyepoxybutanol, which was utilized as a common starting material for syntheses of chiral epoxy pheromones, including disparlure and others (Brevet and Mori, 1992; Mori 1994; Mori and Argade, 1994). This synthetic method may be applied to the preparation of chiral epoxydiene pheromones. The enzymatic desymmetrization produces the epoxy intermediate with rather high enantiomeric purity, but its conversion into the epoxydiene is still laborious. This led us to search for an easier procedure for the preparation of optically active epoxydienes. This paper deals with the direct resolution of the racemic epoxydienes on a chiral HPLC column and the determination of the absolute configuration of separated enantiomers after methanolysis of the epoxy ring. The methanolysis produced two major separable methoxyalcohols. Their chemical structures were confirmed by a two-dimensional (2D) NMR technique, and they were utilized for studying the absolute configuration of the parent epoxides by Mosher's method (Dale and Mosher, 1973). Furthermore, we tried to set up an analytical method using the methoxyalcohols to measure the enantiomeric purity of epoxydienes by a common achiral capillary GC column. We also report here the result of a preliminary field test to attract male moths of the family Geometridae using optically pure isomers of the epoxydienes, which were separated by chiral HPLC.

METHODS AND MATERIALS

Chromatography. The HPLC system was a Jasco PU-980 liquid chromatograph equipped with an integrator (System Instrument Chromatocorder 21J). Enantiomers of epoxydienes were separated on Chiralpak AD or AS columns [0.46 cm ID × 50 cm (two 25-cm columns connected in series), Daicel Chem-

ical Industry Co., Ltd., Tokyo, Japan] with 0.1% 2-propanol in *n*-hexane as a solvent at a flow rate of 0.45 ml/min. 3,4-Epoxydienes and the 9,10-epoxydienes were detected with a UV spectrometric detector (JASCO UV-970) operated at 215 nm, and 6,7-epoxydienes were monitored by an RI detector (Labo System RI-98Scope). A medium-pressure liquid chromatographic system (MPLC) was constructed with a solvent pump (Yanaco 14000W liquid chromatograph), a Lobar column (Merck Lichroprep Si 60, 10 mm ID × 240 mm, 40–60 μm), an RI detector (Shimadzu RID-2A), and a UV detector (Shodex M-315). As a solvent, 10% tetrahydrofuran (THF) in *n*-hexane was used at a flow rate of 2.5 ml/min. GC analysis was conducted with an HP GC System 6890 series and a DB-23 capillary column (0.25 mm ID × 30 m, J & W Scientific) and a Chiraldex A-PH column (0.25 mm ID × 20 m, Astec, Inc.). The temperature program for the former column was 40°C for 1 min, 10°C/min to 160°C, and finally 4°C/min to 220°C. For the latter one, the column temperature was constant at 150°C.

Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded on a Jeol EX270 Fourier transform spectrometer at 270.2 and 67.9 MHz, respectively, with $CDCl_3$ solutions containing TMS as internal standard. The 2D spectra of ^{1}H–^{1}H and ^{1}H–^{13}C correlation spectroscopy (COSY) were measured at 500.2 and 125.7 MHz, respectively, on a Jeol Alpha500 spectrometer with ordinal pulse sequences. The specific rotations were measured on a Jasco DIP-4 polarimeter, and the direction of rotation of the effluents from a chiral HPLC column was also directly detected by an HPLC chiral detector (Jasco OR-990).

Chemicals. Epoxydienes with a C_{18}–C_{23} chain were prepared by the unselective MCPBA oxidation of the corresponding trienes, which were synthesized from linolenic acid, following separation with MPLC. The details of the synthesis and purification have been reported (Ando et al., 1993, 1995). Both stereoisomers of α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA, 2-methoxy-2-phenyl-3,3,3-trifluoropropionic acid) and (*S*)-2-acetoxypropionic acid (AP) were purchased from Aldrich Chemical Co., Inc. and Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), respectively. By refluxing with $SOCl_2$ for 50 hr, these reagents were separately converted into the corresponding acid chlorides, which were distilled and kept in glass ampoules until the reaction with methoxyalcohols.

Methanolysis of Epoxydienes 1 and 4. A racemic mixture of (*Z*,*Z*)-6,9-*cis*-3,4-epoxyoctadecadiene (**1**, 53 mg, 0.2 mmol), which was dissolved in MeOH (5 ml), was slowly mixed with additional MeOH (2 ml) containing H_2SO_4 (5 mg) in an ice bath. After 1 hr stirring at 0°C, the reaction mixture was poured into water (20 ml), and the crude products were extracted with ethyl acetate. The extract was washed with aq. $NaHCO_3$ solution, and preparative TLC gave a mixture of 3-methoxy-4-ol (**2**) and 4-methoxy-3-ol (**3**). These two positional isomers were separated by MPLC in a recyclic mode; **2** (R_t 64.9 min, 26 mg,

46% yield) and **3** (R_t 68.1 min, 17 mg, 30% yield) and their chemical structures were identified by NMR analyses. Each enantiomer of 3,4-epoxydiene, which was separated from the racemic mixture utilizing chiral HPLC with a Chiralpak AS column, was converted into methoxyalcohols by the same procedure. From fraction I (**1-I**, R_t 22.00 min, 5.0 mg), chiral 3-methoxy-4-ol (**2-I**, 2.3 mg) and 4-methoxy-3-ol (**3-I**, 1.5 mg) were obtained (see Figure 2 below), and their enantiomers, 3-methoxy-4-ol (**2-II**, 2.2 mg) and 4-methoxy-3-ol (**3-II**, 1.5 mg), were prepared from fraction II (**1-II**, R_t 22.95 min, 5 mg).

Starting from a racemic mixture of (*Z*,*Z*)-3,6-*cis*-9,10-epoxyoctadecadiene (**4**, 53 mg, 0.2 mmol), 9-methoxy-10-ol (**5**, R_t 55.9 min, 19 mg, 34% yield) and 10-methoxy-9-ol (**6**, R_t 60.7 min, 24 mg, 43% yield) were obtained and their structures were confirmed by NMR analyses. From fraction I (**4-I**, R_t 31.92 min, 5.0 mg) of 9,10-epoxydiene separated with an HPLC Chiralpak AD column, optically active 9-methoxy-10-ol (**5-I**, 1.7 mg) and 10-methoxy-9-ol (**6-I**, 2.1 mg) were obtained (see Figure 2 below), and their enantiomers, 9-methoxy-10-ol (**5-II**, 1.5 mg) and 10-methoxy-9-ol (**6-II**, 2.0 mg), were prepared from fraction II (**4-II**, R_t 33.97 min, 5.0 mg).

MTPA Esters of Methoxyalcohols 2, 3, 5, and 6. (*R*)-MTPA chloride (2.5 mg, 10 μmol), which was prepared from (*S*)-MTPA, was added to methoxyalcohol **2-I** (1.0 mg, 3.5 μmol) dissolved in CCl_4 (0.7 ml) and pyridine (0.3 ml). After stirring for 4 hr at room temperature, this reaction mixture was poured into water (2 ml), and the crude product was extracted with ethyl acetate. The extract was washed with 1 N HCl and aq. $NaHCO_3$ solution, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. After purification by preparative TLC, the (*S*)-MTPA ester of **2-I** (1.2 mg, 67% yield) was obtained. In the same manner, the *R* ester of **2-I** was synthesized with (*S*)-MTPA chloride derived form (*R*)-MTPA. Both MTPA esters of other methoxy alcohols, **2-II**, **3-I**, **3-II**, **5-I**, **5-II**, **6-I**, and **6-II**, were also separately prepared, and their ^1H NMR signals were analyzed to determine the stereochemistry of the methoxyalcohols.

(S)-AP Esters of Methoxyalcohols Derived from Chiral 3,4-Epoxydienes 1-I and 1-II. After the methanolysis of fraction I of the 3,4-epoxide (**1-I**, 100% ee, 0.2 mg, 0.7 μmol), the optical purity of which was confirmed by GC with a chiral capillary column (Chiraldex A-PH), the crude methoxyalcohols were treated with (*S*)-AP chloride (0.3 mg, 2.0 μmol) to yield a mixture of their (*S*)-AP esters by the same procedure as that for the MTPA esters. Another mixture of (*S*)-AP esters of methoxyalcohols derived from fraction II of the 3,4-epoxide (**1-II**, 100% ee) were prepared. The components of these two mixtures were analyzed with an achiral capillary GC column (DB-23).

Field Evaluation. Enantiomers of 3,4- and 9,10-*cis*-epoxydienes with a C_{19} chain were separated by chiral HPLC with Chiralpak AS and Chiralpak AD columns, respectively. Each optically pure sample (0.4 mg) was applied to a rubber septum (8 mm OD). The lure was placed in 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 in a mixed forest area in the suburbs of Tokyo (Rolling Land Laboratory, Tokyo University of Agriculture and Technology, Hachioji-shi, Tokyo), and the number of male moths captured by the trap was recorded from October 1995 to September 1996. Some mixtures of the enantiomers (8:2, 5:5, and 2:8 for 3,4-epoxide, and 5:5 for 9,10-epoxides; 0.4 mg/septum) were tested at the same time. Each lure was renewed every three months.

RESULTS

Resolution of Epoxydiene Racemates with Chiral HPLC Columns. Enantiomeric separation of epoxydienes was achieved by two types of chiral HPLC columns: Chiralpak AS and AD. The separation condition was carefully optimized using the racemic *cis*-3,4-, 6,7-, and 9,10-monoepoxy derivatives of (*Z*,*Z*,*Z*)-3,6,9-octadecatriene. Figure 1 shows the chromatograms obtained using 0.1% 2-propanol in *n*-hexane as a solvent at room temperature. Although good separation was observed with the columns cooled to 0°C, the ability of the columns to produce separation easily declined, and the chromatography was only reproduced with difficulty. The Chiralpak AS column was suitable to separate enantiomers of the 3,4-epoxide, and the Chiralpak AD column was indispensable for the resolution of the 6,7- and 9,10-epoxides. The 6,7-epoxide showed only one peak on the AS column even though the operation was carried out at 0°C. Each enantiomer with a shorter R_t, which was recovered from the HPLC as fraction I, showed a positive value of $[\alpha]_D$ (from ca. +3° to +1°) and that with a longer R_t (fraction II) showed a corresponding negative value.

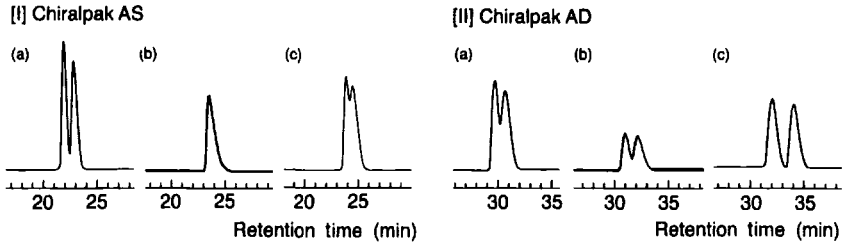


FIG. 1. Chromatography of the racemic *cis*-epoxydienes (50 μg each) derived from (*Z*,*Z*,*Z*)-3,6,9-octadecatriene on chiral HPLC columns, [I] Chiralpak AS and [II] Chiralpak AD. Solvent system: 0.1% 2-propanol in *n*-hexane, flow rate: 0.45 ml/min, (a) 3,4-epoxydiene detected by UV (215 nm), (b) 6,7-epoxydiene detected by RI, and (c) 9,10-epoxydiene detected by UV (215 nm).

Similar chromatographic behavior was observed on other epoxydienes with a C_{19}–C_{23} chain. Table 1 shows the R_t of both enantiomers with a C_{18}, C_{20}, or C_{23} chain and their separation factor (α). An ORD recorder revealed that the (+) isomer eluted faster than the (−) isomer on the separation of every epoxide with these columns without exception.

Chemical Structure of Methoxyalcohols Derived from Epoxydienes. The methanolysis of *cis*-epoxide stereoselectively proceeds from the opposite side of the epoxy oxygen atom to yield the *threo*-methoxyalcohols as major products. Two methoxyalcohols derived from the racemic (Z,Z)-6,9-*cis*-3,4-epoxyoctadecadiene (**1**) were separable on a silica gel MPLC column, and their NMR data in Table 2 revealed that the isomer with a shorter R_t was 3-methoxy-4-ol (**2**), while the isomer with a longer R_t was 4-methoxy-3-ol (**3**). Generally, a hydroxy methine proton resonates in a lower field than the corresponding methoxy methine proton, while the carbons bearing hydroxy and methoxy groups appear in an opposite order. Starting from the proton signal at position-1 [H(1)], H(2)–H(5) are easily correlated by their ^1H–^1H COSY spectra, and the signals at δ 3.02 and 3.59 ppm of the shorter-R_t positional isomer **2** were assigned to H(3) and H(4), respectively. These protons showed the cross-peaks with C(3) at δ 84.3 ppm and C(4) at δ 71.9 ppm in the ^1H–^{13}C COSY spectrum, indicating the 4-hydroxy group. Furthermore, the position of the hydroxy group was also confirmed by the NMR data of the acetate. The hydroxy methine proton usually shifts to a lower field (more than 1 ppm) after acetylation, and the acetate of **2** showed H(3) at δ 3.14 ppm and H(4) at δ 5.01 ppm. On the other hand, the 2D NMR spectra of the longer-R_t positional isomer **3** revealed the 3-hydroxy group.

The other two methoxyalcohols derived from the (Z,Z)-3,6-*cis*-9,10-epoxyoctadecadiene (**4**) were also separable on a silica gel MPLC column, while those from the *cis*-6,7-epoxy analog could not be separated by the MPLC. Their NMR data in Table 2 reveal that the shorter-R_t positional isomer was 9-methoxy-10-ol (**5**), and the longer-R_t positional isomer was a 10-methoxy-9-ol (**6**). Compound **5** showed a hydroxy methine proton at δ 3.49 ppm, which moved to a low field by 1.48 ppm after acetylation. By the ^1H–^1H COSY spectrum, this proton signal was assigned to H(10) because of a cross-peak with H(11) methylene protons at δ 1.47 ppm. In contrast, the hydroxy methine proton at δ 3.57 ppm of **6** was assigned to H(9) because of a cross-peak with H(8) allylic protons at δ 2.30 ppm, which was further correlated with the H(7) vinylic proton at δ ~5.5 ppm.

Stereochemistry of Enantiomers Separated on a Chiral HPLC Column. The absolute configuration of the enantiomers, which were separated from a racemic mixture of 3,4-epoxydiene (**1**) by a chiral HPLC column, was examined by using their methanolysis products. 3-Methoxy-4-ol (**2-I**), which was prepared from the shorter-R_t stereoisomer (**1-I**), was converted into the esters of (*S*)- and

TABLE 1. ENANTIOMETRIC SEPARATION OF EPOXYDIENES DERIVED FROM (Z,Z,Z)-3,6,9-TRIENES (C_{18}, C_{20}, AND C_{23}) ON CHIRAL HPLC COLUMNS

Compound	Chiralpak AS column			Chiralpak AD column		
	Retention time (min)			Retention time (min)		
	Fraction I (+) isomer	Fraction II (−) isomer	Separation factor (α)	Fraction I (+) isomer	Fraction II (−) isomer	Separation factor (α)
3,4-Epoxydiene						
C_{18}	22.00	22.95	1.11	29.66	30.86	1.08
C_{20}	20.43	21.16	1.10	29.62	30.51	1.06
C_{23}	19.23	19.74	1.08	29.83	30.31	1.03
6,7-Epoxydiene						
C_{18}	23.62		1.00	30.95	32.21	1.08
C_{20}	22.34		1.00	31.05	32.38	1.08
C_{23}	20.41		1.00	31.79	33.31	1.09
9,10-Epoxydiene						
C_{18}	23.99	24.60	1.06	31.92	33.97	1.12
C_{20}	22.61	22.99	1.04	31.90	33.82	1.11
C_{23}	20.68		1.00	32.70	34.76	1.12

TABLE 2. NMR ASSIGNMENTS OF METHOXYALCOHOLS **2**, **3**, **5**, AND **6**, OBTAINED BY METHANOLYSIS OF (*Z*,*Z*)-6,9-*cis*-3,4- AND (*Z*,*Z*)-3,6-*cis*-9,10-EPOXYOCTADECADIENES (**1** AND **4**) AND FOLLOWING MPLC SEPARATION

	NMR chemical shifts (ppm) for indicated position														
Comp.	1	2	3	4	5	6	7	8	9	10	11	16	17	18	OMe
^{1}H NMR															
2 (4-ol)[a]	0.93	~1.6	3.02	3.59	2.30	~5.5	~5.5[e]	2.81	~5.3[e]	~5.35	2.05	1.27	1.27	0.88	3.43
3 (3-ol)[b]	0.99	~1.5	3.42	3.08	2.35	~5.45	~5.5[e]	2.81	~5.35[e]	~5.4	2.06	1.27	1.27	0.88	3.43
5 (10-ol)[c]	0.98	2.08	5.40	5.31	2.82	~5.45	~5.45	2.35	3.06	3.49	1.47	1.27	1.27	0.88	3.43
6 (9-ol)[d]	0.97	2.07	5.40	5.31	2.81	~5.5	~5.5	2.30	3.57	3.06	1.54	1.28	1.28	0.88	3.42
^{13}C NMR															
2 (4-ol)	9.3	22.3	84.3	71.9	31.3	125.6[e]	130.6[e]	25.8	127.4[e]	130.6	27.3	31.9	22.7	14.1	58.0
3 (3-ol)	10.1	26.3	73.8	83.5	27.7	124.9[e]	130.6[e]	25.8	127.3[e]	130.5[e]	27.3	31.9	22.7	14.1	58.1
5 (10-ol)	14.3	20.6	132.2	126.8	25.7	130.4[e]	124.9[e]	27.6	83.8	72.5	33.4	31.9	22.7	14.1	58.1
6 (9-ol)	14.3	20.6	132.1	126.9	25.7	130.6[e]	125.6[e]	31.4	72.3	83.3	29.8	31.9	22.7	14.1	58.1

[a] $J_{3,4}$ = 5.5 Hz, acetate of **2**; H(3) 3.14 ppm, H(4) 5.01 ppm.
[b] $J_{3,4}$ = 5.5 Hz, acetate of **3**; H(3) 4.90 ppm, H(4) 3.25 ppm.
[c] $J_{9,10}$ = 5.5 Hz, acetate of **5**; H(9) 3.23 ppm, H(10) 4.97 ppm.
[d] $J_{9,10}$ = 5.5 Hz, acetate of **6**; H(9) 4.98 ppm, H(10) 3.19 ppm.
[e] Assignments for these signals are not unambiguous and may be reversed in the same row.

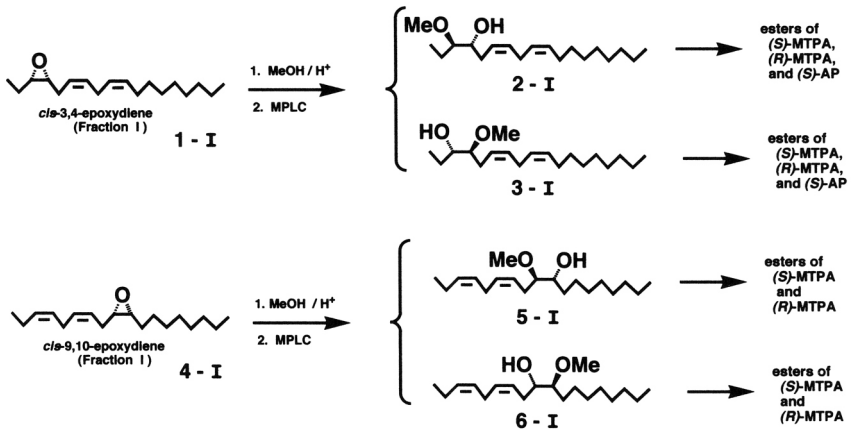


FIG. 2. Conversion of (*Z*,*Z*)-6,9-*cis*-3,4-epoxyoctadecadiene (**1-I**) and (*Z*,*Z*)-3,6-*cis*-9,10-epoxyoctadecadiene (**4-I**), fraction I with a shorter R_f on chiral HPLC, into the methoxyalcohols and their esters with MTPA and AP.

(*R*)-MTPA (Figure 2). ^{1}H NMR data of their alcohol moieties are shown in Table 2 together with the chemical shift difference ($\Delta\delta$) between the (*S*)-MTPA ester (δ_S) and (*R*)-MTPA ester (δ_R). The $\Delta\delta$ of H(1)–H(3) and methoxy protons are negative values, while those of H(5)–H(11) are positive values. Ohtani et al. (1989) reported that the ester carbonyl and trifluoromethyl groups in the MTPA moiety and, further, the α-hydrogen in the secondary alcohol moiety are oriented in the same plane. Thus, these data indicate that the H(1)–H(3) and methoxy protons of the (*S*)-MTPA ester are located on the same side of the benzene ring and accept its anisotropic effect, as shown in Figure 3, leading to the 4*R* configuration of **2-I**, i.e., the 3*S*,4*R* configuration of the parent chiral 3,4-epoxydiene (**1-I**). This result was confirmed with the NMR data of the MTPA esters of 4-methoxy-3-ol (**3-I**) prepared from the shorter-R_f stereoisomer (**1-I**) (Table 3). Their $\Delta\delta$ values indicate the 3*S* configuration of **3-I** i.e., the 3*S*,4*R* configuration of **1-I.** On the contrary, the opposite 3*R*,4*S* configuration was assigned to the longer-R_f stereoisomer **1-II**. The (*S*)-MTPA ester of **2-II** showed the same NMR spectra as the (*R*)-MTPA ester of **2-I**, because they are enantiomers. The (*R*)-MTPA ester of **2-II** showed the same NMR spectra as the (*S*)-MTPA ester of **2-I** for the same reason. The MTPA esters of **3-II** also showed the same spectra as the esters of **3-I** with MTPA possessing the opposite configuration. By a similar method, the absolute configuration of enantiomers separated from a racemic mixture of 9,10-epoxydiene (**4**) was determined (Figure 3). The ^{1}H NMR data of the MTPA esters of **5-I** (Table 3) and **6-I** (Table

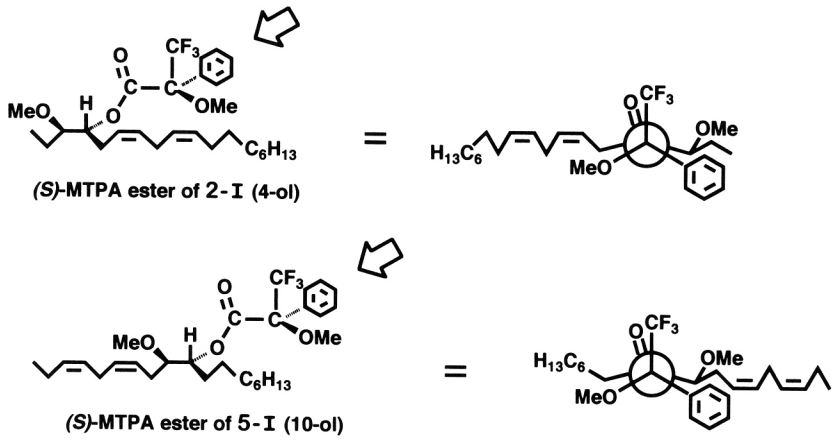


FIG. 3. Chemical structures of (*S*)-MTPA esters of 3-methoxy-4-ol (**2-I**) and 9-methoxy-10-ol (**5-I**), which were derived from (3*S*,4*R*)-(*Z*,*Z*)-6,9-*cis*-3,4-epoxyoctadecadiene (**1-I**) and (9*S*,10*R*)-(*Z*,*Z*)-3,6-*cis*-9,10-epoxyoctadecadiene (**4-I**), respectively.

3) revealed the 9*S*,10*R* configuration of the shorter-R_t stereoisomer (**4-I**), and those of **5-II** and **6-II** showed the 9*R*,10*S* configuration of the longer-R_t stereoisomer (**4-II**).

GC Analysis of (S)-AP Esters of Methanolysis Products. In order to establish a method for estimating the enantiomeric purity of epoxydienes with achiral chromatography, the (*S*)-AP esters of methanolysis products derived from each stereoisomer of 3,4-epoxydiene (**1-I** and **1-II**, 100% ee) were analyzed by GC with an ordinary capillary column (DB-23). (*S*)-AP esters of (3*R*,4*R*)-3-methoxy-4-ol (**2-I**), (3*S*,4*S*)-4-methoxy-3-ol (**3-I**), (3*S*,4*S*)-3-methoxy-4-ol (**2-II**), and (3*R*,4*R*)-4-methoxy-3-ol (**3-II**) were separately detected in a ratio of 55:38:4:3 from the 3*S*,4*R* isomer (**1-I**), and of 4:3:57:36 from the 3*R*,4*S* isomer (**1-II**) as shown in Figure 4. The selectivity of the methanolysis was not perfect, but rather high. This result indicates that the optical purity of epoxydienes can be estimated briefly after the methanolysis of the epoxy ring without depending on any chiral chromatography.

Field Attraction. Field evaluation of both enantiomers of (*Z*,*Z*)-6,9-*cis*-3,4- and (*Z*,*Z*)-3,6-*cis*-9,10-epoxynonadecadienes and their mixtures revealed specific attraction of three geometrid species (Table 4). While male moths of *Pachyerannis obliquaria*, which belongs to the subfamily Ennominae, were not attracted to any lures in the random screening tests with racemic mixtures (Ando et al., 1995), more than 100 males were captured by traps baited with only the 3*R*,4*S* isomer of 3,4-epoxydiene. The enantiomer of this isomer attracted another

TABLE 3. ^{1}H NMR CHEMICAL SHIFTS OF (*S*)-MTPA ESTERS [δ_S] AND (*R*)-MTPA ESTERS [δ_R] OF METHOXYALCOHOLS **2-I**, **3-I**, **5-I**, AND **6-I**, AND THEIR DIFFERENCES [$\Delta\delta = \delta_S - \delta_R$]

	^{1}H chemical shift (ppm) for indicated position												
Compound	1	2	3	4	5	6	7	8	9	10	11	12-17	OMe
2-I (4-ol)													
(*S*)-MTPA [δ_S]	0.88	1.37	3.16	5.25	2.45	5.40	5.51	2.77	5.31	5.40	2.02	1.27	3.31
(*R*)-MTPA [δ_R]	0.96	1.57	3.24	5.25	2.40	5.29	5.39	2.69	5.25	5.39	2.01	1.27	3.38
$\Delta\delta = \delta_S - \delta_R$	−0.08	−0.20	−0.08	0.00	0.05	0.11	0.12	0.08	0.06	0.01	0.01	0.00	−0.07
3-I (3-ol)													
(*S*)-MTPA [δ_S]	0.83	1.65	5.16	3.35	2.30	5.47	5.48	2.71	5.32	5.40	2.03	1.27	3.37
(*R*)-MTPA [δ_R]	0.96	1.72	5.14	3.27	2.20	5.40	5.43	2.71	5.29	5.39	2.02	1.27	3.29
$\Delta\delta = \delta_S - \delta_R$	−0.13	−0.07	0.02	0.08	0.10	0.07	0.05	0.00	0.03	0.01	0.01	0.00	0.08
5-I (10-ol)													
(*S*)-MTPA [δ_S]	0.96	2.05	5.36	5.27	2.71	5.43	5.38	2.16	3.27	5.19	1.31	1.26	3.30
(*R*)-MTPA [δ_R]	0.97	2.06	5.40	5.29	2.77	5.47	5.43	2.29	3.33	5.20	1.26	1.21	3.37
$\Delta\delta = \delta_S - \delta_R$	−0.01	−0.01	−0.04	−0.02	−0.06	−0.04	−0.05	−0.13	−0.06	−0.01	0.05	0.05	−0.07
6-I (9-ol)													
(*S*)-MTPA [δ_S]	0.96	2.04	5.25	5.28	2.69	5.37	5.40	2.41	5.21	3.30	1.49	1.27	3.37
(*R*)-MTPA [δ_R]	0.96	2.05	5.26	5.38	2.78	5.40	5.50	2.47	5.21	3.23	1.43	1.24	3.31
$\Delta\delta = \delta_S - \delta_R$	0.00	−0.01	−0.01	−0.10	−0.09	−0.03	−0.10	−0.06	0.00	0.07	0.06	0.03	0.06

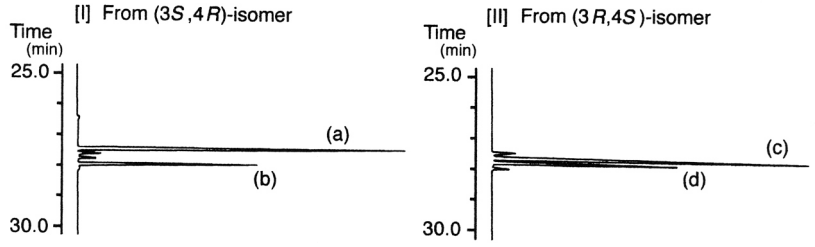


FIG. 4. Chromatography of (*S*)-AP esters of methoxyalcohols derived from (3*S*,4*R*)-(*Z*,*Z*)-6,9-*cis*-3,4-epoxyoctadecadiene (**1-I**) [I] and the 3*R*,4*S* isomer (**1-II**) [II] on a capillary GC column, DB-23 (0.25 mm ID × 30 m). (a) Ester of (3*R*,4*R*)-3-methoxy-4-ol (**2-I**), (b) ester of (3*S*,4*S*)-4-methoxy-3-ol (**3-I**), (c) ester of (3*S*,4*S*)-3-methoxy-4-ol (**2-II**), and (d) ester of (3*R*,4*R*)-4-methoxy-3-ol (**3-II**).

species of Ennominae, *Zethenia albonotaria nesiotis*. Attractants for these polyphagous forest defoliators have not been reported (Arn et al., 1992; Ando, personal data base). Specific attraction by optically pure isomers of 9,10-epoxydiene was not observed, but it was confirmed that both isomers are necessary for strong attraction of *Operophtera relegata* in the subfamily Larentiinae, as expected from previous field tests (Ando et al., 1993).

TABLE 4. FIELD ATTRACTION OF SOME GEOMETRID SPECIES TO LURES BAITED WITH OPTICAL ISOMERS OF EPOXYNONADECADIENES AND THEIR MIXTURES

Attractant and species	Total number of males attracted to indicated ratio of (+)-*S*,*R* isomer: (−)-*R*,*S* isomer[a]					Time of flight
	10:0	8:2	5:5	2:8	0:10	
3,4-Epoxydiene C_{19}						
Pachyerannis obliquaria (Motschulsky)[b]	0	0	0	0	122	Nov–Dec
Zethenia albonotaria nesiotis Wehrlic[b]	20	1	0	0	0	April–June
9,10-Epoxydiene C_{19}						
Operophtera relegata Prout[c]	37		169		0	Nov–Dec

[a]Two traps were used for each lure. 3*S*,4*R* isomer:3*R*,4*S* isomer for (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene, and 9*S*,10*R* isomer:9*R*,10*S* isomer for (*Z*,*Z*)-3,6-*cis*-9,10-epoxynonadecadiene.
[b]Ennominae.
[c]Larentiinae.

DISCUSSION

The resolution of *cis*-epoxydiene racemates derived from (*Z*,*Z*,*Z*)-3,6,9-trienes with a C_{18}–C_{23} chain was accomplished by HPLC with Chiralpak AS and AD columns. We observed the attraction of several geometrid and noctuid male moths by random screening tests with racemic mixtures of the epoxydienes, which were easily synthesized starting from linolenic acid in a rather high yield (Ando et al., 1993). It is interesting to examine whether or not both enantiomers are necessary for attraction and to find new attractants for other species using optically pure samples. For these purposes HPLC with chiral column packings is one of the most useful techniques. Our preliminary field test revealed that two geometrid species were specifically attracted to the separated enantiomers with opposite configurations, whereas one geometrid species preferred a racemic mixture. Both species attracted to optically pure isomers have not been captured in the random screening tests of racemic mixtures. We are now systematically evaluating the field attractancy of optically active epoxydienes.

A chiral GC column may separate enantiomers of epoxydienes, but information is very limited. There is only one application of a chiral GC column packed with a β-cyclodextrin derivative as a liquid phase for the stereochemical identification of a natural 6,7-epoxydiene pheromone (Szöcs et al., 1993). We successfully separated the enantiomers of 3,4-epoxides by another GC column of the α-cyclodextrin type (Chiraldex A-PH), but failed in the resolution of 6,7- and 9,10-epoxydienes despite an effort with several chiral columns that were commercially available (Ando et al., unpublished data).

Without exception, the (+)-enantiomers of the epoxydienes eluted faster than the (−)-enantiomers from the chiral HPLC columns. The optical rotation of the epoxydienes stereoselectivity synthesized by Sharpless asymmetric epoxidation has been reported for C_{17-21} 3,4-epoxydienes (Cossé et al., 1992; Millar et al., 1990; Szöcs et al., 1993), C_{19} 6,7-epoxydiene (Mori and Ebata, 1986), and C_{17-22} 9,10-epoxydiene (Mori and Brevet, 1991; Wong et al., 1985). All the *S*,*R* isomers (3*S*,4*R*, 6*S*,7*R*, and 9*S*,10*R* isomers) of these epoxydienes are dextrorotatory compounds, and all of the *R*,*S* isomers (3*R*,4*S*, 6*R*,7*S*, and 9*R*,10*S* isomers) are levorotatory. Therefore, it was expected that the shorter-R_t stereoisomers separated by the chiral HPLC columns possessed the *S*,*R* configuration and the longer-R_t enantiomers possessed the opposite *R*,*S* configuration. In order to confirm the absolute configuration of the enantiomers, we studied the stereochemistry of the methanolysis products derived from C_{18} epoxydienes. Although unfortunately the methanolysis products of the 6,7-epoxydiene could not be separated because of their structural similarities, two major methoxyalcohols prepared from each of the 3,4- and 9,10-epoxydienes were isolated, and their structures were reliably defined by 2D NMR. The methanolysis products included a secondary hydroxy group; thus their absolute configurations were

empirically determined applying a modified Mosher's method (Ohtani et al., 1989). Configurations of the parent epoxydienes, which were estimated from the result of the methoxyalcohols, coincided with those predicted by another empirical rule by the Sharpless epoxidation reaction.

The optical purity of a chiral compound can be detected without any chiral chromatography by analyzing the diastereomers that are prepared by coupling with a chiral reagent. The enantiomeric excess of synthetic epoxy pheromones has been estimated by achiral GC or HPLC analyses of the esters between the epoxyalcohol intermediates and chiral acids, AP, MTPA, and so on. This estimation is based on the hypothesis that epimerization does not happen during the synthetic process after chiral epoxidation. It is interesting to determine the optical purity of the epoxy pheromones utilizing their derivatives that are prepared by a stereoselectively controlled reaction. Oliver and Waters (1995) have reported a method to convert epoxyalkane to the corresponding *N*-(α-methylbenzyl)aziridine and successfully determine the optical purity of disparlure with an achiral GC column. We separately detected the (*S*)-AP esters of the methanolysis products of C_{18} 3,4-epoxydiene on a capillary column, indicating the optical purity of the parent epoxide. However, the selectivity of the methanolysis catalyzed with a limited amount of H_2SO_4 was not perfect even under cooling conditions. Minor products produced by an attack of the solvent on the same side of the epoxy oxygen atom were detected. To estimate the exact purity of epoxydienes, perfect selectivity is necessary for the solvolysis.

Acknowledgments—The authors are grateful to Prof. Y. Ambe of Tokyo University of Agriculture and Technology for his assistance in the field tests and Dr. S. Hashimoto of Meijo University for identification of geometrid species. This work was partly supported by a Grant-in-Aid for Scientific Research (C) (No. 07660053) from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

ANDO, T., OHSAWA, H., UENO, T., KISHI, H., OKAMURA, Y., and HASHIMOTO, S. 1993. Hydrocarbons with a homoconjugated polyene system and their monoepoxy derivatives: Sex attractants of geometrid and noctuid moths distributed in Japan. *J. Chem. Ecol.* 19:787–798.

ANDO, T., KISHI, H., AKASHIO, N., QIN, X.-R., SAITO, N., ABE, H., and HASHIMOTO, S. 1995. Sex attractants of geometrid and noctuid moths: Chemical characterization and field test of monoepoxides of 6,9-dienes and related compounds. *J. Chem. Ecol.* 21:299–311.

ARN, H., TÖTH, M., and PRIESNER, E. 1992. List of sex pheromones of Lepidoptera and related attractants. OILB-SROP/IOBC-WPRS, Secrétariat Général, F-84143 Montfavet, France, ISBN 92-9067-044-4, 179 pp.

BREVET, J.-L., and MORI, K. 1992. Pheromone synthesis; CXXXIX. Enzymatic preparation of (2*S*,3*R*)-4-acetoxy-2,3-epoxybutan-1-ol and its conversion to the epoxy pheromones of the gypsy moth and the ruby tiger moth. *Synthesis* 1992:1007–1012.

COSSÉ, A. A., CYJON, R., MOORE, I., WYSOKI, M., and BECKER, D. 1992. Sex pheromone com-

ponents of the giant looper, *Boarmia selenaria* Schiff. (Lepidoptera: Geometridae): Identification, synthesis, electrophysiological evaluation, and behavioral activity. *J. Chem. Ecol.* 18:165–181.

DALE J. A., and MOSHER, H. S. 1973. Nuclear magnetic resonance enantiomer reagents. Configurational correlations via nuclear magnetic resonance chemical shifts of diastereomeric mandelate, *O*-methylmandelate, and α-methoxy-α-trifluoromethylphenylacetate (MTPA) esters. *J. Am. Chem. Soc.* 95:512–519.

MILLAR, J. G., and UNDERHILL, E. W. 1986. Synthesis of chiral bis-homoallylic epoxides. A new class of lepidopteran sex attractants. *J. Org. Chem.* 51:4726–4728.

MILLAR J. G., GIBLIN, M., BARTON, D., MORRISON, A., and UNDERHILL, E. W. 1990. Synthesis and field testing of enantiomers of (6*Z*,9*Z*)-*cis*-3,4-epoxydienes as sex attractants for geometrid moths. Interactions of enantiomers and regioisomers. *J. Chem. Ecol.* 16:2317–2339.

MORI, K. 1994. Synthetic and stereochemical aspects of pheromone chemistry. *Pure Appl. Chem.* 66:1991–1998.

MORI, K., and ARGADE, N. P. 1994. Synthesis of (9*Z*,25*S*,26*R*,43*Z*)-25,26-epoxy-9,43-henpentacontadiene and its antipode, components of the nymph recognition pheromone produced by nymphs of the cockroach *Nauphoeta cinerea*. *Liebigs Ann. Chem.* 1994:695–700.

MORI, K., and BREVET, J.-L. 1991. Pheromone synthesis; CXXXIII. Synthesis of both the enantiomers of (3*Z*,9*Z*)-*cis*-6,7-epoxy-3,9-nonadecadiene, a pheromone component of *Erannis defoliaria*. *Synthesis* 1991:1125–1129.

MORI, K., and EBATA, T. 1986. Syntheses of optically active pheromones with an epoxy ring, (+)-disparlure and both the enantiomers of (3*Z*,6*Z*)-*cis*-9,10-epoxy-3,6-heneicosadiene. *Tetrahedron* 42:3471–3478.

OHTANI I., KUSUMI, T., ISHITSUKA, M. O., and KAKISAWA, H. 1989. Absolute configurations of marine diterpenes possessing a xenicane skeleton. An application of an advanced Mosher's method. *Tetrahedron Lett.* 30:3147–3150.

OLIVER, J. E., and WATERS, R. M. 1995. Determining enantiomeric composition of disparlure. *J. Chem. Ecol.* 21:199–211.

SZÖCS, G., TÓTH, M., FRANCKE, W., SCHMIDT, F., PHILIPP, P., KÖNIG, W. A., MORI, K., HANSSON, B. S., and LÖFSTEDT, C. 1993. Species discrimination in five species of winter-flying geometrids (Lepidoptera) based on chirality of semiochemicals and flight season. *J. Chem. Ecol.* 19:2721–2735.

WONG, J. W., UNDERHILL, E. W., MACKENZIE, S. L., and CHISHOLM, M. D. 1985. Sex attractants for geometrid and noctuid moths. Field trapping and electroantennographic responses to triene hydrocarbons and monoepoxydiene derivatives. *J. Chem. Ecol.* 11:727–756.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

DISTRIBUTION AND ACCUMULATION OF THE ALKALOID PERAMINE IN *Neotyphodium lolii*-INFECTED PERENNIAL RYEGRASS

O. J.-P. BALL,[1,*] G. M. BARKER,[2] R. A. PRESTIDGE,[3] and D. R. LAUREN[4]

[1]*AgResearch, Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand*
[2]*Landcare Research, Private Bag 3127, Hamilton, New Zealand*
[3]*AgResearch, Ruakura Agricultural Research Centre*
[4]*Hort Research, Ruakura Agricultural Research Center*
Private Bag 3123
Hamilton, New Zealand

(Received May 6, 1996; accepted January 8, 1997)

Abstract—Through the production of alkaloids and mycotoxins, the endophytic fungus of perennial ryegrass, *Neotyphodium lolii*, confers resistance to the host grass against several insect pasture pests and causes health problems in livestock. The effects of plant component, plant reproductive development, and plant management (trimming) on the concentration and accumulation of peramine, the alkaloid thought to be most important for conferring resistance against Argentine stem weevil, were investigated. Clones of eight different naturally occurring perennial ryegrass-*N. lolii* associations were used in the experiment, which was conducted during spring–summer, which corresponded with the height of plant reproductive activity. Clones were either trimmed regularly to maintain them in a vegetative state (V clones) or not trimmed, allowing them to become fully reproductive (R clones). V and R clones of each grass/endophyte association were destructively harvested at times that corresponded roughly with the start, middle, and end of the plant reproductive phase. Harvested material was dissected into a number of plant components that were analyzed for peramine concentration by HPLC. All clippings from the V clones were also analyzed. Peramine concentrations were usually similar in leaf sheaths and leaf blades, but they decreased as leaf age increased. The seed from R clones and younger sheaths and blades of leaves from vegetative tillers contained the highest concentrations, while the root, crown, and dead leaf tissue contained the lowest. During the inflorescence phase, tissues from reproductive tillers generally contained lower concentrations than corresponding tissues from vegetative tillers. Also at this time, mean peramine concentrations were higher in V clones than in R clones. By the end of the plant

*To whom correspondence should be addressed.

0098-0331/97/0500-1419$12.50/0 © 1997 Plenum Publishing Corporation

reproductive phase, at the mature seed stage, mean peramine concentrations in R and V clones were similar. The seed component, which contained approximately 75% of the total peramine present in the plant (at a mean concentration of 63.7 μg/g), probably accounted for much of the recovery in the R clones.

Key Words—Peramine, perennial ryegrass, endophyte, *Neotyphodium lolii*, *Acremonium*.

INTRODUCTION

Perennial ryegrass (*Lolium perenne* L.) may be infected with the symptomless clavicipitaceous endophytic fungus, *Neotyphodium lolii* (Latch, Christensen and Samuels), Glenn, Bacon, and Hanlin, previously assigned to the genus *Acremonium*. In countries reliant on perennial ryegrass as the major forage species such as New Zealand, *N. lolii* has important repercussions for the agricultural industry. Due to the production of a number of alkaloids and mycotoxins, *N. lolii* infection confers resistance to the host grass against attack from a number of insect pasture pests including Argentine stem weevil [*Listronotus bonariensis* (Kuschel) (Coleoptera: Curculionidae)], one of the most serious insect pasture pests in New Zealand (Prestidge et al., 1982). *N. lolii* infection has also been shown to be responsible for health problems in livestock such as ryegrass staggers (a neuromuscular toxicosis), reduced live weight gains, hormone imbalance, and heat stress (Fletcher, 1993). Thus, there are both advantages and disadvantages to its presence.

Argentine stem weevil resistance in infected plants is largely due to the production of the alkaloid peramine by *N. lolii* (Rowan and Gaynor, 1986), while the animal ill-thrift problems appear to be largely caused by mycotoxins and alkaloids such as lolitrem B (Gallagher et al., 1984) and ergovaline (Piper and Fletcher, 1990). Peramine is not thought to be toxic to livestock (Pownall et al., 1995) and is viewed as a desirable alkaloid. Through selection of nontoxic strains or genetic manipulation of endophytes and perennial ryegrass, it may therefore be possible to develop an association that is resistant to Argentine stem weevil but that does not cause health problems in livestock.

This paper reports on a study that investigated the effects of plant component, plant reproductive development, and plant management (clipping) on the concentration and accumulation of peramine in *N. lolii*-infected perennial ryegrass.

METHODS AND MATERIALS

Plant Origin and Maintenance

Eight genetically different *N. lolii*-infected perennial ryegrass plants (genotypes) were used in the study. These were progeny of paired crosses per-

formed between ryegrass plants collected from a variety of New Zealand North Island habitats and that had been characterized according to their alkaloid content (Wedderburn et al., 1989). Each plant was split into five approximately equally sized clones which were planted outdoors in a sandy loam in mid-winter (July 7, 1991) approximately 50 cm apart at the Ruakura Agricultural Research Centre, Hamilton, New Zealand. Plants were exposed to rainfall and did not require additional irrigation. The harvests were timed to coincide with the major part of the reproductive phase of the plant (October–January).

On September 16, 1991 (time zero for all treatments), plants were trimmed to 8 cm stubble height. A regular clipping regime was continued for three of the five clones of each genotype for the remainder of the experiment. These randomly chosen clones, were termed V (vegetative) clones as the trimming regime largely prevented the plants from becoming reproductive. All trimmed material (regrowth) was retained for chemical analysis. The remaining two clones of each genotype were left untrimmed and were termed R (reproductive) clones as full reproductive development was possible.

Harvest and Dissection of V Plants

One V clone of each grass genotype was destructively harvested on October 1, 1991 (mid-spring), November 6, 1991 (late-spring), and January 14, 1992 (mid-summer). These times corresponded roughly with the start, middle (some mature inflorescences), and end (seed heads mature) of the plant reproductive phase. Plants were removed from soil and washed thoroughly under cold tap water. After trimming plants to a height of 8 cm, the tillers of each plant were separated and dissected into the following components: second, third, and fourth leaf sheaths; second, third, and fourth leaf blades; first leaf (not sectioned into sheath and blade); dead leaf; reproductive stem tissue; crown; and root. The fourth leaf refers to the oldest (outermost) leaf and the first leaf refers to the youngest (innermost). Due to the nature of the sample, there were no fourth leaf blade and fourth leaf sheath components in the January 14, 1992, harvest. During the experimental period, the V clones yet to be harvested were trimmed to 8 cm above ground level on October 2, 14, 24, and 30, 1991; November 14 and 22, 1991; and December 2, 17, and 31, 1991.

Harvest and Dissection of R Plants

On November 6, 1991 (late-spring) and January 14, 1992 (mid-summer), one R clone of each grass genotype was destructively harvested.

Late-Spring Harvest. Tillers from plants harvested on November 6, 1991 were separated and classed as either vegetative, reproductive, or intermediate. Vegetative tillers were classed as such if they showed no, or only early signs, of developing reproductive structures. Tillers were classed as reproductive if the

inflorescence was present and had emerged fully from the sheath. Tillers that were neither fully vegetative nor reproductive were classed as intermediate. A large proportion of the vegetative tillers were dissected into the same components as described for the mid-spring and late-spring harvests of the V clones. The root components from all three classes of tiller from the R plants were bulked to form one root category. The vegetative tillers that were not dissected were trimmed to 8 cm to form two further categories called lower (ground level to 8 cm) and upper vegetative tiller (leafy material from 8 cm upwards). Intermediate tillers were not dissected, but were trimmed to 8 cm to form two categories, lower (ground level to 8 cm) and upper intermediate tiller material (from 8 cm upwards). A large proportion of the reproductive tillers were dissected into the following categories: first, second, and third leaf sheaths and blades; inflorescence; dead leaf; lower reproductive stem (reproductive stem minus leaf sheath material up to the end of the second to last internode); upper reproductive stem (the last internode before the inflorescence); crown; and root. As for the vegetative and intermediate tillers, the remaining undissected reproductive tillers were trimmed to a height of 8 cm to form lower and upper reproductive tiller categories.

Mid-Summer Harvest. Vegetative tillers from R clones harvested on January 14, 1992, were dissected into the same components described for the late-spring harvest of the R clones, except that there were no fourth leaf sheath and blade components and no intermediate tillers were present. Reproductive tillers were dissected into seed, seed head (minus seed), lower reproductive stem (culm up to the end of the second to last internode), upper reproductive stem (the last internode before the seed head), dead leaf, crown, and lower and upper reproductive tiller categories. By January 14, 1992, there was no green leaf material remaining on the reproductive tillers. Dead material which could not be assigned to vegetative or reproductive tillers was included in a separate category (mixed dead leaf).

Sample Treatment and Analysis

All harvested plant material, including all trimmings (regrowth taken from V clones) was immediately frozen at $-20°C$ and subsequently freeze-dried, weighed, and ground to a fine powder (1.0 mm mesh size). Plant samples were analyzed for peramine content using HPLC as described in Ball et al. (1995).

Statistical Analysis and Calculations

Data were log transformed and subjected to analysis of variance. Least significant ratios (LSR), calculated by multiplying the mean standard error of the differences by the value of t at the 5% level and back-transforming the resulting value (Steel and Torrie, 1980), are presented with all figures. Com-

parisons between two values are significantly different when the larger value divided by the smaller value exceeds the LSR. The total quantity of peramine in each plant component, and within each entire plant, was estimated. The peramine contained in the clippings removed from the V clones during the experiments was included. Dividing the total amount of peramine in each plant by the total weight of the plant yielded the mean peramine concentration for the entire plant. Roots were excluded from this calculation as concentrations of peramine in this tissue were negligible.

RESULTS

Peramine concentrations differed widely between genotypes. These differences generally were maintained over the different harvest dates, management regimes, and plant components.

V Clones

Mid-Spring Harvest (October 1). Peramine concentrations were highest in leaf sheath tissues (Figure 1A). Leaf blade concentrations were significantly ($P < 0.001$) lower than those in the corresponding leaf sheaths. As leaf age increased, concentrations, particularly in the blade, decreased ($P < 0.001$). The lowest concentrations were present in the roots [where levels were usually below the level of detection (approximately 0.5 μg/g)], dead leaf, and fourth blade. Regrowth, second to fourth leaf sheaths, and the first leaf components contained the largest quantities of peramine (Figure 2A). Only small quantities were found in roots or dead tissue.

Late-Spring Harvest (November 6). Peramine concentrations were generally below the level of detection in the root and were low in the dead leaf and crown (Figure 1B). The highest concentrations were registered in the first leaf and second leaf sheath and blade. As leaf age increased, concentrations decreased ($P < 0.001$). Sheath and blade concentrations were similar at all leaf ages. Peramine concentrations at this harvest were higher in most plant components compared to those on October 1 (Figure 1A and B). The largest quantity of peramine was present in the accumulated regrowth category (Figure 2B). Compared to other plant components, the quantity of peramine present in the reproductive stem tissue was also high.

Mid-Summer Harvest (January 14). The highest peramine concentrations were recorded in the first leaf, second and third leaf sheaths and blades, and regrowth, while the lowest were in the dead leaf and root categories (Figure 1C). As leaf age increased, concentrations decreased ($P < 0.001$). Sheath and blade concentrations were similar at all leaf ages. Peramine concentrations were generally similar to those recorded in plants harvested on November 6 (Figure

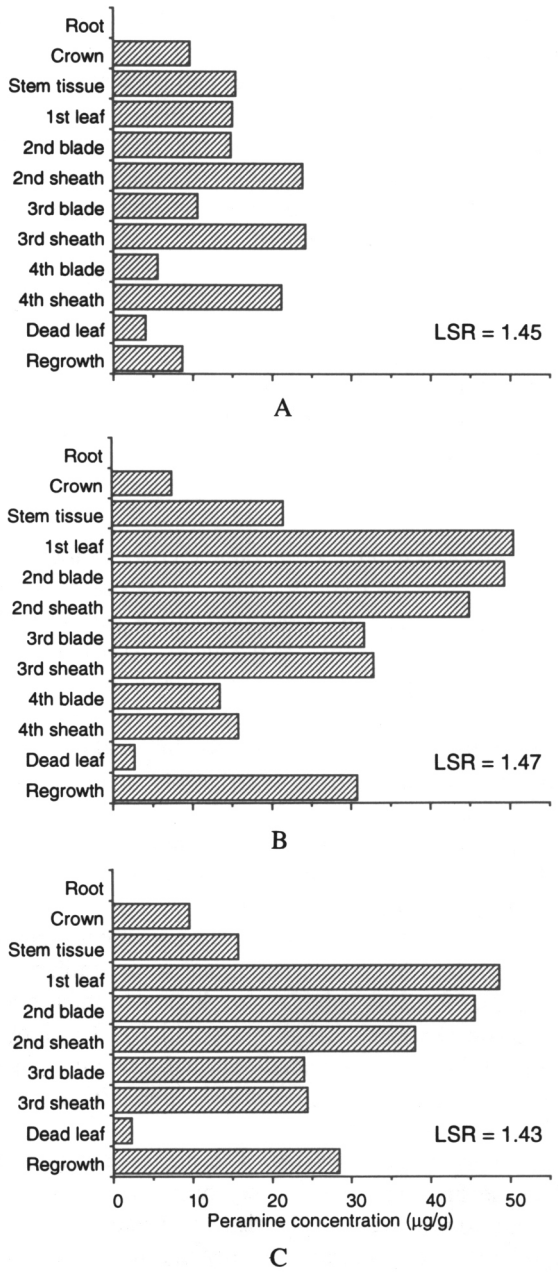


FIG. 1. Concentrations of peramine in different components of *N. lolii*-infected V perennial ryegrass plants sampled on October 1, 1991 (A), November 6, 1991 (B), and January 14, 1992 (C) (N = 8 plants).

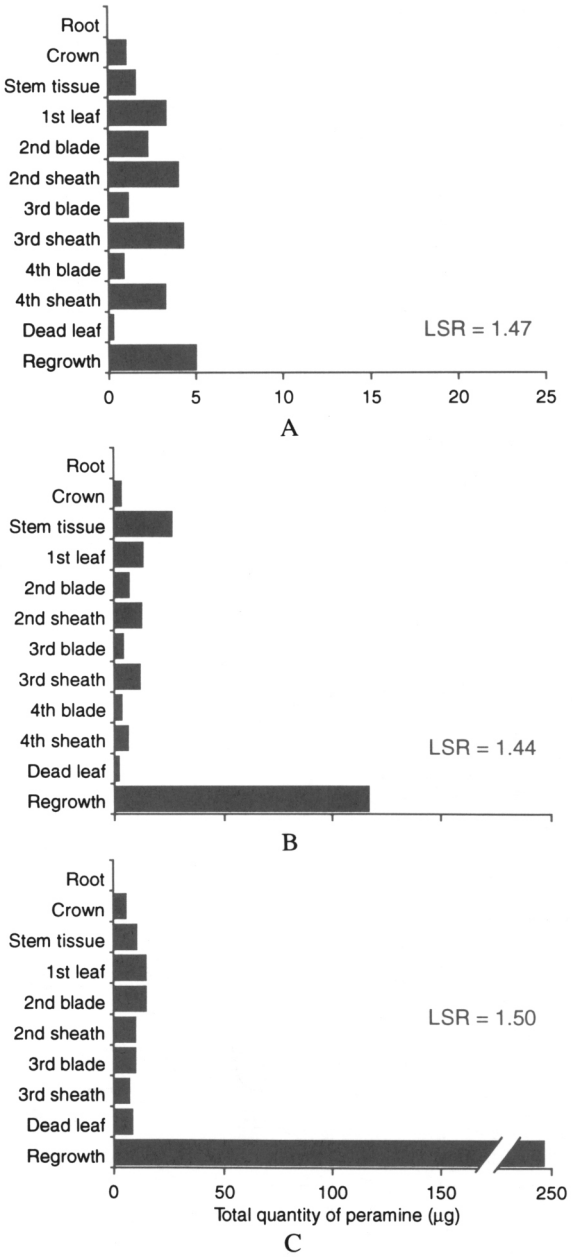


FIG. 2. Total quantities of peramine in different components of *N. lolii*-infected V perennial ryegrass plants sampled on October 1, 1991 (A), November 6, 1991 (B), and January 14, 1992 (C) (N = 8 plants). The peramine removed during the clipping regime prior to the destructive harvest of the plants is included in the regrowth category.

1B and C). The total amount of peramine in the accumulated regrowth tissue was much greater than in any other plant component (Figure 2C).

R Clones

Late-Spring Harvest (November 6). Peramine concentrations in the root were generally below the limit of detection (Figure 3A). Low concentrations were also registered in the dead leaf, leaf blades, and crown from reproductive tillers. The highest concentrations were recorded in the first leaf and the second and third leaf sheaths and blades from vegetative tillers. Crown, dead leaf, and

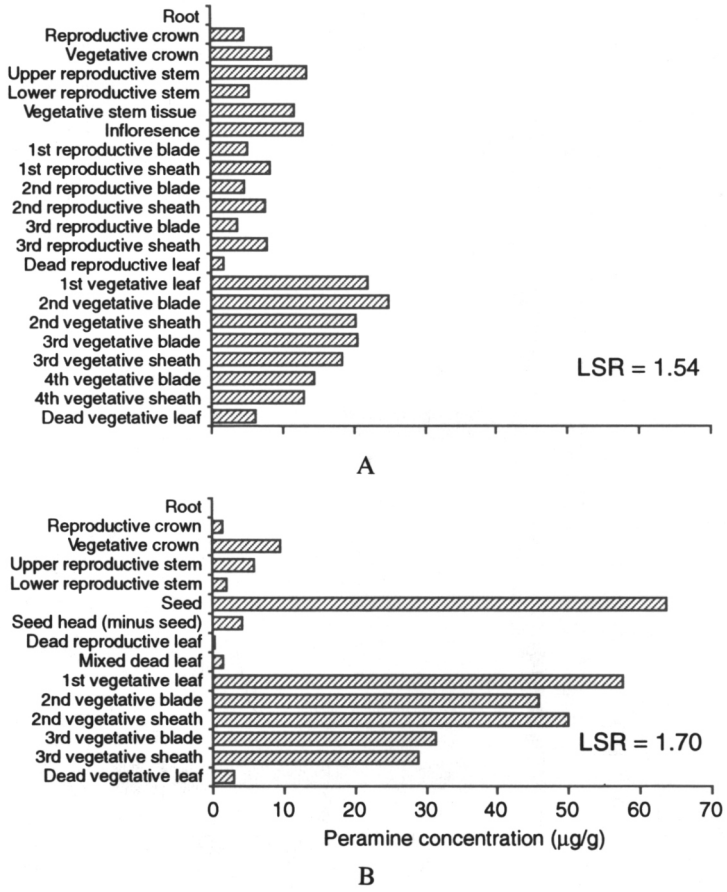


FIG. 3. Concentrations of peramine in different components of vegetative and reproductive tillers from *N. lolii*-infected R ryegrass plants sampled on November 6, 1991 (A), and January 14, 1992 (B) (N = 8 plants).

leaf sheaths and blades from vegetative tillers contained higher ($P < 0.001$) concentrations than the same tissues from reproductive tillers of the same plants. Similarly, in the bulked undissected herbage, peramine concentrations were higher in vegetative tillers than in reproductive tillers (Figure 4).

In leaves from reproductive tillers, peramine concentrations were not affected by age (Figure 3A). Concentrations in leaves from vegetative tillers declined ($P < 0.001$) with increasing age. In reproductive tillers, leaf sheaths contained higher ($P < 0.001$) concentrations than leaf blades. In the bulked undissected herbage, peramine concentrations were highest in the upper part of the tillers in all three tiller categories (Figure 4).

Leaf sheaths of vegetative tillers derived from V clones harvested on November 6 (Figure 1B) contained higher concentrations of peramine ($P < 0.05$ using a t test) than sheaths of vegetative tillers derived from *R* clones harvested on the same day (Figure 3A). The greatest quantity of peramine in the R clones was present in the inflorescence, while leaf sheaths and blades from vegetative tillers contained more peramine than the same tissues from reproductive tillers (Figure 5A).

Mid-Summer Harvest (January 14). The highest concentration of peramine was recorded in the seed (Figure 3B). Excluding the dead leaf material, leaf

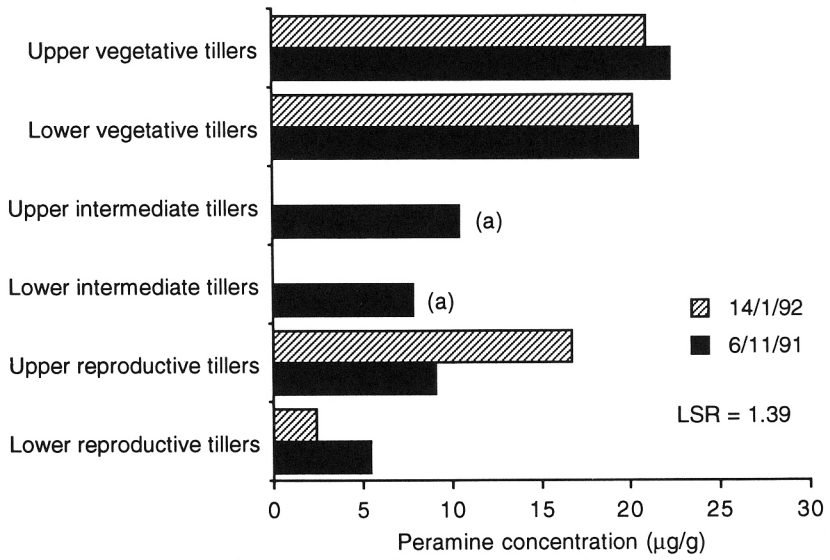


FIG. 4. Peramine concentrations in lower (from ground level to 8 cm) and upper (above 8 cm) components of vegetative, intermediate, and reproductive tillers from *N. lolii*-infected R ryegrass plants sampled on November 6, 1991, and January 14, 1992 (N = 8 plants). (a) denotes that no intermediate tillers were present on January 14, 1992.

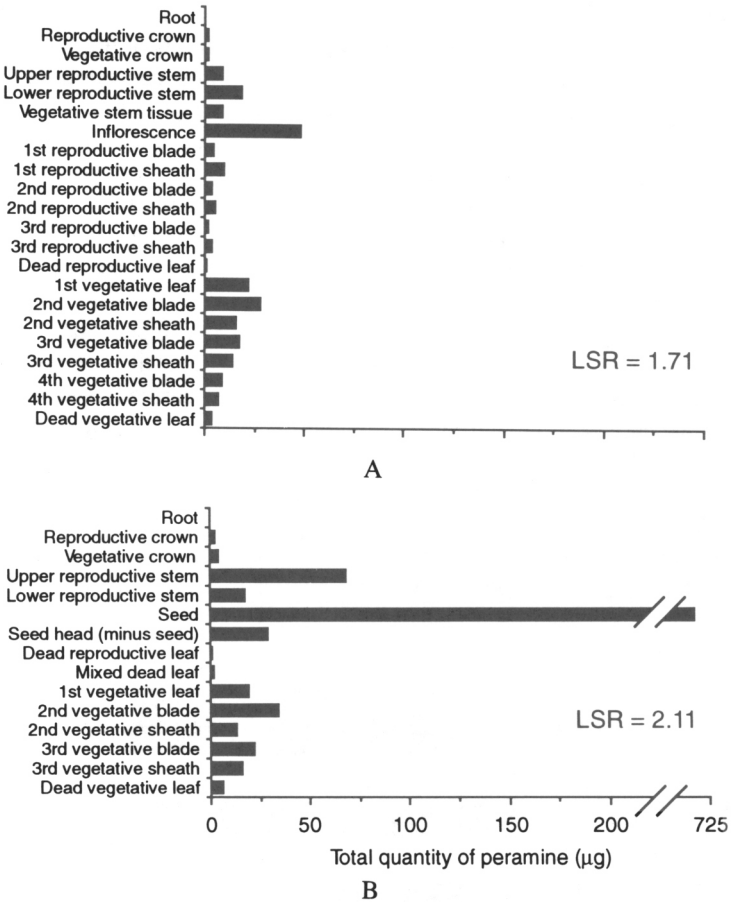


FIG. 5. Total quantities of peramine in different components of vegetative and reproductive tillers from *N. lolii*-infected R ryegrass plants sampled on November 6, 1991 (A), and January 14, 1992 (B) ($N = 8$ plants).

tissues (sheaths and blades) from vegetative tillers also had comparatively high concentrations. Concentrations in all other plant components were below 10 μg/g. Roots (where it was rarely detected) and dead leaf tissue from reproductive tillers had particularly low concentrations of peramine. Crown and dead leaf material from vegetative tillers contained significantly ($P < 0.001$) higher concentrations than the same tissue categories from reproductive tillers. In bulk undissected herbage, peramine concentrations were also higher in vegetative tillers compared to reproductive tillers (Figure 4), particularly in the basal (lower) category.

Peramine concentrations in vegetative tillers were significantly ($P < 0.001$)

TABLE 1. PERAMINE CONCENTRATION OF HARVESTED V AND R PERENNIAL RYEGRASS CLONES[a]

	Concentration (μg/g)			
	November 6, 1991[b]		January 14, 1992[b]	
	V clones	R clones	V clones	R clones
Mean (8 plants)	18.6	10.6	16.1	17.6
SEM	2.7	1.8	2.2	2.6
	**			

[a]The root component was excluded from the calculations while the accumulated regrowth of the V clones was included (**$P < 0.01$).
[b]Harvest date.

higher in the second leaf compared to the third leaf, although there were no differences between sheaths and blades (Figure 3B). In the first, second, and third leaf categories from vegetative tillers, concentrations were higher at this harvest (Figure 3B) than at the November 6 harvest (Figure 3A). Concentrations in the upper reproductive tiller component of undissected herbage were also higher than recorded at the previous harvest (Figure 4). On January 14, concentrations were higher in upper reproductive tillers of undissected herbage than in lower reproductive tillers. The plant component containing the greatest quantity of peramine was the seed, which accounted for 75.5% of the total peramine content of the plant (Figure 5B).

Mean Concentration of Peramine in Individual Plants

In late spring, the mean concentration of peramine was higher ($P < 0.01$) in V compared to R clones, while the mean concentrations in V and R clones in mid-summer were similar (Table 1).

DISCUSSION

Plant Component Effects

With the exception of the mid-spring harvest, at which time concentrations were higher in the leaf sheath than in the blade (Figure 1A), there were no consistent differences in peramine concentration between leaf sheaths and leaf blades of vegetative tillers, irrespective of whether the tillers were derived from V or R clones (Figures 1B and C, 3A and B). Unlike lolitrem B, which consistently occurs in higher concentrations in the sheath than in the blade (di Menna et al., 1992; Davies et al., 1993; Keogh and Tapper, 1993; Ball et al., 1997),

peramine appears to be more uniformly distributed throughout the vegetative leaf, although this is likely to be dependent upon time of year (Figure 1A). These results are in general agreement with those of Davies et al. (1993) and Keogh and Tapper (1993), who also measured peramine levels in vegetative tillers. However, in late spring, peramine concentrations in leaves from reproductive tillers were significantly higher in sheaths than in blades (Figure 3A). Thus, it appears that the distribution of peramine in leaves from reproductive tillers is different from that in leaves from vegetative tillers, even if the tillers are derived from the same plant.

Although there was no consistent difference in peramine concentrations between sheaths and blades in leaves from vegetative tillers, there was a consistent effect of leaf age. In all harvests of V and R clones, concentrations in leaves from vegetative tillers decreased as leaf age increased (Figures 1A–C, 3A and B). In most cases, this leaf age effect was maintained through to the youngest (first) leaf, and the dead leaf material (the oldest leaf tissue) consistently had the lowest peramine concentration of any of the components derived from leafy tissues. Leaf age has been shown to have the opposite effect on lolitrem B concentrations (Keogh and Tapper, 1993; Ball et al., 1997). A similar effect of leaf age on peramine concentration in vegetative tillers was previously reported by Davies et al. (1993) and Keogh and Tapper (1993), who harvested their plants in autumn. Assuming that it has arisen through some kind of selection pressure, this peramine concentration gradient probably ensures that young developing leaves receive greater protection against peramine-sensitive invertebrate herbivores. Protection of younger leaves from attack is more important than protection of older leaves, as it ensures the continued growth and persistence of the plant. Although the Argentine stem weevil is sensitive to peramine (Rowan and Gaynor, 1986), it is unlikely to have provided the necessary selection pressure as Argentine stem weevil and perennial ryegrass have coexisted for only a short time (< 100 years). Other invertebrates more likely have provided the selection pressure. Alternatively, it is possible that there is a simple physiological explanation for this gradient, such as the age of the fungus. There was no effect of leaf age on peramine concentrations in leaf sheaths from reproductive tillers, although concentrations in the first blade were significantly ($P < 0.05$) higher than those in the third blade (Figure 3A).

Comparing all components from V or R clones, peramine concentrations were at their highest in the seed (Figure 3B), where they ranged from 40.5 μg/g to 105.4 μg/g in the eight grass genotypes. This is very high compared to the 10–50 μg/g normally found in *N. lolii*-infected perennial ryegrass herbage (Tapper et al., 1989; Fannin et al., 1990; Ball et al., 1995). Studies have indicated that high alkaloid and mycotoxin concentrations in the seed of *N. lolii*-infected perennial ryegrass and in the seed of tall fescue (*Festuca arundinacea* Schreb.) infected with *Neotyphodium coenophialum* (Morgan-Jones and Gams)

Glenn, Bacon, and Hanlin, are a general characteristic (Bush et al., 1982; Gallagher et al., 1982; Ball et al., 1997; O. J.-P. Ball and G. A. Lane, unpublished data). It has been demonstrated that the presence of endophyte, and therefore alkaloid, in perennial ryegrass and tall fescue seed can protect the seed and/or developing seedling against attack from herbivores (Stewart, 1985; Madej and Clay, 1991; Knoch et al., 1993), possibly explaining the high seed concentrations of peramine observed in this study.

In relation to other plant components, peramine concentrations were also high in the first leaf, second and third leaf sheaths and blades from vegetative tillers, and, with the possible exception of the mid-spring harvest, the regrowth (Figures 1A–C, 3A and B). Although there is variability, reproductive stem and inflorescence components generally contained intermediate concentrations, while the lowest concentrations were recorded in the roots (where it was often not detectable), crown, and dead leaf tissues. Low peramine concentrations in root and senescent leaf tissue have also been reported by Fannin et al. (1990) and Davies et al. (1993), respectively. If peramine proved to be a relatively broad-spectrum insect deterrent, the scarcity of this alkaloid in roots could have major implications on the vulnerability of this tissue (and therefore the plant) to attack from soil-borne invertebrate herbivores. However, apart from the Argentine stem weevil (Prestidge et al., 1982), the greenbug (*Schizaphis graminum* Rondani) (Siegel et al., 1990), and to a lesser extent the cutworm (*Graphania mutans* Walker) (Dymock et al., 1988), an adverse effect of peramine has not been demonstrated on other insects, although the chemical basis of the endophyte-mediated resistance against most insects has not been studied.

Several factors could determine the concentrations of peramine in different plant components. Since peramine is synthesized by the endophyte, the *N. lolii* concentration of the plant component may be important. However, with the exception of the seed, which contained very high concentrations of peramine (Figure 3B) and *N. lolii* (Ball, 1994), there appears to be little correlation between these two parameters in most plant components (Ball, 1994), even though such a relationship exists at the whole-tiller level (Ball et al., 1995). This suggests that translocation [as it is hydrophilic (Rowan and Gaynor, 1986), peramine is thought to be highly translocatable] and/or rate of degradation and synthesis probably play major roles in determining the observed distribution. For example, this study indicates that there may be a net translocation from older to younger vegetative leaves. Alternately, the rates of peramine synthesis and degradation may occur more rapidly in young and old leaves, respectively (Figures 1–3, 5). It also appears that peramine is translocated from the leaf sheath to the leaf blade of vegetative tillers (Figures 1B and C, 3A and B).

The data indicate that the eight different plant/endophyte genotypes contained different concentrations of peramine and that these relative differences were generally maintained over the different plant components, harvest dates,

and plant management systems. Genotype differences in peramine concentration, which were maintained over three years, have been observed previously (Ball et al., 1995). Regression analysis also showed that, with the exception of the V clones harvested in late spring, peramine concentrations in the eight plant/endophyte genotypes were highly correlated with lolitrem B concentrations in the same plants. Such a correlation also has been observed previously (Ball et al., 1995).

Plant Reproductive Status Effects

Plant reproductive development was often associated with a decline in peramine concentration, particularly in late spring. For example, concentrations in late spring and mid-summer were consistently higher in components associated with vegetative tillers (i.e., leaf blades and sheaths, dead leaf, and crown) compared to corresponding components on reproductive tillers from the same plants (Figure 3A and B), and in late spring, concentrations in the first, second, and third leaves of V clones were significantly higher than those in vegetative tillers of R clones (Figures 1B and 3A respectively). Furthermore, in whole undissected tillers, peramine concentrations in late spring and mid-summer decresed as tillers became reproductively more mature (Figure 4). The increase in peramine in the upper reproductive tillers in mid-summer compared to late spring (Figure 4) was due almost entirely to the large contribution from the seed component (Figures 3B and 5B). The clearest effect of plant reproductive activity on peramine concentration in late spring can be seen at the individual plant level. The mean concentration of R clones sampled in late spring was nearly half that of V clones (Table 1). Although the accumulated regrowth contained 56.1% of the total peramine present (Figure 2B), the exclusion of this component from calculations did not significantly change the concentration of whole V clones. The mean concentration of V clones calculated on this basis was therefore still significantly higher than the mean concentration of R clones. A greater rate of production of dry matter, a lower rate of peramine synthesis, or a faster rate of peramine degradation by the R clones in relation to the V clones, would explain the differences in peramine concentration between V and R clones. However, it is difficult to draw conclusions as these three parameters were not monitored during the experiment.

Peramine concentrations did not always decline as a result of plant reproductive development. For example, the highest concentrations in any plant component were recorded in the seed (Figure 3B), and concentrations in vegetative tillers from V and R clones harvested in mid-summer were similar (Figures 1C and 3B). Furthermore, by mid-summer, mean peramine concentrations in V and R clones were similar (Table 1). This recovery may be largely attributed to the seed component, which contained 75.5% of the total peramine of the R clones (Figure 5B); concentrations were three times higher ($P < 0.001$) in the V clones

compared to the R clones when the contribution from the seed was removed in calculations.

This study has shown that peramine concentrations vary with plant component, time of year, plant reproductive status, and clipping regime. Concentrations were higher in younger parts of the vegetative tiller than in older parts. In late spring, concentrations were lower when plant reproductive development was allowed to occur. However, in mid-summer, concentrations in reproductive plants were similar to those in plants that had been clipped to maintain them in a vegetative state. The increase in peramine concentration in reproductive plants relative to clipped plants in mid-summer was almost entirely due to the high concentrations of peramine contained in the seed.

Acknowledgments—We thank Drs. Brian A. Tapper and Daryl D. Rowan for their help with the peramine assay, Dr. Neil Cox for his assistance with the statistics, and Ms. Pauline Hunt for her help with the figures.

REFERENCES

BALL, O. J.-P. 1994. Interrelationships between perennial ryegrass (*Lolium perenne*), the endophytic fungus *Acremonium lolii* and black beetle (*Heteronychus arator*). PhD thesis. Department of Biological Sciences, University of Waikato, Hamilton, New Zealand.

BALL, O. J.-P., PRESTIDGE, R. A., and SPROSEN, J. M. 1995. Interrelationships between *Acremonium lolii*, peramine, and lolitrem B in perennial ryegrass. *Appl. Environ. Microbiol.* 61(4):1527–1533.

BALL, O. J.-P., BARKER, G. M., PRESTIDGE, R. A., and SPROSEN, J. M. 1997. Distribution and accumulation of the mycotoxin lolitrem B in *Neotyphodium lolii*-infected perennial ryegrass. *J. Chem. Ecol.* 23:1435–1449.

BUSH, L. P., CORNELIUS, P. L., BUCKNER, R. C., VARNEY, D. R., CHAPMAN, R. A., BURRUS, P. B., KENNEDY, C. W., JONES, T. A., and SAUNDERS, M. J. 1982. Association of *N*-acetyl loline and *N*-formyl loline with *Epichloe typhina* in tall fescue. *Crop Sci.* 22:941–943.

DAVIES, E., LANE, G. A., LATCH, G. C. M., TAPPER, B. A., GARTHWAITE, I., TOWERS, N. R., FLETCHER, L. R., and POWNALL, D. B. 1993. Alkaloid concentrations in field-grown synthetic perennial ryegrass endophyte associations, pp. 72–76, *in* D. E. Hume, G. C. M. Latch, and H. S. Easton (eds.). Proceedings of the Second International Symposium on *Acremonium*/Grass Interactions. AgResearch, Palmerston North, New Zealand.

DI MENNA, M. E., MORTIMER, P. H., PRESTIDGE, R. A., HAWKES, A. D., and SPROSEN, J. M. 1992. Lolitrem B concentrations, counts of *Acremonium lolii* hyphae, and the incidence of ryegrass staggers in lambs on plots of *Acremonium lolii*-infected perennial ryegrass. *N.Z. J. Agric. Res.* 35:211–217.

DYMOCK, J. J., ROWAN, D. D., and MCGEE, I. R. 1988. Effects of endophyte produced mycotoxins on Argentine stem weevil and the cutworm *Graphania mutans*, pp. 35–43, *in* P. P. Stahle (ed.). Proceedings of the Fifth Australasian Conference on Grassland Invertebrate Ecology: Melbourne, Australia. D. and D. Printing, Victoria, Australia.

FANNIN, F. F., BUSH, L. P., SIEGEL, M. R., and ROWAN, D. D. 1990. Analysis of peramine in fungal endophyte-infected grasses by reversed-phase thin layer chromatography. *J. Chromatogr.* 503:288–292.

FLETCHER, L. R. 1993. Grazing ryegrass/endophyte associations and their effect on animal health

and performance, pp. 115–120, *in* D. E. Hume, G. C. M. Latch, and H. S. Easton (eds.). Proceedings of the Second International Symposium on *Acremonium*/Grass Interactions: Plenary Papers. AgResearch, Palmerston North, New Zealand.

GALLAGHER, R. T., SMITH, G. S., DI MENNA, M. E., and YOUNG, P. W. 1982. Some observations on neurotoxin production in perennial ryegrass. *N.Z. Vet. J.* 30:203–204.

GALLAGHER, R. T., HAWKES, A. D., STEYN, P. S., and VLEGGAAR, R. 1984. Tremorgenic neurotoxins from perennial ryegrass causing ryegrass staggers disorder of livestock: Structure elucidation of lolitrem B. *J. Chem. Soc. Chem. Commun. (London)* 1984:614–616.

KEOGH, R. G., and TAPPER, B. A. 1993. *Acremonium lolii*, lolitrem B, and peramine concentrations within vegetative tillers of perennial ryegrass, pp. 81–84, *in* D. E. Hume, G. C. M. Latch, and H. S. Easton (eds.). Proceedings of the Second International Symposium on *Acremonium*/Grass Interactions. AgResearch, Palmerston North, New Zealand.

KNOCH, T. R., FAETH, S. H., and ARNOTT, D. L. 1993. Endophytic fungi alter foraging and dispersal by desert seed-harvesting ants. *Oecologia* 95:470–473.

MADEJ, C. W., and CLAY, K. 1991. Avian seed preference and weight loss experiments: The effect of fungal endophyte-infected tall fescue seeds. *Oecologia* 88:296–302.

PIPER, E. L., and FLETCHER, L. R. 1990. Influence of a dopamine antagonist on ryegrass staggers, pp. 248–250, *in* S. S. Quisenberry and R. E. Joost (eds.). Proceedings of the First International Symposium on *Acremonium*/Grass Interactions. Louisiana Agricultural Experiment Station, Baton Rouge, Louisiana.

POWNALL, D. B., FAMILTON, A. S., FIELD, R. J., FLETCHER, L. R., and LANE, G. A. 1995. The effect of peramine ingestion in pen-fed lambs. *Proc. N.Z. Soc. Anim. Prod.* 55:186.

PRESTIDGE, R. A., POTTINGER, R. P., and BARKER, G. M. 1982. An association of *Lolium* endophyte with ryegrass resistance to Argentine stem weevil. *Proc. N.Z. Weed Pest Control Conf.* 35:119–122.

ROWAN, D. D., and GAYNOR, D. L. 1986. Isolation of feeding deterrents against Argentine stem weevil from ryegrass infected with the endophyte *Acremonium loliae*. *J. Chem. Ecol.* 12:647–658.

SIEGEL, M. R., LATCH, G. C. M., BUSH, L. P., FANNIN, F. F., ROWAN, D. D., TAPPER, B. A., BACON, C. W., and JOHNSON, M. C. 1990. Fungal endophyte-infected grasses: Alkaloid accumulation and aphid response. *J. Chem. Ecol.* 16:3301–3315.

STEEL, R. G. D., and TORRIE, J. H. 1980. Principles and Procedures of Statistics. McGraw-Hill, New York.

STEWART, A. V. 1985. Perennial ryegrass seedling resistance to Argentine stem weevil. *N.Z. J. Agric. Res.* 28:403–407.

TAPPER, B. A., ROWAN, D. D., and LATCH, G. C. M. 1989. Detection and measurement of the alkaloid peramine in endophyte-infected grasses. *J. Chromatogr.* 463:133–138.

WEDDERBURN, M. E., PENGELLY, W. J., TUCKER, M. A., and DI MENNA, M. E. 1989. Description of ryegrass removed from New Zealand North Island hill country. *N.Z. J. Agric. Res.* 32:521–529.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

DISTRIBUTION AND ACCUMULATION OF THE MYCOTOXIN LOLITREM B IN *Neotyphodium lolii*-INFECTED PERENNIAL RYEGRASS

O. J.-P. BALL,[1,*] G. M. BARKER,[2] R. A. PRESTIDGE,[3] and J. M. SPROSEN[3]

[1]*AgResearch, Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand*
[2]*Landcare Research, Private Bag 3127, Hamilton, New Zealand*
[3]*AgResearch, Ruakura Agricultural Research Centre Private Bag 3123 Hamilton, New Zealand*

(Received May 6, 1996; accepted January 8, 1997)

Abstract—The symbiotic relationship between perennial ryegrass and its endophytic fungus, *Neotyphodium lolii*, is of considerable agronomic significance in New Zealand. Livestock ingesting *N. lolii*-infected perennial ryegrass may succumb to a neuromuscular condition, ryegrass staggers, the probable cause of which has been identified as the endophyte-produced indole-diterpenoid mycotoxin lolitrem B. The effects of plant component, plant reproductive development, and plant management (clipping) on the concentration and accumulation of lolitrem B were investigated. Eight different naturally occurring perennial ryegrass–*N. lolii* associations were cloned. They were either trimmed regularly to maintain their vegetative state (V clones) or not trimmed at all to enable full plant reproductive development (R clones). At times that corresponded approximately with the start, middle, and end of the plant reproductive phase, V and R clones of each grass-endophyte association were destructively harvested. After dissection into a number of plant components, harvested material was analyzed for lolitrem B content by HPLC. Analyses were also performed on all clippings taken from the V clones. Highest lolitrem B concentrations in vegetative parts were found in older leaf sheaths and dead leaves. Towards the end of the plant reprodutive phase, dead leaves also contained a large proportion of the total lolitrem B content of the V clones. The highest concentration of lolitrem B was found in the seed, which accounted for nearly 60% of the total quantity in R clones. Leaf blades, young leaf sheaths, roots, and crowns generally contained low to moderate concentrations. In late spring, mean concentrations in R and V clones were similar. By the time mature seed was present (mid-summer), mean concentrations in R clones were three times higher than in V clones. This difference

*To whom correspondence should be addressed.

0098-0331/97/0500-1435$12.50/0 © 1997 Plenum Publishing Corporation

was attributed largely to the contribution by the seed in the R clones. The implications of these findings in relation to ryegrass staggers in livestock are discussed.

Key Words—Lolitrem B, ryegrass staggers, perennial ryegrass, endophyte, *Neotyphodium lolii*, *Acremonium*.

INTRODUCTION

The endophyte of perennial ryegrass (*Lolium perenne* L.), *Neotyphodium lolii* (Latch, Christensen, and Samuels) Glenn, Bacon, and Hanlin, produces many alkaloids and mycotoxins in the host grass. Some of these confer resistance against a number of insect species, and some cause health problems in livestock. The most obvious endophyte-mediated health problem that may afflict livestock is the neuromuscular condition known as ryegrass staggers. This condition occurs in sheep, cattle, horses, and deer. Symptoms of ryegrass staggers can differ slightly between species and have been described in detail by Cunningham and Hartley (1959). Although mortality is generally low, and the condition is considered more a nuisance than an economic concern, the financial cost to the individual farmer in areas prone to ryegrass staggers may be substantial (Everest, 1983; Hewitt, 1983).

The mycotoxin lolitrem B, a substituted indole–diterpenoid, was identified as a compound likely to be important in the etiology of ryegrass staggers (Gallagher et al., 1984). Although the emphasis of recent research has largely centered on removing the lolitrem B-producing ability of *N. lolii*, it is important to study the dynamics of the major endophyte-associated toxins such as lolitrem B in order to gain a fuller understanding of the symbiosis between host plant and endophyte. Research also has shown that lolitrem B is toxic to larvae of the Argentine stem weevil [*Listronotus bonariensis* (Kuschel)] (Prestidge and Gallagher, 1985; Dymock et al., 1989), a serious pest of New Zealand pastures, indicating a possible useful role of lolitrem B in the control of this insect. This paper reports on a study that examined the effects of plant component, plant reproductive development, and plant management (clipping) on the concentration and accumulation of lolitrem B in *N. lolii*-infected perennial ryegrass. The distribution of the alkaloid peramine, an important insect deterrent, is reported in Ball et al. (1997).

METHODS AND MATERIALS

A detailed description of the experimental methodology, with the exception of the lolitrem B HPLC analysis, can be found in Ball et al. (1997). Briefly, eight genetically different naturally occurring *N. lolii*-infected perennial ryegrass

plants (genotypes) were each split into five approximately equal-sized clones. These were planted in mid-winter (July 7, 1991) and maintained outdoors at the Ruakura Agricultural Research Centre, Hamilton, New Zealand. After September 16, 1991 (time zero for the experiment), a regular clipping regime to a stubble height of 8 cm was continued for the remainder of the experiment for three of the five clones of each genotype. Since the clipping regime prevented their becoming reproductive, these plants are termed V (vegetative) clones. After September 16, 1991, all grass trimmings (regrowth) were retained for chemical analysis. The remaining two clones were not trimmed any further, allowing full reproductive development to occur, and are termed R (reproductive) clones.

On October 1, 1991 (mid-spring), November 6, 1991 (late spring), and January 14, 1992 (mid-summer), one of the three V clones of each grass genotype was destructively harvested. These times corresponded approximately to the start, middle, and end of the plant reproductive phase. Harvested plants were trimmed, and each tiller dissected into the following components: second, third, and fourth leaf sheaths; second, third, and fourth leaf blades; first leaf (not split into sheath and blade); dead leaf; reproductive stem tissue; crown; and root. The fourth leaf refers to the oldest (outermost) leaf and the first leaf refers to the youngest (innermost). There were no fourth leaf components in the mid-summer harvest.

One R clone of each grass genotype was destructively harvested on November 6, 1991 (late spring) and January 14, 1992 (mid-summer). Tillers from plants harvested on November 6, 1991 were classed as either vegetative, reproductive, or intermediate. Most vegetative tillers from November 6, 1991, were dissected as described for the October and November harvests of the V clones. Root material from vegetative, reproductive, and intermediate tillers was pooled. Most reproductive tillers from November 6, 1991, were dissected into the following components: first, second, and third leaf sheaths and blades; inflorescence; dead leaf; lower reproductive stem (reproductive stem excluding leaf sheath material up to the end of the second to last internode); upper reproductive stem (the last internode before the inflorescence); crown; and root. Undissected vegetative and reproductive tillers, as well as all intermediate tillers, were trimmed to a height of 8 cm to form lower and upper vegetative, reproductive, and intermediate tiller categories, respectively. Vegetative tillers from R clones harvested on January 14, 1992, were dissected into the same components, except there were no fourth leaf components and no intermediate tillers. Reproductive tillers were dissected into seed, seed head (minus seed), lower reproductive stem, upper reproductive stem, dead leaf, crown, and lower and upper reproductive tiller categories. By January 14, 1992, there was no green leaf material on the reproductive tillers. A mixed dead leaf category was included for material that could not be assigned to vegetative or reproductive tillers.

Freeze-dried and finely ground plant samples were analyzed for lolitrem B content by a modification of the Gallagher et al. (1985) HPLC methodology described in Ball et al. (1995).

Least significant ratios (LSR) (Steel and Torrie, 1980) at the 5% level are presented with all figures. A number exceeding the LSR, after dividing the larger value by the smaller value, indicates that the comparison is significantly different. Statistical analyses used and the calculations made are as described in Ball et al. (1997).

RESULTS

Large genotype differences in lolitrem B concentration were observed. These generally were maintained regardless of plant component, harvest date, and clipping regime.

V Clones

Mid-Spring Harvest (October 1). Lolitrem B concentrations were low in the root, regrowth, and leaf blade components, and high in the third and fourth leaf sheaths and dead leaf (Figure 1A). Leaf sheath concentrations were significantly ($P < 0.001$) higher than those in corresponding leaf blades, while concentrations in leaf sheath 4 were significantly ($P < 0.05$) higher than in leaf sheath 2. The largest quantities were contained in the third and fourth leaf sheaths (Figure 2A).

Late-Spring Harvest (November 6). The highest concentrations of lolitrem B were found in the third and fourth leaf sheaths and dead leaf (Figure 1B); root and regrowth contained the lowest. In addition to leaf sheaths containing significantly ($P < 0.001$) higher concentrations than the leaf blades, older leaves contained significantly ($P < 0.001$) higher concentrations than younger leaves. The highest quantities were recorded in the dead leaf component, while the accumulated regrowth, reproductive stem, and third and fourth leaf sheaths also contained relatively high quantities (Figure 2B).

Mid-Summer Harvest (January 14). The third leaf sheath and dead leaf components contained the highest concentrations of lolitrem B, while the lowest were in the root, first leaf, and regrowth components (Figure 1C). As leaf age increased, concentrations also increased ($P < 0.05$) and were significantly ($P < 0.001$) higher in the leaf sheath than the leaf blade. Dead leaf contained almost half (49.5%) the lolitrem B present in the whole plant, although the accumulated regrowth component also contained a relatively high proportion (Figure 2C).

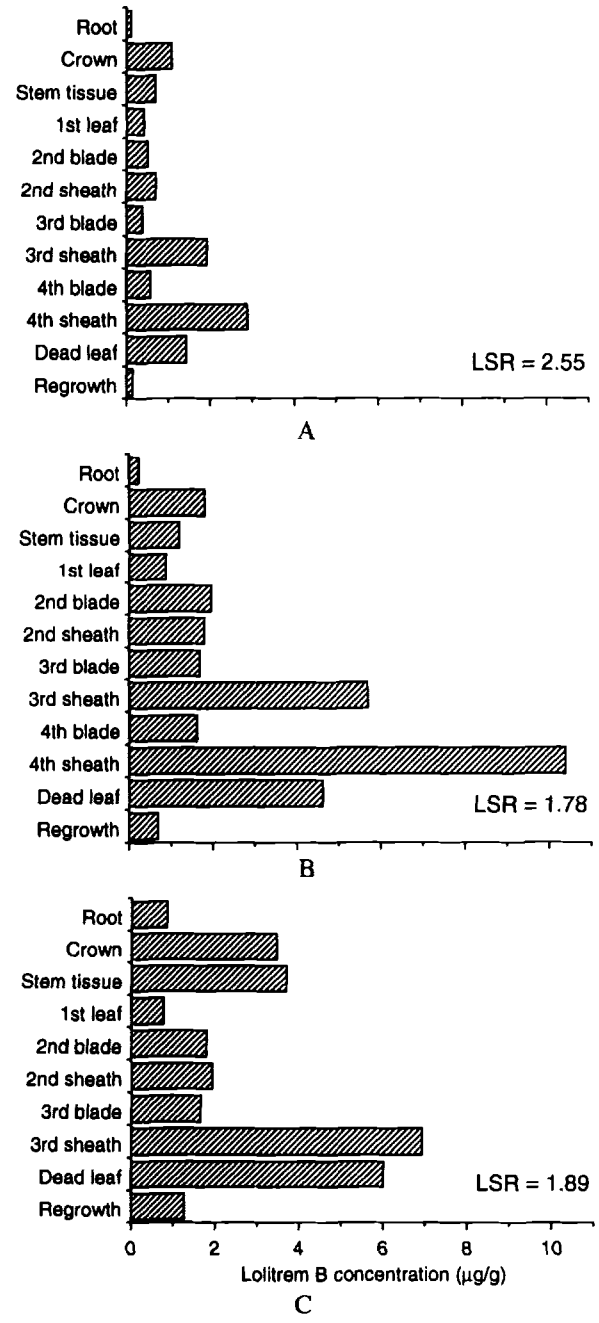


FIG. 1. Concentrations of lolitrem B in different components of *N. lolii*-infected V perennial ryegrass plants sampled on October 1, 1991 (A), November 6, 1991 (B), and January 14, 1992 (C) (N = 8 plants).

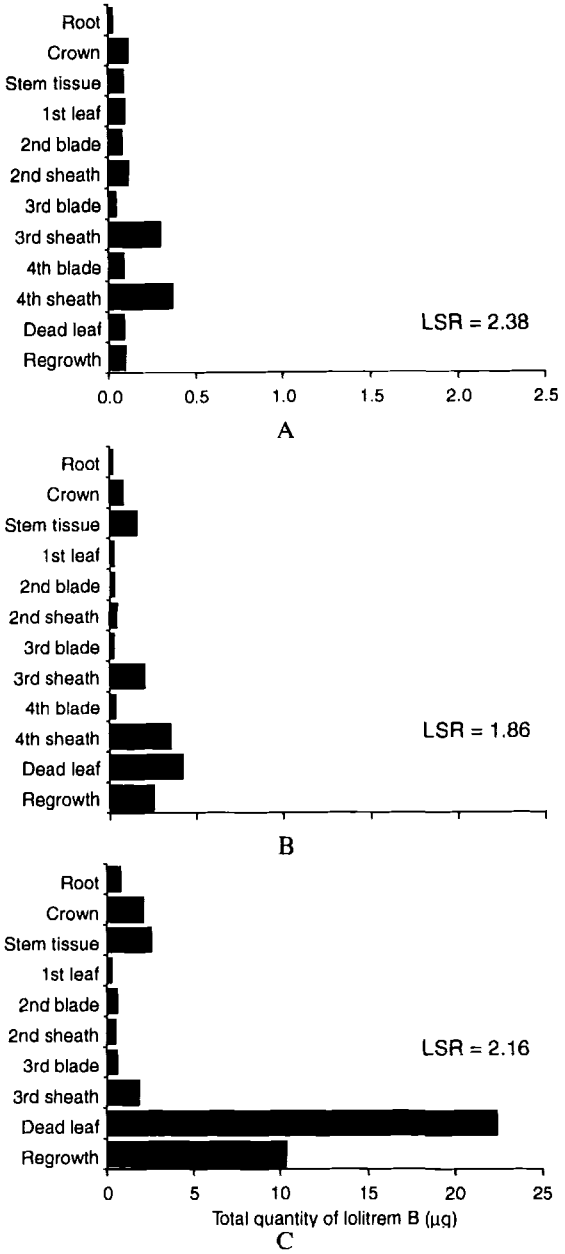


FIG. 2. Total quantities of lolitrem B in different components of *N. lolii*-infected V perennial ryegrass plants sampled on October 1, 1991 (A), November 6, 1991 (B), and January 14, 1992 (C) (N = 8 plants). Lolitrem B removed during the clipping regime prior to the destructive harvest of the plants is included in the regrowth category.

R Clones

Late-Spring Harvest (November 6). Lolitrem B concentrations were low in the root, leaf blades from reproductive tillers, and first leaf and second leaf sheath from vegetative tillers and were high in the fourth leaf sheath and dead leaf from vegetative tillers (Figure 3A). Herbage from the lower part of whole undissected vegetative, intermediate, and reproductive tillers contained higher concentrations than herbage from the upper portions (Figure 4). In older leaves, concentrations were higher in sheaths and blades from vegetative tillers than in the corresponding tissues from reproductive tillers (Figure 3A). In leaves from vegetative tillers, concentrations increased ($P < 0.001$) with increasing leaf

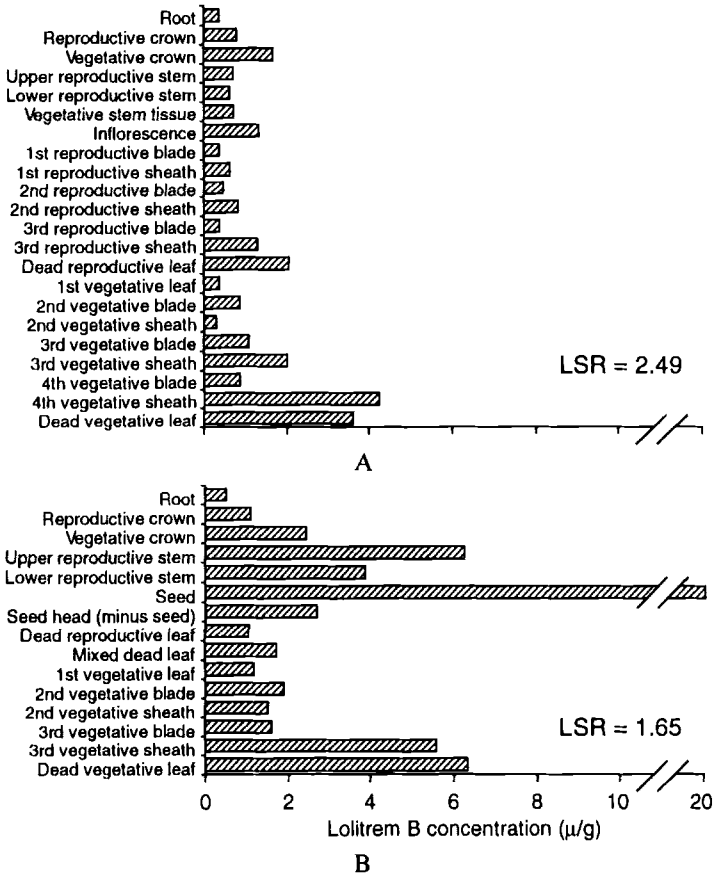


FIG. 3. Concentrations of lolitrem B in different components of vegetative and reproductive tillers from *N. lolii*-infected R ryegrass plants sampled on November 6, 1991 (A), and January 14, 1992 (B) ($N = 8$ plants).

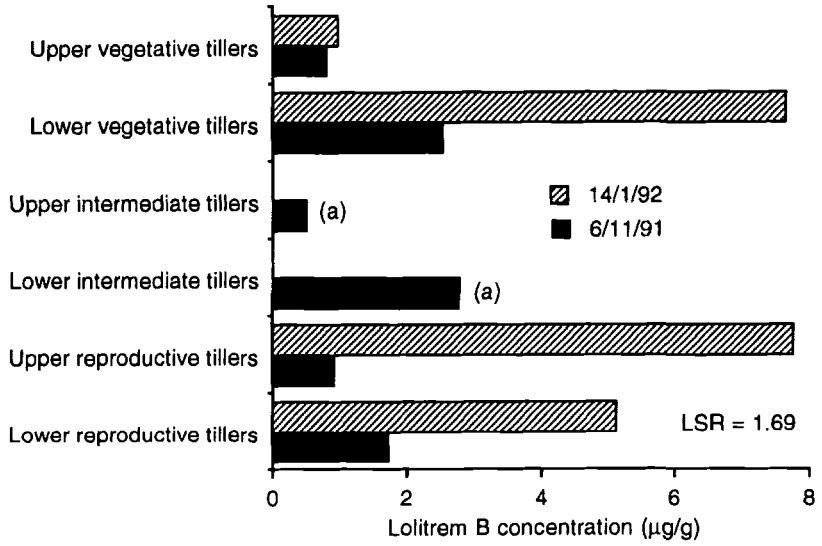


FIG. 4. Lolitrem B concentrations in lower (from ground level to 8 cm) and upper (above 8 cm) components of vegetative, intermediate, and reproductive tillers from *N. lolii*-infected R ryegrass plants sampled on November 6, 1991, and January 14, 1992 (N = 8 plants). (a) denotes that no intermediate tillers were present on January 14, 1992.

age. There was no such effect in leaves from reproductive tillers. Leaf sheaths from both vegetative and reproductive tillers contained significantly higher ($P < 0.05$ and $P < 0.01$ respectively) concentrations than corresponding leaf blades. The inflorescence had the greatest amount (Figure 5A). The lower reproductive stem and dead leaf and the fourth leaf sheath components from vegetative tillers also contained appreciable quantities.

Mid-Summer Harvest (January 14). The seed contained the highest concentration of lolitrem B (Figure 3B). High concentrations were also present in the reproductive stem from reproductive tillers and in dead leaf and third leaf sheath from vegetative tillers. The lowest concentrations were present in roots, dead leaf, and crown from reproductive tillers and in first leaf from vegetative tillers. Amounts were significantly higher in the crown ($P < 0.01$) and dead leaf ($P < 0.001$) tissues from vegetative compared to reproductive tillers. In general, lolitrem B concentrations in undissected vegetative and reproductive tillers rose substantially between the late-spring and mid-summer harvests (Figure 4). The seed component contained the greatest quantity, accounting for 59.3% of the total content of the plant (Figure 5B).

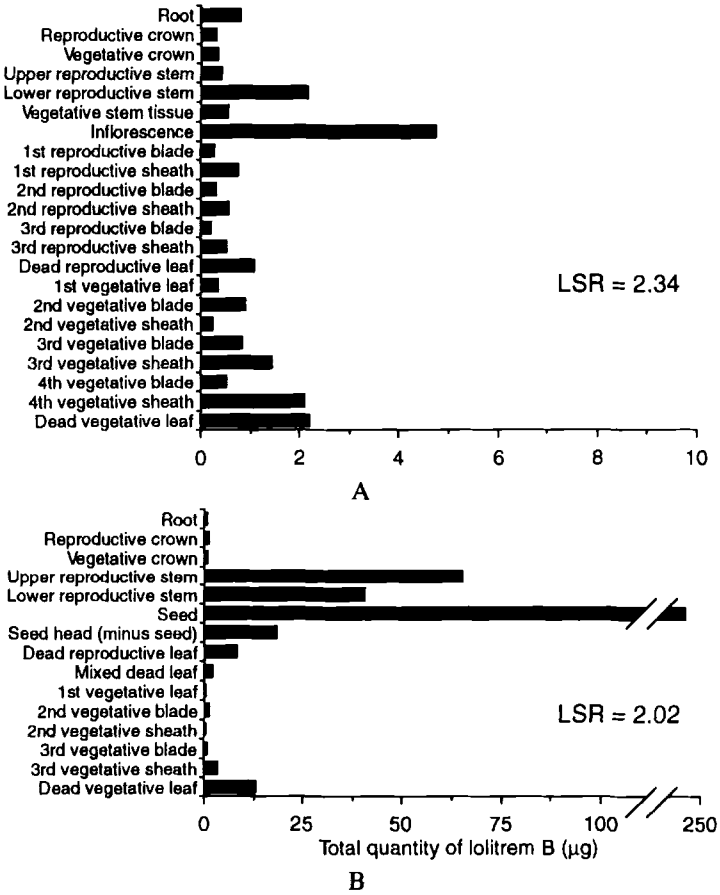


FIG. 5. Total quantities of lolitrem B in different components of vegetative and reproductive tillers from *N. lolii*-infected R ryegrass plants sampled on November 6, 1991 (A), and January 14, 1992 (B) (N = 8 plants).

Mean Concentration of Lolitrem B in Individual Plants

In late spring, mean concentrations of lolitrem B in V and R clones were similar, while in mid-summer, the mean concentration of R clones was greater than V clones (Table 1).

DISCUSSION

The large genotype differences in lolitrem B recorded in this study have been observed previously with this and other mycotoxins and alkaloids in both

TABLE 1. LOLITREM B CONCENTRATION OF HARVESTED V AND R PERENNIAL RYEGRASS CLONES[a]

	Concentration (μg/g)			
	November 6, 1991[b]		January 14, 1992[b]	
	V clones	R clones	V clones	R clones
Mean (8 plants)	1.5	1.2	2.2	7.0
SEM	0.2	0.2	0.4	0.8
			***	

[a]The root component has been excluded from the calculations, while the accumulated regrowth of the V clones is included (***$P < 0.001$).
[b]Harvest date.

N. lolii-infected perennial ryegrass and *N. coenophialum*-infected tall fescue (Belesky et al., 1989; Davies et al., 1993; Agee and Hill, 1994; Ball et al., 1995). As observed with peramine (Ball et al., 1997), genotype differences were generally maintained within plant components, harvest dates, and clipping regimes.

Plant Component Effects

In older leaves (third and fourth), lolitrem B concentrations were consistently higher in leaf sheaths compared to leaf blades of vegetative tillers, irrespective of whether the tillers were derived from R or V clones (Figures 1A–C, 3A and B). The total amount was also higher in older leaf sheaths of vegetative tillers (Figures 2A–C, 5A and B). A similar distribution has been reported previously for lolitrem B in vegetative tillers (Gallagher et al., 1982, 1985, 1987; di Menna et al., 1992; Davies et al., 1993; Keogh and Tapper, 1993). The same difference in concentration and quantity between sheath and blade was also observed in leaves from reproductive tillers (Figures 3A and 5A). *N. lolii* concentrations are higher in sheaths than blades of both vegetative (Musgrave, 1984) and reproductive (Ball, 1994) tillers, suggesting a possible explanation for the similar distribution of lolitrem B. *N*-formyl loline and *N*-acetyl loline alkaloids (Bush et al., 1993), as well as ergovaline, (Rottinghaus et al., 1991) in tall fescue (*Festuca arundinacea* Schreb.) infected with *Neotyphodium coenophialum* (Morgan-Jones and Grams) Glenn, Bacon and Hanlin are also higher in sheaths than blades.

Lolitrem B concentrations in sheaths from vegetative and reproductive tillers increased with age (Figures 1A–C, 3A and B). A similar distribution pattern was reported by Gallagher et al. (1987) and Keogh and Tapper (1993) (although

only for vegetative tillers) and is opposite to the situation with peramine, the concentration of which decreases with age (Ball et al., 1997). In vegetative, and sometimes reproductive tillers, the oldest leaf component (dead leaf) also contained relatively high concentrations of lolitrem B (e.g., Figures 1C and 3A), suggesting that it is unlikely that much, if any, lolitrem B was translocated from the dying leaf into the living plant, as may be the case with peramine (Ball et al., 1997). This indicates that either lolitrem B accumulates over time or that the production increases towards the end of the life of the endophyte and/or plant tissue. There was no effect of leaf age on lolitrem B concentrations in leaf blades.

In relation to all plant components, lolitrem B concentrations in V and R plants were consistently highest in seed and in dead leaf and outermost leaf sheaths from vegetative tillers. With a mean concentration of 20.0 μg/g, more than three times higher than in any other component, seed contained particularly high amounts (Figure 3B). Seed also contained very high concentrations of peramine (Ball et al., 1997). Additionally, Bush et al. (1982), found that *N*-acetyl loline and *N*-formyl loline concentrations in *N. coenophialum*-infected tall fescue were higher in seed than in vegetative tissue. High mycotoxin and alkaloid concentrations in seed may have arisen as a defense against seed- and/or seedling-eating herbivores (Stewart, 1985; Knoch et al., 1993). It is also possible that high toxin concentrations in seed are simply a function of the large quantity of *N. lolii* mycelium that resides there (Musgrave, 1984; Ball, 1994). The crown, inflorescence, and reproductive stem generally contained intermediate concentrations of lolitrem B, while the lowest were recorded in roots, first vegetative leaf, leaf blades from vegetative and reproductive tillers, and the regrowth from vegetative plants.

There may be several factors that control lolitrem B concentrations and distribution. The lipophilic character suggests that translocation is not important. However, transportation via the phloem cannot be ruled out and may explain the presence in roots. As discussed, the age of the plant tissue appears to be significant, with older herbage often containing higher concentrations than younger. High concentrations in seed and higher concentrations in sheaths compared to blades were accompanied by correspondingly high concentrations of *N. lolii* (Ball, 1994), indicating that endophyte concentration is also likely important.

Plant Reproductive Status Effects

Examination of individual plant components in late spring indicates that lolitrem B concentrations generally declined with reproductive development. For example, components from vegetative tillers of R clones sampled in late spring contained lower concentrations than the same components from V clones sampled at the same time (Figures 3A and 1B, respectively). Furthermore, in late

spring, concentrations in R clones were lower in structures associated with reproductive tillers compared to corresponding structures from vegetative tillers (Figure 3A). This has been observed previously with *N*-acetyl loline and *N*-formyl loline in *N. coenophialum*-infected tall fescue (Bush et al., 1993). However, due to large differences in the quantity of lolitrem B in various plant components (Figures 2B and 5A), this trend was not apparent at the whole tiller and whole plant levels. In late spring, there were no differences in concentrations between whole vegetative, intermediate, and reproductive tillers (Figure 4), and concentrations in whole V and R clones were similar (Table 1).

By mid-summer, leaf sheath and blade lolitrem B concentrations in vegetative tillers of R clones were similar to those found in V clones (Figures 3B and 1C, respectively). At this time, there also was no significant difference in concentration between whole lower reproductive tillers and whole lower vegetative tillers from R clones (Figure 4). However, lolitrem B concentrations in whole upper reproductive tillers were substantially higher than those in whole upper vegetative tillers (Figure 4), and mean concentrations in R clones were three times higher than in V clones (Table 1). This was due primarily to the seed, which contributed nearly 60% of the total content (Figures 3B and 5B). However, the seed component was not solely responsible for the increase in R clones. Calculations showed that lolitrem B concentrations in R clones were still significantly ($P < 0.02$) higher than in V clones when the lolitrem B accounted for in the seed of the R clones was removed. The upper and lower reproductive stem components are also likely to have contributed to the higher concentrations in R clones (Figures 3B and 5B). In contrast, plant reproductive development had a different effect on overall peramine concentrations. In late spring, whole plant peramine concentrations were significantly lower in R than V clones, while they were similar in mid-summer (Ball et al., 1997).

Implications for Livestock Management

As Keogh (1973, 1978) and Keogh and Tapper (1993) have reported, knowing that lolitrem B concentrations are high both near the base of the vegetative plant and in the outermost leaves is important in terms of devising management strategies for avoiding ryegrass staggers in livestock. These authors have identified simple grazing management practices, such as shifting stock daily (which enables animals to avoid the higher lolitrem B concentrations in the basal tissues) and spelling pastures for short periods (to maintain them at a young leafy stage), which could, in some situations, be effective in reducing the incidence of ryegrass staggers.

The results of the present study may help to identify further the factors that are important in the etiology of ryegrass staggers. It is important to note that the conditions under which the current experiment was performed are different from those that may be encountered in grazed mixed-species pastures. Further-

more, the time of year that the study was undertaken did not include the period during which ryegrass staggers outbreaks tend to be most prevalent (February–April). With these experimental limitations in mind, it is interesting to note that although the outer leaf sheath in the V clones sampled in mid-summer contained the highest lolitrem B concentrations (Figure 1C), the absolute quantity contained in that component was relatively small (Figure 2C). The largest quantity was in the dead leaf (Figure 2C), indicating that this component, rather than the mature leaf sheaths, may become more important as the ryegrass staggers season approaches. An increase in the incidence of ryegrass staggers in lambs has been observed following an increase in dead matter content of pasture after herbicide treatment (Fletcher and Piper, 1990). The large amount of lolitrem B in the accumulated regrowth (Figure 2C) was present in a large quantity of herbage (i.e., is very dilute) and was produced over a long period of time, suggesting that the regrowth component would probably be of less importance in ryegrass staggers.

The observations that serious outbreaks of staggers occurred in animals grazing ryegrass allowed to bolt with seed head formation (Mortimer and di Menna, 1985) and that the incidence of staggers was significantly higher on untopped (flowering stems present) compared to topped (flowering stems removed) subplots (di Menna et al., 1992) are also interesting in the context of the present study. Prestidge and Gallagher (1988) and di Menna et al. (1992) suggested that a lolitrem B concentration of more than 2.0–2.5 μg/g was required for ryegrass staggers symptoms to develop in lambs. In the mid-summer harvest, concentrations in most parts of the R clones (equivalent to the untopped herbage), particularly in the upper reproductive tiller component, which includes the seed (Figure 4), were well in excess of this figure and considerably more than those in the V clones (equivalent to the topped herbage). Thus, the reproductive state of the ryegrass plant in the pasture may affect the amount of lolitrem B ingested by livestock and hence the incidence of early season staggers. The study of di Menna et al. (1992) and the results of the current experiment indicate that short spelling periods (which are also recommended to reduce the amount of mature and dead sheath material) are desirable, as the lolitrem B increase associated with stalk and seed head development could be largely avoided. However, detailed field experiments are required to confirm these recommendations.

Acknowledgments—We thank Dr. Neil Cox for his assistance with the statistics, and Ms. Pauline Hunt for her help with the figures.

REFERENCES

AGEE, C. S., and HILL, N. S. 1994. Ergovaline variability in *Acremonium*-infected tall fescue due to environment and plant genotype. Crop. Sci. 34:221–226.

BALL, O. J.-P. 1994. Interrelationships between perennial ryegrass (*Lolium perenne*), the endophytic fungus *Neotyphodium lolii* and black beetle (*Heteronychus arator*). PhD thesis. Department of Biological Sciences, University of Waikato, Hamilton, New Zealand.

BALL, O. J.-P., PRESTIDGE, R. A., and SPROSEN, J. M. 1995. Interrelationships between *Acremonium lolii*, peramine, and lolitrem B in perennial ryegrass. *Appl. Environ. Microbiol.* 61(4):1527–1533.

BALL, O. J.-P., BARKER, G. M., PRESTIDGE, R. A., and LAUREN, D. R. 1997. Distribution and accumulation of the alkaloid peramine in *Neotyphodium lolii*-infected perennial ryegrass. *J. Chem. Ecol.* 23:1419–1434.

BELESKY, D. P., STRINGER, W. C., and PLATTNER, R. D. 1989. Influence of endophyte and water regime upon tall fescue accessions. II. Pyrrolizidine and ergopeptine alkaloids. Ann. Bot. 64:343–349.

BUSH, L. P., CORNELIUS, P. L., BUCKNER, R. C., VARNEY, S. R., CHAPMAN, R. A., BURRUS, II, P. B., KENNEDY, C. W., JONES, T. A., and SAUNDERS, M. J. 1982. Association of *N*-acetyl loline and *N*-formyl loline with *Epichloe typhina* in tall fescue. Crop Sci. 22:941–943.

BUSH, L. P., FANNIN, F. F., SIEGEL, M. R., DAHLMAN, D. L., and BURTON, H. R. 1993. Chemistry, occurrence and biological effects of saturated pyrrolizidine alkaloids associated with endophyte–grass interactions. Agric. Ecosyst. Environ. 44:81–102.

CUNNINGHAM, I. J., and HARTLEY, W. J. 1959. Ryegrass staggers. *N.Z. Vet J.* 7:1–7.

DAVIES, E., LANE, G. A., LATCH, G. C. M., TAPPER, B. A., GARTHWAITE, I., TOWERS, N. R., FLETCHER, L. R., and POWNALL, D. B. 1993. Alkaloid concentrations in field-grown synthetic perennial ryegrass endophyte associations, pp. 72–76, *in* D. E. Hume, G. C. M. Latch, and H. S. Easton (eds.). Proceedings of the Second International Symposium on *Acremonium*/Grass Interactions. AgResearch, Palmerston North, New Zealand.

DI MENNA, M. E., MORTIMER, P. H., PRESTIDGE, R. A., HAWKES, A. D., and SPROSEN, J. M. 1992. Lolitrem B concentrations, counts of *Acremonium lolii* hyphae, and the incidence of ryegrass staggers in lambs on plots of *Acremonium lolii*-infected perennial ryegrass. *N.Z. J. Agric. Res.* 35:211–217.

DYMOCK, J. J., PRESTIDGE, R. A., and ROWAN, D. D. 1989. The effects of lolitrem B on Argentine stem weevil larvae. *Proc. N.Z. Weed Pest Control Conf.* 42:73–75.

EVEREST, P. G. 1983. Ryegrass staggers: An overview of the north Canterbury situation and possible costs to the farmer. *Proc. N.Z. Grassl. Assoc.* 44:228–229.

FLETCHER, L. R., and PIPER, E. 1990. Some factors besides *Acremonium lolii* which influence ryegrass staggers in grazing stock, pp. 216–220, *in* S. S. Quisenberry and R. E. Joost (eds.). Proceedings of the First International Symposium on *Acremonium*/Grass Interactions. Louisiana Agricultural Experiment Station, Baton Rouge, Louisiana.

GALLAGHER, R. T., SMITH, G. S., DI MENNA, M. E., and YOUNG, P. W. 1982. Some observations on neurotoxin production in perennial ryegrass. *N.Z. Vet. J.* 30:203–204.

GALLAGHER, R. T., HAWKES, A. D., STEYN, P. S., and VLEGGAAR, R. 1984. Tremorgenic neurotoxins from perennial ryegrass causing ryegrass staggers disorder of livestock: Structure elucidation of lolitrem B. *J. Chem. Soc. Chem. Commun. (London)* 1984:614–616.

GALLAGHER, R. T., HAWKES, A. D., and STEWART, J. M. 1985. Rapid determination of the neurotoxin lolitrem B in perennial ryegrass by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr.* 321:217–226.

GALLAGHER, R. T., SMITH, G. S., and SPROSEN, J. M. 1987. The distribution and accumulation of lolitrem B neurotoxin in developing perennial ryegrass plants. *Proc. A.A.A.P. Anim. Sci. Cong.* 4:404.

HEWETT, R. L. 1983. Breeding for resistance to ryegrass staggers, the inflation-proof long-term solution? *Proc. N.Z. Grassl. Assoc.* 44:244–247.

KEOGH, R. G. 1973. Induction and prevention of ryegrass staggers in grazing sheep. *N.Z. J. Exp. Agric.* 1:55–57.

KEOGH, R. G. 1978. Development and control of ryegrass staggers in sheep. *Proc. N.Z. Soc. Anim. Prod.* 38:47–52.

KEOGH, R. G., and TAPPER, B. A. 1993. *Acremonium lolii*, lolitrem B, and peramine concentrations within vegetative tillers of perennial ryegrass, pp. 81–84, *in* D. E. Hume, G. C. M. Latch and H. S. Easton (eds.). Proceedings of the Second International Symposium on *Acremonium*/Grass Interactions. AgResearch, Palmerston North, New Zealand.

KNOCH, T. R., FAETH, S. H., and ARNOTT, D. L. 1993. Endophytic fungi alter foraging and dispersal by desert seed-harvesting ants. *Oecologia* 95:470–473.

MORTIMER, P. H., and DI MENNA, M. E. 1985. Interactions of *Lolium* endophyte on pasture production and perennial ryegrass staggers disease, pp. 149–158, *in* J. Lacey (ed.). Trichothecenes and Other Mycotoxins. Wiley, New York.

MUSGRAVE, D. R. 1984. Detection of an endophytic fungus of *Lolium perenne* using enzyme-linked immunosorbent assay (ELISA). *N.Z. J. Agric. Res.* 27:283–288.

PRESTIDGE, R. A., and GALLAGHER, R. T. 1985. Lolitrem B—a stem weevil toxin isolated from *Acremonium*-infected ryegrass. *Proc. N.Z. Weed Pest Control Conf.* 38:38–40.

PRESTIDGE, R. A., and GALLAGHER, R. T. 1988. *Acremonium* endophyte in perennial ryegrass, ryegrass staggers in lambs, and growth rate of Argentine stem weevil larvae, pp. 229–235, *in* P. P. Stahle (ed.). Proceedings of the Fifth Australasian Conference on Grassland Invertebrate Ecology: Melbourne, Australia. D and D Printing, Victoria, Australia.

ROTTINGHAUS, G. E., GARNER, G. B., CORNELL, C. N., and ELLIS, J. L. 1991. HPLC method for quantitating ergovaline in endophyte-infested tall fescue: Seasonal variation of ergovaline levels in stems with leaf sheaths, leaf blades, and seed heads. J. Agric. Food Chem. 39:112–115.

STEEL, R. G. D., and TORRIE, J. H. 1980. Principles and Procedures of Statistics. McGraw-Hill, New York.

STEWART, A. V. 1985. Perennial Ryegrass seedling resistance to Argentine stem weevil. *N.Z. J. Agric. Res.* 28:403–407.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

WOUND ACTIVATION OF PROTOXINS IN MARINE SPONGE *Aplysina aerophoba*

RAINER EBEL,[1] MARKO BRENZINGER,[1] ARNO KUNZE,[1] HANS J. GROSS,[2] and PETER PROKSCH[1,*]

[1]*Julius-von-Sachs-Institut für Biowissenschaften*
Universität Würzburg
Mittlerer Dallenbergweg 64
D-97082 Würzburg, Germany

[2]*Institut für Biochemie*
Universität Würzburg,
Biozentrum, Am Hubland
D-97074 Würzburg, Germany

(Received July 29, 1996; accepted January 8, 1997)

Abstract—The marine sponge *Aplysina aerophoba* accumulates brominated isoxazoline alkaloids, which include aerophobin-2, aplysinamisin-1, and isofistularin-3 as major constituents. Following disruption of compartmentalization, the isoxazoline alkaloids are enzymatically converted to aeroplysinin-1, which in turn gives rise to a dienone. The described bioconversions were demonstrated for the first time in vitro using an enzyme preparation from *A. aerophoba*. Cell-free extracts of other *Aplysina* species were capable of performing the same bioconversions, whereas enzyme extracts of sponges from other orders, which lack isoxazoline alkaloids, were inactive with regard to the reactions analyzed. These findings suggest that the enzyme activities studied are linked to the accumulation of suitable substrates and hence represent a specific biochemical property of sponges from the genus *Aplysina*. Time-course experiments with *A. aerophoba*, performed in situ, demonstrated that wound-induced bioconversions of isoxazoline alkaloids proceeded rapidly. Within 40 sec after mechanical damage of a tube of *A. aerophoba*, both aerophobin-2 and aplysinamisin-1 were completely converted to the dienone. The wound activation of protoxins results in a pronounced increase of the fish deterrent activity of *A. aerophoba* as shown in bioassays employing the common Caribbean wrasse *Thalassoma bifasciatum*.

Key Words—*Aplysina aerophoba*, sponges, natural products, bioconversion, fish deterrency, chemical defense.

*To whom correspondence should be addressed.

0098-0331/97/0500-1451$12.50/0 © 1997 Plenum Publishing Corporation

INTRODUCTION

Marine organisms are rich sources of bioactive natural products that often have unique structural features unprecedented in the terrestrial environment (Attaway and Zaborsky, 1993). The frequent occurrence of bioactive compounds, especially in sessile or slow-moving marine invertebrates such as sponges, bryozoans, or holothurians, is usually interpreted as chemical defense that protects these soft-bodied organisms against biotic stress factors such as predation, infection by pathogens, or overgrowth by fouling organisms (Bakus et al., 1986; Paul, 1992; Pawlik, 1993; Proksch, 1994). Chemical defense systems of marine invertebrates that have been studied so far are static. They rely on preformed toxic, antibiotic, or deterrent compounds that are constitutively present in the respective organisms and are usually liberated from the cells following mechanical damage resulting from attack by predators or invasion by pathogens.

The brightly yellow-colored sponge *Aplysina aerophoba* (Verongida: Aplysinidae) (syn. *Verongia aerophoba*) that occurs in the Mediterranean Sea as well as around the Canary Islands was recently introduced as the first example of a marine invertebrate featuring a more dynamic chemical defense that relies on wound activation of storage compounds or protoxins (Teeyapant and Proksch, 1993). Following mechanical damage of tissue, resulting in disturbance of their compartmentalization, brominated isoxazoline alkaloids (e.g., compounds **1–3,** Figure 1) are converted to aeroplysinin-1 (**5**) and the dienone (**6**) (Teeyapant and Proksch, 1993). This wound-induced bioconversion is paralleled by a marked increase of biological activity of the products (**5** and **6**) compared to their substrates (**1–3**), indicating an involvement of this reaction in the chemical defense of *A. aerophoba* (Weiss et al., 1996). Evidence for the enzymatic nature of the wound-activated conversion of protoxins in *A. aerophoba* was previously provided only through in situ experiments involving whole sponges (Teeyapant and Proksch, 1993). Furthermore, no information on the speed of the described bioconversions, which is important in order to evaluate their ecological significance, was available so far.

In the present study we have specifically addressed the enzymatic nature as well as the speed of the defense reaction of *A. aerophoba*. Furthermore, we were able to show that enzyme extracts of other *Aplysina* species catalyze the same reactions as shown previously for *A. aerophoba*, whereas enzyme extracts of sponges from other orders do not. Additionally, we could prove that the described bioconversions of brominated isoxazoline alkaloids in *Aplysina* species result in a pronounced increase of the deterrency of these sponges towards the common Caribbean wrasse *Thalassoma bifasciatum*, thus corroborating the defensive nature of the studied reactions.

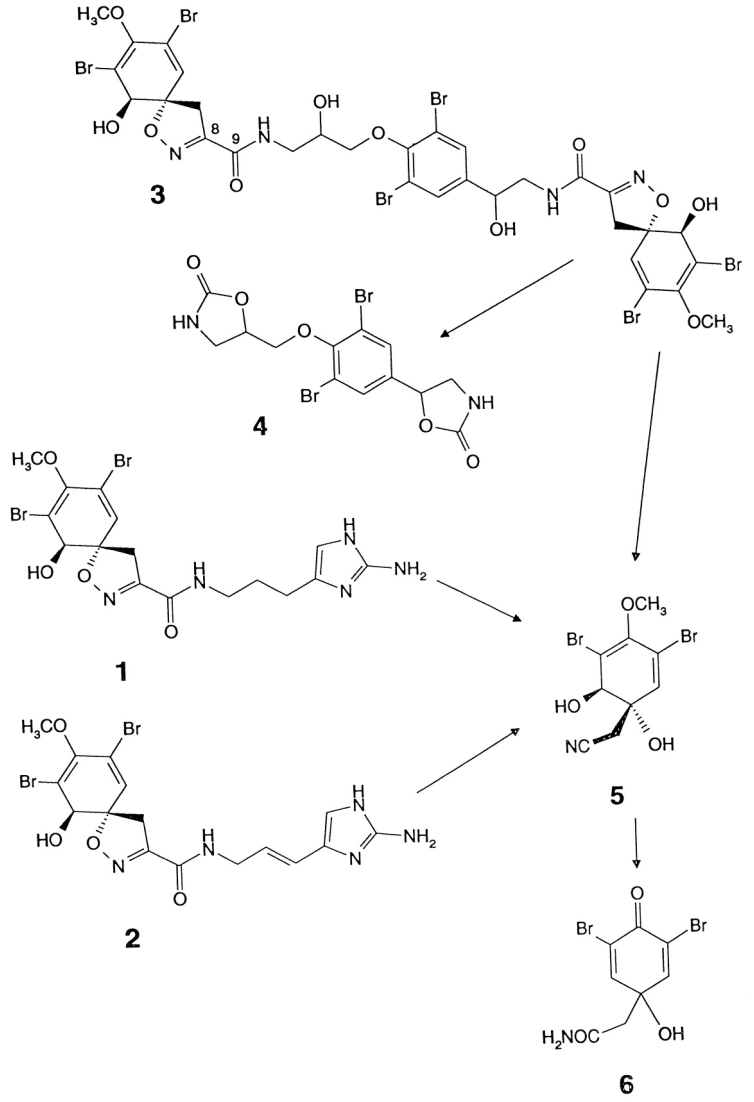


FIG 1. Wound-induced bioconversion of the brominated isoxazoline alkaloids aerophobin-2 (**1**), aplysinamisin-1 (**2**), and isofistularin-3 (**3**) to aeroplysinin-1 (**5**) and the dienone (**6**) in the marine sponge *Aplysina aerophoba*. When isofistularin-3 (**3**) is used as a substrate, the bisoxazolidinone derivative (**4**) is recovered as a second product of the reaction.

METHODS AND MATERIALS

Origin of Sponges. *Aplysina* sponges employed in this study were collected around the Canary Islands (*A. aerophoba*), in the Mediterranean Sea (*A. aerophoba* and *A. cavernicola*), or in the Caribbean Sea (other *Aplysina* species) either by snorkeling or by Scuba diving in May and July 1995. Voucher specimens are deposited in the senior author's laboratory. All sponges were immediately frozen after collection (−20°C) and freeze-dried prior to extraction. Other sponges used in this study were from previous collections in the Indo-Pacific Ocean. Like *Aplysina* sponges, these latter specimens were immediately frozen following collection and freeze-dried prior to extraction.

In Vitro Assays. For the preparation of crude enzyme extracts, 2.5 g of freeze-dried sponge tissue was pulverized in a mortar and extracted at 4°C with 10 ml 20 mM imidazole/HCl buffer, pH 6.8. After centrifugation (15,000*g*), the supernatant was desalted using a Sephadex PD-10 column. The eluent was directly used as the enzyme source. A typical enzyme assay consisted of 8 μl 20 mM imidazole/HCl buffer, pH 6.8, 4 μl of substrate (compound **1**, **2**, or **3**, Figure 1) dissolved in 96% ethanol (final concentration of substrates in assay was 8 mM), and 8 μl of the cell free extract (representing approximately 80 μg total protein as measured by Bradford assay). Incubation was usually for 20 min at 50°C. Following incubation, the assays were directly injected into a HPLC column coupled to a photodiode array detector. Separation was achieved using a C-18 reverse-phase column and a linear gradient from A (10% methanol in water adjusted to pH 2.0 with phosphoric acid) to B (100% methanol) in 15 min. Flow was at 1 ml/min, and detection at 254 nm. Identification of the products was by coinjection of previously isolated compounds (Weiss et al., 1996) as well as by comparison of the on-line UV spectra. Identification of compound **4** was by coinjection of the isolated natural product using a similar HPLC gradient as described above with actonitrile instead of methanol (detection at 220 nm).

Time-Course Experiment. Part of a tube (ca. 2.5 g) of a freshly collected specimen of *A. aerophoba* was crushed in seawater (ca. 10 ml) at 20°C. Over a period of 2 min tissue samples were collected and immediately frozen in liquid nitrogen. Following lyophilization and extraction with methanol, the samples were analyzed by HPLC. The amount of each individual compound in the analyzed samples was calculated from the detector response and the molar extinction coefficients.

Fish-Deterrency Assays. Fish assays were performed in an aquarium on board the *R/V Seward Johnson* as recently described (Pawlik et al., 1995). In short, sponge extracts were incorporated at their natural concentrations (as present in a 2-ml volume of *A. aerophoba*) into 2 ml of a matrix consisting mainly of alginic acid and powdered, freeze-dried squid mantle. After vigorous stirring,

the mixture was loaded into a syringe. The tip of the syringe was inserted into a solution of calcium chloride and the syringe emptied, allowing the contents to form a long noodle. After hardening, the noodle was removed and cut into 4-mm-long pellets. A control pellet (treated with the same amount of solvent as the treated pellets) followed by a treated pellet (containing a methanolic or an aqueous extract prepared from freeze-dried sponge) was offered to a group of five to six wrasses (*T. bifasciatum*) held in a laboratory aquarium. A pellet was considered to be rejected when it was spit out for the third time by one or more fish. Each concentration of sponge extract was tested with 10 different groups of fishes in 10 different aquariums. Such a series of experiments was repeated on two other days.

RESULTS

Bioconversion of Brominated Metabolites in A. aerophoba. Unequivocal proof of the enzymatic nature of the wound-induced bioconversion of brominated isoxazoline alkaloids in *A. aerophoba* was obtained through in vitro experiments using a cell-free extract of *A. aerophoba.* This crude enzyme extract is able to convert several isoxazoline alkaloids including aerophobin-2 (**1**), aplysinamisin-1 (**2**), and isofistularin-3 (**3**) from *A. aerophoba* to the dienone (**6**). Figure 2 shows representative HPLC charts for aerophobin-2 (**1**) used as a substrate in the in vitro assays. In similar assays, aplysinamisin-1 (**2**) and isofistularin-3 (**3**) were used as enzyme substrates (data not shown). When isofistularin-3 (**3**) is used as a substrate, the bisoxazolidinone derivative **4** is recovered as a second product of the reaction in addition to aeroplysinin-1 (**5**), which in turn gives rise to the dienone (**6**) (Figure 1). The bioconversion of aeroplysinin-1 (**5**) to the dienone (**6**) is also an enzymatically catalyzed reaction as shown in a further in vitro experiment using aeroplysinin-1 as a substrate (Figure 2).

No bioconversions of the isoxazoline alkaloids **1**–**3** or of aeroplysinin-1 (**5**) were observed when known enzyme inhibitors such as trichloroacetic acid were added to the enzyme assay or when the cell-free extract was boiled prior to incubation. The enzymes involved in the described bioconversions, however, were shown to retain their catalytic activity in the presence of comparatively high concentrations of ethanol or methanol (up to 30% of total assay volume) in the in vitro assays. When fresh sponges are immersed into methanol or ethanol after collection (as is done in many cases when working on marine natural products), the described bioconversions of isoxazoline alkaloids proceed even in 50–60% aqueous ethanol (Teeyapant and Proksch, 1993). Purification and characterization of the enzymes involved in the described bioconversions are in progress.

Screening for Enzyme Activity in Other Sponges. The capability to perform the described bioconversions of isoxazoline alkaloids is not limited to *A. aero-*

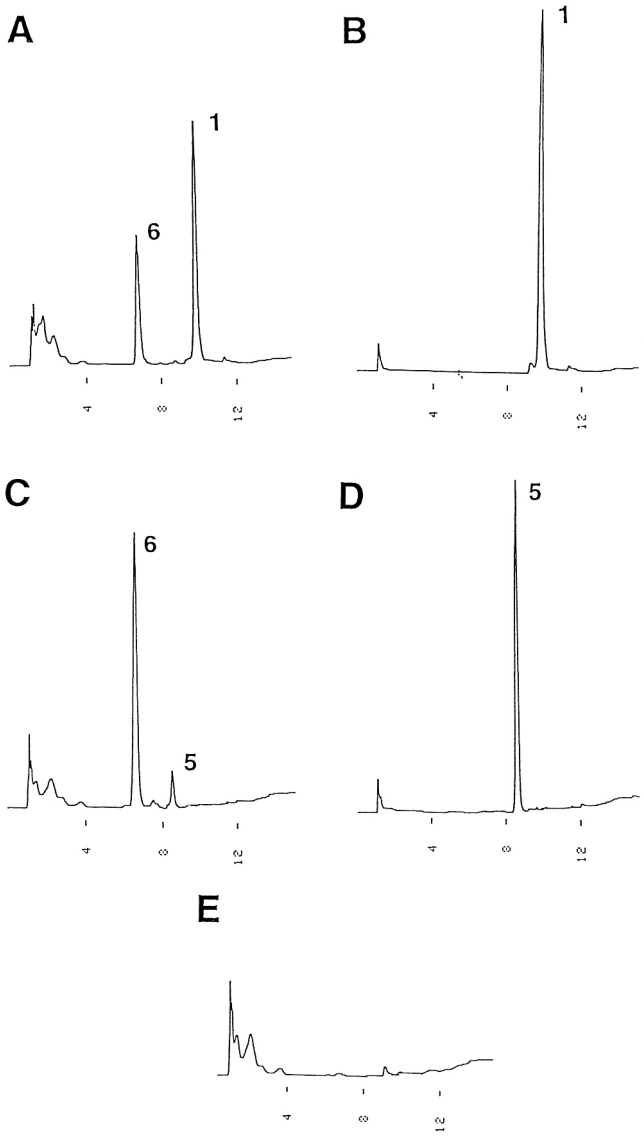


FIG. 2. Bioconversion of aerophobin-2 (**1**) and aeroplysinin-1 (**5**) to the dienone (**6**) in vitro. (A) Enzyme assay using aerophobin-2 (**1**) as a substrate; (B) control (similar assay without cell free extract); (C) enzyme assay using aeroplysinin-1 (**5**) as a substrate; (D) control (similar assay without cell free extract); and (E) control (enzyme assay without substrates). Product formation was measured by HPLC, retention times are given in minutes.

phoba but extends also to other species in this genus, as shown in in vitro assays using cell-free extracts of *A. cavernicola* (from the Mediterrean Sea), *A. archeri*, *A. cauliformis*, *A. fistularis*, *A. fulva*, and *A. lacunosa* (all from the Caribbean Sea) as enzyme source. Crude enzyme preparations of all of the latter sponges converted aerophobin-2 (**1**) to the dienone (**6**) (data not shown). *A. cavernicola* as well as the Caribbean *Aplysina* species analyzed in this study accumulate brominated isoxazoline alkaloids similar to those of *A. aerophoba* (Acosta and Rodriguez, 1992; Ciminiello et al., 1994; Gopichand and Schmitz, 1979; Gunasekara and Cross, 1992) as confirmed by HPLC-MS analysis of the respective methanolic extracts prepared from freeze-dried sponges. Aeroplysinin-1 (**5**) and the dienone (**6**), however, were not found when freeze-dried sponges were used for extraction with methanol. The latter compounds were only detected when aqueous alcoholic solvents (e.g., 50% aq. MeOH) were used for extraction of freeze-dried material or when freeze drying of sponges was omitted prior to extraction with ethanol.

Cell-free extracts prepared from various sponges outside the order Verongida that accumulate no brominated isoxazoline alkaloids (e.g., *Stelleta globostellata*, order Astrophorida; *Axinella carteri*, order Halichondrida; *Theonella swinhoei*, order Lithistida) were inactive in our in vitro enzyme assay. Thus, the enzyme activity studied appears to be linked to the accumulation of suitable substrates and hence may represent a specific biochemical property of sponges from the genus *Aplysina* (and perhaps of other sponges from the order Verongida, which were not available for this study).

Time Course of Bioconversions in A. aerophoba. HPLC analysis of the sponge tissues collected at different times after injuring revealed that the major alkaloids aerophobin-2 (**1**) and isofistularin-3 (**3**) are converted within seconds into aeroplysinin-1 (**5**) and the dienone (**6**) (Figure 3). An almost complete turnover of the substrates **1** and **2** is observed after the first 40–50 sec. The concentration of dienone (**6**) in the treated sponge samples reaches a maximum after 40 sec and remains stable until the observation is terminated (120 sec) (Figure 3).

Fish Deterrency of Different Extracts from A. aerophoba. Fish deterrency of crude organic extracts from several Caribbean reef sponges of the genus *Aplysina* towards the common Caribbean wrasse *T. bifasciatum* was recently reported. In a similar experimental setup, we incorporated two different extracts prepared from *A. aerophoba* into artificial food pellets and offered the pellets to *T. bifasciatum* in a choice bioassay (Figure 4). One of the sponge extracts was a methanolic extract prepared from freeze dried *A. aerophoba* that was rich in compounds **1–3** but did not contain aeroplysinin-1 (**5**) or the dienone (**6**). The other was an aqueous extract that was rich in compounds **5** and **6** but lacked metabolites **1–3**. Food pellets treated with the aqueous extract were considerably more deterrent to the wrasses than food pellets treated with the methanolic

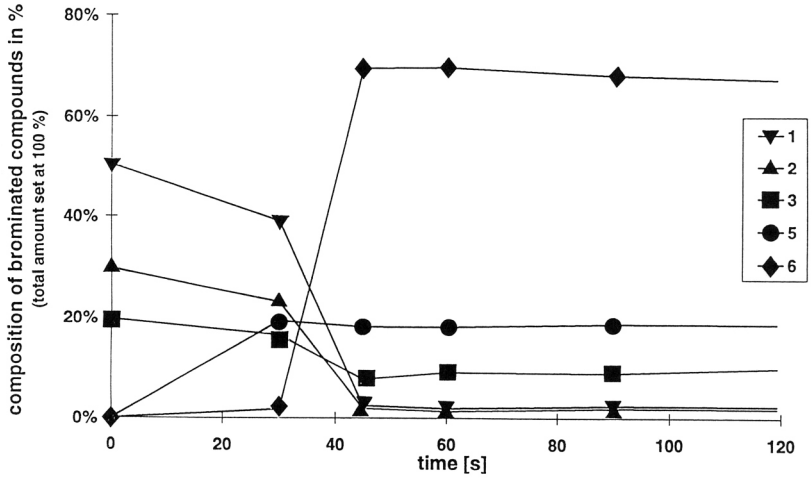


FIG. 3. Time course of the enzymatic defense reaction in a crushed tube of *Aplysina aerophoba*. Data given for $t = 0$ are from a second tube of the same sponge that was immediately frozen following collection and freeze-dried prior to extraction with methanol. Compound numbers follow Figure 1. The amount of each compound present in the samples was calculated from the molar extinction coefficients. Total amount of brominated compounds was set at 100%.

extract (Figure 4) suggesting that the bioconversion of sponge metabolites is paralleled by increased biological activity.

DISCUSSION

Disturbance of the compartmentation of *A. aerophoba* results in a rapid enzymatic conversion of isoxazoline alkaloids (**1–3**) to aeroplysinin-1 (**5**) and the dienone (**6**), which is paralleled by a pronounced increase in the biological activity of the products versus their substrates, as suggested by the fish deterrency assay. Since neither aeroplysinin-1 (**5**) nor dienone (**6**) are observed in intact specimens of *A. aerophoba*, it has to be assumed that substrates and converting enzymes are stored in different, so far unknown, cellular compartments (or even different cell types), and substrate and enzyme will only get in contact upon wounding of the sponge tissue. Similar defense strategies are known from terrestrial higher plants and insects. An example is provided by the cyanogenic glycosides, which are metabolized upon breakdown of the cellular compartment releasing HCN (Jones, 1988). For marine invertebrates, the sponge *A. aerophoba* is the first known example for a wound-activated chemical defense

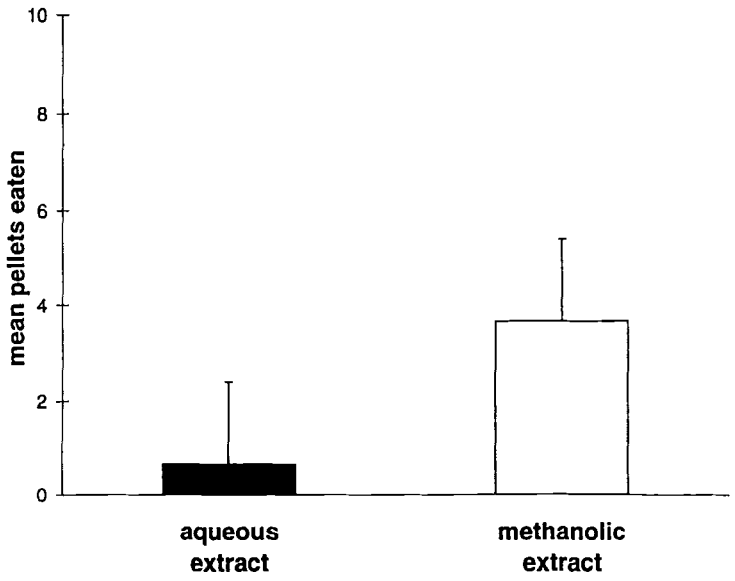


FIG. 4. Deterrency of an aqueous extract [containing aeroplysinin-1 (**5**) and dienone (**6**) as major constituents] and of a methanolic extract [containing aerophobin-2 (**1**) and isofistularin-3 (**3**) as major constituents] of *Aplysina aerophoba* to the common Caribbean wrasse (*Thalassoma bifasciatum*).

involving enzymatic conversion of protoxins. For marine green algae of the genus *Halimeda*, it was shown recently that wounding of the thalli results in a conversion of the diterpene halimedatetraacetate to the biologically more active halimedatrial (Paul and van Alystyne, 1992). Present evidence suggests that the wound-induced conversion of secondary metabolites in *Halimeda* spp. is also caused by enzymes as shown unequivocally in this study for *A. aerophoba.*

Aeroplysinin-1 (**5**) and the dienone (**6**) are not only deterrent to *T. bifasciatum*, but both compounds also are active in bioassays involving other marine organisms such as bacteria, microalgae, or mollusks, whereas their precursors (**1–3**) are always inactive (Weiss et al., 1996). Aeroplysinin-1 (**5**) and the dienone (**6**) suppress, for example, the growth of a plethora of gram-positive and gram-negative marine bacteria including *Alteromonas*, *Moraxella*, and *Vibrio* spp. (Weiss et al., 1996). Towards the marine *Photobacterium phosphoreum*, which is a well-established model for the analysis of waterborne toxins (Bulich et al., 1990), the EC_{50}s of aeroplysinin-1 (**5**) and of the dienone (**6**) were 3.45 and 1.37 μM, respectively (Weiss et al., 1996). In this experimental system both sponge constituents are comparable in their toxicity to strong environmental toxins such as pentachlorophenol or cadmium ions. Aeroplysinin-1 (**5**) and the

dienone (**6**) are also active against marine microalgae such as *Coscinodiscus wailesii* or *Prorocentrum minimum*. At micromolar concentrations both compounds are growth inhibitory to the microalgae and cause membrane damage (Weiss et al., 1996), and the marine snail *Littorina littorea* was repelled when aeroplysinin-1 (**5**) or dienone (**6**) was added to tanks containing the snails (Weiss et al., 1996). The broad bioactivity of the defense metabolites **5** and **6** can at least partly be explained by their capacity to form free radicals that can destroy cells, as we were able to show in a recent study on the cytotoxicity of these metabolites towards human cancer cell lines (Koulman et al., 1996).

The speed of the defense reaction of *A. aerophoba* was one of the key questions addressed by the present study. To be of ecological advantage for the sponge, the conversion of protoxins must proceed rapidly in order to minimize tissue loss to predators or to prevent pathogens from invading through a wound. Within 40 sec after mechanical damage of the sponge tissue, both aerophobin-2 (**1**) and aplysinamisin-1 (**2**) are converted to products **5** and **6.** The slower turnover of isofistularin-3 (**3**) compared to that of **1** or **2** is probably due to the higher lipophilicity of isofistularin-3 (**3**), which is an obstacle for diffusion of the compound in the crushed sponge tube. The speed of the bioconversions measured in situ appears to be sufficiently fast to provide *A. aerophoba* with an effective chemical protection. This hypothesis is corroborated by field observations, which indicate that, at least around the Canary Islands, *A. aerophoba* is among the dominating sponge species and shows only rare signs of damage attributable to attack by predators (Teeyapant and Proksch, 1993; Proksch, unpublished results).

The enzymes responsible for activation of brominated metabolites are not only operating in *A. aerophoba* but were also found in all other *Aplysina* sponges analyzed in this study. No enzyme activity was detected in sponges from other orders that lack brominated isoxazoline alkaloids. Thus, the enzymes involved in the bioconversions appear to be linked to the accumulation of suitable substrates and hence may represent a specific biochemical property of sponges from the genus *Aplysina* (and perhaps of other sponges from the order Verongida not available for this study).

Based on the in vitro studies conducted so far, it appears that at least three different types of enzymes are involved in the activation of protoxins in *A. aerophoba.* The first enzyme, which generates aeroplysinin-1 (**5**), cleaves the bond between C-8 and C-9 of the investigated isoxazolines, as indicated by the detection of the bisoxazolidinone derivative **4** as a product of the biotransformation of isofistularin-3 (**3**). Conversion of aeroplysinin-1 (**5**) to the dienone (**6**) requires demethylation of the OCH_3 substituent (second postulated enzyme) and hydration of the nitrile moiety (third postulated enzyme). *O*-Dealkylations may be catalyzed by microsomal P_{450}-dependent monooxygenases, which are ubiquituously present in prokaryotes as well as eukaryotes. Enzymes that cat-

alyze the hydration of nitrile groups (nitrile hydratases) have been isolated from various bacteria (e.g., *Rhodococcus* strains) (e.g., Moreau et al., 1993) as well as from fungi (Maier-Greiner et al., 1991) but were hitherto unknown in sponges. Hence, it is possible that the conversion of aeroplysinin-1 (**5**) to the dienone (**6**), which involves hydration of the nitrile group, is catalyzed by an enzyme produced by a symbiont rather than by the sponge. Ongoing work is focusing on the purification and characterization of enzymes involved in the wound-activated chemical defense of *A. aerophoba.*

Acknowledgments—Financial support from the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 251 der Universität Würzburg) and from the Fonds der Chemischen Industrie to P.P. is gratefully acknowledged. R.E. wishes to thank Dr. Hilmer-Stiftung im Stifterverband für die Deutsche Wissenschaft for a scholarship. We are also grateful to Prof. W. Fenical (Scripps Institution of Oceanography, San Diego, California) for the opportunity to participate in a scientific cruise of the *R/V Seward Johnson* to the Caribbean Sea in 1995 and to Prof. J. R. Pawlik, B. Chanas, G. McFall and D. C. Swearingen III (all University of North Carolina at Wilmington, North Carolina) for their help in sponge collection and in performing fish bioassays aboard of the *R/V Seward Johnson.*

REFERENCES

ACOSTA, A. L., and RODRIGUEZ, A. D. 1992. 11-Oxoaerothionin: A cytotoxic antitumor bromotyrosine-derived alkaloid from the Caribbean marine sponge *Aplysina lacunosa. J. Nat. Prod.* 55:1007–1012.

ATTAWAY, D. H., and ZABORSKY, O. R. (eds.) 1993. Marine Biotechnology, Vol. 1. Plenum Press, New York.

BAKUS, G. J., TARGETT, N. M., and SCHULTE, B. 1986. Chemical ecology of marine organisms: an overview. *J. Chem. Ecol.* 12:951–987.

BULICH, A. A., KER-KONG, T., and SCHEIBNER, G. 1990. The luminescent bacteria toxicity test: Its potential as an in vitro alternative. *J. Bioluminesc. Chemoluminesc.* 5:71–77.

CIMINIELLO, P., COSTANTINO, V., FATTORUSSO, E., MAGNO, S., MANGONI, A., and PANSINI, M. 1994. Chemistry of Verongida sponges, II. Constituents of the Caribbean sponge *Aplysina fistularis* forma *fulva. J. Nat. Prod.* 57:705–712.

GOPICHAND, Y., and SCHMITZ, F. J. 1979. Marine natural products: Fistularin-1, -2, and -3 from the sponge *Aplysina fistularis* forma *fulva. Tetrahedron. Lett.* 41:3921–3924.

GUNASEKERA S. P., and CROSS S. S. 1992. Fistularin-3 and 11-ketofistularin-3. Feline leukemia virus active bromotyrosine metabolites from the marine sponge *Aplysina archeri. J. Nat. Prod.* 55:509–512.

JONES, D. A. 1988. Cyanogenesis in animal-plant interactions, pp. 151–170, *in* D. Evered and S. Harnett (eds.). Cyanide Compounds in Biology. John Wiley, Chichester, UK.

KOULMAN, A., PROKSCH, P., EBEL, R., BEEKMAN, A. C., VAN UDEN, W., KONINGS, A. W. T., PEDERSEN, J. A., PRAS, N., and WOERDENBAG, H. J. 1996. Cytotoxicity and mode of action of aeroplysinin-1 and a related dienone for the sponge *Aplysina aerophoba. J. Nat. Prod.* 59:591–594.

MAIER-GREINER, U. H., OBERMAIER-SKROBRANEK, B. M. M., ESTERMAIER, L. M., KAMMERLOHER, W., FREUND, C., WUELFIG, C., BURKERT, U. I., MATERN, D. H., and BREUER, M. 1991. Isolation and properties of a nitrile hydratase from the soil fungus *Myrothecium verrucaria* that

is highly specific for the fertilizer cyanamide and cloning of its gene. *Proc. Natl. Acad. Sci. U.S.A.* 88:4260-4264.

MOREAU J. L., AZZA, S., ARNAUD, A., and GALZY, P. 1993. Purification and characterization of nitrile hydratase of a mutant strain of *Brevibacterium* sp. *J. Basic Microbiol.* 33:323-329.

PAUL, V. J. 1992. Chemical defenses of benthic marine invertebrates, pp. 164-188, *in* V. J. Paul (ed.). Ecological Roles of Marine Natural Products. Comstock, Ithaca, NY.

PAUL, V. J., and VAN ALSTYNE, K. L. 1992. Activation of chemical defenses in the tropical green algae *Halimeda* spp. *J. Exp. Mar. Biol. Ecol.* 160:191-203.

PAWLIK, J. R. 1993. Marine invertebrate chemical defenses. *Chem. Rev.* 93:1911-1922.

PAWLIK, J. R., CHANAS, B., TOONEN, R . J., and FENICAL, W. 1995. Defenses of Caribbean sponges against predatory reef fish. I. Chemical deterrency. *Mar. Ecol. Progr. Ser.* 127:183-194.

PROKSCH, P. 1994. Defensive roles for secondary metabolites from marine sponges and sponge-feeding nudibranchs. *Toxicon* 32:639-655.

TEEYAPANT, R., and PROKSCH, P. 1993. Biotransformation of brominated compounds in the marine sponge *Verongia aerophoba*: Evidence for an induced chemical defense? *Naturwissenschaften* 80:369-370.

WEISS, B., EBEL, R., ELBRÄCHTER, M., KIRCHNER, M., and PROKSCH, P. 1996. Defense metabolites from the marine sponge *Verongia aerophoba*. *Biochem. Syst. Ecol.* 24:1-12.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

SYSTEMIC APPLICATION OF L-PHENYLALANINE INCREASES PLANT RESISTANCE TO VERTEBRATE HERBIVORY

CLARE B. GREY,* DAVE P. COWAN, STEVE D. LANGTON, and RICHARD W. WATKINS

Central Science Laboratory
Ministry of Agriculture, Fisheries and Food
Sand Hutton, York, YO4 1LZ, UK

(Received May 13, 1996; accepted January 12, 1997)

Abstract—The systemic application of L-phenylalanine (PHE), a phenolic precursor, significantly increases the phenolic pool of oilseed rape (*Brassica napus* var. Bristol). In a two-choice test with captive feral pigeons (*Columba livia*), PHE-treated plants sustained significantly less damage than control plants. This was supported by the results of behavioral studies, where video analyses showed that the PHE-treated plants received significantly fewer pecks than the controls. This is the first report of increased resistance to damage by a vertebrate pest following the systemic application of precursors for plant defense compounds.

Key Words—*Brassica napus*, oilseed rape, *Columba livia*, pigeon, vertebrate, herbivores, resistance, phenolics, defense, crop protection.

INTRODUCTION

Plant secondary compounds have long been recognized to be a major determinant in the acceptability of plants to their herbivores (Rogers, 1978). Phenolic compounds have been shown to be important factors in the defense of plants against fungal infection (Retig and Chet, 1974; Carrasco et al., 1978), insect herbivory (Hartley and Firn, 1989; Harborne, 1989), and parasitic nematodes (Birch et al., 1993; Feldman and Hanks, 1971). They are also implicated in the resistance of plants to vertebrate damage. Phenolic compounds display both primary and secondary deterrent modes of action (Cooper and Owen-Smith, 1993; Palo, 1984; Barry et al., 1986). Primary deterrents cause the plant to be

*To whom correspondence should be addressed.

0098-0331/97/0500-1463$12.50/0 © 1997 Plenum Publishing Corporation

rejected because of their bitter taste or offensive odor, while secondary deterrents induce a malaise that consequently leads to rejection of the plant through a learned aversion (Rogers, 1978; Crocker and Perry, 1990).

Plants may constitutively accumulate these phenolic compounds as a means of passive defense (Barry et al., 1986; Barry and Manley, 1986), or they may be rapidly and systemically accumulated in response to attack by pests and diseases (Chappell and Hahlbrock, 1984). In both constitutive and systemically induced defense systems the magnitude of the response is due, in part, to the supply of the primary precursor L-phenylalanine (PHE) (Da Cunha, 1987). A number of studies have shown that the resistance of plants to both disease and insect pests can be enhanced by systemically applying phenolic precursors and thereby boosting the production of phenolics (Carrasco et al., 1978). Experiments were carried out to determine if the resistance of oilseed rape plants to vertebrate damage can be increased through the systemic application of PHE. The feeding behavior of feral pigeons, a major bird pest, was assessed to determine the efficacy of the treatment and its mode of action (i.e., primary or secondary activity).

METHODS AND MATERIALS

Feral Pigeons. Naive feral pigeons were housed as four pairs in outside aviaries (size 2.5 × 1.8 × 2.5 m). Each pair was visually isolated from the other experimental birds and maintained on a diet of chicken layer pellets, corn, maize, and maple peas (2:2:2:1). Water was available ad libitum throughout the trial.

Growth and Treatment of Rape Plants. Rape plants were cultivated at a temperature of 22± 5°C in a greenhouse lit by Phillips Son-T Agri lamps on a 12-hr light/dark photoperiod. Rape seeds were germinated on blotting paper soaked in a nutrient solution (Carrasco et al., 1978). A new batch of seeds was sown each day over eight consecutive days. Germination occurred 15 days after sowing with comparable development between batches. Fourteen days after germination, the seedlings were planted in horticultural sand contained in perforated seed trays lined with landscaping material. Fifteen seedlings were planted in each tray. The tray was placed in a slightly larger tray containing the nutrient solution. Forty days after germination, trays of plants were treated systemically with either tap water (pH 5, 400 ml) or PHE (20 mM, pH 5, 400 ml), for 10 hr/day over three consecutive days (Carrasco et al., 1978). Consequently, each tray of treated plants received 8 mmol PHE per day (33 μmol PHE/g fresh weight). The treatment of the rape plants was staggered over eight consecutive days. This ensured that all plants selected for presentation to feral pigeons or

for the analysis of free phenolic compounds were used immediately after the three-day PHE (or water) treatment. Plants to be analyzed for free phenolics were placed in an empty outside aviary for the duration of the day's test period prior to harvesting. This ensured that all plants were maintained under the same daily test conditions.

Phenolic Extraction and Analysis. Rape plants were harvested at the end of each day by cutting just above sand level and immediately processed to minimize any breakdown of cellular material. Plant material within each tray was combined, cut into small pieces, frozen with liquid nitrogen, roughly ground using a mortar and pestle, and then lyophilized ($-60°C$ at 10^{-1} atm, for 48 hr or until fully dried). After lyophilization, the plant material was milled (hammer mill, 0.25 mm mesh) and stored at $-20°C$ until required for analysis.

Lyophilized, milled plant material (0.5 g) was then extracted in a Tecator Soxtec system (Perstorp Analytical, Berkshire, UK) with 80% aqueous acetone (HPLC grade; 2×40 ml) for a total of 5 hr. The extracts were combined and concentrated (to 15 ml) using a Zymark Turbo-Vap 2 evaporator (Zymark Ltd., Cheshire, UK). The resulting extract was then refluxed for a few seconds to facilitate the precipitation of the chlorophyll. The chlorophyll was removed by filtering while hot (Whatman No. 1 filter paper) and the filtrate diluted to 50 ml with distilled water. Free phenolic compounds were quantified from the cooled filtrate using the Prussian blue assay (Price and Butler, 1977). The results were expresed in gallic acid equivalents. The mean concentration of phenolics for control and treated plants were compared using a one-way ANOVA.

Bioassay. The trial was carried out using a two-choice test for a period of 3 hr (09:00–12:00) on eight consecutive days. After this trial period (12:00), the birds were presented with their maintenance diet regime until 17:00, when all food was removed. On days 1, 3, 5, and 7 (nontreatment days) the birds were presented with two trays of control plants. On days 2, 4, 6, and 8 (treatment days) the birds were presented with one tray of the PHE-treated plants and one tray of control plants. To remove any positional preferences, the location of the treated and control trays was alternated on the treatment days.

Behavioral Analysis. The feeding behavior of the birds was monitored using a video camera. The number of pecks received by plants, 15 min after the first peck, was recorded. During this period, any feeding behaviors indicating an aversion to the rape, such as head shaking and bill wiping (Gill et al., 1994) were also noted. Treatment and day effects were tested by ANOVA.

Damage Assessment. A damage assessment was carried out on all trays of plants immediately after each trial. Individual plants were assessed for the number of damaged and undamaged leaves (a leaf was considered damaged if it had received one or more pecks). A typical example of pigeon damage to rape can be seen in Figure 1. Treatment and day effects were tested by ANOVA.



FIG. 1. A typical example of pigeon damage to oilseed rape.

RESULTS

Induction of Free Phenolic Compounds. The PHE treatment resulted in a significant increase ($F = 31.07$, $df = 1, 16$, $P < 0.001$) in the phenolic content of the rape plants (Table 1); an increase of 13% above the levels observed for the control plants.

Resistance of Treated Plants to Pigeon Damage. Video analysis showed that the number of pecks sustained by the rape (Figure 2) was significantly reduced on the treated plants ($F = 25.30$, $df = 1, 15$, $P < 0.001$). Those birds that fed on the treated plants showed behavioral responses immediately after sampling suggesting distaste, such as head shaking, bill wiping and increased drinking. One bird was observed to regurgitate treated plant material soon after ingestion. These behavioral responses are reflected by the results of the plant

TABLE 1. EFFECT OF L-PHENYLALANINE TREATMENT ON PHENOLIC CONTENT OF RAPE PLANTS[a]

PHE-treated	Control (untreated)
4.7 ± 0.09	4.1 ± 0.08

[a]Results are expressed in gallic acid equivalents (mg/g dry weight ± SE). A one-way ANOVA was used to compare the means ($F = 31.07$, $df = 1, 16$, $P < 0.001$).

damage assessment (Figure 3). PHE-treated plants received significantly less damage than the control plants ($F = 14.26$, $df = 1, 15$, $P < 0.01$). Analysis of variance of the data for nontreatment days ($F = 0.01$, $df = 1, 14$, $P > 0.1$) showed that there were no residual preferences for the side where the control plants had previously been. The total number of pecks sustained by the rape (both treated and control) declined linearly over the course of the trial ($F =$

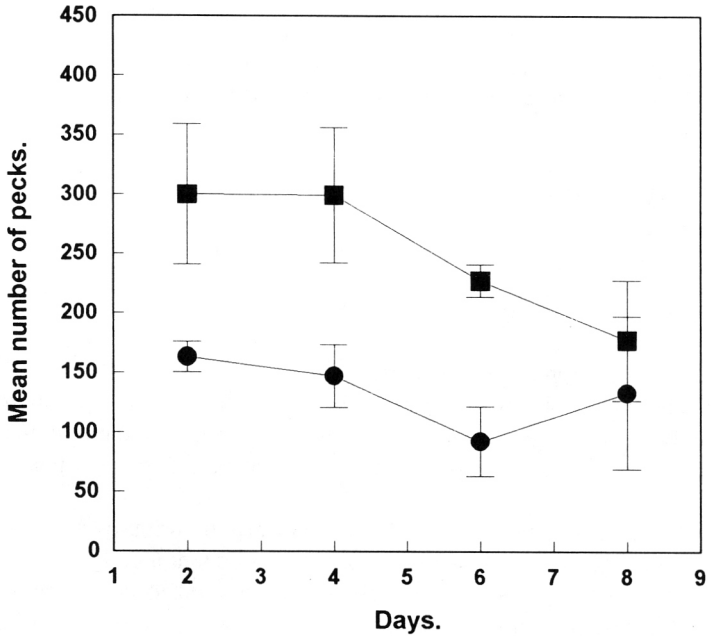


FIG. 2. Mean number of pecks recorded on the phenylalanine-treated (●) and control (untreated; ■) plants over a 15-min period.

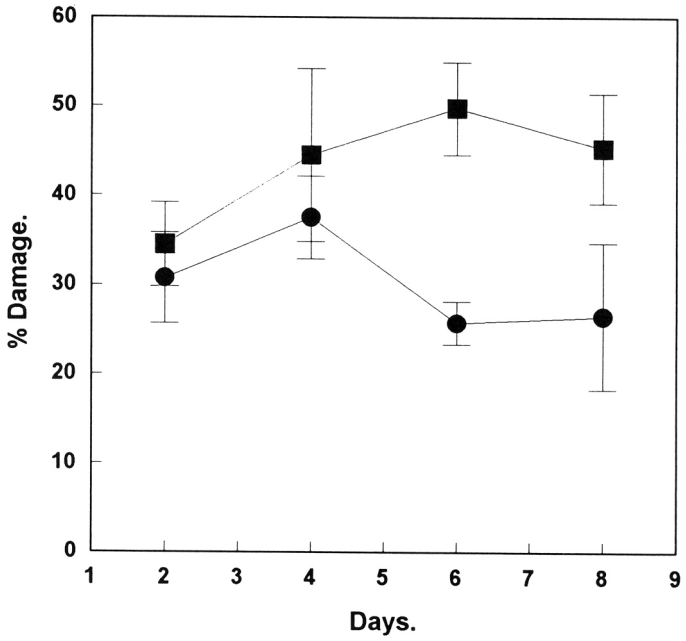


FIG. 3. Damage assessment: Mean number of leaves damaged per tray (%) from phenylalanine-treated (●) and control (untreated; ■) plants.

7.88, $df = 1, 15$, $P < 0.05$); however, this was not reflected in the damage assessment.

DISCUSSION

The systemic application of PHE significantly increases the levels of free phenolic compounds in oilseed rape plants. The treated plants also received significantly less damage than the control plants, supporting the hypothesis that increased levels of free phenolic compounds enhance a plant's resistance to pigeon damage.

An effective feeding deterrent may require both primary and secondary activity, since pigeons are likely to habituate to a food that merely tastes unpleasant (Crocker and Perry, 1990). The observed behavioral responses suggest that the birds could readily distinguish between treated and untreated plants. The immediate decline in the consumption of the treated plants may be attributed to a primary response in which the birds detect an offensive taste or odor cue. The decline in the overall consumption of both treated and control plants over the

course of the trial may be due to a generalized learned aversion to the rape. Further experiments are planned to determine whether the decline in the number of pecks is solely mediated by the treated rape, thereby indicative of a secondary response.

This technique may provide a novel and effective method of deterring not only pigeons from damaging crops, but other vertebrate pests as well. Traditional methods of deterrence such as the auditory and visual scarers have only transitory effects, as the pigeons soon habituate to their presence. Indeed, in some cases, they may encourage damage as the birds learn to associate the device with a good source of food (I. R. Inglis, unpublished observations).

This systemic approach has considerable advantages over the topological application of feeding deterrents. Specifically, the products of the treatment may be translocated throughout the growing plant, isolated from any external environmental degradation (e.g., weathering), and provide a unique cocktail of defense chemicals that could not be synthesized in vitro. A further advantage of this approach is that it does not rely on reallocation of plant PHE resources away from primary metabolism to the production of secondary (defense) metabolites. Therefore the trade-off between the benefits of increased resistance and the costs (reduced fitness, growth) may be minimized (Fineblum and Rausher, 1995). The longevity of the observed effect has yet to be determined in oilseed rape. However, there is evidence to suggest that the effect may be persistent. Carrasco et al. (1978) showed that pretreatment of tomato plants with quinic acid and PHE delayed the onset of *Fusarium* wilt for two weeks after a single application. The symptoms, when they did develop (i.e., wilting, yellowing, and foliar abscission) were less severe than those observed in the control plants. The resistance mechanism involved in the PHE-treated plants still remains to be identified, although we hope to study this and determine the minimum effective application rate for PHE. However, we provide here the first evidence that the systemic application of PHE decreases the palatability of rape plants to feral pigeons.

Acknowledgments—We thank the Land Use & Rural Economy Division and Pesticides Safety Directorate of the UK Ministry of Agriculture, Fisheries and Food for funding this work, E. Gill, S. Richardson, and D. Grey for their assistance in the experimental design and execution; and N. Price, J. Edwards, and I. R. Inglis for their comments on the manuscript.

REFERENCES

BARRY, T. N., and MANLEY, T. R. 1986. Interrelationships between concentrations of total condensed tannins, free condensed tannin and lignin in *Lotus* sp. and their possible consequences in ruminant nutrition. *J. Sci. Food. Agric.* 37:248–254.

BARRY, T. N., ALLSOP, T. F., and REDEKOPP, C. 1986. The role of condensed tannins in the

nutritional value of *Lotus pedunculatus* for sheep. % Effects on the endocrine system and on the adipose tissue metabolism. *Br. J. Nutr.* 56:607-614.

BIRCH, A. N., ROBERTSON, W. M., and FELLOWS, L. E. 1993. Plant products to control plant parasitic nematodes. *Pestic. Sci.* 39:141-145.

CARRASCO, A., BOUDET, A. M., and MARIGO, G. 1978. Enhanced resistance of tomato plants to *Fusarium* by controlled stimulation of their natural phenolic production. *Physiol. Plant Pathol.* 12:225-232.

CHAPPELL, J., and HAHLBROCK, K. 1984. Transcription of plant defence genes in response to UV light or fungal elicitor. *Nature* 311:76-78.

COOPER, S. M., and OWEN-SMITH, N. 1993. Condensed tannins deter feeding by browsing ruminants in a South African savanna. *Oecologia* 67:142.

CROCKER, D. R., and PERRY, S. M. 1990. Plant chemistry and bird repellents. *Ibis* 132:300-308.

DA CUNHA, A. 1987. The estimation of L-phenylalanine ammonia-lyase shows phenylpropanoid biosynthesis to be regulated by L-phenylalanine supply and availability. *Phytochemistry* 26:2723-2727.

FELDMAN, A. W., and HANKS, R. W. 1971. Attempts to increase tolerance of grapefruit seedlings to the burrowing nematode (*Radopholus similis1) by application of phenolics. Phytochemistry* 10:701-709.

FINEBLUM, W. L., and RAUSHER, M. D. 1995. Trade-off between resistance and tolerance to herbivore damage in a morning glory. *Nature* 377:517-520.

GILL, E. L., SERRA, M. B., CANAVELLI, S. B., FEARE, C. J., ZACCAGNINI, M. E., NADIAN, A. K., HEFFERNAN, M. L., and WATKINS, R. W. 1994. Cinnamamide prevents captive chestnut-capped blackbirds (*Agelaius ruficapillus*) from eating rice. *Int. J. Pest Manage.* 40:195-198.

HARBORNE, J. B. 1989. Methods in Plant Biochemistry: Plant Phenolics. Academic Press, London.

HARTLEY, S. E., and FIRN, R. D. 1989. Phenolic biosynthesis, leaf damage and insect herbivory in birch (*Betula pendula*). *J. Chem. Ecol.* 15:275-283.

PALO, R. T. 1984. Distribution of birch (*Betula* spp.), willow (*Salix* spp.), and poplar (*Populus* spp.) secondary metabolites and their potential role as chemical defence against herbivores. *J. Chem. Ecol.* 10:499-519.

PRICE, M. L., and BUTLER, L. G. 1977. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *J. Agric. Food Chem.* 25:1268-1273.

RETIG, N. and CHET, I. 1974. Catechol-induced resistance of tomato plants to *Fusarium* wilt. *Physiol. Plant Pathol.* 4:469-475.

ROGERS, J. G. 1978. Repellents to protect crops from vertebrate pests: Some considerations for their use and development, pp. 150-165, *in* R. W. Bullard (ed.). Flavour Chemistry of Animal Foods. American Chemical Society, Washington, D.C.

Journal of Chemical Ecology, Vol. 23, No. 5, 1997

IMPROVED CONTROLLED-RELEASE FORMULATIONS FOR A NEW TRAP DESIGN FOR MALE MEDITERRANEAN FRUIT FLIES: THE C&C TRAP

J. D. WARTHEN,[1,*] R. T. CUNNINGHAM,[2] B. A. LEONHARDT,[1] J. M. COOK,[3] J. W. AVERY,[1] and E. M. HARTE[1]

U.S. Department of Agriculture, Agricultural Research Service
[1]*Insect Chemical Ecology Laboratory*
Beltsville, Maryland 20705-2350
[2]*Tropical Fruit and Vegetable Research Laboratory*
Hilo, Hawaii 96720
[3]*Farma Tech International*
Fresno, California 93710

(Received 13 September, 1996; accepted 12 January, 1997)

Abstract—The Mediterranean fruit fly, *Ceratitis capitata*, is a major pest of fruits and vegetables in the world. With the help of an extensive trapping program, the continental United States has remained free of established medfly populations. In an effort to increase trapping efficiency, controlled-release polymeric panels with trimedlure and ceralure were developed in conjunction with the development of a new trap, the C&C trap. This study contrasts the effectiveness of different panel formulations with the standard trimedlure polymeric plug and describes the use of the panels in the C&C trap. Field tests in Hawaii using released sterile flies showed consistently higher male medfly catches with panels in C&C traps than with the standard trimedlure polymeric plug in Jackson traps.

Key Words—Mediterranean fruit fly, *Ceratitis capitata*, medfly, C&C trap, Jackson trap, trimedlure, ceralure, attractant, parapheromone, panels.

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), commonly called the medfly, is a worldwide agricultural pest of fruits, nuts, and vegetables (Liquido et al., 1991). The fly's establishment in the continental United States

*To whom correspondence should be addressed.

0098-0331/97/0500-1471$12.50/0 © 1997 Plenum Publishing Corporation

has been prevented through vigorous detection, quarantine, and eradication in subtropical areas where hosts exist (Anonymous, 1996a, b). A network of traps using both parapheromones or separate trap types with food baits is used to detect any accidental introductions of the medfly.

The parapheromone that is used in the Jackson traps (Harris et al., 1971) is trimedlure (TML), *tert*-butyl 4- and 5-chloro-2-methyl cyclohexane carboxylate (Beroza et al., 1961). Commercial TML is a mixture of 90–95% *trans* isomers (McGovern et al., 1986), designated A, B1, B2, and C (McGovern and Beroza, 1966; Leonhardt et al., 1982). The most attractive isomer is TML-C (McGovern et al., 1966, 1987), or more specifically one of its enantiomers, (1*S*,2*S*,4*R*)-TML-C (Sonnet et al., 1984; Doolittle et al., 1991). USDA specifications for commercial TML require a 38–45% TML-C content. The controlled release of TML from cotton wicks and polymeric materials has been studied extensively in an effort to develop an optimal formulation of parapheromone (Leonhardt et al., 1984, 1986, 1987, 1989, 1994; Rice et al., 1984).

Another parapheromone that is used to monitor the male medfly is ceralure (CER) (McGovern and Cunningham, 1987, 1988), ethyl 4- and 5-iodo-2-methylcyclohexane-1-carboxylate. Commercial CER is also a mixture of 90–95%

TABLE 1. CATCH OF MALE MEDITERRANEAN FRUIT FLIES WITH TRIMEDLURE PANELS

	Mean[a] catch (±SE)/trap for			
Formulation	0–1	2–3	4–5	6–7
2.0 g TML fresh[b]	58.80 ± 10.63 c	108.70 ± 24.17 bc	119.50 ± 16.45 ab	89.20 ± 11.14 bc
2.0 g TML aged[c]	85.90 ± 13.87 c	112.30 ± 29.18 bc	153.30 ± 18.37 a	122.90 ± 10.50 b
1.9 g TML TNO panel[d]	100.90 ± 16.88 bc	77.40 ± 17.29 c	82.70 ± 25.26 b	59.50 ± 15.50 c
9.5 g TML TNO panel[e]	167.90 ± 31.50 ab	242.10 ± 44.68 a	154.20 ± 29.56 a	239.90 ± 28.21 a
2.1 g TML CDFA panel[f]	211.20 ± 38.20 a	171.30 ± 28.73 b	154.90 ± 29.25 a	82.40 ± 16.31 bc
F	6.92	8.34	1.72	17.06
EMS	5697.12	5111.30	5891.66	2988.74
P	<0.001	<0.001	0.1670	<0.001

[a] Means followed by the same letter within a column are not significantly different ($P > 0.05$; Fisher PLSD test), randomized complete block field plot design with five replicates. Statistics are on untransformed data for all weeks.
[b] AgriSense plug in standard Jackson trap (replaced once a month).
[c] AgriSense plug in standard Jackson trap.
[d] 1.4% TML in Farma Tech 15 × 25 cm panel covered with Hold Fast Stikem.
[e] 7% TML in Farma Tech 15 × 25 cm panel covered with Hold Fast Stikem.
[f] 14% TML in 15 g Hold Fast Stikem coated on 14 × 23 cm panel.

trans isomers (McGovern et al., 1986), which are also designated A, B1, B2, and C (DeMilo et al., 1994). The most attractive isomer is CER-B1 (McGovern and Cunningham, 1987, 1988; Leonhardt et al., 1994, 1996; Avery et al., 1994; Warthen et al., 1994). USDA specifications for commercial CER require a 26% CER-B1 content. The controlled release of CER (Leonhardt et al., 1994; Avery et al., 1994) has not been as extensively investigated as that of TML.

The purpose of the work reported here was to develop a new controlled-release trap for mass-trapping of released sterile male medflies and to compare the efficacy of new polymeric panels of TML and CER under field conditions. Catches of flies were compared with those caught in Jackson traps baited with standard TML polymeric plugs. Residual TML and CER from the panels were determined at various points of aging to define the release profile.

METHODS AND MATERIALS

Chemicals. TML (Lot 505010249) in AgriSense (Columbia, Maryland) plugs, on California Department of Food and Agriculture (CDFA) panels (Table 1), and in polyvinyl chloride (PVC) panels (Table 2) was supplied by Biosys/

VERSUS STANDARD TRIMEDLURE PLUGS IN JACKSON TRAPS (HILO, HAWAII)

each formulation after indicated weeks aged in field

8–9	10–11	12–13	14–15	16–17
112.80 ± 17.86 a	192.30 ± 12.10 a	190.00 ± 16.68 a	217.10 ± 4.24 a	128.70 ± 16.17 a
111.30 ± 20.59 a	106.90 ± 19.07 b	113.70 ± 19.39 b	110.70 ± 16.76 b	39.20 ± 8.29 b
52.70 ± 15.15 b	89.50 ± 23.56 b	112.90 ± 18.59 b	112.10 ± 29.16 b	66.60 ± 8.68 b
119.40 ± 22.36 a	230.20 ± 26.54 a	195.60 ± 26.80 a	246.60 ± 47.47 a	68.60 ± 12.85 b
41.80 ± 10.16 b	20.10 ± 4.28 c	10.80 ± 6.13 c	2.80 ± 1.31 c	0.00 ± 0.00 c
5.55	25.99	22.37	14.53	20.28
2488.17	2707.42	2517.61	6494.47	1093.19
0.0014	<0.001	<0.001	0.001	0.001

AgriSense (Columbia, Maryland). TML (Lot 10292) in the Farma Tech TNO panels (Tables 1–3) was provided by Farma Tech International (Fresno, California). Both lots of TML met the USDA specifications of 38–45% w/w TML-C. The CER in the PVC panel (Table 2) was supplied by AgriSense (Dow-Corning, UK) and met USDA specifications of 26% w/w CER-B1.

Plugs and Panels. Creamy-white AgriSense fresh and aged plugs (Biosys/AgriSense) containing the standard 2 g of TML (creamy white insert) were used in each test. Plastic TNO panels (15 × 25 cm) (Tables 1 and 2) and TNO panels A, B, and C (15 × 15 cm, proprietary compositions) (Table 3) of a light yellow color containing TML were supplied by Farma Tech International (developed under license from TNO of Holland). The TNO panels (Table 1) were coated with Hold Fast Stickem (Seabright Laboratory, Emeryville, California). The 14- × 23-cm CDFA panel (Seabright) (Table 1), coated with a mixture of TML and Hold Fast Stickem, was a plastic-coated light cardboard similar in composition to that of the Jackson trap. The 14- × 23-cm PVC panels (Table 2) containing parapheromone were provided by AgriSense, Dow Corning, UK.

Traps. Standard Jackson traps with unobstructed ends (Harris et al., 1971) were utilized for the 2-g TML AgriSense fresh and aged plugs. A trap was designed utilizing a 14- × 23-cm Seabright (long axis fold) bifold yellow cardboard coated with Hold Fast Stikem to envelope each of the TNO and PVC panels (Table 2). The bottoms of the cardboard enveloping the panel were stapled, and the hanging wire placed in the bifold at the opposite top end. The new C&C trap (patent pending) was used for the 15- × 15-cm panels impregnated with parapheromone (Table 3). This trap is a device invented by R. T. Cunningham (USDA) and J. M. Cook (Farma Tech International). The C&C trap consists of a metal hook or hanger and a plastic holder, about the size of a chalkboard eraser, that has three panels hanging down from it. The center plastic panel is impregnated with the parapheromone. Creamy white paperboard panels coated on both sides with Hold Fast Stickem hang down on either side of the center panel, approximately 2.5 cm away from the parapheromone panel. All the panels slide neatly into the holder, making them easy to remove, inspect, and replace (Anonymous, 1996c). The paperboard panels were replaced weekly if the fly catch was high or cleaned by removal of flies if the fly catch was low.

Field Bioassay. Three studies were conducted in a mature *Macadamia integrifolia* Maiden & Betche orchard near Hilo, Hawaii. Treatments in all studies were hung from consecutive trees 1.5 m above the ground and 20 m apart in a randomized complete-block design (Cochran and Cox, 1957). Blocks were arranged in two parallel rows, and approximately 36,000 sterile adult male and female laboratory-reared flies (3–5 days old) were released for each bioassay period from holding box cages that were carried between the two rows of blocks. Reference treatments of 2.0 g TML (AgriSense plug) in a Jackson trap were included in each study; in one series of traps, the TML plug was replaced

TABLE 2. CATCH OF MALE MEDITERRANEAN FRUIT FLIES WITH TRIMEDLURE AND CERALURE PANELS VERSUS STANDARD TRIMEDLURE PLUGS IN JACKSON TRAPS (HILO, HAWAII)

Formulation	Mean[a] catch (±SE)/trap for each formulation after indicated weeks aged in field						
	0–1	2–3	4–5	6–7	8–9	10–11	12–13
2 g TML fresh[b]	81.12 ± 10.59 bc	136.75 ± 15.23 a	95.62 ± 12.89 b	123.88 ± 12.32 ab	70.25 ± 8.34 a	136.50 ± 11.24 a	188.00 ± 11.40 a
2 g TML aged[c]	77.94 ± 18.45 bc	84.56 ± 11.98 b	77.19 ± 10.41 bc	71.69 ± 8.57 c	41.88 ± 7.59 bc	64.81 ± 8.12 bc	53.81 ± 8.45c
10 g TML TNO panel[d]	99.44 ± 12.27 ab	130.19 ± 20.22 a	82.62 ± 12.15 bc	116.88 ± 17.40 b	34.44 ± 4.70 c	57.25 ± 9.46 c	33.81 ± 5.00 cd
20 g TML TNO panel[e]	111.88 ± 11.01 a	138.69 ± 18.17 a	139.69 ± 16.24 a	145.56 ± 16.21 ab	54.88 ± 9.65 ab	84.94 ± 13.12 b	41.25 ± 5.43 cd
10 g TML CDFA panel[f]	80.88 ± 14.41 bc	82.94 ± 9.63 b	54.75 ± 10.02 c	107.56 ± 9.74 bc	34.38 ± 3.37 c	41.00 ± 5.42 c	28.88 ± 6.93 d
10 g CER PVC panel[f]	69.94 ± 10.67 c	130.75 ± 24.25 a	81.31 ± 13.44 bc	161.06 ± 22.64 a	69.75 ± 7.74 a	111.44 ± 13.55 a	98.00 ± 13.86 b
F	2.90	3.07	7.83	4.31	7.46	15.17	59.45
EMS	1344.07	3580.37	1645.22	3598.58	588.06	1357.11	1006.66
P	0.019	0.014	<0.001	0.002	<0.001	<0.001	0.001

[a] Means followed by the same letter within a column are not significantly different ($P > 0.05$; Fisher PLSD test), randomized complete block field plot design with eight replicates. Statistics are on untransformed data for all weeks.
[b] AgriSense plug in standard Jackson trap (replaced once/month).
[c] AgriSense plug in standard Jackson trap.
[d] 7% TML in Farma Tech 15 × 25 cm panel enveloped in 14 × 23 cm Seabright (long axis fold) bifold yellow cardboard coated with Hold Fast Stikem.
[e] 15% TML in Farma Tech 15 × 25 cm panel enveloped in 14 × 23 cm Seabright (long axis fold) bifold yellow cardboard coated with Hold Fast Stikem.
[f] Parapheromone in AgriSense 14 × 23 cm panel enveloped in 14 × 23 cm Seabright (long axis fold) bifold yellow cardboard coated with Hold Fast Stikem.

TABLE 3. CATCH OF MALE MEDITERRANEAN FRUIT FLIES WITH TRIMEDLURE PANELS IN C&C TRAPS VERSUS STANDARD TRIMEDLURE PLUGS IN JACKSON TRAPS (HILO, HAWAII)

Formulation	Mean[a] catch (±SE)/trap for each formulation after indicated weeks aged in field						
	0	1–3	4–6	7–9	10–12	13–15	16–18
2 g TML fresh[b]	38.60 ± 15.91 b	64.87 ± 12.32 c	57.67 ± 9.83 d	92.47 ± 18.38 bc	181.20 ± 12.98 b	131.53 ± 15.82 b	160.60 ± 18.76 c
2 g TML aged[c]	37.40 ± 18.16 b	53.00 ± 12.56 c	41.87 ± 6.66 d	37.80 ± 13.63 d	69.67 ± 14.34 c	27.80 ± 9.15 c	32.53 ± 10.91 d
20 g TML panel A[d]	172.60 ± 37.34 b	232.40 ± 41.19 b	111.13 ± 19.93 c	75.07 ± 12.14 cd	251.40 ± 35.86 ab	153.60 ± 32.61 b	118.60 ± 23.54 c
20 g TML panel B[d]	318.40 ± 82.90 a	302.20 ± 33.94 ab	169.13 ± 17.45 b	135.13 ± 21.23 ab	292.60 ± 40.18 a	272.87 ± 48.66 a	361.00 ± 40.31 b
20 g TML panel C[d]	313.00 ± 62.61 a	332.80 ± 50.28 a	216.67 ± 25.83 a	143.60 ± 24.12 a	221.93 ± 25.88 ab	271.33 ± 32.66 a	447.40 ± 54.16 a
F	8.94	22.36	23.97	6.95	11.40	11.43	42.20
EMS	10,781.33	11,565.02	3,414.60	4,120.18	9,533.26	14,047.04	10,703.97
P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a] Means followed by the same letter within a column are not significantly different ($P > 0.05$; Fisher PLSD test), randomized complete block field plot design with five replicates. Statistics are on untransformed data for all weeks.
[b] AgriSense plug in standard Jackson trap (replaced once/month).
[c] AgriSense plug in standard Jackson trap.
[d] Farma Tech TNO 15 × 15 cm panel in C&C trap.

monthly (fresh). In another series, the plug was allowed to age without replacement.

The first study, which lasted 17 weeks (Table 1) (March 24 to July 26, 1993), included five replicates of 1.9 g and 9.5 g TML in TNO panels and 2.1 g TML in 15 g Hold Fast Stikem coated on a CDFA panel.

The second study, which lasted 13 weeks (Table 2) (May 18 to August 23, 1993), included eight replicates of 10 g and 20 g TML in TNO panels and 10 g TML and 10 g CER in PVC panels, all of which were enveloped in the Seabright bifold yellow cardboard. The use of the Seabright cardboard was the first attempt at a trap designed to accommodate the panels for mass-trapping of medflies.

The third study, which lasted 18 weeks (Table 3) (August 25 to December 27, 1993), included five replicates of 20 g TML in TNO panels A, B, and C, all of which were in C&C traps.

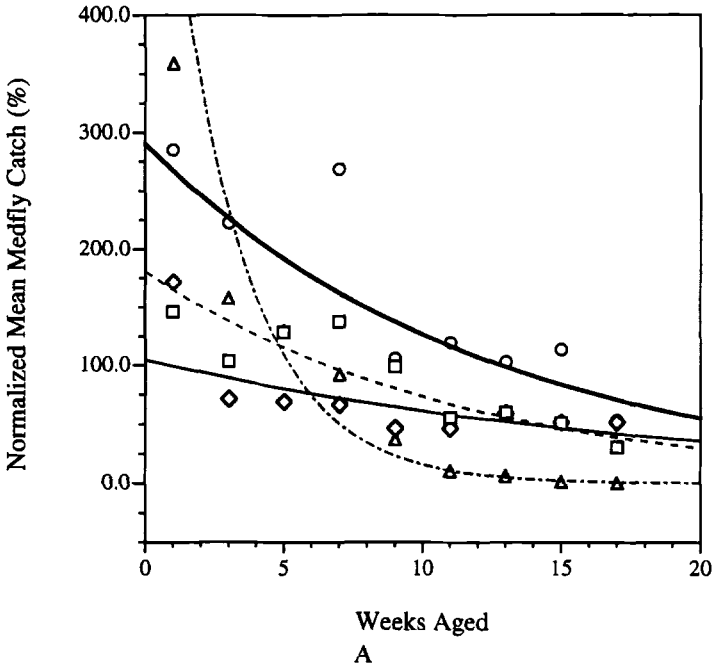
Trap catches for all three studies were counted weekly and recorded by the procedure of Rice et al. (1984). The untransformed data for each study were subjected to ANOVA and the Fisher's PLSD test (Fisher, 1966). Because of the variations in field environment during the bioassay, the fly capture data were normalized by dividing the mean captures of the treatments in the weeks aged by the mean captures of the fresh 2 g TML control in the weeks aged and multiplying by 100 (Figures 1A–3A below).

Residual Content Analysis. The 2-g AgriSense aged plugs and small samples of the TML and CER panels were taken on varying days (at least in triplicate) during the aging process for GC analysis of residual parapheromone content. These samples were soaked in 600 ml of acetone and hexane (1 : 1 v/v) for one to three days before GC analysis. The residual parapheromone contents (Leonhardt et al., 1994, 1996) of the plugs and panels were then graphed (Figures 1B–3B below).

RESULTS AND DISCUSSION

In the first study (Table 1, Figure 1A and B), fly captures with ca. 2 g TML in either a TNO panel or on a CDFA panel were compared to those with the 2-g TML AgriSense aged plug. The 1.9-g TML TNO panel caught significantly fewer medflies than the aged plug at four to nine weeks. The 2.1-g CDFA panel caught significantly greater numbers of flies at weeks 0–1 compared to the aged plug, but significantly less at 8–17 weeks. The superior efficacy of the TNO panel to the CDFA panel was confirmed when fly catches were compared, particularly after nine weeks. Therefore, the dose of TML in the TNO panel should be greater than the 2 g TML in the aged plugs for mass-trapping of medflies. Increasing the TML content of the TNO panel fivefold to 9.5 g

compared to the 1.9 g resulted in significantly greater fly catches at 2–15 weeks aged. Normalized data (Figure 1A) effectively show that the 1.9-g TML TNO panel, with a release of 1.166 g of TML over four months (Figure 1B), was inferior in fly captures compared to the 2-g TML AgriSense aged plug, which released 1.69 g of TML over four months (Figure 1B). The release of parapheromone from the 2.1-g TML CDFA panel occurred too quickly [overall, 4.3× (0.167/0.039 from exponential equations, Figure 1A) that of 2-g TML, aged], resulting in a significantly greater fly capture compared to the 2-g TML aged plug and 2-g TML AgriSense fresh standard for weeks 0–1, but catching significantly fewer flies than the 2-g TML aged plug or the 2-g TML AgriSense



A

FIG. 1. (A) Data from Table 1: means were normalized by dividing by the means of the 2.0-g TML fresh standard at the weeks aged and multiplying by 100; the 2.0-g TML fresh standard was replaced on the days of medfly catch determination. Curves were determined by exponential regression by the method of least squares. (- - - - □: 2g TML aged plug, $y = 180.399 \times 10^{-0.039x}$, $r^2 = 0.833$; ——◇: 1.9 g TML TNO panel, $y = 104.515 \times 10^{-0.023x}$, $r^2 = 0.534$; ——○: 9.5 g TML TNO panel, $y = 290.725 \times 10^{-0.036x}$, $r^2 = 0.716$; - · - · - · - △: 2.1 g TML CDFA panel, $y = 739.809 \times 10^{-0.167x}$, $r^2 = 0.952$). (B) Residual TML (grams) in AgriSense aged plugs and panels (from Table 1) initially (T-0) and at four months.

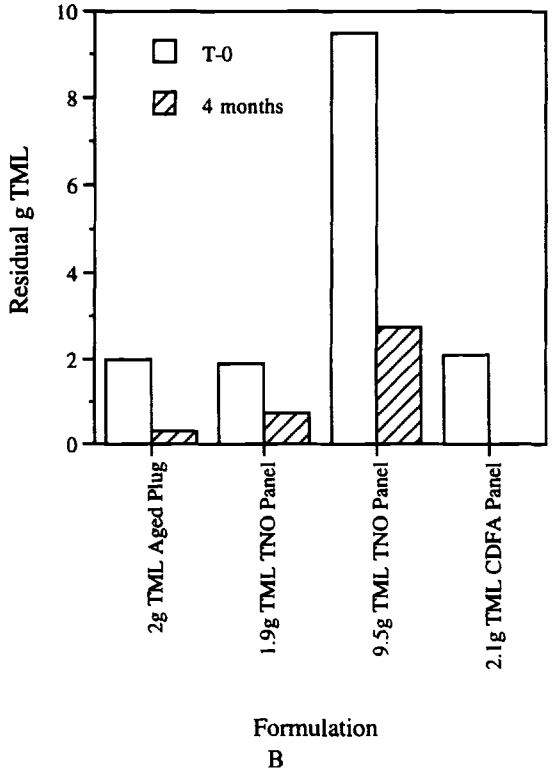
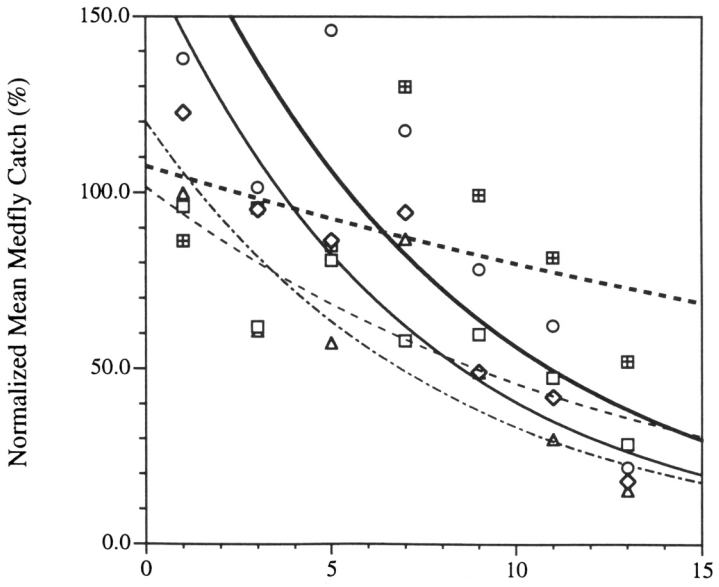


FIG. 1. Continued.

fresh standard from 8 to 17 weeks. The 9.5-g TML TNO panel caught a significantly greater number of flies than the 2-g TML AgriSense aged plug except for weeks 4–5, 8–9, and 16–17; overall captures were even larger than with fresh standard plugs, but not for every week. The 9.5-g TML TNO panel released 71.2% (6.8 g) TML over the four-month period (Figure 1B) compared to the release of 84.5% (1.69 g) from the 2-g TML AgriSense aged standard and the release of 61.4% (1.166 g) from the 1.9-g TML TNO panel.

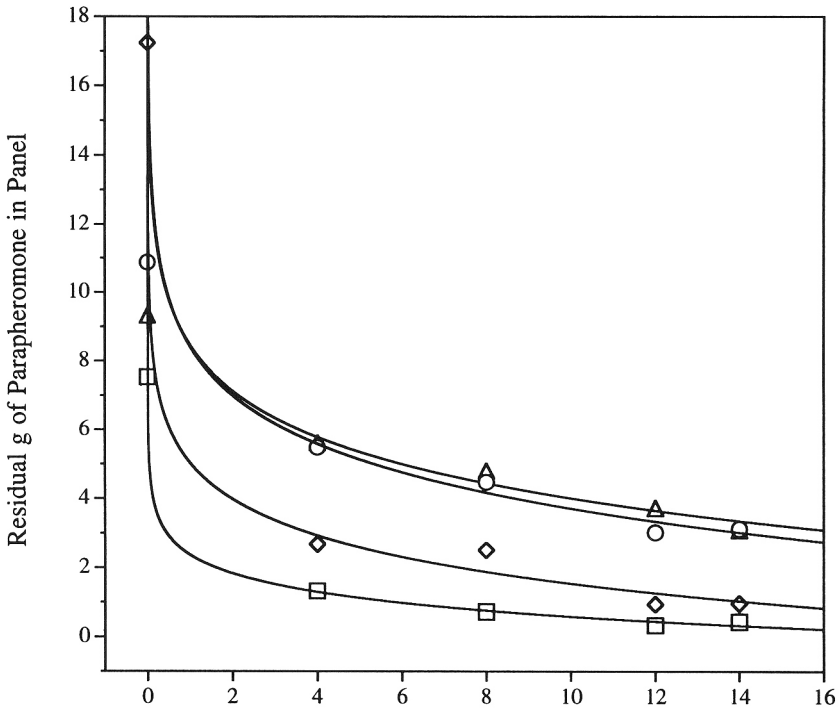
In the second study (Table 2, Figures 2A and B), each of the TML panels was enveloped in the Seabright bifold yellow cardboard, coated with Hold Fast Stikem. Under these conditions, the 10-g TML TNO panel caught significantly more flies only at weeks 2–3 and 6–7, with no other significant differences, compared to the 2-g TML AgriSense aged plug. Compared to the 2-g TML AgriSense fresh plug, this 10-g TML TNO panel caught significantly less flies at weeks 8–13. With this bifold, yellow cardboard trap design, the 20-g TML TNO panel caught significantly greater numbers of flies at weeks 0–7 compared



A

FIG. 2. (A) Data from Table 2: means were normalized by dividing by the means of the 2.0-g TML fresh standard at the weeks aged and multiplying by 100; the 2.0-g TML fresh standard was replaced on the days of medfly catch determination. Curves were determined by exponential regression by the method of least squares. (---- □: 2 g TML aged plug, $y = 101.606 \times 10^{-0.035x}$, $r^2 = 0.784$; ——— ◇: 10 g TML TNO panel, $y = 167.755 \times 10^{-0.062x}$, $r^2 = 0.847$; ——— ○: 20 g TML TNO panel, $y = 200.395 \times 10^{-0.055x}$, $r^2 = 0.697$; -·-·-· △: 10 g TML PVC panel, $y = 120.137 \times 10^{0.056x}$, $r^2 = 0.739$; ——— □: 10 g CER PVC panel, $y = 107.518 \times 10^{-0.013x}$, $r^2 = 0.219$). (B) Residual parapheromone (grams) in panels (from Table 2) initially (T-0) and at 4, 8, 12, and 14 weeks aged as a logarithmic regression with correlation coefficient. (□: 10 g TML TNO panel, $y = -1.787\text{LOG}(x) + 2.367$, $r^2 = 0.956$; ◇: 20 g TML TNO panel, $y = -3.488\text{LOG}(x) + 5.023$ $r^2 = 0.791$; ○: 10 g TML PVC panel, $y = -4.710\text{LOG}(x) + 8.414$, $r^2 = 0.949$; △: 10 g CER PV panel, $y = -4.474\text{LOG}(x) + 8.475$ $r^2 = 0.938$).

to the 2-g TML AgriSense aged plug and significantly greater numbers of flies than the 10-g TML TNO panel at 4–5 and 8–11 weeks. Therefore, medfly catches with this trap design were clearly not delineating the twofold difference in parapheromone dosage, except for three out of seven treatments. There was a significant difference in fly catch at weeks 2–3 between the 10-g TML PVC panel and the 10-g TML TNO panel, but essentially there was no overall dif-



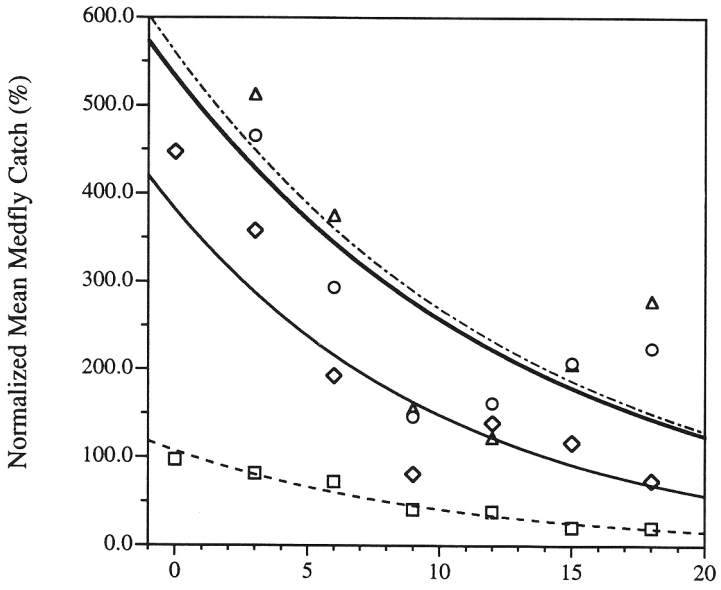
B

FIG. 2. Continued.

ference between these panels. There were, however, significantly greater fly catches for the 10-g CER PVC panel over the 10-g TML PVC panel at 2–3 and 6–13 weeks; unfortunately, a poor correlation coefficient (R^2) for the exponential regression curve for normalized mean medfly catch (percent) versus weeks aged was observed for this CER panel. Linear, power, or logarithmic regression curves for the CER PVC panel produced even poorer R^2, but a first-order polynomial regression curve yielded an $R^2 = 0.678$ with a curve quite different from that for TML release. The magnitude of the significant fly catches of the 10-g CER PVC panel over those of the 10-g TML PVC panel, except for weeks 0–1 and 4–5, is striking when one considers that an equal weight of these two parapheromones is being compared. Initially, there was actually 21.6% less CER present on a molar basis compared to TML because of the difference in their molecular weights. If one considers the amount of active ingredient in the

parapheromones (ca. 40% TML-C and 26% CER-B1) (Warthen et al., 1994; McGovern et al., 1990), one is actually comparing 4.0 g TML-C to 2.6 g CER-B1. Thus, there is almost twice as much active ingredient on a molar basis of TML-C versus CER-B1 in equal weights of the parapheromones (Leonhardt et al., 1996). This demonstrates how persistent CER is compared to TML.

The residual parapheromone data (Figure 2B) revealed that the TNO panels released TML more rapidly than the PVC panel in a logarithmic fashion; the normalized data reflect this in fly captures with this bifold, yellow cardboard

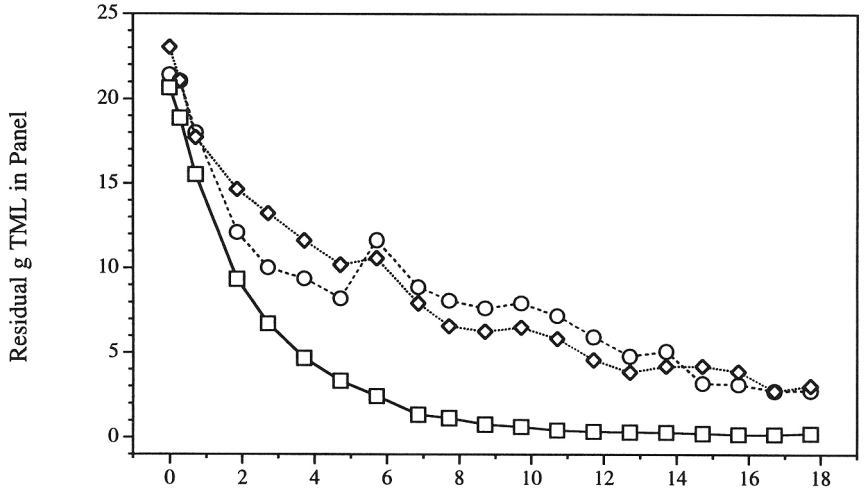


A

FIG. 3. (A) Data from Table 3: means were normalized by dividing by the means of the 2.0-g TML fresh standard at the weeks aged and multiplying by 100; the 2.0-g TML fresh standard was replaced on the days of medfly catch determination. Curves were determined by exponential regression by the method of least squares. (---- □: 2 g TML aged plug, $y = 107.017 \times 10^{-0.042x}$, $r^2 = 0.955$; ——◇: 20 g TML, A Panel, C&C trap, $y = 382.079 \times 10^{-0.041x}$, $r^2 = 0.778$; ——○: 20 g TML, B Panel, C&C trap, $y = 532.768 \times 10^{-0.032x}$, $r^2 = 0.582$; -·-·-· △: 20 g TML, C Panel, C&C trap, $y = 559.724 \times 10^{-0.032x}$, $r^2 = 0.500$). (B) Residual TML (grams) in panels (from Table 3) initially (T-0) to 18 weeks. (—□—: 20g TML, A panel, C&C trap; ····◇····: 20g TML, B panel, C&C trap; ----○---- 20g TML, C panel, C&C trap).

trap design. The 10-g and 20-g TNO panels released TML overall 1.8× and 1.6× (0.062/0.035 and 0.055/0.035, respectively, from exponential equations, Figure 2A) faster than the 2-g TML aged plugs. This resulted in significantly greater fly catches for weeks 2–3 and 6–7 for the 10-g TNO panel and significantly greater fly catches for weeks 0–7 for the 20-g TNO panel compared to the 2.0-g TML aged plugs. The 10-g TML PVC panel had a slower release rate compared to the TNO panels and did not catch as well. The trap design with the Seabright cardboard surrounding the polymeric panels was not effective in delineating dosages of TML in the TNO panels, nor was a 10-g dose of TML sufficient for mass-trapping since only two treatments of seven had significantly greater medly catches than the 2-g TML aged plugs.

In the third study (Table 3, Figures 3A and B), the amounts of TML in all the various proprietary polymeric TNO panel formulations were increased to 20 g compared to 10 g in the second study, and the C&C trap was utilized to hold the panels. This trap design evolved from observations (R.T.C.) in the second study. The first C&C traps (J.M.C.) were made of wood, including the framelike panel holders. For this study, the trap was simplified so that the panels slid into grooves in the body of a plastic holder. The C&C trap containing the 20-g TML TNO panel A caught significantly more flies at 1–6 and 10–18 weeks than the 2-g TML AgriSense aged plug. The C&C trap containing the 20-g



B

FIG. 3. Continued.

TML TNO panel B caught significantly more flies at every sampled week than the aged plug. The C&C trap containing the 20-g TML TNO panel C caught significantly more flies at every sampled week than the aged plug. Utilization of panels B and C in this study (Table 3) resulted in significant fly captures at every sampled week exceeding those of the 2.0-g TML plug that was replenished monthly, except for weeks 7–9 for panel B and for weeks 10–12 for panel C. The normalized data (Figure 3A) showed that the utilization of the C&C trap with a controlled-release parapheromone dispenser was extremely effective in attracting flies and was superior to 100%, which represents the medfly catch of the 2-g TML AgriSense fresh plug. It was also far better than the 2-g TML AgriSense aged plug. The residual parapheromone data (Figure 3B) revealed that panel A released parapheromone too quickly over 18 weeks in the field. About 3.5 g of TML remained in panels B and C at 18 weeks, whereas this residual level occurred in panel A in only five weeks. Since the C&C trap with panel C caught 6.0% more flies during the 18-week study than with panel B, panel C was considered slightly superior to panel B.

The C&C trap with the 20-g TML TNO panel C in the third study caught 2.5× and 6.3× as many total flies as the 2-g TML AgriSense fresh plug and the 2-g TML AgriSense aged plug, respectively. In contrast, the 20-g TML TNO panel enveloped with the Seabright cardboard in the second study caught 0.86× and 1.5× as many total flies as the 2-g TML AgriSense fresh plug and the 2-g TML AgriSense aged plug, respectively. The increase in the fly capture with the C&C trap over the trap design utilizing the Seabright cardboard is probably due to the design of the C&C trap. This design allows the parapheromone to be evenly released from the entire surface of the polymeric panel into the air, since the cardboards coated with Hold Fast Stikem on either side are separated from it by a gap. When the parapheromone-containing polymeric panel is enveloped by and touching the Seabright cardboard, as in the second study, the parapheromone escapes only from the edges of that trap.

CONCLUSIONS

An improved controlled-release formulation in a new trap (C&C) was developed to maximize effectiveness for mass-capture and monitoring of male Mediterranean fruit flies. The TML attractant in the polymeric TNO panel C was highly effective in delivering attractant doses of parapheromone for at least 18 weeks. The combination of this trap design and polymeric panel dispenser increased fly capture 6.3× above that of the standard 2-g TML AgriSense aged plug over an 18-week period. The design of this trap allows the panels to slide neatly into the holder, making them easy to remove, inspect, and replace. The 18-week effectiveness of the panel will permit less labor-intensive surveillance.

The superiority of CER over TML as an attractant was also demonstrated with a significantly greater number of fly catches using a molar quantity half that of TML.

Acknowledgments—We thank Dr. J. J. deVlieger and D. P. W. Alkema, BSc, of TNO Plastics & Rubber Research Institute, Delft, The Netherlands, for development of the various test formulations and providing the test sample panels.

REFERENCES

ANONYMOUS. 1996a. http://www.aphis.usda.gov/oa/medfly.html

ANONYMOUS. 1996b. http://www.ars-grin.gov:80/ars/Beltsville/barc/psi/icel/icel.html

ANONYMOUS. 1996c. To zap medflies—red dye, updated traps. *Agric. Res.* January: 20–22.

AVERY, J. W., CHAMBERS, D. L., CUNNINGHAM, R. T., and LEONHARDT, B. A. 1994. Use of ceralure and trimedlure in Mediterranean fruit fly (Diptera: Tephritidae) mass-trapping tests. *J. Entomol. Sci.* 29:543–556.

BEROZA, M., GREEN, N., GERTLER, S. I., STEINER, L. F., and MIYASHITA, D. M. 1961. New attractants for the Mediterranean fruit fly. *J. Agric. Food Chem.* 9:361–365.

COCHRAN, W. G., and COX, G. M. 1957. Experimental Designs, 2nd ed. Wiley, New York.

DEMILO, A. B., WARTHEN, J. D., JR., and LEONHARDT, B. A. 1994. Capillary gas chromatography method for the analysis of the *trans* isomers of ceralure, a medfly attractant. *J. Chromatogr. A* 673:295–298.

DOOLITTLE, R. E., CUNNINGHAM, R. T., MCGOVERN, T. P., and SONNET, P. E. 1991. Trimedlure enantiomers: Differences in attraction for Mediterranean fruit fly, *Ceratitis capitata* (Wied.) (Diptera: Tephritidae). *J. Chem. Ecol.* 17:475–484.

FISHER, R. A. 1966. The Design of Experiments, 8th ed. Hafner Press, New York.

HARRIS, E. J., NADAGAWA, S., and URAGO, T. 1971. Sticky traps for detection and survey of three tephritids. *J. Econ. Entomol.* 64:62–65.

LEONHARDT, B. A., MCGOVERN, T. P., and PLIMMER, J. R. 1982. Capillaray GC analysis of trimedlure, the attractant for the medfly. *J. High Resol. Chromatogr. Chromatogr. Commun.* 5:430–431.

LEONHARDT, B. A., RICE, R. E., HARTE, E. M., and CUNNINGHAM, R. T. 1984. Evaluation of dispensers containing trimedlure, the attractant for the Mediterranean fruit Fly (Diptera: Tephritidae). *J. Econ. Entomol.* 77:744–749.

LEONHARDT, B. A., CUNNINGHAM, R. T., HARTE, E. M., and MCGOVERN, T. P. 1986. Effect of additives on the evaporation of the most active isomer of trimedlure, the attractant for *Ceratitis capitata* (Diptera: Tephritidae). *J. Econ. Entomol.* 79:1050–1054.

LEONHARDT, B. A., CUNNINGHAM, R. T., RICE, R. E., HARTE, E. M., and MCGOVERN, T. P. 1987. Performance of controlled-release formulations of trimedlure to attract the Mediterranean fruit fly, *Ceratitis capitata. Entomol. Exp. Appl.* 44:45–51.

LEONHARDT, B. A., CUNNINGHAM, R. T., RICE, R. E., HARTE, E. M., and HENDRICHS, J. 1989. Design, effectiveness, and performance criteria of dispenser formulations of trimedlure, an attractant of the Mediterranean fruit fly (Diptera: Tephritidae). *J. Econ. Entomol.* 82:860–867.

LEONHARDT, B. A., CUNNINGHAM, R. T., CHAMBERS, D. L., AVERY, J. W., and HARTE, E. M. 1994. Controlled-release panel traps for the Mediterranean fruit fly (Diptera: Tephritidae). *J. Econ. Entomol.* 87:1217–1223.

LEONHARDT, B. A., CUNNINGHAM, R. T., AVERY, J. W., DEMILO, A. B., and WARTHEN, J. D.,

JR. 1996. Comparison of ceralure and trimedlure attractants for the male Mediterranean fruit fly (Diptera: Tephritidae). *J. Entomol. Sci.* 31:183–190.

LIQUIDO, N. J., SHINODA, L. A., and CUNNINGHAM, R. T. 1991. Host plants of the Mediterranean fruit Fly: An annotated world review. *Misc. Publ. Entomol. Soc. Am.* 77:1–52.

MCGOVERN, T. P., and BEROZA, M. 1966. Structure of the four isomers of the insect attractant trimedlure. *J. Org. Chem.* 31:1472–1477.

MCGOVERN, T. P., and CUNNINGHAM, R. T. 1987. New medfly attractants: Halogen analogs of trimedlure. National Conference of the Entomological Society of America, Boston, Massachusetts, Paper #1283, November 29–December 3.

MCGOVERN, T. P., and CUNNINGHAM, R. T. 1988. Persistent attractants for the Mediterranean fruit fly, the method of preparation and method of use. U.S. Patent 4,764,366. Issued August 16.

MCGOVERN, T. P., BEROZA, M., OHINATA, K., MIYASHITA, D., and STEINER, L. F. 1966. Volatility and attractiveness to the Mediterranean fruit fly of trimedlure and its isomers, and a comparison of its volatility with that of seven other insect attractants. *J. Econ. Entomol.* 59:1450–1455.

MCGOVERN, T. P., CUNNINGHAM, R. T., and LEONHARDT, B. A. 1986. *cis*-Trimedlure: Attraction for the Mediterranean fruit fly (Diptera: Tephritidae) and isomeric structural assignments. *J. Econ. Entomol.* 79:98–102.

MCGOVERN, T. P., CUNNINGHAM, R. T., and LEONHARDT, B. A. 1987. Attractiveness of *trans*-trimedlure and its four isomers in field tests with the Mediterranean fruit fly (Diptera: Tephritidae). *J. Econ. Entomol.* 80:617–620.

MCGOVERN, T. P.,WARTHEN, J. D., JR., and CUNNINGHAM, R. T. 1990. Relative attraction of the Mediterranean fruit fly (Diptera: Tephritidae) to the eight isomers of trimedlure. *J. Econ. Entomol.* 83:1350–1354.

RICE, R. E., CUNNINGHAM, R. T., and LEONHARDT, B. A. 1984. Weathering and efficacy of trimedlure dispensers for attraction of Mediterranean fruit flies (Diptera: Tephritidae). *J. Econ. Entomol.* 77:750–756.

SONNET, P. E., MCGOVERN, T. P., and CUNNINGHAM, R. T. 1984. Enantiomers of the biologically active components of the insect attractant trimedlure. *J. Org. Chem.* 49:4639–4643.

WARTHEN, J. D., JR., CUNNINGHAM, R. T., DEMILO, A. B., and SPENCER, S. 1994. *trans*-Ceralure isomers: Differences in attraction for Mediterranean fruit fly, *Ceratitis capitata* (Wied.) (Diptera: Tephritidae). *J. Chem. Ecol.* 20:569–578.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

A GENETIC ANALYSIS OF POPULATION DIFFERENCES IN PHEROMONE PRODUCTION AND RESPONSE BETWEEN TWO POPULATIONS OF THE TURNIP MOTH, *Agrotis segetum*

SIANA LAFOREST,[1,2,*] WENQI WU,[2] and CHRISTER LÖFSTEDT[2]

[1]*Department of Ecology and Evolution, SUNY at Stony Brook*
Stony Brook, New York 11794-5145

[2]*Department of Ecology, Lund University*
S-223 62 Lund, Sweden

(Received December 29, 1995; accepted January 17, 1997)

Abstract—The genetic basis of the differences in female pheromone blend ratio and male behavioral response in the turnip moth, *Agrotis segetum*, was examined by crossing individuals derived from Scandinavian and Zimbabwean populations. These two populations differ both in the ratio of the three major female pheromone components, Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc and in the behavioral response of the males in both wind-tunnel and field-trapping assays. The female pheromone blend in this study is treated as the log ng Z5-10:OAc/ng Z7-12:OAc and log ng Z9-14:OAc/ng Z7-12:OAc for statistical analysis. The mean log ng Z5-10:OAc/ng Z7-12:OAc, is under control by a major autosomal factor or factors, but it is unclear what genetic factor or factors may control the mean log ng Z9-14:OAc/ng Z7-12:OAc. Frequency distributions of the proportions of each component show wide individual variation and also suggest control of Z5-10:OAc and Z7-12:OAc by major autosomal factors, which for Z5-10:OAc may show partial dominance. Analysis of male behavioral response to synthetic blends in the wind tunnel yields inconclusive results, but suggests that *A. segetum* may have a broad window of response that reflects the range of individual variation in female blends.

Key Words—Turnip moth, *Agrotis segetum*, Lepidoptera, Noctuidae, sex pheromone, genetics, population variation, pheromone production, behavioral response.

*To whom correspondence should be addressed.

0098-0331/97/0600-1487$12.50/0 © 1997 Plenum Publishing Corporation

INTRODUCTION

Moth sex pheromone communication systems provide an excellent opportunity for biologists to examine genetic variation in characters related to mate recognition. Because they play the major role in the process of mate location in moths, sex pheromone systems are likely to be under strong selection (Cardé and Baker, 1984; Phelan, 1992). Since many species show a high degree of species specificity in both pheromone production and response, it has been suggested that these systems play a role in reproductive isolation (Löfstedt et al., 1991). A number of species show interesting patterns of geographical and individual variation in spite of this high species specificity (Löfstedt, 1990). If we are to understand the processes of mate recognition and speciation in moths, an understanding of the genetic basis for this variation is crucial.

The genetic basis of pheromone production and response has been studied in very few species. The most detailed study thus far is that of the European corn borer, *Ostrinia nubilalis.* Two distinct strains occur in Europe and North America: one in which females produce and males respond optimally to a pheromone blend of approximately 97 : 3 (*E*)-11-tetradecenyl acetate (*E*11-14 : OAc) to (*Z*)-11-tetradecenyl acetate (*Z*11-14 : OAc), and the other producing and responding optimally to a blend ratio of 3 : 97 *E*11-14 : OAc to *Z*11-14 : OAc (Klun and Cooperators, 1975; Kochansky et al., 1975; Anglade et al., 1984; Barbattini et al., 1985; Peña et al., 1988). By examining the pheromone blend and response of F_1, F_2, and backcross hybrids, it was found that the difference in female pheromone production between these two strains is controlled by two alleles at a single autosomal locus, with F_1 females producing an intermediate blend (Klun and Maini, 1979; Zhu et al., 1996). Furthermore, it was also found that male electrophysiological response is controlled by a single autosomal locus (Hansson et al., 1987) and male behavioral response in wind-tunnel experiments is controlled by a single *Z*-linked locus (Roelofs et al., 1987). Individual variation in this blend ratio is quite low, which is typical for moth sex pheromone blends consisting of a ratio of *E* and *Z* isomers (Löfstedt, 1990). By contrast, moth sex pheromone blends consisting of a mixture of acetates show a broader range of individual variation. The noctuid *Trichoplusia ni* uses a mixture of acetates in its pheromone blend, and crosses between laboratory-reared and field-derived *T. ni* (Haynes and Hunt, 1990; Hunt et al., 1990) reveal that the female pheromone blend ratio is controlled primarily by multiple autosomal factors and possible sex-linked factors (Haynes and Hunt, 1990; Hunt et al., 1990).

The turnip moth, *Agrotis segetum*, also has a sex pheromone system with a broad range of individual variation. Females from Scandinavia produce a blend of *Z*5-10 : OAc, *Z*7-12 : OAc, and *Z*9-14 : OAc in a ratio of 1 : 5 : 2.5 (Löfstedt and Odham, 1984). In this population, there is a high coefficient of variation for the percentage of the three major pheromone components: approximately

127% for Z5–10:OAc, 32% for Z7–12:OAc, and 59% for Z9–14:OAc (Löfstedt et al., 1985b). Recently, Z5–12:OAc was demonstrated to be both electrophysiologically and behaviorally active and thus was identified as a fourth sex pheromone component (Wu et al., 1997). However, this compound is produced in very small quantities by Scandinavian females, and only trace amounts can be found in Zimbabwean females (Wu, 1995).

In *A. segetum*, the ratio of the three major components and the corresponding degree of male preference varies across geographic regions (Arn et al., 1983; Löfstedt et al., 1986; Hansson et al., 1990; Tóth et al., 1992). The most distinct interpopulational differences can be found between Eurasian and sub-Saharan populations, including one from Zimbabwe and one from South Africa (Tóth et al., 1992). In South Africa and Zimbabwe, females produce a 1:0.25:0.03 blend of Z5–10:OAc, Z7–12:OAc, and Z9–14:OAc. In the flight tunnel and in the field, Zimbabwean males are significantly more attracted to the three-component Zimbabwean blend than the three-component Scandinavian blend. In the field, Zimbabwean males are much more strongly attracted to Z5–10:OAc alone than are Scandinavian males (Wu, 1995).

The breadth of population and individual variation in this species suggested to us a polygenic basis of inheritance of both female production and male response. We hypothesized that female production would be controlled by autosomal factors, with intermediate blends produced by F_1 hybrid females, and that male response in the wind tunnel would be controlled by sex-linked factors. To determine the mode of inheritance, we crossed laboratory raised Scandinavian and Zimbabwean moths to create F_1, F_2, and backcross hybrids and analyzed individual pheromone gland contents as well as male response in the wind tunnel to a range of blends.

METHODS AND MATERIALS

Insect Materials. Scandinavian *Agrotis segetum* were collected from southern Sweden and Denmark and maintained in the laboratory for over 10 years (approximately 70 generations). Periodically, adults emerging from wild-collected pupae were added to the Scandinavian stock. The Zimbabwean *Agrotis segetum* culture was established from pupae collected in Zimbabwe, courtesy of Dr. C. B. Cottrell. The Zimbabwean colony was started from approximately 50 individuals and had been maintained for two generations prior to this study. For both populations, each new generation was produced in the laboratory by mating 10–15 each of randomly selected males and females. The larvae were reared on a Hinks and Byers semisynthetic diet, and maintained at 25°C on a reversed 18L:6D cycle. Pupae were sexed and kept in separate chambers, with a 16L:8D photoperiod at 23°C.

Crosses. To create the F_1 generation, 10 pairs of males and females from both Scandinavian and Zimbabwean strains were kept separately in individual mating cages for each pair. Eggs, larvae, and adults produced from each pair were kept separately as families and reared as described above. Four families of F_1 insects were then used for subsequent data analysis and production of F_2 and backcross strains. The F_2 generation was produced by sibling matings within one family of F_1 insects from each reciprocal cross (Scandinavian female × Zimbabwean male, Zimbabwean female × Scandinavian male). F_1 females from within two families (one from each reciprocal cross) were mated to males of each parental strain to produce the backcross generation (Figure 1). The F_1, F_2, and backross males used in the analysis of behavior were derived from a second generation of parental lines, using the same crossing patterns as described above.

Analysis of Pheromone Gland Extracts. Terminal segments containing pheromone glands were dissected from 2 to 4-day-old females during the third to fifth hour of the scotophase, and extracted in 8 μl of hexane containing 1 ng internal standard for 30 min. The extracts were analyzed on a Hewlett Packard 5890 gas chromatograph equipped with a DB-wax column (30 m × 0.25 mm

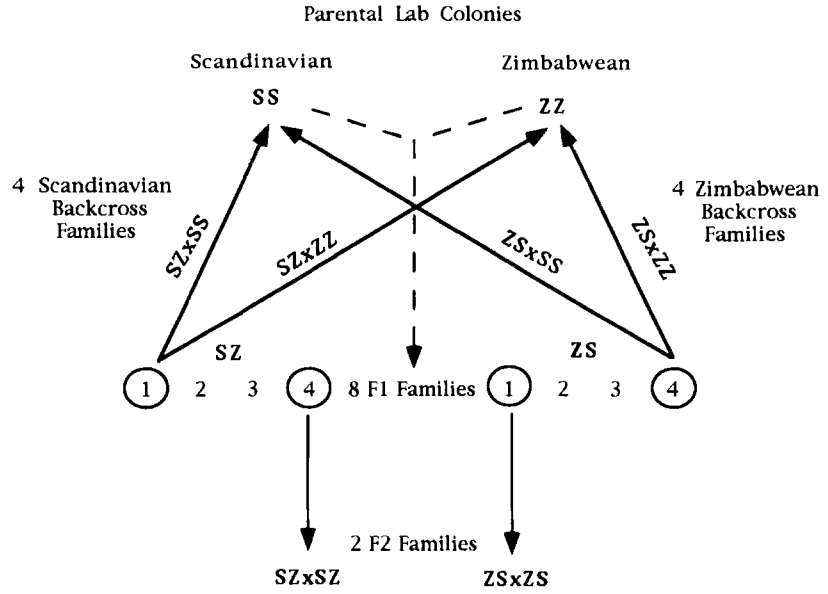


FIG. 1. Diagram of crossing patterns used to create F_1, F_2, and backcross families. Bold capitals indicate direction of cross, with maternal lines indicated first.

ID, 0.5-μm film, J&W Scientific, Folsom, California) and an automatic injector tray. Hydrogen was used as a carrier gas. Samples were injected splitless, and the split valve was opened 1 min after injection. The injector temperature was 225°C. Column temperature was maintained at 80°C for 2 min after injection and linearly increased to 230°C at a rate of 10°C/min.

Statistical Analysis of Female Data. Because the total amount of the three major compounds can vary so widely among individuals (Löfstedt et al., 1985b), one may calculate the proportion of each component, so that comparisons can be made independent of absolute amounts. However, because the proportions of pheromone components must all add up to one, there are no satisfactory statistical methods for modeling variation (Aitchison, 1986; Neems and Butlin, 1995). Aitchison (1986) recommends replacing the vector of components ($X_1 X_2$, . . . , X_n) with a vector of log contrasts: log (X_1/X_n), log (X_2/X_n), . . . , log (X_{n-1}/X_n) where X_1, X_2, . . . , $X_{x=1}$ (see also Neems and Butlin, 1995). When the analysis deals with the proportions of only three components that sum to one, only two of the three proportions of pheromone components (X_1 and X_2) can vary independently. If we treat the pheromone blend as a ratio of absolute amounts rather than proportions (log X_1/X_2 and log X_3/X_2, where X_1 = ng Z5-10:OAc, X_2 = ng Z7-12:OAc and X_3 = ng Z9-14:OAc), variation in the blend can be treated by standard univariate rather than multivariate techniques.

Behavioral Tests. Flight tunnel experiments were performed in two rectangular Plexiglas flight tunnels (Löfstedt et al., 1985a), one of which was 2.5 m long × 0.9 m wide × 0.9 m high, and the other of which was 3 m × 0.9 m × 0.9 m. Two- to 3-day-old male moths were transferred individually to 250-ml screen cages before the onset of the dark period and allowed to acclimate to wind tunnel room conditions for 1 hr before they were tested 3–5 hr into the scotophase. The wind tunnel was kept at 21–23°C, 40–60% relative humidity, 0.3 m/s wind velocity, and 1 lux intensity for all experiments. Fifty nanograms of synthetic bait in 10 μl hexane were applied to rubber septa and placed on a metal stand at one end of the wind tunnel. One bait contained Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc in the same ratio as that produced by Scandinavian females (1:5:2.5), another used the three compounds in the ratio produced by Zimbabwean females (1:0.25:0.03), and a third bait contained Z5-10:OAc alone, since this compound has been shown to be as attractive alone as a three component blend to Zimbabwean males in the field (Tóth et al., 1992; Wu et al., 1997). Males were released individually into the plume at the opposite end, and the presence/absence of three behavioral steps (orientation, upwind flight half the distance between the release cage and the pheromone source, and source contact) were scored. Males were tested once and discarded. After testing, release cages and bait holders were heated at 200°C for 1 hr to completely remove all traces of bait.

RESULTS AND DISCUSSION

Matings. The frequency of matings between Scandinavian and Zimbabwean parental strains and between hybrids and both parental strains (40–60%) was similar to that within each parental strain under the conditions described above. Hybrid larvae showed no sign of inviability, but rearing difficulties made it necessary to pool data collected across families within each of the hybrid groups.

Female Data. The mean of the total amount of Z5–10:OAc, Z7–12:OAc, and Z9–14:OAc was 0.88 ng and varied from 0.20 to 2.5 ng across all samples. Using the log-transformed ratios of the absolute amount of each component (log ng Z5–10:OAc/ng Z7–12:OAc and log ng Z9–14:OAc/ng Z7–12:OAc), Bartlett's test for heterogeneity of variances revealed significant heterogeneity, so the nonparametric Mann-Whitney U statistic was used for both characters.

The mean log ng Z5–10:OAc/ng Z7–12:OAc of both parental strains was significantly different (Figure 2). Female Lepidoptera are heterogametic; if this character is controlled by a major sex-linked factor, the means of the F_1 and subsequent backcross females would be identical to or closer to females of the original paternal line in the parental strain in both sets of reciprocal crosses. Since both F_1 reciprocal crosses are intermediate and not significantly different, and there were no significant differences between reciprocal crosses within either class of the backcross and F_2 groups (Figure 2), these results suggest an autosomal mode of inheritance with no dominance for the log ng Z5–10:OAc/ng Z7–12:OAc. For the remaining analysis, the reciprocal crosses were pooled within the F_1, backcross, and F_2 groups. The means of the pooled F_1 and F_2 groups were intermediate and significantly different from the parental groups, and both pooled backcross groups were significantly different from but closer to the parental strain that produced them (Figure 2). Thus we assumed that differences in the mean blend ratio of log ng Z5–10:OAc/ng Z7–12:OAc between these groups were due mainly to genetic rather than environmental causes. Although the mean of the Zimbabwean backcross group is closer to the Zimbabwean parental strain than the F_1 and F_2 groups, we found that comparisons between the mean of these three groups were not significantly different.

Examination of the mean log ng Z9–14:OAc/ng Z7–12:OAc reveals more ambiguous results. First, there are no significant differences between reciprocal crosses, which would indicate an autosomal pattern of inheritance. Although the mean log ng Z9–14:OAc/ng Z7–12:OAc differs significantly between the two parental strains, the mean of the pooled F_1 group is not intermediate, but resembles the Scandinavian parental strain (Figure 2). This implies a major dominant autosomal factor or factors. However, if we assume the trait is controlled by a major dominant autosomal factor, the mean of the backcross Scan-

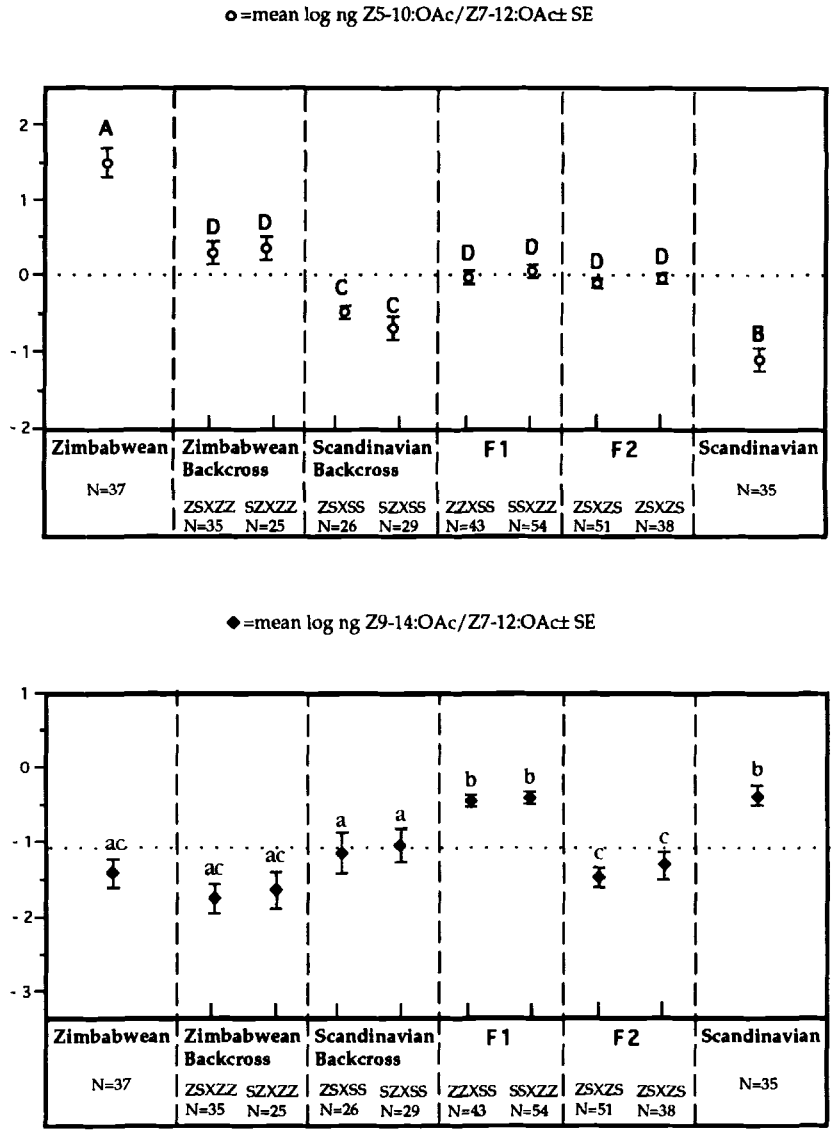


FIG. 2. Comparison of means and standard errors across groups. Comparisons of the mean log ng Z5-10:OAc/ng Z7-12:OAc across strains are indicated by bold uppercase letters, comparisons of the mean log ng Z9-14:OAc/ng Z7-12:OAc across strains are indicated in lowercase letters. Shared letters indicate nonsignificant comparisons using Mann-Whitney U, $\alpha = 0.05$, with the Dunn Sidák method of adjustment of significance levels.

dinavian group should be at least as close to the Scandinavian parental strain as the F_1, whereas the mean of the backcross Zimbabwean and F_2 groups should be closer to the midpoint than the backcross Scandinavian group because of the presence of the Zimbabwean and Scandinavian phenotypes. Subsequent comparisons of the means between the parental, backcross and F_2 groups reveal that the mean log ng Z9-14:OAc/ng Z7-12:OAc of the backcross Zimbabwean group is not significantly different from the parental Zimbabwean, and the backcross Scandinavian is not significantly different from the F_2 or backcross African means. Thus, it is difficult to conclude from this analysis the extent to which differences in the mean log ng Z9-14:OAc/ng Z7-12:OAc are affected by genetic factors.

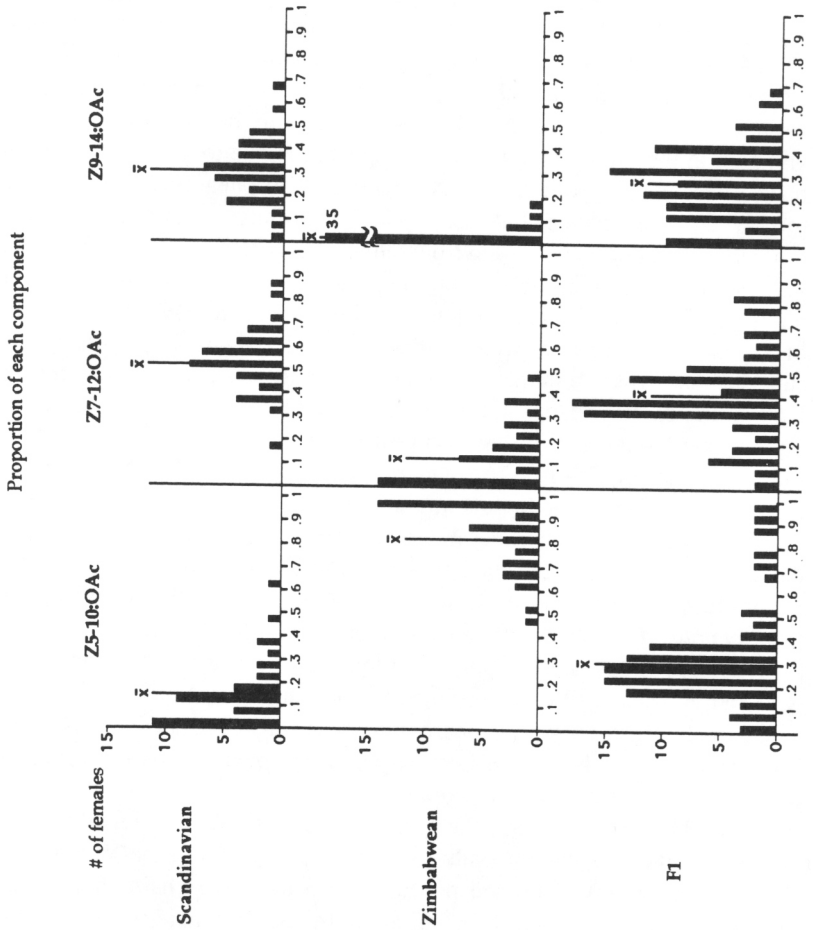
To examine the patterns of variation of the three components further, the proportion of the total blend represented by each component was calculated, and separate frequency distributions were generated from each within each of the groups (Figure 3). As with the analysis of the log ng Z5-10:OAc/ng Z7-12:OAc, the mean proportion of Z5-10:OAc and the mean proportion of Z7-12:OAc are intermediate in F_1 and F_2; the mean proportion of Z5-10:OAc and proportion of Z7-12:OAc for the backcross African group are closer to the Zimbabwean parental strain than the F_1, and the mean proportion of Z5-10:OAc and proportion of Z7-12:OAc in the backcross Scandinavian group are closer to the Scandinavian parental strain than the F_1. This also supports the model of control of these two components by an autosomal factor or factors. However, the mean proportion of Z5-10:OAc in the F_1 is not exactly intermediate and is closer to the Scandinavian parental strain. Furthermore, the mean proportion of Z5-10:OAc of the backcross Zimbabwean and the F_2 lie close to the midpoint (0.5), while the backcross Scandinavian mean is quite close to the mean of Scandinavian parental strain (Figure 3). This suggests some partial Scandinavian dominance of the gene or genes that control the proportion of Z5-10:OAc. The frequency distribution of the backcross Zimbabwean and F_2 groups is quite broad, which may be due to the segregation of alleles resulting in Scandinavian and Zimbabwean phenotypes. The distribution patterns of the proportion of Z7-12:OAc also shows broadening in the F_2 and backcross Zimbabwean groups, but the F_1 mean lies close to the midpoint (0.35), which does not suggest partial Scandinavian dominance.

Examination of the mean and variation in proportion of Z9-14:OAc yields more ambiguous results and explains the lack of any clear pattern of inheritance in the significance tests of the mean log ng Z9-14:OAc/ng Z7-12:OAc (Figure 2). The mean proportion of Z9-14:OAc for the F_1 is close to that of the Scandinavian parental strain, again suggesting Scandinavian dominance, but the mean for the backcross Scandinavian group is closer to the midpoint than the F_1, and the mean for the Zimbabwean backcross and F_2 is closer to the Zimbabwean parental strain. The frequency distributions show that the majority of

F_2, Zimbabwean backcross, and Zimbabwean parental females produce from very little or no Z9–14:OAc, whereas there is greater variation in the proportion of this compound in the F_1 and backcross Scandinavian and Scandinavian parental females. We cannot determine an obvious mode of inheritance for the proportion of Z9–14:OAc from these data. The frequency distributions of the proportion of Z9–14:OAc and the statistical analysis of the mean log ng Z9–14:OAc/ng Z7–12:OAc together suggest that variation in the proportion of Z9–14:OAc may not be under tight genetic control.

The proportions of each of these three components show wide individual variation (Figure 3). It is unclear, however, exactly how much of the extensive variation in the proportions of each component is due to genetic causes, or how many genes may be responsible. A broad range of phenotypes can be attributed to polygenic inheritance, but also could be attributed to the influence of environmental and genotype by environmental affects (Falconer, 1989). Individual variation in the proportions of the pheromone components has been shown to be even higher in wild-caught females (Löfstedt et al., 1985b). The presence of a broad range of phenotypes rather than a segregation into distinct classes suggests that variation in this character, if genetic, is due to multiple (polygenic) factors. Increased variance in the backcross and F_2 generations is expected under a polygenic model, but in these data this is only the case for the distribution of the proportion of Z5–10:OAc and proportion of Z7–12:OAc in the F_2 and Zimbabwean backcross. In general, sex pheromones that consist of mixtures of acetates tend to show a broader range of individual variation in blend ratios than those composed of geometric isomers, and this may in part be due to the different vapor pressures of the acetates (Schlyter and Birgersson, 1989; Löfstedt, 1990). In *Trichoplusia ni*, pheromone blend ratios containing Z7- and Z9–14:OAc and Z7–12:OAc varied significantly with temperature and time during scotophase (Liu and Haynes, 1994). This suggests that a significant proportion of the variation in *A. segetum*, whose pheromone blend also contains Z9–14:OAc and Z7–12:OAc, may be due to fluctuations in temperature and differing time of collection while preparing the samples.

The difference between the blend ratios in the two parental strains may originate in the biosynthetic pathway of the three components (Wu, 1995). Z7–12:CoA and Z5–10:CoA are produced by successive chain shortening steps from Z9–14:CoA through β-oxidation, and these fatty acid precursors are converted to the homologous acetates through reduction (reductase) and acetylation (acetyltransferase). Scandinavian females rapidly convert Z9–14:CoA into Z9–14:OAc and Z7–12:CoA into Z7–12:OAc, whereas Zimbabwean females more rapidly convert Z9–14:CoA into Z7–12:OAc and Z7–12:CoA into Z5–10:OAc. Zimbabwean females convert Z5–10:CoA more effectively into Z5–10:OAc than Scandinavian females, reflecting the greater propotion of this compound in the blends of females from this population. Control of the pher-


Proportion of each component
# of females
Z5-10:OAc
Z7-12:OAc
Z9-14:OAc
Scandinavian
Zimbabwean
F1
35

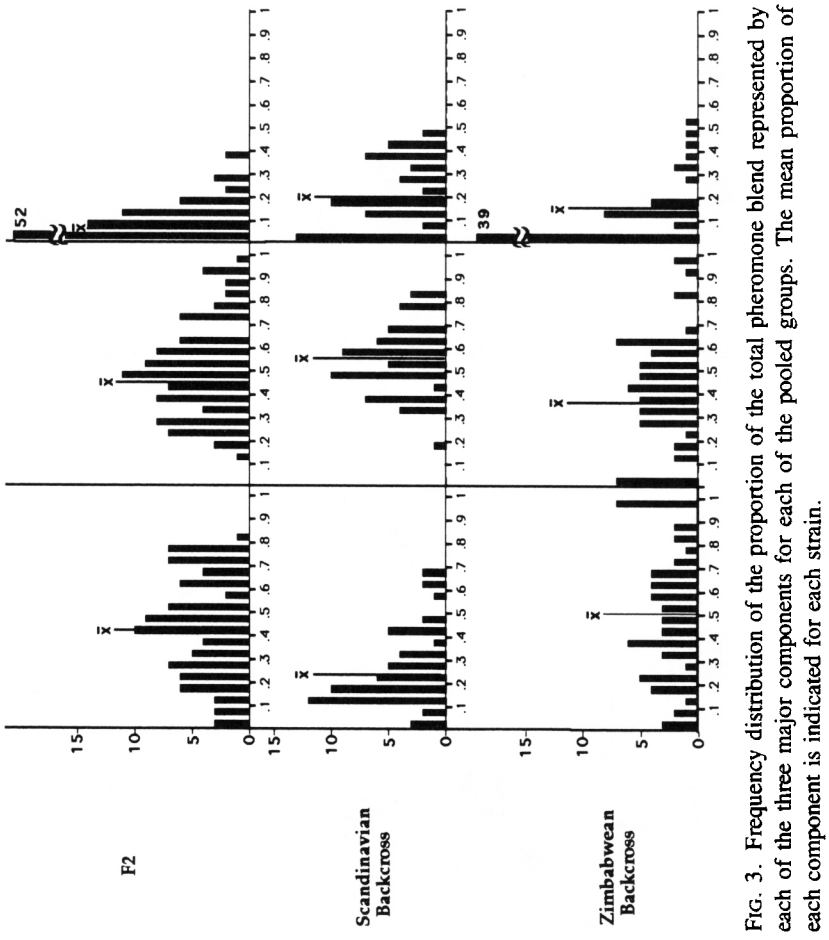
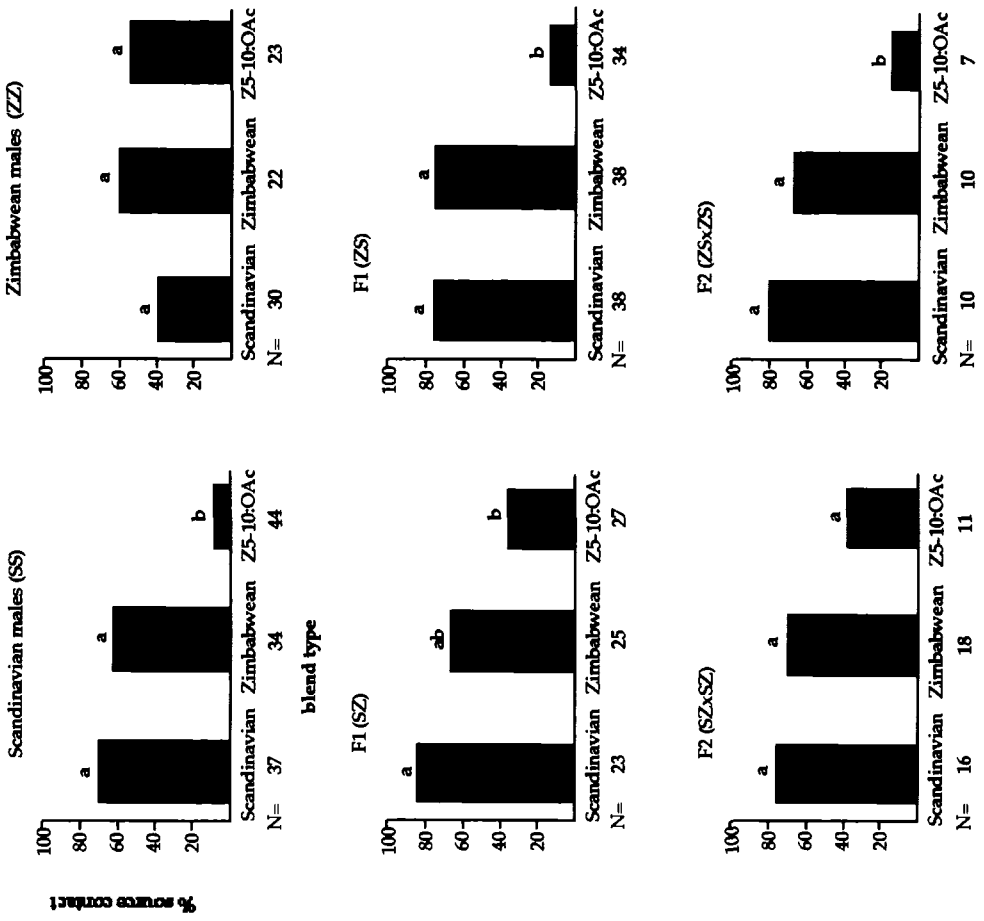


FIG. 3. Frequency distribution of the proportion of the total pheromone blend represented by each of the three major components for each of the pooled groups. The mean proportion of each component is indicated for each strain.


Scandinavian males (SS)
Zimbabwean males (ZZ)
F1 (SZ)
F1 (ZS)
F2 (SZxSZ)
F2 (ZSxZS)
% source contrib.
100
80
60
40
20
a
ab
b
Scandinavian
Zimbabwean
Z5-10:OAc
N= 37 34 44
N= 30 22 23
N= 23 25 27
N= 38 38 34
N= 16 18 11
N= 10 10 7
blend type

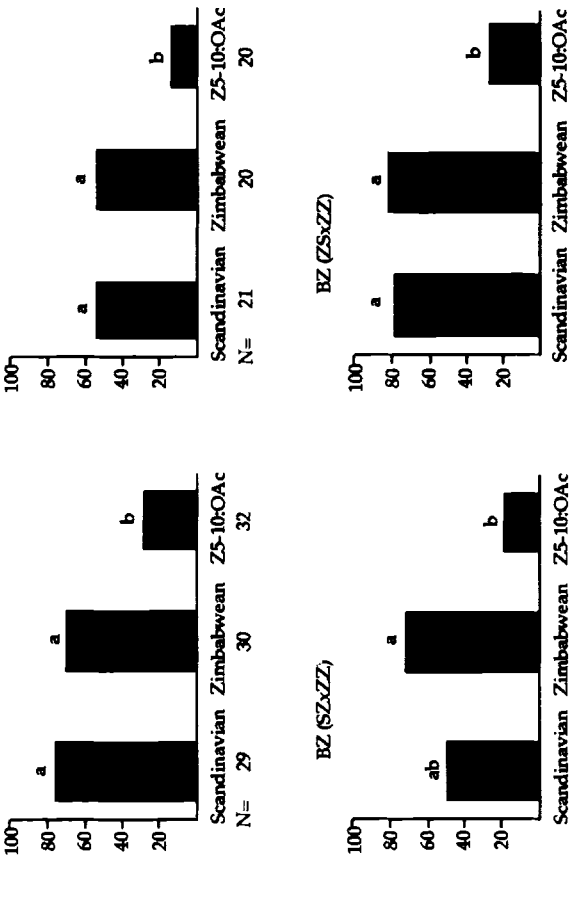


FIG. 4. The percent of males reaching source contact when tested by each of three blend types. Shared letters indicate non-significant differences in pairwise χ^2 comparisons at $\alpha = 0.05$ for each blend type, with adjustment for multiple comparisons (Ryan, 1960).

omone blend by a single autosomal locus, even given the broad individual variation, is feasible if this locus codes for a β oxidase, reductase, or acetyltransferase. The effect of Scandinavian dominance in controlling the proportion of Z5–10:OAc may be due to dominance of alleles coding for one or more of those enzymes. Swedish females reduce Z9–14:CoA to Z9–14:OAc and Z7–12:CoA to Z7–12:OAc more effectively than Zimbabwean females, thus reducing the pool of Z7–12:CoA available for conversion to Z5–10:OAc through β-oxidation.

Male Data. Across all males tested, 94–100% of those placed in the wind tunnel exhibited wing fanning and took flight. There were no significant differences in the proportion of males that took flight across all groups and blends ($\chi^2 = 11.195$, $df = 20$, $\alpha = 0.05$, or in the proportion of males exhibiting orientation at the 95% level of significance, using a χ^2 test with adjustment for multiple comparison of proportions (Figure 4) (Ryan, 1960). There were few differences in the ability of males from different groups to reach source contact when responding to the three different baits. Males from most groups were significantly less able to respond to Z5–10:OAc alone in comparison to the three Scandinavian (S) and Zimbabwean (Z) blends, with the exception of the Zimbabwean and F_2 groups from the SZ × SZ reciprocal cross (Figure 1). Furthermore, none of the tests conducted show significant differences between any of the F_1, F_2, and backcross groups in their response to each blend. Low numbers of males tested in some groups prevent a clear conclusion. Since there are few detectable differences in the ability of the different groups to respond to different pheromones, we cannot determine the mode of inheritance. However, lack of significant differences in the ability of males to respond to Scandinavian versus Zimbabwean pheromone blends suggests that males have a broadly tuned window of response.

Reliable assessments of variation in mate preferences are rare, and in addition to various environmental influences, different laboratory strains derived from the same original wild culture may not reflect identical genetic variation or reflect the range of genetic variation in the field (Ritchie, 1992). The fact that distinct difference between strains in male response could not be found raises the issue of the degree to which this homogeneity may reflect wind-tunnel experimental conditions or the fact that our laboratory population does not represent the range of genetic variation in wild males. In an earlier study, there were no significant differences in the ability of laboratory Scandinavian males to respond to a blend ratio of 1:5:2.5 or 1:1:1 (Z5–10:OAc/Z7–12:OAc/Z9–14:OAc) in the wind tunnel at 0.3, 3, and 30 μg (Löfstedt et al., 1985a). However, Scandinavian males in the field also show broad ranges of response. Bait ratios varying from 1:5:2.5 to 1:0.25:0.03 (identical to the Scandinavian and Zimbabwean blend ratios used in our study) have also been used in the field in Sweden with no significant differences in the number of wild, presumably

outbred males they attracted (Wu et al., 1997). Thus, the Scandinavian laboratory males in our study show response patterns similar to wild males tested on the same baits in the field. However, significantly more Zimbabwean males were attracted to the Zimbabwean blend than to the Scandinavian blend in both field tests in Zimbabwe and in the wind tunnel (Wu, 1995). In our study, Zimbabwean laboratory males showed the same response to these two blends in the wind tunnel. Thus, it may be possible that the laboratory populations did not possess identical genetic variation to wild populations or that conditions in the wind-tunnel experiments influenced male behavior so as to produce patterns that would not be found in the field.

As sexual selection acts more strongly on males than females, we may expect the range of variation in male response to track the range of variation in female pheromone blend (Phelan, 1992). Wide individual variation in the female blend of *A. segetum* and the lack of precise genetic control may allow broad response windows in males. Thus, the ability of *A. segetum* males to respond fully to both Zimbabwean and Scandinavian blends may enable males to mate with many females, since one may find, at a low frequency, females in a Zimbabwean population that produce blends similar to Scandinavian females, and vice versa. Additionally, the ability of Zimbabwean males in this study and in previous field work (Tóth et al., 1992; Wu et al., 1997) to respond to Z5-10:OAc alone may reflect selection to respond to a broad range of Zimbabwean female pheromone blends, which in some females can consist almost entirely of this compound.

Acknowledgments—In Lund, we thank Anna Tunlid for help in collecting female data, Junwei Zhu for help with GC operation, and Erling Jirle for help with insect rearing. In Stony Brook, we thank Dennis Slice and Dean Adams for help with statistical analysis of data, Anthony Lentz for literary criticism, and Glenn Prestwich for funding travel expenses (NSF Sweden Cooperative Research Grant). This research was also supported by grants from the Scandinavian Research Councils (SIJFR, NFR) to Christer Löfstedt.

REFERENCES

AITCHISON, J. 1986. Statistical Analysis of Compositional Data. Chapman and Hall, New York.

ANGLADE, P., STOCKEL, P., and Cooperators. 1984. Intraspecific sex-pheromone variability in the European corn borer, *Ostrinia nubilalis* HBN. (Lepidoptera, Pyralidae). *Agronomie* 4:183-187.

ARN, H., ESBJERG, P., BUES, R., TÓTH, M., SZÖCS, G., GUERIN, P., and RAUSCHER, S. 1983. Field attraction of *Agrotis segetum* males in four European countries to mixtures containing three homologous acetates. *J. Chem. Ecol.* 9:267-276.

BARBATTINI, R., MARCHETTI, S. PRAVISANI, L., and ZANDIGIACOMO, P. 1985. Attrazione di feromoni sessuali di sintesi nei confronti di *Ostrinia nubilalis* Hb in Friuli. *Frustula Entomol.* 7:1-21.

CARDÉ, R. T. and BAKER, T. C. (1984). Sexual communication with pheromones. Chemical Ecology of Insects. Chapman and Hall, London.

Falconer, D. S. 1989. Introduction to Quantitative Genetics. Longman, London.

Hansson, B. S., Löfstedt, C., and Roelofs, W. L. 1987. Inheritance of olfactory response to sex pheromone components in the European corn borer, *Ostrinia nubilalis*. *Naturwissenschaften* 73:497–499.

Hansson, B. S., Tóth, M., Löfstedt, C., Szöcs, G., Subchev, M., and Löfqvist, J. 1990. Pheromone variation among eastern European and a western Asian population of the turnip moth *Agrotis segetum*. *J. Chem. Ecol.* 16:1611–1622.

Haynes, K. F., and Hunt, R. E. 1990. A mutation in pheromonal communication system of cabbage loop moth, *Trichoplusia ni*. *J. Chem. Ecol.* 16:1249–1257.

Hunt, R. E., Zhao, B.-G., and Haynes, K. F. 1990. Genetic aspects of interpopulational differences in pheromone blend of cabbage looper moth. *Trichoplusia ni*. *J. Chem. Ecol.* 16:2935–2946.

Klun, J. A., and Cooperators. 1975. Insect sex pheromones: Intraspecific pheromonal variability of *Ostrinia nubilalis* in North America and Europe. *Environ. Entomol.* 4:891–894.

Klun, J. A., and Maini, S. 1979. Genetic basis of an insect chemical communication system: The European corn borer. *Environ. Entomol.* 8:423–426.

Kochansky, J., Cardé, R. T., Liebherr, J., and Roelofs, W. L. 1975. Sex pheromone of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae), in New York. *J. Chem. Ecol.* 1:225–231.

Liu, Y.-B., and Haynes, K. F. 1994. Temporal and temperature-induced changes in emission rates and blend ratios of sex pheromone components in *Trichoplusia ni*. *J. Insect Physiol.* 40:341–346.

Löfstedt, C. 1990. Population variation and genetic control of pheromone communication systems in moths. *Entomol. Exp. Appl.* 54:199–218.

Löfstedt, C., and Odham, G. 1984. Analysis of moth pheromone acetates by selected ion monitoring using electron impact and chemical ionization mass spectrometry. *Biomed. Mass Spectrom.* 11:106–113.

Löfstedt, C., Linn, C., and Lofqvist, J. 1985a. Behavioural responses of male turnip moths *Agrotis segetum* to sex pheromone in a flight tunnel and in the field. *J. Chem. Ecol.* 11:1209–1223.

Löfstedt, C., Lanne, B. S., Löfqvist, J., Appelgren, M., and Bergström, G. 1985b. Individual variation in the pheromone of the turnip moth, *Agrotis segetum*. *J. Chem. Ecol.* 11:1181–1196.

Löfstedt, C., Löfqvist, J., Lanne, B. S., Van der Pers, J. N. C., and Hansson, B. S. 1986. Pheromone dialects in European turnip moths *Agrotis segetum*. *Oikos* 46:250–257.

Löfstedt, C., Herrebout, W. M., and Menken, S. B. J. 1991. Sex pheromones and their potential role in the evolution of reproductive isolation in small ermine moths (Yponomeutidae). *Chemoecology* 2:20–28.

Neems, R. M., and Butlin, R. K. 1995. Divergence in cuticular hydrocarbons between parapatric subspecies of the meadow grasshopper, *Chorthippus parallelus* (Orthoptera, Acrididae). *Biol. J. Linn. Soc.* 54:139–134.

Peña, A., Arn, H., Buser, H.-R., Rauscher, S., Bigler, F., Brunetti, R., Maini, S., and Toth, M. 1988. Sex pheromone of the European corn borer, *Ostrinia nubilalis:* Polymorphism in various laboratory and field strains. *J. Chem. Ecol.* 14:1359–1366.

Phelan, P. L. 1992. Evolution of sex pheromones and the role of asymmetric tracking. Insect Chemical Ecology. Chapman and Hall, New York, pp. 265–314.

Ritchie, M. G. 1992. Setbacks in the search for mate-preference genes. *Tree* 7(10):328–329.

Roelofs, W., Glover, T., Tang, X.-H., Sreng, I., Robbins, P., Eckenrode, C., Löfstedt, C., Hansson, B. S., and Bengtsson, B. O. 1987. Sex pheromone production and perception in European corn borer moths is determined by both autosomal and sex-linked genes. *Proc. Natl. Acad. Sci. U.S.A.* 84:7585–7589.

RYAN, T. A. 1960. Significance tests for multiple comparison of proportions, variances, and other statistics. *Psychol. Bull.* 57:318–328.

SCHLYTER, F., and BIRGERSSON, G. 1989. Individual variation in bark beetle and moth pheromones—a comparison and an evolutionary background. *Holarct. Ecol.* 12:457–465.

TÓTH, M., LÖFSTEDT, C., BLAIR, B. W., CABELLO, T., FARAG, A. I., HANSSON, B. S., KOVALEV, B. G., MAINI, S., NESTEROV, E. A., PAJOR, I., SAZONOV, P., SHAMSHEV, I. V., SUBCHEV, M., and SZÖCS, G. 1992. Attraction of male turnip moths *Agrotis segetum* (Lepidoptera: Noctuidae) to sex pheromone components and their mixtures at 11 sites in Europe, Asia and Africa. *J. Chem. Ecol.* 18:1337–1347.

WU, W.-Q. 1995. Mechanisms of specificity in moth pheromone production and response. PhD thesis, Lund University, Sweden.

WU, W., COTTRELL, C. B., HANSSON B. S., and LÖFSTEDT, C. 1997. A comparative study of pheromone production and response in two populations of the turnip moth. *Agrotis segetum* from North Europe and South Africa. *J. Chem. Ecol.* In press.

ZHU, J. W., LÖFSTEDT, C., and BENGTSSON, B. O. 1996. Genetic variation in the strongly canalized sex pheromone communication system of the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera; Pyralidae). *Genetics* In press.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

Cotesia glomerata FEMALE WASPS USE FATTY ACIDS FROM PLANT-HERBIVORE COMPLEX IN HOST SEARCHING

MAYUMI HORIKOSHI,[1] JUNJI TAKABAYASHI,[2,*] SHUICHI YANO,[2] RYOHEI YAMAOKA,[1] NAOTA OHSAKI,[3] and YOSHIBUMI SATO[4]

[1]*Department of Applied Biology, Kyoto Institute of Technology Matsugasaki, Sakyo-ku, Kyoto 606, Japan*

[2]*Laboratory of Ecological Information Graduate School of Agriculture, Kyoto University Kyoto 606-01, Japan*

[3]*Laboratory of Insect Ecology Graduate School of Agriculture, Kyoto University Kyoto 606-01, Japan*

[4]*Kyoto College of Medical Technology Sonobe, Funai-gun, Kyoto 622, Japan*

(Received February 28, 1996; accepted January 18, 1997)

Abstract—*Cotesia glomerata* parasitizes early instars of the cabbage butterfly, *Pieris rapae*, in Japan. Female wasps antennated *Rorippa indica* leaves damaged by feeding of *P. rapae* larva, but ignored artificially damaged leaves. Females also antennated filter paper containing *R. indica* leaf juice plus *P. rapae* regurgitant. Chemical analysis revealed five compounds in higher amounts in the infested edges of leaves than in artificially damaged edges. Among them, we identified palmitic acid, oleic acid, and stearic acid. Female wasps antennated filter paper containing each of these three acids. We discuss the function of these acids in the tritrophic context.

Key Words—*Cotesia glomerata*, *Pieris rapae*, *Rorippa indica*, plant-herbivore complex, palmitic acid, oleic acid, stearic acid, tritrophic interaction, antennation.

*To whom correspondence should be addressed.

0098-0331/97/0600-1505$12.50/0 © 1997 Plenum Publishing Corporation

INTRODUCTION

Arthropod carnivores (predators and parasitoids) often use volatile and/or contact allelochemicals produced by plants or herbivores to find their host/prey (reviewed in Whitman 1988). Carnivores also use volatile allelochemicals originating from the plant-herbivore complex (PHC) in their long- or short-range host habitat location. For example, volatile chemicals that attract natural enemies of herbivores are emitted by leaves in response to herbivore damage (Dicke et al., 1990; Geervliet et al., 1994; Mattiacci et al., 1994; Turlings et al., 1991, 1993; Takabayashi et al., 1991, 1994ab, 1995; Whitman and Eller, 1990; Shimoda et al., 1997). Wasps may also use allelochemicals from PHC to find hosts on a plant. In this paper, we investigate the role of contact allelochemicals of PHC origin in host location by a parasitic wasp, *Cotesia glomerata* (Hymenoptera: Braconidae), that attacks *Pieris rapae crucivora* (Lepidoptera: Pieridae) on cruciferus plants.

C. glomerata is an endoparasitoid of first to third instars of *P. rapae*, which has five instars in its larval development. The larvae feed on crucifer plants, especially *Rorippa indica* in natural ecosystems in Japan. *R. indica* are ephemeral plants that grow in temporary habitats. When host-searching, a female *C. glomerata* usually lands on a leaf of *R. indica* or another crucifer (Sato, 1988), and walks around slowly with her antennal tips touching the leaf surface. When she comes within ca. 10 mm of a part damaged by larval feeding (hereafter called "host-fed edge"), she raises her antennae and turns towards the host-edge. She then starts intensive searching with antennal contact, raised wings, and/or bending the abdomen forward (Sato, 1979). We refer to this antennal searching as "antennation."

This paper reports the presence and chemical nature of contact allelochemicals of PHC origin that elicit antennation in *C. glomerata*. We also discuss the function of the allelochemicals in the tritrophic interaction of foraging *C. glomerata*, *P. rapae* larva, and the host-food plant.

METHODS AND MATERIALS

Insects and Plants

Adult *P. rapae* were collected in Kino district, Kyoto City, and maintained with their larval host plant, *R. indica*. Eggs laid on *R. indica* leaves were collected, and hatched larvae were reared in the climate room (25°C, 16L:8D, 50–70% relative humidity) at the laboratory of Kyoto University.

Cocoons of *C. glomerata* were obtained from a stock culture at the laboratory or collected in the field at Matsugasaki, Kyoto City. Emerged wasps were kept in the laboratory (20–30°C) in glass tubes (40 mm diam. × 150 mm long) with honey as food. Mated females were isolated in the incubator (24D,

10–13°C, 50–70% relative humidity) in glass tubes (20 mm diam. × 120 mm long) with honey until used. Females were tested when 5–10 days old. Each female was tested only once a day.

Uninfested *R. indica* plants were obtained from a stock culture at a greenhouse at the laboratory. The plants were separately planted in clay pots (800 ml) in the greenhouse under natural light conditions at 20–30°C.

Response of Parasitoids to R. indica *Leaves*

The response of wasps toward various samples was tested (20–30°C) at the Department of Applied Biology, Kyoto Institute of Technology. The following samples were prepared.

Leaf Infested by Early Instars. The surface of a detached leaf (ca. 50 mm long) was covered with filter paper with a hole (20 mm circumference) in a glass petri dish (50 mm diam.). Five to 10 second-instar larvae were placed on the leaf. The larvae fed only on the leaf surface exposed under the paper hole, making 20-mm-long feeding edge. Immediately after the feeding, the leaf was used for the experiment.

Leaf Infested by Late Instars. A detached leaf (ca. 50 mm long) was offered to one fifth instar. When the larva completed the 20-mm-long feeding edge, we carefully removed the larva and its associated products such as feces and silk from the leaf with a small fine brush. Immediately after the treatment, the leaf was used for the experiment.

Leaf Artificially Damaged with Scissors. We prepared artificially damaged leaves (ca. 50 mm long) by cutting with scissors a part of the leaf to create a damaged site 20 mm long.

Uninfested leaves, the artificially damaged leaves, and the leaves infested by *P. rapae* larva were separately placed in the glass tubes with one female wasp to observe behavior.

Response of Parasitoids to Extracts

We used five heavily infested leaves (more than 80% of the surface was infested by fourth to fifth instars) for extraction. The leaves (780 mg) were soaked in diethylether (5 ml) for 30 min. We separated the leaves and diethylether solution by filtration through filter paper (Toyo No. 2). The solution was called the infested leaf extract.

Five uninfested leaves (1930 mg) were also extracted with diethylether (5 ml) using the same method to produce the uninfested leaf extract.

Both extracts (1.6 mg leaf equivalent dose) were separately applied to a dry donut-shaped paper ring (7 mm outer diam., 6 mm inner diam., Toyo filter paper No. 2). After evaporation of the solvent, 4 μl of water was added, and the paper ring was offered to a wasp in the glass tube.

When the female parasitoid showed the same degree of antennation to the ring as to the infested site of the leaf, the sample was judged to be active. We also measured the duration of antennation of the ring. When a wasp left the ring for more than 30 sec, the observation was terminated.

Response of Parasitoids to Filter Paper with Leaf Juice and Regurgitant

Ten leaves of *R. indica* were homogenized and leaf juice (50 μl) was collected with a glass micropipet (Micropet 50 μl). Regurgitant (25 μl) from *P. rape* larvae was collected with a glass micropipet (Micropet 50 μl) from the mouth of fifth instars previously starved for 24 hr by gently pinching the body with forceps.

To test thermolability of plant juice and regurgitant on the production of chemicals for the wasp, a flame-sealed Micropet (50 μl capacity) containing 25 μl of regurgitant or plant juice was placed in boiling water for 5 min and then tested.

A piece of filter paper (10 $\times$ 10 mm) was used as a substrate. The leaf juice and the regurgitant (5 μl each) were impregnated into the filter paper. Immediately after the treatment, we offered the paper to the wasp in the glass tube and the behavior was observed.

Effect of Parasitization on the Feeding Rate of P. rape *Larva*

A second instar and a female wasp were confined in a glass tube (11 mm diam., 100 mm long). When the wasp parasitized the host, they were separated and the host was reared in a plastic cup (120 mm diam., 50 mm height) in the climate room with *R. indica* leaves as food. Ten parasitized larvae were used. As a control, 10 unparasitized second instars were reared under the same conditions.

Uninfested *R. indica* leaves were collected in the greenhouse. The area of each leaf was measured with a Digitizer (MYPAD-A3, Logitec), and they were offered to the parasitized or unparasitized larvae. The infested leaves were collected the next day, and the remaining area was determined with the Digitizer. The experiment was terminated when a larva became a prepupa. Mean total leaf area eaten by the parasitized larvae and by the unparasitized larvae were compared by a *t*-test.

Chemical Analysis

Solvent Extraction. We carefully cut the margin (ca. 1 mm width) of the host-fed edge of leaves made by fifth instars. As a control, we cut the uninfested leaves in 1-mm widths along the original leaf margin. These cut edges (ca. 58 cm long for each leaf) were soaked in diethylether (1 ml) for 12 hr. The extracts

were called the host-fed edge extract and the artificial edge extract, respectively. We evaporated the diethylether solution to 100 μl with a rotary evaporator for the chemical analysis.

Regurgitant (100 μl) of the fifth instar hosts was extracted with 500 μl of diethylether (the regurgitant extract). Five fifth instar *P. rapae* were soaked in diethylether (50 ml) for 5 min to extract the cuticular wax (the host surface extract). Both extracts were used for chemical analysis.

Instrument. Extracts were analyzed by GC (Shimadzu GC-14A with an OV-1 capillary column, 30 m long, 0.25-μm film thickness, injection temperature: 300°C, detector temperature: 300°C) and GC-MS (GC—Shimadzu GC-14A with OV-1 capillary column: 30 m long, 0.25-μm film thickness, injection temperature: 300°C); MS—Shimadzu QP-1000 EX, 70 eV). The oven temperature of the GC and GC-MS was programmed from 130°C (2 min hold) to 300°C at 5°C/min.

Identification. We identified compounds in comparison with mass spectra and retention time of synthetic chemicals. Pure chemicals were obtained from Nakarai Chemicals Co. Ltd.

RESULTS

Response of Parasitoids to R. indica *Leaves*

When female parasitoids encountered a host-fed edge made by early instars, all wasps antennated it. This behavior, with significantly longer duration, was also observed at a fed-upon edge made by a late instar. When wasps encountered an artificially damaged part or an uninfested leaf, no wasp antennated it (Table 1).

TABLE 1. RESPONSE OF FEMALE *Cotesia glomerata* TO *Rorripa indica* LEAVES WITH DIFFERENT TREATMENTS

Leaf	*N*	Antennation (%)	Duration (sec) of antennation (mean ± SE)
Uninfested	20	0	
Artificially damaged	20	0	
Infested by early instars	20	100	88.6 ± 36.7 a[a]
Infested by late instars	30	100	187.5 ± 41.2 b[a]

[a]Antennation time values with different letters are significantly different ($P < 0.01$: F test).

TABLE 2. RESPONSE OF FEMALE *Cotesia glomerata* TO DIETHYLETHER EXTRACTS OF INFESTED AND UNINFESTED LEAVES

Sample	*N*	Antennation (%)	Duration (sec) of antennation (mean ± SE)
Infested leaf extract	20	0	
Infested leaf extract + water	20	70	50.2 ± 13.7
Uninfested leaf extract + water	20	0	
Pure ether + water	20	0	
Water	20	0	

Response of Parasitoids to Leaf Extracts

The parasitoids antennated a paper ring containing the infested leaf extract and water. The infested leaf extract without water did not elicit antennation. When the wasps encountered a paper ring with the uninfested leaf extract and water, the wasps did not antennate it. A paper ring with pure diethylether plus water or with water alone did not elicit the behavior (Table 2).

Response of Parasitoids to Filter Paper with Regurgitant and Leaf Juice

The wasps antennated the filter paper treated with leaf juice and regurgitant. However, the wasps antennated neither a filter paper with leaf juice plus heated regurgitant nor that with heated leaf juice plus regurgitant. Regurgitant or leaf juice alone did not elicit antennation (Table 3).

Effect of Parasitization on the Feeding Rate of P. rapae *Larva*

Two unparasitized hosts died during the experiment. The mean leaf area (mm^2 ± SE) eaten by the parasitized hosts (8522 ± 681; $N = 10$) was signif-

TABLE 3. RESPONSE OF FEMALE *Cotesia glomerata* TO LEAF JUICE PLUS REGURGITANT

Sample	*N*	Antennation (%)	Duration (sec) of antennation (mean ± SE)
Regurgitant + leaf juice	20	80	18.0 ± 5.4
Heated regurgitant + leaf juice	20	0	
Regurgitant + heated leaf juice	20	0	
Regurgitant	20	0	
Leaf juice	20	0	

icantly larger than that by the unparasitized hosts (6796 ± 250; $N = 8$: $t = 2.37$, $df = 11.34$, $P < 0.05$).

Chemicals Eliciting Intensive Searching in Parasitoids

GC analysis of the host-fed edge extract and the artificial edge extract showed 15 major peaks (Table 4). Among them, we found five peaks that were larger in the host-fed edge extract than in the artificial edge extract. From GC-MS analyses followed by comparison of mass spectra and retention times of GC-MS standards, three peaks (3, 6, and 7) were identified as palmitic acid, oleic acid, and stearic acid. The chemical structure of the other two peaks (8 and 10) are unknown. The acids and two unknown compounds were not found in the regurgitant extract and the host surface extract.

We bioassayed synthetic palmitic acid, oleic acid, and stearic acid dissolved in diethylether (1 mg/ml). The activity of each acid was tested separately with the moist paper ring; all were active. Both palmitic acid and stearic acid elicited antennation at 1 μg/ring and 10 μg/ring. In contrast, oleic acid elicited antennation at 10 ng/ring and 100 ng/ring (Table 5). The acids in a dry paper ring did not elicit antennation.

TABLE 4. GC PEAK AREA OF INFESTED EDGE EXTRACT AND ARTIFICIAL EDGE EXTRACT

Peak number	Mean peak area (arbitrary unit)[a] of Infested edge (A)	Artificial edge (B)	A/B	Identification[b]
1	10,972	10,512	1.0	
2	3,351	3,452	1.0	
3	15,464	5,601	2.8	palmitic acid
4	16,652	16,660	1.0	
5	118,833	107,730	1.1	
6	8,660	4,257	2.0	oleic acid
7	18,804	4,480	4.2	stearic acid
8	28,050	12,697	2.2	unknown
9	8,077	15,606	0.5	
10	251,403	78,915	3.2	unknown
11	21,734	18,126	1.2	
12	10,591	10,366	1.0	
13	15,764	16,805	0.9	
14	13,192	17,912	0.7	
15	10,980	9,708	1.1	

[a] 10-mm-long edge equivalent, $N = 4$.
[b] Peaks that showed larger area in infested edge extract than in artificial edge extract.

TABLE 5. RESPONSE OF FEMALE *Cotesia glomerata* TO PALMITIC ACID, STEARIC ACID, AND OLEIC ACID IN A PAPER RING

Amount (ng)	*N*	Antennation (%)	Duration (sec) of antennation (mean ± SE)
Palmitic acid			
Moist paper ring			
10000	10	60	12 ± 6
1000	10	20	4 ± 1
100	10	0	
10	10	0	
Dry paper ring			
10000	10	0	
Stearic acid			
Moist paper ring			
10000	40	87.5	31 ± 19
1000	20	55	20 ± 12
100	20	0	
10	20	0	
Dry paper ring			
10000	20	0	
Oleic acid			
Moist paper ring			
1000	10	0	
100	10	0	
10	20	45	13 ± 21
1	20	50	12 ± 8
0.1	20	40	13 ± 12
Dry paper ring			
10	20	0	

DISCUSSION

Our results suggest that female *C. glomerata* can distinguish host-damaged leaves from artificially damaged ones by the compounds that are present in increased amounts on the edge of host-damaged leaves. This hypothesis is supported by the fact that the wasps antennated at least three of the five compounds involved (Table 4): palmitic acid (*n*-hexadecanoic acid), oleic acid (*cis*-9-octadecanoic acid) and stearic acid (*n*-octadecanoic acid). As we did not find these compounds on the body surface of *P. rapae* larva or in its regurgitant, the possibility that the host deposited the compounds on the fed edge is unlikely.

Immediately after *P. rapae* larva infested a leaf, the wasps responded to

the fed edge. In addition, immediately after the plant juice plus the regurgitant were impregnated into a filter paper, the wasp responded to it. From these data, we postulate that the acids and unknown compounds are produced locally along the host-fed edge as the interaction of the plant juice and the regurgitant by chemical breakdown of precursors. As larvae were starved for 24 hr before collecting the regurgitant, chemicals in the regurgitant should be of larval origin. The production of the acids and the two unidentified compounds are thus due to an interaction of chemicals from the larva and from the plant. The precursors and/or agents involved in the production of the allelochemicals seem to be thermolabile, since heating the regurgitant or leaf juice resulted in loss of activity.

The wasps responded longer to the fed edge made by the later instars than that made by the early instars (Table 1), although the late instars were not a suitable host stage. The data suggest that the wasps do not discriminate between the suitable host stage and the unsuitable host stage of *P. rapae* through the contact cue along the fed-upon edge. *Cotesia glomerata* is, however, reported to discriminate young and old instars of *P. brassicae* by contacting the fed-upon edge (Mattiacci and Dicke, 1995).

Palmitic acid, oleic acid, and stearic acid are long-chain free fatty acids and are commonly found as triacylglycerols in fats and oils in both the plant and the animal kingdoms. Long-chain free fatty acids of C_{14} to C_{18} and their esters have been reported to have several ecological functions: as ovipositional repellents for mosquitoes (Hwang et al., 1984), in oviposition site avoidance by Lepidoptera (Thiéry and Gabel, 1993; Thiéry et al., 1995; Gabel and Thiéry, 1996), and in attraction of *Varroa jacobsoni*, a parasite of honeybees (Le Conte et al., 1989; Trouiller et al., 1992; Rickli, 1992). Long-chain fatty acids also mediate herbivore–plant interactions (Hibbard et al., 1994). All these studies focused on ditrophic interactions. This paper shows an ecological function of long-chain fatty acids in a tritrophic context: they are produced in the plant–herbivore complex and function to mediate interaction between plants (first trophic level) and parasitic wasps (third trophic level).

There is a difference between the infochemicals in our study and those in previously reported tritrophic systems in terms of function. In previously reported tritrophic systems, the volatile chemicals of the plant–herbivore complex function as synomones that are adaptively favorable to both emitter (plant) and receiver (carnivore) (Dicke, 1994; Dicke et al., 1990; Takabayashi et al., 1994a,b, 1955; Takabayashi and Dicke, 1996; Vet and Dicke, 1992). In this study, parasitized hosts eat more than unparasitized hosts. Obviously, this does not benefit the individual plant and therefore the production of the five chemicals is detrimental to both the caterpillar and the plant. The reasons why the complex of the *R. indica* plant and the *P. rapae* larva produced compounds with three functions remains unanswered.

Acknowledgments—We thank Marcel Dicke for comments on a previous version of the manuscript. We also thank two anonymous reviewers for their comments. This study was partly supported by a Grant-in Aid (Bio Cosmos Program IBP-97-1-B-2) from the Ministry of Agriculture, Forestry and Fisheries, Japan and by Research for the Future (JSPS-RFTF96L00603) Program.

REFERENCES

DICKE, M. 1994. Local and systemic production of volatile herbivore-induced terpenoids: Their role in plant-carnivore mutualism. *J. Plant Physiol.* 143:465–472.

DICKE, M., VAN BEEK, T. A., POSTHUMUS, M. A., BEN DOM, N., VAN BOKHOVEN, H., and DE GROOT, Æ. 1990. Isolation and identification of a volatile kairomone that effects acarine predator-prey interactions: Involvement of host plant in its production. *J. Chem. Ecol.* 16:381–396.

GABEL, B., and THIÉRY, D. 1996. Oviposition response of *Lobsia botrana* females to long-chain free fatty acids and esters from its eggs. *J. Chem. Ecol.* 22:161–171.

GEERVLIET, J. B. F., VET, L. E. M., and DICKE, M. 1994. Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid *Cotesia rubecula*. *Entomol. Exp. Appl.* 73:289–297.

HIBBARD, B. E., BERNKLAU, E. J., and BJOSTAD, L. B. 1994. Long-chain free fatty acids: Semiochemicals for host location by western corn rootworm larvae. *J. Chem. Ecol.* 20:3335–3344.

HWANG, Y.-S., SCHULTS, G. W., and MULLA, M. S. 1984. Structure-activity relationship of unsaturated fatty acids as mosquito ovipositional repellents. *J. Chem. Ecol.* 10:145–151.

LE CONTE, Y., ARNORD, G., TROUILLER, J., MASSON, C., CHAPPE, B., and OURISSON, G. 1989. Attraction of the parasitic mite *Varroa* to the drone larvae of honey bees by simple aliphatic esters. *Science* 245:638–639.

MATTIACCI, L., and DICKE, M. 1995. Host-age discrimination during host location by *Cotesia glomerata*, a larval parasitoid of *Pieris brassicae*. *Entomol. Exp. Appl.* 76:37–48.

MATTIACCI, L., DICKE, M., and POSTHUMUS, M. A. 1994. Induction of parasitoid attracting synomone in Brussels sprouts plants by feeding of *Pieris brassicae* larvae: Role of mechanical damage and herbivore elicitor. *J. Chem. Ecol.* 20:2229–2247.

RICKLI, M. 1992. Palmitic acid released from honeybee worker larvae attracts the parasitic mite *Varroa jacobsoni* on a servosphere. *Naturwissenshaften* 79:320–322.

SATO, Y. 1979. Experimental studies on parasitization by *Apanteles glomeratus*. V. Factors leading a female to the host. *Physiol. Entomol.* 4:63–70.

SATO, Y. 1988. The World of Parasitic Wasps. Tokai University Press, Tokai (in Japanese).

SHIMODA, T., TAKABAYASHI, J., ASHIHARA, W. and TAKAFUJI, A. 1997. Response of the predatory insect *Scolothrips takahashii* toward herbivore-induced plant synomone under both laboratory and field conditions. *J. Chem. Ecol.* 23:(in press).

TAKABAYASHI, J., and DICKE, M. 1996. Plant-carnivore mutualism through herbivore-induced carnivore attractants. *Trends Plant Sci.* 1:109–113.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M. A. 1991. Induction of indirect defense against spider-mites in uninfested Lima bean leaves. *Phytochemistry* 30:1459–1462.

TAKABAYASHI, J., DICKE, M., TAKAHASHI, S., and POSTHUMUS, M. A. 1994a. Leaf age affects composition of herbivore-induced synomones and attraction of predatory mites. *J. Chem. Ecol.* 20:373–386.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M. A. 1994b. Volatile herbivore-induced terpenoids in plant-mite interactions: variation caused by biotic and abiotic factors. *J. Chem. Ecol.* 20:1329–1354.

TAKABAYASHI, J., DICKE, M., TAKAHASHI, S., and POSTHUMUS, M. A. 1995. Effect of the developmental stage of the herbivore on the production of herbivore-induced synomone. *J. Chem. Ecol.* 21:273–287.

THIÉRY, D., and GABEL, B. 1993. Inter-specific avoidance of egg-associated semiochemicals in four tortricids. *Experientia* 49:998–1001.

THIÉRY, D., GABEL, B., FARKAS, P., and JARRY, M. 1995. Egg dispersion in codling moth: Influence of egg extract and of its fatty acid constituents. *J. Chem. Ecol.* 21:2015–2026.

TROUILLER, J., ARNOLD, G., CHAPPE, B., LE CONTE, Y., and MASSON, C. 1992. Semiochemical basis of infestation of honey bee brood by *Varroa jacobsoni*. *J. Chem. Ecol.* 18:2041–2053.

TURLINGS, T. C. J., TUMLINSON, J. H., HEATH, R. R., PROVEAUX, A. T., and DOOLITTLE, R. E. 1991. Isolation and identification of allelochemicals that attract the larval parasitoid, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts. *J. Chem. Ecol.* 17:2235–2251.

TURLINGS, T. C. J., WÄCKERS, F. L., VET, L. E. M., LEWIS, W. J., and TUMLINSON, J. H. 1993. Learning host-finding cues by hymenopterous parasitoids, pp. 51–78, *in* D. R. Papaj and A. C. Lewis (eds.). Insect Learning. Chapman & Hall, New York.

VET, L. E. M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

WHITMAN, D. W. 1988. Allelochemical interactions among plants, herbivores and their predators, pp. 11–64, *in* P. Barbosa and D. K. Letourneau (eds.). Novel Aspects of Insect-plant Interactions. Wiley Interscience, New York.

WHITMAN, D. W., and ELLER, F. J. 1990. Parasitic wasps orient to green leaf volatiles. *Chemoecology* 1:69–75.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

METHYL ESTER OF *p*-COUMARIC ACID: A PHYTOALEXIN-LIKE COMPOUND FROM LONG ENGLISH CUCUMBER LEAVES

FOUAD DAAYF, RACHID BEL-RHLID, and RICHARD R. BÉLANGER*

Département de phytologie
Centre de Recherche en Horticulture, Université Laval,
Ste-Foy, Québec, G1K 7P4, Canada

(Received July 11, 1996; accepted January 21, 1997)

Abstract—Methanolic extracts from long English cucumber leaves were fractionated and tested for their fungitoxicity by direct bioassay against *Cladosporium cucumerinum* on TLC plates. After separation by several preparative TLCs and flash chromatography and purification through reverse-phase HPLC, a crystalline compound possessing antifungal properties was isolated and identified as *p*-coumaric acid methyl ester. This antifungal compound was found to increase following infection and treatment with Milsana®, which could explain in part the prophylactic properties of the natural inducer. As such, *p*-coumaric acid methyl ester is the first identified compound to be associated with phytoalexin-like behavior in cucumber.

Key Words—*Cucumis sativus*, phenolic, phytoalexin, milsana, induced resistance, *p*-coumaric acid methyl ester.

INTRODUCTION

Higher plants offer an excellent source of biologically active products, which are generally secondary metabolites (e.g., Benner, 1993; Rhodes, 1994). Among them, phenols, possessing pesticidal capacity in general or antifungal activity in particular, have been shown to be produced by several plants investigated so far (e.g., Nicholson and Hammerschmidt, 1992; Bennett and Wallsgrove, 1994). Many examples of such antifungal compounds are stress metabolites or phyto-

*To whom correspondence should be addressed.

0098-0331/97/0600-1517$12.50/0 © 1997 Plenum Publishing Corporation

alexins. Others are constitutive compounds referred to as phytoanticipins (VanEtten et al., 1994) that may play a role in plant resistance as preformed inhibitors of invaders (Benner, 1993).

In cucumber plants (*Cucumis sativus* L.) infected by pathogens, activities of enzymes associated with defense reactions, such as chitinases, β-1,3-glucanases (Dalisay and Kuc, 1995; Schneider and Ullrich, 1994), and enzymes involved in secondary metabolite pathways such as phenylalanine ammonia lyase (Schneider and Ullrich, 1994), were shown to increase significantly, but defense reactions were reported to occur mostly through lignification (Hammerschmidt and Kuc, 1982; Siegrist et al., 1994). Recently, however, using chemical or natural inducers, Chérif et al. (1994) and Daayf et al. (1995) observed an increase in soluble antibiotic phenolics as a response to invasion by *Pythium ultimum* and *Sphaerotheca fuliginea*, respectively. Some of these compounds were found to be present in both free and conjugated form in control healthy plants (Daayf et al., 1995), albeit in lower concentrations than in powdery mildew-infected cucumber leaves treated with a natural inducer (Daayf et al., 1997). While no phytoalexins have ever been described in cucumber, these observations suggest that some antifungal phenolic compounds are indeed part of its defense reactions. In this context, the objectives of this work were to isolate a biologically active compound found in control cucumber leaves, to show variation of its concentration in both control and elicited plant material, and to establish its chemical structure.

METHODS AND MATERIALS

Extraction. A modified extraction method (Daayf et al., 1995) of Dercks and Buckenauer (1986) was adopted. The procedure allowed for the determination of free and glycosidic-linked phenolics.

Fresh foliar material from 10-week-old *Cucumis sativus* cv. Mustang was mixed in 10 ml acidified methanol 80% per gram of fresh foliar material, protected from oxidation by replacing air with nitrogen and eliminating light, and extracted for 24 hr. The mixture was then filtered, and the filtrate was extracted several times with petroleum ether to remove chlorophyll, carotenoids, lipids, and waxes (fraction FI). The methanolic fraction was then rota-evaporated, and the aqueous fraction was extracted several times with anhydrous ethyl ether (fraction FII). The aqueous fraction was diluted with an equal volume of 4 N HCl and hydrolysis was performed for 90 min in a water bath at 100°C. After cooling, the hydrolysate was partitioned against anhydrous ethyl ether (fraction FIII). Extractions were carried out for both control plants and plants infected with powdery mildew and treated with Milsana® (Compo, Münster, Germany) (extracts from *Reynoutria sachalinensis*), a natural inducer known for its pro-

tective role against the disease (Daayf et al., 1995; Dik and Van Der Straay, 1995; Herger and Klingauf, 1990; Herger et al., 1988; Schneider and Ullrich, 1994).

Screening of Molecules with Antifungal Activity. The different extracted fractions were evaluated for fungitoxicity directly on thin-layer chromatograms (TLC). All fractions were spotted on TLC plates and developed with solvent systems s1, hexane–EtOAc (8:2, v/v); s2, hexane–$CHCl_3$–EtOAc (5:3:2, v/v/v); and s3, dichloromethane–EtOAc–MeOH (6:4:1, v/v/v). To localize zones of antifungal activity, silica gel plates were sprayed with a sporal suspension of *Cladosporium cucumerinum* Ellis & Arth. as described by Bailey and Burden (1973). Plates were then incubated in a humid chamber for 48 hr, and zones of inhibition appeared as white zones against a dark background formed by spores and mycelium of the fungus.

Chromatography. TLC was carried out, using S1, S2, and S3, on 0.2-mm precoated silica gel 60 F254 aluminum sheets (Merck) for analytical TLC and on 0.5-mm silica gel 60 F254 on glass plates for preparative TLC. Chromatograms were visualized under ultraviolet illumination at 254 nm and 366 nm and after spraying one of the following reagents: solution 1—ammonium molybdate (75 g) and ceric sulfate (4 g) in 100 ml of an aqueous solution of H_2SO_4 (10%) or solution 2—vanillin (6 g), and H_2SO_4 (3 ml) in MeOH (197 ml).

HPLC was performed in a reverse-phase mode using a Nova-Pak C_{18} column (60 A, 4 μm, 3.9 $\times$ 300 mm) and a photodiode array detector (Waters 991). The eluent was acetonitrile–H_2O, pH 3, from 0 to 80% acetonitrile using the gradient indicated in Table 1.

Isolation and Identification of Bioactive Compound. After extraction and localization of zones with antifungal activity, the fraction having exhibited fungitoxicity was purified by preparative TLC, followed by flash column chromatography with hexane–EtOAc as eluent. For each eluted fraction, assessment of antifungal activity was carried out with the TLC bioassay as described above. The bioactive fraction was then purified by HPLC using the conditions described above. NMR spectroscopy was recorded in MeOH-d_4 with TMS as internal standard in a Bruker 300 MHz spectrometer. GC-MS spectra were recorded with a Hewlett-Packard (HP) 5890 series II gas chromatograph, coupled with MS

TABLE 1. SOLVENT GRADIENT USED IN HPLC ELUTIONS

Time (min)	0	10	20	40	50	60	70	80	85	90	95	100
Acetonitrile (%)	0	0	15	15	25	25	40	50	70	80	80	0
Flow (ml/min)	1.0	0.9	0.9	0.9	0.9	0.65	0.5	0.5	0.5	0.9	1.0	1.0

HP mass selective detector 5972. EI-MS spectra were recorded at 70 eV (ionizing potential) with a direct inlet system.

RESULTS AND DISCUSSION

After extraction of mixed healthy cucumber leaves in methanolic solution for 24 hr the total extract was fractionated into FI (chlorophylls, carotenoids, waxes, lipids), FII (free phenolics), and FIII (aglycones from conjugated phenolics). Individual fractions were tested for their bioactivity against *Cladosporium cucumerinum* directly on TLC plates. No toxicity was observed with FI, and no differential bioactivity was detected between FII from control and treated plants. On the other hand, qualitative and quantitative differences were observed in FIII between control and treated plants. We were interested in the zone of toxicity present in the control and which showed a variation in treated plants.

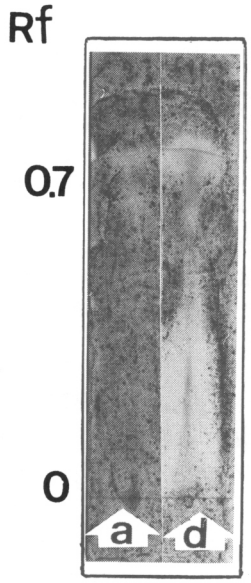


FIG. 1. Thin-layer chromatography of aliquots from fraction III, extracted from control healthy (a) and infected and Milsana®-treated (d) long English cucumber leaves. The plate was developed with dichloromethane–hexane–methanol (6:4:1, v/v/v) and after drying was sprayed with a conidial suspension of *Cladosporium cucumerinum* and incubated for 72 hr in a humid chamber. White spots indicate zones of fungitoxicity. The spot at R_f 0.7 represents the fungitoxic zone observed in both treatments, with higher concentration in the treatment (d).

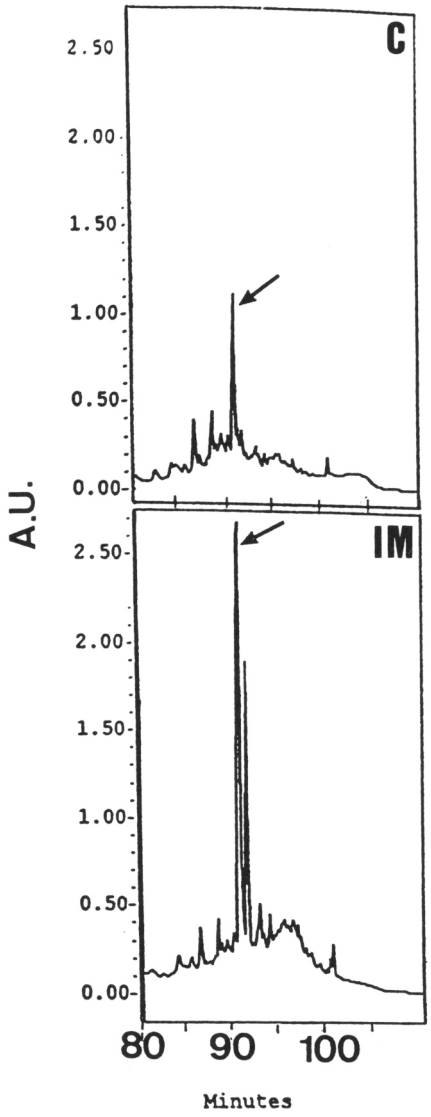


FIG. 2. Eighty- to 100-min section of the HPLC chromatogram showing *p*-coumaric acid methyl ester (*p*-hydroxycinnamic acid methyl ester) peak from analysis on C_{18} column of fraction III from control (C) and infected Milsana®-treated (IM) long English cucumber leaves, one day after milsana application. Elution was performed with a gradient of 0 to 80% acetonitrile. Volume of sample from (C) and (IM) used for these injections corresponded to the same quantity of fresh leaf material.

In control leaves, one major and one minor zones of inhibition were detected. Concentration of the major zone appeared to be more important in treated and infected leaves [30 ± 5 μg/g fresh material (FM)] than in control leaves (13 ± 2 μg/g FM) (Figures 1 and 2). Its R_f values were 0.32, 0.63, and 0.7 in solvent systems s1, s2, and s3, respectively). After separation by successive preparative TLC and flash liquid chromatography, followed by purification through reverse-phase HPLC, a crystalline compound was isolated. Analytical and spectral data identified this compound as *p*-coumaric acid methyl ester (Figure 3). Its retention time was ca. 92 min (see Figures 2 and 4), with a maximum absorbance at ca. 310 nm. Acetone extracts at standard pH and at pH 2.0 both contained the active compound, thus eliminating the possibility of artifacts being formed in the methanol extraction (Figure 4). By putting water droplets containing fungal spores on cucumber leaf surface, we were also able to extract this compound, as indicated by HPLC analyses (results not shown).

p-*Coumaric Acid Methyl Ester.* High-resolution fast atom bombardment MS *m/z* [M + H] 178.1872 ($C_{10}H_{10}O_3$ requires 178.1870). EI-MS (70 eV), *m/z* 178 $[M]^+$ (70), 147 $([M\text{-}OCH_3]^+$ (100), 119 $[M\text{-}COOCH_3]^+$ (43), 91 $[C_7H_7]^+$ (33), 77 $[C_6H_5]^+$ (3), 65 $[C_5H_5]^+$ (15). Crystalline solid mp: 129–130°C. ^{1}H NMR (300 MHz, MeOH-d_4) d (ppm): 3.8 (s, 3H, $COOCH_3$); 5.98 (d, 1H, HO—C_6H_4—CH=CH-); 6.72 (2d, 2H, H_3 and H_5); 7.14 (2d, 2H, H_2 and H_6); 7.18 (d, 1H, HO—C_6H_4—CH=CH—).

Comparison of our results with data available on *p*-coumaric acid methyl ester in the literature (Bandara et al., 1988) and on NMR of hydroxycinnamic derivatives (Hiroaki et al., 1993) confirmed the validity of the chemical structure. In addition, chemical shifts and multiplicity of benzenic protons corroborated the identification of the molecule. We have also synthesized this molecule, as well as *m*-hydroxycinnamic acid methyl ester, through methylation of *p*-coumaric and *m*-coumaric acids, respectively. Synthetic *p*-coumaric acid gave the same R_t and R_f as our compound, and their cochromatography gave only one peak (Figure 4), confirming the nature of the purified compound.

While free acids are widely distributed throughout nature as secondary metabolites of plants and exhibit considerable antimicrobial effects against fungi (Ndubizu, 1976) and gram-positive and gram-negative bacteria and viruses (Grodzinska-Zachwieja et al., 1976), this methyl ester, as well as alkyl esters

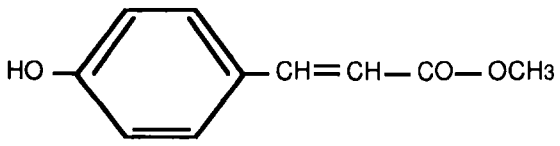


FIG. 3. *p*-Coumaric acid methyl ester (*p*-hydroxycinnamic acid methyl ester).

in general, are somewhat rare and are found only occasionally in nature. However, they were reported to inhibit bacteria (Baranowski and Nagel, 1982, 1983), diseases due to molds (Gupta and Banerjee, 1976), and rusts (Macko et al., 1972) and also to have antioxidant activities in food products (Baranowski and Nagel, 1984).

p-Coumaric acid methyl ester has been found before in the rhizome of *Costus speciosus* as a constitutive principle with antifungal activity against *Cladosporium cladosporioides*, *Colletotrichum gleosporioides*, *Curvularia* sp., and *Penicillium* sp. (Bandara et al., 1988), thus supporting the fungitoxicity of the compound reported here. However, these authors did not report on the role of this compound as a defense weapon. This is the first time that a specific antifungal compound from cucumber has been characterized. The fact that it was found to occur constitutively in control plants and to increase in concentration following infection and treatment with a natural inducer indicates that it plays

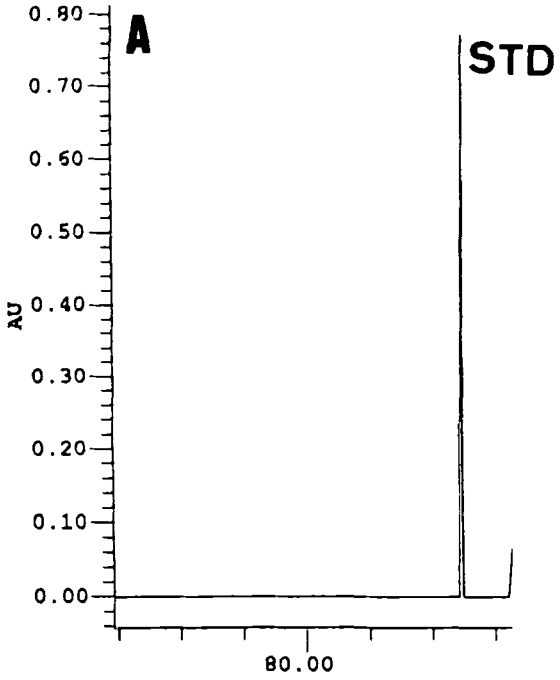


FIG. 4. HPLC chromatogram of (A) synthesized *p*-coumaric acid methyl ester standard (STD), (B) STD cochromatographed with acetone-extracted fraction III from cucumber leaves infected and treated with Milsana® (IM), and (C) acetone-extracted fraction III from IM. Note the increase from C (height = 0.6) to B (height = 1.8) of only peak at 92 min.

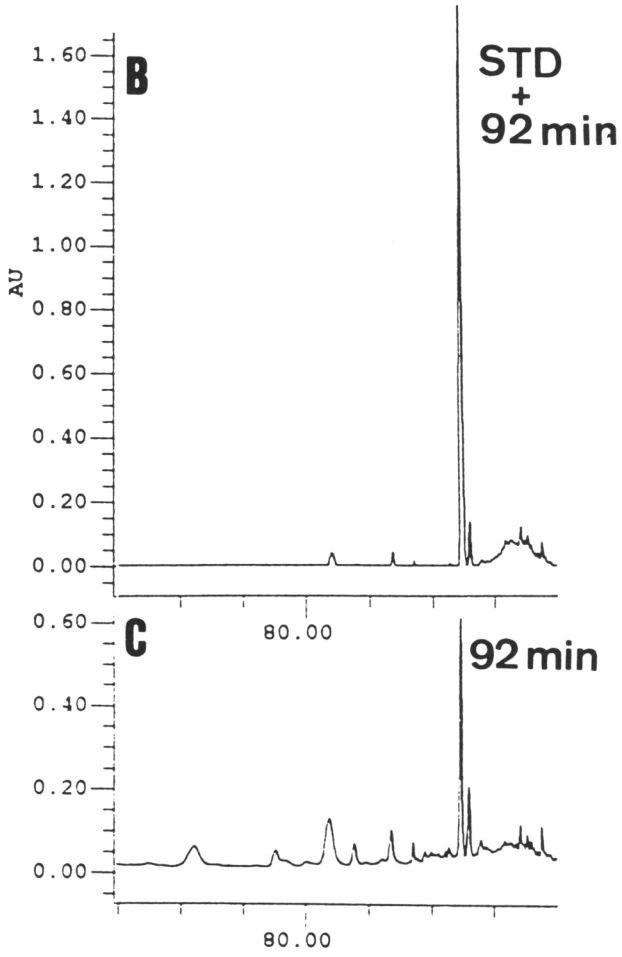


FIG. 4. Continued.

a role in both constitutive and induced resistance in cucumber. It was found in higher concentrations following hydrolysis, implying the synchronized occurrence of hydrolytic reactions in planta. This phenomenon is now reported to be quite common in the plant kingdom and is believed to be of key importance in a plant's defense reactions (Geibel, 1995; Higgins et al., 1995).

Phytoalexins have not been found previously to be associated with resistance in cucumber, but lignification and production of hydrolytic enzymes have been reported as the main mechanisms. Hammerschmidt and Kuc (1982) did

suggest that coniferyl alcohol may act as a phytoalexin in cucumber, but they could not find detectable amounts in either control or treated plants. In the case of *p*-coumaric acid methyl ester, it was found in control plants and was observed to increase markedly in treated plants. In addition, Daayf et al. (1997) reported that yet-unidentified phenolic compounds were induced in powdery-mildew infected leaves following Milsana® treatment. This indicates that induced resistance in cucumber is mediated in part by the production of phytoalexin-like compounds.

Acknowledgments—The authors thank Caroline Labbé, Dr. Reynald Boulanger, and Tyler Avis for their technical assistance. Financial assistance from NSERCC and the program Synergie is gratefully acknowledged.

REFERENCES

BAILEY, J. A., and BURDEN, R. S. 1973. Biochemical changes and phytoalexin accumulation in *Phaseolus vulgaris* following cellular browning caused by tobacco necrosis virus. *Physiol. Plant Pathol.* 3:171–177.

BANDARA, R. B. M., HEWAGE, C. M., KARUNARATNE, V., and ADIKARAM, N. K. B. 1988. Methyl ester of para-coumaric acid: Antifungal principle of the rhizome of *Costus speciosus*. *Planta Med.* 54:477–478.

BARANOWSKI, J. D., and NAGEL, C. W. 1982. Inhibition of *Pseudomonas fluorescens* by hydroxycinnamic acids and their alkyl ester. *J. Food Sci.* 47:1587–1589.

BARANOWSKI, J. D., and NAGEL, C. W. 1983. Properties of alkyl hydrocinnamates and effects on *Pseudomonas fluorescens*. *Appl. Environ. Microbiol.* 45:218–222.

BARANOWSKI, J. D., and NAGEL, C. W. 1984. Antimicrobial and antioxidant activities of alkyl hydroxycinnamates (alkacins) in model systems and food products. *Can. Inst. Food. Sci. Technol. J.* 17:79–85.

BENNER, J. P. 1993. Pesticidal compounds from higher plants. *Pestic. Sci.* 39:95–102.

BENNETT, R. N., and WALLSGROVE, R. 1994. Secondary metabolites in plant defense mechanisms. *New Phytol.* 127:617–633.

CHÉRIF, M., ASSELIN, A., and BÉLANGER, R. R. 1994. Defense responses induced by soluble silicon in cucumber roots infected by *Pythium* spp. *Phytopathology* 84:236–242.

DAAYF, F., SCHMITT, A., and BÉLANGER, R. R. 1995. The effects of plant extracts of *Reynoutria sachalinensis* on powdery mildew development and leaf physiology of long English cucumber. *Plant Dis.* 79:577–580.

DAAYF, F., SCHMITT, A., and BÉLANGER, R. R. 1997. Evidence of phytoalexins in cucumber leaves infected with powdery mildew following treatment with leaf extracts of *Reynoutria sachalinensis*. *Plant Physiol.* 113:719–727.

DALISAY, R. F., and KUC, J. A. 1995. Persistence of induced resistance and enhanced peroxidase and chitinase activities in cucumber plants. *Physiol. Mol. Plant Pathol.* 47:315–327.

DERCKS, W., and BUCHENAUER, H. 1986. Untersuchungen zum Einfluss von Aluminiumfosetyl auf den pflanzlichen Phenolstoffwechsel in den Pathogen-Wirt Beziehungen *Phytophthora/Fragaria*-Erdbeere und *Bremia/Lactuca*-Salat. *J. Phytopathol.* 115:37–55.

DIK, A. J., and VAN DER STRAAY. 1995. The effect of milsana on cucumber powdery mildew under Dutch conditions. *Med. Fac. Landbouww. Rijksuniv. Gent.* 59:1027–1034.

GEIBEL, M. 1995. Sensitivity of the fungus *Cytospora persoonii* to the flavonoids of Prunus cerasus. *Phytochemistry* 3:599–601.

GRODZINSKA-ZACHWIEJA, Z., ZHORNIAK-NOWOSIELSKA, I., MARCISZEWSKA, M., and GATKIEWICZ, A. 1976. Antiviral activity of caffeic acid in in vitro studies. *Acta Biol. Cracov. Ser. Bot.* 19:29–33.

GUPTA, S. K., and BANERJEE, A. B. 1976. Isolation of ethyl *p*-methoxycinnamates, the major antifungal principle of *Curcuma zedoaria*. *Lloydia* 39:218–222.

HAMMERSCHMIDT, R., and KUC, J. 1982. Lignification as a mechanism for induced systemic resistance in cucumber. *Physiol. Plant Pathol.* 20:61–71.

HERGER, G., and KLINGAUF, F. 1990. Control of powdery mildew fungi with extracts of the giant knotweed. *Reynoutria sachalinensis* (Polygonaceae). *Med. Fac. Landbouww. Rijksuniv. Gent.* 55:1007–1014.

HERGER, G., KLINGAUF, F., MANGOLD, D., POMMER, E. H., and SCHERER, M. 1988. Efficacy of extracts of *Reynoutria sachalinensis* (F. Schmidt) Nakai (Polygonaceae) against fungal diseases, especially powdery mildews. (in German), *Nachr. Dsch. Pflanzenschutzdeinst (Braunschweig)* 40:56–60.

HIGGINS, V. J., HOLLANDS, J., and BATES, D. K. 1995. Phytoalexins in forage legumes: Studies on detoxification by pathogens and the role of glycosidic precursors in roots, pp. 391–403, *in* M. Daniel and R. P. Purkayastha (eds.). Handbook of Phytoalexin Metabolism and Action. Marcel Dekker, New York.

HIROAKI, O., YOSHINORI, N., HIROKO, J., TAKESHI, K., and GENJI, I. 1993. Preparation of *p*-hydroxycinnamic acid derivatives and skin-lightening cosmetics containing them (patent). Kao Corp., Jpn. Kokai Tokkyo Koho 05105620 (93105620), CI.: A61K7/48, Appl.: 91/266405.

MACKO, V., SAPLES, R. C., RENWICK, J. A. A., and PIRONE, J. 1972. Germination self-inhibitors of rust uredospores. *Physiol. Plant Pathol.* 2:347–350.

NDUBIZU, T. O. C. 1976. Relations of phenolic inhibitors to resistance of immature apple fruits to rot. *J. Hortic. Sci.* 51:311–316.

NICHOLSON, R. L., and HAMMERSCHMIDT, R. 1992. Phenolic compounds and their role in disease resistance. *Annu. Rev. Phytopathol.* 30:369–389.

RHODES, M. J. C. 1994. Physiological roles for secondary metabolites in plants: Some progress, many outstanding problems. *Plant Mol. Biol.* 24:1–20.

SCHNEIDER, S., and ULLRICH, W. 1994. Differential induction of resistance and enhanced enzyme activities in cucumber and tobacco caused by treatment with various abiotic and biotic inducers. *Physiol. Mol. Plant Pathol.* 45:291–304.

SIEGRIST, J., JEBLICK, W., and KAUSS, H. 1994. Defense responses in infected and elicited cucumber (*Cucumis sativus*) hypocotyl segments exhibiting acquired resistance. *Plant Physiol.* 105:1365–1374.

VANETTEN, H., MANSFIELD, J. W., BAILEY, J. A., and FARMER, E. E. 1994. Two classes of plant antibiotics: Phytoalexins versus phytoanticipins. *Plant Cell* 6:1191–1192.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

MULTIPLE FUNCTIONS FOR SECONDARY METABOLITES IN ENCRUSTING MARINE INVERTEBRATES

MIKEL A. BECERRO,[1] XAVIER TURON,[2,*] and MARIA J. URIZ[1]

[1]*Centre for Advanced Studies (C.S.I.C.)*
Camí de Sta. Bàrbara, s/n. 17300 Blanes (Girona) Spain
[2]*Department of Animal Biology*
Faculty of Biology, University of Barcelona
645, Diagonal Ave. 08028 Barcelona, Spain

(Received December 29, 1995; accepted January 22, 1997)

Abstract—We used three chemical fractions (spanning a wide range of polarities) from the extracts of four marine invertebrates, the sponges *Crambe crambe* and *Hemimycale columella* and the ascidians *Cystodytes dellechiajei* and *Polysyncraton lacazei*, to test inhibition of cell division, photosynthesis, and settlement. We used assay organisms from the same habitat, seeking to determine whether a species may display diverse, ecologically relevant bioactivities and, if so, whether the same types of compound may be responsible for such activities. Cell division was strongly inhibited by the sponge *C. crambe*. A dichloromethane fraction from *C. crambe* prevented development of sea urchin *Paracentrotus lividus* eggs at a concentration of 10 μg/ml, as did the butanolic fraction, but at higher concentrations (50 and 100 μg/ml). At 50 μg/ml, the aqueous fraction of *C. crambe* allowed cell division but prevented eggs from developing beyond the gastrula stage. Similar results were recorded with the dichloromethane fraction of *P. lacazei* and from the aqueous fraction of *H. columella*. Photosynthesis was unaffected by any of the species at 50 μg/ml. Larval settlement was inhibited by one or another fraction from the four species surveyed at a concentration of 50 μg/ml, although *C. crambe* exhibited the greatest amount of activity. We therefore found that various fractions displayed the same type of bioactivity, while compounds from the same fraction were responsible for multiple activities, suggesting that secondary metabolites are multiple-purpose tools in nature, which is relevant to our understanding of species ecology and evolution. Moreover, results showed that the assessment of the role of chemical compounds is significantly influenced by the assay organism, fractionation procedure, concentration, and

*To whom correspondence should be addressed.

0098-0331/97/0600-1527$12.50/0 © 1997 Plenum Publishing Corporation

duration of experiments. All these factors should be carefully considered when testing ecological hypotheses of the roles of chemically-mediated bioactivities.

Key Words—Secondary metabolites, chemical defense, evolution, ascidians, sponges.

INTRODUCTION

The field of natural products research has uncovered a vast source of new compounds in marine organisms (Faulkner, 1996, and previous reviews by the same author), many of which show antimitotic, cytotoxic, antibacterial, and antiviral properties that may play important ecological roles (Bhakuni and Jain, 1990). Despite the number of compounds reported, comparatively few of them have been properly tested for an ecological role, and so the biological significance of most secondary metabolites remains largely unknown. Indeed, the bioactivity of a metabolite has frequently been described much later than its chemical characteristics. For instance, avarol, a sesquiterpenoid hydroquinone, was first isolated from the sponge *Dysidea avara* (Schmidt) in 1974 (Minale et al., 1974), but it was not until several years later that avarol was reported to produce aberrations in the development of sea urchins (Cariello et al., 1980) and to display antibacterial, antifungal, and antiviral activities (Seibert et al., 1985, Uriz et al., 1992). Additional ecological functions of avarol have been suggested (Martin and Uriz, 1993; Uriz et al., 1992a), although only the antipredatory role has been experimentally tested (Uriz et al., 1996a). Similar stories can be told for a variety of bioactive marine natural products.

The importance, however, of predator–prey interactions in the production of chemical defenses has been highlighted both in terrestrial and benthic organisms. Chemical defenses may have evolved to avoid predation (Rosenthal and Berenbaum, 1992), resulting, in many instances, in a chemically mediated predator–prey coevolution with ecological advantages for both parties (Paul et al., 1990). However, the importance of antipredation in the evolution of chemical defenses in marine environments has been, in our opinion, overemphasized, perhaps because many predictions stem from works on chemical adaptations to herbivory in terrestrial plants. The idea that the antipredatory function is the sole or the most important selective process in the evolution of chemical defenses underlies most of the literature on seaweeds (Hay and Steinberg, 1992; Steinberg and van Altena, 1992; Yates and Peckol, 1993; Hay et al., 1994, to cite some recent examples) and also benthic animals (Van Alstyne et al., 1994; Pawlik et al., 1987, 1995; Paul et al., 1990; Uriz et al., 1996a). Historically, comparatively less attention has been given to other possible roles of chemical substances, such as competition for space or antifouling mechanisms, although more recently they have been the subject of a growing number of studies, especially

those considering benthic animal groups (Coll et al., 1987; Sammarco and Coll, 1988; Porter and Targett, 1988; Davis et al., 1989; Coll, 1992; Maida et al., 1993; Becerro et al., 1995; Clare, 1996; Turon et al., 1996a). It would seem that the most realistic scenario is that secondary metabolites have evolved in response to a panoply of selective pressures and that they fulfill a variety of ecological roles in nature.

Whether a species may display multiple chemically mediated types of interaction is a suggestive hypothesis to test. Such multiple functions could provide a range of ecological advantages in addition to that of the well-known antipredator role. For instance, space is usually a limiting resource in rocky littoral communities; and for encrusting filter-feeding organisms certain basic physiological parameters, such as feeding, reproduction, and mortality, are a function of their area (Jackson, 1979; Ryland and Warner, 1986; Sebens, 1987). Thus, the need for mechanisms for space acquisition and maintenance would seem paramount, and the use of "chemical warfare" can be expected. Furthermore, fouling organisms may interfere with the filtering activity of the basibionts and diminish their efficiency, especially in those filter-feeding organisms in which water is pumped through the free surfaces, as in sponges and some compound ascidians. In organisms of this kind, mechanisms that fight against fouling could also be expected. If secondary metabolites have multiple ecological functions, then changes in chemical defenses could not be modeled as a response to changes in a particular competitor, predator, or fouler, since the producer may be constrained by the other functions of the metabolites (Schmitt et al., 1995). The possibility that particular secondary metabolites have multiple functions has recently been considered, but as yet only occasionally tested in marine environments (Paul, 1992; Schmitt et al., 1995).

This study seeks to determine whether a species may display diverse chemically mediated functions, focusing on functions related to antifouling and space competition. Four common encrusting, filter-feeding species on hard substrata were extracted and their chemical compounds fractionated according to their polarity. Each group of compounds was tested for the inhibition of cell division, photosynthesis, and settlement using ecologically relevant organisms. These activities were selected since they may be involved in growth inhibition, competition with algae, and antifouling processes.

METHODS AND MATERIALS

Previously gathered chemical and ecological data allowed the following species to be selected from among a pool of Mediterranean species whose bioactivity was already known (Uriz et al., 1992a; Martin and Uriz, 1993; and authors' unpublished research):

Crambe crambe (Schmidt) is a red encrusting sponge (Poecilosclerida). It can reach surface areas of 0.5 m^2 in the study zone and is always found free of macroepibionts. This species has been reported to have an array of bioactivities (Berlinck et al., 1990, 1992a,b; Jares-Erijman et al., 1991; Becerro, 1994; Becerro et al., 1994). *C. crambe* is one of the most abundant species in Mediterranean littoral waters (Uriz et al., 1992b). It inhabits both well-illuminated and dark habitats at depths ranging from 1 to 60 m and can be found on hard substrata as well as seagrass (*Posidonia oceanica* Delile) beds; it is one of the few species able to grow among the "new colonizing alga" in the western Mediterranean, *Caulerpa taxifolia* (Vahl) C. Agardh (authors' personal observation).

Hemimycale columella (Bowerbank) is a pinkish encrusting sponge (Halichondrida). Maximum size in the study area is about 500 cm^2. It is also a common species in the Mediterranean, where it inhabits *P. oceanica* beds and hard substrata at depths ranging from 1 to 300 m (Uriz, 1978).

Cystodytes dellechiajei (Della Valle) is a polycitorid ascidian, with a blue tunic (occasionally white, green, or brown) free of epibionts and sand. It has a maximum size in the study area of about 400 cm^2. Although *C. dellechiajei* is preferentially a sciaphilous species, it can also be observed in well-lit habitats (Turon, 1988, 1990). Specimens collected in Japanese waters have been reported to have alkaloids with antineoplastic activity (Kobayashi et al., 1988). Mediterranean specimens featured antimitotic and cytotoxic activities (authors' unpublished research).

Polysyncraton lacazei (Giard) is a red encrusting didemnid ascidian that can reach a surface area of 400 cm^2 in the zone studied. It is usually clean of macroepibionts (Wahl and Lafargue, 1990). It is one of the most abundant colonial ascidians in the zone of study between 0 and 20 m of depth (Turon, 1990). *P. lacazei* has been reported to have antimitotic and cytotoxic activities, and its antifouling mechanisms have been extensively studied (Wahl and Lafargue, 1990; Wahl and Banaigs, 1991).

All specimens were collected in the locality of Tossa de Mar (Northwestern Mediterranean; 41°43.2′N, 2°56.0′E) from a vertical wall at a depth ranging from 1 to 20 m, by scuba diving.

Extraction Procedure. Several specimens of each of the four species selected were freeze-dried and ground together. This was necessary to obtain sufficient amounts of extract. Interspecimen variation was, therefore, not addressed in this work. The samples were extracted three times [1/2 hr, 1/2 hr, and overnight (13 hr)] with a mixture of dichloromethane (DCM) and methanol (MeOH) (1 : 1) in the proportion of 20 ml of solvents per gram (dry weight) of sample. The solvent was evaporated to dryness under reduced pressure and the residue was dissolved in 20 ml of a mixture of DCM and distilled water (1 : 1). These solvents yielded two layers (DCM and aqueous phases) in which the extracted compounds

were separated according to their polarity. The DCM phase was then isolated. Addition to the water phase of 10 ml of DCM yielded new DCM/aqueous layers. The new DCM phase was isolated and a further 10 ml of DCM was added to the aqueous phase, obtaining a third pair of DCM/aqueous layers, from which the DCM layer was isolated again. The three DCM phases were then pooled, evaporated under reduced pressure and weighed. This residue contained the lowest polarity compounds of the crude extract, and it will hereafter be referred to as the DCM fraction. The remaining aqueous phase was extracted with 10 ml of *n*-butanol (BuOH). Two new layers (aqueous and butanolic phases) were obtained. The butanolic layer was isolated and 10 ml BuOH was again added to the aqueous phase. The formation of this layer was performed for a third time. The three butanolic phases were then pooled and evaporated under reduced pressure. This residue contained the mid-range polarity compounds, and it will hereafter be referred to as the BuOH fraction. The remaining aqueous fraction was also evaporated, and the residue, containing the highest polarity compounds of the initial crude extract, will hereafter be referred to as the aqueous fraction. Consequently, this separation procedure gave three fractions of the crude extract—DCM, aqueous, and BuOH fractions—which were used to test the inhibition of cell division, photosynthesis, and settlement in three bioassays using organisms that share the habitat with the four species being investigated.

Inhibition of Cell Division. The bioassay was run using the eggs and embryos of the sea urchin *Paracentrotus lividus* (Lamarck), commonly found in the same habitats as the species analyzed, to detect both antimitotic and cytotoxic properties in the samples. These properties may be useful in competing for space, as antifouling mechanisms or in those processes in which the inhibition of another's growth is a determining factor. A general description of this method can be found in Reish and Oshida (1987), Dinnel et al. (1987), and Martin and Uriz (1993). Adult specimens of *P. lividus* were collected in the field and carried to the laboratory. Spawning was induced by peristomic injection of 1 ml of 0.5 M potassium chloride and both sperm and eggs were released into filtered (0.45-μm pore diameter) seawater (FSW). Cross-fertilization was carried out by adding a small quantity of sperm to the eggs. Tests were run with eggs and sperm from the same couple of sea urchins, since they deliver sufficient numbers of gametes for the simultaneous testing of several samples. Fertilization was usually successful—over 80%. Otherwise the eggs were rejected and the cross-fertilization process was repeated with other specimens. The eggs were then placed in a vessel containing the necessary FSW to a concentration of ca. 200 eggs/ml. Each of the fractions (DCM, BuOH, and aqueous) for each species was tested at three concentrations: (1) 100 μg of extract per ml, (2) 50 μg of extract per milliliter, (3) 10 μg of extract per milliliter. These concentrations were obtained by diluting 100, 50, and 10 μg of extract per 100 μl of ethanol (EtOH). The final solution was obtained by adding 0.2 ml of this ethanolic

solution to 1.8 ml of FSW with eggs. These steps were followed to ensure a good solution of the solid residue in the FSW, i.e., to ensure that the active compounds, if any, diffuse adequately in the aqueous solution. Three replicates were used for each treatment and control. The control was performed by adding 0.2 ml of EtOH to 1.8 ml of FSW. Experiments were stopped after 80 min by adding a drop of formaldehyde (40%). The number of eggs [non-fertilized (NF)] and embryos [fertilized without division (ND), in two-cell stage (TC), and in four-cell stage (FC)] were counted and the proportion of each category was calculated. A three-way analysis of variance of the proportion of embryos at the first cellular division (TC) was performed using species, fraction (nested within species), and concentration (orthogonal to fraction) as factors. A treatment (i.e., any combination of the factors investigated) was referred to as bioactive whenever it differed significantly from the control, as assessed by one-way ANOVA. The proportion of TC was the variable selected on which to run the analyses since this category was the most abundant in the controls. Data were arcsine transformed in order to meet the assumptions of normality and homoscedasticity (as tested by Kolmogorov-Smirnov and Bartlett tests, respectively).

A second experiment was performed as previously described but with a final concentration of only 50 μg of fraction per milliliter. The aim of this second bioassay was to follow the embryonic development of the eggs to the pluteus stage, thus allowing the compounds to act at different stages of the larval development. This may also help to clarify the mechanisms of action of the compounds tested. The number of NF, ND, nonpluteus (NP), and pluteus (P) stages were recorded after 48 hr, and their relative proportions were calculated. To test the effect of time in the detection of fraction activity, data from the two experiments described (experiment 1: concentration = 50 μg/ml and time = 80 min; experiment 2: concentration = 50 μg/ml and time = 48 hr) were combined. Since the most abundant stage in the controls changed between the two experiments (two-cell embryos after 80 min, and pluteus after 48 hr), it was necessary to formulate a new standardized variable that would allow for comparison. This standardized variable was calculated by dividing the proportion of TC and P in the treatments after 80 min and 48 hr, respectively, by the mean proportion of TC or P in their respective controls. Thus, at both times, controls have a mean value of 1, while the treatments may have values higher than 1 (indicating a higher proportion of TC or P than in controls, 1 (no effect), or less than 1 (lower proportion of TC and P). In practice, however, values ranged from about 1 to 0, indicating inhibition of development. A three-way ANOVA was performed on this variable using species, fraction (nested within species), and time (orthogonal to fraction) as factors. As in the shorter-term experiment, and in order to detect bioactivity, data from all combinations of species and fractions were compared with the respective controls with one-way ANOVAs (one per observation time). No data transformation was necessary since the standardized variable met the assumptions on which the ANOVAs were run.

Photosynthesis Inhibition. The test was conducted with the green alga *Ulva rigida* C. Agardh. This test shows whether photosynthesis is affected by the samples. Generally speaking, algae are fast-growing organisms when compared to most zoobenthic species. Thus, inhibition of the photosynthetic ratio may favor zoobenthic organisms competing with algae for space. *U. rigida* was collected in the field and taken to the laboratory to measure oxygen production. This alga was selected because of its morphology, which allowed a clean thallus to be cut in uniform pieces of approximately 1–2 cm^2. These pieces were individually introduced in 280-ml flasks. Oxygen content was measured with an oxygen indicator (Orbisphere Laboratories) after 90 min. Incubation was performed in a Mk X Incubator Shaker (LH Fermentation) at 100 rpm, 20°C, and 500 $\mu mol/m^2/s$ of light intensity. The extraction procedure was as described above. Treatments were tested at the concentration of 50 μg of extract per milliliter. Three replicates were used for each treatment and control. Oxygen content was adjusted to grams dry weight of algae (four days at 80°C, constant weight). No data transformation was necessary since normality and homoscedasticity assumptions were met by the data. Therefore, two-way ANOVA of the oxygen content was performed with species and fraction (nested within species) as factors. As before, bioactivity was assessed by one-way ANOVA using all combinations of species and fractions, together with the controls, as levels of the main factor.

Settlement Inhibition. The settlement inhibition bioassay was run with larvae of the bryozoan *Bugula neritina* (Linné). This bioassay records larval settlement success and posterior survival under the effect of the extracts. Bryozoans are significant components of fouling communities, so this test can be considered as an indicator of general antifouling properties. Colonies of *B. neritina* were collected in the sea, taken to the laboratory, and placed in a completely dark, areated aquarium for one day. After this period, release of larvae was achieved in response to exposure to natural light (Keough, 1984). The colonies were collectively subjected to the photic shock, and consequently larvae from all the colonies were sufficiently mixed to ensure that no colony-dependent differences in the settlement behavior were present among treatments. Treatments were obtained as previously described and consisted of 50 μg of extracts per milliliter. Between 12 and 34 larvae were pipetted into 5-ml Petri dishes where the experiment was performed. Three replicates were used for each treatment and control. The number of larvae settled [settled (ST) and ancestrula (AN)] and not settled (NS), and their movements, were recorded at 1 hr, 30 min; 5 hr, 30 min; 9 hr, 30 min; and 20 hr (times 1, 2, 3, and 4, respectively) and the proportion of each category was calculated. A three-way ANOVA was performed using species, fraction (nested within species), and time (orthogonal to time) as factors. Bioactivity was assessed by one-way ANOVA (one for each observation time) comparing all levels of species and fractions with the controls. As in the experiment of cell division inhibition, the most abundant variable in the controls

changed as a function of time, so it was necessary to use a standardized variable. Here, the standardized variable was calculated by dividing the proportion of ST (time 1 and 2) and AN (time 3 and 4) in the treatments by the mean proportion of ST or AN in their respective controls. As before, at any time, controls have a mean value of 1 while in the treatments it ranged between about 1 (no effect) and 0 (strongest inhibition). No data transformation was necessary since the assumptions of normality and homoscedasticity were met by the standardized variable.

RESULTS

Inhibition of Cell Division. Results from the inhibition of cell division after 80 min are given in Figure 1, while the corresponding ANOVA output is shown in Table 1. Bioactive treatments (significantly different from the controls—$P = 0.05$) are indicated by asterisks in the figure (note that they correspond to the three DCM concentrations and the two highest BuOH concentrations of *C. crambe*). At the conclusion of the experiment, most embryos in the controls (over 80%) had accomplished the first or second cellular division (mainly the former). The low number of unfertilized eggs (<5%) and undivided embryos (<15%) approximated that expected under normal experimentation conditions (Reish and Oshida, 1987). All factors showed a significant effect in the three-way ANOVA (Table 1). The effect of species was due to the inhibition of the sponge *C. crambe* (Tukey multiple comparison $P < 0.001$ with the other species). The effect of fraction did not change significantly with concentration (nonsignificant interaction, Table 1), although this might be due to our sample size since the BuOH fraction was inactive at a concentration of 10 μg/ml but active at higher levels (Figure 2). The effect of fraction was due to the aqueous fraction (Tukey, $P < 0.001$ with the other fractions), which was the only fraction inactive in *C. crambe* (Figure 2). The effect of concentration was due to the significant differences found between 10 and 100 μg/ml (Tukey, $P = 0.002$).

When the eggs of the sea urchin were allowed to reach the pluteus stage, and the data for both experiments combined, new activities were detected (Figure 2, ANOVA output in Table 2). The aqueous fraction of *C. crambe* and *Hemimycale columella* as well as the DCM fraction of *Polysyncraton lacazei*, inactive in the first experiment, became active in the second (bioactive treatments indicated by asterisks in the figure). Accordingly, a significant fraction × time term was detected in the three-way ANOVA (Table 2). In the presence of a significant interaction term, no conclusion can be drawn for the main factors, and data should be reanalyzed for one variable within fixed values of the other variables (Underwood, 1981). We used two-way ANOVAs to look for effects

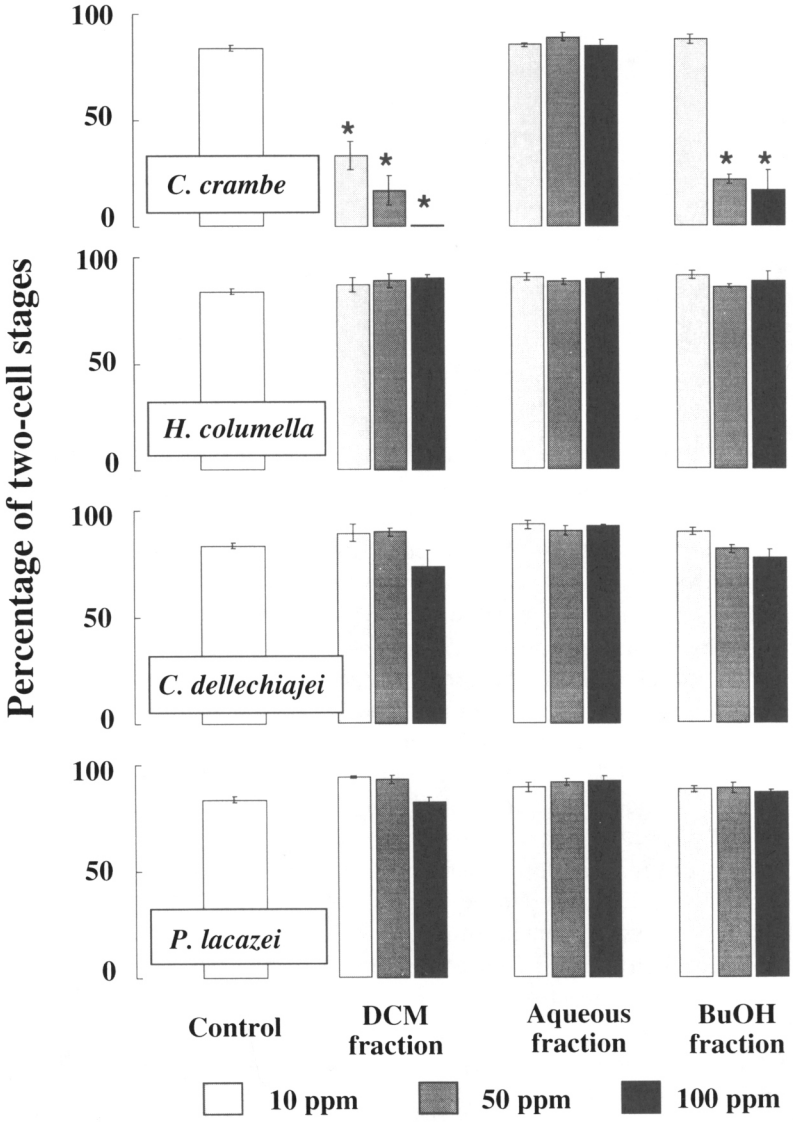


FIG. 1. Percentage of embryos at the two-cell stage 80 min after fertilization when incubated with extracts at different concentrations. Asterisks mark the treatments that proved significantly different from the controls in one-way ANOVA.

TABLE 1. RESULTS OF THREE-WAY ANOVA OF EFFECT OF SPECIES, FRACTION, AND CONCENTRATION ON PROPORTION OF SEA URCHIN EMBRYOS AT TWO-CELL STAGE (ARCSINE TRANSFORMED) AFTER 80 MINUTES OF INCUBATION

Source	SS	DF	MS	F	P
Species	4.791	3	1.597	39.013	<0.001
Fraction	1.181	2	0.590	14.425	<0.001
Concentration	0.472	2	0.236	5.773	0.004
Fraction × concentration	0.387	4	0.096	2.366	0.058
Error	3.930	96	0.040		

of species and fraction within the two experiments. In the first experiment, both species and fraction had a significant effect (Table 3). The species effect was due to the sponge *C. crambe* (Tukey, $P < 001$ with the rest of species), while the fraction effect was due to the aqueous fraction (Tukey, $P = 0.028$ with the other fractions), which remained inactive in this experiment (Figure 3). In the second experiment, the species factor had the same results (Table 3, *C. crambe* responsible for significant effect; Tukey, $P < 0.01$ with the remaining species). Fraction had no effect in the longer experiment (Table 3). Time comparisons were made between each combination of species and fraction by t tests. Significant differences between the activity reported for the two times were found in the aqueous and BuOH fractions of *C. crambe* ($P < 0.001$ and $P = 0.014$, respectively), the aqueous fraction of *H. columella* ($P = 0.002$), and the DCM fraction of *P. lacazei* ($P < 0.001$).

Photosynthesis Inhibition. Results of the oxygen production of *Ulva rigida* are summarized in Figure 3. Neither species nor fraction had a significant effect (Table 4) on the oxygen content (milligrams O_2 per liter per gram) at the concentration tested in this experiment (50 μg of extract per milliliter) and all treatments remained inactive when compared with the controls (one-way ANOVA, $P = 0.716$).

Settlement Inhibition. All factors investigated affected the larval settlement of the bryozoan *Bugula neritina* (results summarized in Figure 4 and ANOVA output in Table 5). The effect of species was due to the strong inhibitory effects of the sponge *C. crambe* (Tukey, $P < 0.001$ with the rest of species) and the ascidian *Polysyncraton lacazei* (Tukey, $P < 0.001$ with the rest of species). The aqueous fraction showed the weakest inhibitory effect and accounted for the significant differences detected in the fraction factor (Tukey, $P < 0.001$ with the other two fractions). Time also showed a significant effect. Multiple comparisons showed no significant differences between times 1 and 2 (Tukey,

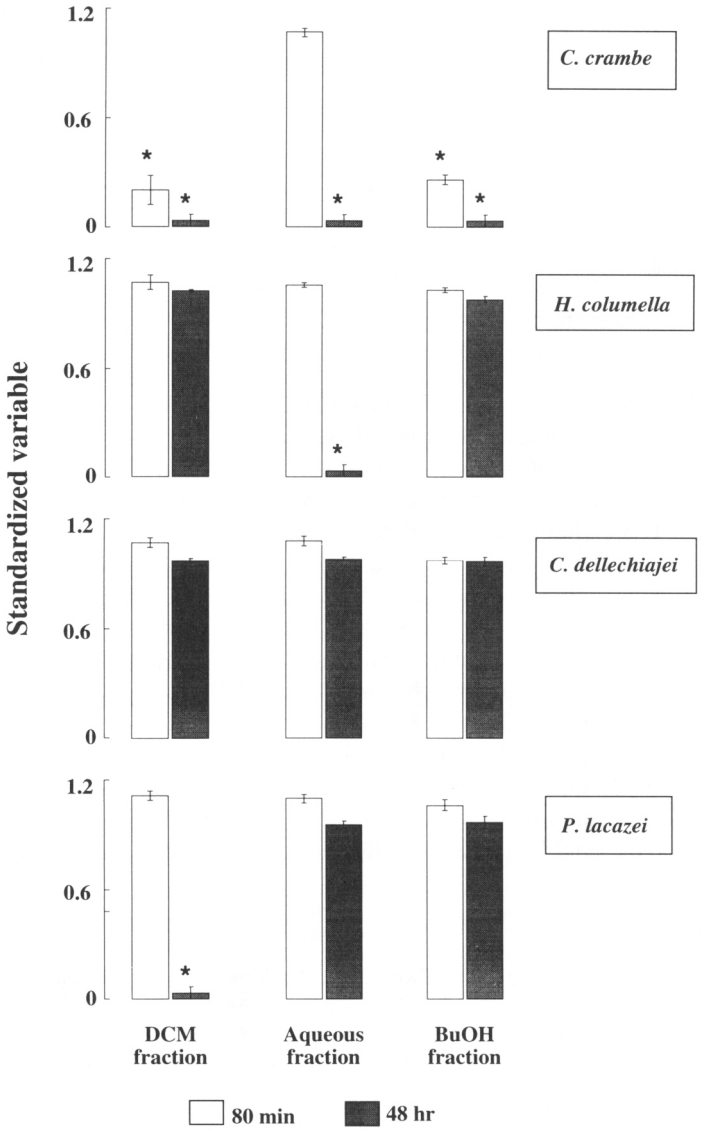


FIG. 2. Values of the standardized variable (see Methods and Materials) measuring the effect of the treatments with respect to the controls in the *Paracentrotus lividus* experiment 80 min and 48 hr after fertilization. The concentration of extracts was 50 μm/ml. Asterisks mark the treatments that proved significantly different from the controls in one-way ANOVA.

TABLE 2. RESULTS OF THREE-WAY ANOVA ON STANDARDIZED VARIABLE MEASURING EFFECT OF SPECIES, FRACTION, AND TIME ON DEVELOPMENT OF SEA URCHIN EMBRYOS

Source	SS	DF	MS	*F*	*P*
Species	5.871	3	1.957	25.712	<0.001
Fraction	0.149	2	0.074	0.983	0.379
Time	2.067	1	2.067	27.154	<0.001
Fraction × time	0.693	2	0.346	4.558	0.014
Error	4.795	63	0.076		

$P = 0.998$) and between times 3 and 4 (Tukey, $P = 0.877$). The effect was due to significant differences between these two groups ($P < 0.001$).

Bioactive treatments are indicated by asterisks in Figure 4. It is worth noting that some of the fractions that did not prevent settlement at the beginning of the experiment (i.e., after 1 hr and 30 min) became active after a longer period. This trend could be observed in the aqueous and BuOH fractions of *H. columella*. It was also observed that some fractions produced detachment of settled larvae, a trend that was particularly clear in the DCM fraction of *P. lacazei*. After 5 hr and 30 min no significant differences were detected in this fraction when compared to the controls, but at 9 hr and 30 min larvae had become detached and were lying dead on the bottom of the Petri dish, giving a highly significant effect compared to the controls (Figure 4). This trend could also be observed in the BuOH fractions of *P. lacazei*, the three fractions of *C. crambe*, and the DCM fraction of *Cystodytes dellechiajei*. In the remaining treatments, nonsettled larvae could be observed alive and moving around during the whole duration of the experiment.

TABLE 3. RESULTS OF SEPARATE TWO-WAY ANOVAS OF EFFECT OF SPECIES AND FRACTION ON DEVELOPMENT OF SEA URCHIN EMBRYOS (STANDARDIZED VARIABLE) 80 MINUTES AND 48 HOURS AFTER FERTILIZATION

Time	Source	SS	DF	MS	*F*	*P*
1 hr, 20 min	Species	2.096	3	0.698	19.040	<0.001
	Fraction	0.426	2	0.213	5.815	0.007
	Error	1.100	30	0.036		
48 hr	Species	4.237	3	1.412	13.106	<0.001
	Fraction	0.416	2	0.208	1.933	0.162
	Error	3.233	30	0.107		

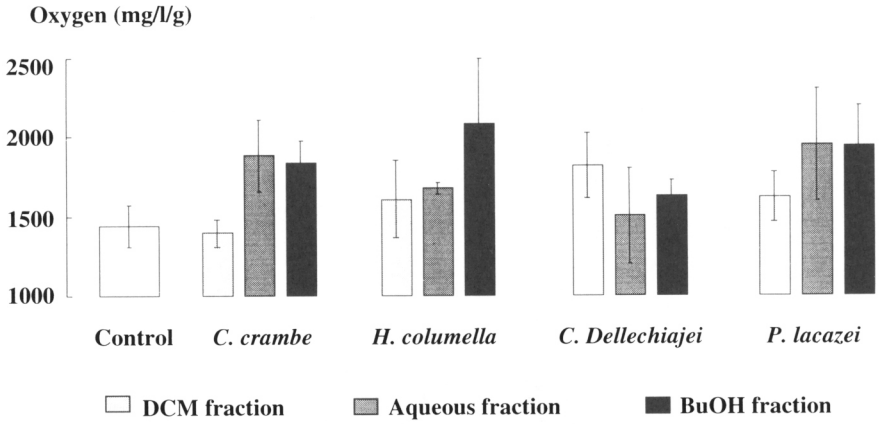


FIG. 3. Oxygen production of *Ulva rigida* when incubated for 90 min with extracts (50 μm/ml) of the different species.

TABLE 4. TWO-WAY ANOVA TABLE OF EFFECT OF SPECIES AND FRACTION ON OXYGEN PRODUCTION BY ALGA *Ulva rigida*

Source	SS	DF	MS	*F*	*P*
Species	0.194×10^6	3	0.648×10^5	0.341	0.795
Fraction	0.420×10^6	2	0.210×10^6	1.107	0.343
Error	0.570×10^7	30	0.190×10^6		

TABLE 5. RESULTS OF THREE-WAY ANOVA ON STANDARDIZED VARIABLE MEASURING EFFECT OF SPECIES, FRACTION, AND TIME ON SETTLEMENT OF BRYOZOAN LARVAE

Source	SS	DF	MS	*F*	*P*
Species	7.922	3	2.640	45.912	<0.001
Fraction	1.204	2	0.602	10.467	<0.001
Time	2.311	3	0.770	13.392	<0.001
Fraction × time	0.240	6	0.040	0.697	0.652
Error	7.420	129	0.057		

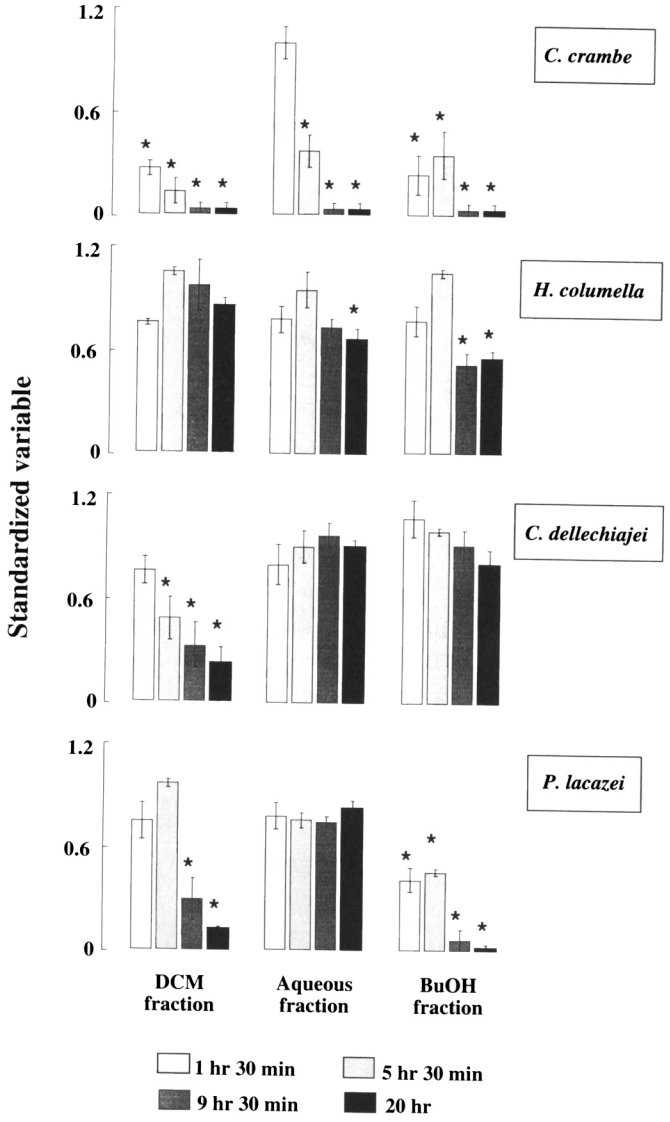


FIG. 4. Values of the standardized variable (see Methods and Materials) measuring settlement inhibition with respect to the controls after different times of incubation with extracts (50 μm/ml) of the species under study. Asterisks mark the treatments that proved significantly different from the controls in one-way ANOVA.

DISCUSSION

The results obtained showed that: (1) different fractions of the same species displayed similar ecologically relevant activities in certain cases, (2) different fractions displayed different activities in other instances, and (3) the same fraction could also display diverse activities. "Fraction" is not the same as "compound," of course, but compounds belonging to diverse fractions can be assumed to be different (given the range of polarities covered), and the fact that one single fraction was able to display diverse activities strongly suggests that the same compound, or a group of related compounds, can have multiple ecological roles. To the extent that the active chemicals in our fractions corresponded to single (or groups of related) compounds, which are unrelated to those of the other fractions, our results indicate that the relationship between secondary metabolites and ecological functions is indeed complex. This is consistent with the idea that secondary metabolites have evolved as a response to diverse selective pressures from a variety of competitors.

The sponge *Crambe crambe* was overall the most active species. It showed a strong inhibition of cell division in its DCM and BuOH fractions. These fractions even caused cell destruction in the eggs of the sea urchin *Paracentrotus lividus*. Egg destruction could be due to cytotoxic rather than antimitotic activity of the fractions, as cytotoxic activity has previously been described for this sponge (Berlink et al., 1992b; Jares-Erijman et al., 1991; Uriz et al., 1992a). The development of pluteus larvae was inhibited by the three fractions, which prevented the formation of the first skeletal rods. Settlement in *Bugula neritina* was also inhibited by all fractions from *C. crambe*; the DCM and BuOH fractions did so at the first observation time, while the aqueous fraction had a significant effect after 5 hr and 30 min of incubation.

Apparently *C. crambe* has low-, middle-, and high-polarity compounds that are able both to inhibit cell division and to prevent settlement. Over 15 compounds have already been described for this sponge (Berlinck et al., 1990, 1992a,b; Jares-Erijman et al., 1991). Response to chemical defenses has been shown to be usually species-dependent (Schupp and Paul, 1994). A high number of compounds sharing the same biological activity could be interpreted as an attempt to increase the number of species targeted by chemical defenses. They could have additive or even synergistic effect (Van Alstyne et al., 1994), making them more effective in their role(s). Compounds from *C. crambe* have been reported to reduce predation, fouling, and competitors' growth effectively (Becerro et al., 1994; Turon et al., 1996a; Uriz et al., 1996a). *C. crambe* is one of the most widespread benthic invertebrates in Mediterranean littoral waters (Uriz et al., 1992b), where it successfully inhabits a great variety of contrasting habitats. Thus, *C. crambe* encounters more diverse competitors than any other species dwelling in a narrower range of habitats. Particular responses (coevo-

lution) to one of the competitors would be constrained by this fact (Schmitt et al., 1995). The diverse biological activities that this species displays may be responsible for such a widespread distribution in nature. In this sense, the evolution of "chemical warfare" in *C. crambe* may have favored the acquisition of generalist, multiple-purpose compounds to effectively protect the sponge against a wide array of competitors.

Information on the other species investigated in this work is more limited. Antifouling properties in the ascidian *Polysyncraton lacazei* have been reported (Wahl and Lafargue, 1990; Wahl and Banaigs, 1991). Our findings are consistent with the idea of an ascidian that is chemically defended against fouling, since the BuOH fraction prevents settlement and successful development of the larvae of *B. neritina*. Even if larvae are able to contact the *P. lacazei* surface and settle, they still have to avoid being detached, a trend that was particularly clear in the DCM fraction of this ascidian. Sloughing off the external layer of the tunic has been reported in this species (Wahl and Lafargue, 1990; Turon, 1992). This process and the settlement inhibition activities found here may act together and contribute to the maintenance of a epibiont-free surface. Whether the inhibition of cell division found in the DCM fraction in the *P. lacazei* experiment strengthens the antifouling effect or serves other functions remains unknown.

Results with the sponge *Hemimycale columella* are similar to those from the ascidian *P. lacazei*, although the active compounds of the sponge are more polar than those in the ascidian. High polar compounds (aqueous fraction) of *H. columella* inhibited cell division in *P. lividus*, while the formation of ancestrula in *B. neritina* was retarded by less polar compounds (BuOH compounds, although the aqueous fraction also had an effect at the end of the experiment). As in *P. lacazei*, the activities found in the sponge *H. columella* may also play a role as an antifouling mechanism of this (macroscopically) epibiont-free species.

Active compounds from the ascidian *Cystodytes dellechiajei* are restricted to the lowest polar fraction (DCM fraction). Several toxic alkaloids with antineoplastic activity have been described from this species (Kobayashi et al., 1988). These compounds may be responsible for the antimitotic and cytotoxic activities featured by Mediterranean specimens (authors' unpublished research). Our data exclusively show an inhibitory effect on the settlement of *B. neritina* that may suggest a chemically mediated antifouling mechanism in this ascidian, which also features macroscopically clean surfaces.

None of the four species investigated inhibited photosynthesis of the alga *Ulva rigida*. Whether this is due to a true lack of activity against photosynthesis or is due to an inappropriate concentration or another methodological problem in the experiments remains unclear. An equivalent ecological function could be achieved by increasing respiration rates (Porter and Targett, 1988), but unfortunately no data are available to support this possibility.

Overall, the aqueous fractions tended to have lower activity than the less polar compounds. This may indicate energetic constraints related to the cost of production of substances readily diffusible in water. There would be a continuous waste of these compounds unless they are retained or encapsulated in some way, while apolar compounds, even if released, would remain on the animals' surfaces for longer times.

The assays run in this work were chosen in an attempt to select biological tests with ecological relevance. The activities were tested against marine organisms that are abundant in the communities where the selected species live, which allows for an ecological interpretation. However, it is difficult to determine the concentrations at which the compounds in our fractions may operate in nature. The metabolites were extracted and placed in the water to check their activity. Whether these compounds are naturally released to the water and contact other organisms is at present unknown (but see Uriz et al., 1996b). Furthermore, while intraspecific and intraspecimen variations in the production of secondary metabolites have been described for a variety of benthic organisms, they were not considered here (Paul and Van Alstyne, 1988; Harvell and Fenical, 1989; Wylie and Paul, 1989; Maida et al., 1993; Turon et al., 1996b). In spite of these cautionary remarks, our experiments showed variability in the activities of the fractions within the range of concentrations tested, which may indicate that we were not far from the ecologically relevant concentrations.

The results obtained in this study suggested that *C. crambe* is a highly active species that uses its activities against fouling and in competition for space, while *P. lacazei*. *H. columella*, and *C. dellechiajei* may preferentially use toxicity as an antifouling mechanism. For *C. crambe* and *P. lacazei*, studies carried out previously provide firm support for the proposed ecological roles and also for an antipredation role in *C. crambe* (Wahl and Lafargue, 1990; Wahl and Banaigs, 1991; Becerro, 1994; Becerro et al., 1994; Uriz et al., 1996a; Turon et al., 1996a). In the case of *H. columella* and *C. dellechiajei*, the extrapolation of our laboratory results to ecological functions is at present an assumption that should be tested with more specific work.

Methodological procedures may play a major role in evaluating both compound levels and ecological functions. Cronin et al. (1995) showed how storage and extraction procedures determine the levels of secondary metabolites found in two *Dictyota* species. Selection of an ecologically relevant organism does not guarantee a correct interpretation of the ecological role. Responses to chemical defenses vary depending on test organisms. Schupp and Paul (1994) reported that the response to *Halimeda macroloba*'s secondary metabolites differed among fish species. Pawlik et al. (1986) concluded that diterpenes defended the limpet *Collisella limatula* against natural predators, while Manker and Faulkner (1996) found that the diterpenes did not prevent predation by the starfish *Astrometis* sp., a well-known limpet predator. The test organism used should vary for each life-history stage of the species whose activity is being studied (Uriz et al.,

1996a). Our results confirm that final activity (either in ecologically relevant experiments or in more general screenings) is significantly affected by assay organism, fractionation procedure, concentration, and duration of experiments. Therefore, all these factors should be carefully selected to test ecological roles. Selection should be based on: observational work in the field that leads to ecologically relevant hypotheses and, accordingly, set up extraction procedures, ecologically relevant concentrations and appropriate time lengths, and a range of laboratory or field experiments with a variety of ecologically relevant organisms on which to test these hypotheses.

Acknowledgments—The authors thank Dr. D. M. Tapiolas (James Cook University of North Queensland, Australia) for her chemical expertise and advice on the design of the extraction procedure followed in this study. Comments by Dr. V. Paul (University of Guam) greatly improved the manuscript. The study was funded by the projects DGICYT PB-94-0015 and CICYT MAR95-1764 of the Spanish Government and a Basque Government predoctoral fellowship to M.A.B.

REFERENCES

BECERRO, M. A. 1994. Chemically mediated bioactivity of the encrusting sponge *Crambe crambe* and its ecological consequences. PhD thesis. University of Barcelona.

BECERRO, M. A., LÓPEZ, N. I., TURON, X., and URIZ, M. J. 1994. Antimicrobial activity and surface bacterial film in marine sponges. *J. Exp. Mar. Biol. Ecol.* 179:195–205.

BECERRO, M. A., TURON, X., and URIZ, M. J. 1995. Natural variation of toxicity in the encrusting sponge *Crambe crambe* (Schmidt) in relation to size and environment. *J. Chem. Ecol.* 21:1931–1946.

BERLINCK, R. G. S., BRAEKMAN, J. C., DALOZE, D., HALLENGA, K., and OTTINGER, R. 1990. Two new guanidine alkaloids from the Mediterranean sponge *Crambe crambe*. *Tetrahedron Lett.* 31:6531–6534.

BERLINCK, R. G. S., BIFULCO, G., BRAECKMAN, J. C., BRUNO, I., DALOZE, D., PEM, S., RICCIO, R., SPAMPINATO, S., and SPERONI, E. 1992a. Strong Ca^{++} channel blocker activity of crambescidin 816, a cytotoxic guanidine alkaloid from the marine sponge *Crambe crambe*. *In* 7th International Symposium on Marine Natural Products, Capri, Italy.

BERLINCK, R. G. S., BRAEKMAN, J. C., DALOZE, D., BRUNO, I., RICCIO, R., ROGEAU, D., and AMADE, P. 1992b. Crambines C1 and C2: Two further ichthyotoxic guanidine alkaloids from the sponge *Crambe crambe*. *J. Nat. Prod.* 55:528–532.

BHAKUNI, D. S., and JAIN, S. 1990. Bioactive molecules of the marine invertebrates. Part I: Sponges, jelly fish, sea anemones, corals, and bryozoans. *J. Sci. Ind. Res.* 49:330–349.

CARIELLO, L., GIUDICI, M., and ZANETTI, L. 1980. Developmental aberrations in the sea-urchin eggs induced by avarol and two congeners, the main sesquiterpenoid hydroquinones from the marine sponge *Dysidea avara*. *Comp. Biochem. Physiol.* 65:37–41.

CLARE, A. S. 1996. Marine neutral products antifoulants: status and potential. *Biofouling* 9:211–229.

COLL, J. C. 1992. The chemistry and chemical ecology of octocorals (Coelenterata, Anthozoa, Octocorallia). *Chem. Rev.* 92:613–631.

COLL, J. C., PRICE, I. R., KONING, G. M., and BOWDEN, B. F. 1987. Alga overgrowth of alcyonacean soft corals. *Mar. Biol.* 96:129–135.

CRONIN, G., LINDQUIST, N., HAY, M. E., and FENICAL, W. 1995. Effects of storage and extraction procedures on yields of lipophilic metabolites from the brown seaweeds *Dyctiota ciliolata* and *D. menstrualis*. *Mar. Ecol. Prog. Ser.* 119:265–273.

DAVIS, A. R., TARGETT, N. M., MCCONNELL, O. J., and YOUNG, C. M. 1989. Epibiosis of marine algae and benthic invertebrates: natural products chemistry and other mechanisms inhibiting settlement and overgrowth, pp. 85–114, *in* P. J. Scheuer (ed.). Bioorganic Marine Chemistry, Vol. 3. Springer-Verlag, New York.

DINNEL, P. A., LINK, J. M., and STOBER, Q. J. 1987. Improved methodology for a sea urchin sperm biossay for marine waters. *Arch. Environ. Contam. Toxicol.* 16:23–32.

FAULKNER, D. J. 1994. Marine natural products. *Nat. Prod. Rep.* 11:355–394.

HARVELL, C. D., and FENICAL, W. 1989. Chemical and structural defenses of Caribbean gorgonians (*Pseudopterogorgia* spp.): Intracolony localization of defense. *Limnol. Oceanogr.* 34:382–389.

HAY, M. E., and STEINBERG, P. D. 1992. The chemical ecology of plant-herbivore interactions in marine versus terrestrial communities, pp. 371–413, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores: The Interactions with Plant Metabolites, Vol. II. Academic Press, New York.

HAY, M. E., KAPPEL, Q. E., and FENICAL, W. 1994. Synergisms in plant defense against herbivores: Interactions of chemistry, calcification, and plant-quality. *Ecology* 75:1714–1726.

JACKSON, J. B. C. 1979. Morphological strategies of sessile animals, pp. 499–555, *in* G. Larwood and B. R. Rosen (eds.). Biology and Systematics of Colonial Organisms. Academic Press, London.

JARES-ERIJMAN, E. A., SAKAI, R., and RINEHART, K. L. 1991. Crambescidins: New antiviral and cytotoxic compounds from the sponge *Crambe crambe*. *J. Org. Chem.* 56:5712–5715.

KEOUGH, M. J. 1984. Kin-recognition and the spatial distribution of larvae of the bryozoan *Bugula neritina* (L.). *Evolution* 38:142–147.

KOBAYASHI, J., CHENG, J., WALCHLI, M. R., NAKAYAMA, H., HIRATA, Y., SASAKI, T., and OHIZUMI, Y. 1988. Cystodytins A, B and C, novel tetracyclic aromatic alkaloids with potent antineoplastic activity from the Okinawan tunicate *Cystodytes dellechiajei*. *J. Org. Chem.* 53:1800–1804.

MAIDA, M., CARROL, A. R., and COLL, J. C. 1993. Variability of terpene content in the soft coral *Sinularia flexibilis* (Coelenterata, Octocorallia) and its ecological implications. *J. Chem. Ecol.* 19:2285–2296.

MANKER, D. C., and FAULKNER, D. J. 1996. Investigation of the role of diterpenes produced by marine pulmonates *Trimusculus reticulatus* and *T. conica*. *J. Chem. Ecol.* 22:23–35.

MARTIN, D., and URIZ, M. J. 1993. Chemical bioactivity of Mediterranean benthic organisms against embryos and larvae of marine invertebrates. *J. Exp. Mar. Biol. Ecol.* 173:11–27.

MINALE, L., RICCIO, R., and SODANO, G. 1974. Avarol, a novel sesquiterpenoid hydroquinone with a rearranged drimane skeleton from the sponge *Dysidea avara*. *Tetrahedron Lett.* 38:3401–3404.

PAUL, V. J. 1992. Ecological Roles of Marine Natural Products. Comstock, Ithaca, New York.

PAUL, V. J., and VAN ALSTYNE, K. L. 1988. Chemical defense and chemical variation in some tropical Pacific species of *Halimeda* (Halimedaceae, Chlorophyta). *Coral Reefs* 6:263–269.

PAUL, V. J, LINDQUIST, N., and FENICAL, W. 1990. Chemical defenses of the tropical ascidian *Atapozoa* sp. and its nudibranch predators *Nembrotha* spp. *Mar. Ecol. Prog. Ser.* 59:109–118.

PAWLIK, J. R., ALBIZATI, K. F., and FAULKNER, D. J. 1986. Evidence of a defensive role for limatulone, a novel triterpene from the limpet *Collisella limatula*. *Mar. Ecol. Prog. Ser.* 30:252–260.

PAWLIK, J. R., BURCH, M. T., and FENICAL, W. 1987. Patterns of chemical defense among Caribbean gorgonian corals: a preliminary survey. *J. Exp. Mar. Biol. Ecol.* 108:55–66.

PAWLIK, J. R., CHANAS, B., TOONEN, R. J., and FENICAL, W. 1995. Defenses of Caribbean sponges against predatory reef fish. I. Chemical deterrency. *Mar. Ecol. Prog. Ser.* 127:183–194.

PORTER, J. M., and TARGETT, W. M. 1988. Allelochemical interactions between sponges and corals. *Biol. Bull.* 175:230–239.

REISH, D. L., and OSHIDA, P. S. 1987. Manual of methods in aquatic environment research. Part 10. Short-term statistics bioassays. *FAO Fish. Tech. Pap.* 247:269.

ROSENTHAL, G. A., and BERENBAUM, M. R. 1992. Herbivores: Their Interaction with Secondary Plant Metabolites, 2nd ed., Vol. II. Evolutionary and Ecological Processes. Academic Press, San Diego, California.

RYLAND, J. S., and WARNER, G. F. 1986. Growth and form in modular animals: Ideas on the size and arrangement of zooids. *Phil. Trans. R. Soc. London Ser.* B313:53-76.

SAMMARCO, P. W., and COLL, J. C. 1988. The chemical ecology of alcyonacean corals (Coelenterata: Octocorallia), pp. 87-116, *in* P. J. Scheuer (ed.). Bioorganic Marine Chemistry, Vol. 2. Springer-Verlag, New York.

SCHMITT, T. M., HAY, M. E., and LINQUIST, N. 1995. Constraints on chemically mediated coevolution: Multiple functions for seaweed secondary metabolites. *Ecology* 76:107-203.

SCHUPP, P. J., and PAUL, V. J. 1994. Calcium carbonate and secondary metabolites in tropical seaweeds: variable effects on herbivorous fishes. *Ecology* 75:1172-1185.

SEBENS, K. P. 1987. The ecology of indeterminate growth in animals. *Annu. Rev. Ecol. Syst.* 18:371-407.

SEIBERT, G., RAETHER, W., DOGOVIC, N., GASIV, M. J., ZAHN, R. K., and MÜLLER, W. E. G. 1985. Antibacterial and antifungal activity of avarone and avarol. *Zentralbl. Bekt. Hyg.* 260:379-386.

STEINBERG, P. D., and VAN ALTENA, I. 1992. Tolerance of marine invertebrate herbivores to brown algal phlorotannins in temperate Australasia. *Ecol. Monogr.* 62:189-222.

TURON, X. 1988. Distribución ecológica de las ascidias de las costas de Catalunya e islas Baleares (Mediterráneo occidental). *Misc. Zool.* 12:219-236.

TURON, X. 1990. Distribution and abundance of ascidians from a locality on the northeast coast of Spain. *PSZNI Mar. Ecol.* 11:291-308.

TURON, X. 1992. Periods of non-feeding in *Polysyncraton lacazei* (Ascidiacea: Didemnidae): A rejuvenative process? *Mar. Biol.* 112:647-655.

TURON, X., BECERRO, M. A., LLOPIS, J., and URIZ, M. J. 1996a. Small-scale association measures in benthic communities as a clue for allelochemical interactions. *Oecologia (Berlin)* 108:351-360.

TURON, X., BECERRO, M. A., and URIZ, M. J. 1996b. Seasonal patterns of toxicity in benthic invertebrates: The encrusting sponge *Crambe crambe* (Poecilosclerida). *Oikos* 75:33-40.

UNDERWOOD, A. J. 1981. Techniques of analysis of variance in experimental marine biology and ecology. *Ocean Mar. Biol. Annu. Rev.* 19:513-605.

URIZ, M. J. 1978. Contribución a la fauna de esponjas (Demospongia) de Cataluña e islas Baleares (Mediterráneo occidental). *Misc. Zool.* 11:291-308.

URIZ, M. J., MARTIN, D., and ROSELL, D. 1992a. Relationship of biological and taxonomic characteristics to chemically mediated bioactivity in Mediterranean littoral sponges. *Mar. Biol.* 113:287-297.

URIZ, M. J., MARTIN, D., and ROSELL, D. 1992b. The sponge population of the Cabrera archipielago (Balearic Islands): Characteristics, distribution and abundance of the most representative species. *PSZNI Mar. Ecol.* 13:101-117.

URIZ, M. J., TURON, X., BECERRO, M. A., and GALERA, J. 1996a. Feeding deterrence in sponges. The role of toxicity, physical defenses, energetic contents, and life-history stage. *J. Exp. Mar. Biol. Ecol.* 205:187-203.

URIZ, M. J., BECERRO, M. A., TUR, J. M., and TURON, X. 1996b. Location of toxicity within the Mediterranean sponge *Crambe crambe* (Demospongiae: Poecilosclerida). *Mar. Biol.* 124:583-590.

VAN ALSTYNE, K. L., WYLIE, C. R., and PAUL, V. J. 1994. Antipredator defenses in tropical soft corals (Coelenterata: Alcyoncea). II. The relative importance of chemical and structural defenses in three species of *Sinularia*. *J. Exp. Mar. Biol. Ecol.* 178:17-34.

WAHL, M., and BANAIGS, B. 1991. Marine epibiosis. III. Possible antifouling defense adaptations in *Polysyncraton lacazei* (Giard) (Didemnidae, Ascidiacea). *J. Exp. Mar. Biol. Ecol.* 145:49–63.

WAHL, M., and LAFARGUE, F. 1990. Marine epibiosis. II. Reduced fouling on *Polysyncraton lacazei* (Didemnidae, Tunicata) and proposal of an antifouling potencial index. *Oecologia* 82:275–282.

WYLIE, C. R., and PAUL, V. J. 1989. Chemical defenses in three species of Sinularia (Coelenterata, Alcyonacea): Effects against generalist predators and the butterflyfish *Chaetodon unimaculatus* Bloch. *J. Exp. Mar. Biol. Ecol.* 129:141–160.

YATES, J. L., and PECKOL, P. 1993. Effects of ambient availability and herbivory on polyphenolics in the seaweed *Fucus vesiculosus*. *Ecology* 74:1757–1766.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

SEQUENCE DATA OF MITOCHONDRIAL 16S rDNA OF ARCTIIDAE AND NYMPHALIDAE: EVIDENCE FOR A CONVERGENT EVOLUTION OF PYRROLIZIDINE ALKALOID AND CARDIAC GLYCOSIDE SEQUESTRATION

MICHAEL WINK* and EVA VON NICKISCH-ROSENEGK

Universität Heidelberg
Institut für Pharmazeutische Biologie
Im Neuenheimer Feld 364
D-69120 Heidelberg, Germany

(Received April 22, 1996; accepted January 24, 1997)

Abstract—The mitochondrial 16S rRNA gene of several arctiids (nine species of the Arctiinae, three Callimorphinae, one Nyctemerinae, and five Lithosiinae), three taxa of the Ctenuchidae, and three of the Danainae (family Nymphalidae) was amplified by PCR and sequenced directly (537 bp). DNA sequences were used to reconstruct phylogenetic relationships between and within Arctiidae, Ctenuchidae, and Nymphalidae. The Ctenuchidae share a close ancestry with Arctiidae and, like the Lithosiinae, should be treated as subfamilies of the Arctiidae. Most species studied are able to sequester dietary pyrrolizidine alkaloids (PAs) and/or cardiac glycosides (CGs) for chemical defenses or (PAs only) as pheromones. The molecular data imply that CG sequestration evolved independently in a few genera of the Nymphalidae (*Danaus*) and Arctiidae (*Syntomeida*): CG sequestration can be regarded as a convergent trait and is not based on common descent. PA sequestration also appears to have evolved independently and convergently in the Nymphalidae and Arctiidae. Within the Arctiidae, PA sequestration occurs in the subfamilies Arctiinae, Callimorphinae, and Nyctemerinae, but not in the Ctenuchinae or Lithosiinae. Whether PA storage evolved independently in each of the subfamilies Arctiinae, Callimorphinae, and Nyctemerinae or in a common ancestor of all Arctiidae could not be resolved with certainty. If the latter case is true, than the Lithosiinae and Ctenuchinae must have lost the capacity of PA sequestration but acquired the sequestration of lichen compounds (Lithosiinae) or cardiac glycosides (*Syntomeidae*) instead.

*To whom correspondence should be addressed.

0098-0331/97/0600-1549$12.50/0 © 1997 Plenum Publishing Corporation

Key Words—Arctiidae, Nymphalidae, pyrrolizidine alkaloids, cardiac glycosides, 16S rDNA, molecular phylogeny, sequestration, convergence.

INTRODUCTION

A number of insects, especially moths and butterflies, sequester secondary metabolites from their host plants and use them for their own protection against predators (reviews: Bernays and Chapman, 1994; Brown and Trigo, 1995; Harborne, 1993; Rosenthal and Berenbaum, 1991, 1992; Wink, 1993). Due to the pioneering work of M. Rothschild, T. Eisner, T. Brown, and D. Schneider and coworkers, the best studied groups of secondary metabolites that are widely used as acquired defense compounds in insects are pyrrolizidine alkaloids and cardiac glycosides (for review see: Boppré, 1990, 1995; Brown and Trigo, 1995; Harborne, 1993; Hartmann, 1992; Hartmann and Witte, 1995; Rosenthal and Berenbaum, 1991; Rothschild, 1972b; Rothschild et al., 1972; Schneider, 1986, 1991, 1992, 1993).

Pyrrolizidine alkaloids (PA), of which more than 370 structures are known, are produced mainly in the Asteraceae (tribes Eupatorieae, Senecioneae), Boraginaceae, Leguminosae (mainly genus *Crotalaria*) and Orchidaceae. Other families include the Apocynaceae, Celastraceae, Convolvulaceae, Poaceae, Ranunculaceae, Rhizophoraceae, Santalaceae, and Sapotaceae (Hartmann and Witte, 1995; Roeder, 1995). PAs bind to muscarinic and serotoninergic receptors (Schmeller et al., 1997), actions that might be involved in acute PA toxicity. In the liver of vertebrates, PAs are converted to toxic pyrrolic derivatives that are alkylating compounds responsible for the long-term toxicity of PAs. Binding of the alkylating metabolites to proteins and DNA will cause mutations or may even lead to cancer (Cheeke, 1984; Mattocks, 1972; McLean, 1970; Roeder, 1995).

Plant-derived pyrrolizidine alkaloids have been detected in a number of specialized insects, which often advertise their unpalatability by aposematic coloration and/or production and release of pyrazines (Boppré, 1995; Brown and Trigo, 1995; Hartmann and Witte, 1995; Rothschild, 1972a,b; Rothschild et al., 1970a, 1979, 1984), such as aphids [e.g., *Aphis jacobaeae*, *A. cacaliae* (Hartmann and Witte, 1995; Wink and Witte, 1991)], beetles [e.g., *Oreina cacaliae*, *O. speciosissima* (Rowell-Rahier and Pasteels, 1990; Rowell-Rahier et al., 1991)], grasshoppers [e.g., *Zonocerus* (Bernays et al., 1977; Boppré et al., 1992)], and many moths and butterflies, especially within the families Arctiidae, Nymphalidae (subfamilies Danainae and Ithomiinae) (Boppré, 1986, 1990, 1994; Boppré and Schneider, 1982; Brown and Trigo, 1995; Hartmann and Witte, 1995; Rothschild et al., 1979). Utilization of PAs is most complex in Lepidoptera and larvae of some species sequester the dietary PAs and store them throughout all life stages, often in the integument (Boppré, 1990; Egelhaaf

et al., 1990; Ehmke et al., 1990; Nickisch-Rosenegk et al., 1990b; Nickisch-Rosenegk and Wink, 1993, 1994; Rothschild, 1972b; Rothschild et al., 1979; Wink and Schneider, 1990). In *Utetheisa*, *Creatonotos*, and *Danaus gilippus*, PAs are transferred to the eggs, where they serve to protect the clutch (Dussourd et al., 1988; Nickisch-Rosenegk et al., 1990b). In some of these species the males contribute to chemical defense of the offspring by incorporating PAs into the spermatophore, which the females receive as a nuptial gift (Dussourd et al., 1988, 1989; Nickisch-Rosenegk et al., 1990b). In many instances, insects convert the original PAs into new derivatives, such as callimorphine, creatonine, and others (Edgar et al., 1980; Hartmann and Witte, 1995; Trigo et al., 1993). PAs sequestered by larvae from leaves or by adults from nectar or wilted/dead plant material are often transformed into pheromones such as (7*R*)-hydroxydanaidal, danaidal, danaidone, nordanaidone or ithomiinae lactone (reviews: Boppré and Vane-Wright, 1989; Brown and Trito, 1995; Edgar et al., 1976; Eisner and Meinwald, 1987; Hartmann and Witte, 1995) and are released via specialized androconial structures (Boppré, 1981, 1983, 1990; Boppré and Schneider, 1989; Schneider, 1977, 1986, 1992). Male arctiid moths of the genus *Creatonotos* develop large inflatable coremata only when they have the chance to ingest PAs during their larval stages. These hairy coremata have an enlarged surface area and are used to release the PA-derived pheromones (Boppré, 1990; Nickisch-Rosenegk et al., 1990b; Schneider, 1992; Schneider et al., 1982).

Cardiac glycosides (CGs) are produced in many unrelated plant families, such as the Scrophulariaceae, Apocynaceae, Asclepiadaceae, Ranunculaceae, Brassicaceae, Liliaceae, Celastraceae, and a few others (Harborne, 1993). Even a few animals, such as toads and beetles, can produce their own CGs. CGs inhibit Na^+, K^+-ATPase (Vaughan and Jungreis, 1977) and thus destroy the ion gradients that are necessary for many cellular functions, including neuronal activity, secondary active transport, and muscle contraction. It seems noteworthy that arrow poisons are often based on CGs as the effective agents (Neuwinger, 1994).

Similar to the situation in PAs, a number of specialized insects (often aposematically colored) are able to take up and store plant-derived CGs. Examples are grasshoppers [e.g., *Poekilocerus*, *Phytmaeteus* (Harborne, 1993; Rothschild, 1996, 1972b)], aphids [e.g., *Aphis nerium* (Malcolm, 1990; Rothschild et al., 1970b)], lygaeid bugs [e.g., *Oncopeltus*, *Caenocoris*, *Spilostethus*, *Lygaeus*, *Apterola*, *Arocatus*, *Aspilocoryphus*, *Aulacopeltus*, *Graptostethus*, *Haemobaphus*, *Lygaeospilus*, *Melanerythrus*, *Microspilus*, *Horvathiolus* (Malcolm, 1990; Rothschild, 1972b; Rothschild et al., 1971)] beetles [*Tetraopes*, *Epicauta* (Rothschild, 1972b)], Diptera [*Zenilla* (Rothschild, 1972b)], and again Lepidoptera [e.g, *Danaus*, *Euploea*, *Syntomeida*, *Euchaetias*, *Arctia*, *Empyreuma* (Harborne, 1993; Nickisch-Rosenegk et al., 1990a; Rothschild, 1972b; Rothschild et al., 1970a, 1973, 1975, 1978; Schneider, 1993)]. Insects seques-

tering CGs are usually protected from predators such as birds (Brower et al., 1968, 1975; Brower and Van Zandt-Brower, 1964; Rothschild, 1966, 1972a; Rothschild et al., 1972; Rothschild and Kellett, 1972).

The biochemical mechanisms that enable insects to sequester PAs or CGs are certainly intricate, and so far only partly known (if at all). Specializations should involve among others, (1) an insensitivity towards the defense compounds (which are toxic to nonspecialized animals, (2) a transport system across biomembranes if compounds are polar or charged molecules, and (3) a specialized storage compartment to store them at the right concentration and site. Monarch butterflies, *D. plexippus*, are rather insensitive towards CGs because Na^+, K^+-ATPase cannot be inhibited by CGs (Holzinger et al., 1992; Vaughan and Jungreis, 1977). On a molecular level, we showed recently that an amino acid substitution in the CG binding site in the first extramembrane loop of Na^+, K^+-ATPase is responsible for this insensitivity (Holzinger et al., 1992; Holzinger and Wink, 1996). Since a common derivative of PAs, PA N-oxides, and CGs are polar compounds, they cannot pass biomembranes by simple diffusion. A carrier-mediated uptake system has been proposed for both groups of substances (Detzel and Wink, 1995; Frick and Wink, 1995; Nickisch-Rosenegk et al., 1990a; Wink and Schneider, 1988), and other mechanisms have been discussed also for PAs (Hartmann and Witte, 1995). Most defense chemicals are sequestered in the integument (Egelhaaf et al., 1990; Frick and Wink, 1995; Nickisch-Rosenegk et al., 1990a,b; Nickisch-Rosenegk and Wink, 1993; Wink et al., 1991) or in special epidermal glands (Rowell-Rahier et al., 1991), which appears to be a further specialization and adaptation.

The close interactions between insects and plant-derived allelochemicals suggests that a number of substantial biochemical modifications must have occurred in those insects that sequester toxic compounds, including specially adapted and modified proteins (e.g., receptors and carriers). The evolutionary question is whether specialized insects have acquired these adaptations only once or several times independently during evolution. If these traits evolved only once, we should expect to find, by independent means, that species that share these traits are also phylogenetically related.

Molecular markers provide a chance to test this assumption (Avise, 1994; Hillis et al., 1996). DNA sequences are especially useful to reconstruct the underlying phylogeny of any group of organisms since convergent traits are less common as compared to morphological characters. In insects, sequences of mitochondrial genes (among them genes for rDNA) have been frequently used for phylogenetic reconstructions (Brown et al., 1994; Fang et al., 1993; Friedrich and Tautz, 1995; Kambhampati, 1995; Moran et al., 1994; Pashley and Ke, 1992; Salle, 1992; Sperling and Hickey, 1994; Van Zandt-Brower, 1994; Vogler et al., 1993).

In this study we have selected a number of taxa from the Arctiidae and Nymphalidae, in which PA and CG sequestration has been previously analyzed in our or other laboratories. Using mitochondrial 16S rDNA as a marker we have evaluated whether PA- and/or CG-sequestering species share common ancestry.

METHODS AND MATERIALS

Animals. Most species were collected in the wild and provided by a number of colleagues: M. Boppré, K. Cerny, E. A. Diehl, M. Malicki, E. Priesner, D. Schneider, and J. Settele.

DNA Extraction. Adult moths were dissected prior to extraction; the contents of the abdomen or thoracic muscles were separated from the cuticle to avoid sample contamination with colored scale pigments that may inhibit the Taq polymerase. Such isolated tissues or whole animals were stored in 100% ethanol or in a special EDTA buffer (Arctander, 1988) that preserves DNA from degradation. Samples were homogenized in SDS buffer (25 mM EDTA, 75 mM NaCl, 10 mM Tris, pH 7.5, and 1% SDS) to which 0.5 mg/ml proteinase K was added, and the homogenate incubated at 37°C overnight. These homogenates were extracted two times with phenol–chloroform, and DNA was precipitated with ethanol following standard procedures.

PCR and DNA Sequencing. Experiments were carried out according to protocols established in our laboratory (Heidrich et al., 1995; Seibold et al., 1996). The 16S rRNA was amplified by polymerase chain reaction (PCR) using specific eukaryotic primers (kindly provided by S. Pääbo, Munich): 5′-CGCCTGTTTATCAAAAACCA, and 5′-CTCCGGTTTGAACTCAGATC. A final volume of 100 μl contained 1 μg total DNA, 75 μM dNTPs, 1.5 mM $MgCl_2$, 10 μl 10× Taq buffer, 20 pmol primers, and 1–2 units Taq polymerase. The first cycle of the PCR program requires 4 min at 94°C (denaturing), 1 min at 45°C (primer annealing), and 2 min at 72°C (primer extension). The next 30–40 cycles required 30 sec at 93°C, 1 min at 45°C, 2 min at 72°C, and finally 10 min at 72°C. When this program was not successful or produced only weak bands, a two-step PCR was performed with better results in most cases, as follows. The first cycle required 3 min at 94°C, 1 min at 40°C, and 2 min at 72°C. The next 10 cycles required 30 sec at 93°C, 1 min at 40°C, and 2 min at 72°C, followed by a further 30 cycles of 30 sec at 93°C, 1 min at 50°C, 2 min at 72°C, and finally 10 min at 72°C. PCR products were separated from primers, nonincorporated nucleotides, and genomic DNA by agarose gel electrophoresis. The appropriate band was excised, and the DNA isolated with the QiaEx kit (Diagen, Düsseldorf) as described in the kit protocol.

Double-stranded DNA was directly sequenced using one of the PCR primers

as a sequencing primer. Didesoxy-terminated sequencing reactions (Sanger et al., 1977) were carried out with the T7-kit from Pharmacia (Freiburg). DNA was denatured prior to sequencing at 95°C for 10 min in 10% DMSO and quickly frozen to −70°C. The primer annealing step followed immediately after the samples had thawed on ice. All further reactions were as outlined in the Kit-protocol using [α-^{35}S]dATP as a tracer. The labeled sequencing products were electrophoretically separated on a 6% acrylamide–8 M urea gel. Gels were dried and exposed to X-ray films.

Processing of DNA Sequences. Sequences were determined manually from the autoradiograms. Sequences were aligned (total length 537 bp) using the program Clustal (implemented in the program package HUSAR) and corrected for missing informations or gaps. For phylogeny reconstructions both the distance matrix method neighbor joining (NJ), implemented in the program package MEGA 1.0 (Kumar et al., 1993), and the character state method maximum parsimony (MP), implemented in the program package PAUP 3.1 (Swofford, 1993) were employed. Bootstrap analyses (Felsenstein, 1985) were carried out to assess how well the furcations found are supported by the data. Nucleotide sequences will be deposited at the EMBL nucleotide sequence data base.

RESULTS AND DISCUSSION

Phylogeny Inferred from Nucleotide Sequences of 16S rDNA

The data set analyzed included 14 samples of the Arctiinae, three of the Callimorphinae, one of Nyctemerinae (genera *Creatonotos*, *Arctia*, *Panaxia*, *Spilosoma*, *Tyria*, *Nyctemera*, *Diaphora*, and *Utetheisa*), six samples of the Lithosiinae (a subfamily of the Arctiidae specialized on lichens), three taxa of the Ctenuchidae (*Syntomis* and *Syntomeida*), which are often regarded as a subfamily of the Arctiidae (or at least related to them), three taxa of the Danainae (*Danaus* and *Euploea*; family Nymphalidae), and several insects unrelated both in phylogeny and secondary chemistry such as *Manduca sexta* (family Sphingidae), *Papilio machaon* (family Papilionidae), and *Spodoptera frugiperda* (family Noctuidae).

The phylogeny reconstruction with the neighbor-joining method (using the Jukes Cantor distance algorithm) and the maximum parsimony method produced almost congruent phylogenetic trees (Figure 1A and B) in which the Arctiidae and the Danainae are separated as monophyletic clades with significant bootstrap values (NJ: 97% or 98%, respectively). As expected, *Manduca*, *Papilio*, and *Spodoptera* appear unrelated to both groups. The placement of the noctuid *Spodoptera* in the clade of Nymphalidae in NJ reconstructions (Figure 1A) has no phylogenetic relevance (low bootstrap value of 51%), since moths and butterflies are unrelated and not sister groups (Scoble, 1996). The lygaeid bug, *Oncopeltus fasciatus*, is totally unrelated to Lepidoptera. Since its incorporation as an out-

group did not change tree topology (identical trees were obtained with *Manduca* (Lepidoptera, Sphingidae) as an outgroup, *Oncopeltus* was included because it sequesters CGs (Detzel and Wink, 1995; Rothschild, 1972b) and thus could be useful for the discussion of evolution of CG storage.

The members of the Lithosiinae and Ctenuchidae are in a common clade with the Arctiinae, Callimorphinae, and Nyctemerinae (Figure 1). The molecular data would suggest that the Ctenuchidae represent a subgroup of the Arctiidae and, similar to the situation of the Lithosiinae, should be treated as an arctiid subfamily, Ctenuchinae. The Arctiinae cluster in a well-supported clade (bootstrap value 97%), which includes *Arctia*, *Spilosoma*, *Diaphora*, and *Creatonotos*. *Diaphora mendica* had been separated from *Spilosoma* previously as a monotypic genus, but our data (Figure 1) indicate that *Diaphora* falls in the *Spilosoma* clade. Since genetic distances (Table 1) are in the same range as distances within the genus *Creatonotos* and also morphologically both taxa appear rather closely related, it remains doubtful whether *Diaphora* deserves a genus of its own.

Tyria and *Panaxia* have been grouped by some authors in the subfamily Callimorphinae, which is also supported by the molecular data. Larval morphology and food plant selection would imply a closer relatedness between *Tyria* and the Nyctemerinae (K. Cerny, personal communication) which is not apparent from the 16S rRNA tree (Figure 1A and B). It is surprising that *Panaxia dominula* and *P. quadripunctaria* do not occur in a common clade, as did all other species of this study that belong to a common genus. Further studies with more samples of *Panaxia* must corroborate whether *Panaxia* really represents a paraphyletic taxon.

Creatonotos constitutes a group of arctiid moths that are widely distributed in southern Asia. *C. transiens* has been employed in several PA studies (Boppré, 1985, 1990; Nickisch-Rosenegk et al., 1990b; Nickisch-Rosenegk and Wink, 1993, 1994; Schneider et al., 1982). As can be seen from Figure 1 and Table 1, animals from Thailand, Sumatra, and Bali differ significantly from moths from the Philippines. Whereas Nakamura (1976) distinguished four subspecies within the *C. transiens* complex, Inoue (1988) separated *C. t. philippinense* from the Philippines as a true species, *C. wilemani*. This taxon has been widely used in our laboratory but classified as *C. transiens* (Nickisch-Rosenegk et al., 1990b; Nickisch-Rosenegk and Wink, 1993, 1994). The genetic distances in Table 1 support species status for *C. wilemani*, whereas a subspecies allocation for the moths from Thailand, Sumatra, and Bali appears to be difficult.

Correlation of Molecular Phylogeny with Ability to Sequestrate PAs and CGs

The phylogenetic relationships found in Figure 1 agree quite well with morphological data and the traditional classification of the Arctiidae (Tikhomirov, 1979). Do they also agree with biochemical traits?

A

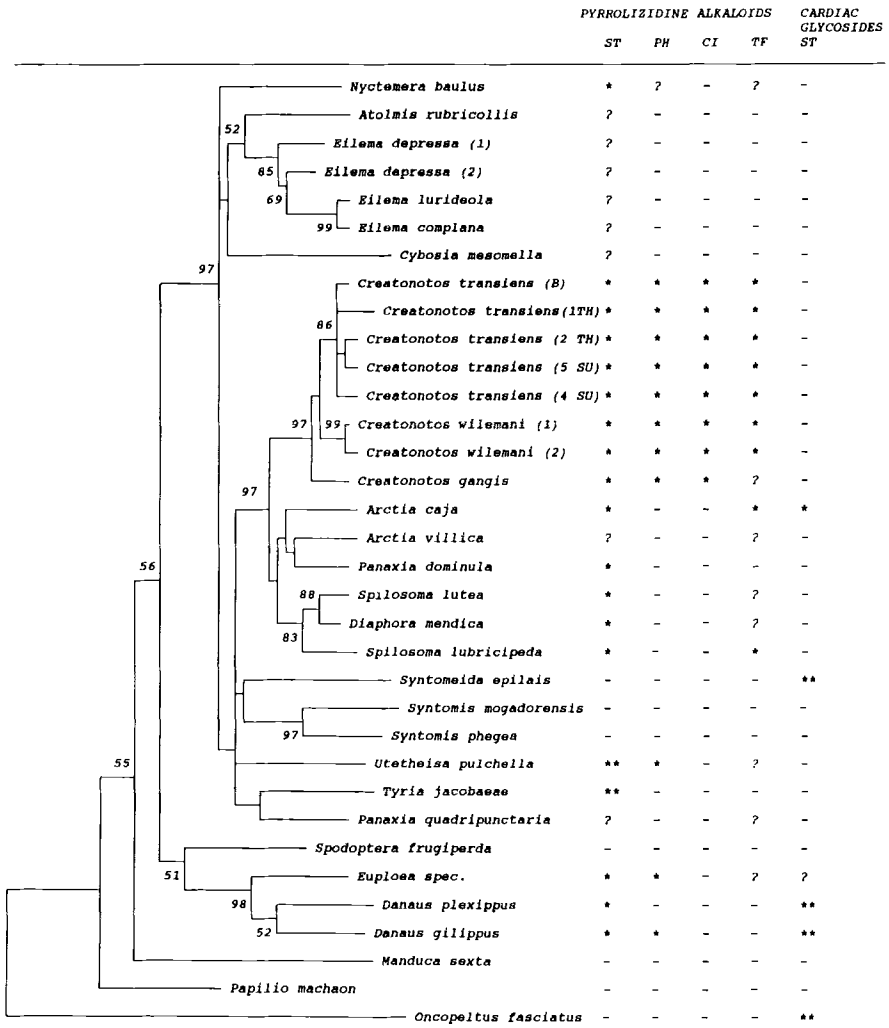


FIG. 1. Phylogeny reconstruction of PA- and CG-sequestering taxa of the Arctiidae and Nymphalidae based on nucleotide sequences of 16S rDNA. (A) Neighbor-joining analysis (Jukes Cantor distance algorithm); illustration as a phylogram in which branch lengths are proportional to genetic distances. Data for PA and CG sequestration were obtained from Boppré (1986, 1995), Brown and Trigo (1995), Hartmann and Witte (1995), Rothschild (1972b), Rothschild et al. (1970a, 1973, 1975, 1978, 1979), Nickisch-Rosenegk and Wink (1993), and Wink (1993). (B) Maximum-parsimony analysis (branch swapping algorithm; TBS; sequence addition: simple); one tree of 688 steps (min. 308, max. 1170 steps) was obtained, which is illustrated as a bootstrap cladogram. Retention index (RI) 0.559, consistency index (CI) 0.448, and homoplasy index (HI) 0.552; 1000 random

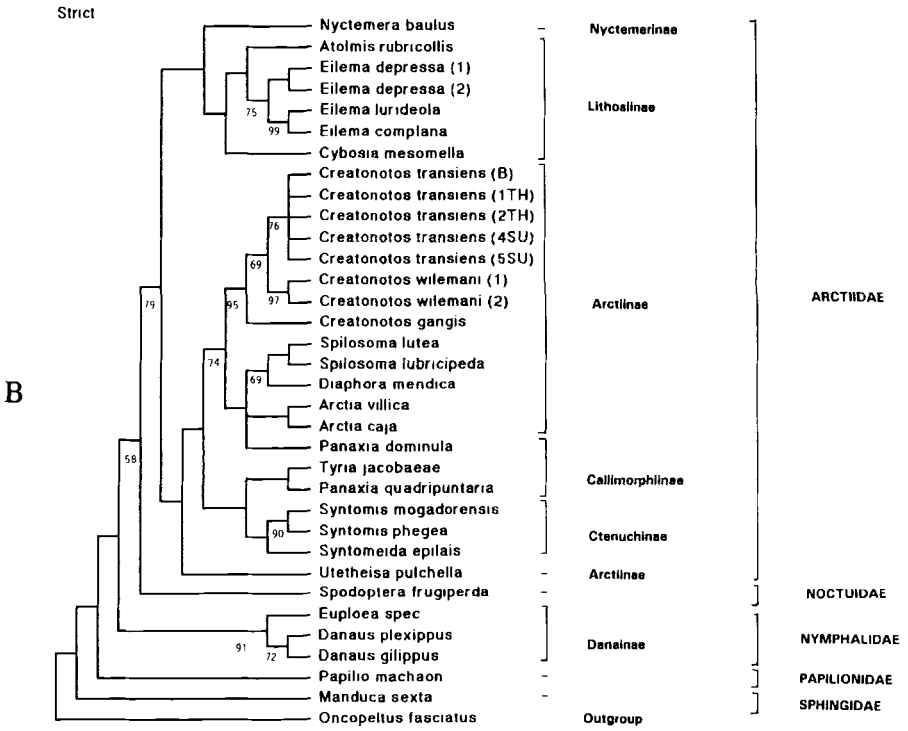


FIG. 1. Continued.

trees had an unsymmetrical distribution (g1 = −0.512), indicating a significant phylogenetic signal in the data set (Hillis and Huelsenbeck, 1992). The data set contains 537 base pairs, of which 230 are variable and 144 phylogenetically informative. Bootstrap values (500 replicates) above 50% are given at each furcation. ST = storage, PH = pheromone derived from PAs; CI = induction of corema formation by dietary PAs; TF = stereochemical inversion of dietary alkaloids; * = trait found; ** = more or less obligatory sequestration; ? = possible, but not tested; − trait not found.

CG Sequestration. As shown in Figure 1A, CGs are naturally sequestered by *Oncopeltus*, *Danaus*, and *Syntomeida*. Rothschild reported that *Arctia caja*, which was reared on *Digitalis* and other CG plants, also accumulated CGs (Rothschild et al., 1970a). We have tested *Creatonotos* and *Syntomis mogadorensis* for CG sequestration, but these species either did not ingest CGs or eliminated them with the feces (Wink and Schneider, 1990). *Danaus plexippus* gains insensitivity towards CGs through an amino acid substitution in the CG binding site of the Na^+, K^+-ATPase (Holzinger et al., 1992; Holzinger and

TABLE 1. GENETIC DISTANCES (NUCLEOTIDE SUBSTITUTIONS) BETWEEN MEMBERS OF THE ARCTIIDAE, NYMPHALIDAE, AND OUTGROUPS[a]

	1	2	3	4	5	6	7	8	9	10	11
1 *Nyctemera baulus*	—	0.103	0.138	0.153	0.144	0.112	0.118	0.108	0.118	0.111	0.108
2 *Creatonotos transiens* (B)	45	—	0.135	0.148	0.130	0.075	0.078	0.066	0.113	0.065	0.060
3 *Euploea* sp.	60	59	—	0.072	0.097	0.162	0.157	0.160	0.169	0.163	0.161
4 *Danaus plexippus*	64	62	30	—	0.074	0.179	0.184	0.173	0.160	0.172	0.165
5 *Danaus gilippus*	61	55	41	31	—	0.166	0.187	0.159	0.171	0.158	0.153
6 *Spilosoma lutea*	48	32	69	75	70	—	0.035	0.016	0.098	0.049	0.063
7 *Spilosoma lubricipeda*	51	34	68	77	79	15	—	0.040	0.111	0.067	0.078
8 *Diaphora mendica*	46	28	68	72	67	7	17	—	0.096	0.047	0.056
9 *Tyria jacobaeae*	51	49	73	67	72	42	48	41	—	0.096	0.115
10 *Arctia villica*	48	28	70	72	67	21	29	20	41	—	0.055
11 *Arctia caja*	47	26	70	69	65	27	34	24	50	24	—
12 *Atolmis rubricollis*	45	48	68	67	71	46	46	44	45	49	45
13 *Eilema depressa* 1	37	43	65	65	64	38	43	35	42	41	39
14 *Eilema depressa* 2	37	41	61	63	65	36	38	32	42	40	37
15 *Eilema lurideola*	44	44	64	68	66	44	47	41	42	46	38
16 *Eilema complana*	43	45	63	67	67	45	48	42	43	47	39
17 *Cybosia mesomella*	49	54	70	68	69	56	58	56	49	57	53
18 *Panaxia dominula*	46	29	69	75	69	21	25	18	46	20	27
19 *Panaxia quadripunctaria*	46	40	63	70	69	35	36	36	34	40	41
20 *Syntomis mogadorensis*	50	50	79	72	74	50	49	46	60	51	51
21 *Syntomis phegea*	47	45	71	72	71	41	46	38	56	50	47
22 *Syntomeida epilais*	46	47	68	72	69	46	47	44	53	45	50
23 *Utetheisa pulchella*	51	45	63	68	68	35	43	39	50	44	50
24 *Creatonotos transiens* 1 TH	49	8	65	68	62	34	34	30	47	30	28
25 *Creatonotos transiens* 2 TH	45	6	61	63	57	31	32	27	46	27	26
26 *Creatonotos transiens* 4 SU	47	6	63	66	59	32	32	28	47	28	26
27 *Creatonotos transiens* 5 SU	44	5	60	63	58	31	31	27	45	29	27
28 *Creatonotos gangis*	46	14	65	65	61	28	30	26	46	28	27
29 *Creatonotos wilemani* 1	42	10	58	64	61	27	26	25	46	31	31
30 *Creatonotos wilemani* 2	42	10	57	64	61	28	26	26	47	32	31
31 *Spodoptera frugiperda*	53	62	49	46	60	55	56	56	60	58	65
32 *Papilio machaon*	58	61	54	48	53	64	64	60	64	64	60
33 *Manduca sexta*	80	77	73	79	80	70	70	70	79	75	73
34 *Oncopeltus fasciatus*	111	114	112	106	112	111	105	107	115	111	112

	12	13	14	15	16	17	18	19	20	21	22
1 *Nyctemera baulus*	0.105	0.087	0.086	0.103	0.100	0.119	0.106	0.111	0.117	0.110	0.110
2 *Creatonotos transiens* (B)	0.112	0.101	0.095	0.103	0.105	0.131	0.067	0.097	0.117	0.105	0.112
3 *Euploea* sp.	0.159	0.152	0.142	0.150	0.147	0.170	0.159	0.153	0.185	0.166	0.163
4 *Danaus plexippus*	0.160	0.156	0.151	0.163	0.160	0.165	0.179	0.169	0.172	0.172	0.173
5 *Danaus gilippus*	0.168	0.152	0.154	0.156	0.159	0.168	0.163	0.167	0.175	0.168	0.165
6 *Spilosoma lutea*	0.107	0.089	0.084	0.103	0.105	0.136	0.049	0.085	0.117	0.096	0.110
7 *Spilosoma lubricipeda*	0.107	0.100	0.088	0.110	0.111	0.141	0.058	0.087	0.114	0.107	0.112
8 *Diaphora mendica*	0.103	0.082	0.075	0.096	0.098	0.136	0.042	0.087	0.108	0.089	0.105
9 *Tyria jacobaeae*	0.105	0.098	0.098	0.098	0.100	0.119	0.106	0.082	0.141	0.131	0.126
10 *Arctia villica*	0.114	0.096	0.093	0.108	0.109	0.139	0.046	0.097	0.119	0.117	0.107
11 *Arctia caja*	0.105	0.091	0.086	0.089	0.090	0.129	0.062	0.099	0.119	0.110	0.119
12 *Atolmis rubricollis*	—	0.079	0.072	0.077	0.070	0.117	0.100	0.109	0.136	0.124	0.129
13 *Eilema depressa* 1	34	—	0.030	0.054	0.051	0.107	0.093	0.087	0.126	0.105	0.122
14 *Eilema depressa* 2	31	13	—	0.037	0.035	0.109	0.090	0.089	0.121	0.098	0.107
15 *Eilema lurideola*	33	23	16	—	0.007	0.102	0.103	0.099	0.133	0.121	0.177
16 *Eilema complana*	30	22	15	3	—	0.097	0.104	0.101	0.126	0.114	0.119
17 *Cybosia mesomella*	48	44	45	42	40	—	0.138	0.124	0.126	0.135	0.149
18 *Panaxia dominula*	43	40	39	44	45	57	—	0.082	0.119	0.117	0.107
19 *Panaxia quadripunctaria*	45	36	37	41	42	51	34	—	0.097	0.109	0.105
20 *Syntomis mogadorensis*	58	54	52	57	54	52	51	40	—	0.072	0.114
21 *Syntomis phegea*	53	45	42	52	49	56	50	45	31	—	0.121
22 *Syntomeida epilais*	54	51	45	49	50	61	45	43	48	51	—
23 *Utetheisa pulchella*	43	45	41	48	47	52	44	38	56	43	50
24 *Creatonotos transiens* 1 TH	50	44	44	47	48	57	31	42	54	49	51
25 *Creatonotos transiens* 2 TH	47	42	40	43	44	51	28	39	49	46	48
26 *Creatonotos transiens* 4 SU	48	43	43	46	47	54	29	38	52	47	48
27 *Creatonotos transiens* 5 SU	47	42	40	44	45	53	28	39	49	46	46
28 *Creatonotos gangis*	43	42	40	44	45	54	26	36	48	46	41
29 *Creatonotos wilemani* 1	43	40	38	44	45	55	28	37	50	46	44
30 *Creatonotos wilemani* 2	44	41	39	45	46	56	29	38	51	47	45
31 *Spodoptera frugiperda*	56	55	52	57	56	60	59	54	69	68	61
32 *Papilio machaon*	63	59	55	62	63	65	62	62	65	71	61
33 *Manduca sexta*	78	76	78	75	76	79	71	76	81	77	80
34 *Oncopeltus fasciatus*	117	117	119	119	119	115	112	110	112	118	112

TABLE 1. Continued

	23	24	25	26	27	28	29	30	31	32	33	34
1 *Nyctemera baulus*	0.119	0.116	0.104	0.110	0.105	0.107	0.097	0.096	0.121	0.141	0.185	0.265
2 *Creatonotos transiens* (B)	0.106	0.019	0.014	0.014	0.012	0.033	0.023	0.023	0.142	0.148	0.178	0.272
3 *Euploea* sp.	0.148	0.154	0.141	0.147	0.143	0.151	0.134	0.131	0.112	0.131	0.169	0.269
4 *Danaus plexippus*	0.163	0.164	0.151	0.158	0.151	0.156	0.153	0.153	0.110	0.117	0.189	0.257
5 *Danaus gilippus*	0.162	0.148	0.135	0.140	0.138	0.145	0.144	0.144	0.142	0.129	0.190	0.268
6 *Spilosome lutea*	0.082	0.080	0.072	0.075	0.073	0.065	0.063	0.065	0.129	0.155	0.164	0.266
7 *Spilosoma lubricipeda*	0.101	0.080	0.074	0.075	0.073	0.070	0.060	0.060	0.129	0.155	0.162	0.259
8 *Diaphora mendica*	0.092	0.071	0.063	0.066	0.064	0.061	0.059	0.061	0.131	0.146	0.164	0.257
9 *Tyria jacobaeae*	0.117	0.111	0.106	0.110	0.107	0.107	0.106	0.109	0.138	0.155	0.182	0.275
10 *Arctia villica*	0.103	0.071	0.063	0.065	0.069	0.065	0.072	0.074	0.134	0.156	0.175	0.264
11 *Arctia caja*	0.117	0.066	0.060	0.061	0.064	0.063	0.071	0.071	0.148	0.146	0.168	0.266
12 *Atolmis rubricollis*	0.100	0.118	0.110	0.112	0.111	0.100	0.100	0.103	0.131	0.153	0.181	0.280
13 *Eilema depressa* 1	0.105	0.104	0.098	0.100	0.100	0.098	0.094	0.096	0.129	0.143	0.178	0.280
14 *Eilema depressa* 2	0.096	0.104	0.093	0.100	0.095	0.093	0.088	0.090	0.121	0.133	0.181	0.285
15 *Eilema lurideola*	0.112	0.111	0.100	0.107	0.104	0.103	0.103	0.105	0.133	0.150	0.176	0.285
16 *Eilema complana*	0.110	0.113	0.102	0.110	0.107	0.104	0.105	0.107	0.130	0.153	0.177	0.285
17 *Cybosia mesomella*	0.127	0.140	0.124	0.131	0.129	0.131	0.134	0.136	0.146	0.159	0.192	0.283
18 *Panaxia dominula*	0.103	0.073	0.065	0.068	0.066	0.060	0.064	0.066	0.135	0.150	0.164	0.266
19 *Panaxia quadripunctaria*	0.092	0.103	0.094	0.092	0.094	0.087	0.090	0.092	0.130	0.151	0.184	0.270
20 *Syntomis mogadorensis*	0.131	0.128	0.114	0 121	0.116	0.112	0.117	0.119	0.161	0.158	0.190	0.267
21 *Syntomis phegea*	0.100	0.116	0.107	0.110	0.109	0.107	0.107	0.110	0.159	0.172	0.180	0.282
22 *Syntomeida epilais*	0.119	0.123	0.115	0.115	0.110	0.098	0.105	0.107	0.145	0.149	0.191	0.271
23 *Utetheisa pulchella*	—	0.109	0.103	0.101	0.105	0.101	0.101	0.103	0.129	0.151	0.192	0.285
24 *Creatonotos transienes* 1 TH	46	—	0.017	0.024	0.022	0.047	0.036	0.038	0.151	0.160	0.190	0.271
25 *Creatonotos transiens* 2 TH	44	7	—	0.012	0.005	0.035	0.023	0.025	0.143	0.146	0.181	0.263
26 *Creatonotos transiens* 4 SU	43	10	5	—	0.012	0.037	0.026	0.028	0.145	0.153	0.180	0.268
27 *Creatonotos transiens* 5 SU	44	9	2	5	—	0.031	0.019	0.021	0.145	0.146	0.181	0.267
28 *Creatonotos gangis*	43	20	15	16	13	—	0.026	0.028	0.128	0.153	0.163	0.268
29 *Creatonotos wilemani* 1	43	15	10	11	8	11	—	0.002	0.130	0.148	0.176	0.262
30 *Creatonotos wilemani* 2	44	16	11	12	9	12	1	—	0.130	0.148	0.176	0.261
31 *Spodoptera frugiperda*	55	64	62	62	61	55	57	57	—	0.131	0.164	0.271
32 *Papilio machaon*	62	65	60	63	60	63	61	61	54	—	0.175	0.232
33 *Manduca sexta*	82	80	78	77	76	70	76	76	71	72	—	0.259
34 *Oncopeltus fasciatus*	119	112	110	112	111	112	110	110	115	94	108	—

[a]Pairwise distances between taxa; below diagonal; absolute distances; above diagonal: mean distances (adjusted for missing data; 1 = 100%); TH = Thailand, SU = Sumatra, B = Bali.

Wink, 1996). We could not discover the same substitution in other CG-sequestering insects, including another member of the genus *Danaus*, *D. gilippus* (Holzinger and Wink, 1996). These data and the molecular phylogeny suggest that CG sequestration has evolved several times and independently. A similar conclusion can be drawn for plants that produce CGs. They occur in families that are not close relatives according to morphological or molecular evidence (Chase et al., 1993). This implies that the structures of cardenolides and bufodienolides evolved independently in different plant families, i.e., cardenolide production represents a convergent trait. Since CG storage provides a selective advantage against herbivores, it is likely that secondary metabolites were shaped in several families in such a way that they can tightly interfere with the ouabain binding site of Na^+, K^+-ATPase, which constitutes an important target in animals. Thus the structural similarities of CGs (especially in the aglycone part of the molecule) and the distribution in unrelated families can be explained by a process that could be termed evolutionary molecular modeling.

PA Sequestration. PA sequestration by some members of the Danainae and the Arctiidae has been interpreted as reflecting a common genetic base (Harborne, 1993), although Arctiidae and Nymphalidae are not closely related and are not sister groups (Scoble, 1996). As expected, the phylogenetic data (Figure 1) clearly favor the alternative, that PA sequestration evolved independently in moths and butterflies. In this context it should be recalled that even a few beetles, such as *Oreina cacaliae* (Pasteels et al., 1992; Rowell-Rahier et al., 1991; Rowell-Rahier and Pasteels, 1990), sequester PAs, which also can be explained by convergence only. There are a number of fine differences between PA sequestration in Danainae and Arctiidae. For example, Danainae ingest PAs as adult butterflies from wilting or dead plant parts that are rich in PAs, whereas members of the Arctiidae obtain PAs through their larval diet. *Danaus* spp. convert PAs mainly to the pheromones danaidal and danaidone, whereas Arctiidae convert PAs predominantly to (7*R*)-hydroxydanaidal. If PAs are present with the 7*S* configuration, they are often inverted to the 7*R* series by arctiids (Hartmann and Witte, 1995; Schulz et al., 1993; Wink et al., 1988). Thus, it is certain that PA sequestration in the Nymphalidae and Arctiidae represents a convergent trait. Furthermore, the production of PAs by plants that belong to different and not closely related families, such as Boraginaceae, Asteraceae, Leguminosae, or Orchidaceae, appears to be adaptive and not linked through common descent.

As can be seen from Figure 1A, the members of the Arctiinae, Nyctemerinae, and Callimorphinae sequester PAs if these are present in the larval diet. Within this group a number of differences can be seen in the way they deal with PAs. For a few taxa, PA storage is almost obligatory (for example, *Utetheisa* and *Tyria*) [reviewed by Brown and Trigo (1995) and Hartmann and Witte (1995)], although even here populations exist that do not sequester PAs in the wild. *Tyria jacobaeae* normally feeds on *Senecio jacobaea* or *Adenostyles*

glabra, and sequesters and stores the dietary PAs, but in the Alps we found *T. jacobaeae* on *Petasites albus* and *P. paradoxus* plants, which are almost devoid of PA. We could not detect PAs in the larvae. To our surprise, *T. jacobaeae* was not attracted to PA-rich *Senecio fuchsii* or *Adenostyles alliariae* plants, which were growing in the immediate neighborhood of *Petasites* (Nickisch-Rosenegk and Wink, 1993). Other genera of the Arctiidae are polyphagous and not specialized on PA plants. Under laboratory conditions most of them are able to sequester dietary PAs and other compounds (Rothschild, 1972b). Some Arctiinae are highly attracted to PAs and even ingest filter paper soaked in pure PA solutions (Boppré, 1986, 1990). Members of the monophyletic genus *Creatonotos* share a close common ancestry (bootstrap values of 95 or 97%) (Figure 1), which is reflected in a most peculiar way of dealing with PAs. In addition to specific PA uptake and PA storage in the integument, *Creatonotos* can invert the stereochemistry of PAs of the 7*S* series, and males can convert 7*R*-configured PAs into (7*R*)-hydroxydanaidal, which is dissipated via large inflatable coremata. These coremata only develop if larvae have the chance to ingest a PA-rich diet (Boppré, 1990; Boppré and Schneider, 1989; Ehmke et al., 1990; Hartmann et al., 1990; Nickisch-Rosenegk et al., 1990b; Nickisch-Rosenegk and Wink, 1993, 1994; Schneider, 1986, 1991; Schneider et al., 1982; Wink and Schneider, 1988, 1990; Wink et al., 1988).

PA sequestration is obligatory in a few species, but most modern Arctiinae, although they have maintained the capacity to take up, store, and transform PAs, are more opportunistic feeders and are no longer strictly bound to PA plants. Most of the species concerned are aposematically colored, indicating that at least a proportion of the animals should contain defensive chemicals. It is unclear at present whether PA sequestration found in a small proportion of the population (if at all) is responsible for aposematic coloration or whether other toxins are additionally present. It should also be recalled that at least some of the arctiids (such as *Arctia caja*) produce pyrazines, acetylcholine, and other signal compounds and are able to sequester a wide variety of other dietary allelochemicals (Rothschild, 1972b).

The Lithosiinae (including *Cybosia*, *Atolmis*, and *Eilema*) are specialized on lichens; all members of *Eilema* and probably also *Cybosia* and *Atolmis* belong to a common monophyletic clade (although the bootstrap values are not significant for the latter taxa). This would suggest that the adaptation to feed on lichens and to handle their numerous secondary metabolites has evolved once and was maintained throughout this group. As most other members of the Arctiidae store dietary allelochemicals (Rothschild, 1972b), it is not surprising that Lithosiinae sequester lichen secondary metabolites.

It could be speculated that PA sequestration in Arctiinae, Callimorphinae, and Nyctemerinae evolved independently, since Lithosiinae and Ctenuchinae share ancestry with them but do not accumulate PAs (Figure 1A). Alternatively,

there might have been a common ancestor to all of them that evolved the capacity to sequester a wide variety of dietary secondary metabolites, but only Arctiinae, Callimorphinae, and Nyctemerinae specialized on PAs, while Lithosiinae specialized on lichen compounds and *Syntomeidae* (a ctenuchid) on cardiac glycosides.

In conclusion, molecular markers are valuable tools that aid in interpretation of PA and CG sequestration in specialized insects. The data imply that sequestration of defensive chemicals evolved independently in Arctiidae and Danainae. This first analysis only included a limited number of taxa. Furthermore, since a phylogeny based on a single gene does not necessarily reflect the phylogeny of the species concerned, our analysis should be corroborated and extended using another gene, preferably a nuclear marker, and a wider range of taxa.

Acknowledgments—We like to thank M. Boppré, K. Cerny, E. A. Diehl, M. Malicki, E. Priesner, D. Schneider, and J. Settele for providing insects for this study and K. Cerny and J. Nation for helpful comments. This study was financially supported by grants of the Deutsche Forschungsgemeinschaft (to M. W.) and the Land Baden-Württemberg (Landes-Schwerpunktprogramm "Molekulare Evolution").

REFERENCES

ARCTANDER, P. 1988. Comparative studies on avian DNA by restriction length polymorphism analysis: convenient procedures based on blood samples from live birds. *J. Ornithol.* 129:205–216.

AVISE, J. C. 1994. Molecular Markers, Natural History and Evolution. Chapman & Hall, New York.

BERNAYS, E. A., and CHAPMAN, R. F. 1994. Host-Plant Selection by Phytophagous Insects. Chapman & Hall, New York.

BERNAYS, E., EDGAR, J. A., and ROTHSCHILD, M. 1977. Pyrrolizidine alkaloids sequestered and stored by the aposematic grasshopper. *Zonocerus variegatus. J. Zool. London* 182:85–87.

BOPPRÉ, M. 1981. Adult Lepidoptera feeding at withered *Heliotropium* plants (Boraginaceae) in East Africa. *Ecol. Entomol.* 6:449–452.

BOPPRÉ, M. 1983. Leaf-scratching—a specialized behaviour of danaine butterflies (Lepidoptera) for gathering secondary plant substances. *Oecologia* 59:414–416.

BOPPRÉ, M. 1985. The biology of *Creatonotos* (Lepidoptera: Arctiidae) with special reference to the androconial system. *Zool. J. Linn. Soc.* 96:97–103.

BOPPRÉ, M. 1986. Insects pharmacophagously utilizing defensive plant chemicals (pyrrolizidine alkaloids). *Naturwissenschaften* 73:17–26.

BOPPRÉ, M. 1990. Lepidoptera and pyrrolizidine alkaloids: Exemplification of complexity in chemical ecology. *J. Chem. Ecol.* 16:1–20.

BOPPRÉ, M. 1994. Sex, drugs and butterflies. *Nat. Hist.* 1:27–32.

BOPPRÉ, M. 1995. Pharmakophagie: Drogen, Sex und Schmetterlinge. *Biol. Z.* 25:8–17.

BOPPRÉ, M., and SCHNEIDER, D. 1982. Insects and pyrrolizidine alkaloids. Proceedings, 5th International Symposium on Insect–Plant Relationships, Pudoc, Wageningen, pp. 373–374.

BOPPRÉ, M., and SCHNEIDER, D. 1989. The biology of *Creatonotos* (Lepidoptera Arctiidae) with special reference to the androconial system. *Zool. J. Linn. Soc.* 96:339–356.

BOPPRÉ, M., and VANE-WRIGHT, V. 1989. Androconial systems in Danainae (Lepidoptera): functional morphology of *Amauris*, *Danaus*, *Tirumala* and *Euploea*. *Zool. J. Linn. Soc.* 97:101–133.

BOPPRÉ, M., BILLER, A., FISCHER, O. W., and HARTMANN, T. 1992. The non nutrional relationship of *Zonocerus* (Orthoptera) to *Chromolaena* (Asteraceae). Proceedings, 8th International Symposium on Insect-Plant Relationships, pp. 89–90. Kluwer Academic Publishers, Dordrecht.

BROWER, L. P., and VAN ZANDT-BROWER, J. 1964. Birds, butterflies and plant poisons: A study in ecological chemistry. *Zoologica* 49:137–159.

BROWER, L. P., RYERSON, W. N., COPPINGER, L. L., and GLAZIER, S. C. 1968. Ecological chemistry and the palatability spectrum. *Science* 161:1349–1351.

BROWER, L. P., EDMUNDS, M., and MOFFITT, C. M. 1975. Cardenolide content and palatability of a population of *Danaus chrysippus* butterflies from West Africa. *J. Entomol.* 49:183–196.

BROWN, J. M., PELLMYR, O., THOMPSON, J. N., and HARRISON, R. G. 1994. Phylogeny of *Greya* (Lepidoptera: Prodoxidae). *Mol. Biol. Evol.* 11:128–141.

BROWN, K. S., and TRIGO, J. R. 1995. The ecological activity of alkaloids. *Alkaloids* 47:227–354.

CHASE, M. W., SOLTIS, D. E., OLMSTEAD, R. G., MORGAN, D., LES, D. H., MISHLER, B. D., DUVALL, M. R., PRICE, H. G., HILLIS, H. G., QIU, Y. L., KRON, K. A., RETTIG, J. H., CONTI, E., PALMER, J. D., MANHART, J. R., SYTSMA, K. J., MICHAELS, H. J., KRESS, W. J., KAROL, K. G., CLARK, W. D., HEDREN, M., GAUT, B. S., JANSEN, R. K., KIM, K. J., WIMPEE, C. F., SMITH, J. F., FURNIER, G. R., STRAUSS, S. H., XIANG, Q. Y., PLUNKETT, G. M., SOLTIS, P. S., SWENSEN, S., WILLIAMS, S. E., GADEK, P. A., QUINN, C. J., EGUIARTE, L. E., GOLENBERG, E., LEARN, G. H., JR., GRAHAM, S. W., BARRETT, S. C. H., DAYANANDIAN, S., and ALBERT, V. A. 1993. Phylogenetics of seed plants: An analysis of nucleotide sequences from the plastid gene rbcL. *Ann. Mo. Bot. Gard.* 80:528–580.

CHEEKE, P. R. 1984. Comparative toxicity and metabolism of pyrrolizidine alkaloids in ruminants and nonruminant herbivores. *Can. J. Anim. Sci.* 64:201–202.

DETZEL, A., and WINK, M. 1995. Evidence for a cardenolide carrier in *Oncopeltus fasciatus* (Dallas) (Insecta: Hemiptera). *Z. Naturforsch.* 50c:127–134.

DUSSOURD, D., UBIK, K., HARVIS, C., RESCH, J., MEINWALD, J., and EISNER, T. 1988. Biparental defensive endowment of eggs with acquired plant alkaloid in the moth *Utetheisa ornatrix*. *Proc. Natl. Acad. Sci. U.S.A.* 85:5992–5996.

DUSSOURD, D. E., HARVIS, C. A., MEINWALD, J., and EISNER, T. 1989. Paternal allocation of sequestered plant pyrrolizidine alkaloid to eggs in the Danaine butterfly *Danaus gilippus*. *Experientia* 45:896–898.

EDGAR, J. A., CULVENOR, C. C. J., and PLISKE, T. E. 1976. Isolation of a lactone, structurally related to the esterifying acids of pyrrolizidine alkaloids, from the costal fringers of male ithomiinae. *J. Chem. Ecol.* 3:263–270.

EDGAR, J. A., CULVENOR, C. C. J., COCKRUM, P. A., SMITH, L. W., ROTHSCHILD, M., and PETERBOROUGH, A. 1980. Callimorphine: Identification and synthesis of the cinnabar moth metabolite. *Tetrahedron Lett.* 21:1383–1384.

EGELHAAF, A., CÖLLN, K., SCHMITZ, B., BUCK, M., WINK, M., and SCHNEIDER, D. 1990. Organ-specific storage of dietary pyrrolizidine alkaloids in the arctiid moth *Creatonotos transiens*. *Z. Naturforsch.* 45:115–120.

EHMKE, A., WITTE, L., BILLER, A., and HARTMANN, T. 1990. Sequestration, N-oxidation and transformation of plant pyrrolizidine alkaloids by the arctiid moth *Tyria jacobaeae* L. *Z. Naturforsch.* 45c:1185–1192.

EISNER, T., and MEINWALD, J. 1987. Alkaloid-derived pheromones and sexual selection in Lepidoptera, pp. 251–269, *in* G. D. Prestwich and G. J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, Orlando, Florida.

FANG, Q., BLACK, W. C. I., BLOCKER, H. D., and WHITCOMB, R. F. 1993. A phylogeny of new world *Deltocephalus*-like leafhopper genera based on mitochondrial 16S ribosomal DNA sequences. *Mol. Phylogen. Evol.* 2:119–131.

FELSENSTEIN, J. 1985. Phylogenies and the comparative method. *Am. Nat.* 125:1–15.

FRICK, C., and WINK, M. 1995. Uptake and sequestration of ouabain and other cardiac glycosides in *Danaus plexippus* (Lepidoptera: Danaidae): Evidence for a carrier-mediated process. *J. Chem. Ecol.* 21:557–575.

FRIEDRICH, M., and TAUTZ, D. 1995. Ribosomal DNA phylogeny of the major extant arthropod classes and the evolution of myriapods. *Nature* 376:165–167.

HARBORNE, J. B. 1993. Introduction to Ecological Biochemistry. Academic Press, London.

HARTMANN, T. 1992. Alkaloids: Biochemistry, physiology and chemical ecology by example of the pyrrolizidine alkaloids, pp. 65–76, *in* K. Takai (ed.). Frontiers and New Horizons in Amino Acid Research. Elsevier, Amsterdam.

HARTMANN, T., and WITTE, L. 1995. Chemistry, biology and chemoecology of the pyrrolizidine alkaloids, pp. 155–233, *in* S. W. Pelletier (ed.). Alkaloids: Chemical and Biological Perspectives. Pergamon, Oxford.

HARTMANN, T., BILLER, A., WITTE, L., ERNST, L., and BOPPRÉ, M. 1990. Transformation of plant pyrrolizidine alkaloids into novel insect alkaloids by arctiid moths. *Biochem. Syst. Ecol.* 18:1–12.

HEIDRICH, P., KÖNIG, C., and WINK, M. 1995. Bioacoustics, taxonomy and molecular systematics in American pygmy owls (Strigidae: *Glaucidium* spp.). *Stuttgarter Beitr. Naturk. A* 534:1–47.

HILLIS, D. M., and HUELSENBECK, J. P. 1992. Signal, noise, and reliability in molecular phylogenetic analysis. *J. Hered.* 83:189–195.

HILLIS, D. M., MORITZ, C., and MABLE, B. K. 1996. Molecular Systematics. Sinauer Press, Sunderland, Massachusetts.

HOLZINGER, F., and WINK, M. 1996. Mediation of cardiac glycoside insensitivity in the monarch (*Danaus plexippus*): Role of an amino acid substitution in the ouabain binding site of Na^+, K^+-ATPase. *J. Chem. Ecol.* 22:1931–1947.

HOLZINGER, F., FRICK, C., and WINK, M. 1992. Molecular basis for the insensitivity of the monarch (*Danaus plexippus*) to cardiac glycosides. *Febs Lett.* 314:477–480.

INOUE, H. 1988. Three new species and some synonymic notes on the Arctiidae from Japan, Taiwan, and the Phillippines. *Tyo to Ga* 39:99–118.

KAMBHAMPATI, S. 1995. A phylogeny of cockroaches and related insects based on DNA sequence of mitochondrial ribosomal RNA genes. *Proc. Natl. Acad. Sci. U.S.A.* 92:2017–2020.

KUMAR, S., TAMURA, K., and NEI, M. 1993. MEGA—Molecular Evolutionary Genetics Analysis. Version 1.0. Pennsylvania State University.

MALCOLM, S. B. 1990. Chemical defence in chewing and sucking insect herbivores: plant derived cardenolids in the monarch butterfly and oleander aphid. *Chemoecology* 1:12–21.

MATTOCKS, A. R. 1972. Acute hepatotoxicity and pyrrolic metabolites in rats dosed with pyrrolizidine alkaloids. *Chem. Biol. Interactions* 5:227–242.

MCLEAN, E. 1970. The toxic actions of pyrrolizidine (*Senecio*) alkaloids. *Pharmacol. Rev.* 22:430–483.

MORAN, N. A., BAUMANN, P., and VON DOHLEN, C. 1994. Use of DNA sequences to reconstruct the history of the association between numbers of the Sternorrhyncha (Homoptera) and their bacterial endosymbionts. *Eur. J. Entomol.* 91:79–83.

NAKAMURA, M. 1976. Notes on the geographical variations of *Creatonotos transiens* Walker with descriptions of a new species and two subspecies (Lepidoptera: Arctiidae). *Trans. Lepid. Soc. Jpn.* 27:11–117.

NEUWINGER, R. 1994. Afrikanische Heilpflanzen und Jagdgifte. Wiss. Verlagsges., Stuttgart.

NICKISCH-ROSENBEGK, E. VON, and WINK, M. 1993. Sequestration of pyrrolizidine alkaloids in several arctiid moths (Lepidoptera Arctiidae). *J. Chem. Ecol.* 19:1889–1903.

NICKISCH-ROSENEGK, E. VON, and WINK, M. 1994. Influence of previous feeding regimes and ambient temperatures on degradation and storage of pyrrolizidine alkaloids in the moth species *Creatonotos transiens* (Lepidoptera: Arctiidae). *Entomol. Gen.* 19:159–170.

NICKISCH-ROSENEGK VON, E., DETZEL, A., WINK, M., and SCHNEIDER, D. 1990a. Carrier-mediated uptake of digoxin by larvae of the cardenolide squestering moth *Syntomeida epilais*. *Naturwissenschaften* 77:336–338.

NICKISCH-ROSENEGK, E. VON, SCHNEIDER, D., and WINK, M. 1990b. Time-course of pyrrolizidine alkaloid processing in the alkaloid exploiting arctiid moth *Creatonotos transiens*. *Z Naturforsch Sect. C Biosci.* 45:881–894.

PASHLEY, D. P., and KE, L. D., 1992. Sequence evolution in mitochondrial ribosomal and Nd-1 genes in Lepidoptera: Implications for phylogenetic analyses. *Mol. Biol. Evol.* 9:1061–1075.

PASTEELS, J. M., EGGENBERGER, F., ROWELL-RAHIER, M., EHMKE, A., and HARTMANN, T. 1992. Chemical defense in chrysomelid leaf beetles. *Naturwissenschaften* 79:521–523.

ROEDER, E. 1995. Medicinal plants in Europe containing pyrrolizidine alkaloids. *Pharmazie* 50:83–98.

ROSENTHAL, G. A., and BERENBAUM, M. R. 1991. Herbivores. Their Interactions with Secondary Plant Metabolites, Vol. 1. Academic Press, San Diego.

ROSENTHAL, G. A., and BERENBAUM, M. R. 1992. Herbivores. Their Interactions with Secondary Plant Metabolites, Vol. 2. Academic Press, San Diego.

ROTHSCHILD, M. 1966. Experiments with captive predators and the poisonous grasshopper *Poekilocerus bufonius*. *Proc. R. Entomol. Soc. London* 31:32–33.

ROTHSCHILD, M. 1972a. Colour and poisons in insect protection. *New Sci.* 318–320.

ROTHSCHILD, M. 1972b. Secondary plant substances and warning colouration in insects, pp. 59–83, *in* H. F. van Emden (ed.). Insect/Plant Relationships. Blackwell, Oxford.

ROTHSCHILD, M., and KELLETT, D. 1972. Reactions of various predators to insects storing heart poisons (cardiac glycosides) in their tissues. *J. Entomol.* 46:103–110.

ROTHSCHILD, M., REICHSTEIN, T., EUW, J., and APLIN, R. 1970a. Toxic Lepidoptera. *Toxicon* 8:293–299.

ROTHSCHILD, M., VON EUW, J., and REICHSTEIN, T. 1970b. Cardiac glycosides in the oleander aphid. *Aphis nerii*. *Insect Physiol.* 16:1141–1145.

ROTHSCHILD, M., VON EUW, J., and REICHSTEIN, T. 1971. Heart poisons (cardiac glycosides) in the lygaeid bugs *Caenocoris nerii* and *Spilostethus pandorus*. *Insect Biochem.* 1:373–384.

ROTHSCHILD, M., VON EUW, J., and REICHSTEIN, T. 1972. Some problems connected with warningly coloured insects and toxic defense mechanism. *Mitt. Basler Afr. Bibliogr.* 4:135–158.

ROTHSCHILD, M., VON EUW, J., and REICHSTEIN, T. 1973. Cardiac glycosides (heart poisons) in the polka-dot moth *Syntomeida epilais* Walk. with some observations on the toxic qualities of *Amata* (= *Syntomis*) *phegea*. *Proc. R. Soc. London Ser. B* 183:227–247.

ROTHSCHILD, M., SMITH, D. A. S., and PIERRE, J. 1975. Cardenolide storage in *Danaus chrysippus* with additional notes on *D. plexippus*. *Proc. R. Soc. London* 190:1–31.

ROTHSCHILD, M., MARSH, N., and GARDINER, B. 1978. Cardioactive substances in the monarch butterfly and *Euploea core* reared on leaf-free artificial diet. *Nature* 275:649–650.

ROTHSCHILD, M., APLIN, R. T., COCKRUM, P. A., EDGAR, J. A., FAIRWEATHER, P., and LEES, R. 1979. Pyrrolizidine alkaloids in arctiid moths (Lepidoptera) with a discussion on host plant relationships and the role of these secondary plant substances in the Arctiidae. *Biol. J. Linn. Soc.* 12:305–326.

ROTHSCHILD, M., MOORE, B., and BROWN, W. 1984. Pyrazines as warning odour components in the Monarch butterfly, *Danaus plexippus*, and in moths of the genera *Zygaena* and *Amata*. *Biol. J. Linn. Soc.* 23:375–380.

ROWELL-RAHIER, M., and PASTEELS, J. 1990. Chemical specialization on toxic plants provides increased protection from natural enemies. *Symp. Biol. Hung.* 39:343–347.

ROWELL-RAHIER, M., WITTE, L., EHMKE, A., and HARTMANN, T. 1991. Sequestration of plant

pyrrolizidine alkaloids by chrysomelid beetles and selective transfer into the defensive secretions. *Chemoecology* 2:41–48.

SALLE, R. 1992. The phylogenetic relationship of flies in the family Drosophilidae deduced from mtDNA sequences. *Mol. Phylogenet. Evol.* 1:31–40.

SANGER, F., NICKLEN, S., and COULSON, A. R. 1977. DNA-sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74:5463–5467.

SCHMELLER, T., EL-SHAZLY, A., and WINK, M. 1997. Binding of pyrrolizidine alkaloids to acetylcholine, serotonin and dopamine receptors. *J. Chem. Ecol.* 23(2):399–416.

SCHNEIDER, D. 1977. Plant alkaloids as pheromone percursors in danaid butterflies. *Colloq. Int. C.N.R.S.* 265:353–355.

SCHNEIDER, D. 1986. The strange fate of pyrrolizidine alkaloids, pp. 123–142, *in* R. F. Chapman, E. A. Bernays, and J. G. Stoffolano (eds.). Perspectives in Chemoreception and Behavior. Springer-Verlag, New York.

SCHNEIDER, D. 1991. Pflanzen and Pflanzenfresser: Ein Kapitel der chemischen Ökologie mit Beispielen von Insekten. *Verh. Westd. Entomol.* 1–15. Düsseldorf.

SCHNEIDER, D. 1992. 100 years of pheromone research. *Naturwissenschaften* 6:241–250.

SCHNEIDER, D. 1993. Danaine butterflies, a didactic story about chemical ecology. *Nat. Hist. Mus. Los Angeles County Sci. Bull.* 19–28. Los Angeles.

SCHNEIDER, D., BOPPRÉ, M., ZWEIG, J., HORSLEY, S. B., BELL, T. W., and MEINWALD, J. 1982. Scent organ development in *Creatonotos* moth: Regulation by pyrrolizidine alkaloids. *Science* 215:1264–1265.

SCHULZ, S., FRANCKE, W., BOPPRÉ, M., EISNER, T., and MEINWALD, J. 1993. Insect pheromone biosynthesis: Stereochemical pathways of hydroxydanaidal production from alkaloidal precursors in *Creatonotus transiens* (Lepidoptera, Arctiidae). *Proc. Natl. Acad. Sci. U.S.A.* 90:6834–6838.

SCOBLE, M. J. 1996. In search of butterfly origins: morphology and molecules. *Tree* 11:274–275.

SEIBOLD, I., HELBIG, A. J., MEYBURG, B.-U., NEGRO, J. J., and WINK, M. 1996. Genetic differentiation and molecular phylogeny of European *Aquila* eagles (Aves: Falconiformes) according to cytochrome *b* nucleotide sequences, pp. 1–15, *in* B. U. Meyburg and R. Chancellor (eds.). Eagle Studies. WWGBP, Berlin.

SPERLING, F. A. H., and HICKEY, D. A. 1994. Mitochondrial DNA sequence variation in the spruce budworm species complex (Choristoneura: Lepidoptera). *Mol. Biol. Evol.* 11:656–665.

SWOFFORD, D. L. 1993. PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1.1. Illinois Natural History Survey, Champaign.

TIKHOMIROV, A. M. 1979. Phylogenetic links and classification of European Arctiidae (Lepidoptera) on the basis of the functional morphology of the male genitalia. *Entomol. Obozr.* 58:116–127.

TRIGO, J., WITTE, L., BROWN, K., HARTMANN, T., and BARATA, L. 1993. Pyrrolizidine alkaloids in the arctiid moth. *J. Chem. Ecol.* 19:669–679.

VAN ZANDT-BROWER, A. 1994. Phylogeny of *Heliconius* butterflies inferred from mitochondrial DNA sequences (Lepidoptera: Nymphalidae). *Mol. Phylogenet. Evol.* 3:159–174.

VAUGHAN, G. L., and JUNGREIS, A. M. 1977. Insensitivity of lepidopteran tissues to ouabain. Physiological mechanisms for protection from cardiac glycosides. *J. Insect Physiol.* 23:585–589.

VOGLER, A. P., DESALLE, R., ASSMANN, T., KNISLEY, C. B., and SCHULTZ, T. D. 1993. Molecular population genetics of the endangered tiger beetle *Cicindela dorsalis* (Coleoptera: Cicindelidae). *Ann. Entomol. Soc. Am.* 86:142–152.

WINK, M. 1993. Allelochemical properties or the raison d'être of alkaloids. *Alkaloids* 43:1–118.

WINK, M., and SCHNEIDER, D. 1988. Carrier-mediated uptake of pyrrolizidine alkaloids in larvae of the aposematic and alkaloid-exploiting moth *Creatonotos*. *Naturwissenschaften* 75:524–525.

WINK, M., and SCHNEIDER, D. 1990. Fate of plant-derived secondary metabolites in three moth species (*Syntomis mogadorensis*, *Syntomeida epilais*, and *Creatonotos transiens*). *J. Comp. Physiol. B.* 160:389–400.

WINK, M., MONTLLOR, C. B., BERNAYS, E. A., and WITTE, L. 1991. *Uresiphita reversalis* (Lepidoptera: Pyralidae): Carrier-mediated uptake and sequestration of quinolizidine alkaloids obtained from the host plant *Teline monspessulana*. *Z. Naturforsch.* 46c:1080–1088.

WINK, M., and WITTE, L. 1991. Storage of quinolizidine alkaloids in *Macrosiphum albifrons* and *Aphis genistae* (Homoptera: Aphididae). *Entomol. Gen.* 15:237–254.

WINK, M., SCHNEIDER, D., and WITTE, L. 1988. Biosynthesis of pyrrolizidine alkaloid-derived pheromones in the arctiid moth *Creatonotos transiens*. Stereochemical conversion of heliotrine. *Z. Naturforsch. Sect. C Biosci.* 43:737–741.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

ABSOLUTE CONFIGURATION OF 4-METHYL-3-HEPTANONE AND 4-METHYL-3-HEPTANOL FROM MANDIBULAR GLANDS OF VIRGIN MALES AND FEMALES OF *Atta sexdens rubropilosa*

RUTH R. DO NASCIMENTO,[1,*] E. DAVID MORGAN,[2] W. A. KÖNIG,[3] and TEREZINHA M. C. DELLA LUCIA[4]

[1]*Departamento de Química, Universidade Federal de Alagoas BR 101 Norte, Km 14, Tabuleiro, 57072-970, Maceió-Alagoas, Brasil*

[2]*Department of Chemistry, Keele University Keele, Staffordshire ST5 5BG, England*

[3]*Institut für Organische Chemie der Universität Hamburg D-20146 Hamburg, Germany*

[4]*Departamento de Biologia Animal, Universidade Federal de Viçosa 36570, Viçosa, Minas Gerais, Brasil*

(Received June 12, 1996; accepted January 25, 1997)

Abstract—(*S*)-(+)-4-Methyl-3-heptanone is the principal component in the secretion from the mandibular glands of unmated and mated males and females of *Atta sexdens rubropilosa*. In mated and unmated females, it is essentially the only component in the glandular secretion. In unmated males up to 50% of the secretion is a mixture of (3*S*, 4*S*)- and (3*R*, 4*S*)-4-methyl-3-heptanol. After mating, the amount of alcohol in the males decreases markedly.

Key Words—4-Methyl-3-heptanone, 4-methyl-3-heptanol, *Atta sexdens rubropilosa*, male, female, chirality, Hymenoptera, Formicidae, gas chromatography-mass spectrometry, chiral column gas chromatography.

INTRODUCTION

The mandibular glands are a pair of thin-walled sacs situated in the head of an ant. These glands may be filled with an enormous diversity of chemicals such as straight- and branched-chain alcohols, aldehydes, ketones, and pyrazines

*To whom correspondence should be addressed.

0098-0331/97/0600-1569$12.50/0 © 1997 Plenum Publishing Corporation

(Attygalle and Morgan, 1984). Mandibular gland volatiles quite often play an important role in the alarm-defense behavior of the species. Pasteels et al. (1980, 1981) described 4-methyl-3-heptanol and 4-methyl-3-heptanone in the mandibular glands of sexuals of the ant *Tetramorium caespitum*, but in general, few studies have been carried out on the mandibular glands of sexuals in ants.

The secretion from the mandibular glands of leaf-cutting ant workers, whose activities are limited to the New World, are predominantly composed of straight- and branched-chain ethyl ketones, although monoterpenoids are also found there. 4-Methyl-3-heptanone was present in the secretion from the mandibular glands of some *Atta* species, namely *Atta sexdens*, *A. laevigata*, *A. robusta*, *A. capiguara*, *A. bisphaerica*, *A. cephalotes*, and *A. texana* (Blum et al., 1968; Riley et al., 1974b).

The activity of sexuals outside the nest is restricted to a few days of the year, a factor that contributes to making the study of their exocrine secretions a difficult task. The secretion from the mandibular glands of the sexual forms of leaf-cutting ants has been analyzed only for *A. s. rubropilosa* (Do Nascimento et al., 1993). 4-Methyl-3-heptanone is the principal component of the secretions of unmated females and mated females and males, while the secretion from unmated males contains almost equal amounts of 4-methyl-3-heptanone and its corresponding alcohol, 4-methyl-3-heptanol. As part of this study, Bento et al. (unpublished results) demonstrated the role in laboratory colonies that a mixture of 4-methyl-3-heptanone and 4-methyl-3-heptanol plays in attracting workers to protect sexual forms prior to the nuptial flight.

4-Methyl-3-heptanone possesses an asymmetric carbon atom at C-4. In ants, this ketone, like other methyl-branched ketones, can be synthesized with a very precise degree of specificity. For example, workers of *Atta cephalotes* and *A. texana* produce (*S*)-(+)4-methyl-3-heptanone and respond more readily to this enantiomer than to the (*R*)-(−)-enantiomer (Riley et al., 1974a). (*S*)-4-Methyl-3-heptanone is the most active component in the mandibular glands of *A. cephalotes* and *A. texana*, acting as an alarm pheromone (Riley et al., 1974a). It is also reported as a component in the secretion of a number of species of ants, and in the Mutillidae within Hymenoptera and in glands of Opiliones outside the Insecta (Blum, 1981).

4-Methyl-3-heptanol possess two asymmetric centers on adjacent carbon atoms, and thus can exist in four enantiomeric forms. It is found, together with methylheptanone, in *A. cephalotes* and *A. texana* mandibular glands (Riley et al., 1974b) and in five species of *Pogonomyrmex* (Blum, 1981). We recently found the alcohol and ketone in the venom glands of *Aphaenogaster albisetosus* and *A. cockerelli* (Hölldobler et al., 1995). In most cases, its chirality has not been determined. Steghaus-Kovac et al. (1992) showed that the ant *Leptogenys diminuta*, which uses 4-methyl-3-heptanol as its trail pheromone, responds only to the 3*R*,4*R* isomer. Wadhams et al. (1982) showed that the bark beetle *Scolytus scolytus* had antennal receptors for both the (−)-*threo* and (−)-*erythro* isomers

but responded differently to them. In *Aphaenogaster albisetosus* an 8:2 mixture of (*S*)- and (*R*)-4-methyl-3-heptanone constitute the trail pheromone.

In this paper we report the absolute configuration of 4-methyl-3-heptanol and 4-methyl-3-heptanone from the mandibular glands of sexuals of *A. s. rubropilosa* as part of our attempts to understand the function of these compounds in the reproductive stage of this species.

METHODS AND MATERIALS

Unmated males and females of *A. s. rubropilosa* were collected in Viçosa (Minas Gerais, Brazil) during the nuptial flight. Their heads were removed, sealed in glass capillaries, and sent to Keele for analysis.

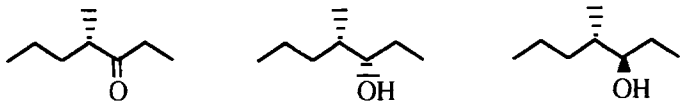
Extracts of the mandibular glands of unmated females and males were obtained by macerating each head in hexane (200 μl) with a tissue grinder, consisting of a Pyrex test tube (3 cm × 8 mm ID) and a glass rod with a spherical knob at one end, which had been roughened by griding with carborundum paste. One part of each extract was used for gas chromatography–mass spectrometry (GC-MS) analyses and the other part was placed in glass ampoules and sent to Hamburg to be analyzed by gas chromatography using a chiral-phase capillary column.

Gas chromatography–mass spectrometry was performed on a Hewlett Packard 5890 gas chromatograph and 5970 B mass selective detector (quadrupole mass spectrometer using 70 eV electron impact ionization). The system was controlled by the Hewlett Packard Series 300 computer with HP 59970 C ChemStation. Chromatography was carried out on a 5% phenyl–95% methylsiloxane phase (nonpolar) in a fused silica capillary column (12 m × 0.32 m × 0.25 μm film thickness) linked directly to the mass spectrometer source by a 5-m length of deactivated fused silica capillary tubing (0.2 mm ID). Helium was used as the carrier gas at 1 ml/min. The GC injection port was held at 150°C, and the oven was programmed from 30 to 200°C at 8°C/min. The split vent was closed before injection of the sample and reopened 50 sec later.

Chiral column gas chromatography was carried out on an octakis [6-O-methyl-2,3-di-*O*-pentyl-γ-cyclodextrin phase (50% w/w in polysiloxane OV 1701, 25 m × 0.25 mm ID) fused silica capillary column]. The oven was held isothermally at 60°C for the analyses of 4-methyl-3-heptanone and 4-methyl-3-heptanol.

RESULTS AND DISCUSSION

Secretions of unmated females and mated males and females are mainly composed of 4-methyl-3-heptanone (94.8, 91.7, and 81.8%, respectively) (Figures 2 and 3 in do Nascimento et al., 1993). Secretions from unmated males



[1] [2] [3]

FIG. 1. Structures of ketone (**1**) and heptanols (**2** and **3**).

contain almost equal amounts of the alkanone and its corresponding alcohol, 4-methyl-3-heptanol. Chiral column gas chromatography demonstrated that the *S* enantiomer of the ketone (Figure 1, **1**) dominates the secretion of both sexes with an enantiomeric excess of at least 98.8% with trace amounts of the *R* enantiomer (Figures 2 and 3). The 4-Methyl-3-heptanol in the secretion from mandibular glands of unmated males consisted of equal proportions of two of the possible four isomers, namely (3*S*,4*S*)- and (3*R*,4*S*)-4-methyl-3-heptanol (Figure 1, **2** and **3**).

Very likely, 4-methyl-3-heptanone is produced in an enantiomerically pure form, and the small amount of the *R* isomer may be formed through enolization after the ants were collected. Reduction to the alcohol appears to occur in a

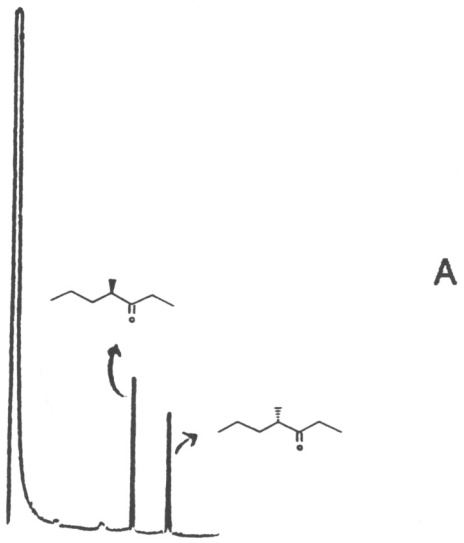


FIG. 2. Chiral column gas chromatography of (A) a solution of racemic 4-methyl-3-heptanone, (B) (*S*)-4-methyl-3-heptanone, and (C) a hexane extract of a single head of an unmated female of *A. s. rubropilosa.*

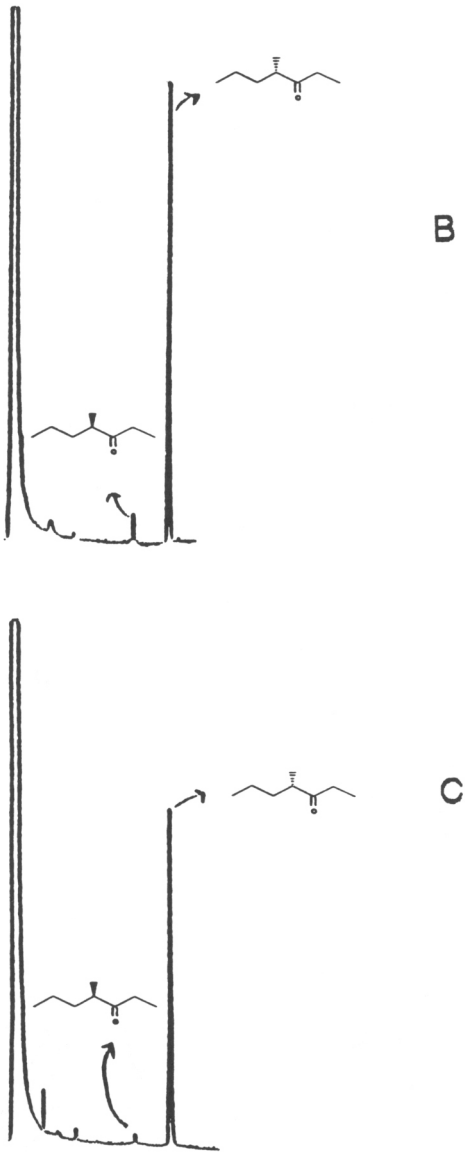


FIG 2. Continued.

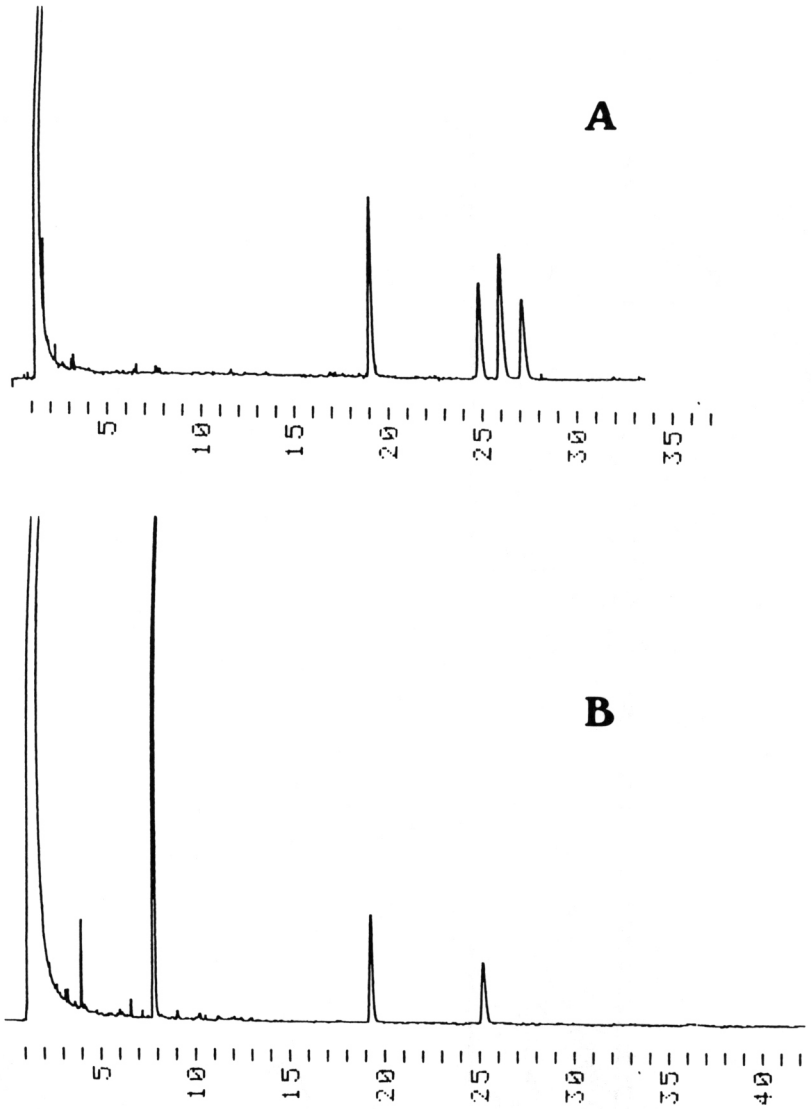


FIG. 3. Chiral column gas chromatograms of (A) racemic 4-methyl-3-heptanol and (B) a hexane extract of a single head of an unmated male of *A. s. rubropilosa* showing that only two of the four possible isomers are present; the two *erythro* isomers elute first and third, the two *threo* isomers elute second and fourth.

completely achiral way to give both enantiomers at C-4 but preserving the purity of the chiral center at C-3.

Acknowledgments—R. R. do N. thanks the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-RHAE) for financial support (grant 260073/90.3) and E. D. M. thanks the Science and Engineering Research Council for a grant to purchase equipment.

REFERENCES

ATTYGALLE, A. B., and MORGAN, E. D. 1984. Chemicals from the glands of ants. *Chem. Soc. Rev.* 13:245–278.

BLUM, M. S. 1981. Chemical Defenses of Arthropods. Academic Press, New York, pp. 96, 145–146.

BLUM, M. S., PADOVANI, F., and AMANTE, E. 1968. Alkanones and terpenes in the mandibular glands of *Atta* species (Hymenoptera: Formicidae). *Comp. Biochem. Physiol.* 16:291–299.

DO NASCIMENTO, R. R., MORGAN, E. D., BILLEN, J., SCHOETERS, E., DELLA LUCIA, T. M. C., and BENTO, J. M. S. 1993. Variation with caste of the mandibular gland secretion in the leaf-cutting ant, *Atta sexdens rubropilosa*. *J. Chem. Ecol.* 19(5):907–918.

HÖLLDOBLER, B., OLDHAM, N. J., MORGAN, E. D., and KÖNIG, W. A. 1995. Recruitment pheromones in the ants *Aphaenogaster albisetosus* and *A. cockerelli* (Hymenoptera: Formicidae). *J. Insect Physiol.* 41(9):739–744.

PASTEELS, J. M., VERHAEGHE, J. C., BRAECKMAN, J. C., DALOZE, D., and TURSCH, B. 1980. Caste dependent pheromones in the head of the ant *Tetramorium caespitum*. *J. Chem. Ecol.* 6:467–472.

PASTEELS, J. M., VERHAEGHE, J. C., OTTINGER, R., BRAECKMAN, J. C., and DALOZE, D. 1981. Absolute configuration of (3*R*,4*S*)-4-methyl-3-hexanol—a pheromone from the head of the ant *Tetramorium impurum* Foerster. *Insect Biochem.* 11:675–678.

RILEY, R. G., SILVERSTEIN, R. M., and MOSER, J. C. 1974a. Biological responses of *Atta texana* to its alarm pheromone and the enantiomer of the pheromone. *Science* 183:760–762.

RILEY, R. G., SILVERSTEIN, R. M., and MOSER, J. C. 1974b. Isolation, identification, synthesis and biological activity of volatile compounds from the heads of *Atta* ants. *J. Insect Physiol.* 20:1629–1637.

STEGHAUS-KOVAC, S., MASCHWITZ, U., ATTYGALLE, A. B., FRIGHETTO, R. T. S., FRIGHETTO, N., VOSTROWSKY, O., and BESTMANN, H. J. 1992. Trail-following responses of *Leptogenys diminuta* to stereoisomers of 4-methyl-3-heptanol. *Experientia* 48:690–694.

WADHAMS, L. J., ANGST, M. E., and BLIGHT, M. M. 1982. Responses of the olfactory receptors of *Scolytus scolytus* (F.) (Coleoptera: Scolytidae) to the stereoisomers of 4-methyl-3-heptanol. *J. Chem. Ecol.* 8:477–492.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

DEFINING SOURCES OF COFFEE PLANT ODOR ATTRACTIVE TO *Ceratitis capitata* FLIES

RONALD J. PROKOPY,[1,*] THOMAS W. PHILLIPS,[2] ROGER I. VARGAS,[2] and ERIC B. JANG[2]

[1]*Department of Entomology*
University of Massachusetts
Amherst, Massachusetts 01003

[2]*USDA Tropical Fruit and Vegetable Laboratory*
Box 4459
Hilo, Hawaii 96720

(Received September 9, 1996; accepted January 16, 1997)

Abstract—We evaluated attraction of released mature laboratory-cultured Mediterranean fruit flies to different sources of coffee plant odor placed in potted nonfruiting guava trees in outdoor field cages. Volatiles from crushed medium or dark red fruit of *Coffea arabica* cv. arabica plants were significantly more attractive than volatiles from cut leaves or stems of such plants, volatiles from less-ripe crushed *C. arabica* fruit, and volatiles from crushed red fruit of *C. racemosa*, *C. canephora*, or *C. dewevari*. Volatiles from *C. arabica* cv. arabica crushed red fruit were equally attractive as volatiles from crushed red fruit of *C. congensis* or *C. arabica* cv. mundo, cv. bourbon, cv. kents or cv. catura. Volatiles from as little as 2 g of crushed red *C. arabica* fruit (= 1 fruit) were as attractive as volatiles from 32 g of such fruit, demonstrating sensitivity of the bioassay approach used to a small amount of source material. Odor of *C. arabica* red fruit refrigerated for 1–10 days after picking was significantly more attractive than odor of fresh-picked fruit, while odor of a 24-hr water extract of intact red *C. arabica* fruit was significantly more attractive than odor of 24-hr extracts of such fruit with methanol, methylene chloride, or hexane or 1- or 6-hr extracts with water. Extraction studies suggested that at least some of the volatiles of red coffee fruit attractive to medflies may be polar water-soluble molecules. In our final test, volatiles from crushed red *C. arabica* fruit trapped on Super Q and eluted with methylene chloride proved just as attractive as volatiles emanating directly from crushed fruit of the same type. Together, our findings define optimal source

*To whom correspondence should be addressed.

0098-0331/97/0600-1577$12.50/0 © 1997 Plenum Publishing Corporation

material and effective handling procedures of source material for future identification of volatile components of coffee fruit attractive to medflies.

Key Words—Coffee, *Ceratitis capitata*, odor, attractants.

INTRODUCTION

Several species of tephritid flies are known to be attracted to odors emanating from food sources, mates, and ovipositional sites (reviewed in Jang and Light, 1996). Among the most economically important tephritids is the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), known worldwide to infest more than 300 species of fruits and vegetables (Liquido et al., 1991). Recent studies have uncovered specific compounds from food, mates, and ovipositional sites that are attractive to medflies (reviewed in Millar, 1995; Jang and Light, 1996; Light and Jang, 1996), but to date there has been no systematic investigation of the identity of potentially attractive compounds emitted from the presumed ancestral host of medflies, fruit of coffee plants (*Coffea* spp.). That odor of coffee fruit is in fact attractive to medflies has been demonstrated in wind-tunnel, field-cage, and field assays (Prokopy and Vargas, 1996; Lance et al., unpublished data). Indeed, Prokopy and Vargas (1996) found that odor of coffee fruit was significantly more attractive to medflies than fruit odor of five other host plants and three nonhost plants.

In patches of nonfruiting host trees in outdoor field cages, we conducted experiments aimed at defining optimal source material and effective handling procedures of source material for future identification of volatile components of coffee attractive to medflies. Specifically, we evaluated different structures of coffee plants (fruit, foliage, and woody tissue), different ripeness stages of coffee fruit, different parts of coffee fruit (skin and flesh, seeds), ripe fruit of different species of *Coffea*, ripe fruit of different cultivars of *Coffea arabica*, and different dosage equivalents of ripe *C. arabica* fruit. We also evaluated different periods and methods of cold storage, different solvents for extracting attractive components, and different approaches to and durations of water-based solvent extraction of ripe coffee fruit. Finally, we evaluated the attractiveness of a head-space collection of volatiles from ripe coffee fruit.

METHODS AND MATERIALS

Source of Flies. All medflies used in our tests originated from a laboratory colony in culture for about 20 generations at the USDA Tropical Fruit and Vegetable Research Laboratory in Honolulu, Hawaii. Until tested when mature at 7–10 days of age, both sexes were held together from eclosion onward in 30 × 30 × 30-cm screened cages supplied with protein (enzymatic yeast hydro-

lysate), sucrose, and water, but not fruit, under conditions of about 24°C, 50% relative humidity and 13 hr natural daylength. Mature females from this same culture tested one year earlier were found to respond similarly to medfly females of wild origin to coffee fruit odor (Prokopy and Vargas, 1996).

Bioassay. All trials were carried out from 09:00 to 12:00 hr and from 13:00 to 16:00 hr in two cylindrical, 3-m-tall × 3-m-diam. clear nylon-screen enclosures placed beneath the protection of a partly opaque plastic roof on the grounds of the University of Hawaii field station at Kainaliu (Kona), Hawaii. Flow of wind through the cages was not regulated but rarely exceeded 2 km/hr. Each cage contained five potted nonfruiting guava trees, whose bases were grouped near the cage center and which together formed a foliar canopy of 2.2 m diam., extending to within 40 cm of the cage wall and reaching the cage ceiling. Before use, the foliage and branches of each tree were rinsed thoroughly with water.

At each of six positions within each cage, we hung a single container of test substance. The positions were about 75 cm apart along the circumference of an imaginary circle about 30 cm inward from the periphery of the canopy and about 20 cm below the cage ceiling. In all but one experiment, containers of test substances were cylindrical clear-plastic cups (9 cm diam. × 6 cm tall) whose interior walls and bottoms were covered with aluminum foil to obscure visibility of contents within. All but the outer 1 cm of the clear-plastic lid of a cup consisted of nylon netting that effectively obscured visibility of contents but permitted air flow. In two experiments (Table 3 below, experiments 1 and 2) clear glass Petri dishes (9 cm diam. × 1.5 cm tall) were substituted for clear plastic containers.

At 08:30 and 12:30 hr each test day, about 200 flies of each sex were released as a group at the base of the canopy in each cage. Flies that arrived on the screened area of the lid of a container within the 5-min period allotted each replicate were considered as responding to odor of the contents, were sexed, and were removed within 30–60 sec of arrival via an aspirator held by one of us, who continuously circled the perimeter of the foliar canopy watching for flies arriving on treatment containers. Even though we released approximately as many males as females for each experiment, comparatively fewer males responded to test odors. Among males that did respond, response patterns to the same treatment were highly variable across experiments. Hence, we chose to exclude from consideration here all data on male responders. In previous tests in field cages, medfly males from the same culture tested here likewise proved inconsistent in response pattern to coffee odor (Prokopy and Vargas, 1996). For the first replicate, treatment positions were randomized. Treatments were rotated clockwise within the canopy after each replicate until each treatment occupied each position once, requiring a total of about 35 min to complete set of six replicates. For the next set of six replicates, we used freshly prepared sources of treatment odor that were deployed in a newly arranged random order. From

09:00 to 12:00 hr, we were able to complete three sets of replicates of the same experiment per field cage, provided weather conditions were favorable (i.e., no rainfall and temperatures between 24 and 33°C). The same was true for trials conducted between 13:00 and 16:00 hr. By the end of 3 hr, the number of flies in each field cage that responded to treatments had reached a low level, with most nonresponders having flown to the cage wall. We did not collect such nonresponding flies before the start of the next set of replicates but instead allowed them to remain in the cage. At the beginning of each set of replicates, we sprinkled canopy foliage with water and dabbed several leaves at the center of the canopy with food (same type as presented in fly maintenance cages) to minimize fly responses to treatments on the basis of thirst and hunger.

Source of Test Material. Except where stated otherwise, odor source material used in all experiments consisted of medium or dark red fruit of *C. arabica* cv. arabica tested when fresh (within 8 hr of picking) or after storage for one to two days at 4°C. Each fruit weighed about 2 g. Previously, we found that odor of crushed coffee fruit was more attractive to medflies than odor of intact fruit (Prokopy and Vargas, 1996). To crush fruit, we placed it beneath an Erlenmeyer flask and pressed and rotated for about 10 sec, until the skin of each was torn open and the seeds exposed. Foliage and stems tested in experiment 1 (Table 1 below) consisted of middle-aged leaves or stems (proximal of the most recent growth) cut into 25 pieces about 4 cm long. All odor source material was rinsed in water and dried before preparation. Each trial was initiated within 5 min following the end of preparation of source material.

For experiments described in Table 3 (below), unless specified otherwise, 60 g of whole fruit was allowed to soak in 60 ml solvent (water, methanol, methylene chloride, or hexane) in an enclosed container for 24 hr at 25°C, after which solvent was poured off and stored at −20°C until use. According to protocol, 2 ml (experiments 1 and 2) or 4 ml (experiments 3 and 4) of solvent extract was pipetted into each test container. In experiment 2, 30 ml of water extract of fruit was mixed thoroughly with 30 ml of methylene chloride, which, within 5 min, formed two clearly separate phases; test material was drawn from the methylene chloride phase on the bottom. Solvent controls consisted of 2 ml each of methanol, methylene chloride, and hexane (experiment 1) or 2 ml of methylene chloride (experiment 2). For experiments 1 and 2, solvent extracts and solvent controls were exposed to air for 20 min to permit complete evaporation of solvent, following which 2 ml of water was added to each container before testing.

For collection of headspace volatiles from coffee fruit, 167 g of fruit was crushed in 167 ml of distilled water. This preparation was placed in a 500-ml Erlenmeyer flask fitted with a cork stopper equipped with two vertical glass tubes, one of 250 × 4 mm that served as an air inlet and extended into the liquid, and the second of 80 × 4 mm that terminated above the liquid and

allowed for air exit. Headspace volatiles were trapped on a glass column, 100 × 4 mm and packed with 175 mg of Super-Q adsorbent (Alltech Assoc., Deerfield, Illinois), connected above the exit tube. Inlet air was filtered through activated charcoal (60/40 mesh) in a 100 × 10-mm glass column and passed through the volatile collection system at 60 ml/min by means of a vacuum pump connected upwind of the Super-Q column. An identical collection setup was made that contained water only to serve as a control. Volatiles were collected from the fruit and the water control for 24 hr, after which each Super-Q column was eluted with 6 ml of methylene chloride. Extracts were not concentrated. For bioassay, 100 μl of fruit volatile extract, equal to an amount from 66 g collected for 1 hr, was applied to filter paper; 5 min was allowed for evaporation of methylene chloride prior to testing. Control extract consisted of 100 μl of methylene chloride extract of volatiles collected from water. Two milliliters of water was added to each test container before assay.

Statistical Analysis. Data were analyzed by two-way analysis of variance (ANOVA). Treatment means were compared using the least significant difference test criterion (0.05 level). For ANOVA, the 36 replicates per treatment per experiment (characteristic of most experiments) were grouped into six sets of six replicates each. For each treatment, the value of a single set of six replicates consisted of the number of females arriving on the screen of a container for all six positions of that treatment combined. We reasoned that grouping the data into sets of replicates for ANOVA was the most valid approach to minimize the varying effects of treatment position within the tree canopy on fly response to replicates within a set.

RESULTS

The first set of experiments characterized sources and amounts of *Coffea* plant material most attractive to medfly females (Table 1). Crushed red fruit of *C. arabica* proved more attractive than cut leaves or cut stems of *C. arabica* and more attractive than crushed red fruit or cut leaves of *Ptychosperma macarthurii* (Wendell) palm trees (experiment 1). Among these sources, only crushed fruit and cut leaves of *C. arabica* proved more attractive than water. Among different ripeness stages of *C. arabica* fruit, medium red and dark red fruit were equally attractive, and both types were more attractive than orange, yellow or green fruit (experiment 2). Fruits of each ripeness stage were more attractive than water. Flies were attracted just as strongly to intact seeds of crushed red *C. arabica* fruit as to the skin and flesh (epicarp) of the fruit (experiment 3). Crushed red fruit of *C. arabica* and *C. congensis* proved equally attractive and more attractive than crushed red fruit of *C. racemosa*, *C. canephora*, and *C. dewevari*, all of which proved more attractive than water (experiment 4).

TABLE 1. ATTRACTION OF RELEASED *C. capitata* FEMALES TO ODOR OF DIFFERENT TYPES OR AMOUNTS OF SOURCE MATERIAL IN CONTAINERS IN NONFRUITING FIELD-CAGED TREES

Experiment	Replicates (*N*)	Source	Treatment	Arriving females per replicate (mean ± SE)[a]
1	36	*C. arabica* cv. arabica	4 g crushed red fruit	2.4 ± 0.8 a
		C. arabica cv. arabica	4 g cut leaves	1.4 ± 0.3 b
		C. arabica cv. arabica	4 g cut stems	0.8 ± 0.2 bc
		P. macarthurii	4 g crushed red fruit	0.5 ± 0.1 c
		P. macarthurii	4 g cut leaves	0.6 ± 0.3 bc
			water	0.2 ± 0.1 c
2	36	*C. arabica* cv. arabica	16 g crushed green fruit	0.9 ± 0.1 b
		C. arabica cv. arabica	16 g crushed yellow fruit	0.9 ± 0.2 b
		C. arabica cv. arabica	16 g crushed orange fruit	1.1 ± 0.2 b
		C. arabica cv. arabica	16 g crushed medium red fruit	2.0 ± 0.4 a
		C. arabica cv. arabica	16 g crushed dark red fruit	2.4 ± 0.2 a
			water	0.3 ± 0.1 c
3	18	*C. arabica* cv. arabica	8 g crushed skin and flesh of red fruit	3.0 ± 0.5 a
		C. arabica cv. arabica	8 g intact seeds of red fruit	4.0 ± 0.7 a
			water	0.6 ± 0.1 b
4	36	*C. arabica* cv. arabica	8 g crushed red fruit	1.8 ± 0.2 a
		C. congensis	8 g crushed red fruit	1.9 ± 0.3 a
		C. racemosa	8 g crushed red fruit	1.3 ± 0.3 b
		C. canephora	8 g crushed red fruit	1.2 ± 0.2 b
		C. dewevari	8 g crushed red fruit	0.8 ± 0.2 c
			water	0.2 ± 0.1 d
5	36	*C. arabica* cv. arabica	8 g crushed red fruit	1.7 ± 0.3 a
		C. arabica cv. mundo	8 g crushed red fruit	1.7 ± 0.4 a
		C. arabica cv. bourbon	8 g crushed red fruit	1.7 ± 0.4 a
		C. arabica cv. kents	8 g crushed red fruit	1.5 ± 0.3 a
		C. arabica cv. catura	8 g crushed red fruit	1.3 ± 0.4 a
			water	0.2 ± 0.1 b
6	36	*C. arabica* cv. arabica	32 g crushed red fruit	1.8 ± 0.5 a
		C. arabica cv. arabica	16 g crushed red fruit	1.9 ± 0.5 a
		C. arabica cv. arabica	8 g crushed red fruit	1.7 ± 0.4 a
		C. arabica cv. arabica	4 g crushed red fruit	1.6 ± 0.5 a
		C. arabica cv. arabica	2 g crushed red fruit	1.4 ± 0.3 a
			water	0.2 ± 0.1 b

[a] Values in each experiment followed by the same letter are not significantly different at the 0.05 level according to least significant difference tests.

Crushed red fruit of five cultivars of *C. arabica* (arabica, mundo, bourbon, kents, catura) were equally attractive; all were more attractive than water (experiment 5). Finally, medflies were equally attracted to 2, 4, 8, 16, or 32 g of crushed red *C. arabica* fruit and more attracted to each of these amounts than to water (experiment 6).

The second set of experiments was designed to characterize procedures for handling red fruit of *C. arabica* that would permit maximum attractiveness of fruit to medflies (Table 2). Among fruit crushed just before testing, those stored at 4°C for 1, 5, or 10 days after picking were equally attractive and more attractive than water (experiment 1). Interestingly, unstored fruit tested within 8 hr after picking was not more attractive than water. Furthermore, of fruit crushed just before testing, fruit stored for one day at 4°C, frozen at −20°C for 24 hr, and then thawed at 25°C for 2 hr before testing proved just as attractive as fruit stored for one day at 4°C and not frozen before testing (experiment 2).

Our third set of experiments concerned identifying an appropriate solvent and procedures for extracting components of red *C. arabica* fruit attractive to medflies (Table 3). Of the four solvents used for a period of 24 hr, water yielded a more attractive extract than did methanol, which in turn was superior to methylene chloride and hexane (experiment 1). In fact, water extract proved more attractive than unextracted freshly crushed fruit, whereas hexane extract proved less attractive than the solvent control. Females were more responsive to a 24-hr water extract of whole fruit than to 6-hr or 1-hr water extracts of

TABLE 2. ATTRACTION OF RELEASED *C. capitata* FEMALES TO ODOR OF CRUSHED RED FRUIT OF *C. arabica* CV. arabica (8 GRAMS PER CONTAINER) HANDLED IN DIFFERENT WAYS BEFORE TESTING IN NONFRUITING FIELD-CAGED TREES

Experiment	Replicates (*N*)	Treatment[a]	Arriving females per replicate (mean ± SE)[b]
1	30	0 days of storage before test	0.8 ± 0.2 bc
		1-day storage before test	1.2 ± 0.4 ab
		5-day storage before test	1.4 ± 0.3 a
		10-day storage before test	1.0 ± 0.2 ab
		Water (= control treatment)	0.3 ± 0.1 c
2	27	Frozen and thawed before test	1.7 ± 0.5 a
		Not frozen before test	1.6 ± 0.3 a
		Water (= control treatment)	0.2 ± 0.1 b

[a]Conditions for storage: 4°C; freezing: −20°C.
[b]Values in each experiment followed by the same letter are not significantly different at the 0.05 level according to least significant difference tests.

TABLE 3. ATTRACTION OF RELEASED *C. capitata* FEMALES TO ODOR OF RED FRUIT OF *C. arabica* CV. arabica FOLLOWING EXTRACTION OF WHOLE FRUIT USING DIFFERENT SOLVENTS, EXTRACT PROCEDURES, OR DURATIONS OF EXTRACTION BEFORE TESTING IN NONFRUITING FIELD-CAGED TREES

Experiment	Replicates (*N*)	Treatment[a]	Arriving females per replicate (mean ± SE)[b]
1	36	24-hr water extract	3.6 ± 0.3 a
		24-hr methanol extract plus water	1.1 ± 0.2 c
		24-hr methylene chloride extract plus water	0.5 ± 0.2 de
		24-hr hexane extract plus water	0.2 ± 0.1 e
		Crushed fruit (no extract)	1.7 ± 0.3 b
		Solvent control plus water	0.8 ± 0.2 cd
2	18	Water extract	2.4 ± 0.4 a
		Methylene chloride extract (of water extract) plus water	1.1 ± 0.3 b
		Solvent control plus water	0.3 ± 0.1 c
3	30	24-hr water extract	2.6 ± 0.5 a
		6-hr water extract	1.0 ± 0.1 bc
		1-hr water extract	0.5 ± 0.1 c
		Crushed fruit (no extract)	1.5 ± 0.2 b
		Water	0.2 ± 0.1 c
4	30	16-hr water extract of whole fruit	1.7 ± 0.3 a
		16-hr water extract of crushed fruit	2.2 ± 0.3 a
		Water	0.3 ± 0.1 b

[a] Fruit equivalents per test container in experiments 1–4, respectively, were 2, 2, 4, and 4 g.
[b] Values in each experiment followed by the same letter are not significantly different at the 0.05 level according to least significant difference tests.

whole fruit (experiment 3). Again, the former was more attractive than unextracted freshly crushed fruit, whereas the 6-hr and 1-hr extracts were not more attractive than water alone. Lastly, a 16-hr extract of whole fruit was as attractive as a 16-hr extract of crushed fruit (experiment 4).

In our final experiment, volatiles from crushed red fruit of *C. arabica* trapped on the adsorbent Super-Q and subsequently eluted with methylene chloride proved just as attractive as volatiles emanating directly from crushed fruit of the same type ($\bar{X}$ + SE = 3.2 ± 0.9 vs. 2.6 ± 0.4, N = 18) and more attractive than volatiles from water alone adsorbed on Super-Q and subsequently eluted with methylene chloride ($\bar{X}$ ± SE = 0.9 ± 0.4, N = 18).

DISCUSSION

Our findings indicate that volatiles from red fruit of *C. arabica* cf. arabica are more attractive to laboratory-cultured medfly females than volatiles from

foliage or stems removed from plants bearing red fruit, are more attractive than volatiles from less-ripe fruit, are equally or more attractive than volatiles from red fruit of four other species of *Coffea* plants tested, and are equally attractive as volatiles from red fruit of four other cultivars of *C. arabica* tested. Our field cage bioassay approach proved sufficiently sensitive that volatiles from as little as 2 g of crushed red *C. arabica* fruit (= 1 fruit) attracted as many females as volatiles from a batch of 32 g of crushed red fruit, thereby allowing us to use, in some experiments subsequent to the above, as little as 2 g of source material per test container. In these experiments, *C. arabica* fruit refrigerated 1–10 after picking proved superior in attractiveness to fresh-picked unstored fruit. Moreover, a water extract of fruit proved more attractive than a methanol, methylene chloride, or hexane extract, and a 24-hr water extract of fruit proved superior to a 6-hr or 1-hr extract.

Our findings contrast in part with those of a recent study of responses of adult coffee berry borers, *Hypothenemus hampei* Ferari (Coleoptera: Scolytidae), to solvent extracts of various structures of *C. canephora* plants (Guttierrez-Martinez and Ondarza, 1996). Coffee berry borers were more attracted to methylene chloride extracts than to methanol or water extracts of mature coffee fruit (hexane was not evaluated as a solvent) and were just as attracted to whole (or components of) immature (green) fruit as to whole (or components of) mature (red) fruit. As in our study, volatiles from coffee fruit were more attractive to coffee berry borers than were volatiles from coffee leaves or stems. It should not be surprising that coffee berry borers are comparatively more responsive than medflies to the odor of green coffee fruit because green fruit is accepted equally as mature fruit for oviposition by coffee berry borer females (Gutierrez-Martinez and Ondarza, 1996), whereas medflies strongly prefer mature fruit for egg laying (Vargas et al., 1995). Interestingly, both coffee berry borers and medflies (Table 1, experiment 3) are just as attracted to freshly exposed seeds (beans) of coffee fruit as to freshly exposed epicarp of coffee fruit.

Two lines of evidence from our findings suggest that at least some of the volatiles of red coffee fruit attractive to medflies may be polar molecules characteristic of or associated with ripe or senescing fruit of many types. First, attraction was greatest to an extract using water, a highly polar solvent, and successively less to extracts using methanol, methylene chloride, and hexane, solvents of successively diminishing polarity. Second, attraction was greater to fully mature fruit than to less mature fruit, fruit stored in a refrigerator for at least one day after picking than to fresh-picked fruit, and was greater to water extracts made over longer (24 hr) than shorter (1–6 hr) durations. Storage of coffee fruits in water or under refrigerator is likely to injure fruit tissue and result in the release or production of compounds related to senescence or degradation (e.g., Lyons, 1973). Medflies often have difficulty drilling through the skin of host fruit to reach ovipositional sites in the flesh and are quick to exploit already existing punctures in fruit for egg laying (Papaj et al., 1992). Conceiv-

ably, polar molecules are produced and released from tissues injured during the puncturing of a fruit. It is unlikely, however, that compounds common to injured fruit of a wide variety of species comprise the entire repertoire of fruit volatiles attractive to medflies. If this were so, then it would be difficult to explain the observed (Prokopy and Vargas, 1996) greater degree of attraction of medflies to intact or crushed ripe coffee fruit compared with intact or crushed ripe fruit of lower-ranking host and nonhost plants. Interestingly, odor of fermented fruit of host chapote, *Sargentia greggii*, is known to attract *Anastrepha ludens* Loew flies (Robacker et al., 1992). Furthermore, water extracts of ripe fruit of host box-orange, *Severinia buxifolia*, are more attractive to *A. suspensa* (Loew) flies than are methanol and hexane extracts (Nigg et al., 1994), and water extracts of ripe olive fruit (*Olea europaea*) are attractive to *Bactrocera oleae* Gmelin flies (Scarpati et al., 1996). With respect to *A. suspensa*, Nigg et al. (1994) suggested that in addition to water solubility of some of the attractive compounds they uncovered, water (and, in nature, dew) may aid volatilization of compounds insoluble in water.

In conclusion, our findings suggest that future attempts to identify components of coffee odor attractive to medflies should use as source material red fruit of *C. arabica* stored at 4°C for one to five days after picking. Freezing and thawing fruit after storage does not affect attractiveness. In capturing volatiles, we recommend using as a source either a 16 to 24-hr water extract of whole or crushed fruit or a 1 : 1 (w/v) mixture of crushed fruit and water. The field cage bioassay described here is sufficiently sensitive to permit use of as little as one fruit equivalent per test container for significant discrimination by medfly females.

Acknowledgments—We thank Tadao Urago, Marc Meisner, and Harold Stene for their assistance in collecting fruit and providing essential supplies, and the California Citrus Research Board for financial support of this work.

REFERENCES

GUTIERREZ-MARTINEZ, A., and ONDARZA, R. N. 1996. Kairomone effect of extracts from *Coffea canephora* on *Hypothenemus hampei*. *Environ. Entomol.* 25:96–100.

JANG, E. B., and LIGHT, D. M. 1996. Olfactory semiochemicals of tephritids, pp. 73–90, *in* B. A. McPheron and G. J. Steck (eds.). Fruit Fly Pests. St. Lucie Press, Delray Beach, Florida.

LIGHT, D. M., and JANG, E. B. 1996. Plant volatiles evoke and modulate tephritid behavior, pp. 123–133, *in* B. A. McPheron and G. L. Steck (eds.). Fruit Fly Pests. St. Lucie Press, Delray Beach, Florida.

LIQUIDO, N. J., SHINODA, L. A., and CUNNINGHAM, R. T. 1991. Host plants of the Mediterranean fruit fly: An annotated world review. *Misc. Public. Entomol. Soc. Am.* 77:1–52.

LYONS, J. M. 1973. Chilling injury in plants. *Annu. Rev. Plant Physiol.* 24:445–446.

MILLAR, J. 1995. An overview of attractants for the Mediterranean fruit fly, pp. 123–143, *in* J. G. Morse, R. L. Metcalf, J. R. Carey, and R. V. Dowell (eds.). The Medfly in California:

Defining Critical Research. College of Natural and Agricultural Sciences, University of California, Riverside.

NIGG, H. N., MALLORY, L. L., SIMPSON, S. E., CALLAHAM, S. B., TOTH, J. P., FRASER, S., KLIM, M., NAGY, S., NATION, J. L., and ATTAWAY, J. A. 1994. Caribbean fruit fly, *Anastrepha suspensa* (Loew), attraction to host fruit and host kairomones. *J. Chem. Ecol.* 20:727–743.

PAPAJ, D. R., AVERILL, A. L., PROKOPY, R. J., and WONG, T. T. Y. 1992. Host marking pheromone and use of previously established oviposition sites by the Mediterrean fruit fly. *J. Insect Behav.* 5:583–598.

PROKOPY, R. J., and VARGAS, R. I. 1996. Attraction of *Ceratitis capitata* flies to odor of coffee fruit. *J. Chem. Ecol.* 22:807–820.

ROBACKER, D. C., WARFIELD, W. C., and FLATH, R. A. 1992. A four-component attractant for the Mexican fruit fly, *Anastrepha ludens*, from host fruit. *J. Chem. Ecol.* 18:1239–1254.

SCARPATI, M. L., SCALZO, R. L., VITA, G., and GAMBARCORTA, A. 1996. Chemotropic behavior of female olive fly (*Bactrocera olea* Gmel.) on *Olea europaea* L. *J. Chem. Ecol.* 22:1027–1036.

VARGAS, R. I., WALSH, W. A., and NISHIDA, T. 1995. Colonization of newly planted coffee fields: Dominance of Mediterranean fruit fly over oriental fruit fly. *J. Econ. Entomol.* 88:620–627.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

COMPARISONS AND CONTRASTS IN HOST-FORAGING STRATEGIES OF TWO LARVAL PARASITOIDS WITH DIFFERENT DEGREES OF HOST SPECIFICITY

A. M. CORTESERO,[1,*] C. M. DE MORAES,[2] J. O. STAPEL,[1] J. H. TUMLINSON,[3] and W. J. LEWIS[1]

[1]*Insect Biology and Population Management Research Laboratory*
Agricultural Research Service, U.S. Department of Agriculture
Tifton, Georgia 31793

[2]*Department of Entomology,*
University of Georgia
Athens, Georgia 30605

[3]*Insect Attractants, Behavior, and Basic Biology Research Laboratory*
Agricultural Research Service, U.S. Department of Agriculture
Gainesville, Florida 32604

(Received July 2, 1996; accepted January 27, 1997)

Abstract—In theory, the degree of specificity of the signals a parasitoid species needs to successfully locate its host correlates with its level of specialization. We examined this question by comparing the foraging strategies of two parasitoids that differ in their host ranges. In wind-tunnel experiments, we investigated how systemically released herbivore-induced volatiles were used by the generalist parasitoid, *Cotesia marginiventris* (Cresson) and the specialist, *Microplitis croceipes* (Cresson). We determined the relative influence of these volatiles as compared to other signals emitted in the host orientation of the two parasitoids. Both the generalist and the specialist parasitoid strongly preferred *Spodoptera exigua* (Hübner) leaf-induced systemic plants over undamaged plants when no other information was available. When wasps were given a choice between leaf-induced and undamaged plants carrying other plant- or host-related materials, the responses differed for the two species. *C. marginiventris* appeared to cue primarily on recent damage volatiles, whereas *M. croceipes* appeared to cue primarily on host frass volatiles. However, recent damage on previously leaf-induced plants, was strongly preferred to recent damage on plants previously damaged by both species. When plants were induced at the squares by *Helicoverpa zea* (Boddie), only *M. croceipes* exhibited a preference for these plants over undamaged plants. The adaptive

*To whom correspondence should be addressed.

0098-0331/97/0600-1589$12.50/0 © 1997 Plenum Publishing Corporation

significance of the behaviors as related to dietary specializations of the parasitoids is discussed.

Key Words—Hymenoptera, Braconidae, *Microplitis croceipes, Cotesia marginiventris, Gossypium hirsutum*, volatile chemicals, systemic, generalist, specialist, host location, wind tunnel.

INTRODUCTION

Parasitoids exhibit multiple foraging strategies that are shaped by the host/plant system with which they interact. One current view of the evolution of foraging strategies in parasitoids is that the degree of specialization at a particular trophic level sets the degree of specificity of the information needed for successful foraging (Vet and Dicke, 1992). The greater the number of hosts or plants a parasitoid is able to use, the less specific the information it needs and vice versa. Because specialists depend upon only a few host species, they are expected to benefit more from a highly efficient host detection system than generalists whose wide host range makes detection of a particular host species less crucial. When compared with generalists, specialist parasitoids are expected to use more specific signals, as they need information on host identity, presence, and availability. A few studies have addressed this aspect of host/parasitoid interactions. Vet et al. (1993) compared the response of the specialist *Leptopilina boulardi* and the generalist *Leptopilina heterotoma* to larval extracts from six different drosophilid larvae. They found that the behavioral response to the kairomone reflected the dietary breadth of the two parasitoids, as the specialist displayed a more specific response than the generalist. Geervliet et al. (1996), however, failed to demonstrate such a relationship when they compared the innate long-range host discriminative abilities of the specialist *Cotesia rubecula* and the generalist *Cotesia glomerata.* Neither could discriminate volatiles from plants infested with host and nonhost species. In a comparative study between the generalist parasitoid, *Campoletis sonorensis*, and the specialist, *Microplitis croceipes*, Elzen et al. (1987) found that the specialist parasitoid was attracted by volatiles from feces of its host, *Heliothis virescens*, whereas the generalist was not. These varying results could be the consequence of differences in the bioassays used but also in the volatiles tested.

For each plant–herbivore complex, volatile signals used by foraging parasitoids can originate from the plant, the host, or from an interaction between the two. In a few plants (Dicke et al., 1990a; Turlings et al., 1990; Dicke, 1994; Loughrin et al., 1994; McCall et al., 1994; Takabayashi et al., 1991, 1994) the latter comprises passive release volatiles (i.e., constitutive volatiles emitted upon mechanical damage) as well as induced volatiles (i.e., volatiles emitted as a delayed response to herbivore feeding damage only). Studies with

lima beans (Dicke et al., 1990b, 1993), corn (Turlings and Tumlinson, 1992), and recently cotton (Turlings et al., 1995; Röse et al., 1996) have shown that induced volatiles were released not only locally by the damaged leaf, but also systemically in undamaged parts of the plant. Chemical analysis with corn (Turlings et al., 1993a) indicated that the composition of the systemically released herbivore-induced volatiles did not differ significantly with different species of herbivores. Moreover, behavioral experiments with the parasitoids *Cotesia marginiventris* and *M. croceipes* showed that neither species was able to discriminate between volatiles systemically emitted by caterpillar- (*Spodoptera exigua*) or grasshopper- (*Schistocerca americana*) induced plants. Similarly, in cotton, chemical analysis of volatiles systematically released by plants damaged by different species of caterpillars revealed no significant differences, (Röse, unpublished data). Thus, herbivore-induced signals systematically emitted by corn or cotton plants do not appear to carry any specific information on the identity of the herbivore causing the damage. Because of this lack of specificity, systemically released herbivore-induced volatiles do not reliably indicate host presence for specialist parasitoids and therefore could be used differently by generalist and specialist species.

In the present study, we investigated how such volatiles are used in the foraging strategies of two related solitary endoparasitoids that differ in dietary specializations: *M. croceipes* (Hymenoptera: Braconidae), a specialist that parasitizes a limited number of related host species (*H. virescens, Heliothis subflexa, Helicoverpa zea*), and *C. marginiventris* (Hymenoptera: Braconidae), a generalist with a wide range of lepidopterous hosts, including *S. exigua, S. frugiperda* and *Heliothis/Helicoverpa* spp. We questioned the role of systemically released volatiles in the long-range orientation of the two parasitoids and their shifting influence as wasps are exposed to other plant- or host-related signals.

We restricted the study to two lepidopterous herbivores, *S. exigua* and *H. zea*. These species are significant pests of cotton but occur also on a wide variety of cultivated and noncultivated plants (Pearson, 1982; Stadelbacher et al., 1986). *M. croceipes* attacks caterpillar hosts on as many as 24 different host plants (Eller, 1990) while *C. marginiventris* attacks hosts on 30 different species (Turlings, 1990). The two herbivore species exhibit different feeding behaviors. The first three instars of *S. exigua* feed in groups, usually on the underside of the leaves (Poe et al., 1973). *H. zea* larvae, conversely, are solitary feeders. On cotton, their preferred plant parts are squares, flowers, and bolls (Wilson and Gutierrez, 1980). The feeding behavior of early *S. exigua* instars evokes a systemic response in cotton plants (Röse et al., 1996). After 48 hr of continuous damage of the lower leaves, the upper undamaged leaves systemically released (*Z*)-3-hexenyl acetate, (*E*)-β-ocimene, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E*)-β-farnesene, (*E,E*)-α-farnesene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. All these compounds are induced by herbivore damage

and are not released in detectable amounts by undamaged plants. Based on these results, we used *B. exigua* larvae to obtain plants systemically releasing herbivore-induced volatiles.

We investigated whether the specialist parasitoid *M. croceipes* is able to discriminate between volatiles systemically released from nonhost induced plants and volatiles released by undamaged plants. We then determined the primary host-finding cues used by a specialist as compared to a generalist parasitoid. Finally, we questioned whether the natural feeding behavior of *H. zea* larvae triggers the release of parasitoid-attracting volatiles from undamaged parts of cotton plants.

METHODS AND MATERIALS

Hosts

S. exigua and *H. zea* eggs were obtained from the rearing facilities at the IBPMRL, USDA-ARS, Tifton, Georgia. Larvae were fed on a laboratory-prepared pinto bean diet and held in a climatic room at 25°C, 14L:10D, and 70% relative humidity until used for experiments.

Parasitoids

M. croceipes were reared on *H. zea* larvae according to the procedure of Lewis and Burton (1970). *C. marginiventris* were reared similarly on *S. frugiperda* larvae. Both species were reared and held at 25°C, 14L:10D and 70% relative humidity. Under these conditions, adult *M. croceipes* and *C. marginiventris* emerged 17–23 days and 15–21 days after parasitization, respectively.

All experiments were conducted with mated *M. croceipes* and *C. marginiventris* females, 2 and 4 days old, respectively. Unless stated otherwise, females were given an oviposition experience with a *H. zea* larva fed artificial diet immediately prior to being bioassayed.

Plants

Cotton plants (*Gossypium hirsutum*, Deltapine 90 variety) were grown in individual pots in a greenhouse at 25°C ± 10, 15L:9D, 60 ± 20% relative humidity. Seeds were planted in a 1:1 mixture of peatmoss (Promix Bx) and potting soil fertilized with Osmocote. Eight- to 10-week-old plants were used in the experiments. Immediately before beginning the experiments, the stem was submerged in water and cut to remove the terminals consisting of the upper four to five leaves. These terminals were placed in a 125-ml water filled Erlenmeyer flask for use in the bioassays.

Wind Tunnel

A 50 × 50 × 120-cm wind tunnel as described by Drost et al. (1986) was used. Experiments were conducted at a wind speed of 40 ± 2 cm/sec and at 25 ± 2°C and 40 ± 10% relative humidity.

Volatile Sources Tested

The following volatiles sources were used in the wind-tunnel dual choice tests described in the next section:

Terminals from Leaf-Damaged Plants (Leaf-Induced Plants). The three lower leaves were damaged with *S. exigua* larvae, 48 hr prior to bioassay (Figure 1). Four third instars were contained in a 6-cm-diameter screened cage placed on each of the three leaves. Since only the tops of the plants were tested, damaged leaves were always absent in the experiments.

Terminals from Undamaged Plants (Undamaged Plants). Plants were never exposed to caterpillar damage (Figure 1). When they were tested against a square-induced plant, three to four squares were removed and the excisions covered with paraffin. Undamaged plants were kept in close proximity to damaged plants during the 48-hr damage period.

Recently Damaged Leaves (Recent Damage). Depending on treatments, recently damaged leaves either originated from undamaged or leaf-induced plants. One leaf was damaged by 10–15 first and second instar *H. zea* for less than

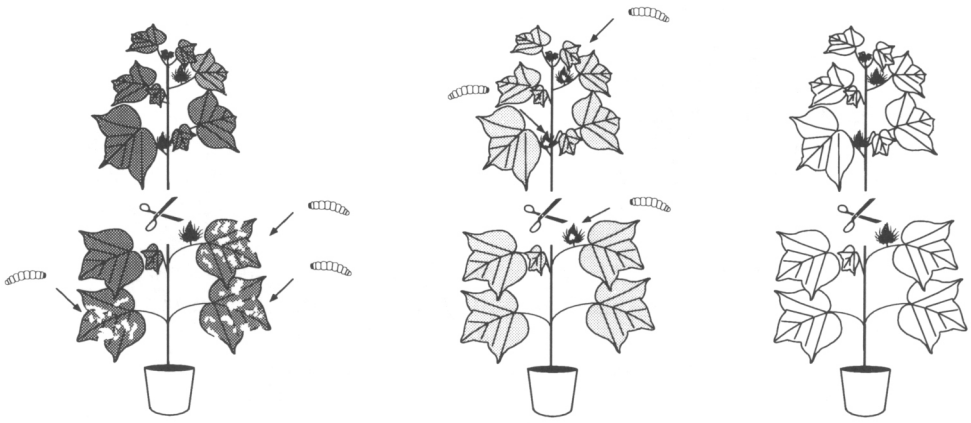


FIG. 1. Schematic representation showing procedure for volatile sources. Cut off terminals of cotton plants were used. (For more details, see "Volatile Sources Tested".)

3 hr. When the leaf was added to another plant, the stem was cut in water and wrapped in moist cotton wool. Host-related products such as feces or silk were removed with a brush.

Frass Obtained from Larvae Feeding on Cotton Plants (Plant Frass). Frass was collected from first and second instar *H. zea* feeding on cotton plants for 24 to 36 hr. Immediately prior to the experiments, the frass was humidified, mashed, and applied with a brush to one leaf of the plant tested. Approximately 3.5 $\pm$ 0.5 mg (dry weight) of frass were applied.

Frass Obtained from Larvae Feeding an Artificial Diet (Artificial Frass). Frass was collected from first and second instar *H. zea* feeding on pinto bean diet for 24–36 hr. Artificial diet frass was prepared and applied as described for plant frass.

Terminals from Square Damaged Plants (Square-Induced Plants). Three to four cotton squares were damaged by *H. zea* larvae 48 hr prior to bioassays (Figure 1). One third instar was placed on each square. In order to avoid possible contamination of plants by host-related products, squares were contained in closed 30 ml plastic cups. Damaged squares were excised immediately prior to testing and the excisions covered with paraffin.

Bioassays

Two plant terminals were placed at the upwind end of the wind tunnel. At the beginning of the test, a female, held in a shell vial, was released at the downwind end. The vial was positioned with its opening directed towards the center of the two volatile plumes generated by the terminals. The first plant on which the female landed was recorded. Females were given a maximum of three chances to land on a plant. If they landed anywhere else in the wind tunnel three consecutive times, they were reported as making an incomplete flight. *M. croceipes* and *C. marginiventris* females were tested alternatively. For each combination tested, 10 females of each species were bioassayed and four replications on different days were made. All females were used only once. Plants were switched from one side to the other after *M. croceipes* and *C. marginiventris* each completed five tests.

In order to assess the role of systemically released signals relative to other signals emitted, the following dual-choice tests were performed using volatile sources obtained as previously described:

(a) leaf-induced plant versus undamaged plant; (b) leaf-induced plant versus undamaged plant + recent damage—a recently damaged leaf obtained from a previously undamaged plant was added to the undamaged plant; (c) leaf-induced plant + recent damage versus undamaged plant + recent damage—a recently damaged leaf obtained from a previously undamaged plant was added to the undamaged plant and to the leaf-induced plant; (d) leaf-induced plant with recent damage versus undamaged plant with recent damage—one leaf of the leaf-induced

plant and one leaf of the undamaged plant were damaged prior to testing; (e) leaf-induced plant versus undamaged plant + plant frass—Plant frass was applied to one leaf of the undamaged plant; (f) leaf-induced plant versus undamaged plant + artificial frass—artificial frass was applied to one leaf of the undamaged plant. In this treatment, only naive females (never exposed to host or host products prior to testing) were used in order to avoid possible learning and orientation to artificial diet related volatiles in the frass. Treatments a and e were repeated using naive females to permit accurate comparisons of results; and (g) square-induced plant versus undamaged plant.

The following treatments were performed on the same days: treatments a, b, c, and d; treatments a and e; treatments a, e, and f (with naive females); and treatments a and g.

In order to verify if the undamaged part of damaged plants emitted herbivore-induced volatiles and to account for eventual daily variations, experiments were always preceded by a control choice test with a leaf-induced and an undamaged plant (treatment a). Only when the leaf-induced plant was preferred over the undamaged plant (which happened in over 95% of the cases), were subsequent tests conducted using these plants. Data obtained during these control choice tests are included in the figures, and serve as a reference for comparisons among treatments.

Data Analysis

Results of all dual choice tests were analyzed with chi-square tests. The Yates correction for continuity was applied (Zar, 1984).

RESULTS

In all the tests, the overall rate of response of both parasitoids was high. When given three chances to land on a plant, only 1.6% of *M. croceipes* and 8.2% of *C. marginiventris* made incomplete flights in the wind tunnel.

Leaf-Induced Plant Versus Undamaged Plant. Volatiles emitted by *S. exigua* induced plants were attractive to female parasitoids. Both *M. croceipes* and *C. marginiventris* exhibited a strong preference for leaf-induced plants (Figures 2A and 3A). They were chosen by over 80% of females that made complete flights. The responses of both parasitoids to this dual choice test were consistent (compare Figures 2, 3, 4, and 6).

Leaf-Induced Plant Versus Undamaged Plant + Recent Damage. When a recently damaged leaf was added to an undamaged plant, no significant difference was found in *M. croceipes* choice between this combination and the leaf-induced plant (Figure 2B). However, female preference for leaf-induced plants appeared to decrease (compare Figures 2A and B) ($\chi^2 = 27.0$, $P < 0.001$,

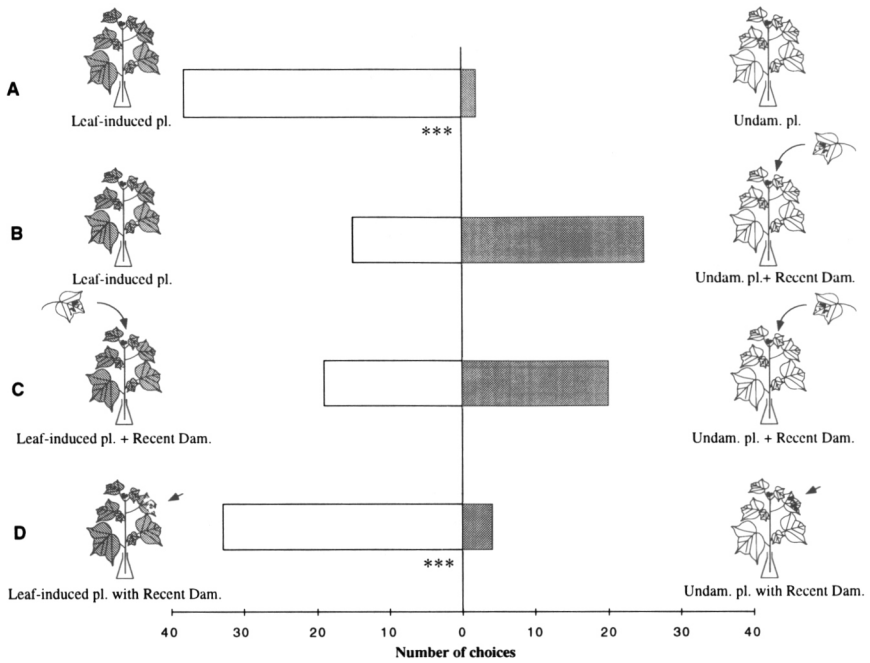


FIG. 2. Flight responses of *M. croceipes* females in wind-tunnel dual-choice tests. Bars indicate the number of complete flights to each volatile source ($N = 40$). Asterisks indicate significant differences within a choice test (χ^2 test, $^{**}P < 0.01$; $^{***}P < 0.001$). Undam. pl. = plants never exposed to caterpillar damage. Leaf-induced pl. = plants damaged on the three lower leaves by *S. exigua* larvae 48 hr prior to tests (damaged leaves removed). Recent Dam. = one leaf damaged by *H. zea* larvae 3 hr prior to tests. Recent damage was either added (+) or conducted (with) on the plants being tested.

$df = 1$). Females that made complete flights chose the leaf-induced plants 95% of the time when no recent damage was present, as opposed to 37.5% when a recently damaged leaf was added to the undamaged plant. *C. marginiventris* showed a clear preference for the undamaged plant + recent damage combination over the leaf-induced plant (Figure 3B).

Leaf-Induced Plant + Recent Damage Versus Undamaged Plant + Recent Damage. When a recently damaged leaf was added to both the leaf-induced and the undamaged plant, no clear preference for any combination was found in either parasitoid species (Figures 2C and 3C).

Leaf-Induced Plant with Recent Damage Versus Undamaged Plant with Recent Damage. When the recent damage was conducted on the leaf-induced plant itself, both parasitoids exhibited strong preference for this combination over the undamaged plant with recent damage combination (Figures 2D and 3D).

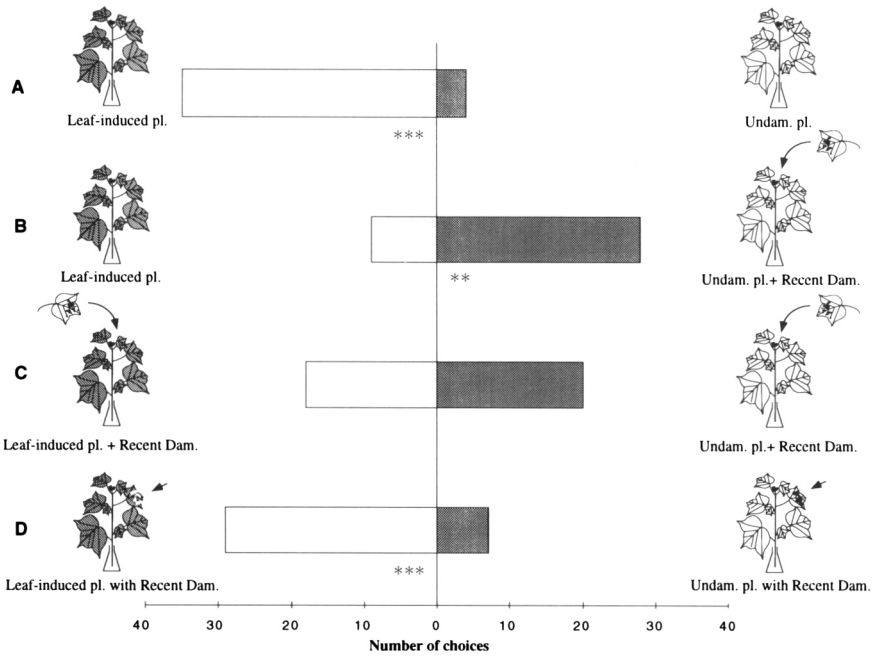


FIG. 3. Flight responses of *C. marginiventris* females in wind-tunnel dual-choice tests. Bars indicate the number of complete flights to each volatile source ($N = 40$). Asterisks indicate significant differences within a choice test (χ^2 test, $^{**}P < 0.01$, $^{***}P < 0.001$). Undam. pl. = plants never exposed to caterpillar damage. Leaf-induced pl. = plants damaged on the three lower leaves by *S. exigua* larvae 48 hr prior to tests (damaged leaves removed). Recent Dam. = one leaf damaged by *H. zea* larvae 3 hr prior to tests. Recent damage was either added (+) or conducted (with) on the plants being tested.

Leaf-Induced Plant Versus Undamaged Plant + Plant Frass. When frass obtained from cotton feeding larvae was added to undamaged plants, the results varied with parasitoid species. *M. croceipes* preferred the undamaged plant + plant frass combination (Figure 4B). Females with no previous oviposition experience exhibited similar preference (Figure 5C). *C. marginiventris*, however, displayed no particular preference (Figure 4D). When plant frass was added to undamaged plants, *C. marginiventris* preference for leaf-induced plants decreased (compare Figure 4C and D) ($\chi^2 = 6.2$, $P < 0.05$, $df = 1$). In that situation, the leaf-induced plant was chosen by only 58.3% of the females that made complete flights, against 86.8% when no frass was present.

Leaf-Induced Plant Versus Undamaged Plant + Artificial Frass (Naive Females). When frass obtained from artificial diet fed *H. zea* was added to undamaged plants, no significant difference was found in *M. croceipes* choices

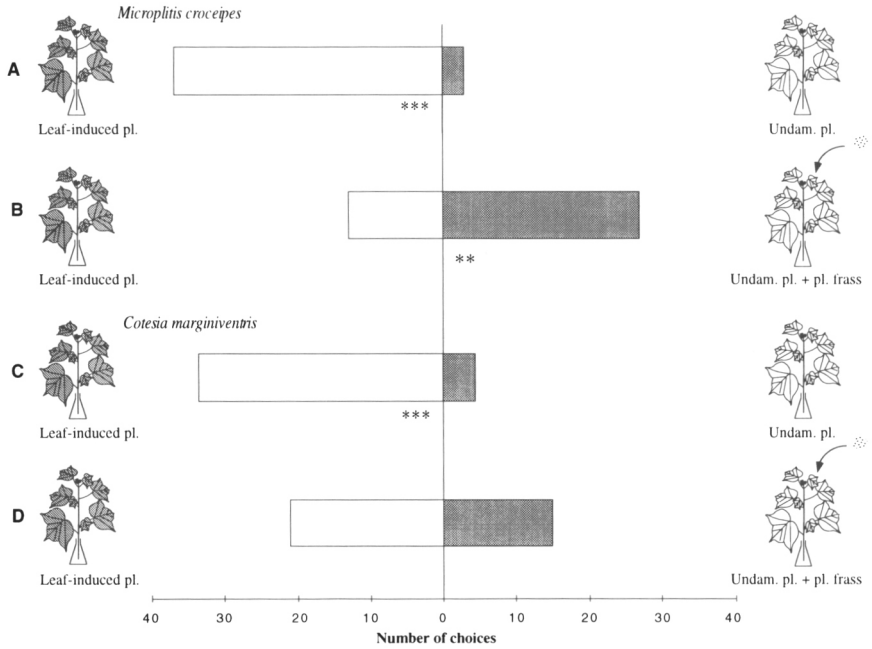


FIG. 4. Flight responses of *M. croceipes* (A, B) and *C. marginiventris* (C, D) females in wind-tunnel dual-choice tests. Bars indicate the number of complete flights to each volatile source ($N = 40$). Asterisks indicate significant differences within a choice test (χ^2 test, $^{**}P < 0.01$, $^{***}P < 0.001$). Undam. pl. = plants never exposed to caterpillar damage. Leaf-induced pl. = plants damaged on the three lower leaves by *S. exigua* larvae 48 hr prior to tests (damaged leaves removed). pl. frass = feces from *H. zea* larvae feeding on cotton plants for 24–36 hr.

between this combination and leaf-induced plants (Figure 5B). However, when artificial diet frass was added to undamaged plants, female preference decreased (compare Figure 5A and B) ($\chi^2 = 6.4$, $P < 0.05$, $df = 1$). Of the naive females that made complete flights, 87.5% chose the systemic plant when no frass was present compared to 60.0% when artificial diet frass was added to the undamaged plant.

Square-Induced Plant Versus Undamaged Plant. Plants induced by *H. zea* larvae feeding on squares had different effects on the two parasitoid species. *M. croceipes* preferred square-induced over undamaged plants (Figure 6B) but *C. marginiventris* showed no clear preference for either choice (Figure 6D). For the latter species, this was the treatment where the highest rate of incomplete flights (17.5%) was observed.

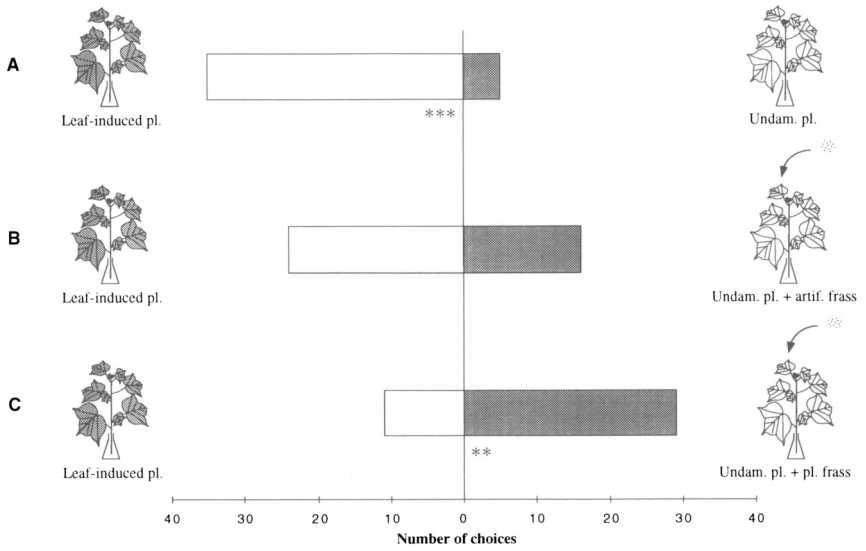


FIG. 5. Flight responses of naive *C. croceipes* females in wind-tunnel dual-choice tests. Bars indicate the number of complete flights to each volatile source ($N = 40$). Asterisks indicate significant differences within a choice test (χ^2 test, $^{**}P < 0.01$, $^{***}P < 0.001$). Undam. pl. = plants never exposed to caterpillar damage. Leaf-induced pl. = plants damaged on the three lower leaves by *S. exigua* larvae 48 hr prior to tests (damaged leaves removed). pl. frass = feces from *H. zea* larvae feeding on cotton plants for 24–36 hr. artif. frass = feces from *H. zea* larvae feeding on pinto bean diet for 24–36 hr.

DISCUSSION

Response to Leaf-Induced Plants. Despite the lack of previous plant experience, both parasitoid species were strongly attracted to volatiles released by undamaged terminals from cotton plants damaged by *S. exigua* larvae. When no other information was available, both *M. croceipes* and *C. marginiventris* strongly preferred leaf-induced plants over undamaged plants. Previous chemical analysis of cotton plants receiving similar *S. exigua* damage on the lower leaves demonstrated that such plants released herbivore-induced volatiles systemically (Röse et al., 1996). Our experimental design does not exclude the possibility of volatiles from damaged leaves being adsorbed on undamaged leaves and reemitted during wind-tunnel experiments. However, in all experiments, the induced plants and the undamaged plants were kept very close to each other. Any adsorption of volatiles from damaged sites would have occurred equally on both induced and undamaged plants. Thus, only systemically released vol-

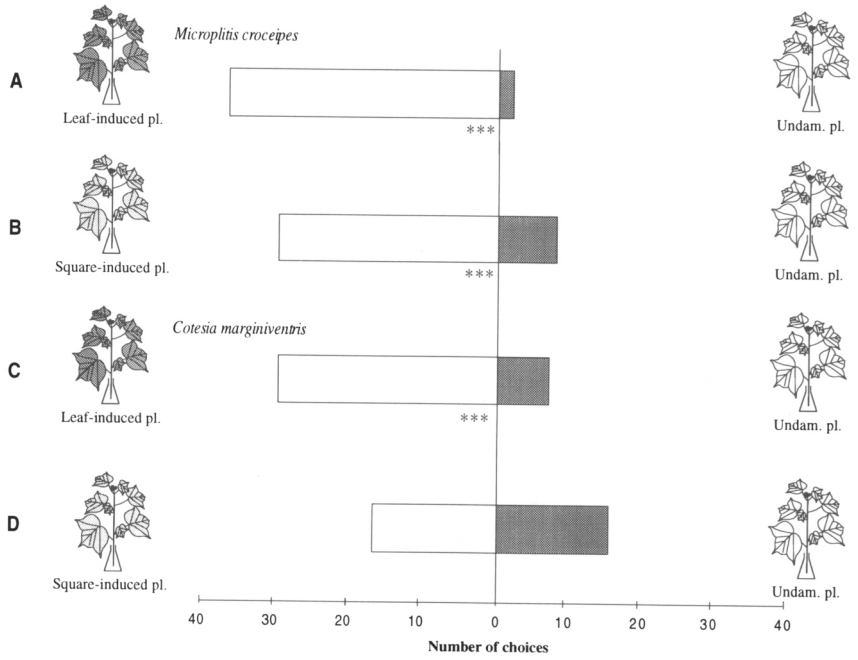


FIG. 6. Flight responses of *M. croceipes* (A, B) and *C. marginiventris* (C, D) females in wind-tunnel dual-choice tests. Bars indicate the number of complete flights to each volatile source ($N = 40$). Asterisks indicate significant differences within a choice test (χ^2 test, $***P < 0.001$). Undam. pl. = plants never exposed to caterpillar damage. Leaf-induced pl. = plants damaged on the three lower leaves by *S. exigua* larvae 48 hr prior to tests (damaged leaves removed). Square-induced pl. = cotton plants damaged on three or four squares by *H. zea* larvae 48 hr prior to the tests (damaged squares were removed).

atiles, as demonstrated by Röse et al. (1996), can reasonably account for the consistently stronger attraction of leaf-induced over undamaged plants.

Herbivore-induced systemically released volatiles are emitted from a large source and therefore, under natural conditions, may be detected over relatively large distances by parasitoids. These volatiles, which indicate herbivore damage, are likely to serve as long-range cues for the location of potential host patches. However, there is no evidence that volatiles emitted by undamaged portions of damaged plants carry information about the nature of the herbivore causing the damage. In our experiments, plants were damaged by *S. exigua* larvae, which are not suitable hosts for *M. croceipes*, and still these plants were very attractive. Turlings et al. (1993a) found similar results with corn seedlings induced with

S. exigua regurgitate. In these experiments, *M. croceipes* also showed strong attraction to non-host-induced corn seedlings. Furthermore, females were not able to distinguish between caterpillar- and grasshopper-induced seedlings. At the level of the actual damaged site, however, some plants emitted different volatiles when damaged by different herbivore species (Takabayashi et al., 1991; Agelopoulos and Keller, 1994b; Dicke, 1994). A number of parasitoids and predators are able to differentiate plants damaged by different species. The predatory mites *Phytoseiulus persimilis, Amblyseius potentillae*, and *Amblyseius finlandicus* can distinguish leaves infested by different spider-mite species (Sabelis and Van de Baan, 1983). The parasitoid *C. rubecula* showed no preference for cabbage plants damaged by hosts (*Pieris rapae*) or non-host caterpillars (*Plutella xylostella*) but preferred caterpillar- over snail- (*Helix aspera*) damaged plants (Agelopoulos and Keller, 1994a). However, host discriminative abilities at the damaged site level are no assurance of discriminative abilities at the systemic level, since the nature and the quantity of volatiles emitted locally and systemically may differ. Furthermore, learning can play an important role in parasitoids' abilities to discriminate between host and nonhost infested plants. Naive *C. glomerata* females did not discriminate between volatiles from plants infested by different caterpillar species (Geervliet et al., 1996). However, multiple experiences with a particular host plant complex led to clear preferences for this complex over others in wind-tunnel dual-choice tests (Geervliet, 1997). Similarly, the parasitoid *C. marginiventris* was able to distinguish between corn plants damaged by *S. exigua* or by *S. frugiperda* larvae after having an oviposition experience on either one of these host/plant complexes (Turlings et al., 1993b; Turlings et al., 1995). Therefore, further experimentation using *M. croceipes* and *C. marginiventris* with different learning experiences are needed to determine if they are able to distinguish between volatiles systemically released by plants damaged by different herbivore species.

Response to Other Plant- and Host-Related Signals. Once parasitoids enter potential host habitats, they cue on information more directly related to the presence of the host. Even if undamaged terminals from damaged plants are very attractive, parasitoids can still discriminate between signals emitted by these and signals indicating host presence more reliably. The nature of the signals used by the two parasitoid species studied here varied. Recent damage volatiles seemed to constitute the primary orientation cue for *C. marginiventris*. When a leaf damaged for less than 3 hr was added to an undamaged plant, this combination became more attractive to *C. marginiventris* than the systemic plant alone. On the other hand, when frass from *H. zea* larvae feeding on cotton leaves was added to an undamaged plant, *C. marginiventris* females exhibited no clear preference for either this combination or the systemic plant. Our results concur with previous findings by Turlings et al. (1991), where damaged corn seedlings were the primary source of volatiles attracting this parasitoid, whereas frass

volatiles appeared to have only a minor role. Different results were found with *M. croceipes*, in which frass volatiles were the primary orientation cue. However, volatiles from recently damaged leaves also appeared to have some effect. When provided a choice between a leaf-induced plant and either an undamaged plant + a recently damaged leaf or an undamaged plant, *M. croceipes* females chose the undamaged plant + recent damage 12.5 times more often. In our experiments, we used leaves damaged for less than 3 hr. According to previous chemical analysis of cotton (McCall et al., 1994), volatiles emitted by recently damaged leaves are mainly composed of green-leaf volatiles and constitutive terpenes. These are emitted at the damaged site concomitant with caterpillar feeding (Loughrin et al., 1994) but are not emitted systemically (Röse et al., 1996). Herbivores such as lepidopterous larvae can move from previously damaged locations or fall prey to other predators. Such factors can reduce the predictability of discovering a host when cueing only on induced volatiles. However, because emission wanes with time, volatiles released immediately upon damage could constitute reliable indicators of proximate damage and therefore indicate actual host presence to foraging parasitoids.

Both *C. marginiventris* and *M. croceipes* were attracted by leaf-induced plants receiving new damage. At the plant patch level, these results indicate that induced plants receiving secondary damage may have some advantage over newly damaged neighbors by attracting more natural enemies and attracting them quicker. It is likely that leaves from plants already induced by previous herbivore damage release inducible volatiles faster when they receive new damage. Furthermore, recent chemical analysis with cotton (Röse et al., 1996) showed that artificially damaged systemic plants released larger amounts of inducible and constitutive volatiles than artificially damaged control plants. Previous studies with *M. croceipes* (McCall et al., 1993) and *C. marginiventris* (Turlings et al, 1993b) showed that both parasitoids preferred old damage to fresh damage. According to these authors, the absence of inducible volatiles in the recently damaged leaves could account for the preferences observed.

Response of Naive Females to Leaf-Induced Plants and to Host Frass. In our experiments, the number of *M. croceipes* failing to achieve complete flights remained low even when we used naive females. This confirms the strong effect of systemically released herbivore-induced volatiles on this species.

The active component in caterpillar frass appeared to have two origins. Although no preference was found between leaf-induced and undamaged plants + artificial frass, artificial diet frass volatiles appeared to have some attractive effect on *M. croceipes* females. When tested against a leaf-induced plant, an undamaged plant where artificial diet frass was added attracted this parasitoid 3.2 times more than an undamaged plant alone. Since artificial diet is not attractive to *M. croceipes* females, it can therefore be assumed that volatile compounds originating from the host itself and present in the frass are responsible

for the attraction observed. However, adding plant frass resulted in a stronger attraction than adding artificial diet frass. Whether this resulted from an additive effect of plant and host products present in the frass or from an absence of modified plant-related products in the artificial diet frass remains unclear. The use of frass volatiles in the host location process of the specialist *M. croceipes* has already been reported (Jones et al., 1971; Eller et al., 1988; Lewis and Tumlinson, 1988). Previous experience with frass appeared to have a crucial influence on female response to these volatiles. Several studies (Lewis and Tumlinson, 1988; Lewis et al., 1991; Eller et al., 1992) showed that few naive *M. croceipes* would fly upwind to host feces even when the hosts were plant fed. However, our results demonstrate an innate attraction to host frass volatiles in *M. croceipes*. Because only choice experiments were conducted here and systemic volatiles (highly attractive even to naive females) were always present, it is conceivable that these volatiles were responsible for the flight initiation and initial orientation of naive females and that frass volatiles influenced females' choices at the shorter range. By being closer to situations encountered by foraging parasitoids in nature (frass volatiles would seldom be alone), our experiments allow a more realistic view of the use of multiple information. It should be noted that only humidified frass was used. This action increased the amount and modified the composition of volatiles detected by chemical analysis from *P. rapae* and *Pieris brassicae* frass (Agelopoulos et al., 1995).

In *M. croceipes*, frass volatiles represent a source of specific information allowing discrimination between host and nonhost species from a distance (Alborn et al., 1995). The presence of herbivore-specific cues for parasitoids in caterpillar frass has also been demonstrated for other species (Smith et al., 1994; Agelopoulos et al., 1995). For specialist parasitoids such as *M. croceipes*, the ability to detect volatiles specifically associated with their hosts could have a double advantage. Not only could it give indications at a distance of the identity of the species attacking a plant, but also, because of the extremely polyphagous nature of their hosts, it could help decrease the need to resort to multiple plant signals emanating from all the different species they feed on.

Response to Square-Induced Plants. M. croceipes can only parasitize *Heliothis/Helicoverpa* species that, on cotton, typically do not feed on leaves, but prefer squares, flowers, and bolls (Wilson and Gutierrez, 1980). As there is a strong correlation between this site being damaged and presence of a suitable host, volatiles specifically released by square-induced plants would be reliable cues for foraging *M. croceipes*. Our experiments show that the natural feeding behavior of *H. zea* larvae on cotton triggers the release of parasitoid-attracting volatiles from the undamaged parts of the plant. When cotton plants were induced by *H. zea* larvae feeding on squares, only *M. croceipes* showed a strong preference for these plants over undamaged plants. Since no chemical analysis has been done yet, the question remains whether the difference in volatile production

between leaf-damaged plants and square-damaged plants is quantitative, qualitative, or both. At the damaged site level, Turlings et al. (1993b) found both qualitative and quantitative differences in the volatiles released by leaves, flowers, and bolls of cotton plants fed upon by *H. zea* larvae. So far, however, there is no evidence that such differences also exist at the systemic level. When *H. zea* larvae were contained on the squares with screened instead of closed cups, the responses of both parasitoids remained similar (Cortesero, unpublished data). Therefore, the difference between the response of *C. marginiventris* to leaf-induced and square-induced plants is not related to the method used to damage the plants. However, the origin of the lack of discriminative ability of *C. marginiventris* between *H. zea* square-induced plants and undamaged plants remains unclear. It may be related to differences in the species used to induce the plants as well as differences in the sites damaged. We hope to clarify this point in future experiments.

Acknowledgments--We are grateful to Thoris Green for rearing the parasitoids. We thank P. Barbosa and M. Dicke for useful comments on the initial manuscript. Financial support was provided in part by a Lavoisier grant from the French Minister of Foreign Affairs to A. M. Cortesero. Mention of a proprietary product does not constitute an endorsement of the product by the USDA.

REFERENCES

AGELOPOULOS, N. G., and KELLER, M. A. 1994a. Plant-natural enemy association in tritrophic system, *Cotesia rubecula–Pieris rapae*–Brassicaceae (Cruciferae). II. Preference of *C. rubecula* for landing and searching. *J. Chem. Ecol.* 20:1735–1748.

AGELOPOULOS, N. G., and KELLER, M. A. 1994b. Plant-natural enemy association in tritrophic system, *Cotesia rubecula–Pieris rapae*–Brassicaceae (Cruciferae). III: Collection and identification of plant and frass volatiles. *J. Chem. Ecol.* 20:1955–1967.

AGELOPOULOS, N. G., DICKE, M., and POSTHUMUS, M. A. 1995. Role of volatile infochemicals emitted by feces of larvae in host-searching behavior of parasitoid *Cotesia rubecula* (Hymenoptera: Braconidae): A behavioral and chemical study. *J. Chem. Ecol.* 21:1789–1811.

ALBORN, H. T., LEWIS, W. J., and TUMLINSON, J. H. 1995. Host-specific recognition kairomone for the parasitoid *Microplitis croceipes* (Cresson). *J. Chem. Ecol.* 21:1697–1708.

DICKE, M. 1994. Local and systemic production of volatile herbivore-induced terpenoids: Their role in plant-carnivore mutualism. *J. Plant Physiol.* 143:465–472.

DICKE, M., VAN BEEK, T. A., POSTHUMUS, M. A., BEN DOM, N., VAN BOKHOVEN, H., and DE GROOT, A. E. 1990a. Isolation and identification of volatile kairomone that affects acarine predator–prey interactions. Involvement of host plant in its production. *J. Chem. Ecol.* 16:381–396.

DICKE, M., SABELIS, M. W., TAKABAYASHI, J., BRUIN, J., and POSTHUMUS, M. A. 1990b. Plant strategies of manipulating predator-prey interactions through allelochemicals: Prospects for application in pest control. *J. Chem. Ecol.* 16:3901–3118.

DICKE, M., VAN BARLEN, P., WESSEL, R., and DIJKMAN, H. 1993. Herbivory induces systemic production of plant volatiles that attract predators of the herbivore—extraction of endogenous elicitor. *J. Chem. Ecol.* 19:581–599.

DROST, Y. C., LEWIS, W. J., ZANEN, P. O., and KELLER, M. A. 1986. Beneficial arthropod behavior mediated by airborne semiochemicals. I. Flight behavior and influence of preflight handling of *Microplitis croceipes* (Cresson). *J. Chem. Ecol.* 12:1247–1262.

ELLER, F. J. 1990. Foraging behavior of *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae) a parasitoid of *Heliothis* (Lepidoptera: Noctuidae) species. PhD dissertation. University of Florida, Gainesville, 221 pp.

ELLER, F. J., TUMLINSON, J. H., and LEWIS, W. J. 1988. Beneficial arthropod behavior mediated by airborne semiochemicals. Source of volatiles mediating the flight behavior of *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae), a parasitoid of *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae). *Environ. Entomol.* 17:745–753.

ELLER, F. J., TUMLINSON, J. H., and LEWIS, W. J. 1992. Effect of host diet and preflight experience on the flight response of *Microplitis croceipes* (Cresson). *Physiol. Entomol.* 17:235–240.

ELZEN, G. W., WILLIAMS, H. J., VINSON, S. B., and POWELL, J. E. 1987. Comparative flight behavior of parasitoid *Campoletis sonorensis* and *Microplitis croceipes*. *Entomol. Exp. Appl.* 9:113–123.

GEERVLIET, J. B. F., VET, L. E. M., and DICKE, M. 1996. Innate responses of the parasitoids *Cotesia glomerata* and *C. rubecula* (Hymenoptera: Braconidae) to volatiles from different plant-herbivore complexes. *J. Insect Behav.* 9:525–538.

GEERVLIET, J. B. F. 1997. Infochemical use by insect parasitoids in a tritrophic context: Comparison of a generalist and a specialist. PhD dissertation. Wageningen Agricultural University, Wageningen, pp. 77–93.

JONES, R. L., LEWIS, W. J., BOWMAN, M. C., BEROZA, M., and BIERL, B. A. 1971. Host-seeking stimulant for parasite of corn earworm: isolation, identification, and synthesis. *Science* 173:842–843.

LEWIS, W. J., and BURTON, R. L. 1970. Rearing *Microplitis* in the laboratory with *Heliothis zea* as hosts. *J. Econ Entomol.* 63:656–658.

LEWIS, W. J., and TUMLINSON, J. H. 1988. Host detection by chemically mediated associative learning in a parasitic wasp. *Nature* 331:257–259.

LEWIS, W. J., TUMLINSON, J. H., and KRASNOFF, S. 1991. Chemically mediated associative learning an important function in the foraging behavior of *Microplitis croceipes* (Cresson). *J. Chem. Ecol.* 17:1309–1325.

LOUGHRIN, J. H., MANUKIAN, A., HEATH, R. R., TURLINGS, T. C. J., and TUMLINSON, J. H. 1994. Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plants. *Proc. Natl. Acad. Sci. U.S.A.* 91:11836–11840.

MCCALL, P. J., TURLINGS T. C. J., LEWIS W. J., TUMLINSON, J. H. 1993. Role of plant volatiles in host location by the specialist parasitoid *Microplitis croceipes* Cresson (Braconidae: Hymenoptera). *J. Insect Behav.* 6:625–639.

MCCALL, P. J., TURLINGS, T. C. J., LOUGHRIN, J., PROVEAUX, A. T., and TUMLINSON, J. H. 1994. Herbivore-induced volatile emission from cotton (*Gossypium hirsutum* L.) seedlings. *J. Chem. Ecol.* 20:3039–3050.

PEARSON, A. C. 1982. Biology, population dynamics, and pest status of the beet armyworm (*Spodoptera exigua*) in the Imperial Valley of California. PhD dissertation. University of California, Riverside, 282 pp.

POE, S. L., CRANE, G. L., and COOPER, D. 1973. Bionomics of *Spodoptera exigua* Hüb., the beet armyworm, in relation to floral crops. *Proc. Trop. Reg. Am. Soc. Hortic. Sci.* 17:389–396.

RÖSE, U. S. R., MANUKIAN, A., HEATH, R. R., and TUMLINSON, J. H. 1996. Volatile semiochemicals released from undamaged cotton leaves: A systemic response of living plants to caterpillar damage. *Plant Physiol.* 8:487–495.

SABELIS, M. W., and VAN DE BAAN, H. E. 1983. Location of distant spider mite colonies by phytoseiid predators: Demonstration of specific kairomones emitted by *Tetranychus urticae* and *Panonychus ulmi*. *Entomol. Exp. Appl.* 33:303–314.

SMITH, G. S., ALLISON, J. C. S., and PAMMENTER, N. W. 1994. Bioassay study of response by a parasitoid to frass and feeding substrates of its host, the stalk borer *Eldana saccharina*. *Ann. Appl. Biol.* 125:439–446.

STADELBACHER, E. A., GRAHAM, H. M., HARRIS, V. E., LOPEZ, J. D., PHILLIPS, J. R., and ROACH, S. H. 1986. *Heliothis* populations and wild host plants in the Southern U.S., *in* S. J. Johnson, E. G. King, and J. R. Bradley, Jr. (eds.). Theory and Tactics of *Heliothis* Population Management: I—Cultural and Biological Control. South *Coop. Ser. Bull.* 316:54–74.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M. A. 1991. Variation in composition of predator-attracting allelochemicals emitted by herbivore-infested plants: Relative influence of plant and herbivore. *Chemoecology* 2:1–6.

TAKABAYASHI, J., DICKE M., TAKAHASHI, S., POSTHUMUS, M. A., and VAN BEEK, T. A. 1994. Leaf age affects composition of herbivore-induced synomones and attraction of predatory mites. *J. Chem. Ecol.* 20:373–386.

TURLINGS, T. C. J. 1990. Semiochemically mediated host searching behavior of the endoparasitic wasp *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae). PhD dissertation. University of Florida, Gainesville, 178 pp.

TURLINGS, T. C. J., and TUMLINSON, J. H. 1992. Systemic release of chemical signals by herbivore-injured corn. *Proc. Natl. Acad. Sci. U.S.A.* 89:8399–8402.

TURLINGS, T. C. J., TUMLINSON, J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250:1251–1253.

TURLINGS, T. C. J., TUMLINSON, J. H., ELLER, F. J., and LEWIS W. J. 1991. Larval-damaged plants: source of volatile synomones that guide the parasitoid *Cotesia marginiventris* to the micro-habitat of its hosts. *Entomol. Exp. Appl.* 58:75–82.

TURLINGS, T. C. J., MCCALL, P. J., ALBORN, H. T., and TUMLINSON, J. H. 1993a. An elicitor in caterpillar oral secretions that induces corn seedlings to emit chemical signals attractive to parasitic wasps. *J. Chem. Ecol.* 19:411–425.

TURLINGS, T. C. J., WACKERS, F. L., VET, L. E. M., LEWIS, W. J., and TUMLINSON, J. H. 1993b. Learning of host-finding cues by hymenopterous parasitoids, pp. 51–78, *in* D. R. Papaj and A. C. Lewis (eds.). Insect Learning: Ecological and Evolutionary Perspectives. Chapman and Hall, New York.

TURLINGS, T. C. J., LOUGHRIN, J. H., MCCALL, P. J., ROSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. U.S.A.* 92:4169–4174.

VET, L. E. M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

VET, L. E. M., SOKOLOWSKI, M. B., MACDONALD, D. E., and SNELLEN, H. 1993. Responses of a generalist and a specialist parasitoid (Hymenoptera: Eucoilidae) to drosophilid larval kairomones. *J. Insect Behav.* 6:615–624.

WILSON, L. T., and GUTIERREZ, A. P. 1980. Fruit predation sub-model: *Heliothis* larvae feeding upon cotton fruiting structures. *Hilgardia* 48:24–36.

ZAR, J. H. 1984. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, New Jersey.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

ICHTHYODETERRENT PROPERTIES OF LIPOPHILIC EXTRACTS FROM BERMUDIAN SPONGES

B. McCLINTOCK,[1,*] D. SWENSON,[1] H. TRAPIDO-ROSENTHAL,[2] and L. BANGHART[2]

[1]*Department of Biology, University of Alabama at Birmingham Birmingham, Alabama 35294-1170*

[2]*Bermuda Biological Station for Research Ferry Reach, Saint Georges, Bermuda*

(Received July 2, 1996; accepted January 28, 1997)

Abstract—The feeding deterrent properties of hexane, ethyl acetate, and butanol lipophilic extracts of 16 species of Bermudian sponges presented to the sergeant major, *Abudefduf saxatilus*, and the Bermuda bream, *Diplodus bermudensis*, were investigated in the laboratory. The sponges investigated spanned a variety of different color morphs and were representative of patch reef, mangrove, rock wall, soft bottom, seagrass, and cave habitats. Six of the 16 sponges (36%) had at least one extract that deterred feeding by *A. saxatilus*, while only one of 16 sponges (6%) had an extract that deterred feeding by *D. bermudensis*. Among those sponges that deterred feeding in one or both fish, deterrence was most frequently associated with compounds extracted in ethyl acetate (moderately polar). Butanol extracts (most polar) were active in only half as many instances, while in only one case was a hexane extract (least polar) active. Both brighly colored (yellow, orange-yellow, gray-blue, purple-brown) and weakly colored (dull brown, pinkish white) sponges had hexane, ethyl acetate, or butanol extracts that were deterrent. Generally the numbers of sponges with bioactive lipophilic extracts that caused feeding deterrence in fish was low. This may be related to Bermuda being located just within or beyond the extreme northern range of many tropical fish and invertebrates, and therefore sponges are exposed to reduced levels of predation.

Key Words—Sponges, Bermuda, feeding deterrent, lipophilic extract, defense.

*To whom correspondence should be addressed.

0098-0331/97/0600-1607$12.50/0 © 1997 Plenum Publishing Corporation

INTRODUCTION

Sponges are conspicuous and abundant members of marine benthic communities (Koltun, 1970; Harrison and Cowden, 1976; Bergquist, 1978). Due to their sessile nature and high relative abundance, sponges are potentially attractive prey for any number of marine invertebrate and vertebrate predators. Nonetheless, the incidence of spongivory appears to be lower than would be expected. Low levels may be attributable to the nutritional quality of sponges or to their ability to defend themselves mechanically or chemically. Studies that have examined the nutritional content have concluded that while the organic content of sponges can be quite variable, generally levels of organic nutrients are low (McClintock, 1987; Chanas and Pawlik, 1995). Moreover, much of the organic material is in the form of insoluble protein that may be difficult to digest (McClintock, 1986, 1987). Nonetheless, the extreme abundance of sponges may offset their relatively low nutritional content, making them attractive prey. For example, in Antarctica a variety of sea stars focus their feeding efforts on the particularly abundant sponges (Dayton et al., 1974; McClintock, 1987, 1994). Sponges harbor numerous minute calcium carbonate or silicious spicules. While the spicules may be potential deterrents, predators do not appear to be hindered by them (Dayton et al., 1974; McClintock, 1987; Meylan, 1988; Pawlik et al., 1995).

Marine sponges harbor a wide variety of bioactive natural products (Faulkner, 1995, and references within). Although few empirical studies have been conducted, it is evident that these metabolites may serve a variety of functions including mediating allelochemical interactions (competition for space), preventing microbial and macroinvertebrate fouling, and defending against predation (reviewed by Bakus et al., 1986; Paul, 1992; Pawlik, 1993). In many instances the roles of sponge metabolites remain unknown. While several studies have examined the toxicological properties to fish of marine sponge extracts (Bakus and Green, 1974; Green, 1977; Thompson et al., 1985; McClintock, 1987), only a few have examined the chemical feeding deterrent properties employing ecologically relevant invertebrate or fish predators (Bobzin and Faulkner, 1992; McClintock et al., 1994; Pennings et al., 1994; Pawlik et al., 1995). Clearly, there is a need to continue to assess and evaluate the chemical feeding deterrent properties of marine sponges.

The incidence of chemical defense in marine sponges may be related to the intensity of predation. On a broad biogeographic scale, Bakus and Green (1974) suggested that there is an inverse correlation between latitude and toxicity in marine sponges. Sponges occurring at high latitudes, where levels of predation by fish are reduced (Vermeij, 1978), may be depauperate in defensive chemistry. Bakus and Green (1974) provided support for their argument by comparing the toxicity of temperate and tropical sponges exposed to fish. How-

ever, recent evidence suggests that there is no clear relationship between sponge toxicity and feeding deterrence (Pawlik et al., 1995). Moreover, high-latitude sponges in Antarctica are chemically defended (Baker et al., 1994), even in an environment where levels of fish spongivory are extremely low, but where invertebrate spongivores such as sea stars are abundant (Dayton et al., 1974; McClintock et al., 1994). It is likely that sponges occurring in spongivore-rich environments are under greater selective pressure to develop defensive chemistry and that this will vary across a variety of spatial scales. However, to date there are few data with which to evaluate this hypothesis.

Bermuda offers a unique opportunity to test the incidence of chemical defense in marine sponges in an environment characterized by low numbers of spongivores. Both the numbers and species diversity of spongivorous invertebrates (e.g., sea stars) and fish are reduced when compared with the Caribbean (Briggs, 1974; Sterrer, 1992). The shallow waters of Bermuda support a relatively abundant sponge fauna. Although diversity is approximately half that of the West Indian regions, there are more than 70 species of Bermudian sponges, approximately 75% of which also occur throughout the Caribbean (De Laubenfels, 1950). The reduction in species diversity of both sponges and sponge predators is due to Bermuda's unique geography, located in mid-Atlantic waters, yet fed by the warm waters of the Gulfstream (De Laubenfels, 1950). Bermuda lies at the extreme northern range of most of its tropical fauna and beyond the range of many species common throughout the Caribbean.

The purpose of the present study is to evaluate the incidence of ichthyodeterrence in lipophilic extracts of a suite of Bermudian sponges using sympatric fish and to evaluate whether sponges occurring in predator-poor seas of Bermuda are depauperate in defensive chemicals.

MATERIALS AND METHODS

Collection of Material and Extract Preparation. Sponges were collected by hand using snorkeling or scuba in shallow (1–8 m depth) coastal waters of Bermuda (32°19′N; 64°14′W). Samples were placed in zip-lock plastic bags, transported at ambient seawater temperature to the Bermuda Biological Station, and processed immediately. We focused on lipophilic extracts as these are considered the most likely to contain bioactive compounds (B. Baker, personal communication). In order to prepare three extracts of increasing polarity (hexane, ethyl acetate, butanol), tissue was processed as follows. Each 100 g wet weight of tissue was placed in 400 ml of HPLC-grade methanol and homogenized in a blender. The homogenate was mixed with 50 g of celite to make a slurry and subsequently vacuum filtered through course filter paper. The sponge–celite mixture was mixed into 400 ml of an equal volume of methanol and

methylene chloride, filtered, and then recombined with the methanol. The sample (750–800 ml) was evaporated on a rotary evaporator until approximately 100 ml remained. This was mixed with 100 ml hexane, 90 ml methanol, and 10 ml water in a separatory flask. The lower aqueous phase (approximately 200 ml) was collected and evaporated on a rotary evaporator until 100 ml remained. The upper hexane phase was collected and evaporated almost to dryness, resolubilized with 10 ml fresh solvent, and dried completely on a speed-vac to yield a hexane extract.

The aqueous phase (100 ml) was mixed with 100 ml water and 100 ml ethyl acetate and placed in a separatory funnel. The lower aqueous phase was collected (200 ml) and 200 g of sodium sulfate added to precipitate proteins. Sodium sulfate and precipitated proteins were removed by centrifugation. The upper ethyl acetate phase was evaporated on a rotary evaporator, resuspended in 10 ml solvent, and dried on a speed-vac to yield the ethyl acetate extract. The aqueous phase was mixed with 100 ml butanol and 100 ml water in a separatory funnel. The aqueous phase was discarded and the remaining butanol phase (120 ml) made azeotropic by adding 70 ml water. This was mixed and evaporated on a rotary evaporator until approximately 3 ml remained and air-dried at 35–40°C to yield the butanol extract. The final weights of dried extracts were determined.

Bioassays. To test the ichthyodeterrent properties of lipophilic extracts, alginate pellets were prepared by releasing 0.1 ml of 2% alginic acid from an autopipette into a 1 M solution of calcium chloride. Dried krill powder (2%) was added as a feeding stimulant, and red food coloring was added to make the pellets conspicuous. Experimental pellets were prepared by mixing alginic acid-containing krill and food coloring with sponge extracts resolubilized in a small volume of the appropriate solvent. The mixture was stirred sufficiently to allow the solvent to evaporate prior to pelletizing in the $CaCl_2$ solution. The ratio of the final extract yield to the initial wet tissue weight of each sponge was used to determine the appropriate concentration of sponge extract to be added to pellets. Therefore, alginate krill pellets contained chemicals extracted from sponges at the same concentrations that these chemicals occurred naturally in sponge tissues. Control pellets contained krill, red food coloring, and the same volume of the appropriate solvent that was used to resolubilize each sponge extract. As food quality may influence the effectiveness of chemical deterrents in sponges (Duffy and Paul, 1992), we employed a level of krill powder that yielded a final pellet concentration of soluble protein of 13.4%. We used this concentration because soluble protein is a good measure of prey quality (McClintock, 1986), and this concentration falls within the range of soluble protein values for 17 species of marine sponges (McClintock, 1987).

The common reef fish *Abudefduf saxatilus* (sergeant major) (9–10 cm length) and *Diplodus bermudensis* (Bermuda bream) (13–17 cm length) were used as

model predators. While neither fish is a sponge feeder, both are generalist carnivores and are common in Bermudian waters (Sterrer, 1992). Fish were collected from the waterway in front of the Bermuda Biological Station using a small hook baited with squid. Fish were not injured during collection. Nine fish of each species were maintained in aquaria (30 × 30 × 45 cm) equipped with equally spaced plastic mesh divisions such that three fish occupied each of three separate aquaria. Aquaria were supplied with fresh flowing seawater pumped directly from the sea (20°C and 36 parts per thousand salt) except during fish feeding trials, when the seawater flow was shut off. In order to standardize nutritional condition, fish were fed a maintenance diet of fish-meal pellets. Feeding trials consisted of presenting an individual fish either a control or experimental pellet in a predetermined random sequence and recording whether the pellet was accepted (ingested) or rejected (mouthed and then spit out). Nine feeding trials were conducted for each sponge extract or control. Each fish was only tested once against a given extract. Levels of experimental and control pellet acceptance were statistically compared using a Fisher's exact test (Zar, 1984).

RESULTS

Of the 16 species of marine sponges tested, six (38%) had at least one extract that caused significant ($P < 0.05$) deterrence in the sergeant major (Figure 1). These included the butanol extracts of *Adocia amphioxa* and *Dysidea etheria*; the ethyl acetate extracts of *Cinachyra alloclada*, *D. etheria*, *Ircinia felix acuta*, and *Leucetta microraphis*; and the hexane extract of *Aplysina fistularis* (Figure 1). Both the butanol and ethyl acetate extracts of *D. etheria* were strongly deterrent to *A. saxatilus* (Figure 1), while only the ethyl acetate extract of *D. etheria* was deterrent to the Bermuda bream (Figure 2). No other sponge extracts were deterrent to the Bermuda bream. Control pellets were, almost without exception, ingested readily by both species of fish (Figures 1 and 2).

The Bermuda sponges examined were representative of a wide variety of color morphs and habitat types (Table 1). As none of the sponges were devoid of at least some pigmentation, it was not possible to assess whether pigmentation per se was correlated with ichthyodeterrence. Nonetheless, there appeared to be no correlation between the degree of coloration and feeding deterrence, as representatives of both weakly and strongly colored species were ichthyodeterrent.

DISCUSSION

The results show that lipophilic extracts of Bermudian sponges cause low levels of feeding deterrence in the two species of fish tested. Ichthyodeterrence

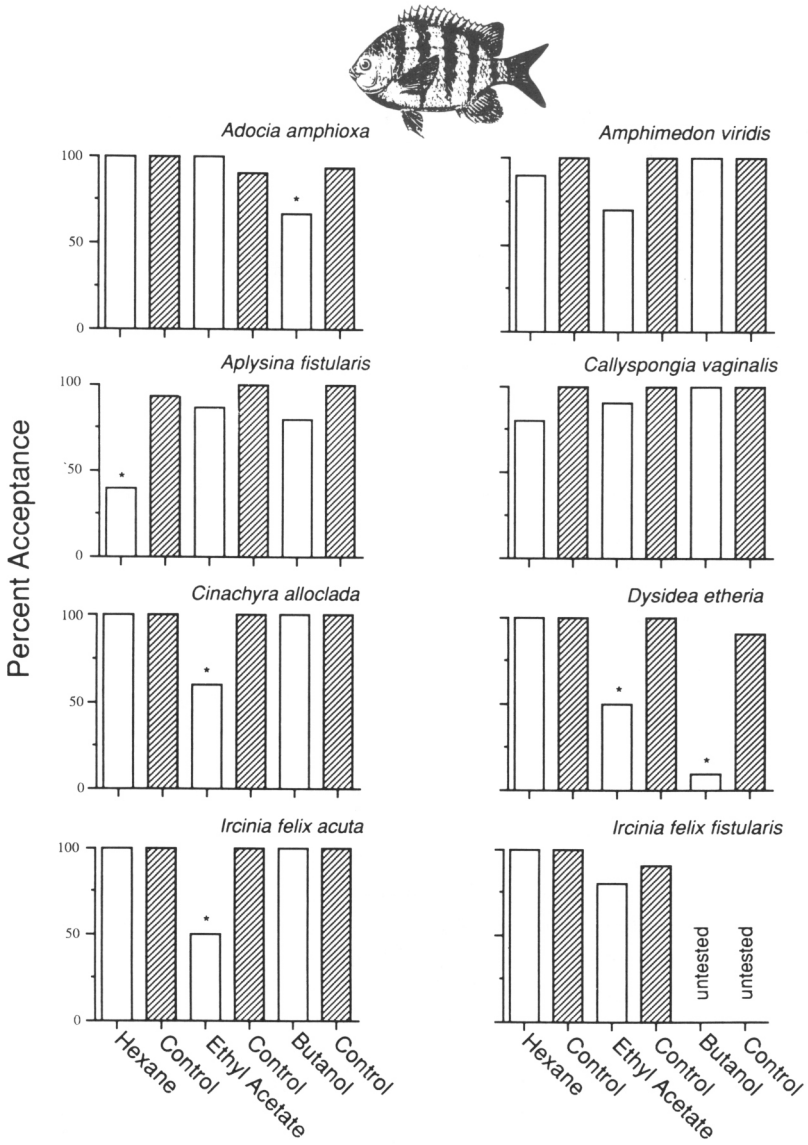


FIG. 1. *Abudefduf saxatilus*. Histograms showing percent acceptance of experimental (krill pellets containing organic sponge extracts) and control (krill pellets containing solvent alone) pellets presented in a random sequence to the sergeant major *Abudefduf saxatilus*. $N = 9$. Asterisks indicate experimental treatments that were significantly different from controls ($P < 0.05$).

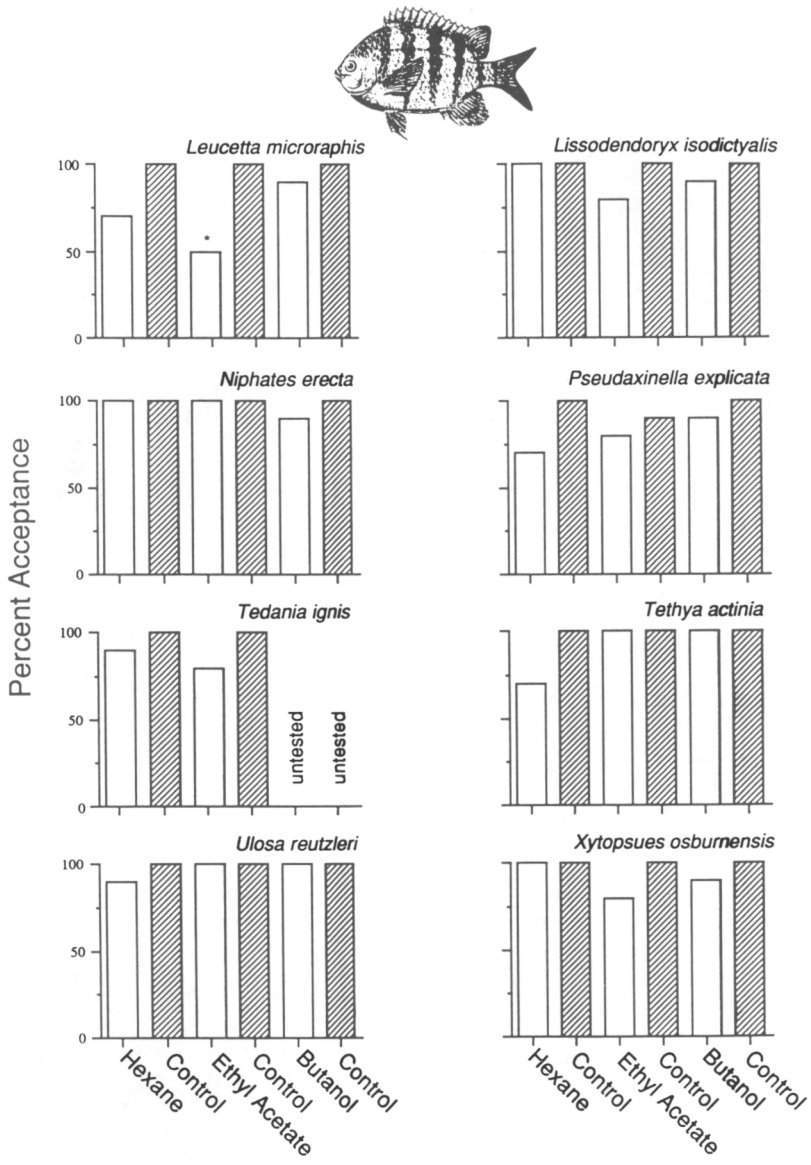


FIG. 1. Continued.

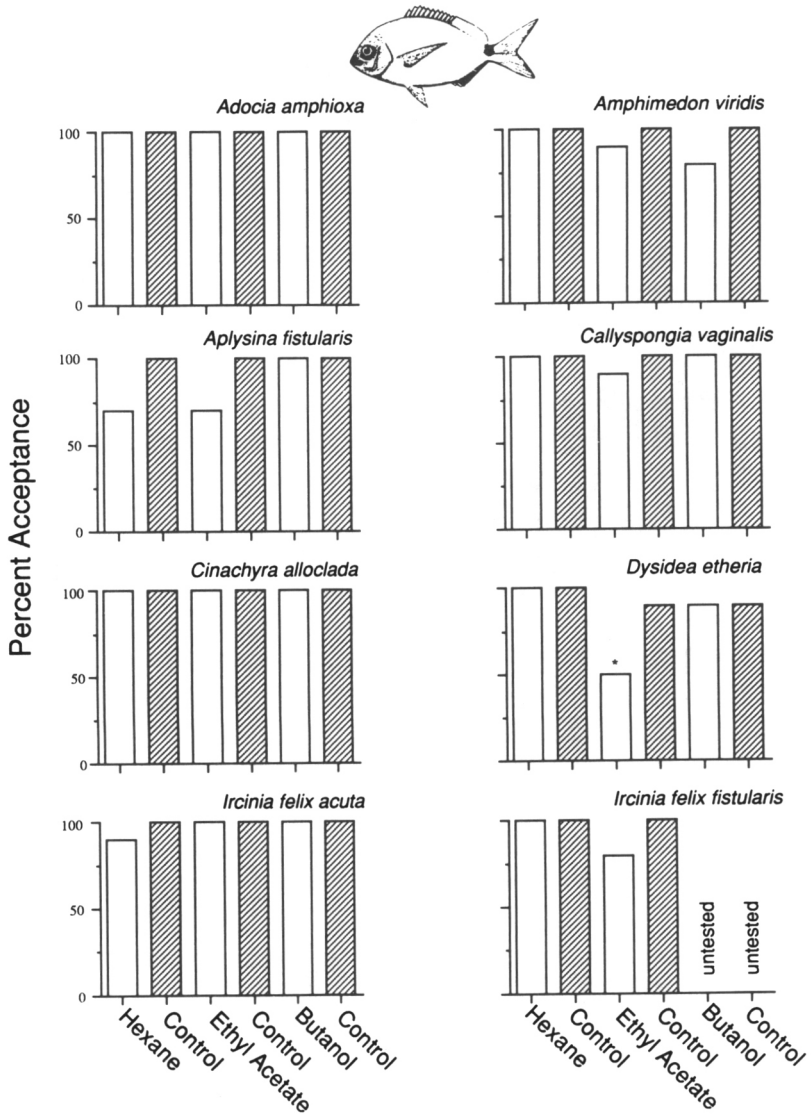


FIG. 2. *Diplodus bermudensis.* Histograms showing percent acceptance of experimental (krill pellets containing organic sponge extracts) and control (krill pellets containing solvent alone) pellets presented in a random sequence to the Bermuda bream *Diplodus bermudensis.* $N = 9$. Asterisks indicate experimental treatments that were significantly different than controls ($P < 0.05$).

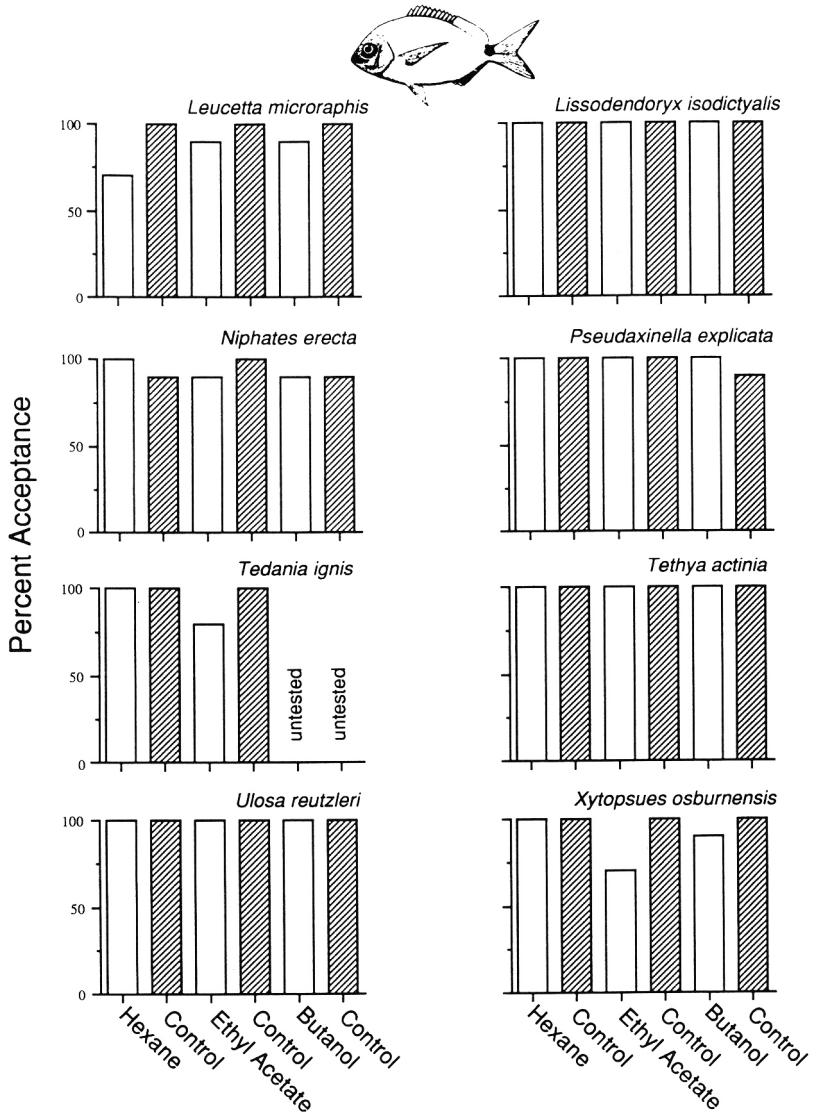


FIG. 2. Continued.

TABLE 1. TAXONOMIC CLASSIFICATION, HABITAT TYPE, AND COLOR MORPH OF BERMUDIAN SPONGES EXAMINED FOR FEEDING DETERRENT PROPERTIES

	Habitat	Color
Haplosclerida		
Callyspongiidae		
Callyspongia vaginalis	patch reefs	gray-green
Niphatidae		
Amphimedon viridis	patch reefs, rock walls, soft bottoms	green
Niphates erecta	patch reefs, rock walls	blue-green
Adociidae		
Adocia amphioxa	rock walls	dull brown
Dictyoceratida		
Spongidae		
Ircinia felix acuta	patch reefs, rock walls, soft bottoms	purple-brown
Ircinia felix fistularis	patch reefs, rock walls, soft bottoms	purple-brown
Dysideidae		
Dysidea etheria	patch reefs, rock walls	gray-blue
Verongida		
Aplysinidae		
Aplysina fistularis	patch reefs, rock walls, soft bottoms	sulfur yellow
Poecilosclerida		
Tedaniidae		
Tedania ignis	mangroves, soft bottoms, seagrass beds	orange-red
Desmaerdonidae		
Xytopsues osburnensis	seagrass beds	dark purple, black
Myxillidae		
Lissodendoryx isodictyalis	rock walls, mangroves	yellow
Hadromerida		
Tethidae		
Tethya actinia	rock walls, caves mangroves	green
Axinellida		
Axinellidae		
Pseudaxinella explicata	rock walls, mangroves, caves	red
Ullosa reutzleri	patch reefs, mangroves, rock walls	orange
Spirophorida		
Tetillidae		
Cinachyra alloclada	rock walls, caves	orange-yellow
Leucettida		
Leucetta microraphis	caves	white-pink

ranged from 6 to 38%. In contrast, 69% of 71 species of Caribbean sponges caused feeding deterrence in fish (Pawlik et al., 1995), although the inclusion of aqueous extracts and the use of a different species of fish model preclude direct comparisons with the present study. These investigators used the Caribbean wrasse, *Thalasoma bifasciatum*, in their assays, an abundant generalist carnivore similar to the sergeant major and Bermuda bream. Although we found overall fewer Bermudian sponges chemically defended, we did observe some similarities between our study and the Caribbean one. Both studies found that *A. fistularis*, *D. etheria* and *I. felix* were unpalatable, and that *Callyspongia vaginalis* and *Niphates erecta* were palatable. Of particular significance was our finding that *Lissodendoryx isodictyalis*, *Niphates erecta*, *Tedania ignis*, and *Tethya actinia* were palatable to generalist fish, while Pawlik et al. (1995) found these same Caribbean species deterrent to a generalist fish. As noted, this may reflect the presence of feeding deterrents in aqueous extracts, the feeding preferences of a different fish model (see also Pennings et al., 1994), or may indicate that chemical defense can be labile over short times scales (Teeyapant and Proksch, 1993). Considerable intraspecific variation in defensive chemistry has been noted in some marine sponges (Pawlik et al., 1995), suggesting that chemical deterrents may be produced in response to ecological variables such as predation. Intraspecific variations in chemistry may also be attributable to differences in symbiont load, genetics, or automimicry (Pawlik et al., 1995).

The natural products responsible for the observed ichthyodeterrence are generally unknown. Grode and Cardellina (1984) detected a novel sesquiterpene (furodysinin lactone) from the Bermudian sponge *D. etheria*, a species we found deterrent to both sergeant majors and Bermuda bream. Furodysinins isolated from nudibranchs cause feeding deterrence in fish (Thompson et al., 1982). It is possible that these compounds are responsible for the feeding deterrence we detected in both species of fish. Grode and Cardellina (1984) demonstrated that the Bermudian nudibranch, *Hypselodoris zebra*, may derive its defensive chemistry from its diet of *D. etheria* and that sergeant majors rejected the digestive tract and outer skin of this nudibranch. Kong and Faulkner (1993) isolated two antimicrobial compounds, leucettamol A and B, from *Leucetta microraphis* in Bermuda, a sponge we found active against sergeant majors. However, the ichthyodeterrent properties of these pure compounds were not investigated.

While vulnerable to predators such as fish, marine sponges in general appear rarely to be selected as preferred prey (Randall and Hartman, 1968). This pattern holds in Bermuda where De Laubenfels (1950) observed very few instances of spongivory by fish. Low levels may be attributable not only to chemical, mechanical, or nutritive defenses, but also to the paucity of spongivorous fish. For example, three common spongivorous groups of fish, the puffers, angel, and spade fish (Randall and Hartman, 1968) are more common, both quantitatively and qualitatively, in the Bahamas than in Bermuda. Only three species of

angel fish and three species of puffer fish are found in Bermuda, whereas six and five species occur in the Bahamas, respectively (William F. Smith-Vaniz, personal communication). Moreover, no spade fish occur in Bermuda (Sterrer, 1992). The reduction in species number is associated with both the extreme northern geographic location of Bermuda that excludes many tropical species, combined with the lasting effects of a tropical fish fauna decimated by the most recent glaciation event some 11,000 years ago (Briggs, 1974). Decades of heavy fish-pot usage (only recently stopped; Butler et al., 1993) also may have contributed to reduced numbers of individuals and species.

While a number of invertebrates are found in association with Bermuda sponges (De Laubenfels, 1950; Sterrer, 1986), they are primarily commensals and do not damage tissues. There has been only one report of a marine polychaete feeding on Bermuda sponges; *Branchiosyllis oculata* feeds on a wide diet of sponges but causes little damage (Pawlik, 1983). In contrast, sea stars in the Caribbean and particularly in Antarctica may specialize on diets of sponges, in some cases causing significant damage (Wulff, 1995; Dayton et al., 1974; Sloan, 1980; McClintock, 1994). Also capable of causing massive damage to sponges are sea turtles such as the hawksbill (Meylan, 1988). Few spongivorous hawksbill turtles occur in Bermuda, and most, if not all, individuals are immature (Anne Meylan, personal communication). Thus, predation on Bermudian sponges would probably be low for both invertebrates and turtles, similar to patterns noted for fish.

It is likely that some sponges rely on either mechanical defenses or very low levels of nutrients to make themselves unattractive as prey. Although we did not investigate the role of spicules as defensive agents, a number of studies indicate they are unlikely deterrents. Meylan (1988) found that hawksbill turtles can consume large amounts of sponge tissue with no detrimental effects. Dayton et al. (1974) suggested that antarctic spongivorous sea stars would not be deterred by spicules as they extrude their cardiac stomach against the outer pinacocytic tissues and digest their prey extraorally. McClintock (1987) found no correlation between those sponges included in antarctic sea star diets and ash content (an indirect measure of spicule content) in 17 species of antarctic sponges. Recently, Chanas and Pawlik (1995) examined five species of Caribbean reef sponges and demonstrated that spicules did not alter food palatability to fish. Thus, there is little direct evidence that spicules are defensive. Food quality may affect the effectiveness of chemical deterrents in sponges (Duffy and Paul, 1992; Pennings et al., 1994). Krill alginate pellets employed in our assays contained concentrations of soluble protein (an acceptable measure of food quality) (McClintock, 1986) that fall within the range of soluble protein values for marine sponges (McClintock, 1987), and it is unlikely that artificially high concentrations of feeding stimulants masked any effects of chemical deterrents.

The relatively small number of sponge species examined makes an evalu-

ation of chemical defense and habitat difficult. We found that chemically active sponges occurred in all habitat types examined except seagrass beds. However, only one sponge was examined from this habitat type. There appeared to be no relationship between chemical defense and degree of coloration: both drab and brightly colored sponges were unpalatable, similar to findings by Pawlik et al., (1995). Baker et al. (1994), however, detected sponge pigments that cause deterrence in sea stars, suggesting that there may be a relationship between pigmentation and feeding deterrence in some marine sponges.

The paucity of spongivory in the shallow waters of Bermuda may provide the basis for the low incidence in lipophilic chemical deterrents present in sponge tissue. Considered together with the results of other studies, our data indicate that chemical defense in sessile and sluggish marine invertebrates is best viewed within the context and as a result of local ecological conditions, rather than as a consequence of latitudinal location.

Acknowledgments—We thank the Bermuda Biological Station for Research (BBSR) for providing laboratory facilities. We also acknowledge a BBSR Graduate Fellowship awarded to D.S. Financial support was provided to J.B.M. by a faculty grant from the University of Alabama at Birmingham and a Research Grant-in-Aid from BBSR. Annie F. Glasspool of the Bermuda Aquarium kindly assisted with fish collections. We also thank Wolfgang Sterrer for graciously allowing us to use illustrations in the 1992 edition of his book on Bermuda's marine life. This is contribution 1445 of the Bermuda Biological Station for Research, Inc.

REFERENCES

BAKER, B. J., YOSHIDA, W. Y., and MCCLINTOCK, J. B. 1994. Chemical constitutents of four antarctic sponges in McMurdo Sound, Antarctica. *Antarct. J. U.S.* 29:153–155.

BAKUS, G. J., and GREEN, G. 1974. Toxicity in sponges and holothurians: A geographic pattern. *Science* 185:951–953.

BAKUS, G. J., TARGETT, N. M., and SCHULTE, B. 1986. Chemical ecology of marine organisms: An overview. *J. Chem. Ecol.* 12:951–987.

BERGQUIST, P. R. 1978. Sponges. University of California Press, Los Angeles, California, 268 pp.

BOBZIN, S. C., and FAULKNER, J. 1992. Chemistry and chemical ecology of the Bahamian sponge *Aplysina glacialis*. *J. Chem. Ecol.* 18:309–332.

BRIGGS, J. C. 1974. Marine Zoogeography. McGraw-Hill, New York, 475 pp.

BUTLER, J. N., BURNETT-HERKES, J., KATES, R. W., MCGOWAN, A., and O'RIORDAN, T. 1993. The Bermuda fisheries: A tragedy of the commons averted? *Environment* 35:6–15.

CHANAS, B., and PAWLIK, P. R. 1995. Defenses of Caribbean sponges against predatory reef fish: II. Spicules, tissue toughness and nutritional quality. *Mar. Ecol. Prog. Ser.* 127:195–211.

DAYTON, P. K., ROBILLIARD, G. A., PAINE, R. T., and DAYTON, L. B. 1974. Biological accommodation in the benthic community at McMurdo Sound, Antarctica. *Ecol. Monogr.* 44:105–128.

DE LAUBENFELS M. W. 1950. An ecological discussion of the sponges of Bermuda. *Trans. Zool. Soc.* 27:155–200.

DUFFY, J. E., and PAUL, V. J. 1992. Prey nutritional quality and the effectiveness of chemical defenses against tropical reef fish. *Oecologia* 90:333–339.

FAULKNER, D. J. 1995. Marine natural products. *Nat. Prod. Rep.* 12:223–269.

GREEN, G. 1977. Ecology of toxicity of marine sponges. *Mar. Biol.* 40:207–215.

GRODE, S. H., and CARDELLINA, J. H. II. 1984. Sesquiterpenes from the sponge *Dysidea etheria* and the nudibranch *Hypselodoris zebra*. *J. Nat. Prod.* 47:76–83.

HARRISON, F. W., and COWDEN, R. R. 1976. Aspects of Sponge Biology. Academic Press, New York.

KONG, F., and FAULKNER, D. J. 1993. Leucettamols A and B, two antimicrobial lipids from the calcareous sponge *Leucetta microraphis*. *J. Org. Chem.* 58:970–971.

KOLTUN, V. M. 1970. Sponges of the arctic and antarctic: A faunistic review. *Symp. Zool. Soc. London* 25:285–297.

McCLINTOCK, J. B. 1986. On estimating energetic values of prey: Implications in optimal diet models. *Oecologia* 70:161–162.

McCLINTOCK, J. B. 1987. An investigation between the relationship between invertebrate predation and the biochemical composition, energy content, spicule armament and toxicity of sponges in the benthic community at McMurdo Sound, Antarctica. *Mar. Biol.* 94:479–487.

McCLINTOCK, J. B. 1994. The trophic biology of antarctic echinoderms. *Mar. Ecol. Prog. Ser.* 111:191–202.

McCLINTOCK, J. B., BAKER B. J., HAMANN, M., SLATTERY, M., KOPITZKE, R. W., and HEINE, J. N. 1994. Tube-foot chemotactic responses of the spongivorous sea star *Perknaster fuscus* to organic extracts of antarctic sponges. *J. Chem. Ecol.* 20:859–870.

MEYLAN, A. 1988. Spongivory in hawksbill turtles: A diet of glass. *Science* 239:393–395.

PAUL, V. J. 1992. Ecological Roles of Marine Natural Products. Comstock, Ithaca, New York.

PAWLIK, J. R. 1983. A sponge eating worm from Bermuda: *Branchiosyllis oculata* (Polychaeta: Syllidae). *PSZNI Mar. Ecol.* 4:65–79.

PAWLIK, J. R. 1993. Marine invertebrate chemical defenses. *Chem. Rev.* 93:1911–1922.

PAWLIK, J. R., CHANAS, B., TOONER R. J., and FENICAL W. 1995. Defenses of Caribbean sponges against predatory reef fish: I. Chemical deterrency. *Mar. Ecol. Prog. Ser.* 127:183–194.

PENNINGS, S. C., PABLO, S. R., PAUL, V. J., and DUFFY, J. E. 1994. Effects of secondary metabolites in different diets on feeding by three groups of consumers. *J. Exp. Mar. Biol. Ecol.* 180:137–149.

RANDALL, J. E., and HARTMAN, W. D. 1968. Sponge feeding fishes of the West-Indies. *Mar. Biol.* 1:216–225.

SLOAN, N. A. 1980. Aspects of feeding biology of asteroids. *Oceanogr. Mar. Biol. Annu. Rev.* 18:57–124.

STERRER, W. 1986. Marine Fauna and Flora of Bermuda. Systematic Guide to the Identification of Marine Organisms. John Wiley & Sons, New York.

STERRER, W. 1992. Bermuda's Marine Life. Island Press, Hamilton, Bermuda.

TEEYAPANT, R., and PROKSCH, P. 1993. Biotransformation of brominated compounds in the marine sponge *Verongia aerophoba*—evidence for an induced chemical defense? *Naturwissenschaften* 80:369–370.

THOMPSON, J. E., WALKER R. P., WRATTEN, S. J., FAULKNER, D. J. 1982. A chemical defense mechanism for the nudibranch *Cadlina luteomarginata*. *Tetrahedron* 38:1865–1873.

THOMPSON, J. E., WALKER, R. P., FAULKNER, D. J. 1985. Screening and bioassays for biologically-active substances from forty marine sponge species from San Diego, California, USA. *Mar. Biol.* 88:11–21.

VERMEU, G. J. 1978. Biogeography and Adaptation. Harvard University Press, Boston.

WULFF, J. L. 1995. Sponge-feeding by the Caribbean starfish *Oreaster reticulatus*. *Mar. Biol.* 123:313–325.

ZAR, J. 1984. Biostatistical Analysis. Prentice Hall, Englewood Cliffs, New Jersey.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

HOW EFFICIENT ARE ALL-GLASS SYSTEMS FOR COLLECTION OF AIRBORNE VOLATILES?

ARNON SHANI[1,3,*] and JOHN CLEARWATER[2]

[1]*Department of Chemistry, Ben-Gurion University of the Negev*
Be'er Sheva 84105, Israel

[2]*The Horticulture and Food Research Institute of New Zealand*
Mt. Albert Research Centre
Private Bag 92169, Auckland, New Zealand

(Received August 5, 1996; accepted January 28, 1997)

Abstract—Airborne volatiles of saturated and unsaturated acetates and alcohols with the dodecane, tetradecane, and hexadecane chains released from fresh polyethylene pheromone dispensers were collected in Pasteur pipets attached to an all-glass collecting system. A maximum of 300–500 ng of pheromone in the first pipet (in a row of three or four) could be accumulated. The total amount that can be collected from an active rich source in 10–15 min of aeration (at 40–50 ml/min/cage) can reach 0.5–1 μg. Breakthrough from a pipet starts after an accumulation of 40–50 ng. There is a direct relationship among airflow rate in the system, the period of collection, and the vapor pressure of the chemicals on the amount adsorbed on the glass surface of the pipets. Polar functionalities, such as hydrogen bonding and π electrons play a significant role in the strength of adsorption to the glass surface. The system and technique are simple, fast, and easy to handle for collection of airborne volatiles from plants, animals, and nonliving sources.

Key Words—All-glass volatile collecting system, volatiles, aeration period, vapor pressure, polar effects, surface adhesion, volatiles breakthrough, insect sex pheromones.

INTRODUCTION

The efficiency of collection of airborne volatiles in a system is dependent on several factors, including the vapor pressure of the chemicals collected, the

*To whom correspondence should be addressed.
[3]On sabbatical leave at The Horticulture and Food Research Institute of New Zealand, Mt. Albert Research Centre, Auckland, New Zealand.

0098-0331/97/0600-1621$12.50/0 © 1997 Plenum Publishing Corporation

polarity of the chemicals, the flow rate of the gas carrying volatiles, the temperature, the polarity, and the surface area of the adsorbing material (charcoal, Tenax, Porapak, or a glass surface for an all glass unit) (Golub and Weatherston, 1984). When a glass surface is used as the adsorbent to trap the molecules, the efficiency is more sensitive to the flow rate of the gas (air), as well as the polarity of the chemicals and their interaction with the glass (the adhesion properties of the glass). The vapor pressure of the chemicals should have an effect on the amount adsorbed and the onset of breakthrough (loss of material that passes through the system without being collected or trapped) from the collecting glass surface.

The all-glass collecting system for airborne volatiles (Shani and Lacey, 1984; Shani, 1990), hereafter referred to as the Shani-Lacey method, is efficient enough to collect nanogram and subnanogram quantities released by live insect females (Barrer et al., 1987; Witzgall and Frerot, 1989; Shani, 1990; Lacey and Sanders, 1992), as well as from artificial sources such as glass beads (Shani and Lacey, 1984). When larger quantities are to be collected one needs to determine the efficiency of the system. Therefore, we performed measurements on the release of volatiles from fresh commercial pheromone dispensers and collected them in the all-glass unit in order to answer the following questions: (1) How much material can be collected on a Pasteur pipet? (2) At what amount per pipet will there be breakthrough? (3) What is the dependence of the collection on the flow rate of the passing air? (4) Is there a direct relationship between the period of air passage and volatile collection? (5) Is there a direct relationship between the vapor pressure of the chemicals studied and the efficacy of adsorption on the glass surface? (6) What is the effect of polar functionalities in the molecules on the adsorption on the glass surface?

In order to investigate these points we selected saturated and unsaturated pheromone components varying in chain length [dodecyl (C_{12}), tetradecyl (C_{14}), and hexadecyl (C_{16}) chains] and with different functionalities (alcohols and acetates). Thus, we could compare the effect of vapor pressure and polarity (hydrogen bonding and π electrons in double bonds) of the chemicals studied, while varying the flow rate of the passing air.

METHODS AND MATERIALS

General. The all-glass collecting system used for airborne volatiles has been described earlier (Shani and Lacey, 1984; Shani, 1990). The major difference was the number of Pasteur pipets connected to the cage, in which a twisted polyethylene Shin Etsu dispenser was housed. The tip of one pipet was introduced into the mouth of the next (usually three pipets in a row), with adhesive tape holding the pipets together, thus producing a 35-cm-long pipet of which 33 cm constituted the active collecting surface (each pipet had an 11-cm

length of active collecting surface). Disposable glass Pasteur pipets were used (John Poulton; D812, 230 mm length, 1 mm ID).

The temperature during the collection of volatiles was 23–24°C, and the flow rate was usually 40 ± 2 ml/min unless otherwise mentioned. The pipets were sealed with a fine flame at their far tip before washing with pentane (3 × 3–5 μl). All samples were kept at 0–5°C until injected into the GC.

Chemicals. Standard solutions of pheromone components (provided by Shin Etsu) were prepared in pentane (nanograde; Mallinckrodt) as concentrations of nanograms and tens of nanograms per microliter and kept at 0–5°C. Air for aeration with less than 1 ppm hydrocarbons (Zerograde BOC Gases) was used.

Dispensers. New commercial Shin Etsu polyethylene rope dispensers were used (Isomate-LBAM, Isomate-C-Plus, Isomate-OFM-Plus, Isomate-C. obliquana, Isomate-P. excessana, and P.B. rope-L). They were introduced into the glass cage after twisting into a double helix, in order to fit them into an egg-shaped cage 3 cm long and 2.5 cm wide.

Gas Chromatography. A Hewlett Packard 5890 series II gas chromatograph, with FID and a DB 1701 capillary column, 30 m × 0.25 mm ID, 0.25-μm film (J&W Scientific, Inc., Folsom, California 95630), operating in splitless mode and H_2 flow (carrier gas) of 2.5 ml/min, was used. The injector temperature was 175°C, detector 225°C. The following oven programming was applied for the C_{14} pheromone components: injection at 100°C (hold 1 min), heating to 200°C at 20°C/min and hold at 200°C for 4 min (2 min for C_{12} components and 6 min for C_{16} components). Retention times (R_t) of the studied chemicals were as follows (minutes): dodecan-1-ol (12:OH), 5.89; (*E*,*E*)-8,10-dodecadienol (*E*,*E*8,10-12:OH), 6.49; (*E*)-8-dodecenyl acetate (*Z*8-12:OAc), 6.58; (*Z*)-8-dodecenyl acetate (*Z*8-12:OAc), 6.63; (*Z*)-5-tetradecenyl acetate (*Z*5-14:OAc), 8.28; (*Z*)-7-tetradecenyl acetate (*Z*7-14:OAc), 8.32; (*Z*)-8-tetradecenyl acetate (*Z*8-14:OAc), 8.36; tetradecyl acetate (14:OAc), 8.40; (*Z*)-9-tetradecenyl acetate (*Z*9-14:OAc), 8.42; (*E*)-11-tetradecenyl acetate (*E*11-14:OAc), 8.44; (*Z*)-11-tetradecenyl acetate (*Z*11-14:OAc), 8.51; (*E*,*E*)-9,11-tetradecadienyl acetate (*E*,*E*9,11-14:OAc), 9.45; (*Z*,*E*)-7,11-hexadecadienyl acetate (*Z*,*E*7,11-16:OAc), 11.16; and (*Z*,*Z*)-7,11-hexadecadienyl acetate (*Z*,*Z*)-7,11-16:OAc), 11.30. Two injections of 1 μl each were made from each sample, and the range and average are shown in the tables and figures below. Standard solutions were injected from time to time to ascertain that the detector response was constant.

RESULTS AND DISCUSSION

Three groups of pheromone components were studied, based on their vapor pressure and release rate from dispensers (Butler and McDonough, 1981; McDonough and Butler, 1983; Heath et al., 1986; McDonough et al., 1989):

the more volatile dodecyl chain (C_{12}), the tetradecyl chain (C_{14}) as the moderately volatile group, and the hexadecyl chain (C_{16}) as the least volatile group among the three. Most of our studies were performed on the tetradecyl chain, and comparisons were then made with the C_{12} and C_{16} chain chemicals.

Maximal Amount of Volatiles Adsorbed and Breakthrough from Pipets. Aeration of several dispensers loaded with different pheromone compositions of C_{14} chain length, for short periods (5, 10, and 15 min, see Table 1) showed that relatively large amounts are adsorbed on a pipet (close to 300 ng) (Table 1, A1, A2, B1, and B2 in 15 min); breakthrough starts at about 40–50 ng (Table 1, A1–C1, 5 min), and an amount that is about 10% of that collected in the first pipet is collected in the second pipet in the series of three pipets in a row. No volatiles were found in the third pipet (at 5 min, except for C1, which might be transferred from the second pipet). This means that airborne volatiles up to 40–50 ng can be safely collected on a single pipet surface at the appropriate flow rate (see later), as well as with the appropriate vapor pressure. The amount of 300 ng collected in one pipet may represent a monolayer of organic molecules covering most of the glass surface. This calculation is based on the area occupied by a tetradecenyl acetate molecule, which, when spread on a surface is ca. 100 $Å^2$ (18 Å × 6 Å, as measured from a model and from an energy minimized model, as implicated within Alchemy III, and visualized using Ras Mol), or 30 $Å^2$ when attached perpendicular to the surface, and the total inner surface area of the pipet (0.1 cm diameter, 11 cm length): $2\pi rh = 2 \times 3.14 \times 0.05 \times 10^8 \times 11 \times 10^8 = 3\text{–}3.5 \times 10^{16}$ $Å^2$.

The number of adsorbed parallel spread molecules on the surface is $3\text{–}3.5 \times 10^{16}/100 = 3\text{–}3.5 \times 10^{14}$, and $3\text{–}3.5 \times 10^{16}/30 = 1\text{–}1.2 \times 10^{15}$ in the case of perpendicular stacking.

Thus, the amount of parallel spread of C_{14} acetate molecules (mol wt ca. 250) is $250 \times 3\text{–}3.5 \times 10^{14}/6 \times 10^{23} = 126\text{–}146$ ng, and in the case of perpendicular stacking, is $250 \times 1\text{–}1.2 \times 10^{15}/6 \times 10^{23} = 420\text{–}500$ ng.

The amount found experimentally shows that most of the surface is covered (if monolayered molecules are assumed), with all combinations of parallel spread and/or perpendicular stacking being possible. By the same token, we may say that breakthrough starts when ca. 10–15% of the surface is covered by the adsorbed molecules.

Based on the 5-min aeration, where the amount collected in the first pipet is below 100 ng and the breakthrough is low, it seems that at 10 min and 40 ± 2 ml/min flow rate there is no breakthrough (or very little) at the third pipet. Testing this in a separate experiment (A3 in Table 1), we found that about 10% of the amount collected in the third pipet is collected in the fourth pipet. The same result is obtained when dispenser C (C3 in Table 1) is aerated for 15 min, and the leak from the third to the fourth pipet is 10% (when the amount in the

TABLE 1. AIRBORNE VOLATILES FROM PHEROMONE DISPENSERS COLLECTED AT SEVERAL TIME PERIODS IN 3 OR 4 PASTEUR PIPETS CONNECTED AS AN ELONGATED PIPET[a]

Dispenser[b]	Pipet in series	Amount collected (ng) as function of aeration period			
		5 min	10 min	15 min	20 min
A1	i	65–70	221–293	252–290	[d]—
	ii	6–7	160–172	145–160	—
	iii	nd[c]	45–48	147–168	—
	Total	71–77	426–513	544–618	
A2	i	43–48	145–157	280–290	—
	ii	4–5	158–192	190–209	—
	iii	nd	42–47	132–148	—
	Total	47–53	345–396	602–647	
A3	i	—	196–224	—	218–236
	ii	—	116–132	—	130–163
	iii	—	57–68	—	102–120
	iv	—	4–6	—	66–69
	Total		373–430		516–588
B1	i	68–73	225–261	275–345	—
	ii	21–24	178–192	162–168	—
	iii	nd	105–124	61–88	—
	Total	89–97	508–577	498–601	
B2	i	85–95	135–203	258–274	—
	ii	10–12	135–142	181–188	—
	iii	nd	16–20	165–170	—
	Total	95–107	286–365	604–632	
C1	i	86–118	134–136	151–161	—
	ii	10–11	54–63	75–79	—
	iii	2–3	48–50	74–89	—
	Total	98–132	236–249	300–329	
C2	i	—	77–91	120–136	—
	ii	—	56–69	53–64	—
	iii	—	28–32	47–52	—
	Total		161–192	220–252	
C3	i	—	—	106–109	—
	ii	—	—	58–95	—
	iii	—	—	60–62	—
	iv	—	—	6–8	—
	Total			230–274	

[a]Air flow 40 ± 2 ml/min in each cage; temperature 23–24°C.
[b]Dispenser A: Z-5-14:OAc/Z-8-14:OAc (1:3); B: Z-5-14:OAc/Z-7-14:OAc/14:OAc (35:35:30); C: *E*-11-14:OAc/Z-11-14:OAc/*E*,*E*-9,11-14:OAc (70:27:3).
[c]Not detected.
[d]Not measured.

pipet is less than 100 ng). Significant breakthrough should occur at 15 min of aeration, as the amounts collected in three of four of the A and B dispensers at the third pipet are above 140–150 ng. A similar amount is also found in the second pipet with 10-min aeration and breakthrough amounts of 40–50 ng. Thus, we may conclude that at 15 min of aeration we should add ca. 40–50 ng to those samples in which the third pipet contains >100–150 ng. This is demonstrated in the fourth pipet at 20 min of aeration of A3 (Table 1). This brings the total amount aerated from each dispenser cage into the pipets into the range of 600–700 ng in a period of 15 min, or 2.5–3 μg/hr.

Amount Collected as a Function of Aeration Time. Figure 1 shows the accumulated amount collected as a function of time. From Figure 1 and Table 1, it can be seen that during the first 5 min the amount collected is much less than expected when compared with the 10-min aeration. This could be the result of a slow saturation process of the glass surface in the first few minutes before a more rapid movement of molecules occurs on the already-deposited monolayer and significant breakthrough starts. The first pipet at 10 min is about three times richer in material than the first pipet at 5 min. The second pipet at 10 min is 10–30 times richer than the corresponding pipet at 5 min. On the other hand,

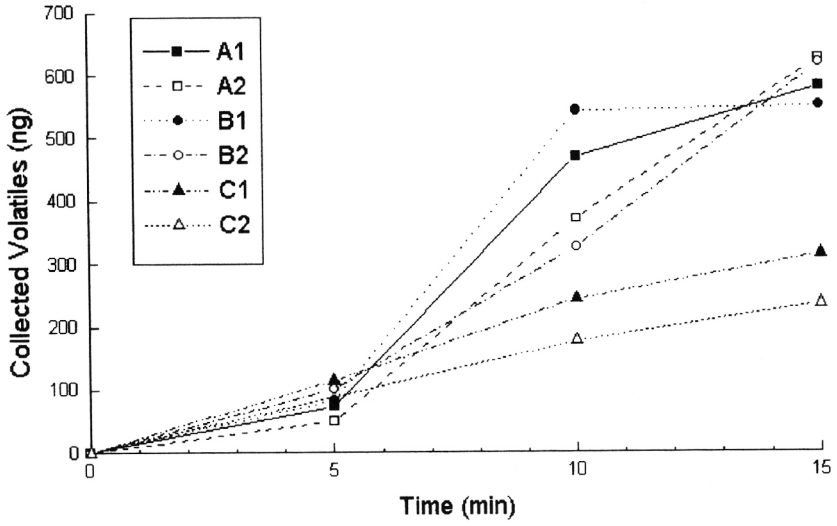


FIG. 1. Total amount of tetradecyl and tetradecenyl acetates collected in three or four Pasteur pipets in a row as a function of aeration period at 40 ml/min at 23–24°C. The data points are the average of two GC measurements of each sample. Dispenser A: *Z*5–14:OAc and *Z*8–14:OAc (1:3); B: *Z*5–14:OAc, *Z*7–14:OAc, and 14:OAc (35:35:30); C: *E*11–14:OAc, *Z*11–14:OAc, *E*,*E*9,11–14:OAc (70:27:3).

the slope between 10 and 15 min has decreased somewhat, as not much space is left available for monolayer adsorption on the glass and less added material is collected. It can be seen that at 20 min (A3 in Table 1), there is leveling off of the collected material in the four pipets. Based on the assumption that the organic molecules accumulate as a monolayer adsorbed on the glass, we may estimate that at higher rates of airflow the breakthrough from the already-saturated pipet will be faster. The average total amount collected in A1, A2, B1, and B2 dispensers (Table 1) is 80 ± 20, 427 ± 85, and 593 ± 30 ng (or 2:10.5:15) at 5, 10, and 15 min, respectively, and these averages are significantly different from each other (ANOVA, $P < 0.01$). The nonlinearity of material collected as a function of aeration time is therefore demonstrated, with two opposing processes taking place, slow saturation at the early stage and fast flooding at the later stage.

Less material is collected from dispenser C compared with A and B (Table 1 and Figure 1). This could result from different release rates of the dispensers, less volatility of the components, or a combination of both factors. As the ratio of components in dispenser C remains almost constant in the pipets (see below), there is no loss of components during the aeration.

Flow Rate of Passing Air. The effect of flow rate is demonstrated in Table 2 and Figure 2. Airflow below 30 ± 2 ml/min removes few volatiles from the cage to the pipets. The reason for this is the restriction of airflow in the long narrow pipet (3 in a row). On the other hand, at 50 ± 2 ml/min, the amount accumulated after 10 min is similar or somewhat lower than that collected at a flow of 40 ± 2 ml/min for 15 min (compare Tables 1 and 2 for A1 and B2). One can therefore compensate for time by increasing flow rate and vice versa. The trend in slopes of the lines in Figure 2 is similar to that of Figure 1. Bradley et al. (1995) found that the release rate from Shin Etsu dispensers varied little with wind speed under field conditions. Our collecting conditions were very different from field conditions, and the effect of airflow is dramatic. On the other hand, it could be that above a certain level of airflow the release rate is no longer sensitive to flow rate, and our measurements were conducted below that level. Van der Kraan and Ebbers (1990) found that the release rate of Z9-14:OAc and Z11-14:OAc from different dispensers is affected by the air velocity (up to 360 cm/sec).

As for the maximum amount collected in one pipet we see that the first pipet in A1 (Table 2, 50 ml/min) adsorbed a little more than 300 ng, lending support to our previous conclusion that about 300 ng of these molecules seems to be the maximum load in one pipet.

Effect of Vapor Pressure on Volatiles Collection. Three groups of chemicals in rubber septa were studied, based on the reported half-lives of pheromone in rubber septa dispensers ($t_{1/2}$) (McDonough and Butler, 1983; Heath et al., 1986; McDonough et al., 1989). The 12-carbon molecules have $t_{1/2}$ in the range of

TABLE 2. AIRBORNE VOLATILES COLLECTED FROM PHEROMONE DISPENSERS AT DIFFERENT AIRFLOW RATES[a]

Dispenser[b]	Pipet in series	Volatiles (ng) at air flow of 30 ± 2 (ml/min)	40 ± 2 (ml/min)	50 ± 2 (ml/min)
A1	i	44–50	221–293	323–408
	ii	< 1	160–172	177–186
	iii	nd[c]	45–48	41–42
	Total	44–50	426–513	541–636
B2	i	35–43	135–203	251–323
	ii	< 1	135–142	113–131
	iii	nd	16–20	25–31
	Total	35–43	286–365	389–485
C1	i	48–51	134–136	[d]—
	ii	25–28	54–63	—
	iii	9–10	48–50	—
	Total	82–89	236–249	—

[a] Air flow for 10 min; temperature 23–24°C.
[b] Dispenser A: Z-5-14:OAc/Z-8-14:OAc (1:3); B: Z-5-14:OAc/Z-7-14:OAc/14:OAc (35:35:30); C: *E*-11-14:OAc/*Z*-11-14:OAc/*E*,*E*-9,11-14:OAc (70:27:3).
[c] Not detected.
[d] Not measured.

27–46 days, the C_{14} molecules have much longer $t1/2$ times of 160–320 days, and the heaviest molecules (the C_{16} series) studied have $t1/2$ of 760–960 days at 25°C. The results of airborne volatiles collected from the C_{12} series (Table 3) and the C_{16} chemicals (Table 4) show that the more volatile C_{12}s behave as expected, as does the pink bollworm pheromone (C_{16}). It took a much longer time and a higher flow rate (50 ml/min) to accumulate comparable quantities of the C_{16} acetates. As a result, it seems that the phenomenon of slow and fast processes that we noted in the C_{14} series (Figure 1) is not significant in this case, even though there is more rapid accumulation between 20 and 30 min. The maximum amount adsorbed (of C_{16} molecules) in one pipet does not exceed 300 ng, which fits our calculations for mixed parallel spread and perpendicular stacking (see above).

Polarity of Chemicals Adsorbed. It is interesting to note that the polarity of the molecules and their interaction with the glass surface plays a significant role in the adsorption process. Two factors, among others, that affect polarity are hydrogen bonding and π electrons in double bonds. The effect of hydrogen

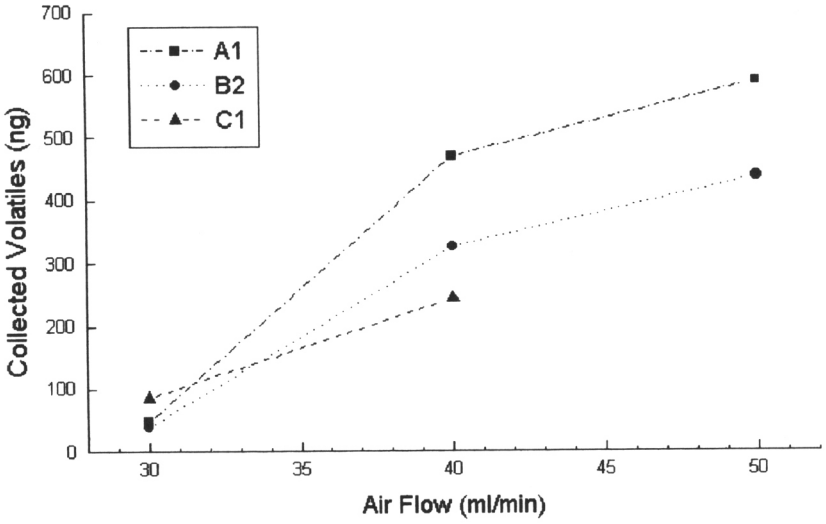


FIG. 2. Total amount of tetradecyl and tetradecenyl acetates collected in three Pasteur pipets in a row as function of airflow rate for 10 min at 23–24°C. The data points are the average of two GC measurements of each sample. Dispenser A: Z5–14:OAc and Z8–14:OAc (1:3); B: Z5–14:OAc, Z7–14:OAc, and 14:OAc (35:35:30); C: E11–14:OAc, Z11–14:OAc, E,E9,11–14:OAc (70:27:3).

TABLE 3. AIRBORNE VOLATILES FROM ORIENTAL FRUIT MOTH AND CODLING MOTH PHEROMONE (C_{12}) DISPENSERS COLLECTED IN 4 PASTEUR PIPETS CONNECTED AS AN ELONGATED PIPET[a]

Pipet in series	Volatiles collected (ng)[b]			
	D1	D2	E1	E2
i	204–228	220–243	460–567	308–335
ii	190–223	189–221	201–209	220–240
iii	170–220	173–184	77–85	90–95
iv	115–136	126–148	3–6	13–18
Total	679–787	708–796	741–867	631–688

[a] Airflow 40 ± 2 ml/min in each cage for 10 min, Temp. 23–24°C.
[b] Dispenser D: Z-8–12:OAc (contains a few percent of the E-isomer); E: E,E-8,10–12:OH/12:OH (60:40).

TABLE 4. AIRBORNE VOLATILES FROM PINK BOLLWORM (C_{16}) DISPENSERS COLLECTED IN 3 AND 4 PASTEUR PIPETS CONNECTED AS AN ELONGATED PIPET[a]

Dispenser[b]	Pipet in series	Amount collected (ng) and ratio[c] as function of aeration period				
		10 min	20 min	30 min	60 min	120 min
F1	i	80–90	164–170	252–255	219–288	304–365
		46.0:54.0	47.2:52.8	47.5:52.5	48.2:51.8	49.3:50.7
	ii	13–18	55–65	99–109	107–119	200–208
		43.0:57.0	44.2:55.8	44.7:55.3	47.2:52.8	49.2:50.8
	iii	2–4	3–5	20–26	89–98	230–238
		40.7:59.3	38.2:61.8	38.6:61.4	46.1:53.9	48.9:51.1
	iv	[d]—	—	—	74–78	152–165
					43.7:56.3	47.6:52.4
	Total	95–112	222–240	371–390	489–583	886–976
F2	i	80–85	115–125	240–287	210–224	278–285
		45.9:54.1	46.6:53.4	47.5:52.5	48.2:51.8	49.2:50.8
	ii	27–32	45–50	121–131	135–167	170–187
		43.4:56.6	44.0:56.0	44.1:55.9	47.8:52.2	49.0:51.0
	iii	3–4	4–6	22–25	85–98	110–127
		40.2:59.8	39.5:60.5	38.6:61.4	46.1:53.9	48.4:51.6
	iv	—	—	—	38–40	141–143
					40.7:59.3	47.4:52.6
	Total	110–121	164–181	383–443	468–529	699–742

[a]Airflow 50 ± 2 ml/min in each cage; temperature 23–24°C.
[b]Dispenser F: *Z,E*/*Z,Z*-7,11-16:OAc (1:1).
[c]Ratio of *Z,E*/(*Z,E* + *Z,Z*)- × 100; ratios are in 0.5–1.0% range between the two injections of each sample.
[d]Not measured.

bonding was studied in the C_{12} series, while the double-bond effect was measured in the C_{14} components, as well as in the C_{16} acetates.

The dispenser of codling moth pheromone consisted of two major components, *E,E*8,10-12:OH and 12:OH, in about a 3:2 ratio. The dispenser of the Oriental fruit moth contained mostly *Z*8-12:OAc (and a few percent of the corresponding *E* isomer). As shown (Table 3), the total amount collected from the C_{12} components in 10 min of aeration is about the same (total of 650–800 ng). There is a big difference, however, in the distribution of the adsorbed material among the pipets in the series of four in a row. While the amounts adsorbed in the first three pipets for the *Z*8-12:OAc collection, where hydrogen bonding is inapplicable, are almost equivalent (D1 and D2, Table 3), the adsorbed yield diminished in the downstream pipets to 30–50% for the *E,E*8,10-12:OH and 12:OH collection (E1 and E2, Table 3), in which hydrogen bonding can occur from the hydroxyl group to the surface. This means that the more polar

molecules are adsorbed strongly on the surface of the first pipet and less material is passed to the second pipet. The maximum amount adsorbed on the first pipet in each cage shows 200–250 ng of the *Z*8-12:OAc and 300–500 ng of the *E*,*E*8,10-12:OH and 12:OH mixture. In one pipet (E1, Table 3) the 500 ng collected even exceeded the 300 ng of the C_{14} components. This quantity fits the calculated amount for monolayer perpendicular stacking molecules with a C_{12} chain, probably due to the hydrogen bonding of the OH group to the glass surface. This is in contrast to the acetates, which adsorbed more as spread molecules (see below).

The ratio between the two C_{12} alcohols (not shown) is slightly different as more of the saturated alcohol is found in the second and third pipets. This could be the result of the effect of π electrons in the double bonds (see below), or because of a higher release rate from the dispenser when compared with the diene alcohol. Brown et al. (1992) found about the same release rate for the two alcohols, but it could be that the dispenser material and thickness differ from those used in this study. On the other hand, McDonough et al. (1992) found that the release rate of 12:OH is about 1.5 times faster than that of *E*,*E*8,10-12:OH.

Careful inspection of the ratio between the three components of dispenser B [*Z*5-14:OAc, *Z*7-14:OAc, and sat. 14:OAc (35:35:30)] reveals that the ratio gradually changes to contain less of the saturated C_{14} acetate. In Table 5, in the second and third pipets of each cage, the ratio markedly changes to 46:45:9 (B1, 5 min), 45:45:10 (B2, 5 min), 47:47:6 (B2, 10 min), and 46:46:8 (B1, 15 min). The greater rate of loss of saturated C_{14} acetate is better demonstrated in the pipets that contain less of the pheromone. Those that have

TABLE 5. RELATIVE COMPOSITION OF PHEROMONE COMPONENTS (C_{14}) COLLECTED FROM DISPENSER B[a] IN SERIES OF 3 PIPETS

		Period of aeration and ratio of components (%)								
		5 min			10 min			15 min		
Dispenser	Pipet in series	(*Z*)-5	(*Z*)-7	Sat.	(*Z*)-5	(*Z*)-7	Sat.	(*Z*)-5	(*Z*)-7	Sat.
B1	i	36	36	28	34	34	32	34	34	32
	ii	46	45	9	37	37	26	36	36	28
	iii		nd[b]		43	43	14	46	46	8
B2	i	38	38	24	33.5	33.5	33	34	34	32
	ii	45	45	10	39	39	22	35.5	35	29.5
	iii		nd		47	47	6	38.5	38	23.5

[a]Original composition Z-5-14:OAc, Z-7-14:OAc, and 14:OAc (35:35:30).
[b]Not detected.

more of the pheromone (compare Table 1, B1, 10 min and B2, 15 min with the corresponding ratios in Table 5) lose a considerable quantity of all three components and the measured ratio is closer to the original. A rough estimation is that above 150 ng per pipet, the loss is not much different among the three components, while at 100 ng and below, the saturated C_{14} acetate is probably the only one to evaporate from the glass surface during the short period of aeration. This phenomenon is possibly due to the lack of π electrons in the hydrocarbon chain that interact with the glass surface. It is probably not the vapor pressure that causes this behavior, since the half-lives of pheromone rubber septa dispensers ($t1/2$), based on vapor pressure, show that saturated 14:OAc has a longer $t1/2$ than monoolefinic 14:OAc (Heath et al., 1986; McDonough et al., 1989, and references within). Support for this explanation is found with dispenser A, where the original ratio is kept constant in all three pipets (no saturated acetates in this dispenser).

An interesting finding is the different adsorption of the *cis Z*, and *trans E* isomeric configurations on the glass surface. Careful inspection of the ratio of the two components of the pink bollworm pheromone (C_{16} acetates), as shown in Table 4, reveals that the *Z,E* isomer is removed faster from the glass surface than the *Z,Z* isomer and the accumulated volatiles become richer with the latter. This phenomenon is more obvious when the total amount in the pipet is small. The reason for this behavior is the greater exposure of the π electrons in the *cis* isomer than in the *trans*. Based on these results, we assume that the acetate molecules, as compared to alcohol molecules, are more likely to be adsorbed flat on the surface rather than stacked (see above).

In dispenser C, which contains both *E*- and Z11-14:OAc, there is a small change in ratio in the pipets (2–3% less of the *E* isomer as compared to the original composition). Again, we see that the *trans* isomer is more weakly adsorbed than the *cis* configuration.

The results obtained in the above series of aeration of fresh dispensers can assist in planning a collection of airborne volatiles in the range of 0.1–1 μg from any appropriate source. Thus, for chemicals with low vapor pressure, a period of 5–15 min with a flow rate of 40–50 ml/min is sufficient to collect 0.1–0.5 μg in two to three Pasteur pipets in a row (or a long pipet of 25–30 cm). The aeration period can be longer than the initial slow stage of saturation of the glass surface, but sufficiently limited to avoid breakthrough, due to an excess of material that cannot be adsorbed on the glass surface.

To summarize, the described method of trapping volatiles has significant advantages over other collecting techniques: The system is simple to construct and to handle; it has many outlets for parallel collections; the period of collection to accumulate enough material for chemical analyses is short; it is efficient for a wide range of quantities from subnanogram to microgram; the trapped volatiles can be recovered as concentrated solutions without any need for extraction and

evaporation; the trapping surface is scrupulously clean; the trapping surface can be readily cooled if required; internal standards can be easily incorporated in the solution to ensure accurate quantification; and the volatiles (in solution) can be stored in the sealed capillaries for months at low temperature, until required for analysis.

Acknowledgments—Many thanks to David Greenwood, The Horticulture and Food Research Institute of New Zealand, Mt. Albert Research Centre, Auckland, for his generosity in allowing us to use his GC unit and for fruitful discussions.

REFERENCES

BARRER, P. M., LACEY, M. J., and SHANI, A. 1987. Variation in relative quantities of airborne sex pheromone components from individual female *Ephestia cautella* (Lepidoptera: Pyralidae). *J. Chem. Ecol.* 13:639–653.

BRADLEY, S. J., SUCKLING, D. M., MCNAUGHTON, K. G., WEARING, C. H., and KARG, G. 1995. A temperature-dependent model for predicting release rates of pheromone from a polyethylene tubing dispenser. *J. Chem. Ecol.* 21:745–760.

BROWN, D. F., KNIGHT, A. L., HOWELL, J. F., SELL, C. R., KRYSAN, J. L., and WEISS, M. 1992. Emission characteristics of a polyethylene pheromone dispenser for mating disruption of codling moth (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 85:910–917.

BUTLER, L. I., and MCDONOUGH, L. M. 1981. Insect sex pheromones. Evaporation rates of alcohols and acetates from natural rubber septa. *J. Chem. Ecol.* 7:627–633.

GOLUB, M. A., and WEATHERSTON, I. 1984. Techniques for extracting and collecting sex pheromones from live insects and from artificial sources, pp. 223–285, *in* H. E. Hummel and T. A. Miller (eds.). Techniques in Pheromone Research. Springer-Verlag, New York.

HEATH, R. R., TEAL, P. E. A., TUMLINSON, J. H., and MENGELKOCH, L. J. 1986. Prediction of release ratios of multicomponent pheromones from rubber septa. *J. Chem. Ecol.* 12:2133–2143.

LACEY, M. J., and SANDERS, C. J. 1992. Chemical composition of sex pheromone of oriental fruit moth and rates of release by individual female moth. *J. Chem. Ecol.* 18:1421–1435.

MCDONOUGH, L. M., and BUTLER, L. I. 1983. Insect sex pheromones: Determination of half-lives from formulations by collection of emitted vapor. *J. Chem. Ecol.* 9:1491–1502.

MCDONOUGH, L. M., BROWN, D. F., and ALLER, W. C. 1989. Insect sex pheromones: Effect of temperature on evaporation rates of acetates from rubber septa. *J. Chem. Ecol.* 15:779–790.

MCDONOUGH, L. M., ALLER, W. C., and KNIGHT, A. L. 1992. Performance characteristics of a commercial controlled-release dispenser of sex pheromone for control of coding moth (*Cydia pomonella*) by mating disruption. *J. Chem. Ecol.* 18:2177–2189.

SHANI, A. 1990. Calling behavior of almond moth (*Ephestia cautella*) females kept in glass cages and airborne pheromone deposited on glass surfaces by airstream. *J. Chem. Ecol.* 16:959–969.

SHANI, A., and LACEY, M. J. 1984. Convenient method applicable to single insects for collection and measurement of blend ratios of airborne pheromones from artificial sources. *J. Chem. Ecol.* 10:1677–1692.

VAN DER KRAAN, C., and EBBERS, A. 1990. Release rates of tetradecen-1-ol acetates from polymeric formulations in relation to temperature and air velocity. *J. Chem. Ecol.* 16:1041–1058.

WITZGALL, P., and FREROT, B. 1989. Pheromone emission by individual females of carnation tortrix, *Cocoecimorpha pronubana*. *J. Chem. Ecol.* 15:707–717.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

INCREASE OF TRAP CATCHES BY A COMBINATION OF MALE SEX PHEROMONES AND FLORAL ATTRACTANT IN LONGHORN BEETLE, *Anaglyptus subfasciatus*

KIYOSHI NAKAMUTA,[1,*] WALTER S. LEAL,[2] TADAKAZU NAKASHIMA,[1] MASAHIKO TOKORO,[1] MIKIO ONO,[3] and MICHITAKA NAKANISHI[4]

[1]*Forestry and Forest Products Research Institute*
P.O. Box 16, Tsukuba Norin Kenkyu Danchi-nai, Tsukuba
Ibaraki 305, Japan

[2]*National Institute of Sericultural and Entomological Science*
Ohwashi, Tsukuba, Ibaraki 305, Japan

[3]*Fuji Flavor Co. Ltd., Midorigaoka*
Hamura, Tokyo 190-11, Japan

[4]*Sankei Chemicals Co. Ltd.*
Hukaya, Saitama, 366, Japan

(Received June 26, 1996; accepted February 2, 1997)

Abstract—A combination of the synthetic sex pheromone [a 125:1 blend of (*R*)-3-hydroxy-2-hexanone and (*R*)-3-hydroxy-2-octanone] with methyl phenylacetate captured significantly more *Anaglyptus subfasciatus* Pic females than either the sex pheromone or methyl phenylacetate did alone.

Key Words—*Anaglyptus subfasciatus*, Coleoptera, Cerambycidae, sex pheromone, methyl phenylacetate, trap catches, synergism.

INTRODUCTION

Anaglyptus subfasciatus Pic (Coleoptera: Cerambycidae) is one of the most harmful insect pests of the Japanese cedar, *Cryptomeria japonica* (Linn. fil.) D. Don, and the Japanese cypress, *Chamaecyparis obtusa* (Sieb. et Zucc.) Endl., the most abundant tree species of forest plantations in Japan. Female

*To whom correspondence should be addressed.

0098-0331/97/0600-1635$12.50/0 © 1997 Plenum Publishing Corporation

beetles lay their eggs on dead twigs of cedar or cypress, and the larvae bore into the twigs and migrate further into the tree trunk. Feeding by the larvae on sapwood followed by fungal infection causes discoloration and decay of the wood, resulting in a decrease of the commercial value of the timber (Kobayashi, 1985). Since forest plantations are usually located near riverheads, spraying insecticides to control the beetles should be avoided to prevent contamination of groundwater. Alternative methods of control are therefore needed.

Since *A. subfasciatus* males and females are known to congregate on several species of flowers for feeding, floral scents and their structural similarities have been screened both in the laboratory and in the field (Ikeda et al., 1993). Methyl phenylacetate has been demonstrated to be attractive and highly specific to *A. subfasciatus* (Nakashima et al., 1994), and it has been used as a floral attractant in practice.

Nakamuta et al. (1994) reported that *A. subfasciatus* females are attracted to males in a wind tunnel and that a male-specific cuticular structure in the pronotum seems to be an organ of pheromone excretion. Furthermore, the male-released sex pheromone constituents of the beetle are identified as a 7:1 blend of (*R*)-3-hydroxy-2-hexanone and (*R*)-3-hydroxy-2-octanone (Leal et al., 1995).

In the present paper we report that trap catches of beetles were increased by a combination of male sex pheromone constituents and the floral attractant.

METHODS AND MATERIALS

Insects. Cryptomeria logs infested by *A. subfasciatus* were collected in the field in Kanagawa Prefecture (35°15′N and 139°5′E, Kanagawa population) in March 1995 and kept in field cages until emergence of the adult beetles. Beetles were collected in cages in April 1995 and then kept individually in plastic cages (12 cm diameter × 5 cm) under a 16L:8D photoperiod and fed a 5% (w/v) sucrose solution.

Collection of Insect Volatiles. Airborne volatiles of *A. subfasciatus* adults supplied only with sucrose solution on pieces of cotton were collected in an all-glass aeration apparatus. This consisted of a glass column (1 cm ID × 25 cm long). A glass collector (1 cm ID) packed to a depth of 1.5 cm with Super Q (Alltech, Deerfield, Illinois) held in place with glass wool. The collector was attached to the column with a ground glass reduction joint. Incoming air that passed through similar glass tubes packed with activated charcoal (1.2 cm ID × 30 cm long) and Tenax TA (Alltech) (1 cm ID × 1 cm) was bubbled into distilled water before entering the collection system. The collection apparatus was placed inside an incubator maintained at 25°C under a 16L:8D photoperiod. Trapped materials were extracted with hexane and the solutions concentrated to 0.1 beetle-equivalent per microliter.

Analysis of Sex Pheromone Constituents. A 7:1 blend was recorded from the beetle that emerged from logs collected in Iwate Prefecture (45°N and 141°5′E, Iwate population) (Leal et al., 1995). Since the field evaluation of synthetic sex pheromone was conducted in Kanagawa Prefecture, the sex pheromone constituents of the Kanagawa population were analyzed by gas chromatography (GC). The two populations are about 600 km apart. GC was carried out on a Hewlett-Packard 5890 II+ instrument equipped with a split/splitless injector, electronic pressure control, a flame ionization detector, and an HP 3365 Series II Chemstation. High-resolution GC analyses were performed with polar and nonpolar capillary columns: HP-Innowax and HP-5MS (30 m × 0.25 mm × 0.25 mm; Hewlett-Packard), respectively. These columns were operated under the following conditions: 50°C for 1 min, increased to 180°C at a rate of 4°C/min and held at this temperature for 1 min, increased again to 230°C at a rate of 10°C/min, and finally held at this temperature for 10 min. Gas chromatography–mass spectrometry (GC-MS) was carried out with an HP 5890 II+ gas chromatograph linked to a mass selective detector MSD 5972 (Hewlett-Packard). Chromatographic resolution was achieved on HP-5MS and HP-Innowax columns operated under the same conditions for GC.

Chemicals and Trap Dispensers. Twenty grams of methyl phenylacetate (>98%, Takasago Perfumery Co., Ltd., Kanagawa, Japan) was mixed and gelled with 5 g of 12-hydroxystearic acid (Matsumoto Oil Co., Ltd., Osaka, Japan) (Nakashima et al., 1994), and packed in a plastic cup (6 cm ID × 2.5 cm in depth). Sex pheromone constituents were synthesized as described by Leal et al. (1995), and 20 mg of pheromone, a 125:1 (w/w) blend of (*R*)-3-hydroxy-2-hexanone (>99%) and (*R*)-3-hydroxy-2-octanone (>99%), was incorporated into a device made of ES fiber (Chisso Co. Ltd., Tokyo, Japan) and covered with a polyethylene film. Traps were baited with a cup of methyl phenylacetate, five samples of synthetic sex pheromone, (100 mg), or a combination of a cup of the two. The dispensers of pheromone and methyl phenylacetate were replaced every two and three weeks, respectively.

Field Trapping. Attractiveness of synthetic sex pheromone and its combination with methyl phenylacetate was evaluated in a cedar forest in Kanagawa Prefecture during the 1995 emergence season. Yellow-colored plastic water pan traps (Sankei Chemicals Co. Ltd., Tokyo, Japan) were suspended about 5 m above the ground from the lowest branches of a cedar tree on April 25, 1995, and inspected every week until June 13. The distance between traps was about 10 m. Traps were randomized after weekly inspections. Captured insects were removed and taken to the laboratory to record their number and sexes. The experiments were conducted with six replicates.

Statistical Analyses. Capture data were transformed to $\sqrt{(\text{trap catches} + 0.5)}$ to meet the assumptions of ANOVA and analyzed using the multivariate general linear hypothesis (MGLH) model of SYSTAT 5.2 (Wilkinson et al.,

1992), followed by Tukey's test. Data of July 6 were omitted from the analysis, since most traps dropped to the ground due to an unusually strong wind.

RESULTS AND DISCUSSION

Analysis of Sex Pheromone Constituents. The sex pheromone system for *A. subfasciatus* was previously identified from the Iwate population as a 7:1 molar mixture of (*R*)-3-hydroxy-2-hexanone and (*R*)-3-hydroxy-2-octanone (Leal et al., 1995). Aeration from beetles of a Kanagawa population gave the two components in a remarkably different ratio, 125:1 (Fig. 1). This difference probably reflects geographical variation of the pheromone system of the two populations. The role of different ratios of pheromone constituents between remote populations and behavioral responses is well documented in the literature (Cardé, 1986). To clarify whether this difference in *A. subfasciatus* reflects a geographical variation, it is necessary to analyze the behavioral responses of the two populations towards each blend. For the present field experiments we used the ratio detected in the Kanagawa population (125:1).

Field Traps. Simultaneous release of synthetic sex pheromone and methyl

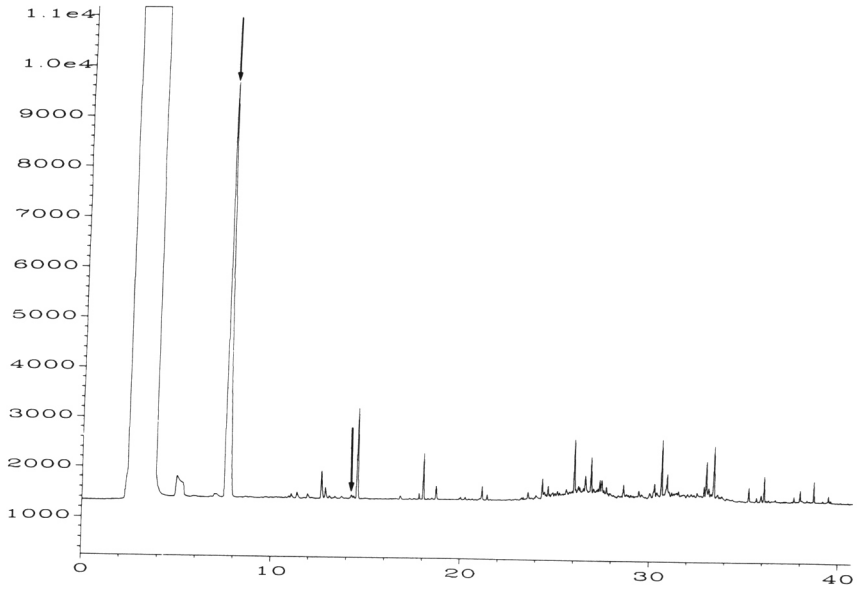


FIG. 1. Gas chromatogram of airborne volatiles from *A. subfasciatus* males emerging from the cedar logs collected in Kanagawa Prefecture (Kanagawa population). Arrows indicate the peak of (*R*)-3-hydroxy-2-hexanone and (*R*)-3-hydroxy-2-octanone, respectively.

TABLE 1. CAPTURE OF *A. subfasciatus* IN TRAPS BAITED WITH METHYL PHENYLACETATE, SYNTHETIC SEX PHEROMONE BLEND, AND BOTH DURING 1995 EMERGENCE SEASON

Lure[a]	Beetle catch/trap/season (mean ± SE)[b]	
	Female	Male
Methyl phenylacetate (MPA)	2.5 ± 0.7a	0.7 ± 0.3a
Synthetic sex pheromone	1.3 ± 0.8b	1.3 ± 0.6a
MPA+ synthetic sex pheromone	5.8 ± 0.9c	2.0 ± 0.9a
Control	0.3 ± 0.3d	0.3 ± 0.2a

[a]The amount of bait was 20 g of methyl phenylacetate and 100 mg of synthesized sex pheromone [125:1 mixture of (*R*)-3-hydroxy-2-hexanone and (*R*)-3-hydroxy-2-octanone (w/w)].
[b]Means within the same columns followed by the different letters are significantly different ($P < 0.05$, Tukey's test).

phenylacetate captured 2.3 times more female beetles than methyl phenylacetate alone, although methyl phenylacetate was 1.9 times as attractive to females as sex pheromone (Table 1). In male catches, however, there was no significant difference between lures ($P > 0.05$, ANOVA).

The enhancement of attraction to synthetic sex pheromone by host-plant volatiles is known from the bean and pea leaf weevil (Blight et al., 1984), Mediterranean fruit fly and smaller European elm bark beetle (Dickens et al., 1990), the corn earworm and codling moth (Light et al., 1993), and the Japanese beetle (Klein et al., 1981). The behavioral mechanisms underlying the enhancement of attraction are not well studied.

We observed that beetles were attracted to traps baited with floral attractant alone, but they walked around the tree trunks or logs near the traps (Nakamuta et al., unpublished). In such cases they are not captured. We, therefore, suggest that addition of male sex pheromone will result in an increase of female trap catches in comparison with floral attractant alone. Male sex pheromone added to floral attractant may guide the beetles to the exact location.

Acknowledgments—We thank Mr. Y. Igarashi, Dr. E. Ohya, and Dr. T. Gotoh of Tohoku Research Center, Forestry and Forest Products Research Institute (FFPRI) for supplying insects. We thank Dr. T. Ikeda of FFPRI for his encouragement throughout the study on *A. subfasciatus*.

REFERENCES

CARDÉ, R. 1986. The role of pheromones in reproductive isolation and speciation of insects. pp. 303–317, *in* M. D. Huettel (ed.). Evolutionary Genetics of Invertebrate Behavior. Plenum Press, New York.

BLIGHT, M. M., PICKETT, J. A., SMITH, M. C., and WADHAMS, L. J. 1984. An aggregation pheromone of *Sitona lineatus*. *Naturwissenschaften* 71:480.

DICKENS, J. C., JANG, E. B., LIGHT, D. M., and ALFORD, A. R. 1990. Enhancement of insect pheromone responses by green leaf volatiles. *Naturwissenschaften* 77:29–31.

IKEDA, T., OHYA, E., MAKIHARA, H., NAKASHIMA, T., SAITOH, A., TATE, K., and KOJIMA, K. 1993. Olfactory responses of *Anaglyptus subfasciatus* Pic and *Demonax transilis* Bates (Coleoptera: Cerambycidae) to flower scents. *J. Jpn. For. Soc.* 75:108–112.

KLEIN, M. G., TUMLINSON, J. H., LADD, T. L., and DOOLITTLE, R. E. 1981. Japanese beetle (Coleoptera: Scarabaeidea): Response to synthetic sex attractant plus phenethyl propionate: Eugenol. *J. Chem. Ecol.* 7:1–7.

KOBAYASHI, F. 1985. Occurrence and control of wood-injuring insects damage in Japanese cedar and cypress plantations. *Z. Angew. Entomol.* 99:94–105.

LEAL, W. S., SHI, X., NAKAMUTA, K., ONO, M., and MEINWALD, J. 1995. Structure, stereochemistry, and thermal isomerization of the male sex pheromone of the longhorn beetle *Anaglyptus subfasciatus*. *Proc. Natl. Acad. Sci. U.S.A.* 92:1038–1042.

LIGHT, D. M., FLATH, R. A., BUTTERY, R. G., ZALOM, F. G., RICE, R. E., DICKENS, J. C., and JANG, E. B. 1993. Host-plant green-leaf volatiles synergize the synthetic sex pheromones of the corn earworm and codling moth (Lepidoptera). *Chemoecology* 4:145–152.

NAKAMUTA, K., SATO, H., and NAKASHIMA, T. 1994. Behavioral and morphological evidence for a male-produced sex pheromone in the *Cryptomeria* twig borer, *Anaglyptus subfasciatus* Pic (Coleoptera, Cerambycidae). *Jpn. J. Entomol.* 62:371–376.

NAKASHIMA, T., NAKAMUTA, K., MAKIHARA, H., OHYA, E., NAKANISHI, M., and IKEDA, T. 1994. Field response of *Anaglyptus subfasciatus* Pic (Coleoptera: Cerambycidae) to benzyl acetate and structurally related esters. *Appl. Entomol. Zool.* 29:421–425.

WILKINSON, L., HILL, M., and VANG, E. 1992. Systat: Statistics. Version 5.2 ed. Systat, Evanston, Illinois.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

BEHAVIORAL AND SUBLETHAL EFFECTS OF STRUCTURALLY RELATED LOWER TERPENES ON *Myzus persicae*

CARMEN GUTIÉRREZ,[1] ALBERTO FERERES,[1] MATÍAS REINA,[2] RAIMUNDO CABRERA,[3] and AZUCENA GONZÁLEZ-COLOMA[1,*]

[1]*Centro de Ciencias Medioambientales, CSIC*
Serrano 115-dpdo., 28006 Madrid, Spain

[2]*Instituto de Productos Naturales y Agrobiologia, CSIC*
Avda. Astrofisico F. Sanchez, 38206 La Laguna, Tenerife, Spain

[3]*UDI Fitopatologia, Facultad de Biologia, Universidad de la Laguna*
La Laguna, Tenerife, Spain

(Received July 15, 1996; accepted February 13, 1997)

Abstract—The sesquiterpenes farnesol, caryophyllene oxide, and 2,10-bisaboladien-1-one (patent #9602748) and the monoterpenes citronellol and geraniol were tested for settling inhibition and chronic effects on the aphid *Myzus persicae* by means of an improved leaf-disk assay. Of these compounds, geraniol, farnesol, and the natural bisabolene significantly inhibit settling in choice tests. Furthermore, application of the bisabolene to intact *Capsicum annuum* leaves did not cause phytotoxicity, but did affect the insects' probing behavior by decreasing the probing activity and the number of intracellular punctures. Both compounds significantly decreased offspring production. Of the compounds tested, the natural product bisabolene could be a promising lead for future development of aphid control agents.

Key Words—*Myzus persicae*, Homoptera: Aphididae, lower terpenes, settling behavior, probing, reproduction.

INTRODUCTION

Aphids are one of European agriculture's main pests, causing damage to crops by feeding and/or transmitting plant viruses. Given the growing problem of insecticide resistance (Ffrench-Constant et al., 1988) and the hazards of insec-

*To whom correspondence should be addressed.

0098-0331/97/0600-1641$12.50/0 © 1997 Plenum Publishing Corporation

ticide use, antifeedants are among the most promising alternative pest-control methods (Pickett et al., 1987).

Aphid feeding is influenced by many plant components (see Pickett et al., 1992) and the lower terpenoids are the most promising behavioral agents and antifeedants against aphid pests (Dawson et al., 1990). Some of the most active ones are the drimane sesquiterpenes (−)-polygodial and (−)-warbuganal (Gibson et al., 1982; Asakawa et al., 1988; Powell et al., 1993, 1995). Furthermore, behavior-controlling natural products such as (−)-polygodial and derivatives of (*E*)-β-farnesene, the main component of the alarm pheromone of aphids (Bowers et al., 1972; Griffith and Pickett, 1980) decrease aphid settling and virus transmission (Dawson et al., 1982; Gibson et al., 1984).

In this work we developed the appropriate bioassay conditions to test for antifeedant and chronic effects of volatile compounds with low water solubility, such as lower terpenes.

To test for behavioral and toxic effects of structurally related lower terpenes on the green peach aphid *Myzus persicae* (Sulzer) (Homoptera: Aphididae), an important plant virus vector, we selected the natural sesquiterpene 2,10-bisaboladien-1-one, a potent antifeedant against the Colorado potato beetle, *Leptinotarsa decemlineata*, isolated from the endemic canarian plant *Senecio palmensis* (González-Coloma et al., 1995), the commercial sesquiterpenes caryophyllene oxide and farnesol, and the monoterpenes citronellol and geraniol (Figure 1).

METHODS AND MATERIALS

Bisabolene was previously isolated in our laboratory from *S. palmensis* (González-Coloma et al., 1995). Citronellol, geraniol, farnesol, and caryophyllene oxide were purchased from Sigma Chemical Co. *M. persicae* apterous adults from a laboratory colony reared on bell pepper (*Capsicum annuum*) plants (22°C, 18L:6D) were used for the experiments.

Choice Tests. Each experiment consisted of 3 × 3 × 1.5-cm plastic boxes with ventilated lids and coated with a thin layer of 2.5% agar. Leaf disks (2 cm^2) were cut from a fully expanded *C. annuum* leaf, divided into two half-disks, and placed on the agar layer before its complete solidification so that the lower leaf surface would not be exposed to the insects. Each half-disk was treated with 10 μl of an acetone solution of the test compound (treatment) or the solvent alone (control). Each treatment consisted of 20 boxes with 10 insects each. The boxes were incubated at 22°C (18L:6D) in an upside-down position under indirect light, and the number of aphids that settled on the treatment and control half-disk were recorded after 24 hr. A settling inhibition index (%SI) was calculated for each compound at an initial dose of 100 $\mu g/cm^2$ [$\%SI = 1 - (\%T/\%C) \times 100$, where %T is the percentage of aphids on treated surface,

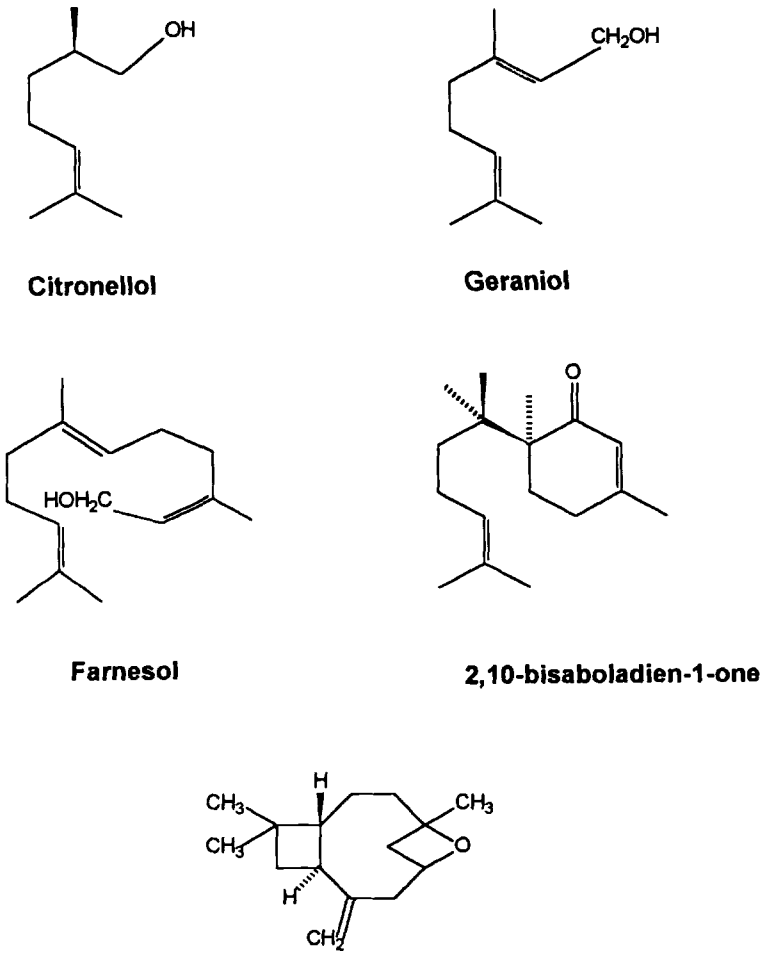


FIG. 1. Molecular structures of the compounds tested.

and %C is the percentage of aphids on control surface]. Dose–response experiments were performed for compounds giving a %SI > 75%, and their effective doses (EC_{50}) calculated by probit analysis (Finney, 1971).

Probing Behavior. To check further for the behavioral effects of those compounds with significant settling inhibition activity, we tested the effect of bisabolene on aphid probing behavior. Aphid probing behavior was assessed by

means of an electrical recording system. The amplifier used was a two-channel DC system (with an input resistance of 1 Ω and a gain of 50×). An adjustable voltage in the range of ±2 V was supplied to the potting soil of the plant. *M. persicae* adults were connected to the circuit using a 20-μm diameter gold wire glued to the dorsum of the aphid by means of silver conducting paint. Each individual aphid was starved for 1 hr and placed on a leaf of the plant previously treated with 60 $\mu g/cm^2$ bisabolene. A different plant with an acetone-treated leaf was used as control. Aphid probing behavior on treated and control plants was recorded simultaneously for 10 min on a two-channel strip chart recorder. The recording signal was also acquired at 100 Hz through a Mac Adios A/C-converter card. Probing behavior was recorded for only 10 min, because nonpersistent viruses are transmitted by aphids during brief superficial probes. A total of 15 paired waveforms (treatment–control) were analyzed with MacStylet software (Febvay et al., 1996) and the following probing behavior variables were calculated: total nonprobing time, total probing time, number of intracellular punctures, mean potential drop (pd) duration, and time elapsed from the first pd to the end of the probe. The data were subjected to one-way ANOVA and the means were separated by Fisher's protected LSD test (Abacus Concepts, 1989).

No-Choice Tests. This assay was run to test for possible toxic and/or sublethal effects associated with significant antifeedant activity. Adult apterous insects of the same age that had begun reproduction were used. Twenty boxes with one 2-cm^2 leaf disk and five insects each were used for each experiment. Each disk was treated with 20 μl of an acetone solution of the test compound (doses of 30 and 60 $\mu g/cm^2$) or the solvent alone. The boxes were incubated under the environmental conditions described above. The nymphs produced during the first 48 hr and the adults were separated, kept in similarly treated boxes, and observed daily for six days to calculate their mortality. The first instars produced by the adults during the following days were observed daily and then removed from the boxes to calculate total offspring production.

Treatment differences in nymphal and adult mortality were determined by contingency table analyses and multiple comparisons for proportions (Neter et al., 1985). Treatment effects on offspring production were analyzed by one-way ANOVA and the means separated by the LSD test.

RESULTS

Choice Assays. The sesquiterpene precursor farnesol, bisabolene, and geraniol gave %SI values >75% against *M. persicae* and were then bioassayed in dose–response experiments (Table 1), while citronellol and caryophyllene oxide both gave %SI values of 57% at the initial dose. The calculation of the effective

TABLE 1. *Myzus persicae* SETTING BEHAVIOR INHIBITION (%SI) CAUSED BY GERANIOL (G), FARNESOL (F), AND 2,10-BISABOLADIEN-1-ONE (B) IN CHOICE ASSAYS

	Average %SI (±SE)[a]		
Dose ($\mu g/cm^2$)	G	F	B
0	21.15 (7.38)	21.15 (7.38)	21.15 (7.38)
10	12.77 (4.95)	39.54 (10.09)	28.99 (12.87)
30	28.86 (7.08)	67.17 (10.45)	63.09 (7.28)
60	51.64 (8.92)	90.57 (3.04)	79.48 (8.29)
100	79.6 (6.61)	97.14 (1.67)	87.86 (4.33)
EC_{50} (95% CL)[b]	47.13 (22.16, 100.25)	14.91 (12.13, 18.33)	20.28 (16.40, 25.07)

[a] %SI = 1 − (%T/%C) × 100, where %T is percentage of aphids on treated disk after 24 hr and %C is percentage of aphids on control disk after 24 hr.
[b] Effective doses (EC_{50}) and 95% confidence limits (lower and upper) of the test compounds.

doses (EC_{50}) showed that the most active compounds were farnesol and bisabolene (Table 1).

Probing Behavior. Bisabolene was the only compound tested because it showed high settling inhibition activity without associated phytotoxic effects, in contrast to farnesol, which had strong phytotoxic effects when applied to intact *C. annuum* leaves at the experimental dose (60 $\mu g/cm^2$). Bisabolene significantly increased the total nonprobing time and reduced the total probing time, the number of intracellular punctures without affecting the number of probes, the mean potential drop duration, and the time elapsed between the first intracellular puncture and the end of the probe (Table 2). These data suggest that bisabolene

TABLE 2. PROBING BEHAVIOR VARIABLES OF *M. persicae* FEEDING ON BISABOLENE-TREATED *C. annuum* PLANTS[a]

Variables	Bisabolene (60 $\mu g/cm^2$)	Control
Probes (*N*)	2.57 ± 0.31a	3.07 ± 0.27a
Total nonprobing time (sec)	330.29 ± 41.14a	198.20 ± 31.32b
Total probing time (sec)	269.46 ± 42.16a	396.08 ± 32.54b
Intracellular punctures (pd) (*N*)	1.57 ± 0.44a	4.21 ± 0.50b
Mean pd duration (sec)	6.28 ± 0.81a	5.03 ± 0.33a
Time from 1st pd to end of probe (sec)	119.02 ± 49.95a	172.93 ± 44.78a

[a] Values are mean ± SE, $N = 15$. Different letters within rows indicate significant differences between treatments, Fisher's protected LSD test, $P < 0.05$.

TABLE 3. DAILY MORTALITY (%) OF ADULT *M. persicae* CAUSED BY BISABOLENE (B) OR FARNESOL (F)[a]

Treatment	Dose ($\mu g/cm^2$)	Day 1	Day 2	Day 3	Day 4
Control	0	0.00 ± 0.00a (20,100)[b]	0.00 ± 0.00a (20,100)	4.73 ± 2.89a (19,94)	8.82 ± 4.97a (17,80)
B	30	0.00 ± 0.00a (19,95)	2.10 ± 1.44a (19,95)	6.31 ± 2.67a (19,93)	18.68 ± 5.93a (19,87)
	60	0.00 ± 0.00a (20,100)	4.00 ± 2.33a (20,100)	7.16 ± 3.15a (20,96)	10.50 ± 4.19a (20,89)
F	30	0.00 ± 0.00a (20,100)	3.00 ± 1.64a (20,100)	4.66 ± 2.22a (20,97)	23.75 ± 6.07b (20,93)
	60	0.00 ± 0.00a (20,100)	26.00 ± 2.55b (20,100)	38.19 ± 3.19b (20,94)	26.58 ± 5.91b (20.88)

[a]Values are mean ± SE. Means within a column followed by the same letter are not significantly different ($P > 0.05$, contingency table analysis).
[b]Number of boxes and total insects.

may have a repellent effect, affecting aphid probing and reducing the probability of making intracellular punctures.

Long-Term No-Choice Tests. Table 3 shows the effect of treated leaf disks on adult aphid mortality. Bisabolene treatment did not increase mortality at any time, while farnesol had a significant effect on adult mortality at a dose of 60 $\mu g/cm^2$ during the second and third day of treatment, and at both doses tested on the fourth day (Table 3). On the sixth day, the mortality of the control adults was >50%, and we have not included these data in the results.

Nymphal mortality was increased by bisabolene concentrations and the highest concentration of farnesol during the first 48 hr of treatment (day 1) (Table 4).

Table 5 shows the effects of bisabolene and farnesol on daily offspring production. Both treatments had a significant negative effect on aphid reproduction, following a dose–response pattern. After four days, nymphal production was reduced in both treatments to less than half the production found in the control adults.

DISCUSSION

In this work we have developed new leaf-disk bioassay conditions that permit testing for settling inhibition activity (24 hr, choice tests) or chronic

TABLE 4. DAILY NYMPHAL MORTALITY (%) CAUSED BY BISABOLENE (B) OR FARNESOL (F)[a]

Treatment	Dose ($\mu g/cm^2$)	Day 1	Day 2	Day 4
Control	0	15.61 ± 5.96a (25,400)	27.58 ± 7.48a (25,393)	31.57 ± 7.47a (21,340)
B	30	35.90 ± 8.27b (24,258)	23.09 ± 8.34a (19,207)	11.59 ± 6.42b (17,186)
	60	33.84 ± 8.24b (24,277)	20.55 ± 7.19a (19,225)	9.01 ± 4.26b (17,199)
F	30	21.33 ± 7.91a (23,370)	7.49 ± 2.08b (19,348)	19.43 ± 6.84a (19,325)
	60	36.89 ± 9.38b (24,172)	23.65 ± 9.12a (17,146)	21.27 ± 7.49a (14,126)

[a]See Table 3 footnotes.

effects (four to six days, no-choice tests) against aphids by using small amounts of nonsystemic (low water solubility) chemicals. This has been made possible by exposing known upper leaf surfaces (1 or 2 cm^2) while keeping the lower surface unavailable to the insects. Under these conditions, the aphids can live and reproduce for up to nine days.

TABLE 5. DAILY OFFSPRING PRODUCTION OF ADULT *M. persicae* TREATED WITH BISABOLENE (B) OR FARNESOL (F)[a]

Treatment	Dose ($\mu g/cm^2$)	Day 1	Day 2	Day 3	Day 4
Control	0	7.60 ± 0.87a (20,100)[b]	6.20 ± 0.81a (20,100)	10.00 ± 1.58a (19,90)	16.64 ± 2.68a (17,73)
B	30	4.45 ± 0.82b (20,95)	4.84 ± 0.99a (19,93)	5.73 ± 1.32b (20,87)	7.31 ± 2.32b (19,71)
	60	3.90 ± 0.68b (20,100)	4.10 ± 0.96a (20,96)	3.45 ± 0.95bc (20,89)	3.45 ± 1.19b (20,79)
F	30	4.95 ± 0.93b (20,100)	9.20 ± 0.97b (20,97)	3.40 ± 1.37bc (20,93)	2.95 ± 1.38b (20,71)
	60	1.45 ± 0.40c (20,100)	4.55 ± 1.00a (20,94)	2.30 ± 0.72c (20,88)	3.60 ± 0.80b (20,67)

[a]Values are mean ± SE. Means within a column followed by the same letter are not significantly different ($P > 0.05$, LSD test).
[b]Number of boxes and living adults per treatment.

Other bioassays currently used to test for the same type of activity are the half-leaf assay (Dawson et al., 1982; Powell et al., 1993) and the leaf-disk assay (Lowery and Isman, 1994). The half-leaf assay requires higher amounts of chemicals than the one described here because the exposed surfaces are bigger and variable. Furthermore, in both cases the insects have access to the lower leaf-surface, making it necessary to treat both upper and lower leaf areas, thus increasing the amount of compound needed. Other methods previously used to prevent access of the insects to the lower leaf surface (floating leaf disks) limited the test duration to a maximum of 15 min (Powell et al., 1995). Additionally, dipping the leaf disks in the test solution can result in variable amounts of the test compound applied to the leaf.

Of the compounds tested, we have shown that the structurally related sesquiterpenes farnesol and bisabolene, a strong antifeedant against the Colorado potato beetle (González-Coloma et al., 1995), had the strongest settling inhibition activity after 24 hr. As previously shown (Griffiths et al., 1989), caryophyllene oxide did not inhibit settling by *M. persicae*. Geraniol was more active than citronellol. Previous results have shown that geraniol was among the most active of the ovicidal monoterpenes tested against *Musca domestica* (Rice and Coats, 1994).

Other plant chemicals and derivatives inhibit settling behavior of *M. persicae* (Gibson et al., 1982a; Asakawa et al., 1988; Powell et al., 1993, 1995; Xie and Isman, 1995), but this is the first report of an antifeedant effect of these compounds on aphids.

Our experiments indicate that bisabolene modified the feeding behavior of *M. persicae* by decreasing probing activity and the number of intracellular punctures (pd), which is a behavioral event essential for the transmission of nonpersistent viruses (Powell, 1991).

Probing and feeding behavior of aphids can result in the uptake of viruses from infected plants and subsequent transmission to healthy plants. It is possible to interfere with virus acquisition and transmission by influencing aphid host-selection and feeding behavior with antifeedant chemicals (Griffiths et al., 1989; Gibson et al., 1982a,b, 1984; Sassen, 1983). Bisabolene has the potential to interfere with plant–virus transmission by the aphid, and we are currently evaluating this possibility.

The long-term feeding bioassays showed that bisabolene did not have any toxic effects on adult *M. persicae*. Similarly, this compound was not toxic to the Colorado potato beetle in no-choice tests (González-Coloma et al., 1995). The highest dose of farnesol increased adult mortality, suggesting that farnesol is also toxic to the aphid.

Nymphal mortality was higher at the beginning of the experiment (48 hr of exposure). This may be due to starvation caused by the antifeedant activity of these compounds. The fact that nymphal mortality decreased during the fol-

lowing days may be due to compound degradation and/or volatilization from the leaf disks. Susceptibility of *M. persicae* to the antifeedant action of bisabolene and farnesol depends on the developmental stage of the insect, and nymphs are more susceptible than adults. Similarly, LC_{50} values for *M. persicae* exposed to azadirachtin increased from first to fourth nymphal instar, and there was no toxicity to adults (Lowery and Isman, 1994).

The offspring production of aphids treated with bisabolene or farnesol was lower than that of controls. Biological parameters of aphids such as fecundity, size, and developmental rate are partly determined during embryo development (Adams and van Emden, 1972). Since bisabolene had no toxic effects on the adults, its effect on offspring production may have been caused by adult starvation resulting from the antifeedant effect. The action of farnesol on offspring production may have been caused by repellency and/or toxicity to the adults.

In summary, our newly developed bioassay conditions have shown that farnesol and 2,10-bisaboladien-1-one inhibit settling and offspring production of adult *M. persicae*. Bisabolene was not phytotoxic to the host plant *C. annuum*, decreased aphid probing activity, and reduced intracellular punctures. Thus, this compound has the potential to interfere with plant virus transmission by the aphid. Compound availability could be a limiting factor, however, when considering its potential use in aphid control.

Acknowledgments—This work has been supported by a grant from DGICYT (PB94-0020). We gratefully acknowledge Prof. B. M. Fraga for kindly supplying commercial terpenes; M. Vieira, M. Duque, and C. González for bioassay assistance and insect rearing; and S. Carlin for language advice.

REFERENCES

ABACUS CONCEPTS. 1989. SuperANOVA. Abacus Concepts, Berkeley, California, 322 pp.

ADAMS, J. B., and VAN EMDEN, H. F. 1972. The biological properties of aphids and their host plant relationships, pp. 47–104, *in* H. F. van Emden (eds.). Aphid Technology. Academic Press, New York.

ASAKAWA, Y., DAWSON, G. W., GRIFFITHS, D. C., LALLEMAND, J.-Y., LEY, S. V., MORI, K., MUDD, A., PEZECH-LECLAIRE, M., PICKETT, J. A., WATANABE, H., WOODCOCK, C. M., and ZHANG, Z.-N. 1988. Activity of drimane antifeedants and related compounds against aphids and comparative biological effects and chemical reactivity of (−)- and (+)-polygodial. *J. Chem. Ecol.* 14:1845–1855.

BOWERS, W. S., NAULT, L. R., WEBB, R. E., and DUTKY, S. R. 1972. Aphid alarm pherome: Isolation, identification, synthesis. *Science* 117:1121–1122.

DAWSON, G. W., GIBSON, R. W., GRIFFITHS, D. C., PICKETT, J. A., RICE, A. D., and WOODCOCK, C. M. 1982. Aphid alarm pheromone derivatives affecting settling and transmission of plant viruses. *J. Chem. Ecol.* 13:1663–1667.

DAWSON, G. W., GRIFFITHS, D. C., MERRIT, L. A., MUDD, A., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1990. Aphid semiochemicals. A review and recent advances on the sex pheromone. *J. Chem. Ecol.* 16:3019–3030.

FEBVAY, G., RAHBÉ, Y., and VAN HELDEN, M. 1996. Macstylet, a Macintosh software to analyse EPG (Electrical Penetration Graph) data. *Entomol. Exp. Appl.* 80:105–108.

FINNEY, D. J. 1971. Probit Analysis. Cambridge University Press.

FFRENCH-CONSTANT, R. M., HARRINGTON, R., and DEVONSHIRE, A. L. 1988. Effect of repeated applications of insecticides to potatoes on numbers of *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) and on the frequencies of insecticide-resistant variants. *Crop Prot.* 7:55–61.

GIBSON, R. W., RICE, A. D., PICKETT, J. A., SMITH, M. C., and SAWICKI, R. M. 1982a. The effects of the repellents dodecanoic acid and polygodial on the acquisition of non-, semi-, and persistent plant viruses by the aphid *Myzus persicae*. *Ann. Appl. Biol.* 100:55–59.

GIBSON, R. W., RICE, A. D., and SAWICKI, R. M. 1982b. Effects of the pyrethroid deltamethrin on the acquisition and inoculation of viruses by *Myzus persicae*. *Ann. Appl. Biol.* 100:49–54.

GIBSON, R. W., PICKETT, J. A., DAWSON, G. W., RICE, A. D., and STRIBLEY, M. F. 1984. Effects of aphid alarm pheromone derivatives and related compounds on non- and semi-persistent plant virus transmission by *Myzus persicae*. *Ann. Appl. Biol.* 104:203–209.

GONZÁLEZ-COLOMA, A., REINA, M., CABRERA, R., CASTAÑERA, P., and GUTIÉRREZ, C. 1995. Antifeedant and toxic effects of sesquiterpenes from *Senecio palmensis* to Colorado potato beetle. *J. Chem. Ecol.* 21:1255–1270.

GRIFFITHS, D. C., and PICKETT, J. A. 1980. A potential application of aphid alarm pheromones. *Entomol. Exp. Appl.* 27:199–201.

GRIFFITHS, D. C., PICKETT, J. A., SMART, L. E., and WOODCOCK, C. M. 1989. Use of insect antifeedants against aphid vectors of plant virus disease. *Pestic. Sci.* 27:269–276.

LOWERY, D. T., and ISMAN, M. B. 1994. Insect growth regulating effects of neem extract and azadirachtin on aphids. *Entomol. Exp. Appl.* 72:77–88.

NETER, J., WASSERMAN, W., and KUTNER, M. H. 1985. Applied Linear Statistical Models: Regression, Analysis of Variance and Experimental Designs, 2nd ed. Richard D. Irwin Inc., Homewood, Illinois.

PICKETT, J. A., DAWSON, G. W., GRIFFITHS, D. C., HASSANALI, A., MERRITT, L. A., MUDD, A., SMITH, M. C., WADHAMS, L. J., WOODCOCK, C. M., and ZHANG, Z. 1987. Development of plant-derived antifeedants for crop protection, pp. 125–128, *in* R. Greenhalgh and T. R. Roberts (eds.). Pesticide Science and Biotechnology. Blackwell Scientific Publications, Oxford.

PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1992. The chemical ecology of aphids. *Annu. Rev. Entomol.* 37:67–70.

POWELL, G. 1991. Cell membrane punctures during epidermal penetrations by aphids: Consequences for the transmission of two potyvirus. *Ann. Appl. Biol.* 119:313–321.

POWELL, G., HARDIE, J., and PICKETT, J. A. 1993. Effect of the antifeedant polygodial on plant penetration by aphids assessed by video and electrical recording. *Entomol. Exp. Appl.* 68:193–200.

POWELL, G., HARDIE, J., and PICKETT, J. A. 1995. Response of *Myzus persicae* to the repellent polygodial in choice and no-choice video assays with young and mature leaf tissue. *Entomol. Exp. Appl.* 74:91–94.

RICE, P. J., and COATS, J. 1994. Structural requirements for monoterpene activity against insects, pp. 92–108, *in* P. A. Hedin (eds.). Bioregulators for Crop Protection and Pest Control. ACS Symposium Series 557. American Chemical Society, Washington, D.C.

SASSEN, B. 1983. The effect of two pyrethroids on the feeding behavior of three aphid species and on transmission of two different viruses. *J. Plant. Dis. Prot.* 90:119–126.

XIE, Y., and ISMAN, M. B. 1995. Toxicity and deterrency of depitched tall oil to the green peach aphid *Myzus persicae*. *Crop Prot.* 14:51–56.

Journal of Chemical Ecology, Vol. 23, No. 6, 1997

EFFECT OF *Solanum* GLYCOALKALOIDS ON POTATO APHID, *Macrosiphum euphorbiae*

CARLOS GÜNTNER, ANDRÉS GONZÁLEZ, RENATO DOS REIS, GABRIEL GONZÁLEZ, ALVARO VÁZQUEZ, FERNANDO FERREIRA,* and PATRICK MOYNA

Cátedra de Farmacognosia y Productos Naturales, Facultad de Química CC1157, General Flores 2124, Montevideo, Uruguay

(Received July 22, 1996; accepted February 13, 1997)

Abstract—*Macrosiphum euphorbiae*, also known as the potato aphid, is a potato virus vector, associated with decreased tuber production. Feeding, toxicity, and reproductive effects of the major potato glycoalkaloids (α-chaconine and α-solanine), their common aglycone (solanidine), and a nonpotato glycoalkaloid (α-tomatine) and its aglycone (tomatidine) on *M. euphorbiae* were studied. As expected, neither α-chaconine nor α-solanine had a strong lethal effect on the aphid, but α-chaconine stimulated feeding at low concentrations. Both aglycones were deterrent and lethal at high concentrations and, like α-chaconine and α-tomatine, they delayed the appearance and decreased the number of nymphs.

Key Words—*Macrosiphum euphorbiae*, Homoptera (Aphididae), *Solanum tuberosum*, Solanaceae, potato, α-chaconine, α-solanine, α-tomatine, solanidine, tomatidine.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the world's fourth most important agricultural product, and several pests, including nematodes, fungi, bacteria, viruses, and insects decrease tuber production. Aphids (Homoptera, Aphididae) are known to transmit several important potato viruses. The green peach aphid, *Myzus persicae* (Sulzer), and the potato aphid, *Macrosiphum euphorbiae* (Thomas), transmit viruses such as potato leafroll virus, PVA, PVM, PVY, and alfalfa mosaic virus (Auclair, 1989). For example, PVY can decrease production up to 80% (de Bokx, 1980). The feeding behavior or performance of aphids could

*To whom correspondence should be addressed.

0098-0331/97/0600-1651$12.50/0 © 1997 Plenum Publishing Corporation

be affected by host secondary metabolites. Many different plant compounds have been shown to be toxic, deterrent, or both (Montllor, 1991). The quinolizidine, pyrrolizidine, and indolizidine alkaloids have significant feeding deterrency against the pea aphid, *Acyrtosiphon pisum* (Dreyer et al., 1985). Some phenolics (Montllor, 1991), quassinoids (Polonsky et al., 1989), and hydroxamic acids (Niemeyer, 1988) also play an important role in the host-plant relationship of sap-feeding insects.

The glycoalkaloids are well-known pest resistance factors (Norris, 1986). Several genera of the Solanaceae are rich in steroidal glycoalkaloids, formed by a steroidal alkaloid conjugated with a tri- or tetrasaccharide residue attached through a glycosidic linkage to the 3-OH group of the aglycone (Schreiber, 1979). Glycoalkaloids exhibit a wide range of biological activities, including antifungal (Sinden et al., 1973; Fewell and Roddick, 1993), inhibition of acetylcholinesterase (Roddick, 1989), membrane-disrupting properties (Fewell et al., 1994) and, in particular, toxic and deterrent activities against insects. *Solanum* glycoalkaloids were implicated as resistance factors against the Colorado potato beetle, *Leptinotarsa decemlineata.* The nature and magnitude of this response varies depending on the specific glycoalkaloid and its concentration. Leptine I is an extremely effective feeding deterrent, and tomatine and demissine are intermediate in activity, followed by solanine and chaconine (Tingey, 1984). Another potato insect pest affected by glycoalkaloids is the potato leafhopper, *Empoasca fabae.* The total foliar content could be related to decreased survival and changed feeding behavior (Tingey, 1984; Sanford et al., 1990).

Although the exact localization of glycoalkaloids is not well known and their translocation is controversial (Roddick, 1982), it is worthwhile to study their interaction with the potato aphid. The adaptation of *M. euphorbiae* to its host plant may mean that the aphid either never gets in contact with and/or is tolerant to the potato glycoalkaloids.

To verify the last possibility, the effects of glycoalkaloids and their aglycones (Figure 1) on feeding, survival, and reproduction rate of *M. euphorbiae* were studied. Different concentrations of the major glycoalkaloids of *S. tuberosum*, α-chaconine (**2**) and α-solanine (**3**), as well as a nonpotato glycoalkaloid, α-tomatine (**5**), and their aglycones, solanidine (**1**) and tomatidine (**4**), were tested against the potato aphid in a no-choice system.

METHODS AND MATERIALS

General. All evaporations and concentrations were performed under vacuum at temperatures below 40°C. GLCs were performed on a Shimadzu 6 AM gas chromatograph fitted with a SE-52 capillary column (0.32 mm × 25 m), at 280°C. GLC-MS was performed on a Shimadzu QP-1100 EX GLC-MS quad-

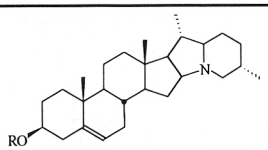
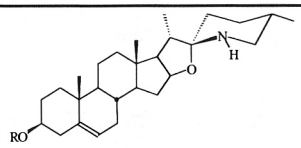
R				
H	solanidine	1	tomatidine	4
chacotriose	chaconine	2		
solatriose	solanine	3		
lycotetraose			tomatine	5

FIG. 1. Structures of tested compounds. Chacotriose: α-L-Rha*p*-(1 → 4)-[α-L-Rha*p*-(1 → 2)]-β-D-glucopiranose; solatriose: β-D-Glc*p*-(1 → 3)-[α-L-Rha*p*-(1 → 2)]-β-D-galactopiranose; and lycotetraose: β-D-Glc*p*-(1 → 2)-[β-D-Xyl*p*-(1 → 3)]-β-D-Glc*p*-(1 → 4)-β-D-galactopiranose.

rupole instrument fitted with a BP 1 capillary column (0.25 mm × 30 m) at 250°C, with the EI ionization source at 70 eV. TLC was performed on silica gel precoated plates of 0.25 mm thickness (Merck, Darmstadt, Germany), using $CHCl_3$–MeOH–2% NH_4OH (70:30:5) or $CHCl_3$–MeOH–AcOEt (95:5:5) mixtures as mobile phase for the glycoalkaloids or the aglycones, respectively.

Biological Assays. M. euphorbiae were kept on potato plants (cv. Kennebeck) in a Phytotron at 24 ± 2°C with a photoperiod of 18L:6D and 15,000 lux. An artificial meridic diet composed of 0.5% soybean protein and 35% sucrose in KH_2PO_4 buffer (pH 7.6, 0.037 M) was used for all the experiments. The solution was filtered through a 0.22-μm filter, stored at −18°C, and used within six months.

Test compounds were dissolved in the diet to give solutions of 50, 100, 250, and 500 ppm. Sachets were made aseptically of two Parafilm membranes containing the control diet or diet plus test compounds. The Parafilm sachets were stretched over glass tubes (diameter 25 mm, height 50 mm) (Febvay et al., 1992). Controls were made with sachets filled only with diet. Three to five replications with 15–30 mixed-aged nymphs were performed for each concentration of the tested products. No-choice assays were performed under ambient light. The aphids were put into the glass tubes with a wet brush or a sucking device as described in Busvine (1957). Feeding was visually estimated by counting the settled aphids on the Parafilm. Wandering aphids were not counted (Dreyer et al., 1981).

Data Analysis. The assays were continued for 72 hr. Every 24 hr the following variables were measured and calculated as indicated below. The number of original aphids refers to the number placed in the test at the beginning and does not include any progeny that may have been produced.

$$\text{Percentage feeding (\%F)} = 100 \times \frac{\text{number original aphids feeding}}{\text{number of original aphids alive}}$$

$$\text{Percentage mortality (\%M)} = 100 \times \frac{\text{number of original aphids dead}}{\text{number of original aphids}}$$

$$\text{Reproduction index (\%N)} = 100 \times \frac{\text{total number of newborn nymphs}}{\text{number of original aphids}}$$

$$\text{Relative nymphal index (\%RN)} = 100 \times \frac{\text{\%N of sample}}{\text{\%N of control}}$$

Sample Distribution. Thirteen sachets were prepared as described above using only diet and %F, %M, and %N were read at 24, 48, and 72 hr. Each data set was tested for standard normal distribution with the D'Agostino test with $\alpha = 0.05$ (Snedecor and Cochran, 1977).

Statistical treatment was done on %F and %M to confirm significant differences between control and test substances by the Student's t test with $\alpha = 0.05$ (Snedecor and Cochran, 1977).

Equivalence of Meridic and Holidic Diet. Two sets of sachets were prepared as described and each was filled with one type of diet. Data were read periodically and compared as described in Snedecor and Cochran (1977).

Tested Compounds. α-Chaconine, α-solanine, α-tomatine, and tomatidine were purchased from Sigma, St. Louis, Missouri. Solanidine was obtained from solanine by hydrolysis, as in Friedman et al. (1993), and the product was identified by TLC and GLC against an authentic sample and by GC-MS.

RESULTS AND DISCUSSION

A meridic diet was developed for the assays for economy and practicality, avoiding the use of the more complex holidic diet (Srivastava and Auclair, 1971). The use of the former diet was validated by comparison of feeding and mortality data obtained on both holidic and meridic diets at each time interval (Srivastava and Auclair, 1971). The new diet did not show significant differences when compared with the holidic diet (t test, $\alpha = 0.05$). The percentage of aphids feeding at 24 hr was 95% and 83% for the holidic and meridic diet, and fell to 82% and 61% at 72 hr, respectively. Mortality was 0% and 6% at 24 hr and 0% and 16% at 72 hr, respectively.

The percentage of feeding and mortality, as well as the relative reproduction indices (%F, %M, and %RN), present a standard normal distribution according to the D'Agostino test ($N = 13$, $\alpha = 0.05$) (Snedecor and Cochran, 1977).

α-Chaconine (2) and α-solanine (3) are the major potato glycoalkaloids. Commercial potato cultivars contain up to 200 ppm of total glycoalkaloids, while

wild *Solanum* species could contain levels several times higher (Maga, 1994). Both glycoalkaloids share the same steroidal aglycone, solanidine (**1**), and differ only in the trisaccharide residue attached to the aglycone's 3-OH. Neither glycoalkaloid showed a significant effect on the feeding of aphids (Table 1). However, α-chaconine at intermediate concentrations (100 and 250 ppm) produced significantly higher mortality after 72 hr, while α-solanine had no lethal effect (Table 2).

A more sensitive measurement of the deleterious effects of these compounds is achieved by measuring their effect on aphid reproduction. A relative reproduction index (%RN) value higher or lower than 100 reflects an increase or decrease in the reproduction rate, respectively.

α-Solanine influenced the reproduction rate at 72 hr at concentrations of 50 and 250 ppm, but had no effect at 100 ppm. α-Chaconine produced up to a fivefold decrease of the reproduction rate at 100 and 250 ppm (Table 3).

To identify the specificity of *M. euphorbiae*'s tolerance to α-solanine and α-chaconine, α-tomatine (**5**), a nonpotato glycoalkaloid was tested. This glycoalkaloid, composed of a spirosten aglycone (tomatidine, **4**) glycosidically

TABLE 1. PERCENTAGE OF APHIDS FEEDING (%F) ON DIET WITH VARIOUS CONCENTRATIONS OF POTATO GLYCOALKALOIDS AND THEIR AGLYCONES[a]

Tested compound	Time (hr)	Concentration of tested compounds (ppm)				
		0	50	100	250	500
α-Chaconine	24	62*	75	63*	74*	72*
	48	68	73*	62*	55*	66
	72	65*	72*	56*	68*	69
α-Solanine	24	57*	41*	57	42	NA
	48	57	46*	68*	52*	NA
	72	57*	60	51*	69	NA
α-Tomatine	24	61	64*	63*	71	88
	48	89	80*	72‡	88	83
	72	62	78*	31*	89	81*
Solanidine	24	88	91	81*	77	67*
	48	92	98	95	91	79*
	72	86	78	87	93	78‡
Tomatidine	24	71*	72*	68	79	67*
	48	96	92	93	93	82‡
	72	87	91*	77*	92*	97

[a]*Data with $\sigma_n > 10$ (%F), otherwise $\sigma_n \leq 10$ (%F). ‡Data significantly different from the control ($\alpha < 0.05$). NA: data not available.

TABLE 2. PERCENTAGE OF MORTALITY (%M) OF APHIDS FEEDING ON DIET CONTAINING VARIOUS CONCENTRATIONS OF POTATO GLYCOALKALOIDS AND THEIR AGLYCONES[a]

Tested compound	Time (hr)	Concentration of the tested compounds				
		0	50	100	250	500
α-Chaconine	24	3	6	8	5	4
	48	16	12	15	18	13
	72	17	12	25‡	27‡*	15
α-Solanine	24	8	11	11	7	NA
	48	20	23	26	15*	NA
	72	26*	38	27	27	NA
α-Tomatine	24	7	5	6	6	4
	48	8	15	11	8*	4
	72	22*	38‡	45*	16*	25*
Solanidine	24	6	7	5	5	9
	48	10	11	10	16	36*
	72	13	12	14	20‡	59‡*
Tomatidine	24	6	8	8	9	14
	48	6	25‡*	11	18*	20‡
	72	10	28‡	13	27‡*	36‡*

[a]* Data with $\sigma_n > 10$ (%M), otherwise $\sigma_n \leq 10$ (%M). ‡Data significantly different from the control ($\alpha < 0.05$). NA: data not available.

linked to a tetrasaccharide residue, is produced by tomato (*Lycopersicon esculentum* Mill.) and several other Solanaceae, but is not present in potato.

α-Tomatine had no appreciable effect on feeding except at 100 ppm, where a small but significant deterrent effect was detected. However, it was more toxic at 50 and 100 ppm in comparison with the control than both potato glycoalkaloids (Table 2). α-Tomatine also decreased the reproduction rate at all tested concentrations (Table 3) and increased the time at which the first nymphs appeared.

The aglycones solanidine and tomatidine were also tested. Solanidine had no significant effect on feeding at 48 and 72 hr at all tested concentrations (Table 1). Solanidine is not toxic to *M. euphorbiae* at low concentrations, but it shows a concentration-related toxicity at higher levels (Table 2). At the highest concentration tested, it produced a fourfold increase in mortality. It also decreased the reproduction rate (Table 3) and delayed the appearance of newborn nymphs.

Tomatidine did not show any important deterrent effect (Table 1). However, clear and significant lethal effects were observed at all concentrations

TABLE 3. INFLUENCE OF POTATO GLYCOALKALOIDS AND AGLYCONES IN DIET ON RELATIVE REPRODUCTION INDEX (%RN) OF *Macrosiphum euphorbiae*

Tested compound	Time (hr)	Concentration of the tested compounds (ppm)			
		50	100	250	500
α-Chaconine	24	57	3	0	90
	48	123	19	35	67
	72	96	17	15	45
α-Solanine	24	100	100	100	NA
	48	NA[a]	NA	NA	NA
	72	213	120	241	NA
α-Tomatine	24	11	14	34	15
	48	29	44	28	41
	72	71	47	61	70
Solanidine	24	34	19	33	11
	48	56	41	68	14
	72	56	69	103	16
Tomatidine	24	88	157	139	62
	48	46	67	55	45
	72	46	39	42	45

[a]NA: data not available.

(Table 2), except 100 ppm. After 48 hr, a decrease in the reproduction rate was observed for all concentrations (Table 3).

The tolerance of *M. euphorbiae* to the potato glycoalkaloids tested in the present work suggests that this aphid is well adapted to potato glycoalkaloids that are likely to be present in the natural diet of this sap-feeding insect. The alternative hypothesis—that the aphid never contacts these metabolites—cannot be eliminated by the present work, but this possibility seems quite unlikely. Definitive elucidation of this question will require further studies on the transport of these metabolites in plants.

Although both glycoalkaloids only differ in the trisaccharide moiety of the molecule, α-solanine increased the reproduction rate while α-chaconine produced a strong decrease, especially at 100 and 250 ppm. The membrane-disrupting properties of glycosides are well known, and although all the glycoalkaloids produce destabilization of cholesterol-containing biomembranes, α-chaconine has a greater effect than α-solanine (Keukens et al., 1996; Steel and Drysdail, 1988; Roddick and Rijnenberg, 1987; Roddick et al., 1990). α-Chaconine is more toxic against *Xenopus* embryos (Rayburn et al., 1994) and has stronger antifungal action (Fewell and Roddick, 1993; Fewell et al., 1994).

α-Tomatine only had moderate deterrent, toxic, and reproductive inhibitory effects at intermediate concentrations in the present study. This is in agreement with previous results on the action of these compounds on *L. decemlineata* (Tingey, 1984).

All of our data indicate that although these compounds did not strongly affect *M. euphorbiae*, which seems quite resistant to them, they did indeed show some action on the insect's vital parameters. The effects found are dependent on the structure of both the aglycone and the oligosaccharide moieties of these compounds.

The role of these compounds in plant resistance to insects is of practical interest because it has been shown that the glycoalkaloids are heritable and depend on the parental glycoalkaloidal composition (Van Gelder et al., 1988; Gregory, 1984). Testing the role of the potato glycoalkaloids in resistance of potato hybrids by breeding with wild *Solanum* species may contribute to increased potato resistance to insects.

Acknowledgments—This work was supported by grants from Comisión Sectorial de Investigación Científica (CSIC), Consejo Nacional de Ciencia y Tecnologia (Project CONICYT-BID 030), Instituto Nacional de Investigación Agropecuaria (INIA-Project 021), Programa de Desarrollo de Ciencias Básicas (PEDECIBA), and the International Program in Chemical Sciences, Sweden (IPICS, Project URU 03). Dr. L. Carballo is gratefully acknowledged for technical assistance. The authors also thank Ms. M. P. Cerdeiras for proof corrections.

REFERENCES

AUCLAIR, J. L. 1989. Host plant resistance, pp. 248–250, *in* A. K. Minks (ed.). Aphids, Their Biology, Natural Enemies and Control. Elsevier Science Publishers.

BUSVINE, J. R. 1957. A Critical Review of the Techniques for Testing Insecticides, Eastern Press, London, pp. 6–11.

DE BOKX, J. A. 1980. Virosis de la papa y de la semilla de papa, Editorial Hemisferio Sur S.A., Buenos Aires, pp. 147–165.

DREYER, D. L., REESE, J. C., and JONES, K. C. 1981. Aphid feeding deterrents in sorghum. *J. Chem. Ecol.* 7:273–284.

DREYER, D. L., JONES, K. C., and MOLYNEAUX, R. J. 1985. Feeding deterrency of some pyrrolizidine, quinolizidine and indolizidine alkaloids towards pea aphid *Acyrthosiphum pisum* and evidence for phloem transport of indolizidine alkaloid swainsonine. *J. Chem. Ecol.* 11:1045–1051.

FEBVAY, G., PUGEAUX, J. F., and BONNOT, G. 1992. Lipid composition of the pea aphid, Acyrtosiphum pisum (Harris) (Homoptera: Aphididae), reared on host plant and on artificial media. *Arch. Insect Biochem. Physiol.* 21:103–118.

FEWELL, A. M., and RODDICK, J. G. 1993. Interactive antifungal activity of the glycoalkaloids α-solanine and α-chaconine. *Phytochemistry* 33:323–328.

FEWELL, A. M., RODDICK, J. G., and WEISSENBERG, M. 1994. Interaction between the glycoalkaloids solasonine and solamargine in relation to inhibition of fungal growth. *Phytochemistry* 37:1007–1011.

FRIEDMAN, M., MCDONALD, G., and HADDON, W. F. 1993. Kinetics of acid-catalyzed hydrolysis of carbohydrate groups of potato glycoalkaloids α-chaconine and α-solanine. *J. Agric. Food Chem.* 41:1397–1408.

GREGORY, P. 1984. Glycoalkaloid composition of potato: Diversity and biological implications. *Am. Potato J.* 61:115–122.

KEUKENS, E. A. J., DE VRIJE, T., JANSEN, L. A. M., DE BOER, H., JANSSEN, M., DE KROON, A. I. P. M., JONGEN, W. M. F., and DE KRUIJFF, B. 1996. Glycoalkaloids selectively permeabilize cholesterol containing biomembranes. *Biochim. Biophys. Acta* 1279:243–250.

MAGA, J. 1994. Glycoalkaloids in Solanaceae. *Food Rev. Int.* 10:285–314.

MONTLLOR, C. B. 1991. The influence of plant chemistry on aphid feeding behavior, pp. 125–175, *in* E. Bernays (ed.). Insect Plant Interactions, Vol. III. CRC Press, Boca Ratón.

NIEMEYER, H. M. 1988. Hydroxamic acids defense chemicals in the Graminaceae. *Phytochemistry* 27:3349–3358.

NORRIS, D. M. 1986. Antifeedant Compounds, pp. 97–146, *in* H. Haug and H. Hoffman (eds.). Chemistry of Plant Protection. Springer, Berlin.

POLONSKY, J., BHATNAGER, S. C., GRIFFITHS, D. C., PICKETT, J. A., and WOODCOCK, C. M. 1989. Activity of quassinoids as antifeedants against aphids. *J. Chem. Ecol.* 15:993–998.

RAYBURN, J. R., BANTLE, J. A., and FRIEDMAN, M. 1994. Role of carbohidrate side chains of potato glycoalkaloids in the developmental toxicity. *J. Agric. Food. Chem.* 42:1511–1515.

RODDICK, J. G. 1982. Distribution of steroidal glycoalkaloids in reciprocal grafts of *Solanum tubersosum* and *Lycopersicon esculentum*. *Experientia* 38:460–462.

RODDICK, J. G. 1989. The acetilcholinesterase inhibiting activity of steroidal glycoalkaloids and their aglycones. *Phytochemistry* 28:2631–2634.

RODDICK, J. G., and RIJNENBERG, A. L. 1987. Synergistic interaction between the potato glycoalkaloids α-solanine and α-chaconine in relation to lysis of phospholipid/sterol liposomes. *Phytochemistry* 26:1325–1328.

RODDICK, J. G., RIJNENBERG, A. L., and WEISSENBERG, M. 1990. Membrane disrupting properties of the steroidal glycoalkaloids solasonine and solamargine. *Phytochemistry* 31:1951–1954.

SANFORD, L. L., DEAHL, K. L., SINDEN, S. L., and LADD, T. L. 1990. Foliar solanidine glycoside levels in *Solanum tuberosum* populations selected for potato leafhopper resistance. *Am. Potato J.* 67:461–466.

SCHREIBER, K. 1979. The steroid alkaloids of *Solanum*, pp. 193–202, *in* J. G. Hawkes (ed.). The Biology and Taxonomy of the Solanaceae, Linnean Society Symposium Series No. 7. Academic Press, New York.

SINDEN, S. L., GOTH, R. H., and O'BRIEN, J. 1973. Effect of potato glycoalkaloids on the growth of *Alternaria solanii* and their possible role as factors in potatoes. *Phytopathology* 63:303–307.

SNEDECOR, G. W., and COCHRAN, W. G. 1977. Métodos Estadísticos, Co. Editorial Mexicana, Mexico, pp. 123–156.

SRIVASTAVA, P. N., and AUCLAIR, J. L. 1971. An improved chemically defined diet for the pea aphid, *Acyrtosiphum pisum*. *Ann. Entomol. Soc. Am.* 64:474–478.

STEEL, C. C., and DRYSDAIL, R. B. 1988. Electrolicage from plant and fungal tissues and disruption of liposome membranes by α-tomatine. *Phytochemistry* 27:1025–1028.

TINGEY, W. M. 1984. Glycoalkaloids as pest resistance factors. *Am. Potato J.* 67:461–466.

VAN GELDER, W. M. J., VINKE, J. H., and SCHEFFER, J. J. C. 1988. Steroidal glycoalkaloids in tubers and leaves of *Solanum* species used in potato breeding. *Euphytica* 37:147–158.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

ANALYSIS OF EPG RECORDED PROBING BY CABBAGE APHID ON HOST PLANT PARTS WITH DIFFERENT GLUCOSINOLATE CONTENTS

B. GABRYS,[1,*] W. F. TJALLINGII,[2] and T. A. VAN BEEK[3]

[1]*Department of Agricultural Entomology, Agricultural University Cybulskiego 32. 50-205 Wrocław, Poland*

[2]*Department of Entomology, Wageningen Agricultural University PO Box 8031, 6700 EH Wageningen, The Netherlands*

[3]*Department of Organic Chemistry, Phytochemical Section Wageningen Agricultural University Dreijenplein 8, 6703 HB Wageningen, The Netherlands*

(Received June 5, 1996; accepted February 13, 1997)

Abstract—Probing behavior of adult apterous *Brevicoryne brassicae* (L.) was monitored with the electical penetration graph (EPG) technique on *Sinapis alba* L. Old, young, and mature leaves, as well as the generally preferred stems were compared with respect to probing activity and glucosinolate content. Compared to leaves, EPGs on stems showed much prolonged initial probes, which more often led to phloem-phase waveforms and successively to sustained phloem ingestion. However, reaching the first phloem phase within a probe was delayed on stems so that the time to the first phloem phase within the experiment was rather similar on the different plant parts. This suggests that superficial (epidermal and mesophyll) factors are more favorable in stems than in leaves but for deeper tissues, i.e., mesophyll and vascular bundle cells, the situation seems reversed, except for the phloem sieve elements, which seemed more or at least equally suitable. 3-Butenyl, *p*-hydroxybenzyl, and benzyl glucosinolates were found in all parts. It is concluded that further research on distribution and compartmentation of glucosinolates within plant tissues is needed for a better understanding of the correlations found with some *B. brassicae* probing parameters.

Key Words—Aphids, Homoptera, *Sinapis*, Cruciferae allelochemicals, feeding, electrical monitoring, electrical penetration graph technique.

*To whom correspondence should be addressed.

0098-0331/97/0700-1661$12.50/0 © 1997 Plenum Publishing Corporation

INTRODUCTION

The cabbage aphid, *Brevicoryne brassicae* (L.), occurs throughout all temperate and warm parts of the world. Its host range is limited to plants of the family *Brassicaceae* (=*Cruciferae*) (Blackman and Eastop, 1985). All *Brassicaceae* contain specific secondary metabolites, the glucosinolates, which stimulate host acceptance and feeding by the cabbage aphid (Nault and Styer, 1972; Fenwick et al., 1983). Introducing sinigrin into young leaves of the broad bean, *Vicia faba* L., a nonhost plant of *B. brassicae*, made it initially "acceptable" to the aphid (Wensler, 1962). Sinigrin was a specific stimulus for host selection by *B. brassicae* and was perceived after the stylets had penetrated the leaf surface. Likewise, addition of sinigrin to 20% sucrose solution substantially enhanced its uptake by the cabbage aphid (Klingauf, 1987). Van Emden and Bashford (1969) found higher concentrations of glucosinolates in young leaves than in mature and old leaves of Brussels sprouts, but there were no differences in performance of *B. brassicae* on these leaves. On the other hand, Van Emden (1978) stated that although glucosinolates in a particular plant-aphid combination might affect the rate of fluid uptake, *B. brassicae* might be sensitive to the amino acid spectrum of the plant as well. Dodd and Van Emden (1979) observed that the susceptibility of the high glucosinolate Brussels sprout varieties decreased with plant age, which was correlated with a decrease in total glucosinolate content. Weber et al. (1986), however, did not find any general correlation between the glucosinolate content of a cultivar of oilseed rape, *B. napus*, and the cabbage aphid population increase on this cultivar. There was no difference in suitability of young and old leaves for *B. brassicae*. However, on *Sinapis alba* L., the cabbage aphid shows a strong behavioral preference to settle and start colonies on the young parts of the growing stems (Gabrys, 1991). It is generally believed that high turgor pressure, high amino acid content, and high glucosinolate concentrations characteristic of young parts of the plant are favored by the cabbage aphid and can explain this distribution (Klingauf, 1987).

The data on glucosinolates and their relations to aphid preference, performance, or feeding stimulation do not seem to explain how they interact with feeding behavior. Glucosinolates occur predominantly in the vacuoles of mesophyll cells (Matile, 1984; Chew, 1988). They are present also in the phloem sap, although possibly in lower concentrations (Weber et al., 1986; Merritt, 1996). In order to understand the relation between glucosinolates and aphid behavior, more precise information is needed on the distribution of these compounds inside the plant and the duration and sequence of separate events in feeding behavior, especially of the events during stylet penetration (probing). The analysis of probing behavior, using the electrical penetration graph (EPG) technique (Tjallingii, 1988), revealed many details and relationships of probing events at separate tissue locations (Tjallingii and Hogen Esch, 1993). In studies

on host plant selection and mechanisms of plant resistance, the EPG technique has been applied by several authors (Givovich and Niemeyer, 1991; Montllor and Tjallingii, 1989; Van Helden and Tjallingii, 1993; Caillaud et al., 1995), and also in relation to glucosinolates (Cole, 1994). In the latter study it was found that some glucosinolates were probably not involved in the resistance of some cabbage cultivars against *B. brassicae*. From the analysis of EPG waveforms, the factors interfering with probing activities at different tissue levels can be inferred, for example, at the epidermis, mesophyll/vascular tissue, or at phloem level (Tjallingii, 1990, 1995).

Our aim was to access the relationship between feeding site preferences by *B. brassicae* and the distribution of glucosinolates in its host plant *S. alba* L. In this study, which is preliminary, we tried to correlate parameters derived from *B. brassicae* probing in different plant parts with the glucosinolate content in these parts.

METHODS AND MATERIALS

Aphids and Plants. The stock culture of *B. brassicae* was maintained on *S. alba* in 16L:8D photoperiod, 20°C, and 60% relative humidity. For EPG recording and chemical analyses, we used 5-week-old *S. alba* plants having seven to eight fully expanded true leaves and closed flower buds grown under the conditions noted above. Leaves 1, 4, and 7 were referred to as old, mature, and young, respectively. The apical part of the stem, just below the flower buds, was referred to as the stem in our treatments. All experiments were done in summer and within a period of two months. Fresh cross sections of these plant parts were examined with a light miroscope at 160× using an eyepiece micrometer to measure the relevant distances. Further staining with aniline blue and phloroglucinol HCl was used (PVA as the mounting medium) to detect cellulose and lignified cell walls, respectively.

EPG Recording and Parameters. Young apterous *B. brassicae* females, one to four days after the final molt, were collected at about 10.00 AM and attached to a 2-cm-long, 20-μm-diameter gold wire with water soluble silver glue. Within 1 hr these aphids were connected to the EPG amplifier (DC system) (Tjallingii, 1988), given access to the plant, and EPG recording was started immediately. Eight aphids were recorded simultaneously by using two four-channel systems, and signals were put on a PC hard disc (100 Hz conversion rate) with STYLET 2.2 software (Tjallingii and Mayoral, 1992). Successful recordings were obtained from 17 aphids on stems, 19 on young leaves, 19 on mature leaves, and 20 on old leaves.

In the EPGs, we distinguished probing and nonprobing. Within probing, three phases were distinguished based on waveforms described and correlated

with probing activity earlier (Tjallingii, 1988; Prado and Tjallingii, 1993). The three phases were: (1) pathway phase (lumped waveforms A, B, and C), (2) xylem phase (only waveform G), and (3) phloem phase (waveforms E1 and E2). Durations of waveform E2 for 10 min or more are referred to as sustained phloem ingestion and considered to reflect sieve element acceptance (Tjallingii and Mayoral, 1992). In order to evaluate aphid reactions to the four different plant parts, probing behavior was divided into three periods by three "natural landmarks" as shown in Figure 1. Nonprobing before the first probe was not used as a parameter as it appeared highly sensitive to the aphid's pretreatment (Tjallingii and Mayoral, 1992). We designated the beginning of the first probe (= first landmark, see Figure 1) the start of the experiment. Durations from then on are referred to as "within experiment" as follows: period I–from start of first probe (first landmark) to first phloem phase (second landmark), i.e., E1 waveform whether followed by E2 or not; period II–from first phloem phase to first sustained phloem sieve element ingestion, i.e., a period of E2 longer than 10 min (third landmark); and period III–from first sustained phloem ingestion until the end of experiment.

The probes were classified according to their duration, as follows: probes equal to or less than 2 min long, which, according to Van Hoof (1958), would

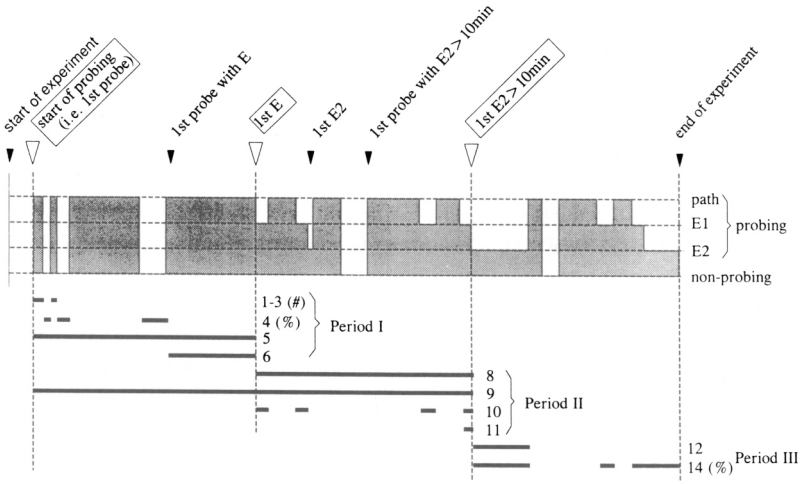


FIG. 1. Diagram of EPG parameters used. All waveforms are represented by a certain level (top diagram). Horizontal bars (bottom diagram) reflect duration or summed durations of parameters 5–12. In other cases, the number of events (#) is used (parameters 1–3), or a relative value (%) (parameters 4 and 14). Arrows indicate points used in parameter definition (Table 2) and open arrows show natural landmarks used to divide probing behavior into three periods.

not go beyond about one cell layer; probes of 2–10 min considering that 10 min was about the minimum time to first phloem phase within a probe in the present study; and probes longer than 10 min. For calculations of the time to reach the first phloem phase, aphids that did not show the first phase were classified as if 8 hr were needed to achieve it (in fact, the total experimental time or more).

The data were analyzed statistically with Kruskal-Wallis non-parametric test at $P = 0.05$.

Chemical Analyses. Whole-leaf samples of young (seventh), mature (fourth), old (first) leaves, and ca. 3 cm of the apical part of the stem (without flower buds) were cut off the plant, weighed, and put immediately into 10 or 25 ml (the volume depending on the leaf size) of boiling water to inactivate the native myrosinases and avoid hydrolysis of glucosinolates into their decomposition products. After boiling for 10 min, an extract was cooled to room temperature, filtered, and water was added to make the final volume 10 or 25 ml based on the original volume of boiling water used, as noted above. Extracts were stored at −20°C, and were injected into the HPLC without further processing.

The underivatized glucosinolates were analyzed with HPLC according to the method described by Betz and Page (1990). Plant samples were separated on a 250- × 4-mm C18RP-HPLC (Nucleosil 120-5 C18, particle size 5 μm) column (Macherey and Nagel) with a 30-mm precolumn. The eluting solvent was an isocratic mixture of 25% MeOH and 0.005 M TAS (tetrabutylammonium hydrogen sulfate). The flow rate was 1 ml/min and the run time 25 min. The injection volumes were 20 μl. The detection at 227 nm UV was performed with a Varian 2050 UV detector using a HP-3395 integrator or with a Waters 994 Diode Array Detector. The glucosinolates were identified and quantified based on comparison of UV spectra, retention times, and peak areas of pure glucosinolate reference solutions.

RESULTS

Probing Behavior. The total probing time by *B. brassicae* on *S. alba* was at least 7.1 hr on all feeding sites, which was more than 85% of the 8-hr experiment (Table 1). The pathway phase constituted about half of the probing time, and the other half was the phloem phase. The xylem phase did not occur. The pathway phase activities predominated during the first four hours after aphids had been given access to the plant, and time spent in phloem ingestion (waveform E2) increased towards the end of experiment (Figure 2). During pathway phase activities on old leaves, *B. brassicae* showed periods with repetitive potential drops (Tjallingii, 1985) frequently, but on other plant parts this was rather rare (data not shown). All aphids on stems showed sustained phloem

TABLE 1. PROBING ACTIVITIES OF CABBAGE APHID ON YELLOW MUSTARD DURING 8-HOUR EXPERIMENT (MEAN PER APHID)[a]

Totals	Stems (N = 17)	Leaves		
		Young (N = 19)	Mature (N = 19)	Old (N = 20)
Probing (hr)	7.4 a	7.1 a	7.1 a	7.1 a
Probes (N)	6.0 a	30.2 b	21.4 b	20.7 b
Stylet pathway phases (hr)	3.2 a	3.7 a	3.8	3.9 a
Phloem phase (all E) (hr)	4.2 a	3.4 a	3.3 a	3.2 a
Phloem ingestion (hr)	4.1 a	3.3 a	3.2 a	3.1 a
Aphids with sustained ingestion (N^b)	17 a	18 a	19 a	17 a

[a] Significant differences marked with different letters (Kruskal-Wallis test, P = 0.05)
[b] Total number.

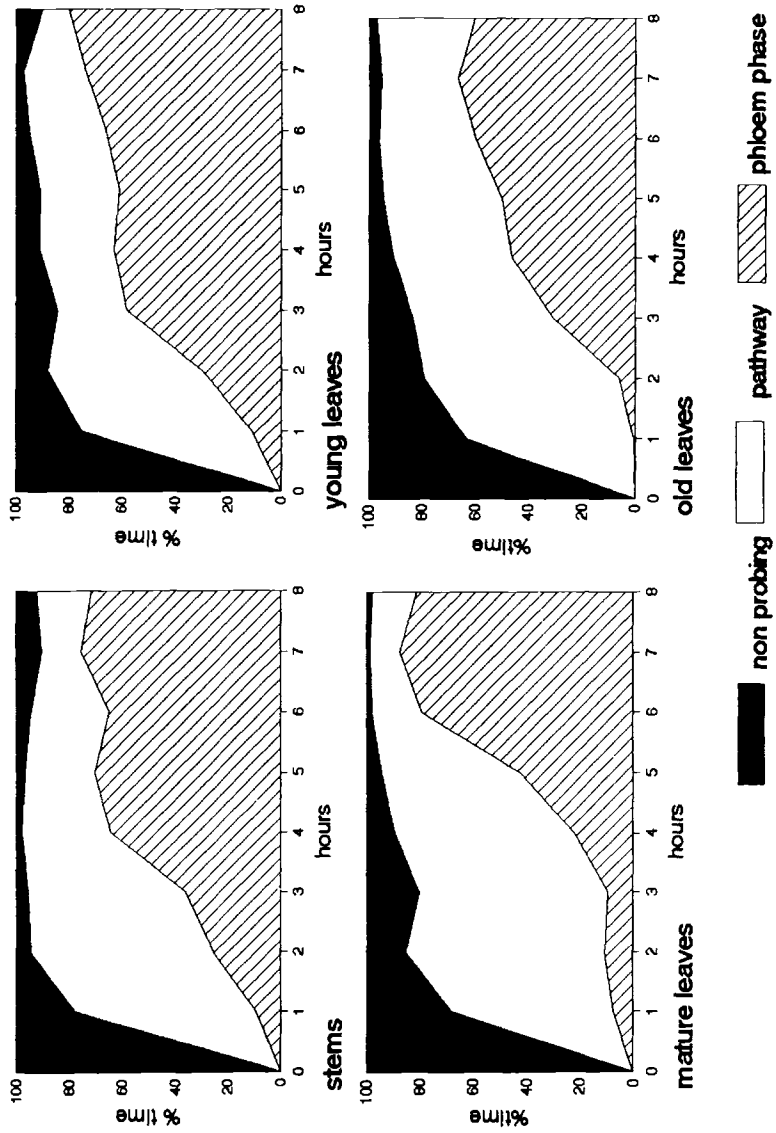


FIG. 2. Total durations of probing phases in course of successive hours by *B. brassicae* on leaves and stems of *S. alba* during 8-hr experiment.

ingestion, while on leaves some individuals failed to do so during the 8-hr duration of an experiment (Table 1).

Period I. Before the first phloem phase, aphids on leaves spent less time probing than those on stems (Table 2). The average number of probes per aphid was 2.7 on stems and 15.6 on leaves. On stems 47% of probes were shorter than 10 min, while on leaves up to 89% were shorter than 10 min. The first phloem phase was reached by 41% of aphids in the first probe. The duration of the pathway phase before the first problem phase within a probe was longer on stems than on young and mature leaves. However, the time to reach the first phloem phase within the experiment was similar on all plant parts tested.

Period II. Aphids on leaves started sustained phloem ingestion more than 1 hr after the first phloem phase had occurred. Period II was rarely observed in aphids on stems. The first phloem phase comprised sustained phloem ingestion in almost all individuals (see also parameter 13, Table 2). The time to the first sustained ingestion was shortest on stems and so was the total sieve element salivation.

Period III. The duration of first sustained ingestion of phloem sap was longest when aphids were on stems. However, the percentage of time spent by aphids on phloem phase, total phloem ingestion (Table 1), and number of new probes was similar on all feeding sites.

Plant Anatomy. Fresh plant sections showed that distances between epidermis and phloem were 250–400 μm in stems and leaves and comparable to feeding sites tested. In stems two to three layers of collenchyma occurred beneath the epidermis and several cells of sclerenchyma at the outer part of vascular bundles. In leaves a collenchyma layer occurred at the midrib.

Chemistry of the Plant. Three glucosinolates, glucosinalbin (*p*-hydroxybenzyl glucosinolate), gluconapin (3-butenyl glucosinolate), and glucotropaeolin (benzyl glucosinolate) were present in plant samples of *S. alba*. The total concentration of glucosinolates (Table 3) was lowest in old leaves and highest in young leaves, whereas in stems it was intermediate. The concentration of sinalbin and tropaeolin changed as described for total glucosinolates, but the concentration of napin was remarkably higher in the stems as compared to leaves. Towards the apex of the plant, the relative amount (percentage of all glucosinolates) of sinalbin decreased in favor of tropaeolin and napin (Table 3).

DISCUSSION AND CONCLUSIONS

The EPG analysis of *B. brassicae* behavior on stems and leaves of *S. alba* showed some general similarities in probing on these plant parts, i.e., similar time spent in total probing, pathway, and phloem phases. There were, however, remarkable differences during early probing. On stems, aphid probing was rarely

TABLE 2. MEAN NUMBER (N), MEAN DURATION (hr), AND PERCENTAGES OF EPG PARAMETERS IN THREE SUCCESSIVE PERIODS OF PROBING AS VISUALIZED IN FIGURE 1[a]

		Leaves		
	Stems (N^a = 17)	Young (N = 19)	Mature (N = 19)	Old (N = 20)
I. Period before first phloem phase				
1. Probes <2 min (N)	0.6 a	7.4 b	5.5 b	4.3 b
2. Probes 2–10 min (N)	0.6 a	5.6 b	5.7 b	5.5 b
3. Probes >10 min (N)	1.5 a	1.7 a	2.7 a	2.9 a
4. Nonprobing (%)	33 a	72 b	72 b	68 b
5. Time to first phloem phase within experiment (hr)	2.3 a	2.2 a	2.9 a	2.7 a
6. Time to first phloem phase within probe (hr)	1.2 c	0.4 a	0.6 ab	1.4 bc
7. Aphids with phloem phase in first probe (N^b)	7 a	0 b	1 b	0 b
II. Period from first phloem phase to first sustained phloem ingestion				
8. Duration of period II (hr)	0.0 b	1.2 a	1.7 a	1.8 a
9. Time to first sustained phloem ingestion within experiment (hr)	2.3 a	3.5 ab	3.9 b	3.9 b
10. Total phloem salivation before first sustained ingestion (min)	1.3 a	2.5 ab	3.7 b	2.7 ab
11. Phloem salivation period just before first sustained ingestion (min)	1.3 b	0.9 a	1.5 b	1.1 ab
III. Period from first sustained phloem ingestion onwards				
12. Duration of first sustained phloem ingestion period (hr)	2.9 c	1.9 bc	1.3 ab	0.7 a
13. Aphids where first phloem phase included sustained ingestion (N^b)	15 a	7 b	8 b	10 b
14. Time in phloem phase after start of first sustained phloem ingestion (%)	77 a	62 a	70 a	57 a
15. New probes after first sustained ingestion (N)	1.8 a	7.3 a	1.4 a	4.1 a

[a]Significant differences marked with different letters. N = number of replicates. Kruskal-Wallis test (P = 0.05).
[b]Total number.

TABLE 3. GLUCOSINOLATE PROFILE OF *Sinapis alba* L.[a]

	Stems	Leaves		
		Young	Mature	Old
G-sinalbin				
mg/g fresh weight	1.9 b	3.5 c	1.5 b	0.45 a
% of all GSLs	83.3 a	85.9 ab	92.5 bc	93.5 c
G-napin				
mg/g fresh weight	0.1 c	0.05 b	0.01 a	0.02 ab
% of all GSLs	5.5 c	1.4 b	0.9 b	5.6 c
G-tropaeolin				
mg/g fresh weight	0.3 b	0.5 b	0.1 c	0.01 a
% of all GSLs	11.3 b	11.8 b	6.6 b	0.9 a
Total GSLs				
mg/g fresh weight	2.3 b	4.0 c	1.61 d	0.48 a

[a]Significant differences marked with different letters. Kruskal-Wallis test ($P = 0.05$).

interrupted, the first probe more often led to phloem phase, and the first phloem phase more often led to sustained phloem ingestion than when the aphids probed leaves. In periods II and III, after reaching phloem phase, there were no differences in aphid behavior.

It is not unlikely that the general similarity in probing behavior of *B. brassicae* on different plant parts was partly due to the intrinsic effect of tethering. Aphids might have walked away after a short probe on a leaf to reach the preferred stems had they been free to move. However, this does not seem to affect our conclusions, as the phloem sap was equally accepted in the different plant parts. We did not compare fecundity of aphids on these parts, but it might well be that they would not differ much as was found for various sites on *B. oleracea* and *B. napus* by Van Emden and Bashford (1969) and Miles et al. (1982), respectively.

The distinctly higher number of short probes on leaves strongly suggests that factors located in the epidermis and outer mesophyll caused discontinuation of probing. This suggestion seems reasonable for probes that stopped within 2 min, as stylet penetration time of the monocellular tissue layer was found to take about 2 min (Van Hoof, 1958). The time needed for stylet penetration from epidermis to phloem may strongly depend on the probing site on the leaf. Leaf penetration can be either in the lamina area or close to a vein—the main vein or a higher order vein. In a lamina probe, the epidermis–phloem distance may be only few cell layers, while on the veins there are more cell layers and the

cells are more densely packed. Given the stylet penetration speed, it was found that the time before the first phloem phase in a probe often appeared much longer than could be explained by the number of cell layers (Tjallingii and Hogen Esch, 1993; Tjallingii and Mayoral, 1992). It has been demonstrated that aphid stylet tracks within plant tissues form several "dead end" branches and that phloem sieve elements are reached and punctured briefly (reflected by the 5-sec potential drops in the EPG) (Tjallingii and Hogen Esch, 1993) before an actual phloem phase is shown. Therefore, the longer time to reach the first phloem phase (parameter 6) on stems and old leaves compared to young leaves could be caused by mesophyll as well as vascular tissue factors, including phloem sieve elements. As we found no obvious differences in the epidermis-phloem distances or the presence of massive sclerenchyma layers between stems and leaf veins, chemical factors could be responsible. Whether these chemicals are in the mesophyll and stimulate pathway activities or whether they are near or in the phloem and prevent switching from pathway to phloem phase activities can not be inferred from our data. From what follows, however, it seems unlikely that this factor is (only) in the phloem sap.

Once phloem phase occurred, it usually ended with sustained phloem ingestion (parameter 13) in the stem probes, while in the leaves it usually did not. Although it may be concluded that phloem sap is more attractive in stems than in leaves, the difference in acceptance of a given plant part seems only initial. The total time spent by aphids on phloem ingestion (parameters 14 and 15) does not differ significantly between plant parts.

Another difference observed between plant parts was the occurrence of "repetitive potential drops." These are potential drops that are repeated at very regular intervals and occur in only a few aphid species (Tjallingi, 1985) occasionally. We found them mainly in probes on old leaves but we will report on them separately elsewhere.

Glucosinolates occurred at highest concentrations in young leaves. Only the gluconapin concentration, highest in stems, corresponded with attractiveness of this site to the aphids, although it was a minor glucosinolate as far as the concentration was concerned. In this study our aim was mainly to identify the glucosinolates in *S. alba* and to demonstrate any differences between plant parts. In terms of concentration in a particular tissue or cell compartment, we still can say little about the separate glucosinolates. With respect to aphid behavior, it seems clear that such information would be very relevant. The differences found in the glucosinolate contents between the plant parts can not fully explain differences in aphid probing behavior. What was presented here as "concentration" (Table 3) was an average of all tissue parts in a certain sample that was not really encountered by aphids in the plant. Decisions in the subsequent phases and periods are presumably based on local stimuli. It is also clear that the

glucosinolates are not the only plant chemicals involved in these decisions. Therefore, determination of the behavioral impact of glucosinolates needs carefully designed experiments with, for example, artificial diets. As we found (unpublished results), simple diet tests may lead to unbalanced conclusions. Future studies should focus on further details in glucosinolate compartmentation as well as on the behavioral impact of glucosinolates in natural and experimental situations.

Acknowledgments—The authors thank Gerrit Gort (Department of Mathematics) for help in statistical analysis and Marijke Bosman for assistance in chemical analyses. Financial support was granted to Beata Gabrys by Wageningen Agricultural University and the Polish Committee for Scientific Research (Project No. 1164/53/93/04).

REFERENCES

BETZ, J. M., and PAGE, S. W. 1990. Liquid chromatographic method for the determination of intact, non-derivatized glucosinolates from Brassicaceae, pp. 103–104, *in* Proceedings of the Symposium Biology and Chemistry of Active Natural Substances. Bonn, July 1990.

BLACKMAN, R. L., and EASTOP, V. F. 1985. Aphids on the World's Crops: An Identification Guide. John Wiley & Sons, New York, pp. 413.

CAILLAUD, C. M., PIERRE, J. S., CHAUBET, B., and DIPIETRO, J. P. 1995. Analysis of wheat resistance to the cereal aphid *Sitobion avenae* using electrical penetration graphs and flow charts combined with correspondence analysis. *Entomol. Exp. Appl.* 75:9–18.

CHEW, F. S. 1988. Searching for defensive chemistry in the *Cruciferae*, or, do glucosinolates always control interactions of *Cruciferae* with their potential herbivores and symbionts? No!, pp. 81–112, *in* K. C. Spencer (ed.), Chemical Mediation of Coevolution. Academic Press, San Diego.

COLE, R. A. 1994. Locating a resistance mechanism to the cabbage aphid in two wild brassicas. *Entomol. Exp. Appl.* 71:32–31.

DODD, G. D., and VAN EMDEN, H. F. 1979. Shifts in host plant resistance to the cabbage aphid (*Brevicoryne brassicae*) exhibited by brussels sprout plant. *Ann. Appl. Biol.* 91:251–262.

FENWICK, G. R., HEANEY R. K., and MULLIN, W. J. 1983. Glucosinolates and their breakdown products in food and foodplants. *CRC Crit. Rev. Food Sci. Nutr.* 18:123–202.

GABRYS, B. 1991. Distribution of *Brevicoryne brassicae* and *Myzus persicae* on yellow mustard plant, pp. 303–305 *in* Proceedings, Conference Insect Chemical Ecology, Tabor, 1990, Academia, Prague, and SPB Academic Publishers, The Hague.

GINOVICH, A., and NIEMEYER, H. M. 1991. Hydroxyamic acids affecting barley yellow dwarf virus transmission by the aphid *Rhopalosiphum padi*. *Entomol. Exp. Appl.* 59:79–85.

KLINGAUF, F. A. 1987. Feeding, adaptation and excretion, pp. 225–248, *in* A. K. Minks and P. Hareewijn (eds.). Aphids, Their Biology, Natural Enemies and Control, Vol. 2A. Elsevier, Amsterdam.

MATILE, P. 1984. Das toxische Kompartiment der Pflanzenzelle. *Naturwissenschaften* 71:18–24.

MERRITT, S. Z. 1996. Within-plant variation in concentrations of amino acids, sugar, and sinigrin in phloem sap of black mustard, *Brassica nigra* (L.) Koch (Curciferae). *J. Chem. Ecol.* 22:1133–1145.

MILES, P. W., ASPINALL, D., and ROSENBERG, L. 1982. Performance of the cabbage aphid, *Brevicoryne brassicae* (L.), on water stressed rape plants, in relation to changes in their chemical composition. *Aust. J. Zool.* 30:337–345.

MONTLLOR, C. B., and TJALLINGII, W. F. 1989. Stylet penetration by two aphid species on susceptible and resistant lettuce. *Entomol. Exp. Appl.* 52:103–111.

NAULT, L. R., and STYER W. E. 1972. Effects of sinigrin on host selection by aphids. *Entomol. Exp. Appl.* 15:423–437.

PRADO, E., and TJALLINGII, W. F. 1993. Aphid activities during sieve element punctures, pp. 109–112, *in* P. Kindlmann and A. F. G. Dixon (eds.). Critical Issues in Aphid Biology. Proceedings, 4th International Symposium on Aphids, Ceske Budejovice.

TJALLINGII, W. F. 1985. Membrane potentials as an indication for plant cell penetration by aphid stylets. *Entomol. Exp. Appl.* 38:187–194.

TJALLINGII, W. F. 1988. Electrical recording of stylet penetration activities, pp. 95–108, *in* A. K. Minks and P. Harrweijn (eds.). Aphids, Their Biology, Natural Enemies and Control, Vol. 2B. Elsevier, Amsterdam.

TJALLINGII, W. F. 1990. Stylet penetration parameters from aphids in relation to host-plant resistance. *Symp. Biol. Hung.* 39:411–419.

TJALLINGII, W. F. 1995. Aphid-plant interactions: What goes on in the depth of the tissues? *Proc. Exp. Appl. Entomol.* 6:163–169.

TJALLINGII, W. F., and HOGEN ESCH, T. 1993. Fine structure of aphid stylet routes in plant tissues in correlation with EPG signals. *Physiol. Entomol.* 18:317–328.

TJALLINGII, W. F., and MAYORAL, A. 1992. Criteria for host-plant acceptance by aphids, pp. 280–282, *in* S. B. J. Menken, J. H. Visser, and P. Harrewijn (eds.). Proceedings 8th International Symposium on Insect-Plant Relationships. Kluwer Academic Publishers, Dordrecht.

VAN EMDEN, H. F. 1978. Insects and secondary plant substances—an alternative viewpoint with special reference to aphids, pp. 309–323, *in* J. B. Harborne (ed.). Biochemical Aspects of Plant and Animal Coevolution. Academic Press, London.

VAN EMDEN, H. F., and BASHFORD, M. A. 1969. A comparison of the reproduction of *Brevicoryne brassicae* and *Myzus persicae* in relation to soluble nitrogen concentration and leaf age (leaf position) in the Brussels sprout plant. *Entomol. Exp. Appl.* 12:351–364.

VAN HELDEN M., and TJALLINGII, W. F. 1993. Tissue localization of lettuce resistance to the aphid *Nasonovia ribisnigri* using electrical penetration graphs. *Entomol. Exp. Appl.* 68:269–278.

VAN HOOF, H. A. 1958. An investigation of the biological transmission of a nonpersistent virus, pp. 81–112, *in* A. Van Putten and B. Oortmeijer. Alkmaar. Academic Press, New York.

WEBER, G., OSWALD, S., and ZOLLNER, U. 1986. Suitability of rape cultivars with a different glucosinolate content for *Brevicoryne brassicae* (L.) and *Myzus persicae* (Sulzer) (Hemiptera, Aphididae). *J. Plant. Dis. Prot.* 93:113–124.

WENSLER, R. J. D. 1962. Mode of host selection by an aphid. *Nature* 195:830–831.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

EPICUTICULAR WAX CHEMICALS IN *Zea mays* INFLUENCE OVIPOSITION IN *Ostrinia nubilalis*

SUJAYA UDAYAGIRI[1,2,*] and CHARLES E. MASON[1]

[1]*Department of Entomology and Applied Ecology*
Delaware Agricultural Experiment Station, University of Delaware
248 Townsend Hall, Newark, Delaware 19717-1303

(Received April 15, 1996; accepted February 24, 1997)

Abstract—The chemical basis of oviposition elicitation in a generalist herbivore was determined by examination of oviposition responses in *Ostrinia nubilalis* to corn (*Zea mays*) chemicals in two-choice laboratory bioassays. A pentane extract of corn leaves stimulated oviposition and the activity persisted for three days, indicating that oviposition in *O. nubilalis* is elicited by low-volatility chemicals. Chemicals in the extract were fractionated by column chromatography on Florisil, using a sequence of solvents of increasing polarity. Bioassays of Florisil fractions indicated that the stimulants were eluted with nonpolar solvents. Positive bioassay results with an extract prepared by dipping corn leaves in pentane for 20 sec for extraction of leaf surface chemicals suggested that some of the active material was present in the leaf epicuticle. Gas chromatographic analyses and comparisons with retention times of standards suggested the presence of several *n*-alkanes in the dip extract. Five *n*-alkanes—hexacosane, heptacosane, octacosane, nonacosane, and tritriacontane—known to be present in the epicuticle of corn leaves were bioassayed, and all five elicited oviposition responses. These results suggest that oviposition elicitation in *O. nubilalis* is influenced by the presence of *n*-alkanes in the host plant epicuticle.

Key Words—*Ostrinia nubilalis*, oviposition stimulants, phytochemical cues, epicuticular chemicals, *n*-alkanes.

INTRODUCTION

In several insect species, especially lepidopterous insects, host selection is a function of the adult female (Renwick, 1989). Oviposition in these species is

*To whom correspondence should be addressed.
[2] Present Address: Department of Environmental Science, Policy & Management, Division of Insect Biology, University of California, 201 Wellman Hall #3112, Berkeley, California 94720.

0098-0331/97/0700-1675$12 50/0 © 1997 Plenum Publishing Corporation

critical as survival of the offspring is dependent on recognition by adult females of host plants that are suitable for larval development. Hence, we can expect females to be influenced by plant-related stimuli for selection of appropriate oviposition sites. Phytochemicals are known to influence feeding behaviors in herbivores, but there is increasing evidence that they are also involved in eliciting oviposition behaviors. Identification of host plant chemicals that act as oviposition stimulants for insect species of economic importance is gaining importance due to their potential for use in manipulation of pest behavior in the field. Most of the research in this area has been conducted with specialist insects that appear to select hosts based on the presence of specific phytochemicals that are characteristic of plant families utilized by larvae (Städler, 1992). For generalist herbivores, the chemical basis of oviposition site selection is largely unknown.

The European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae), is a polyphagous herbivore that was inadvertently introduced into the United States over 80 years ago (Mason et al., 1996). While host plants of *O. nubilalis* include diverse cultivated plants such as potato (*Solanum tuberosum* L.), pepper (*apsicum annum* L.), cotton (*Gossypium hirsutum* L.), and wheat (*Triticum aestivum* L.), in the United States, corn (= maize, *Zea mays* L.) is its principal host (Mason et al., 1996). We have previously examined oviposition behavior in *O. nubilalis* (Udayagiri and Mason, 1995) and determined that foliar extracts of corn, potato, and pepper stimulate oviposition. When the stimulatory factors in these host plants are determined, it may be possible to use them to manipulate *O. nubilalis* oviposition behavior and thereby reduce damage on economically important crops in the field. The present study was conducted to further examine oviposition responses of *O. nubilalis* to active components in corn and determine the source and nature of chemicals that are stimulatory.

METHODS AND MATERIALS

Plants and Insects. Corn (Silver Queen) was raised in the greenhouse at the Delaware Agricultural Experiment Station at Newark. *O. nubilalis* (Z pheromone-type, also known as the Iowa strain) were obtained as pupae from the Corn Insects Research Laboratory, Ames, Iowa, where the colony was started from individuals collected at black light traps the previous year and reared subsequently on an artificial diet (Guthrie et al., 1985). Pupae received from Ames were maintained in an environmental chamber with a photoperiod of 16L:8D and 70 ± 10% relative humidity. The temperature was maintained at 26°C during the light and 22°C during the dark cycle.

Bioassay. Oviposition responses of gravid females were examined in two-choice bioassays in oviposition chambers constructed from 0.47 liter Fonda paper cartons. Treatments [0.5 ml of plant extract = 0.5 g leaf equivalents

(gle)] and controls (0.5 ml of solvent) were applied to half filters (9-cm-diam. Whatman No. 1 filter paper). After evaporation of the solvent, the two half filters were taped together and presented to a gravid female in an oviposition chamber 2 hr before the start of scotophase. Only one female was used in each bioassay to avoid any possible influence on oviposition by conspecific females since females are known to produce an oviposition-deterring pheromone (Thiery and Le Quéré, 1991). Oviposition chambers were held overnight in an environmental chamber with the temperature, photoperiod, and humidity conditions as outlined above. The following morning, egg masses on each half filter were examined, and the number of eggs in each egg mass were recorded. Only egg masses with more than five eggs were included in the analysis. The bioassay was used in the following experiments to identify the nature and source of stimulatory chemicals.

Experiment 1. An extract of corn leaves containing 1 g equivalent of fresh plant material per milliliter of pentane was prepared as described by Udayagiri and Mason (1995). This extract stimulates oviposition in *O. nubilalis* (Udayagiri and Mason, 1995), and the present study was conducted to determine whether the activity decreased with time after exposure. The extract and control (pentane) were applied to half filters as described above, and the taped filter was stored at room temperature (23°C). In a similar manner filters were prepared 24, 28, and 72 hr later. On the third day, filters prepared 0, 24, 48, and 72 hr earlier were bioassayed. The following day, egg laying on half filters of each of the four sets of taped filters was recorded. The experiment was replicated 16 times.

Experiment 2. Chemicals in the pentane extract of corn were separated into different fractions based on their polarity by column chromatography on a 2 × 13-cm column of Florisil (25 g) (activated magnesium silicate) (Sigma Chemical Co., St Louis, Missouri). Corn extract (= 500 g fresh weight of corn leaves) in pentane was concentrated to 5 ml under nitrogen and loaded onto the Florisil column. The column was eluted consecutively with 30 ml (= two void volumes of the column) each of an ascending series of diethyl ether (E) in pentane (P) (100% pentane, 90% P + 10% E, 80% P + 20% E, 50% P + 50% E, 20% P + 80% E, and 100% diethyl ether) followed by 100% acetone and 100% methanol. Eight fractions were collected and bioassayed. When a particular fraction was bioassayed, the solvent used to elute the fraction was used as the control. Bioassay of each fraction was replicated 30 times.

Experiment 3. Location of the source of the stimulatory chemicals was determined by preparation of two extracts from the same plant material. Initially an extract of leaf surface chemicals was prepared by dipping 60 g of intact corn leaves in pentane for 20 sec (dip extract). The solution was evaporated to dryness and the residue was dissolved in 60 ml of pentane. As before, 0.5 ml of the solution was applied to half filters and bioassayed with pentane as the control. A second extract, referred to as the postdip extract, was prepared. The leaves

given the dip treatment were crushed and extracted in pentane as described above. Bioassays were conducted with the postdip extract and pentane was used for the control. Each of these two experiments was replicated 30 times.

For gas chromatographic analysis, four individual-leaf dip extracts were prepared by dipping individual leaf blades in 100 ml of pentane for 20 sec. Leaves closest to the ear from plants at late tassel stage (>50 days) were selected and the surface area of each leaf blade (upper and lower surfaces) was determined with a Li-Cor LI-3000A Portable Area Meter (Li-Cor, Inc., Lincoln, Nebraska). After evaporation of the solvent, the residue from each individual-leaf dip extract was redissolved in 1.5 ml of pentane. Six microliters was removed, reduced to 3.5 μl, and 1 μl (4.5 ng) of an internal standard, *cis*-7-tetradecenyl acetate (Sigma), was added. This was injected into a Varian 3500 gas chromatograph (Varian Associates, Sunnyvale, California) with a Varian 1077 split/splitless injector and a flame ionization detector. The analytical column was a Stabilwax (Restek Corporation, Bellefonte, Pennsylvania) fused silica capillary column (15 m $\times$ 0.25 mm, 0.50 μl film thickness). Chromatography conditions included an initial temperature of 40°C held for 1.5 min, rising 8°C/min to 250°C, and held at the final temperature for 6.25 min. The injector was splitless for 1.5 min. and then held on split at a 50:1 ratio for the remainder of the run. N_2 was the carrier gas at 19 cm/sec. Integration of peaks was initiated 7 min into the run to eliminate the solvent peak from the analysis.

Experiment 4. A reference mixture of hexacosane ($C_{26}H_{54}$), octacosane ($C_{28}H_{58}$), nonacosane ($C_{29}H_{60}$) (Sigma), and heptacosane ($C_{27}H_{56}$) (Aldrich Chemical Co. Inc., Milwaukee, Wisconsin), *n*-alkanes reported to be associated with the leaf surface in corn (Bianchi and Salamini, 1975), was prepared by mixing 100 ng of each *n*-alkane. As a reference, 4.5 ng of 7-tetradecenyl acetate was added. Retention time and size of the peak for each *n*-alkane relative to the total area of the resolved peaks was determined on the gas chromatograph with GC conditions outlined above. Retention times of the four *n*-alkanes were compared with those of peaks on the gas chromatogram of the individual-leaf dip extracts, and *n*-alkanes that appeared to be present in the dip extract were bioassayed. In addition, tritriacontane ($C_{33}H_{68}$) (Aldrich), which was not added to the reference *n*-alkane mixture since it did not separate on the analytical column used in the gas chromatograph, was also bioassayed. Each chemical was diluted in pentane and tested at two doses, 250 and 500 μg of each *n*-alkane applied to a half filter, and each test was replicated 30 times.

Statistical Analysis. Numbers of eggs on both half filters were totaled for each replicate in each experiment. For presentation of the results an index of stimulation, the oviposition stimulation index (OSI), was calculated using the following formula:

$$\text{Oviposition stimulation index} = \frac{(N \text{ eggs on treatment half filter} - N \text{ eggs on control half filter})}{(N \text{ eggs on treatment half filter} + N \text{ eggs on control half filter})} \times 100$$

The null hypothesis was that equal numbers of eggs were laid on both half filters in each bioassay. The nonparametric Wilcoxon signed-rank test or the Kruskal-Wallis test (Ott, 1984) was used to assess significance of differences between egg laying on both halves of the taped filter or (first experiment) for differences in the OSI based on an α level of 0.05.

RESULTS

Corn leaf extract prepared in pentane was applied to half filters, stored at room temperature for zero to three days, and then presented to gravid females. Half filters presented to females 0, 24, 48, and 72 hr after application of the corn extract all received greater numbers of eggs compared to the control (pentane) half filters, but no significant differences in OSI were observed in egg laying across treatments, i.e., on half filters that were prepared 72, 48, 24, or 0 hr before the bioassay (Kruskal-Wallis, $P > 0.05$) (Figure 1). Hence, even after it was set aside at room temperature (23°C) for 72 hr, the extract of corn stimulated oviposition, indicating that stimulatory chemicals in corn have low volatility.

Eight fractions were collected during column chromatography on Florisil. Half filters containing fraction 1 that eluted with pentane, and fractions 2, 3, and 4 that eluted with 90% pentane + 10% diethyl ether (90% P + 10% E), 80% P + 20% E and 50% P + 50% E, respectively, received significantly greater numbers of eggs than control filters (Wicoxon signed-rank test, $P <$

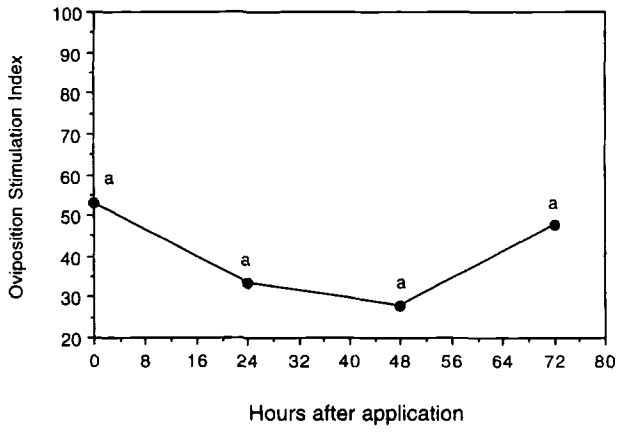


FIG. 1. Comparison of oviposition responses of female *O. nubilalis* to pentane extracts of corn leaves applied to half filters, stored at room temperature from 0 to 72 hr, and then presented to females along with control (pentane) half filters. Oviposition stimulation indices (see text for explanation) with the same letter are not significantly ($P > 0.05$) different as determined by the Kruskal-Wallis test.

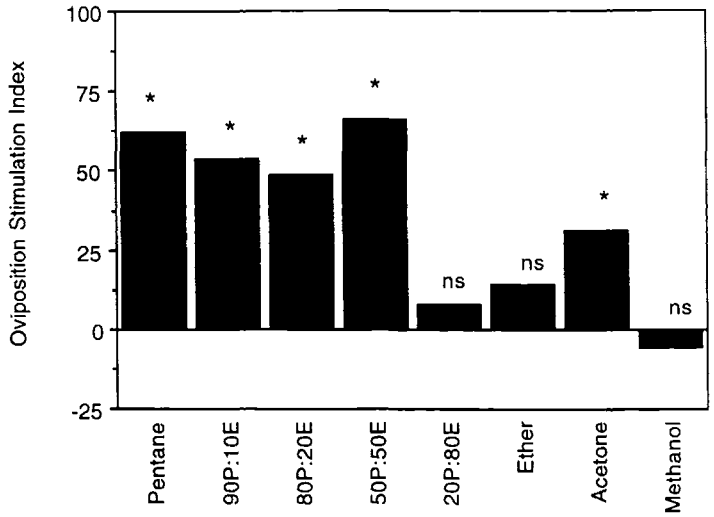


FIG. 2. Oviposition responses of female *O. nubilalis* to Florisil fractions of corn leaf extract. Solvents used to elute fractions are indicated on the abscissa; for example, 90P:10E means 90% pentane + 10% diethyl ether. In each bioassay, the solvent used to elute the fraction was used as the control. See text for explanation of oviposition stimulation index. For each Florisil fraction, an asterisk indicates significant ($P < 0.05$), and ns indicates nonsignificant ($P > 0.05$) difference in egg laying between treatment and control half filters as determined by the Wilcoxon signed-rank test.

0.05) (Figure 2). Half filters with fraction 7, the fraction eluted with acetone, also received significantly greater numbers of eggs compared to control half filters (Wilcoxon signed-rank test, $P < 0.05$). There were no significant differences in egg laying when fractions 5, 6, and 8 eluted with 20% P + 80% E, diethyl ether, and methanol, respectively, were bioassayed (Wilcoxon signed-rank test, $P > 0.05$).

Significantly greater numbers of eggs were laid on half filters containing the dip extract and postdip extract than on control (pentane) half filters (Wilcoxon signed-rank test, $P < 0.05$). Gas chromatographic analysis of the dip extract of a single leaf indicated that the retention times of four of the peaks closely matched those of hexacosane, heptacosane, octacosane, and nonacosane in the *n*-alkane mixture (Figure 3). Based on peak areas, these four chemicals comprised 38.74% of the constituents in the dip extract for the peaks resolved by the gas chromatographic analysis, excluding the internal standard. Similar results were obtained in the gas chromatographic analysis of the other three leaves (Table 1). However, the actual ratio of the four chemicals in the dip extract is expected to be lower, since not all chemicals in the individual-leaf dip

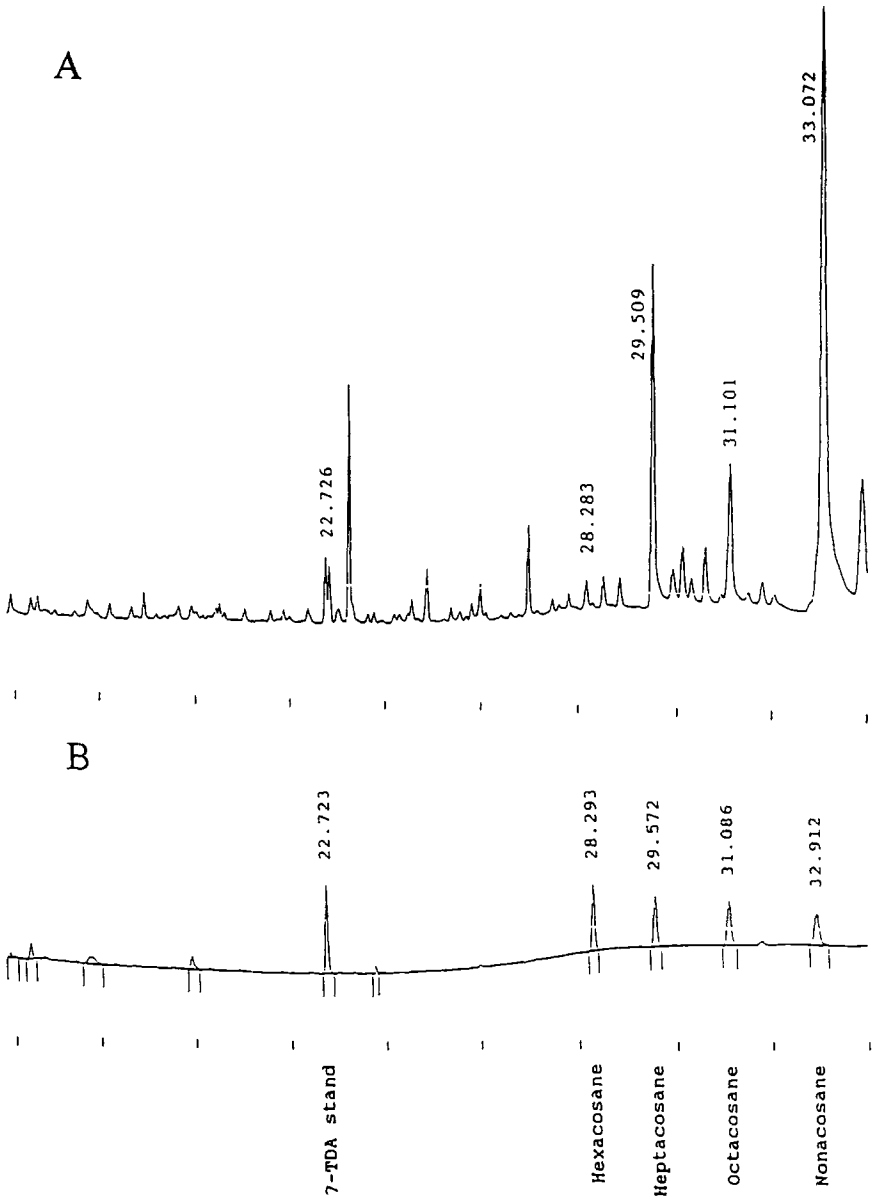


FIG. 3. Gas chromatograms. (A) Extract of an individual leaf from a corn plant at the late tassel stage dipped in pentane for 20 sec and concentrated before injection. (B) A mixture of four *n*-alkanes (100 ng each) from commercial sources. Internal standard used for both analyses was 7-tetradecenyl acetate (4.5 ng). See text for gas chromatography conditions.

TABLE 1. GAS CHROMATOGRAPHIC ANALYSIS OF DIP EXTRACTS OF INDIVIDUAL CORN LEAVES[a]

	Retention time (min) of pure *n*-alkanes[b]	Retention time (min, mean ±SD) of chemicals in individual-leaf dip extracts[c]	Chemicals in individual-leaf dip extracts (relative %, mean ± SD)[d]	Chemicals in individual-leaf dip extracts (ng, mean ± SD)/cm^2 of leaf blade)[e]
Hexacosane	28.293	28.242 ± 0.054	0.5 ± 0.2	3.16 ± 0.30
Heptacosane	29.572	24.495 ± 0.022	9.7 ± 2.2	71.27 ± 43.14
Octacosane	31.086	31.085 ± 0.025	5.4 ± 1.3	40.97 ± 25.78
Nonacosane	32.912	33.048 ± 0.030	21.8 ± 2.8	174.83 ± 83.57

[a]Four individual leaves from corn plants at tassel stage were dipped in pentane for 20 sec and concentrated before injection on the GC. Gas chromatographic conditions provided in text.

[b]Sample containing four pure *n*-alkanes (100 ng each) from commercial sources injected on a GC. Gas chromatographic conditions provided in text.

[c]Retention times of chemicals eluting at times similar to those of four *n*-alkanes in the chromatogram of the *n*-alkane mixture. Values represent the average of four individual-leaf dip extracts.

[d]The ratio of the peak area of the chemical to the total area of all resolved peaks excluding the internal standard. Values represent the average of four individual-leaf dip extracts.

[e]Estimated by comparison of the elution of each chemical with that of the analogous *n*-alkane in the gas chromatogram of the *n*-alkane mixture. Values represent the average of four individual-leaf dip extracts.

extract would have been resolved under the gas chromatographic conditions used in this study. In all four extracts, low quantities (< 1%) of the chemical eluting at ca. 28.242 min and over 20% of the chemical eluting at ca. 33.048 min were present. Amounts (nanogram) of the four chemicals in the extract were estimated by comparing their elution with those of the analogous *n*-alkanes in the gas chromatogram of the *n*-alkane mixture (Table 1).

When heptacosane was bioassayed, no significant differences in egg laying were observed at 250 μg (Wilcoxon signed-rank test, $P > 0.05$), but at the higher dose (500 μg), a half filter treated with it received significantly more eggs than the control. When nonacosane, tritriacontane, hexacosane, and octacosane were tested at 250 μg and 500 μg, significantly greater numbers of eggs were laid on treatment half filters compared to control half filters (Wilcoxon signed-rank test, $P < 0.05$) (Figure 4).

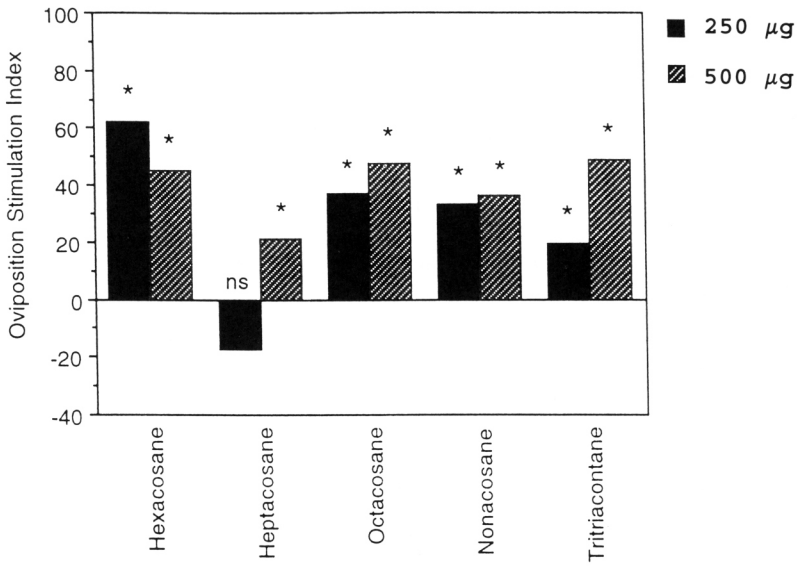


FIG. 4. Oviposition responses of *O. nubilalis* females to odd- and even-numbered chain length *n*-alkanes known to be present in the epicuticle of corn leaves and tested at two doses. Pentane was used for each control. See text for explanation of oviposition stimulation index. For each *n*-alkane, an asterisk indicates significant ($P < 0.05$) and ns indicates nonsignificant ($P > 0.05$) difference in egg laying between treatment and control half filters as determined by the Wilcoxon signed-rank test.

DISCUSSION

Research by Schurr and Holdaway (1970), Cantelo and Jacobson (1979), and Lupoli et al. (1990) indicated that corn odors attracted female *O. nubilalis* to host plants. However, no information was provided on specific chemicals that were involved. Derridj et al. (1986, 1992) further observed that females discriminated between corn cultivars based on differences in carbohydrate content or carbohydrate on plant surfaces. More recently, Binder et al. (1995) reported that farnesene, which is produced when corn plants are damaged by beet armyworms, was stimulatory. Cues from undamaged host plants are more likely to be critical for eliciting oviposition, but specific phytochemical signals inducing oviposition remain unknown. Additionally, for a polyphagous herbivore such as *O. nubilalis* that feeds on plants belonging to taxonomically diverse families, oviposition stimulation may be based on a complex of phytochemicals rather than an individual key compound. Hence, a systematic approach is needed to extract, isolate, and identify constituents in plants that act as oviposition stimulants for *O. nubilalis.*

Udayagiri and Mason (1995) demonstrated that foliar extracts of corn stimulated oviposition in *O. nubilalis* and determined that the stimulatory material was extractable with pentane. The present study provided evidence that nonpolar, low-volatility surface constituents of corn are involved in eliciting oviposition by *O. nubilalis.* When a pentane extract of corn leaves was set aside at room temperature and tested on different days, the extract was still active after 72 hr, indicating that at least some stimulatory chemicals have low volatility.

For further analysis of the nature of the low-volatility stimulatory chemicals, the extract was fractionated on Florisil in a similar system used previously for corn volatiles (Udayagiri and Jones, 1992). An ascending series of diethyl ether in pentane separated chemicals in the plant extract based on their polarity. In our study eight fractions were collected and five elicited oviposition, showing that more than one class of chemicals in corn is stimulatory.

Stimulatory chemicals may be present on the plant surface or in the interior. *Ostrinia nubilalis* ovipositing on the leaf surface is likely to depend on chemical stimuli on the surface to evaluate an appropriate host for ovisposition. To determine whether oviposition in *O. nubilalis* is elicited by leaf surface chemicals, an extract of epicuticular chemicals was prepared. Brief extractions (10–60 sec) of fresh foliage at room temperature are recommended for isolation of epicuticular chemicals so that only surface compounds are extracted (Eigenbrode and Espelie, 1995). In the present study, a 20-sec extract provided evidence that epicuticle chemicals are stimulatory, although the postdip extract of crushed leaves was also stimulatory. It is possible that all epicuticular chemicals were not eluted into the dip extract, and stimulation by the postdip extract may have

been due to residual epicuticular chemicals. Alternatively, internal chemicals may also elicit oviposition.

Gas chromatographic analysis suggested the presence of *n*-alkanes in the stimulatory dip extract. *n*-Alkanes are nonpolar chemicals that are universally present on leaf surfaces (Baker, 1982), and it appeared likely that they could be involved in stimulating oviposition in *O. nubilalis*. *n*-Alkanes that were available commercially were bioassayed. Typically *n*-alkanes in plant epicuticular waxes range from C_{21} to C_{39}, although *n*-alkanes with odd-numbered carbon chain lengths dominate. Bianchi and Salamini (1975) analyzed the epicuticular waxes in corn and reported the presence of *n*-alkanes ranging from C_{20} to C_{33}, with higher percentages of those with odd chain lengths. Blaker and Greyson (1988) observed similar results, but *n*-alkanes reported by them ranged from C_{23} to C_{37}. We selected five *n*-alkanes, ranging from C_{26} to C_{33}, including two with even- and three with odd-numbered carbon chain lengths that occur as major or minor components in the epicuticle of corn. In the leaves used for the gas chromatographic analysis, *n*-alkane quantities ranged from 5.8 ng (hexacosane) to 380 ng (nonacosane)/cm^2 of leaf surface, with high interleaf variation. Our estimates are low due to GC conditions that we used which are not optimal for this analysis. Blaker and Greyson (1988) reported that quantities of C_{26} to C_{33} *n*-alkanes ranged from 1 to 29 μg/cm^2 of leaf blade in 50-day-old corn plants. In the present study, individual females were presented with doses of 250 and 500 μg (= 7.85, and 15.7 μg/cm^2 of filter paper, respectively) of each *n*-alkane. All five *n*-alkanes tested were stimulatory at the higher dose and four were stimulatory at the lower dose, leading us to speculate that females are able to perceive and respond to *n*-alkanes in general, but perhaps they are unable to distinguish differences among C_{26}–C_{33} hydrocarbons. Concentrations of *n*-alkanes in the epicuticle of different parts of a plant can vary (Blaker and Greyson, 1988; Eigenbrode and Espelie, 1995), and within-plant variation in *n*-alkanes could be the basis for females preferring to lay eggs on certain leaves of the corn plant (Lupoli et al., 1990; Mason et al., 1996). While our study shows that *n*-alkanes stimulate oviposition in *O. nubilalis*, other unidentified chemicals present in the postdip extract and active Florisil fractions may also be stimulatory or provide synergistic effects.

There are few reports of lipid components stimulating oviposition behavior. The Hessian fly, *Mayetiola destructor* (Foster and Harris, 1992), the cabbage root fly, *Delia radicum* (Städler and Schöni, 1990), and four *Choristoneura* spp. (Grant and Langevin, 1994) have been reported to oviposit preferentially on host plant epicuticular lipid extracts compared to controls. In none of these studies were the specific lipid components stimulating oviposition identified. Städler and Buser (1984) identified three polar compounds that occur in Umbelliferae and in the surface wax of carrot leaves that stimulated oviposition in the carrot fly, *Psila rosae*. More recently, Renwick et al. (1992) reported that a

polar leaf surface chemical that is present in Cruciferae elicited positive oviposition responses in the cabbage butterfly, *Pieris rapae*.

The n-alkanes found to be stimulatory in this study are widely distributed in plants at various concentrations and combinations depending on the species, genotype, plant tissue, or developmental stage. The presence of these stimulatory lipids in specific ratios and quantities may be critical for oviposition site selection. Differences in epicuticular lipid composition have been correlated with resistance of cultivated plants to herbivores (Eigenbrode and Espelie, 1995) and, hence, by conventional methods or by genetic engineering, it may be possible to develop epicuticular lipid phenotypes of corn that limit *O. nubilalis* by reducing or eliminating oviposition.

Acknowledgments—We are grateful to John D. Pesek, Jr., for assistance with statistical analyses; Laura Iraki, Eva Santana, and Nate Ehresman for assistance in conducting the experiments; and Sulekha Rao for reviewing the manuscript. This research was part of the North Central Regional Research Project NC-205, Integrated Crop Management Effects on Stalk-Boring Lepidoptera. It was supported by funds to C.E.M. from the State of Delaware and from the E. I. DuPont Inc. De Nemours and Company under the auspices of the Delaware Research Partnership Program. Published as Paper No. 1590 in the Journal Series of the Delaware Agricultural Experiment Station, Contribution No. 689 of the Department of Entomology & Applied Ecology, University of Delaware, Newark, Delaware.

REFERENCES

BAKER, E. A. 1982. Chemistry and morphology of plant epicuticular waxes, pp. 139–165, *in* D. F. Cutler, K. L. Alvin, and C. E. Price (eds.). The Plant Cuticle. Academic Press, London.

BIANCHI, G., and SALAMINI, F. 1975. Glossy mutants of maize IV. Chemical composition of normal epicuticular waxes. *Maydica* 20:1–3.

BINDER, B. F., ROBBINS, J. C., and WILSON, R. L. 1995. Chemically mediated ovipositional behaviors of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *J. Chem. Ecol.* 21:1315–1327.

BLAKER, T. W., and GREYSON, R. I. 1988. Developmental variation of leaf surface wax of maize, *Zea mays*. *Can. J. Bot.* 66:839–846.

CANTELO, W. W., and JACOBSON, M. 1979. Corn silk volatiles attract many pest species of moths. *J. Environ. Sci. Health Part A* 14:695–707.

DERRIDJ, S., FIALA, V., and JOLIVET, E. 1986. Increase of European corn borer (*Ostrinia nubilalis*) oviposition induced by a treatment of maize plants with maleic hydrazide: Role of leaf carbohydrate content. *Entomol. Exp. Appl.* 41:305–310.

DERRIDJ, S., FIALA, V., BARRY, P., ROBERT, P., ROESSINGH, P., and STÄDLER, E. 1992. Role of nutrients found in the phylloplane, in the insect host-plant selection for oviposition, pp. 139–140, *in* S. B. J. Menken, J. H. Visser and P. Harrewijn (eds.). Proceedings of the 8th International Symposium of Insect-Plant Relationships. Kluwer Academic Publishers, Dordrecht.

EIGENBRODE, S. D., and ESPELIE, K. E. 1995. Effects of plant epicuticular lipids on insect herbivores. *Annu. Rev. Entomol.* 40:171–190.

FOSTER, S. P., and HARRIS, M. O. 1992. Foliar chemicals of wheat and related grasses influencing oviposition by Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae). *J. Chem. Ecol.* 18:1965–1980.

GRANT, G. G., and LANGEVIN, D. 1994. Oviposition responses of four *Choristoneura* (Lepidoptera: Tortricidae) species to chemical and physical stimuli associated with host and nonhost foliage. *Environ. Entomol.* 23:447–456.

GUTHRIE, W. D., ROBBINS, J. C., and JARVIS, J. L. 1985. *Ostrinia nubilalis*, pp. 407–413, *in* P. Singh and R. F. Moore (eds.). Handbook of Insect Rearing, Vol. 2. Elsevier, Amsterdam.

LUPOLI, R., MARION-POLL, F., PHAM-DELÈGUE, M. H., and MASSON, C. 1990. Effet d'émissions volatiles de feuilles de maïs sur les préférences de ponte chez *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *C.R. Acad. Sci. Ser. 3. Sci. Vie* 311:225–230.

MASON, C. E., RICE, M. E., CALVIN, D. D., VANDUYN, J. W., SHOWERS, W. B., HUTCHISON, W. D., WITKOWSKI, J. F., HIGGINS, R. A., ONSTAD, D. W., and DIVELY, G. R. 1996. European corn borer ecology and management. North Central Regional Publication No. 327. Iowa State University, Ames, Iowa.

OTT, L. 1984. An Introduction to Statistical Methods and Data Analysis, 2nd ed. Duxbury Press, Boston.

RENWICK, J. A. A. 1989. Chemical ecology of oviposition in phytophagous insects. *Experientia* 45:223–228.

RENWICK, J. A. A., RADKE, C. D., SACHDEV-GUPTA, K., and STÄDLER, E. 1992. Leaf surface chemicals stimulating oviposition by *Pieris rapae* (Lepidoptera: Pieridae) on cabbage. *Chemoecology* 3:33–38.

SCHURR, K., and HOLDAWAY, F. G. 1970. Olfactory responses of female *Ostrinia nubilalis* (Lepidoptera: Pyraustinae). *Entomol. Exp. Appl.* 13:455–161.

STÄDLER, E. 1992. Behavioral responses of insects to plant secondary compounds, pp. 45–88, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites, Vol. 2. Academic Press, New York.

STÄDLER, E., and BUSER, H.-R. 1984. Defense chemicals in leaf surface wax synergistically stimulate oviposition by a phytophagous insect. *Experientia* 40:1157–1159.

STADLER, E., and SCHÖNI, R. 1990. Oviposition behavior of the cabbage root fly, *Delia radicum* (L.) influenced by host plant extracts. *J. Insect Behav.* 3:195–209.

THIERY, D., and LE QUÉRÉ, J. L. 1991. Identification of an oviposition-deterring pheromone in the eggs of the European corn borer. *Naturwissenschaften* 78:132–133.

UDAYAGIRI, S., and JONES, R. L. 1992. Role of plant odor in parasitism of European corn borer by braconid specialist parasitoid *Macrocentrus grandii* Goidanich: Isolation and characterization of plant synomones eliciting parasitoid flight response. *J. Chem. Ecol.* 18:1841–1855.

UDAYAGIRI, S., and MASON, C. E. 1995. Host plant constituents as oviposition stimulants for a generalist herbivore: European corn borer. *Entomol. Exp. Appl.* 76:59–65.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

BIOLOGICAL ACTIVITY OF RESVERATROL, A STILBENIC COMPOUND FROM GRAPEVINES, AGAINST *Botrytis cinerea*, THE CAUSAL AGENT FOR GRAY MOLD

MARIELLE ADRIAN,[1] PHILIPPE JEANDET[1,*] JÉRÔME VENEAU,[1] LESLIE A. WESTON,[2] and ROGER BESSIS[1]

[1]*Laboratoire des Sciences de la Vigne*
Institut Jules Guyot, Université de Bourgogne
BP 138, 21004 Dijon Cedex, France

[2]*Department of Horticulture and Landscape Architecture*
N 318 Agricultural Science Building North, University of Kentucky
Lexington, Kentucky 40546

(Received November 26, 1996; accepted February 24, 1997)

Abstract—The biological activity of resveratrol, a stilbenic compound synthesized by grapevines in response to various stresses, was reevaluated against *Botrytis cinerea* using a novel in vitro system that enabled direct observation of the fungus with an inverted microscope. We determined that 90 μg resveratrol/ml reduced germination of *B. cinerea* conidia by ca. 50%. Moreover, resveratrol was shown to significantly reduce mycelial growth of *B. cinerea* at concentrations ranging from 60 to 140 μg/ml. Exposure to resveratrol at concentrations ranging from 60 to 140 μg/ml resulted in cytological changes in *B. cinerea*, such as production of secondary or tertiary germ tubes by conidia, cytoplasmic granulations, protoplasmic retractation in the hyphal tip cells, and formation of curved germ tubes. These data reinforce the role played by this compound in the *B. cinerea*–grapevine interaction.

Key Words—Resveratrol, phytoalexins, grapevines, *Botrytis cinerea*, pterostilbene, *Vitis* spp.

INTRODUCTION

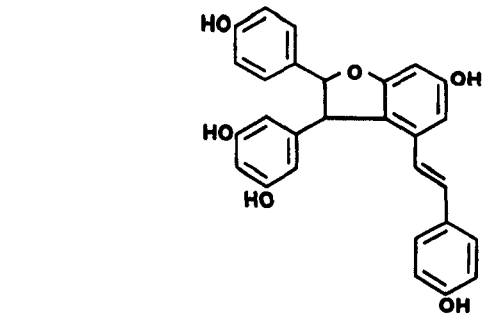
The resistance of grapevines (*Vitis vinifera* L.) to fungal penetration by *Botrytis cinerea* Pers., the causal organism for gray mold, appears to be due in part to

*To whom correspondence should be addressed.

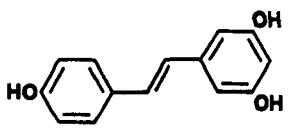
0098-0331/97/0700-1689$12.50/0 © 1997 Plenum Publishing Corporation

an induced resistance mechanism involving the rapid production of several stilbenic compounds, namely resveratrol (*trans*-3,5,4′-trihydroxystilbene) (Langcake and Pryce, 1976; Creasy and Coffee, 1988; Jeandet et al., 1991; Liswidowati et al., 1991; Adrian et al., 1996) and its glucoside (Waterhouse and Lamuela-Raventos, 1994), together with a number of biosynthetically related compounds derived from resveratrol (pterostilbene, *trans*-3,5-dimethoxy-4′-hydroxystilbene, and ϵ-viniferin, a resveratrol dehydrodimer) (Figure 1) (Langcake and Pryce, 1977; Pezet and Pont, 1988, 1990), the last two compounds being considered as phytoalexins. These compounds are typically produced in leaves and berries of grapevines, in response to stresses, which include fungal infection, UV irradiation, and induction by chemicals (Langcake, 1981; Creasy and Coffee, 1988; Liswidowati et al., 1991; Adrian et al., 1996).

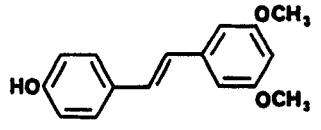
A fundamental question available for investigation concerns the biological activity of resveratrol against *B. cinerea*. Resveratrol is the major stilbenic component that has been identified in grapevine tissues upon infection by *B. cinerea* (Langcake, 1981). Hoos and Blaich (1990) have shown initially that resveratrol inhibited the radial growth of fungal mycelia. Two species of fungi were evaluated, *B. cinerea* and *Phomopsis viticola*. These studies were performed, however, using crystalline suspensions of high concentrations of res-



ε-viniferin



resveratrol



pterostilbene

FIG. 1. Chemical structure of *trans*-resveratrol, *trans*-pterostilbene, and ϵ-viniferin.

veratrol (ranging from 100 to 1000 μg/ml). Thus, solubility problems with resveratrol were encountered, and accurate determination of the actual concentration of resveratrol in solution in the media were impossible to obtain. In addition, studies were performed by adding resveratrol to agar substrates that formed a solid medium. The aim of our investigation was to reevaluate the fungitoxic activity of resveratrol, using a novel in vitro liquid culture system that provides reproducible results and enables rapid direct observations with an inverted microscope. The solubility of resveratrol was ensured by addition of a minute quantity of solvent in our assay system. The reliability of this model was first validated with pterostilbene, a dimethylated derivative of resveratrol, known to possess strong biological activity against *B. cinerea* (ED_{100} = 52 μg/ml) (Pezet and Pont, 1988).

METHODS AND MATERIALS

Culture of B. cinerea *and Preparation of Conidial Suspensions. Botrytis cinerea* Pers. strain SP1 used in this study was collected at the viticultural and enological experiment station at the University of Bourgogne in September 1993. Conidia were collected from infected mature grapes (cv. Pinot noir) and suspended in water before being inoculated on potato dextrose agar (Bio Mérieux, Lyon, France) and incubated at 21°C. Purification of the strain SP1 required several successive subcultures and the use of streptomycin (200 mg/liter). Once purified, the single spore strain was stored at 4°C in tubes containing inclined potato dextrose agar. Prior to experiments, the virulence of strain SP1 was examined and controlled by evaluation of laccase-like stilbene oxidase activity and infection of grapevine in vitro grown plants, i.e., plants grown in sterile conditions, as described in Sbaghi et al. (1996). Virulence of SP1 can be described as moderate to high, based on this evaluation.

For further experimentation, the strain was cultivated on potato dextrose agar in 86-mm Petri dishes and maintained at 21°C with a 12-hr light photoperiod. Conidia were aseptically harvested from 10-day-old cultures by suction (Pezet and Pont, 1990) and transferred into 250-ml Erlenmeyer flasks containing 100 ml of a liquid nutrient-rich medium described by Fournioux and Bessis (1993) and similar to that of Ward et al. (1975). The conidial suspension was vibrated for 2–5 min with a vortex mixer, and the number of conidia was determined with a Malassez counting chamber. After dilution, a final concentration of about 10^5 conidia/ml was obtained. The conidial suspension was then added to sterile 35-mm Petri dishes and immediately incubated with resveratrol or pterostilbene.

Incubation of Pterostilbene and Resveratrol with Conidial Suspension. Prior to incubation, the purity of both stilbenes (>99% purity) was checked by HPLC

analysis using a photodiode array detector (Adrian et al., 1996) and by GC as previously described (Jeandet et al., 1995). Resveratrol (Sigma, St Louis, Missouri) was added as ethanolic solutions to nongerminated conidial suspensions at several concentrations, i.e., 20–160 μg/ml. Pterostilbene (supplied by Drs. Pezet and Pont, Swiss Federal Agricultural Research Station of Changins, Switzerland) was incubated at only three concentrations, 20, 40, and 60 μg/ml, since the ED_{100} (effective dose or concentration required for 100% mortality) of pterostilbene upon dormant conidia of *B. cinerea* was previously reported to be 52 μg/ml (Pezet and Pont, 1988). Resveratrol and pterostilbene are difficult to maintain in aqueous solutions, and this contributes to the difficulty of conducting experiments to assay their biological activities. Thus, ethanol was used at a final concentration of 4%, in the liquid medium to ensure solubility of stilbenes (Sbaghi et al., 1996). Controls were prepared by adding pure ethanol (4% v/v) containing no stilbenes to the conidial suspension. Under these conditions, conidial germination and mycelial growth of *B. cinerea* were not reduced (data not shown). Liquid cultures were protected from light (to avoid photochemical isomerization of the *trans* form of stilbenes to the less active *cis* form) (Langcake and Pryce, 1976), agitated with a reciprocal shaker (120 rpm), and kept at 21°C for 64 hr. There were five replicates per treatment and experiments were carried out 10 times.

Cytological Observations. Conidia were observed by light microscopy directly in Petri dishes, using an inverted microscope (Leitz Fluovert) or on a glass slide with a Leitz Laborlux D microscope. The morphology of conidia and mycelial hyphae and the percentage germination of conidia (obtained by random sampling of 50 individuals) were examined for each treatment. Since standard errors were exceptionally low (< 1% of the means), these were not reported on graphs. The rate of mycelial growth was measured by an estimation of mycelial length over time (30 measurements per treatment), using an ocular micrometer, every 8 hr during 64 hr from culture initiation. The Scheffé F test (a pairwise comparison test adapted for multiple comparisons) was used to compare the mycelial growth (at the 0.05 level) for two different culture conditions at a given time. Trypan blue (Pointet-Girard, Clichy, France) staining was used (1% w/v) to test the viability of conidia since only dead conidia accumulate the stain (Rossall et al., 1980).

RESULTS

Effects of Pterostilbene. Pterostilbene had a significant effect on growth and development of *B. cinerea* conidia over time. The percentage germination of conidia was significantly lower for 20 and 40 μg pterostilbene/ml treatments (42% and 13%, respectively) than the control (96%), after 40 hr. Germination

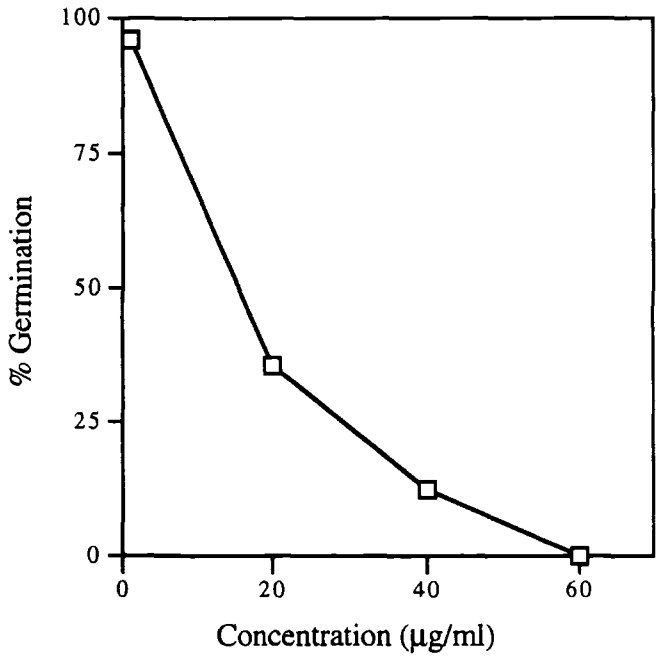


FIG. 2. Effect of increasing pterostilbene concentrations (20–60 µg/ml) on the percentage germination of *B. cinerea* conidia. Each point represents the mean of 30 measurements.

was 0% in the 60 µg/ml treatment (Figure 2). Moreover, staining with trypan blue allowed a determination of the conidial death rate, which was 50, 80, and 100% (ED_{100}) with 20, 40, and 60 µg pterostilbene/ml, respectively. These results are consistent with those obtained by Pezet and Pont (1988), who found 50% germination inhibition with 18 µg pterostilbene/ml and a conidial death rate of 100% with 52 µg pterostilbene/ml.

Pterostilbene also had inhibitory effects on the mycelial growth of *B. cinerea*. Figure 3 shows that the rate of germ-tube elongation dramatically decreased in samples treated with 20 and 40 µg pterostilbene/ml (reductions of 23 and 94%, respectively, within 64 hr) when compared to that of the control. After exposure to 40 µg pterostilbene/ml, there was essentially no growth for the first 40 hr of incubation (latency period) and then growth resumed.

These results provide strong evidence for the reliability of our bioassay, since our results compare favorably with those previously obtained for pterostilbene fungitoxicity (Langcake et al., 1979; Pezet and Pont, 1988). Therefore, the system could be further utilized to assess the potential fungitoxic activity of resveratrol against *B. cinerea*.

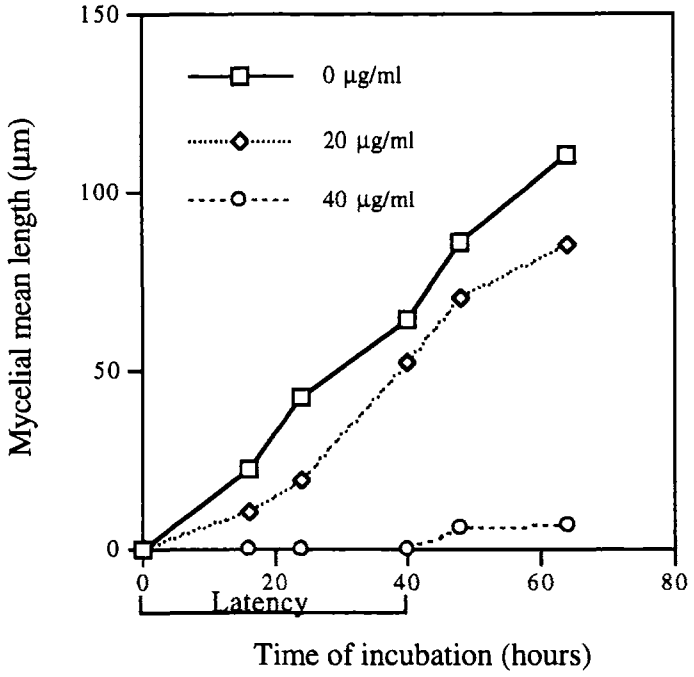


FIG. 3. Effect of pterostilbene at 20 and 40 µg/ml on the germination (latency) and mycelial growth rate of *B. cinerea*. Each data point represents the mean of 30 measurements. Standard errors were not presented on the graph since they do not exceed the size of the symbols.

Effects of Resveratrol. Unlike pterostilbene, resveratrol at the lowest concentrations, 20 or 40 µg/ml, did not affect the percentage germination of conidia or the mycelial growth of *B. cinerea* (Figure 4). However, resveratrol concentrations ranging from 60 to 160 µg/ml caused significant decreases in the percentage germination of conidia when compared to the control (Figure 4). Reduction in conidial germination was correlated with increasing resveratrol concentration; germination was completely inhibited at 160 µg resveratrol/ml (i.e., 7×10^{-4} M). Thus, from these experiments, the ED_{50} for resveratrol was approximately 90 µg resveratrol/ml (3.9×10^{-4} M), (see Figure 4). Beyond 60 µg resveratrol/ml, the germination of conidia was preceded by a latency period, the duration of which increased proportionately with increasing resveratrol concentrations. After 40 hr of incubation, we observed a 70% rate of conidium mortality for 140 µg/ml, nearly 40% for 100 µg/ml and 120 µg/ml,

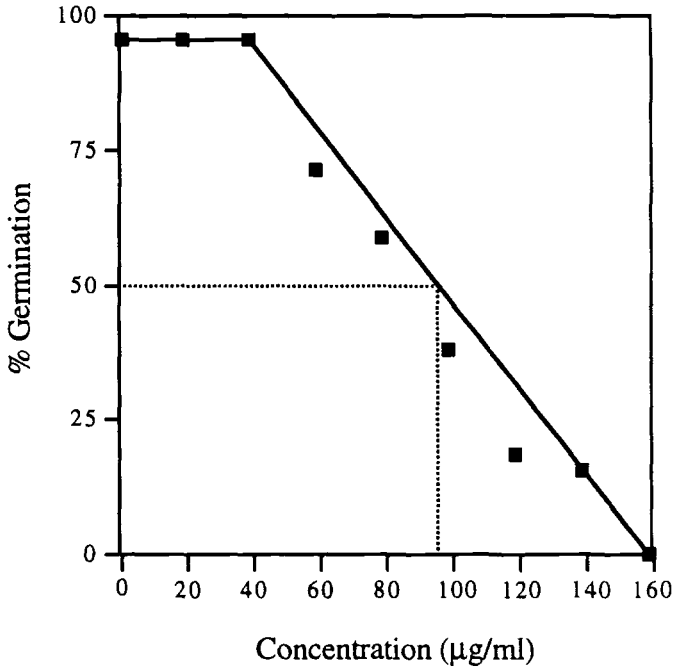


FIG. 4. Effect of increasing resveratrol concentrations (60–160 µg/ml) on the germination of *B. cinerea* conidia. Each point represents the mean of 30 measurements.

and approximately 10% for 60 µg/ml concentrations. After exposure to resveratrol at 160 µg/ml, we frequently observed a disruption of the conidium wall associated with leakage of cellular contents. At 160 µg/ml, the mortality was greater than 95% and conidial germination was completely inhibited. Finally, the growth rate of mycelial hyphae decreased with increasing resveratrol concentration (Figure 5; Tables 1 and 2). Mycelial growth of *B. cinerea* was strongly inhibited in the presence of 100 µg resveratrol/ml, as shown in Figure 6.

Treatment of conidia with resveratrol concentrations ranging from 60 to 140 µg/ml resulted in cytological abnormalities in *B. cinerea*, including the formation of curved germ tubes (Figure 7D), cessation of growth of some germ tubes [with protoplasmic retraction (Figure 7B) in the dead hyphal tip cell as shown by trypan blue staining (Figure 7E)], and regrowth of a secondary (Figure 7A) or tertiary (Figure 7C) germ tube from the surviving conidium. Such modifications were also observed after treatment of conidia with 20 or 40 µg pterostilbene/ml (data not shown), but were never observed in the controls.

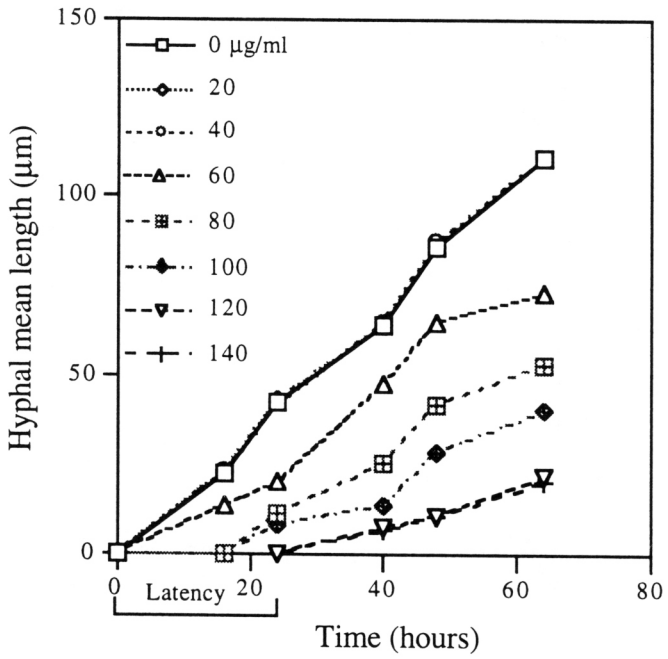


FIG. 5. Effect of increasing resveratrol concentrations (20–140 µg/ml) on the germination and mycelial growth rate of *B. cinerea*. Each point represents the mean of 30 measurements. Standard errors were not presented on the graph since they do not exceed the size of the symbols. Statistical analysis of the data obtained after 64 hr of incubation are presented in Tables 1 and 2. Lines for 0, 20, and 40 µg/ml are superimposed as one line, and the line for 120 µg/ml is superimposed on the line for 140 µg/ml.

TABLE 1. ANOVA, ONE FACTOR WITH REPEATED EXPERIMENTS[a]

Group[a]	Frequency	Mean ± SE
64-0	30	110.27 ± 0.05
64-20	30	110.27 ± 0.05
64-40	30	110.27 ± 0.05
64-60	30	72.7 ± 0.1
64-80	30	53.24 ± 0.09
64-100	30	40.09 ± 0.31
64-120	30	21.18 ± 0.18
64-140	30	19.58 ± 0.15

[a]Time (hr)–resveratrol concentration (µg/ml)

TABLE 2. COMPARISON OF DIFFERENT CONCENTRATIONS

Comparison[a]	Mean difference	Scheffé F test[b]
64-0 vs 64-60	37.57	5040.29
64-0 vs 64-80	57.03	11613.95
64-0 vs 64-100	70.18	17587.32
64-0 vs 64-120	89.09	28342.03
64-0 vs 64-140	90.69	29369.18
64-60 vs 64-80	19.46	1352.26
64-60 vs 64-100	32.61	3797.3
64-60 vs 64-120	51.52	9478.18
64-60 vs 64-140	53.12	10076.02
64-80 vs 64-100	13.15	617.48
64-80 vs 64-120	32.06	3670.29
64-80 vs 64-140	33.66	4045.77
64-100 vs 64-120	18.91	1276.9
64-100 vs 64-140	20.51	1502.12
64-120 vs 64-140	1.6	9.14

[a]Time (hours)-Resveratrol concentration (μg/ml) vs Time (hours)-resveratrol concentration (μg/ml).
[b]All values are significant at 95%.

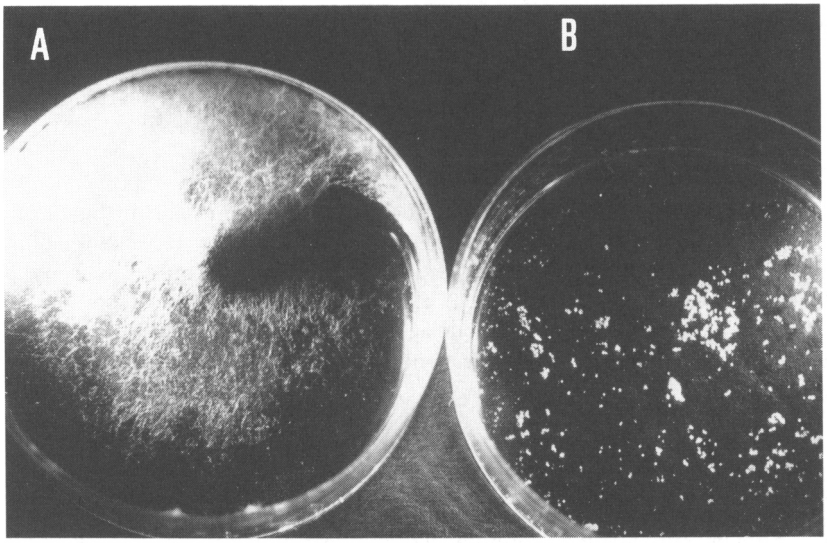


FIG. 6. Inhibition of *B. cinerea* mycelial growth: (A) control; (B) conidia treated with 100 μg resveratrol/ml.

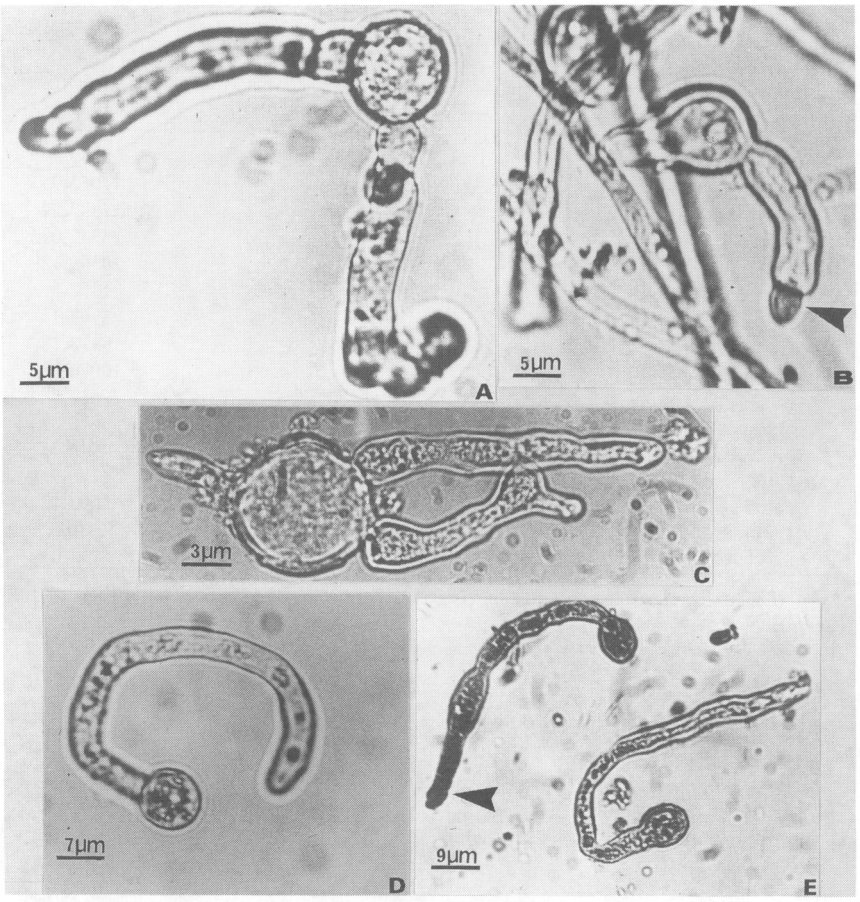


FIG. 7. Observations of resveratrol fungitoxicity on *B. cinerea* conidia by light microscopy. Conidia were grown on a liquid medium incorporating 60 µg resveratrol/ml in 4% ethanol: (A) conidia with two germ tubes; (B) cytoplasmic retraction in the upper part of the germ tube (death of the apical cell); (C) conidia with three germ tubes; (D) conidia with a curved germ tube; and (E) sporelings stained with trypan blue (1% w/v); dead cells accumulate the stain. Note the death of the apical part of the germ tube.

DISCUSSION

Stilbenes are generally biologically active compounds that have antifungal activities against a number of various pathogens: *Plasmopara viticola* (Dercks and Creasy, 1989), *Fusarium solani* (Takasugi et al., 1978). *Cladosporium*

cucumerinum and *Pyricularia oryzae* (Langcake and Pryce, 1976). The aim of the present work was to determine the potential antifungal activity of the stilbene compound, resveratrol, on *B. cinerea* conidia, by using a novel and reproducible conidial assay. The bioassay developed shows that resveratrol has real inhibitory effects on conidial germination and mycelial growth of *B. cinerea* in liquid cultures when used at concentrations ranging from 60 μg/ml (25% inhibition) to 160 μg/ml (100% inhibition), (from 2.6 to 7 × 10^{-4} M). These values correspond to the activity of other phytoalexins, which are generally active at concentrations of 10^{-4}–10^{-5} M (Smith, 1982). Hoos and Blaich (1990) also described inhibitory effects of resveratrol on *B. cinerea* conidia, but they utilized a solid medium and resveratrol in a crystalline suspension, making it difficult to determine the concentrations actually evaluated. The liquid medium in our system was very favorable for the rapid growth of the fungus.

In our assay system, resveratrol (ED_{100} = 160 μg/ml) was threefold less active than pterostilbene, which completely inhibited conidial germination at 60 μg/ml (2.3 × 10^{-4} M). The differences in the fungitoxic activities of two related stilbene compounds indicates that the in vivo methylation of phenol groups can potentially lead to increased biological activity (Pont and Pezet, 1990; Schultz et al., 1990). Despite its high activity, pterostilbene is quantitatively a minor component (usually ranging from 1 to 10 μg/g fresh weight) of the phytoalexin response of grapevines (Langcake et al., 1979), whereas resveratrol is quantitatively a major component (usually ranging from 50 to 400 μg/g fresh weight) (Jeandet et al., 1991; Adrian et al., 1996). Our findings, along with the fact that resveratrol is consistently present in high concentrations during the phytoalexin response, suggest the importance of this compound in regulation of the *B. cinerea*—grapevine interaction.

In addition, several important cytological modifications were observed in conidia and sporelings of *B. cinerea* treated with resveratrol at concentrations ranging from 60 to 140 μg resveratrol/ml, namely, production of secondary or tertiary germ tubes, formation of curved germ tubes, cytoplasmic granulation of conidia, disruption of the plasma membrane, and protoplasmic retraction in hyphal tip cells. Some of these features have previously been described for other phytoalexin/pathogen interactions. For example, zoospores of *Phytophthora infestans*, *Phytophthora porriet*, and *Phytophthora cactorum* can develop cytoplasmic granulations, plasma membrane disruptions, and the leakage of the cellular contents (Harris and Dennis, 1976, 1977) in the presence of four terpenoid-type phytoalexins (rishitin, phytuberin, anhydro-β-rotunol, and solavetivone). These effects have also been observed in fungal cells treated with isoflavonoid-type phytoalexins (Grisebach and Ebel, 1978; Skipp and Bailey, 1976; Smith, 1976; VanEtten and Bateman, 1971; Ward et al., 1975). In particular, phaseollin also caused the death of the apical tip of germ tubes (VanEtten and Bateman, 1971).

Protoplasmic retraction in the hyphal tip cell of *B. cinerea* observed after exposure to resveratrol or pterostilbene can be explained by the fact that apical cells of hyphae are more susceptible to phytoalexins because of their weak wall, which facilitates the entry of these compounds into the cell. In comparison, the entry of phytoalexins into subapical and interstitial hyphal cells (which have mature walls) is generally more restricted (Bartnicki-Garcia and Lippman, 1972; Grisebach and Ebel, 1978; Higgins, 1978; Rossall et al., 1980; Skipp and Bailey, 1976).

The presence of phytoalexins does not always result in the death of fungal invaders. For example, zoospores of *Pythium middletonii* lose their motility but remain viable in the presence of phytuberin (Harris and Dennis, 1977). Our data show that, indeed, the percentage of nongerminated conidia observed in the presence of resveratrol or pterostilbene was generally greater than that of dead conidia. Observations of regrowth from surviving conidia (production of a secondary or tertiary germ tube) also suggest that cells can escape from the action of phytoalexins, with regermination constituting a means of survival for conidia (Higgins, 1978; Rossall et al., 1980). Finally, the formation of curved germ tubes after phytoalexin treatment is likely also a reaction to stress. This asymmetric growth of the germ tube may correspond to the ability of stilbenic compounds, namely resveratrol, to interact with tubulin, with resultant disruption of microtubule assembly at this level, as suggested by Woods et al. (1995). This mode of action is typical for many other fungicides, such as benomyl, a fungicide used for the control of gray mold in the vineyard (Hoang-Van et al., 1989).

In these experiments, we have developed and described an in vitro system enabling direct observation of *B. cinerea* growth in liquid culture that can be utilized successfully to study the fungitoxicity of various phytoalexins. Our data provide strong evidence that resveratrol, at concentrations required for phytoalexin activity, inhibits the germination of conidia and mycelial growth of *B. cinerea*. It also induces several interesting cytological modifications in fungal cells, similar to those described previously by others for various phytoalexin—fungus interactions. Since resveratrol is typically present in high concentrations shortly after *B. cinerea* infection, our findings suggest that resveratrol plays an important role in the regulation of the *B. cinerea*— grapevine interaction.

REFERENCES

ADRIAN, M., JEANDET, P., BESSIS, R., and JOUBERT, J. M. 1996. Induction of resveratrol synthesis in grapevine leaves treated with aluminum chloride. *J. Agric. Food Chem.* 44:1979-1981.

BARTNICK-GARCIA, S., and LIPPMAN, E. 1972. The bursting tendency of hyphal tips of fungi: Presumptive evidence for a delicate balance between wall synthesis and wall lysis in apical growth. *J. Gen. Microbiol.* 73:487-500.

CREASY, L. L., and COFFEE, M. 1988. Phytoalexin production potential of grape berries. *J. Am. Soc. Hortic. Sci.* 113:230–234.

DERCKS, W., and CREASY, L. L. 1989. The significance of stilbene phytoalexins in the *Plasmopara viticola*—grapevine interaction. *Physiol. Mol. Plant Pathol.* 34:189–202.

FOURNIOUX, J. C., and BESSIS, R. 1993. Use of carbon dioxide enrichment to obtain adult morphology of grapevine in vitro. *Plant Cell Tissue Organ Culture* 33:51–57.

GRISEBACH, H., and EBEL, J. 1978. Phytoalexins, chemical defense substances of higher plants. *Angew. Chem. Int. Ed. Engl.* 17:635–647.

HARRIS, J. E., and DENNIS, C. 1976. Antifungal activity of post-infectional metabolites from potato tubers. *Physiol. Plant Pathol.* 9:155–165.

HARRIS, J. E., and DENNIS, C. 1977. The effect of post-infectional potato tuber metabolites and surfactants on zoospores of Oomycetes. *Physiol. Plant Pathol.* 11:163–169.

HIGGINS, V. J. 1978. The effect of some pterocarpanoid phytoalexins on germ tube elongation of *Stemphylium botryosum*. *Phytopathology* 68:338–345.

HOANG-VAN, K., ROSSIER, C., BAIJA, F., and TURIAN, G. 1989. Characterization of tubulin isotypes and of β-tubulin mRNA of *Neurospara crassa* and effects of benomyl on their developmental time courses. *Eur. J. Cell Biol.* 49:42–47.

HOOS, G., and BLAICH, R. 1990. Influence of resveratrol on germination of conidia and mycelium growth of *Botrytis cinerea* and *Phomopsis viticola*. *J. Phytopathol.* 129:102–110.

JEANDET, P., BESSIS, R., and GAUTHERON, B. 1991. The production of resveratrol (3,5,4′-trihydroxystilbene) by grape berries in different developmental stages. *Am. J. Enol. Vitic.* 42:41–46.

JEANDET, P., BESSIS, R., MAUME, B. F., MEUNIER, P., PEYRON, D., and TROLLAT, P. 1995. Effect of enological practices on the resveratrol isomer content of wine. *J. Agric. Food Chem.* 43:316–319.

LANGCAKE, P. 1981. Disease resistance of *Vitis* spp and the production of the stress metabolites resveratrol, ϵ-viniferin, α-viniferin and pterostilbene. *Physiol. Plant Pathol.* 18:213–226.

LANGCAKE, P., and PRYCE, R. J. 1976. The production of resveratrol by *Vitis vinifera* and other membranes of the *Vitaceae* as a response to infection or injury. *Physiol. Plant Pathol.* 9:77–86.

LANGCAKE, P., and PRYCE, R. J. 1977. A new class of phytoalexins from grapevines. *Experientia* 9:77–86.

LANGCAKE, P., CORNFORD, C. A., and PRYCE, R. J. 1979. Identification of pterostilbene as a phytoalexin from *Vitis vinifera* leaves. *Phytochemistry* 18:1025–1027.

LISWIDOWATI, F., MELCHIOR, F., HOHMANN, F., SCHWER, B., and KINDL, H. 1991. Induction of stilbene synthase by *Botrytis cinerea* in cultured grapevine cells. *Planta* 183:307–314.

PEZET, R., and PONT, V. 1988. Mise en évidence de ptérostilbène dans les grappes de *Vitis vinifera*. *Plant Physiol. Biochem.* 26:603–607.

PEZET, R., and PONT, V. 1990. Ultrastructural observations of pterostilbene fungitoxicity in dormant conidia of *Botrytis cinerea* Pers. *J. Phytopathol.* 129:19–30.

PONT, V., and PEZET, R. 1990. Relation between the chemical structure and the biological activity of hydroxystilbenes against *Botrytis cinerea*. *J. Phytopathol.* 130:1–8.

ROSSALL, S., MANSFIELD, J. W., and HUSTON, R. A. 1989. Death of *Botrytis cinerea* and *B. fabae* following exposure to wyerone derivatives in vitro and during infection development in broad bean leaves. *Physiol. Plant Pathol.* 16:135–146.

SBAGHI, M., JEANDET, P., BESSIS, R., and LEROUX, P. 1996. Degradation of stilbene type phytoalexins in relation to the pathogenicity of *Botrytis cinerea* to grapevines. *Plant Pathol.* 45:139–144.

SCHULTZ, T. P., HUBBARD, T. F., JIN, L., FISHER, T. H., and NICHOLAS, D. D. 1990. Role of stilbenes in the natural durability of wood: Fungicidal structure-activity relationships. *Phytochemistry* 29:1501–1507.

SKIPP, R. A., and BAILEY, J. A. 1976. The effect of phaseollin on the growth of *Colletotrichum*

lindemuthianum in bioassays designed to measure fungitoxicity. *Physiol. Plant Pathol.* 9:253–263.

SMITH, D. A. 1976. Some effects of the phytoalexin, kievitone on the vegetative growth of *Aphanomyces euteiches*, *Rhizoctonia solani* and *Fusarium solani* f., sp. *phaseoli*. *Physiol. Plant Pathol.* 9:45–55.

SMITH, D. A. 1982. Toxicity of phytoalexins, pp. 218–252, *in* J. A. Bailey and J. W. Mansfield (eds.). Phytoalexins. Blackie, Glasgow, pp. 218–252.

TAKASUGI, M., MUNOZ, L., MASAMUNE, T., SHIRATA, A., and TAKAHASHI, K. 1978. Stilbene phytoalexins from diseased mulberry. *Chem. Lett.* 1241–1242.

VANETTEN, H. D., and BATEMAN, D. F. 1971. Studies on the mode of action of the phytoalexin phaseollin. *Phytopathology* 61:1363–1372.

WARD, E. W. B., UNWIN, C. H., and STOESSL, A. 1975. Postinfectional inhibitors from plants. XV. Antifungal activity of the phytoalexin orchinol and related phenanthrenes and stilbenes. *Can. J. Bot.* 53:964–971.

WATERHOUSE, A. L., and LAMUELA-RAVENTOS, R. M. 1994. The occurrence of piceid, a stilbene glucoside in grape berries. *Phytochemistry* 37:571–573.

WOODS, J. A., HADFIELD, J. A., PETTIT, G. R., FOX, B. W., and MCGOWN, A. T. 1995. The interaction with tubulin of a series of stilbenes based on combretastatin A-4. *Br. J. Cancer* 71:705–711.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

PYRROLIZIDINE ALKALOIDS MEDIATE HOST-PLANT RECOGNITION BY OVIPOSITING FEMALES OF AN OLD WORLD DANAID BUTTERFLY, *Idea leuconoe*

KEIICHI HONDA,[1,*] NANAO HAYASHI,[1] FUMIKO ABE,[2] and TATSUO YAMAUCHI[2]

[1]*Study of Environmental Sciences*
Faculty of Integrated Arts and Sciences
Hiroshima University, Higashihiroshima 739, Japan

[2]*Faculty of Pharmaceutical Sciences*
Fukuoka University, Jonan-ku, Fukuoka 814-80, Japan

(Received March 28, 1996; accepted February 28, 1997)

Abstract—A giant danaid butterfly, *Idea leuconoe*, specializes on apocynaceous plants such as *Parsonsia laevigata*, which has been reported to contain pyrrolizidine alkaloids. Females of *I. leuconoe* deposited eggs in response to methanolic extract of *P. laevigata*, and subsequent bioassay-guided fractionation of the extract revealed that phytochemicals crucial for host recognition by ovipositing females are *Parsonsia*-specific macrocyclic pyrrolizidine alkaloids including parsonsianine, parsonsianidine, and 17-methylparsonsianidine. Parsonine, another *P. laevigata* pyrrolizidine component with a keto-dihydropyrrolizine moiety that is closely related in structure to male pheromones of the butterfly, and several nonhost pyrrolizidine alkaloids were entirely inactive. We interpret these data as strong evidence for an ancestral association through herbivory between danaid butterflies and pyrrolizidine alkaloids.

Key Words—Oviposition stimulants, *Idea leuconoe*, Lepidoptera, Danaidae, *Parsonsia laevigata*, Apocynaceae, pyrrolizidine alkaloids, parsonsianine, parsonsianidine, 17-methylparsonsianidine.

INTRODUCTION

Close associations of some lepidopterous insects with pyrrolizidine alkaloids (PAs) have long been known and have been studied extensively in regard to pharmacophagy (Boppré, 1984a, 1986, 1990). Adults of many danaid and ithomiid butterflies utilize plant-derived PAs as defensive substances or as precur-

*To whom correspondence should be addressed.

0098-0331/97/0700-1703$12.50/0 © 1997 Plenum Publishing Corporation

sors of male pheromones (Schneider et al., 1975; Eisner and Meinwald, 1987; Kelley et al., 1987; Stelljes and Seiber, 1990). Danaid butterflies use various plants including Asclepiadaceae, Apocynaceae, and Moraceae as their main host plants (Ackery and Vane-Wright, 1984). However, since host plants of the majority of danaid butterflies do not contain PAs, the adult butterflies utilizing those plants ingest PAs from nectar, withered foliage, or roots of PA-containing plants, including plants in the families Boraginaceae, Compositae, and Leguminosae (Edgar, 1975; Pliske, 1975; Boppré, 1981, 1983).

Two controversial hypotheses have been put forward on the origin of Danaidae–PA interactions. One is that the PA requirement of danaid or ithomiid butterflies for sex pheromone and chemical defense stems from an ancestral association involving host plants that contained PAs as defensive chemicals. Intense herbivory by these adapted insects may have led to an evolution of cardenolides or steroidal alkaloids in some of the ancestral plants, with some danaids or ithomiids coevolving to colonize Asclepiadaceae (plants with cardenolides) or Solanaceae (plants with steroidal alkaloids, but no PAs). According to this scenario, the intake of PAs by danaids as adults from nonhost plants is a remnant of their ancestral association with host plants containing PAs (Edgar et al., 1974; Edgar, 1982, 1984).

An alternative hypothesis has been propounded by Boppré (1978, 1984b), who argued that an association may have arisen from accidental uptake of PAs by danaids from PA-containing nectar. Those danaids that could tolerate the toxicity of PAs gained a selective advantage by storing them and subsequently developed a preference for them and a pheromonal system based on them. In time, some species shifted their larval food from PA-lacking plants to PA-containing ones, which also would ensure protection for the larvae.

Sufficient knowledge to choose between these two hypotheses is not available. An investigation of host recognition chemicals in a danaid species that relies on PA-containing plants (e.g., Apocynaceae) for larval growth and for PA acquisition may provide some new insight into the evolution of PA dependence among some Danaidae.

Chemical and biological investigations of phytochemicals involved in egg-laying by butterflies of several families have revealed an array of oviposition stimulants and deterrents (Renwick and Chew, 1994; Honda, 1995a,b; Nishida, 1995a). Relatively little, however, is known about chemical constituents reponsible for host selection by danaid females. To date, two publications have dealt with oviposition stimulants for danaid butterflies feeding on asclepiad plants that do not contain PAs. *Ideopsis similis*, a specialist on *Tylophora tanakae*, was induced to oviposit by phenanthroindolizidine alkaloids (Honda et al., 1995a,b), while certain quercetin glycosides (flavonoid) stimulated oviposition by the monarch butterfly, *Danaus plexippus*, which feeds on plants in *Asclepias* and related genera (Haribal and Renwick, 1996).

Idea leuconoe is one of the Old World butterflies inhabiting Southeast Asia and the Ryukyu Islands of Japan. It specializes on an apocynaceous plant, *Parsonsia laevigata*, in Japan. The present paper reports oviposition stimulants for this butterfly and discusses the origin and the evolutionary process of the PA-danaid association.

METHODS AND MATERIALS

Insects. Females wild-caught in the Yaeyama Islands and their offspring (30- to 50-day-old gravid females) were used for behavioral bioassays. Larvae were raised on *P. laevigata* in a greenhouse at 22–29°C under a natural photoperiod from July to October. After emergence, both sexes were kept together for a month under quasinatural conditions in a greenhouse equipped with flowers as a nectar source. Individual females were examined to determine whether the female laid fertilized eggs. Females fed with 15% aqueous sucrose daily during the experiments were pretested to determine if they responded positively to the foliage of *P. laevigata* and negatively to an artificial leaf (see below) sprayed with water (control). Those that showed a positive response to the control leaf were discarded.

Bioassay for Ovipositional Response. Mature females of *I. leuconoe* (usually more than 30 days after eclosion) search for and select oviposition sites by landing on potential plants and drumming the leaf surface with clubbed forelegs, which contain sensory hairs. Apparently, plant chemicals are perceived by chemoreceptors. If the plant is acceptable, a female immediately curls the abdomen and lays an egg on the underside of the leaf. A similar sequence of oviposition behavior also was observed under experimental conditions.

A heart-shaped green plastic plate (29 cm^2) coated with a test sample was presented to a gravid female as a foliage surrogate. Females were permitted free flight in a transparent plastic chamber (30 cm × 40 cm × 26 cm high) that was externally illuminated with an incandescent lamp (3500 lux). The oviposition-stimulatory activity of test materials at a dose of 100 $\mu g/cm^2$ for crude fractions or 10 $\mu g/cm^2$ for pure chemicals was estimated by a method similar to that given in a previous paper (Honda and Hayashi, 1995). The response was expressed as shown by equations 1 and 2,

$$\text{Percent response} = \frac{\sum R_i}{N} \times 100 \tag{1}$$

$$R_i = \frac{\sum S_j}{n} \tag{2}$$

where N is the total number of females tested, and R_i denotes the response rate of an individual. R_i was calculated by equation 2, where S_j is the score for a single trial of a given female, and n is the number of trials. Trials were replicated four to seven times. S_j was scored as 1.0 for actual egg-laying or an equivalent behavior, 0.5 for half-curling the abdomen after drumming, and 0.0 for drumming only with no positive response. Unresponsiveness to the control leaf was confirmed every three trials of sample presentation. For all trials, merely alighting on ovipositional substrates without drumming was not included.

Extraction, Fractionation, and Identification of Plant Chemicals. Fresh leaves of *P. laevigata* collected in Iriomote Island were extracted with methanol (about 5 ml/g) at room temperature for a week. The methanolic extract was evaporated to dryness in vacuo below 50°C and partitioned between chloroform and water to yield a chloroform fraction (fraction A) and a water-soluble fraction. The water-soluble fraction was further separated into five subfractions (fractions B–F) by column chromatography on a porous polymer gel (MCI Gel CHP20P, 75–150 μm, Mitsubishi Kasei). Fractions were eluted stepwise with water (fraction B), 25% aqueous methanol (fraction C), 50% aqueous methanol (fraction D), 75% aqueous methanol (fraction E), and methanol (fraction F). Identification of constituents was based on direct comparisons of TLC profiles on silicic acid, with chloroform–methanol–water (7 : 3 : 1) as the developer. NMR spectral data of compounds isolated by means of preparative TLC (Merck PLC plate Silica gel 60) were compared with those of authentic compounds that had been isolated and identified previously from *P. laevigata* by Abe and Yamauchi (1989) and Abe et al. (1990, 1991a,b).

Test Chemicals. Pure chemical compounds of host-plant origin used for bioassays were those isolated previously or in this study by reported methods. Monocrotaline, retrorsine, and isatidine (retrorsine *N*-oxide) were purchased commercially (Aldrich).

RESULTS

When an artificial leaf painted with the methanol extract of *P. laevigata* was presented to a flying female, she alighted on the leaf and readily responded positively to the extract, with deposition of an egg after a sequence of drumming. This explicitly implies that a bioactive principle(s) that cues the female to recognize its host is present in the methanol extract of the host plant. The six fractions (A–F) obtained from the methanol extract were bioassayed for their oviposition-stimulatory activity (Table 1). The active substances were localized in fractions A and D, with weak activity in fraction C, which seemed due to contamination.

Chemical analyses of constituents in fractions A and D revealed that three PAs, parsonsianine (**1**), parsonsianidine (**2**), and 17-methylparsonsianidine (**3**),

TABLE 1. OVIPOSITIONAL RESPONSES OF *Idea leuconoe* TO FRACTIONS DERIVED FROM HOST PLANT, *Parsonsia laevigata*

Fraction[a]	Response (%, mean ± SE)	Females (*N*)
A	78.1 ± 6.8	9
B	0	12
C	19.9 ± 7.2	11
D	83.8 ± 6.8	10
E	6.7 ± 4.4	10
F	2.5 ± 2.5	10

[a]The amount of each sample applied on the ovipositional substrate was 100 $\mu g/cm^2$.

were present in fraction A. Fraction D contained in addition to **1** and **2**, two lignan glycosides, lariciresinol-4,4′-bis-*O*-α-L-rhamnoside and secoisolariciresinol-4,4′-bis-*O*-α-L-rhamnoside, and a flavonol glycoside, kaempferol 3-*O*-glucosyl-(1→2)-glucoside, as the major components. The ability of each compound to elicit oviposition was assayed along with three other PAs, monocrotaline, retrorsine, and isatidine, that are common in PA-containing plants but not found in *P. laevigata*. Another PA, parsonine (**4**), that had been isolated from stems of *P. laevigata* (Abe and Yamauchi, 1987) was also tested.

Only three PAs (**1–3**) evoked potent positive responses, and the other compounds were appraised as inactive (Table 2). The active compounds (Figure 1) are 14-membered macrocyclic PAs and are specific, as far as is known, to the genus *Parsonsia* (Edgar et al., 1980; Abe et al., 1990, 1991a,b).

DISCUSSION

Components **1–3** are predominant PAs in the host plant and individually exerted significant stimulatory activities; ovipositing females are highly likely to rely on these PAs as principal cues in recognizing their host plant.

Implication for Host Shift. It is possible that the present-day specialization of *I. leuconoe* on *P. laevigata* is an outcome of host shift. At present, many danaids feed on asclepiad plants that do not contain PAs, which undoubtedly indicates that their host selection is mediated by plant chemicals other than PAs. A recent report has documented that oviposition by an *Asclepias*-feeder, *Danaus plexippus*, is elicited by flavonoids (Haribal and Renwick, 1996). Generally speaking, host shift from one plant species to another seems unlikely to occur if there are no similarities in composition or physiological action of secondary metabolites present in the two. *P. laevigata* may contain some chemicals other

TABLE 2. OVIPOSITIONAL RESPONSES OF *Idea leuconoe* TO HOST-PLANT CONSTITUENTS AND NONHOST PYRROLIZIDINE ALKALOIDS

Compound[a]	Response (%, mean ± SE)	Females (*N*)
1	97.1 ± 1.7	17
2	98.2 ± 1.0	17
3	98.0 ± 1.3	17
4	0	14
5	0.6 ± 0.6	14
6	1.5 ± 1.5	13
7	0	10
8	2.4 ± 1.6	13
9	0.9 ± 0.9	13
10	2.9 ± 1.7	13

[a] **1:** parsonsianine, **2:** parsonsianidine, **3:** 17-methylparsonsianidine, **4:** parsonine, **5:** lariciresinol-4,4′-bis-*O*-α-L-rhamnoside, **6:** secoisolariciresinol-4,4′-bis-*O*-α-L-rhamnoside, **7:** kaempferol 3-*O*-glucosyl-(1→2)-glucoside, **8:** monocrotaline, **9:** retrorsine, **10:** isatidine. The amount of each compound applied on the ovipositional substrate was 10 $\mu g/cm^2$. Compounds **1–7** are present in the host plant (*P. laevigata*) of the butterfly. Compound **7** was dissolved in methanol, the others in acetone. Compounds **1–3**, when dissolved in a protic solvent such as methanol, tended to rapidly lose their biological activities during storage, although there was no sign of appreciable reduction of their titer. Therefore, it seems likely that some uncharacterized degradation products probably formed via solvolysis or decomposition, perhaps only in trace amounts, exerted a notable deterrent effect.

than PAs by which Asclepiadaceae-feeding danaids are stimulated to oviposit, and such plant constituents would be likewise stimulative to *I. leuconoe*. However, females of *I. leuconoe* responded to none of the other coexisting compounds that included a flavonoid (**7**). Flavonoids, among other phytochemicals, are extremely widespread in the plant kingdom. Our finding, therefore, appears somewhat unexpected, assuming that the females remain responsive to ancestral stimulants. It has been demonstrated that certain cyclitols and their derivatives constitute a common subset of oviposition stimulants, although distinct compounds are involved for several papilionid butterflies feeding on Rutaceae, Apiaceae, or Aristolochiaceae, the last of which is a suspected ancestral host-plant family of the Papilionidae (Papaj et al., 1992; Honda, 1995a; Nishida, 1995a).

Although PAs are distributed in many plant families, host plants with PAs presently used by danaid and ithomiid butterflies are confined to apocynaceous plants in the tribe Parsonsieae (Edgar, 1984). Females of *I. leuconoe* may be anticipated to show a broader response spectrum to PAs in oviposition, since most danaid butterflies, irrespective of sex, are considered to have affinity for

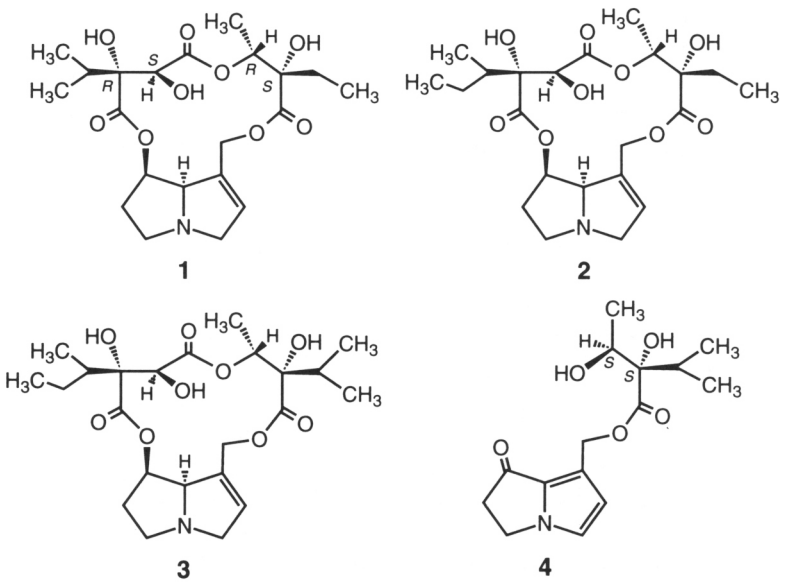


FIG. 1. Major pyrrolizidine alkaloids present in *Parsonsia laevigata*, the host plant of *Idea leuconoe*. Each of compounds **1–3** singly stimulates oviposition by the butterfly, whereas compound **4** does not.

a variety of PAs due to their indiscriminate uptake of unspecified PAs from diverse plants (females of most danaids also visit PA sources, and this behavior is often observed in the field). However, *I. leuconoe* females proved to be narrowly adapted only for host-specific PAs. Therefore, the use of specific PAs in host recognition by the females seems to be a plesiomorphic feature in evolutionary time.

PAs consist of two structurally distinct parts designated necine base and necic acid. Since all PAs tested here (including those not present in *Parsonsia*) except parsonine (**4**) share the same necine base, our results suggest that specific structures of the necic acid moiety are crucial for conferring oviposition-stimulatory activity. It is not clear, however, whether these three PAs are distinguished from one another by the females.

Implication for Pheromone System. Very recently, adult males of *I. leuconoe* were found to secrete danaidone, viridifloric β-lactone, and others from the abdominal hair-pencils. Behavioral and electrophysiological experiments have substantiated that the first two compounds elicit receptive responses from the females (Nishida et al., 1996). By virtue of their structural similarities to the subunits of PAs, i.e., necine base and necic acid, they are believed to be derived

from host-plant PAs sequestered during the larval period. The adults of both sexes have also been shown to store large quantities of characteristic PAs that are suspected to be of host-plant origin (Nishida et al., 1991; Kim et al., 1994), since field observations showed that they almost never visit PA-containing non-host plants. Although the stored PAs are somewhat different in structure from compounds **1–3,** it has been briefly mentioned that they also stimulate oviposition by the females (Nishida, 1995b). Danaidone, a keto-dihydropyrrolizine compound frequently encountered in male androconial secretions of danaids, has been thought to play an important role in mating systems of most danaids (Eisner and Meinwald, 1987; Boppré, 1990; Honda et al., 1995a). Males of *I. leuconoe* also make use of this compound in courtship, but the per capita quantity of danaidone is extremely small; some hundredths to some thousandths as compared with those of other PA-seeking danaids. It seems, therefore, untenable to speculate that a PA-seeking insect that is dependent on them for pheromone biosynthesis (Schneider et al., 1975) came to produce a much smaller amount of pheromone (danaidone) after having switched its host to a PA-containing plant in order to gain easier access to PAs. It also appears curious that, despite that, it ceased to gather more PAs from other plants in the adult.

Furthermore, if pharmacophagous acquisition of PAs as adults has evolutionarily resulted in a development of capacity for producing pheromones, and again if host shift occurred after the establishment of their pheromone system, then parsonine (**4**) present in the host plant would probably be very important to the males, because this unique compound consists simply of two skeletal parts of keto-dihydropyrrolizine and viridifloric acid, which would be readily and efficiently converted to their pheromones, danaidone and viridifloric β-lactone, respectively. From the sole viewpoint of chemical reaction, parsonine appears to be the best precursor of the known PAs (Logie et al., 1994) available for pheromone biosynthesis. The physiological significance of parsonine does not seem limited only to the males. Since males of *I. leuconoe* are considered to rely exclusively on host-plant PAs for pheromone production, the quality and the quantity of PAs including parsonine in the host plant would also be a matter of primary concern to the females that have to achieve successful mating and fertilization. Then ovipositing females of *I. leuconoe* would be expected to show a more or less positive response to parsonine. However, parsonine had no stimulatory activity at all. In view of the well-known male-biased attraction of danaids to PA-containing plants, presumably to get pheromone precursors (Edgar, 1975; Pliske, 1975; Boppré, 1981, 1983), the unresponsiveness of *I. leuconoe* females to parsonine strongly suggests that any later colonization of PA-containing plants by this butterfly as a result of host shift from PA-free plants, if it ever occurred, was independent of their reproductive strategy.

In this butterfly, necic acid-derived viridifloric β-lactone disseminated by males as a sex pheromone is one of the major hair-pencil components (Nishida

et al., 1996). At present, there is no evidence for the use of necic acid-derived pheromones by other danaids feeding on PA-free plants, although males of several ithomiid butterflies have been reported to secrete a γ-lactone structurally related to the necic acid moiety from costal fringes of the hindwings (Edgar et al., 1976; Schulz et al., 1988). The lactone is thought to serve as a territorial marker. Accordingly, it appears unreasonable to assume that such a pheromone system as that represented by *I. leuconoe* has evolved from those of the major lineage of danaid butterflies that sequester PAs as adults and secrete necine base-derived dihydropyrrolizines as principal components (Eisner and Meinwald, 1987). It may be concluded that the establishment of an association of this primitive butterfly with PA-containing plants would have preceded the development of its pheromone system. This is also suggestive of an additional and as yet unknown function of PAs of physiological importance. It may also be hypothesized that ancestors of danaids and ithomiids that had originally utilized two categories of compounds as pheromones might have possibly diverged into two or more groups: one using chiefly necine base derivatives (danaids) and another using chiefly necic acid derivatives (ithomiids).

A number of danaid and ithomiid larvae still maintain the capacity for assimilating and storing PAs (Rothschild and Edgar, 1978; Trigo and Motta, 1990). The present work and other facts discussed above strongly support the hypothesis that the ancestors of danaid butterflies used PA-containing host plants and that the present-day dependence of danaids on PAs originates from their ancestral association with PA-containing host plants. Host shifts from PA-containing plants (e.g., Apocynaceae) eventually to PA-free plants (e.g., Asclepiadaceae) in danaid butterflies that evolved a strategy to acquire PAs as adults from nonhost plants could occur if some phytochemical mediators other than PAs are involved in the exploitation of novel hosts.

Acknowledgments—We thank Dr. M. Nakai of UBE Scientific Analysis Laboratory, Inc., M. Sakamoto of Hiroshima City Forest Park for help in collecting and rearing butterflies, and Dr. R. Nishida of Kyto University for helpful discussion. We are greatly indebted to Dr. James L. Nation, University of Florida, who kindly improved our manuscript linguistically.

REFERENCES

ABE, F., and YAMAUCHI, T. 1987. Parsonine, a pyrrolizidine alkaloid from *Parsonsia laevigata*. *Chem. Pharm. Bull.* 35:4661-4663.

ABE, F., and YAMAUCHI, T. 1989. Lignan glycosides from *Parsonsia laevigata*. *Phytochemistry* 28:1737-1741.

ABE, F., NAGAO, T., OKABE, H., YAMAUCHI, T., MARUBAYASHI, N., and UEDA, I. 1990. Parsonsianine, a macrocyclic pyrrolizidine alkaloid from the leaves of *Parsonsia laevigata* (Studies on *Parsonsia*. III). *Chem. Pharm. Bull.* 38:2127-2129.

ABE, F., NAGAO, T., OKABE, H., and YAMAUCHI, T. 1991a. Macrocyclic pyrrolizidine alkaloids from *Parsonsia laevigata*. *Phytochemistry* 30:1737-1739.

ABE, F., YAMAUCHI, T., YAGA, S., and MINATO, K. 1991b. Pyrrolizidine alkaloids from *Parsonsia laevigata* in Okinawa Island (Studies on *Parsonsia*. V). *Chem. Pharm. Bull.* 39:1576–1577.

ACKERY, P. R., and VANE-WRIGHT, R. I. 1984. Milkweed Butterflies: Their Cladistics and Biology. Cornell University Press, New York, 425 pp.

BOPPRÉ, M. 1978. Chemical communication, plant relationships, and mimicry in the evolution of danaid butterflies. *Entomol. Exp. Eppl.* 24:264–277.

BOPPRÉ, M. 1981. Adult Lepidoptera "feeding" at withered *Heliotropium* plants (Boraginaceae) in East Africa. *Ecol. Entomol.* 6:449–452.

BOPPRÉ, M. 1983. Leaf-scratching—a specialized behaviour of danaine butterflies (Lepidoptera) for gathering secondary plant substances. *Oecologia* 59:414–416.

BOPPRÉ, M. 1984a. Redefining "pharmacophagy." *J. Chem. Ecol.* 10:1151–1154.

BOPPRÉ, M. 1984b. Chemically mediated interaction of butterflies, pp. 259–275, *in* R. I. Vane-Wright and P. R. Ackery (ed.). The Biology of Butterflies. Academic Press, London.

BOPPRÉ, M. 1986. Insects pharmacophagously utilizing defensive plant chemicals (pyrrolizidine alkaloids). *Naturwissenschaften* 73:17–26.

BOPPRÉ, M. 1990. Lepidoptera and pyrrolizidine alkaloids. Exemplification of complexity in chemical ecology. *J. Chem. Ecol.* 16:165–185.

EDGAR, J. A. 1975. Danainae and 1,2-dehydropyrrolizidine alkaloid-containing plants-with reference to observations made in the New Hebrides. *Phil. Trans. R. Soc. B* 272:467–476.

EDGAR, J. A. 1982. Pyrrolizidine alkaloids sequestered by Solomon Island Danaine butterflies. The feeding preferences of the Danainae and Ithomiinae. *J. Zool. London* 196:385–399.

EDGAR, J. A. 1984. Parsonsieae: Ancestral larval foodplants of the Danainae and Ithomiinae, pp. 91–93, *in* R. I. Vane-Wright and P. R. Ackery (ed.). The Biology of Butterflies. Academic Press, London.

EDGAR, J. A., CULVENOR, C. C. J., and PLISKE, T. E. 1974. Coevolution of danaid butterflies with host plants. *Nature* 250:646–648.

EDGAR, J. A., CULVENOR, C. C. J., and PLISKE, T. E. 1976. Isolation of a lactone, structurally related to the esterifying acids of pyrrolizidine alkaloids, from the costal fringes of male Ithomiinae. *J. Chem. Ecol.* 2:263–270.

EDGAR, J. A., EGGERS, N. J., JONES, A. J., and RUSSELL, G. B. 1980. Unusual macrocyclic pyrrolizidine alkaloids from *Parsonsia heterophylla* A. Cunn and *Parsonsia spiralis* Wall. (Apocynaceae). *Tetrahedron Lett.* 21:2657–2660.

EISNER, T., and MEINWALD, J. 1987. Alkaloid-derived pheromones and sexual selection in Lepidoptera, pp. 251–269, *in* G. D. Prestwich and G. J. Blomquist (ed.). Pheromone Biochemistry. Academic Press, London.

HARIBAL, M., and RENWICK, J. A. A. 1996. Oviposition stimulants for the monarch butterfly: Flavonol glycosides from *Asclepias curassavica*. *Phytochemistry* 41:139–144.

HONDA, K. 1995a. Chemical basis of differential oviposition by lepidopterous insects. *Arch. Insect Biochem. Physiol.* 30:1–23.

HONDA, K. 1995b. Plant secondary metabolites implicated for host selection and host specificity in butterflies. *Comp. Physiol. Biochem.* 12:145–165.

HONDA, K., and HAYASHI, N. 1995. A flavonoid glucoside, phellamurin, regulates differential oviposition on a rutaceous plant, *Phellodendron amurense*, by two sympatric swallowtail butterflies, *Papilio protenor* and *P. xuthus*: The front line of a coevolutionary arms race? *J. Chem. Ecol.* 21:1531–1539.

HONDA, K., TADA, A., and HAYASHI, N. 1995a. Dihydropyrrolizines from the male scent-producing organs of a danaid butterfly, *Ideopsis similis* (Lepidoptera: Danaidae) and the morphology of alar scent organs. *Appl. Entomol. Zool.* 30:471–477.

HONDA, K., TADA, A., HAYASHI, N., ABE, F., and YAMAUCHI, T. 1995b. Alkaloidal oviposition stimulants for a danaid butterfly, *Ideopsis similis* L., from a host plant, *Tylophora tanakae* (Asclepiadaceae). *Experientia* 51:753–756.

KELLEY, R. B., SEIBER, J. N., JONES, A. D., SEGALL, H. J., and BROWER, L. P. 1987. Pyrrolizidine alkaloids in overwintering monarch butterflies (*Danaus plexippus*) from Mexico. *Experientia* 43:943–946.

KIM, C. S., NISHIDA, R., ABE, F., YAMAUCHI, T., and FUKAMI, H. 1994. 14-Deoxyparsonsianidine *N*-oxide: A pyrrolizidine alkaloid sequestered by the giant danaine butterfly, *Idea leuconone*. *Biosci. Biotech. Biochem.* 58:980–981.

LOGIE, C. G., GRUE, M. R., and LIDDELL, J. R. 1994. Proton NMR spectroscopy of pyrrolizidine alkaloids. *Phytochemistry* 37:43–109.

NISHIDA, R. 1995a. Oviposition stimulants of swallowtail butterflies, pp. 13–26, *in* J. M. Scriber, Y. Tsubaki and R. C. Lederhouse (ed.). Swallowtail Butterflies: Their Ecology and Evolutionary Biology. *Scientific Publishers, Gainesville.*

NISHIDA, R. 1995b. Sequestration of plant secondary compounds by butterflies and moths. *Chemoecology* 6:127–138.

NISHIDA, R., KIM, C. S., FUKAMI, H., and IRIE, R. 1991. Ideamine *N*-oxides: Pyrrolizidine alkaloids sequestered by the danaine butterfly, *Idea leuconoe*. *Agric. Biol. Chem.* 55:1787–1792.

NISHIDA, R., SCHULZ, S., KIM, C. H., FUKAMI, H., KUWAHARA, Y., HONDA, K., and HAYASHI, N. 1996. Male sex pheromone of a giant danaine butterfly, *Idea leuconoe*. *J. Chem. Ecol.* 22:949–972.

PAPAJ, D. R., FEENY, P., SACHDEV-GUPTA, K., and ROSENBERRY, L. 1992. D-(+)-Pinitol, an oviposition stimulant for the pipevine swallowtail butterfly, *Battus philenor*. *J. Chem. Ecol.* 18:799–815.

PLISKE, T. E. 1975. Attraction of Lepidoptera to plants containing pyrrolizidine alkaloids. *Environ. Entomol.* 4:455–473.

RENWICK, J. A. A., and CHEW, F. S. 1994. Oviposition behavior in Lepidoptera. *Annu. Rev. Entomol.* 39:377–400.

ROTHSCHILD, M., and EDGAR, J. A. 1978. Pyrrolizidine alkaloids from *Senecio vulgaris* sequestered and stored by *Danaus plexippus*. *J. Zool. London* 186:347–349.

SCHNEIDER, D., BOPPRÉ, M., SCHNEIDER, H., THOMNPSON, W. R., BORIACK, C. J., PETTY, R. L., and MEINWALD, J. 1975. A pheromone precursor and its uptake in male *Danaus* butterflies. *J. Comp. Physiol.* 97:245–256.

SCHULZ, S., FRANCKE, W., EDGAR, J., and SCHNEIDER, D. 1988. Volatile compounds from androconial organs of danaine and ithomiine butterflies. *Z. Naturforsch.* 43c:99–104.

STELLJES, M. E., and SEIBER, J. N. 1990. Pyrrolizidine alkaloids in an overwintering population of monarch butterflies (*Danaus plexippus*) in California. *J. Chem. Ecol.* 16:1459–1470.

TRIGO, J. R., and MOTTA, P. C. 1990. Evolutionary implications of pyrrolizidine alkaloid assimilation by danaine and ithomiine larvae (Lepidoptera: Nymphalidae) *Experientia* 46:332–334.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

IDENTIFICATION OF FLORAL VOLATILES INVOLVED IN RECOGNITION OF OILSEED RAPE FLOWERS, *Brassica napus* BY HONEYBEES, *Apis mellifera*

MARGARET M. BLIGHT,[1] MARTINE LE MÉTAYER,[2] MINH-HÀ PHAM DELÈGUE,[2] JOHN A. PICKETT,[1] FRÉDÉRIC MARION-POLL,[3] and LESTER J. WADHAMS[1,*]

[1]*Department of Biological and Ecological Chemistry*
IACR-Rothamsted
Harpenden, Hertfordshire AL5 2JQ, UK

[2]*Laboratoire de Neurobiologie Comparée des Invertébrés INRA-CNRS (URA 1190)*
BP23, 91440 Bures-sur-Yvette, France

[3]*Station de Phytopharmacie*
INRA Route de St. Cyr
78026 Versailles Cedex, France

(Received September 5, 1996; Accepted February 28, 1997)

Abstract—Volatiles from oilseed rape, *Brassica napus*, flowers were sampled by air entrainment and their relevance to the natural odor profile of the flowers was confirmed by conditioned proboscis extension (CPE) assays with honeybee, *Apis mellifera* L., foragers. Coupled gas chromatography (GC)-CPE analysis of the air entrainment samples was used to locate key compounds involved in the recognition of *B. napus* flowers, and the compounds were then identified using coupled gas chromatography-mass spectrometry and comparison with authentic samples. Six regions of the gas chromatograms elicited CPE responses from bees previously conditioned to the total extract, and from these areas 16 compounds were identified that elicited CPE activity from conditioned bees when tested with synthetic samples. Eight of the 16, α-pinene, phenylacetaldehyde, *p*-cymene, α-terpinene, linalool, 2-phenylethanol, (*E*,*E*)-α-farnesene, and 3-carene, gave the highest responses. When the bees were conditioned to the total extract of flower volatiles, a mixture of the eight components elicited responses from 83% of the individuals, suggesting that the eight-component mixture accounted for a major part of the CPE activity of the total extract. In addition, a mixture of the three most active compounds, phenylacetaldehyde, linalool, and (*E*,*E*,)-α-farnesene,

*To whom correspondence should be addressed.

0098-0331/97/0700-1715$12.50/0 © 1997 Plenum Publishing Corporation

evoked responses from 85% of the bees after the latter had been conditioned to the eight-component mixture. Thus, these three compounds appear to play a key role in the recognition of the eight component mixture and, by inference, of oilseed rape flowers.

Key Words—Honeybee, *Apis mellifera*, Hymenoptera, Apidae, *Brassica napus*, oilseed rape, flower volatiles, conditioned proboscis extension, olfactory recognition.

INTRODUCTION

In the honeybee, *Apis mellifera* L., foraging behavior is based on a learning process that associates floral color, shape, and odor with a food reward provided by floral nectar and pollen. Of the stimuli mediating honeybee visits to flowers, olfactory cues are learned most readily (Menzel, 1967; Kriston, 1973). However, flower volatiles comprise complex mixtures of components, and evidence is now accumulating to suggest that mixture recognition by honeybee foragers is based on a few key compounds. Thus, Pham-Delègue et al. (1986) found that the recognition of a sunflower floral extract by free-flying bees visiting an artificial flower feeder was through a limited range of components, comprising about 10% of the total extract. However, in that work, the final active fraction still comprised about 30 components that were not assessed individually for relative behavioral activity. More recently, studies with a synthetic mixture of six compounds, previously identified as oilseed rape flower volatiles, have confirmed that some compounds cue mixture recognition more effectively than others (Pham-Delègue et al., 1993; Wadhams et al., 1994). An olfactory conditioned proboscis extension (CPE) response on restrained bees was used to test the activity of the components. This conditioning procedure has been used to investigate learning processes (e.g. Bitterman et al., 1983; Smith, 1991) and binary mixture discrimination (e.g. Getz and Smith, 1991). Although carried out on restrained bees under unnatural conditions, the CPE response is a valuable technique for the study of complex odor recognition in the honeybee and correlates well with the responses of free-flying foragers (Mauelshagen and Greggers, 1993). However, until now, this bioassay has been applied only to the study of mixtures comprising a limited number of components.

In this paper, we have extended the study of mixture recognition by the honeybee to natural floral blends, using the CPE response to locate biologically active components in the mixture. Oilseed rape was chosen since it is an important melliferous crop frequented by many pollinating insects, particularly honeybees, which are attracted by the large floral mass and abundant pollen and nectar production (Mesquida et al., 1988). We describe the identification of the compounds mediating recognition of oilseed rape flowers by honeybees.

METHODS AND MATERIALS

Insects. Both foraging Italian worker bees and bees that had been maintained in laboratory cages (described as caged bees, see below) were used in this study. The foragers were collected on their return to outdoor hives from June to September and were mounted immediately in the glass holders used in the CPE assay (see below). After being fed *ad libitum* with 30% sugar solution, they were starved for 18 hr at room temperature before use in the assay.

The caged bees were collected from combs of outdoor hives immediately after emergence. They were maintained in groups of about 50 in an incubator at 33°C, 50% relative humidity, and fed with sugar, pollen and water *ad libitum* (Pham-Delègue et al., 1993). Bees were 14–16 days old when used in the CPE assays.

Foragers were used for most of the study, but caged bees were used for three experiments in which CPE responses to 3-carene and to mixtures of three and eight compounds were examined (see Results).

Conditioning. Bees were first habituated to mechanical stimulation by placing them for 15 sec in an airflow of 50 ml/sec and were then conditioned using standard procedures (Bitterman et al., 1983; Pham-Delègue et al., 1993). The conditioning stimulus was presented (6 sec duration), and 3 sec after the odor stimulation began, the antenna was contacted with a sucrose solution (30%). The resulting proboscis extension was rewarded with a drop of the solution. Five conditioning trials were performed, with 15-min intertrial periods. After the first trial, proboscis extension responses to the odor stimulus occurring during the first 3 sec of the conditioning trials were recorded. Following the conditioning process, a control trial, performed by stimulating the insect with hexane (10 μl), was used to determine whether the odor stimulus had been learned specifically or the bee was responding to other stimuli, e.g., residual solvent or airflow. Only bees that gave the conditioned response at least once during the conditioning trials and that did not respond to the control (hexane) stimulation, were used for subsequent testing, either in the coupled gas chromatography (GC)-CPE mode or in the standard CPE assay.

Standard CPE Testing. Those conditioned bees that gave no response to the hexane control were placed in the airflow for 15 sec and then presented with the test stimulus for 6 sec. Test compounds were presented randomly at 15-min intervals and any proboscis extensions occurring during the 6-sec stimulation period were recorded. At the end of the testing protocol, bees were retested with the original conditioning extract and with the odor from six freshly picked flowers of *Brassica napus* cv. Topas. Data from bees that did not respond to the original conditioning stimulus at the end of the experiment were discarded.

Chemicals. Chemicals were purchased from commercial sources (Aldrich Chemical Co. Ltd., Fluka Chemie AG) and were $\geq$98% pure, except for a

α-terpinene, 3-carene, and 1-dodecene (all 95%) and phenylacetaldehyde (>90%). (*E*,*E*)-α-Farnesene ($\geq$98%) was obtained from ether surface washes of apples cv. Granny Smith (Murray, 1969) and purified by liquid chromatography on Florisil (Aldrich Chemical Co.). Pentane and hexane (both HPLC grade, Fisons) were purified as in Blight (1990). The pentane was used to prepare extracts of oilseed rape volatiles collected by air entrainment. Solutions of the individual synthetic compounds and mixtures were prepared in hexane for presentation in the CPE assay.

Isolation of Volatiles from Oilseed Rape. Volatiles were collected from flowering shoots of *Brassica napus*, cv. Topas, a commercial variety containing low levels of erucic acid and glucosinolates. A dynamic headspace (air entrainment) technique was used (Blight, 1990). Nineteen entrainments, each lasting six to seven days, were carried out sequentially in a controlled environment room with a 15L:9D photoperiod, light intensity 300–350 μmol photons/m^2/sec (photosynthetic active radiation), daylight temperature 20°C; nighttime 15°C. In each entrainment, 50–90 cut stems of rape, bearing buds and flowers only, were contained in distilled water in conical flasks (50 ml) in a 5-liter glass culture vessel. The relative humidity in the vessel was 80–90%. Volatiles were drawn from the vessel, using purified air, onto a tube of the adsorbent Porapak Q (1.5 g) and were eluted from it with pentane. The pentane extract was concentrated by fractional distillation of the solvent using a column of glass helices. Final concentration (to 200 μl) was done under nitrogen. Individual extracts were analyzed by GC and were then combined in order to obtain the concentrations of volatiles necessary for GC-CPE analysis. Two samples were prepared: sample A (volatiles from six entrainments) and sample B (volatiles from 13 entrainments). Aliquots of the two samples were stored at −20°C, either in microvials for immediate use or in glass ampoules under nitrogen for longer storage.

Gas Chromatography (GC). Extracts of entrained volatiles were analyzed initially on a 50-m × 0.32-mm-ID dimethylsiloxane bonded-phase fused silica capillary column (HP-1) with either a split/splitless or a cold on-column injector fitted in a Hewlett Packard 5880A GC equipped with a flame ionization detector (FID). In both systems the carrier gas was hydrogen, and the oven temperature was maintained at 40°C for 5 min and then programmed at 5°/min to 150°C, then at 10°/min to 250°C. Coinjections with reference samples and quantitative GC analyses were done using the HP-1 column and also a 30-m × 0.32-mm-ID 35% diphenylsiloxane/65% dimethylsiloxane bonded-phase fused silica capillary column (SPB-35), which was maintained at 30°C for 5 min and then programmed at 5°/min to 150°C, then at 10°/min to 250°C.

Gas Chromatography–Mass Spectrometry (GC-MS). A capillary GC column (50 m × 0.32 mm ID, HP-1) was directly coupled to the MS and integrated

data system (70-250 VG Analytical). Ionization was by electron impact at 70 eV, 230°C. The GC was maintained at 30°C for 5 min and then programmed at 5°/min to 180°C and then held isothermally. Identifications were confirmed by comparison of the mass spectral data with those of authentic samples and by peak enhancement when coinjected with authentic compounds on both the polar and nonpolar GC columns (Pickett, 1990).

Coupled Gas Chromatography–Conditioned Proboscis Extension (GC-CPE). The coupled GC-CPE system, in which the effluent from the GC capillary column is monitored simultaneously by the FID of the GC and by the CPE response of the honeybee, has been described previously (Wadhams et al., 1994).

GC conditions. Samples were separated using an AI 93 GC equipped with a FID and a cold on-column injector and fitted with a 30-m × 0.53-mm-ID, HP-1 bonded-phase fused silica column. The oven temperature was maintained at 40°C for 2 min and then programmed at 10°C/min to 250°C. The carrier gas was hydrogen. The effluent from the GC column was split approximately equally, with one part going to the FID detector of the GC while the remainder was directed to the airflow passing continuously over the bee.

Proboscis Extension Recordings. In preliminary studies with sample A, the CPE activity of the effluent from the GC column was monitored visually and the CPE and FID responses were recorded on a dual-pen chart recorder (Servogor SE-220). Results from separate analyses were transferred manually to one master GC trace. Subsequently, FID and CPE responses to sample B were recorded on a microcomputer. Each bee was scrutinized by an observer, who closed an electrical contact when the CPE response occurred. These electrical signals, together with the output from the FID, were stored on tape (Racal store 4) and simultaneously sampled with a data acquisition card (Data Translation DT 281, 12 bits precision; 200 Hz per channel). The data were stored as binary files using custom software (Marion-Poll and Tobin, 1992). These data were then undersampled to 10 Hz and exported as a series of voltage values to Excel using a Windows data analysis program (Awave) (Marion-Poll, 1995). The responses of the bees were expressed as values of 0 (no response) or 1 (proboscis extension) and summed to build an overall response profile. The amplitude of the profile at any particular time thus represented the number of bees exhibiting a CPE response to the component eluting at that time. This system allowed a more detailed analysis of the GC-CPE results.

Honeybee foragers used for recording CPE responses to the effluent from the GC were first conditioned to 10 μl of the total extract of volatiles (sample A or B). On completion of the recording, the bee was retested with this conditioning stimulus and, if it did not respond, the recorded data were discarded.

RESULTS AND DISCUSSION

As expected from previous studies (Tollsten and Bergström, 1988; Blight et al., 1992, 1995; Evans and Allen-Williams, 1992), both air entrainment samples of volatiles emitted by *B. napus* cv. Topas flowers were shown by GC analysis to be highly complex mixtures (Figure 1). In order to collect quantities of the trace components sufficient for CPE evaluation and chemical identification, the volatiles were collected from a large number of detached shoots standing in water. Some trace compounds were shown subsquently (see below) to be important in the recognition of rape flowers by bees. Collection of trace volatiles from rape plants *in situ* would have been preferable (Mookherjee et al., 1988; Jakobsen et al., 1994), but this was not practicable in this study.

The two samples of volatiles, obtained from six (A) and 13 collections of volatiles (B), respectively, were very similar, but not identical. In the CPE assay, both samples were active, with 85% ($N = 28$) and 70% ($N = 20$) of foragers conditioned to Topas flowers responding to samples A and B, respectively. CPE responses from bees previously conditioned to complex odors are highly specific, e.g., bees conditioned to violet odor did not respond to fennel odor and vice versa (de Jong and Pham-Delègue, 1991). Thus, in the context of this study, the air entrainment samples were a fair reflection of the profile of volatiles emitted by oilseed rape flowers. Preliminary work was carried out with sample A. However, a larger sample size was required for the identification of the active components, and therefore the study was continued using sample B. Subsequent coupled GC-CPE studies (see below) confirmed the validity of this approach, since no significant differences were observed in the responses of the bees to the two samples.

The coupled GC-electroantennogram (EAG)-CPE technique (Wadhams et al., 1994), which enables simultaneous monitoring of peripheral perception and behavioral responses elicited by compounds eluting from a GC column, was developed for location of biologically active compounds in complex natural product extracts. It was not possible to record EAG responses in the present study, however, because the stimulus concentrations required to obtain reliable EAG responses from honeybees were found to be at least an order of magnitude greater than those present in samples A and B (Wadhams et al., 1994). Consequently, areas of activity in the samples were located using the coupled GC-CPE technique. The absence of simultaneous EAG recordings precluded the immediate assignment of activity to specific peaks in the areas of the chromatogram where CPE responses were observed. This was therefore achieved by GC-MS identification of candidate compounds in the areas of CPE activity, with subsequent CPE assays using authentic compounds.

Five areas of activity were observed with sample A (Figure 1A, areas II–VI), in which more than 30% (see below) of the honeybee foragers ($N = 8$)

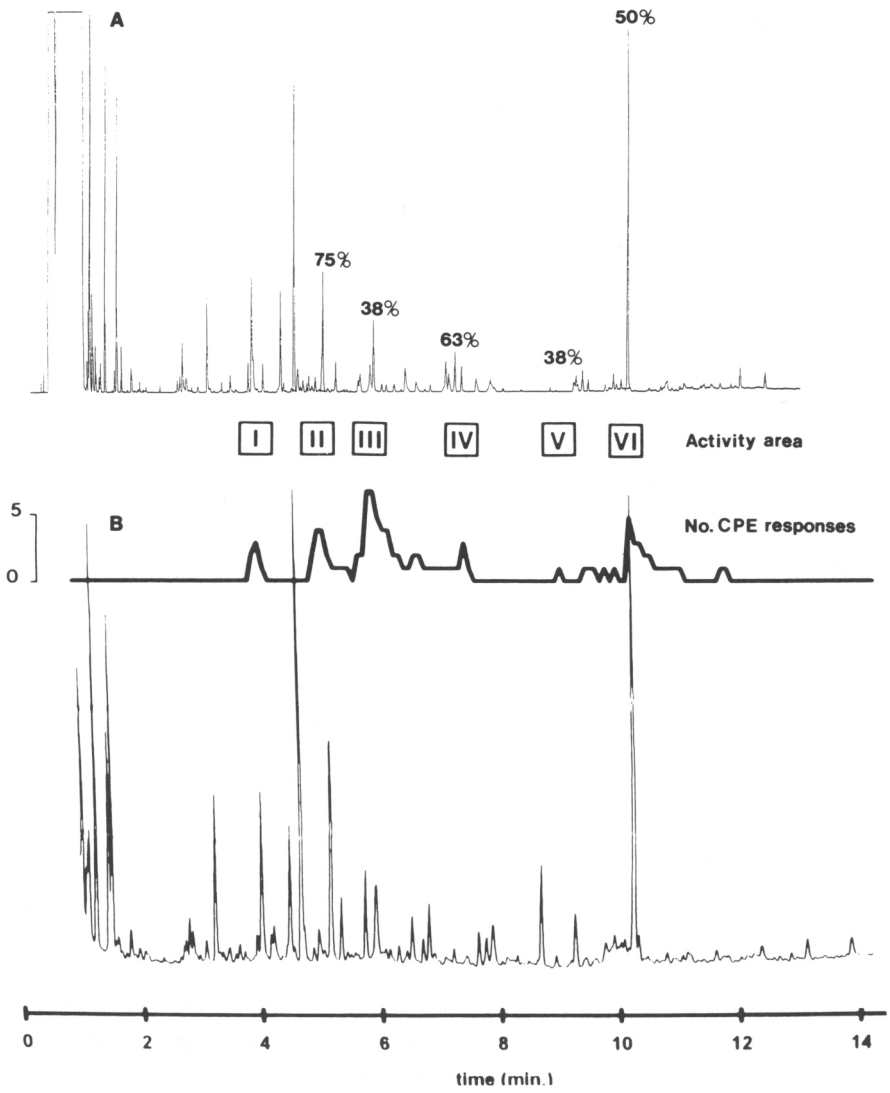


FIG. 1. Coupled gas chromatography–conditioned proboscis extension (GC-CPE) recordings from conditioned honeybees responding to volatiles from *Brassica napus* cv. Topas. No conditioned bees gave responses to the control (hexane). (A) GC trace of sample A volatiles. The numbers of bees responding to the activity areas I–VI on the chromatogram are shown as percentages. Only responses given by more than 30% of the bees are shown. (B) GC of sample B. The graph of the instantaneous CPE responses was obtained by computer accumulation of the responses of individual conditioned bees. (See text for further details.)

showed CPE activity. For sample B, CPE responses were analyzed using a microcomputer in order to provide a more detailed instantaneous response profile for the nine foragers tested. Five main areas of CPE activity (Figure 1B, areas I–IV, VI) were observed, four of which (areas II–IV, VI) were also present in sample A. However, differences were observed between the two samples in the levels of response to these common areas. The major activity in Sample B (area III) elicited a response from 78% of the bees, but only 38% responded to this area in sample A. Similarly, the main CPE activity in A, associated with areas II and IV, which elicited 75% and 63% responses, respectively, elicited only a weak response in sample B. No CPE activity was observed in area I with sample A or in area V with sample B.

The responses to areas II–VI in samples A and B were compared using logistic regression, and no significant differences were observed. A binomial distribution was assumed, but since the data showed slight overdispersion (as measured by the residual mean deviance (rmd) = 1.385), the values were divided by the rmd, and the treatment (trt) terms were tested with F values. For trt (sample) A/B: $F_{1,75} = 0.40$, $P > 0.05$; for area (II–VI): $F_{4,75} = 1.11$, $P > 0.05$; for sample x area interaction: $F_{4,75} = 1.30$, $P > 0.05$.

Coupled GC-MS analysis showed that many of the GC peaks observed in the biologically active areas were multicomponent. Identification of compounds was made by GC-MS and confirmed by GC coinjection with authentic samples. MS results are not detailed since spectra were so similar to those published (NIST, 1990). Fifteen compounds (Table 1) were identified as possibly contributing to the CPE activity.

The activity of these compounds was examined in the standard CPE assay, by conditioning foragers to 10 μl of sample B and then testing them with authentic samples (100 ng in 10 μl hexane) of the compounds. The 100-ng test level was chosen because the 10-μl conditioning stimulus (sample B) was estimated to contain 100–150 ng of biologically active volatile compounds. In addition, previous studies have shown that good discrimination of key components of a mixture is achieved at the 100-ng stimulus level (Pham-Delègue et al., 1993).

The data (Table 1) are derived from the responses of 54 bees. Although all compounds identified in the active areas were found to elicit some CPE responses, test compounds were considered to have significant activity only when they elicited responses from $\geq 30\%$ of the bees. This level was chosen somewhat arbitrarily, but account was taken of the fact that linalool, which is known to elicit high levels of spontaneous response, showed an average of 17% spontaneous activity in a study in which 775 bees were tested (Sandoz et al., 1995). Other compounds may elicit different levels of spontaneous response, but the choice of a threshold of $\geq 30\%$ ensured that all the responses had been fully learned. Thus, seven of the 15 compounds (Table 1) were deemed to have significant activity and were likely to be involved in recognition of the total

TABLE 1. CONDITIONED PROBOSCIS EXTENSION (CPE) RESPONSES OF HONEYBEE FORAGERS[a] TO COMPOUNDS IDENTIFIED AS OILSEED RAPE FLOWER VOLATILES

CPE activity area[b]	Identified compounds[c]	Bees responding (%)
I	Benzaldehyde	19
	α-Pinene	32
II	α-Terpinene	30
	Phenylacetaldehyde	63
	Benzyl alcohol	26
	p-Cymene	32
III	Limonene	26
	1,8-Cineole	28
	2-Nonanone	22
	Linalool	54
	2-Phenylethanol	35
	1-Undecene	26
IV	1-Dodecene	19
	Methyl salicylate	11
V	Unknown	—
VI	(*E*,*E*)-α-Farnesene	46

[a] The foragers ($N = 54$) were conditioned to sample B and gave no response to the control (hexane).
[b] Area of gas chromatogram eliciting CPE activity, from which the compound was identified.
[c] Compounds are listed in order of elution.

floral odor. The weak activity of methyl salicylate in this study was surprising, since in previous studies (Pham-Delègue et al., 1993) it was shown to be a key compound in mixture recognition. This may relate to differences in the hierarchy of the behaviorally active compounds when they are present in mixtures of different compositions.

After tests on the 15 identified compounds had been completed, further work led to the identification of a trace amount of 3-carene in area III of the chromatogram of sample B. In a separate experiment, using caged bees conditioned to sample B (10 μl), this compound was found to elicit responses intermediate in level between those of α-pinene, which gave one of the lower responses, and phenylacetaldehyde, which gave the highest response (Table 1). Accordingly, a synthetic mixture was prepared, comprising the seven most active compounds in Table 1 [α-pinene, α-terpinene, *p*-cymene, phenylacetaldehyde, 2-phenylethanol, linalool, and (*E*,*E*)-α-farnesene] and 3-carene in the approximate proportions and overall concentration in which they were present in sample B (Table 2). Eighty-three percent of caged bees conditioned to sample B ($N = 66$) gave CPE responses to this synthetic mixture. In addition, when caged bees

TABLE 2. COMPOSITION OF EIGHT-COMPOUND MIXTURE ELICITING THE HIGHEST CPE RESPONSES FROM CONDITIONED HONEYBEE FORAGERS

Compound	Concentration (ng/10 μl)
α-Pinene	8.2
Phenylacetaldehyde	0.4
p-Cymene	3.0
α-Terpinene	0.1
Linalool	9.9
2-Phenylethanol	5.3
(*E*,*E*)-α-Farnesene	73.2
3-Carene	0.1

were conditioned to Topas flowers, 61% ($N = 76$) responded to the synthetic eight-component mixture. This latter figure compares well with the overall response (70%, $N = 20$) elicited by sample B from foragers conditioned to Topas flowers and suggests that the eight-component mixture accounts for most of the CPE activity in this sample.

Of the eight most active compounds, phenylacetaldehyde, linalool, and (*E*,*E*)-α-farnesene elicited more CPE responses than did the remaining five (Table 1). When caged bees were conditioned to 100 ng of the eight-component mixture and tested with a mixture of these three compounds (in the proportions and concentrations in which they were found in the original extract, total conc. = 83.5 ng), 83% ($N = 41$) of the bees responded. Thus, these three compounds appear to play a key role in the recognition of the eight-component mixture.

No compounds with significant CPE activity have been identified from areas IV and V, and in sample B these areas appeared to contribute little to the overall mixture recognition process (Figure 1B). Sample A was, however, more effective than sample B in eliciting CPE responses from foragers conditioned to Topas flowers, suggesting that additional, as yet unidentified compounds, probably associated with areas IV and V, may also contribute to oilseed rape flower recognition by foraging worker bees.

The enantiomeric compositions of the chiral compounds, α-pinene, limonene, linalool, and 3-carene, were not investigated in this study. Floral volatiles frequently comprise enantiomeric mixtures (Ohloff, 1994), but there are examples of high stereospecificity. For example, the main constituent (95%) of the floral fragrance of *Daphne mezereum* is (*S*)-(+)-linalool, of enantiomeric purity >99% (Borg-Karlson et al., 1996). Although the importance of chirality to the activity of insect-produced pheromones is fully recognized and has been studied

in detail (Silverstein, 1979, 1988; Mori, 1994), information on its relevance to the activity of plant kairomones is limited. The enantiomers of limonene and the racemate (dipentene) were equally effective in inhibiting the attraction of *Hylobius abietis* (Coleoptera, Curculionidae) to α-pinene and ethanol (Nordlander, 1991), but (−)-limonene, (−)-menthol, and (−)-menthone were more repellent to cockroaches than the respective enantiomers and racemates (Inazuka, 1982). In three studies (Jacobson et al., 1984; Teranishi et al., 1987; Flath et al., 1994) (−)-α-copaene was found to be less attractive to fruit flies than the (+) enantiomer, although Warthen and McInnis (1989) observed no difference in behavioral response to the two chiral forms. The (*S*)-(+) enantiomer of linalool is reported to be more effective than the (*R*) enantiomer in attracting the solitary bee, *Colletes cunicularius* (Borg-Karlson et al., 1996). It should be noted, however, that data to support some of these claims have not been published in full. The significance of chirality in honeybee–floral volatile relationships will be addressed in future studies.

Work is in progress to identify additional CPE-active compounds among oilseed rape flower volatiles and to make further assessments of the contribution of each of the eight compounds so far identified to recognition of floral volatiles by the honeybee.

Acknowledgments—We thank Janine Ryan for technical assistance and Bernard Roger and Norman Carreck for providing the honeybees. We are grateful to Suzanne Clark, Department of Statistics, IACR-Rothamsted, for advice and help with the statistical analysis. M. Le Métayer was supported by the Limagrain Foundation. The cooperative work between England and France was cofunded by the British Council and the French Ministère de la Recherche et de la Technologie (Alliance Program). It was supported in part by the United Kingdom Ministry of Agriculture, Fisheries and Food. IACR Rothamsted receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom.

REFERENCES

BITTERMAN, M. E., MENZEL, R., FIETZ, A., and SCHÄFER, S. 1983. Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* 97:107–119.

BLIGHT, M. M. 1990. Techniques for isolation and characterization of volatile semiochemicals of phytophagous insects, pp. 281–288, *in* A. R. McCaffery and I. D. Wilson (eds.). Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, New York.

BLIGHT, M. M., HICK, A. J., PICKET, J. A., SMART, L. E., WADHAMS, L. J., and WOODCOCK, C. M. 1992. Volatile plant metabolites involved in host plant recognition by the cabbage seed weevil, *Ceutorhynchus assimilis* Payk., pp. 105–106, *in* S. B. J. Menken, J. H. Visser, and P. Harrewijn (eds.). Proceedings of the 8th International Symposium on Insect-Plant Relationships. Kluwer Academic Publishers, Dordrecht.

BLIGHT, M. M., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1995. Antennal perception of oilseed rape, *Brassica napus* (Brassicaceae), volatiles by the cabbage seed weevil *Ceutorhynchus assimilis* (Coleoptera, Curculionidae). *J. Chem. Ecol.* 21:1649–1664.

BORG-KARLSON, A.-K., UNELIUS, C. R., VALTEROVÁ, I., and NILSSON, L. A. 1996. Floral fragrance chemistry in the early flowering shrub *Daphne mezereum*. *Phytochemistry* 41:1477–1483.

DE JONG, R., and PHAM-DELÈGUE, M.-H. 1991. Electroantennogram responses related to olfactory conditioning in the honey bee (*Apis mellifera ligustica*). *J. Insect Physiol.* 37:319–324.

EVANS, K. A., and ALLEN-WILLIAMS, L. J. 1992. Electroantennogram responses of the cabbage seed weevil, *Ceutorhynchus assimilis*, to oilseed rape, *Brassica napus* ssp. *oleifera*, volatiles. *J. Chem. Ecol.* 18:1641–1659.

FLATH, R. A., CUNNINGHAM, R. T., MON, T. R., and JOHN, J. O. 1994. Additional male Mediterranean fruitfly (*Ceratitis capitata* Wied.) attractants from angelica seed oil (*Angelica archangelica* L.). *J. Chem. Ecol.* 20:1969–1983.

GETZ, W. M., and SMITH, K. B. 1991. Olfactory perception in honeybees: Concatenated and mixed odorant stimuli, concentration, and exposure effects. *J. Comp. Physiol. A* 169:215–230.

INAZUKA, S. 1982. Cockroach repellents contained in Japanese mint and Scotch spearmint. *Nippon Noyaku Gakkaishi* 7:145–154 (*Chem. Abstr.* 97:122030).

JACOBSON, M., UEBEL, E. C., and LUSBEY, W. R. 1984. Essential oil yields medfly attractant. *Chem. Eng. News* Dec. 17:24.

JAKOBSEN, H. B., FRIIS, P., NIELSEN, J. K., and OLSEN, C. E. 1994. Emission of volatiles from flowers and leaves of *Brassica napus in situ*. *Phytochemistry* 37:695–699.

KRISTON, I. 1973. Die Bewertung von Duft- und Farbsignalen als Orientierungshilfen an der Futterquelle durch *Apis mellifera* L. *J. Comp. Physiol.* 84:77–94.

MARION-POLL, F. 1995. Object-oriented approach to fast display of electrophysiological data under MS-Windows. *J. Neurosci. Methods* 63:197–204.

MARION-POLL, F., and TOBIN, T. R. 1992. Temporal coding of pheromone pulses and trains in *Manduca sexta*. *J. Comp. Physiol. A* 171:505–512.

MAUELSHAGEN, J., and GREGGERS, U. 1993. Experimental access to associative learning in honey bees. *Apidologie* 24:249–266.

MENZEL, R. 1967. Untersuchungen zum Erlernen von Spektralfarben durch die Honigbiene (*Apis mellifica*). *Z. Vergl. Physiol.* 56:22–62.

MESQUIDA, J., MARILLEAU, R., PHAM-DELÈGUE, M.-H., and RENARD, M. 1988. A study of rapeseed (*Brassica napus* L. var. *oleifera* Metzger) flower nectar secretions. *Apidologie* 19:307–318.

MOOKHERJEE, B. D., TRENKLE, R. W., WILSON, R. A., ZAMPINO, M., SANDS, K. P., and MUSSINAN, C. J. 1988. Fruits and flowers: Live vs dead—which do we want, pp. 415–424, *in* B. M. Lawrence, B. D. Mookherjee, and B. J. Willis (eds.). Flavors and Fragrances: A World Perspective. Proceedings, 10th International Congress of Essential Oils, Fragrances and Flavors. Washington DC, November 16–20, 1986. Developments in Food Science series, Vol. 18, Elsevier, Amsterdam.

MORI, K. 1994. Synthetic and stereochemical aspects of pheromone chemistry. *Pure Appl. Chem.* 66:1991–1998.

MURRAY, K. E. 1969. α-Farnesene: Isolation from the natural coating of apples. *Aust. J. Chem.* 22:197–204.

NIST STANDARD REFERENCE DATA BASE. 1990 (Version 3.01). Office of the Standard Reference Data Base, National Institute of Standards and Technology, Gaithersburg, Maryland.

NORDLANDER, G. 1991. Host finding in the pine weevil *Hylobius abietis*: Effects of conifer volatiles and added limonene. *Entomol. Exp. Appl.* 59:229–237.

OHLOFF, G. 1994. Scent and Fragrances. The Fascination of Odors and their Chemical Perspectives. Springer-Verlag, Berlin.

PHAM-DELÈGUE, M.-H., MASSON, C., ETIÉVANT, P., and AZAR, M. 1986. Selective olfactory choices of the honeybee among sunflower aromas: A study by combined olfactory conditioning and chemical analysis. *J. Chem. Ecol.* 12:781–793.

PHAM-DELÈGUE, M.-H., BAILEZ, O., BLIGHT, M. M., MASSON, C., PICARD-NIZOU, A. L., and WADHAMS, L. J. 1993. Behavioural discrimination of oilseed rape volatiles by the honeybee *Apis mellifera L. Chem. Senses* 18:483–494.

PICKETT, J. A. 1990. Gas chromatography–mass spectrometry in insect pheromone identification: Three extreme case histories, pp. 299–309, *in* A. R. McCaffery and I. D. Wilson (eds.). Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, New York.

SANDOZ, J.-C., ROGER, B., and PHAM-DELÈGUE, M.-H. 1995. Olfactory learning and memory in the honeybee: Comparison of different classical conditioning procedures of the proboscis extension response. *C. R. Acad. Sci. Paris, Ser. III.* 318:749–755.

SILVERSTEIN, R. M. 1979. Enantiomeric composition and bioactivity of chiral semiochemicals in insects, pp. 133–146, *in* F. J. Ritter (ed.). Chemical Ecology: Odour Communication in Animals. Elsevier/North Holland, Amsterdam.

SILVERSTEIN, R. M. 1988. Chirality in insect communication. *J. Chem. Ecol.* 14:1981–2004.

SMITH, B. H. 1991. The olfactory memory of the honeybee *Apis mellifera*. I. Odorant modulation of short- and intermediate-term memory after single-trial conditioning. *J. Exp. Biol.* 161:367–382.

TERANISHI, R., BUTTERY, R. G., MATSUMOTO, K. E., STERN, D. J., CUNNINGHAM, R. T., and GOTHILF, S. 1987. Recent developments in chemical attractants for tephritid fruit flies, pp. 431–438, *in* G. R. Waller (ed.). Symposium Series 330. American Chemical Society, Washington, DC.

TOLLSTEN, L., and BERGSTROM, G. 1988. Headspace volatiles of whole plants and macerated plant parts of *Brassica* and *Sinapis*. *Phytochemistry* 27:4013–4018.

WADHAMS, L. J., BLIGHT, M. M. KERGUELEN, V., LE MÉTAYER, M., MARION-POLL, F., MASSON, C., PHAM-DELÈGUE, M.-H., and WOODCOCK, C. M. 1994. Discrimination of oilseed rape volatiles by honey bee: Novel combined gas chromatographic-electrophysiological behavioral assay. *J. Chem. Ecol.* 20:3221–3231.

WARTHEN, J. D., and MCINNIS, D. O. 1989. Isolation and identification of male medfly attractive components in *Litchi chinensis* stems and *Ficus* spp. stem exudates. *J. Chem. Ecol.* 15:1931–1946.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

WHEAT AND BARLEY INHIBITORS ACTIVE TOWARDS α-AMYLASE AND TRYPSIN-LIKE ACTIVITIES FROM *Spodoptera frugiperda*

JULIO ALFONSO,[1] FELIX ORTEGO,[2] ROSA SANCHEZ-MONGE,[3] GLORIA GARCIA-CASADO,[3] MERARDO PUJOL,[1] PEDRO CASTAÑERA,[2] and GABRIEL SALCEDO[3,*]

[1]*Centro de Ingeniería Genética y Biotecnología Sancti Spiritus, Apartado 83, 60200 Cuba*

[2]*Centro de Investigaciones Biológicas, CSIC Velázquez 144, 28006 Madrid, Spain*

[3]*Unidad de Bioquímica, Departamento de Biotecnología E.T.S. Ingenieros Agrónomos, Ciudad Universitaria 28040 Madrid, Spain*

Abstract—The α-amylase activity was determined throughout the larval development of *Spodoptera frugiperda*. Maximal activities with optimal pH in the range 8.5–9.5 were found in last instars. Protein preparations enriched in heterotetrameric inhibitors from wheat flour were active towards gut amylases from last instars, while those corresponding to homodimeric and monomeric inhibitors showed low inhibition levels. These results were further supported by testing purified members of each inhibitor type and by analyzing the effects of the inhibitors on the amylase isoenzyme pattern from native PAGE. High levels of trypsin-like activity were also found in gut extracts from last instars. Different genetic variants of the major barley trypsin inhibitor were active against this gut enzyme. None of the other larval digestive protease activities (chymotrypsin-like, elastase-like, leucine aminopeptidase-like, and carboxypeptidase A and B-like) were inhibited, indicating that the barley inhibitor is specific towards trypsin-like enzymes.

Key Words—α-Amylase inhibitors, trypsin inhibitors, cereal endosperm, wheat, barley, *Spodoptera frugiperda*, digestive enzymes, midgut proteases.

INTRODUCTION

Studies on plant proteinaceous inhibitors of α-amylases and proteases from phytophagous insects have greatly increased recently. Up to ten different inhibitor

*To whom correspondence should be addressed.

0098-0331/97/0700-1729$12.50/0 © 1997 Plenum Publishing Corporation

families have been described (see García-Olmedo et al., 1987; Richardson, 1991, for reviews), and different lines of evidence point out the potential role of these inhibitors as components of plant resistance mechanisms to pests (Ryan, 1990; Boulter, 1993). The inhibitor genes have been considered useful tools for the improvement of crop protection, and some of them have proved to be effective when expressed in heterologous plants (Boulter, 1993; Shade et al., 1994). In this context, an initial aim would be to select appropriate inhibitors for the major digestive enzymes of the target pest.

The larvae of the fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), preferentially attack cereals and grasses in North, Central, and South America (Metcalf and Metcalf, 1993). However, the characterization of its digestive hydrolases is still minimal (Ferreira et al., 1994a, b), and no inhibitors of the major enzymatic activities have been described. A large family of proteins active against α-amylases from insects and mites, as well as against bovine trypsin, have been characterized in the endosperm of several cereal species, particularly wheat and barley (García-Olmedo et al., 1987; Carbonero et al., 1993). Three types of active α-amylase inhibitors have been described: monomeric, homodimeric, and heterotetrameric (three different subunits, one of them in two copies). Only monomeric forms have been found for the trypsin inhibitors of the family.

The activities of wheat inhibitors towards α-amylases have been tested in coleopteran (Baker and Lum, 1989; Gutiérrez et al., 1990; Briggs and McGregor, 1996; Feng et al., 1996) and lepidopteran species (Baker, 1989; Gutiérrez et al., 1993; Markwick et al., 1996), as well as against house mite enzymes (Sánchez-Monge et al., 1996). Active proteins have been found in all cases. The monomeric and dimeric forms are especially effective against coleopteran α-amylases, and the tetramer against the lepidopteran and house mite enzymes. The effects of antitrypsin proteins belonging to this inhibitor family on digestive proteases of pest insects have not been studied.

We investigate here the α-amylase and protease activities of *S. frugiperda* in order to perform inhibition assays using cereal inhibitors. The purified wheat tetramer and the barley monomer strongly inhibit digestive α-amylase and trypsin-like activities, respectively, of last instars.

METHODS AND MATERIALS

Insects. Neonate *S. frugiperda* larvae were obtained from eggs of a laboratory population maintained in the Laboratorio Provincial de Sanidad Vegetal (Sancti Spiritus, Cuba). The larvae were reared on a semiartificial diet (Poitout and Bues, 1970), modified by the addition of 0.63% (w/w) Wesson's salt mixture. They were reared in an environmental chamber at 22 $\pm$ 2°C, 90 $\pm$ 10% relative humidity, a 16L:8D photoperiod.

Last instars (12 days; 195.4 mg mean weight) were collected, cold-anesthetized, and dissected to remove the whole gut when required.

Insect Extracts. Larvae of different instars, as well as whole guts were homogenized (1:10 w/v) in distilled water with a glass Pyrex homogenizer. After shaking for 20 min, the suspensions were centrifuged at 10,000*g* for 10 min. All the extraction process was performed at 4°C. Supernatants were used for enzymatic activity and inhibition assays.

Extraction and Isolation of Cereal Inhibitors. Flours prepared from *Triticum aestivum* L. (cv. Chinese Spring), *Triticum turgidum* L. (cv. Enano de Andujar), and *Hordeum vulgare* L. (cv. Bomi, Hatif de Grignon and Valticky) were used to isolate the inhibitors tested in this study.

Protein preparations enriched in inhibitors were obtained by flour extraction with 0.15 M NaCl and $(NH_4)_2SO_4$ precipitation, as described by Gómez et al. (1989). These preparations were further subjected to gel filtration on Sephadex G-100 under nondissociating conditions (Gómez et al., 1989) to obtain fractions corresponding to tetrameric (Mr ca. 60,000), dimeric (Mr ca. 25,000), and monomeric (Mr ca. 12,000) inhibitors. Isolation of individual inhibitors from the appropriate gel-filtration fractions was carried out by reverse-phase high-performance liquid chromatography. Columns and chromatographic conditions were according to Gómez et al. (1991) for wheat monomeric α-amylase inhibitor WMAI-1; according to Sánchez-Monge et al. (1989) for wheat homodimeric α-amylase inhibitor WDAI-1; according to Sánchez-Monge et al. (1992) for wheat heterotetrameric α-amylase inhibitor subunits WTAI-CM2, -CM3B, and −CM16; and according Moralejo et al. (1993) for barley trypsin inhibitors BTI-CMe1, -CMe2.1, -CMe3.1, and -CMe3.2. Homogeneity of all isolated proteins was checked by two-dimensional electrophoresis (isoelectrofocusing × starch-gel electrophoresis) as in Gómez et al. (1989). Protein concentration was quantified by the bicinchoninic acid assay (Smith et al., 1985).

Amylase Activity and Inhibition Tests. Amylase activity of whole-larva and whole-gut extracts was assayed following Bernfeld (1955), with the modifications of Gutiérrez et al. (1993). The pH optimum of amylase activity in whole-gut extracts was determined with the following buffers: 0.1 M Tris HCl (pH 7–9) containing 20 mM NaCl and 0.1 mM $CaCl_2$, and 0.1 M glycine–NaOH (pH 8.5–11.5) containing 20 mM NaCl and 0.1 mM $CaCl_2$.

Inhibition assays were performed at the pH optimum. Briefly, gut extracts were diluted 7.7 times with Tris HCl buffer, containing the appropriate amounts of inhibitor preparation, to a final volume of 230 μl and incubated at 25°C for 10 min. After addition of 230 μl of 1% starch solution (incubation at 25°C for 30 min) and 460 μl of 3,5-dinitrosalicylic acid reagent, the final mixture was heated in a boiling water bath (5 min), cooled on ice, diluted with 2 ml of distilled H_2O, and its absorbance measured at 550 nm. All tests were performed with approximately 1 unit of α-amylase, defined as the enzyme activity required

to produce the reducing equivalents of 1 μmol maltose in our experimental conditions.

Native PAGE of Insect Amylases. Whole-gut amylases were separated by nondissociating PAGE (pH 8.8), using the method of Laemmli (1970), but omitting SDS and 2-mercaptoethanol in all buffers. Separation was performed on 160-mm × 150-mm × 1-mm gels, at 4°C and 35 mA for 3 hr 30 min. Specific staining for amylases was carried out with 1% (w/v) soluble starch in 0.1 M Tris HCl, pH 9.0, with 20 mM NaCl and 0.1 mM $CaCl_2$, for 1 hr, followed by a 5 M I_2/10 M KI solution in distilled H_2O for 10 min.

Forty micrograms of protein from gut extracts was inserted in gels. In inhibition tests, the same amount of protein was incubated with different wheat inhibitor preparations (1–5 μg) for 30 min before electrophoretic separation.

Trypsin-like Activity and Inhibition Tests. Trypsin-like activity of whole-gut extracts was assayed using BApNa (*N*α-benzoyl-DL-arginine *p*-nitroanilide) as substrate. Buffers used to determine the pH optimum of activity were: 0.1 M phosphate (pH 6–7), 0.1 M Tris HCl (pH 7–9), 0.1 M glycine NaOH (pH 9–11), and 0.1 M Na_2HPO_4–NaOH (pH 11–12). All buffers contained 0.15 M NaCl and 5 mM $MgCl_2$, except Na_2HPO_4–NaOH (no $MgCl_2$, because it is not maintained in solution at high alkaline pHs).

Tests were carried out according to Ortego et al. (1996), with 1 ml of reaction buffer containing 30 μl of gut extract and 1 mM BApNa (the latter added in 30 μl DMSO). Trypsin inhibitors were included in the reaction buffer (1–20 μg). The reaction mixture was incubated at 30°C for 30 min, and after the addition of 500 μl of 30% acetic acid, centrifuged at 10,000*g* for 5 min. The supernatant was monitored spectrophotometrically at 410 nm. The activity was quantified as nanomoles of substrate hydrolyzed per minute per milligram of protein, using a molar extinction coefficient of 8800 M^{-1} cm^{-1} (Erlanger et al., 1961).

Effect of Barley Trypsin Inhibitor on Other Protease Activities. The following protease activities were assayed in whole-gut extracts: chymotrypsin-like activity with SA_2PPpNa (*N*-succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide), elastase-like activity with SA_3pNa (*N*-succinyl-alanine-alanine-alanine *p*-nitroanilide), leucine aminopeptidase-like activity with LpNa (L-leucine *p*-nitroanilide), carboxypeptidase A-like activity with HPA (hippurylphenylalanine), and carboxypeptidase B-like activity with HA (hippuryl-L-arginine) as substrates. Assays were performed as in Ortego et al. (1996), at pH 8.5 and 10.5, and including 5 μg of barley trypsin inhibitor CMe1 in the reaction mixture.

RESULTS AND DISCUSSION

Amylase Activity and Its Inhibition by Wheat Tetrameric Inhibitor. Alkaline pH optima have been described for amylases of most Lepidoptera (Terra and

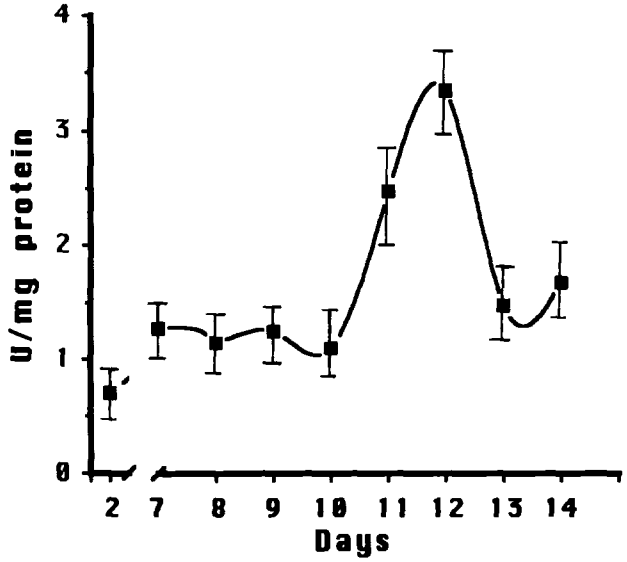


FIG 1. Amylase activity of extracts from larvae of *S. frugiperda* 2–14 days old. An equivalent amount of protein (400 μg) was used in all cases. One unit of α-amylase activity is defined in Methods and Materials section. Values are means $\pm$ SE (N = 4).

Ferreira, 1994). Therefore, the α-amylase was determined at pH 9.0 in extracts from 2- to 14-day-old larvae of *S. frugiperda* (Figure 1). Maximal activities per milligram of protein were found at 11–12 days (last instar) of larval development, when the insect showed its highest food intake. To determine more accurately the pH optimum for activity, extracts of last instars were assayed at different pHs (Figure 2). The optimal was in the pH range 8.5–9.5, in agreement with the data reported by Ferreira et al. (1994a). According to these results, extracts from last instars, as well as from their whole gut, were used for the inhibition tests at pH 9.0.

Previous data (Baker, 1989; Gutiérrez et al., 1993; García-Casado et al., 1994; Markwick et al., 1996) indicate that, while seed proteins from several plant species, including wheat, barley, rye, and kidney bean, inhibit coleopteran α-amylases, only the wheat proteins are truly effective against the lepidopteran enzymes. Thus, gel-filtration fractions enriched in wheat tetrameric, dimeric, and monomeric inhibitors were assayed (Figure 3). The amylases from last instars were mainly affected by the tetrameric inhibitor preparation. It was three- to six-fold more effective than the dimeric and monomeric ones, respectively (Figure 3A). In fact, very low inhibition levels were found for the two latter inhibitor types, even when the highest protein amount (5 μg) was tested.

An alternative approach to support these results was to analyze the whole-

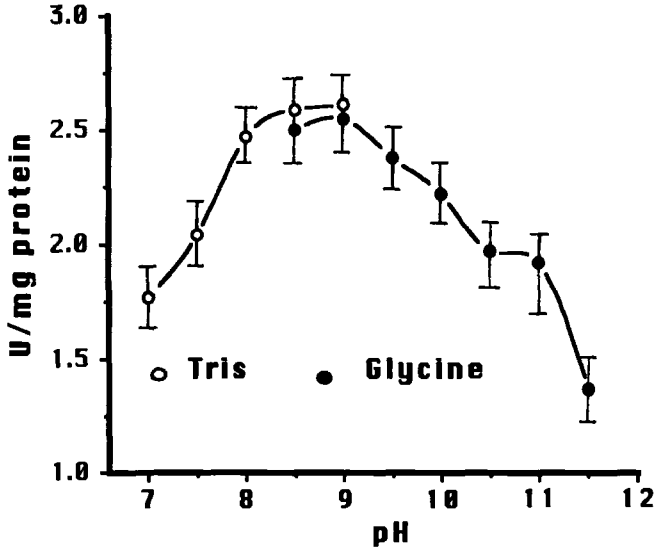


FIG. 2. Activity of *S. frugiperda* amylase in relation to pH. Extracts of last instars (12 days) corresponding to 400 μg of protein were used. Values are means $\pm$ SE ($N = 3$).

gut amylase activity from last instars after separation in native PAGE (Figure 3B). Specific staining revealed a prominent band, plus three minor ones (presumably isozymes). Previous incubation of the gut extract with the tetrameric inhibitor fraction provoked the disappearance of all bands, indicating that inactive enzyme-inhibitor complexes were formed during the incubation period. The effect was not restricted to particular isozymes, but extended to the four bands resolved by electrophoresis. In contrast, the dimeric and monomeric inhibitor fractions showed a negligible reduction of band intensities.

Representative members of each inhibitor type were further purified by reverse-phase HPLC from the appropriate gel-filtration fractions, following procedures previously described (Sánchez-Monge et al., 1989, 1992; Gómez et al., 1991). Only the tetramer, reconstituted from its isolated subunits (a mixture of WTAI-CM2 plus 2 WTAI-CM3 plus WTAI-CM16, where none of the subunits is active by itself; see Gómez et al., 1989), exerted a strong inhibition of the gut amylase activity (Table 1). Neither the homodimeric inhibitor WDAI-1 (synonymous 0.53; Sánchez-Monge et al., 1989) nor the monomeric one WMAI-1 (synonymous 0.28; Gómez et al., 1991) reduced this activity in more than 10%, under similar conditions (up to 5 μg of purified inhibitor per assay). Comparison of these results with the inhibitory levels shown in Figure 3A suggests that partial inactivation and/or reconstitution of the oligomeric forms

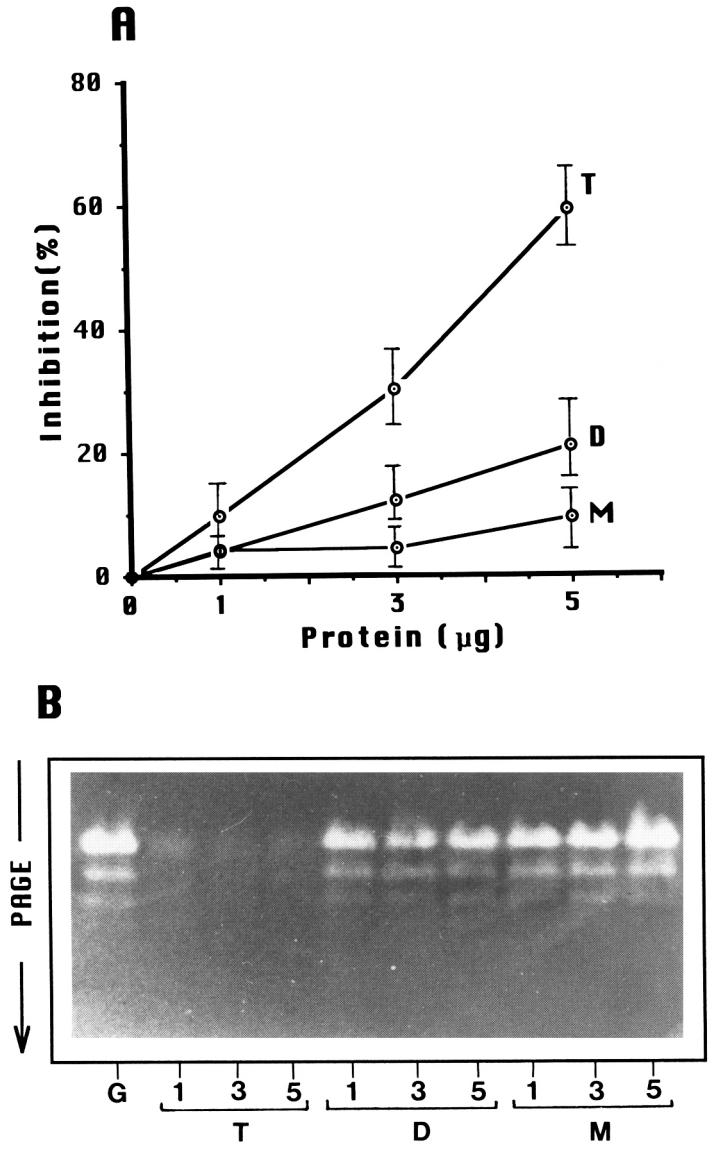


FIG. 3. (A) Inhibition of *S. frugiperda* amylase activity by protein preparations enriched in tetrameric (T), dimeric (D), and monomeric (M) inhibitors from wheat flour. Values are means ± SE ($N = 3$). Tests were performed using 1 unit of amylase. (B) Detection of amylase activity from whole-gut of last instar larvae after native PAGE (pH 8.8). Forty micrograms of protein were directly electrophoresed (lane G) or previously incubated with different amounts (1, 3, or 5 µg) of the protein preparations used in Figure 3A.

TABLE 1. INHIBITION OF GUT AMYLASE ACTIVITY IN LAST INSTARS OF *S. frugiperda* BY PURIFIED TETRAMERIC INHIBITOR FROM WHEAT

Inhibitor[a]	Inhibition (%)[b]		
	1 μg/assay	3 μg/assay	5 μg/assay
WTAI-CM2 + 2WTAI-CM3 + WTAI-CM16	28 ± 3	39 ± 4	50 ± 5

[a] Wheat tetrameric inhibitor was reconstituted from an appropriate mixture of its purified subunits WTAI-CM2, -CM3, and -CM16. No subunit was inhibitory by itself.
[b] Tests were performed with approximately 1 unit α-amylase. Values are means ± SE (N = 3).

might be promoted by the isolation procedures, a methodological limitation previously described for this inhibitor family (Gutiérrez et al., 1990, 1993; Sánchez-Monge et al., 1996).

Joint consideration of the data presented indicates that the wheat tetrameric inhibitor is the most effective towards the digestive amylases of *S. frugiperda*. This is in agreement with results obtained for amylases of other lepidopteran species (Baker, 1989; Gutiérrez et al., 1993). However, Markwick et al. (1996) recently reported a strong effect of a protein preparation enriched in wheat dimeric inhibitors on amylases from the potato tuber moth, but no purified inhibitors were used to confirm this fact.

Inhibition of Trypsin-like Digestive Proteases by Barley Inhibitor CMe. Trypsin-like activity has been detected as one of the major digestive proteases in several lepidopteran species (Terra and Ferreira, 1994; Johnson et al., 1995; Lee and Anstee, 1995; Ortego et al., 1996). Our analysis of gut extracts from last instars of *S. frugiperda* with BApNa as substrate showed high levels of trypsin-like protease activity (around 450 nmol of BApNa hydrolyzed per minute per milligram of protein), with a pH optimum in the range 8.5–10.5 (Figure 4). These data are in agreement with those of Ferreira et al. (1994a), who reported optimal pH between 8.0 and 10.0. Plant inhibitors from various sources, mainly soybean, cowpea, and tobacco have been tested for activity against insect trypsin-like activity. However, no member of the cereal α-amylase/trypsin inhibitor family with anti-trypsin capacity has been assayed against pest proteases so far. In barley flour (but not in wheat), one of the most abundant members of the family is a 13-kDa trypsin inhibitor, designated BTI-CMe (García-Olmedo et al., 1987; Carbonero et al., 1993). BTI-CMe1 was purified by HPLC from the 12-kDa gel-filtration fraction of a barley flour extract enriched in inhibitors, following the method of Moralejo et al. (1993). The isolated inhibitor was then

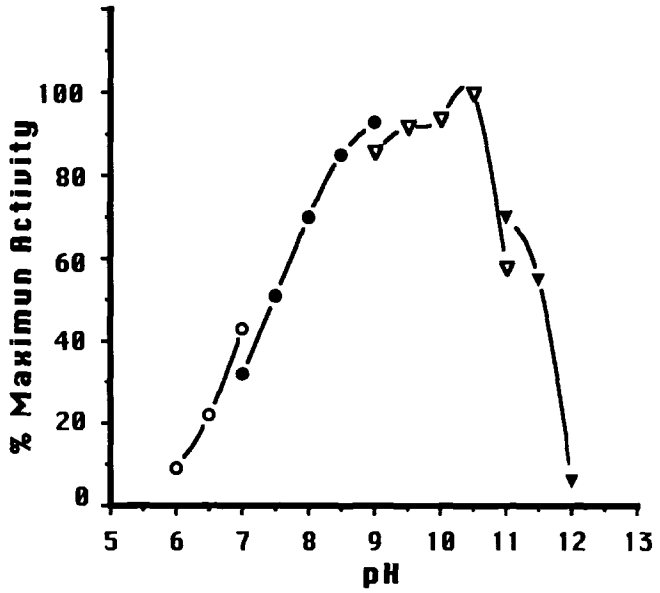


FIG 4. Activity of *S. frugiperda* trypsin-like protease in relation to pH. Larval gut extracts corresponding to 5.7 μg of protein were used. Values are means (N = 3), with SE within 5%. Maximum activity (100%) corresponded to a specific activity of 481 ± 13 nmol of BApNA hydrolyzed per minute per milligram of protein. Reaction buffers were 0.1 M phosphate (○), 0.1 M Tris HCl (●), 0.1 M glycine-NaOH (▽), and 0.1 Na_2HPO_4-NaOH (▼).

tested against the trypsin-like activity of *S. frugiperda* (Figure 5), showing inhibition levels over 50% when 10 μg of inhibitor/assay (0.77 pmol) and pH 8.5 were used. Similar inhibition values were found at pH 6.5, while a decrease of 57% was observed at pH 10.5.

The performance of BTI-CMe1 was further confirmed and extended proving three of its generic variants isolated from different barley cultivars (Moralejo et al., 1993). The trypsin-like activity from gut extracts was sensitive to all variants, being similarly affected by the four inhibitors (Figure 6). These results contrasted with the data previously obtained towards bovine trypsin (Moralejo et al., 1993), where significant differences in anti-tryptic activity (BTI-CMe3.1 and -CME3.2 > BTI-CMe1 and -CMe2.1) were observed. This indicates that the *S. frugiperda* and the mammalian enzymes differ in some properties.

Finally, BTI-CMe1 was assayed against five other protease activities (chymotrypsin-like, elastase-like, leucine aminopeptidase-like, and carboxypep-

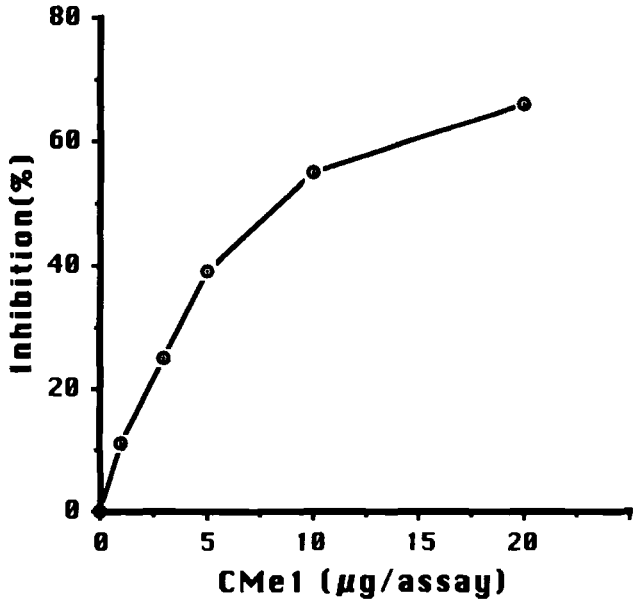


FIG 5. Inhibition of *S. frugiperda* trypsin-like activity by the purified barley inhibitor BTI-CMe1. Values are means ($N = 3$), with SE within 1%. Tests were performed using a protease activity equivalent to 531 ± 6 nmol of BApNa hydrolyzed per minute per milligram of protein.

tidase A and B-like) from *S. frugiperda* gut extracts to evaluate its putative inhibitory effect (Table 2). Three of these digestive proteases (chymotrypsin, elastase, and carboxypeptidase B) are described here for the first time in this noctuid species. None of them was inhibited, at a significant level, by the barley protein, indicating that BTI-CMe1 is specific for trypsin-like enzymes. A previous survey had showed that the inhibitor was inactive towards chymotrypsin, papain, subtilopeptidase A, pepsin, endogenous proteins from green malt and from bacterial and fungal proteinases (Mikola and Soulinna, 1969).

The results presented in this study support the potential use of α-amylase and trypsin inhibitors from cereal seeds to protect crops against *S. frugiperda*. Transgenic tobacco plants expressing some of these inhibitors have been developed (Carbonero et al., 1993; Masoud et al., 1996), and preliminary data suggest that, in some cases, the cereal proteins confer a partial resistance (increase larval mortality) towards some lepidopteran pests.

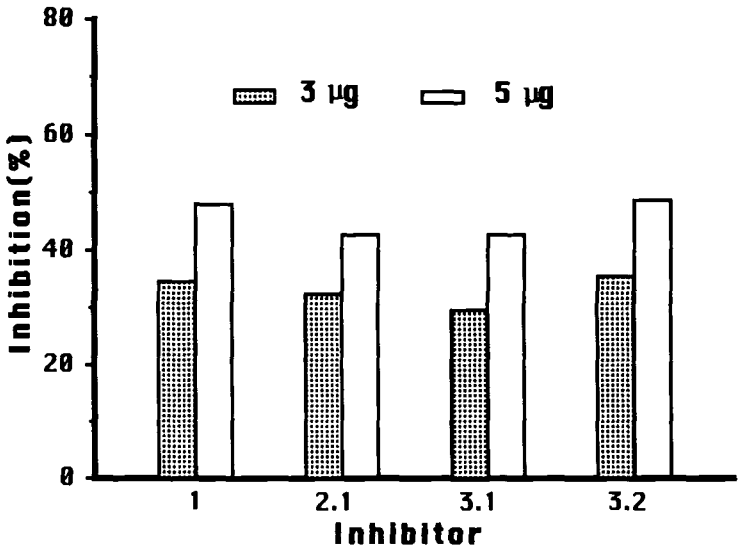


FIG. 6. Inhibition of *S. frugiperda* trypsin-like activity by purified BTI-CMe genetic variants. Gut extracts from last-instar larvae (5.7 µg of protein) and 3 or 5 µg of inhibitor were proved in all tests. Values are means (N = 3) and SE were within 1%. Sample tested were BTI-CMe1 (1), -CME2.1 (2.1), -CME3.1 (3.1), and -CMe3.2 (3.2).

TABLE 2. EFFECT OF BARLEY TRYPSIN INHIBITOR BTI-CMe1 ON DIFFERENT PROTEASE ACTIVITIES FROM LARVAL GUT EXTRACTS OF *S. frugiperda*

Protease (substrate)	pH	Specific activity[a]	Inhibition (%)[b]
Chymotrypsin	8.5	4276 ± 99	−3 ± 1
(0.25 mM SA_2PPpNa)	10.5	8182 ± 270	7 ± 5
Elastase	8.5	71.2 ± 0.3	2 ± 1
(0.5 mM SA_3pNa)	10.5	79.8 ± 0.9	0.2 ± 2
Leucine aminopeptidase (1 mM LpNa)	8.5	586 ± 41	2 ± 4
Carboxypeptidase A (1 mM HPA)	8.5	290 ± 6	−9 ± 7
Carboxypeptidase B (1 mM HA)	8.5	324 ± 3	12 ± 1

[a]Specific activities measured as nanomoles of substrate hydrolyzed per minute per milligram of protein. Values are means ± SE (N = 3).

[b]Inhibition test were carried out using 5 µg of BTI-CMe1/assay. The percentage of inhibition represents the mean ± SE (N = 3).

Acknowledgments—We thank J. L. Ayala for providing the eggs of *S. frugiperda*, and J. García-Guijarro and D. Lamoneda for technical assistance. Financial support was from Comunidad de Madrid, Consejeria de Educación y Cultura (grant COR 0037/94).

REFERENCES

BAKER, J. E. 1989. Interaction of partially purified amylases from larval *Anagasta kuehniella* (Lepidoptera: Pyralidae) with amylase inhibitors from wheat. *Comp. Biochem. Physiol.* 93B:239–246.

BAKER J. E., and LUM, P. T. M. 1989. Multiplicity of albumins from wheat inhibitory to amylase from the rice weevil (Coleoptera: Curculionidae). *J. Econ. Entomol.* 82:1548–1553.

BERNFIELD, P. 1955. Amylases, α and β. *Methods Enzymol.* 1:149–158.

BOULTER, D. 1993. Insect pest control by copying nature using genetically engineered crops. *Phytochemistry* 34:1453–1466.

BRIGGS, D. R., and MCGREGOR, P. G. 1996. Gut pH and amylase and protease activity in larvae of the New Zealand grass grubs (*Costelytra zealandica*; Coleoptera: Scarabaeidae) as a basis for selecting inhibitors. *Insect. Biochem. Mol. Biol.* 26:69–75.

CARBONERO P., SALCEDO G., SÁNCHEZ-MONGE, R., GARCIA-MAROTO, F., ROYO, J., GÓMEZ, L., MENA M., MEDINA, J., and DIAZ, I. 1993. A multigene family from cereals which encodes inhibitors of trypsin and heterologous α-amylases, pp. 333–348, *in* F. X. Aviles (ed.). Innovations of Proteases and Their Inhibitors. Walter de Gruyter, Berlin.

ERLANGER, B. F., KOKOWSKY, N., and COHEN, W. 1961. The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* 95:271–278.

FENG, G. H., RICHARDSON, M., CHEN, M. S., KRAMER, K. J., MORGAN, T. D., and REECK, G. R. 1996. α-Amylase inhibitors from wheat: Amino acid sequences and patterns of inhibition of insect and human α-amylases. *Insect Biochem. Mol. Biol.* 26:419–426.

FERREIRA, C., CAPELLA, A. N., SITNIK, R., and TERRA W. R. 1994a. Digestive enzymes in midgut cells, endo- and ectoperitrophic contents, and peritrophic membranes of *Spodoptera frugiperda* (Lepidoptera) larvae. *Arch. Insect. Biochem. Physiol.* 26:299–313.

FERREIRA, C., CAPELLA A. N., SITNIK, R., and TERRA, W. R. 1994b. Properties of the digestive enzymes and the permeability of the peritrophic membrane of *Spodoptera frugiperda* (Lepidoptera) larvae. *Comp. Biochem. Physiol.* 107A:631–640.

GARCÍA-CASADO, G., SÁNCHEZ-MONGE, R., LOPEZ-OTIN, C., and SALCEDO, G. 1994. Rye inhibitors of animal α-amylases show different specificities, aggregative properties and IgE-binding capacities than their homologous proteins from wheat and barley. *Eur. J. Biochem.* 224:525–531.

GARCÍA-OLMEDO, F., SALCEDO, G., SÁNCHEZ-MONGE, R., GÓMEZ, L., ROYO, J., and CARBONERO, P. 1987. Plant proteinaceous inhibitors of proteinases and α-amylases. *Oxford Surv. Plant Mol. Cell Biol.* 4:275–334.

GÓMEZ, L., SÁNCHEZ-MONGE, R., GARCÍA-OLMEDO, F., and SALCEDO, G. 1989. Wheat tetrameric inhibitors of insect α-amylases: Alloploid heterosis at the molecular level. *Proc. Natl. Acad. Sci. U.S.A.* 86:3242–3246.

GÓMEZ, L., SÁNCHEZ-MONGE, R., LOPEZ-OTIN, C., and SALCEDO, G. 1991. Wheat inhibitors of heterologous α-amylases. Characterization of major components from the monomeric class. *Plant Physiol.* 96:768–774.

GUTIÉRREZ, C., SÁNCHEZ-MONGE, R., GÓMEZ, L., RUIZ-TAPIADOR, M., CASTAÑERA, P., and SALCEDO, G. 1990. α-Amylase activity of agricultural insect pests are specifically affected by different inhibitor preparations from wheat and barley endosperms. *Plant Sci.* 72:37–44.

GUTIÉRREZ, C., GARCÍA-CASADO, G., SÁNCHEZ-MONGE, R., GÓMEZ, L., CASTAÑERA, P., and

SALCEDO, G. 1993. Three inhibitor types from wheat endosperm are differentially active against α-amylases of Lepidoptera pests. *Entomol. Exp. Appl.* 66:47–52.

JOHNSTON, K. A., LEE, M. J., BROUGH, C., HILDER, V. A., GATEHOUSE, A. M. R., and GATEHOUSE, J. A. 1995. Protease activities in the larval midgut of *Heliothis virescens*: Evidence for trypsin and chymotrypsin-like enzymes. *Insect Biochem. Mol. Biol.* 25:375–383.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 227:680–685.

LEE, M. J., and ANSTEE J. H. 1995. Endoproteases from the midgut of larval *Spodoptera littoralis* include a chymotrypsin-like enzyme with an extended binding site. *Insect Biochem. Mol. Biol.* 25:49–61.

MARKWICK, N. P., LAING, W. A., CHRISTELLER, J. T., REID, S. J., and NEWTON, M. R. 1996. α-Amylase activities in larval midgut extracts from four species of Lepidoptera (Tortricidiae and Gelechiidae): Response to pH and to inhibitors from wheat, barley, kidney bean, and *Streptomyces*. *J. Econ. Entomol.* 89:39–45.

MASOUD, S. A., DING, X., JOHNSON, L. B., WHITE, F. F., and REECK, G. R. 1996. Expression of a corn bifunctional inhibitor of serine proteinases and insect α-amylases in transgenic tobacco plants. *Plant Sci.* 115:59–69.

METCALF, R. L., and METCALF, R. A. 1993. Destructive and Useful Insects. Their Habits and Control. McGraw-Hill, New York.

MIKOLA, J., and SOULINNA, E. M. 1969. Purification and properties of a trypsin inhibitor from barley. *Eur. J. Biochem.* 9:555–560.

MORALEJO, M. A., GARCÍA-CASADO, G., SÁNCHEZ-MONGE, R., LOPEZ-OTIN, C., ROMAGOSA, I., MOLINA-CANO, J. L., and SALCEDO, G. 1993. Genetic variants of the tryspin inhibitor from barley endosperm show different inhibitory activities. *Plant Sci.* 89:23–29.

ORTEGO, F., NOVILLO, C., and CASTAÑERA, P. 1996. Characterization and distribution of digestive proteases of the stalk corn borer, *Sesamia nonagrioides* Lef. (Lepidoptera: Noctuidae). *Arch. Insect Biochem. Physiol.* 33:163–180.

POITOUT, S., and BUES, R. 1970. Elevage de plusieurs especes de lepidopteres Noctuidae sur milieu artificial simplifie. *Ann. Zool. Ecol. Anim.* 2:79–91.

RICHARDSON, M. 1991. Seed storage proteins: The enzyme inhibitors, pp. 259–305, *in* L. J. Rogers (ed.). Methods in Plant Biochemistry, Vol. 5. Academic Press, London.

RYAN, C. A. 1990. Protease inhibitors in plants: Genes for improving defenses against insects and pathogens. *Annu. Rev. Phytopathol.* 28:425–449.

SÁNCHEZ-MONGE, R., GÓMEZ, L., GARCÍA-OLMEDO, F., and SALCEDO, G. 1989. New dimeric inhibitor of heterologous α-amylase encoded by a duplicated gene in the short arm of chromosome 3B of wheat. *Eur. J. Biochem.* 183:37–40.

SÁNCHEZ-MONGE, R., GÓMEZ, L., BARBER, D., LOPEZ-OTIN, C., ARMENTIA A., and SALCEDO, G. 1992. Wheat and barley allergens associated with baker's asthma. Glycosylated subunits of the α-amylase inhibitor family have enhanced IgE-binding capacity. *Biochem. J.* 281:401–405.

SÁNCHEZ-MONGE, R., GARCÍA-CASADO, G., BARBER, D., and SALCEDO, G. 1996. Interaction of allergens from house-dust mite and from cereal flours: *Dermatophagoides pteronyssinus* α-amylase (Der P 4) and wheat and rye α-amylase inhibitors. *Allergy* 51:176–180.

SHADE, R. E., SCHROEDER, H. E., PUEYO, J. J., TABE, L. M., MURDOCH, L. L., HIGGINS, T. J. V., and CHRISPEELS, M. J. 1994. Transgenic pea seeds expressing the α-amylase inhibitor of the common bean are resistant to bruchid beetles. *Bio/Technology* 12:793–796.

SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GARTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. W., OLSON, B. J., and KLENK, D. C. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76–85.

TERRA, W. R., and FERREIRA, C. 1994. Insect digestive enzymes: Properties, compartmentalization and function. *Comp. Biochem. Physiol.* 109B:1–62.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

SEX PHEROMONE OF THE MIRID BUG *Phytocoris relativus*

JOCELYN G. MILLAR,[1,*] RICHARD E. RICE,[2] and QIAO WANG[1,3]

[1]*Department of Entomology, University of California Riverside, California 92521*

[2]*Department of Entomology, University of California Davis, California 95616*

(Received November 15, 1996; accepted March 1, 1997)

Abstract—The sex attractant pheromone produced by adult females of the mirid bug *Phytocoris relativus* has been identified as a 2:1 blend of hexyl acetate with (*E*)-2-octenyl butyrate. The pheromone is stage-, sex-, and species-specific, attracting only adult male *P. relativus*. Hexyl acetate was identified in aeration extracts from both sexes, while (*E*)-2-octenyl butyrate was produced only by females. Both males and females also produced hexyl butyrate and octyl acetate, while only females produced (*E*)-2-hexenyl and octenyl acetates, and (*Z*)-3-octenyl acetate. The function(s) of these chemicals were not determined. Attraction of males increased with dose, with doses of 0.1 to 33 mg loaded on grey rubber septa. Attractiveness of rubber septum lures decreased quickly with age due to the volatility of the two pheromone components.

Key Words—*Phytocoris relativus*, Miridae, sex pheromone, pistachio, monitoring, hexyl acetate, (*E*)-2-octenyl butyrate

INTRODUCTION

The plant bug genus *Phytocoris* is large, with an estimated 650 species worldwide (Schuh and Slater, 1995) and approximately 200 species described from western North America (Stonedahl, 1988). At least three species (*P. relativus* Knight, *P. californicus* Knight, and *Phytocoris* new spp., near *conspurcatus*; Rice et al., 1988) are found in California pistachio orchards, where they have been linked to epicarp lesions on developing nuts and loss of immature nuts. *P. relativus* has also been reported infesting apples, almonds, and stone fruits.

*To whom correspondence should be sent.

[3] Current address: Department of Plant Science, Massey University, Private Bag 11222, Palmerston North, New Zealand.

0098-0331/97/0700-1743$12.50/0 © 1997 Plenum Publishing Corporation

However, bugs in this genus are also beneficial, with many being primarily predacious, feeding on scale, mites, and other soft-bodied insects (Rice et al., 1988; Stonedahl, 1988). In California, there are three to four generations a year of *P. relativus*, with the first adults developed from overwintering eggs appearing in early April (Rice et al., 1988).

Mirid bugs and bugs from several other true bug genera can be some of the most damaging pests of pistachio in California. Unfortunately, there is currently no rapid and sensitive method of detecting bug infestations. Monitoring for bug populations in tree crops usually consists of taking beating tray samples, which is laborious and which must be multiply replicated to provide reliable results. Furthermore, many of the pest species are highly mobile and polyphagous, moving rapidly into and out of crops from surrounding fields or uncultivated land. By the time infestations are detected, significant damage may have occurred.

In an attempt to resolve the limitations of current methods of monitoring bug populations in nuts and stone fruits, we embarked on a study of the semiochemistry of several pest species to determine whether compounds such as pheromones could be identified and developed into useful tools for sampling bug populations. We report here the identification of the female-produced sex attractant pheromone of *P. relativus* and the development and optimization of sex pheromone lures for males of this species.

METHODS AND MATERIALS

Insects. Nymphs and adults were collected by beating tray sampling from pistachio and stone fruit orchards in the southern San Joaquin Valley of California. Because of their fragility, all bug shipments were hand-carried rather than mailed or sent by courier. Nymphs and adults were maintained in the laboratory on organically grown green beans, navel orangeworm [*Amyelois transitella* (Walker)] eggs, and shelled sunflower seeds. Bugs were kept in cylindrical clear plastic boxes (15 cm diam × 6.5 cm high; Tri State Plastics, Dixon, Kentucky) with screen-covered ventilation holes cut in the tops, at a temperature of 25°C and ambient humidity. Lighting was provided with overhead fluorescent lights, with a 15L:9D cycle. Insects were segregated by sex daily after the final molt to the adult stage. Adults were sexed visually using a binocular microscope; females are readily identified by a distinct groove along the ventral surface of the abdomen into which the ovipositor fits. Voucher specimens were filed with the University of California, Riverside Entomology Museum. A subsample of voucher specimens was conclusively identified by Dr. G. M. Stonedahl, International Institute of Entomology, Natural History Museum, London, England.

Collection of Insect Volatiles. Virgin adults (>4 days old) were carefully coaxed into the top half of a two-piece cylindrical glass aeration chamber

(20 cm long × 7.5 cm diam) lined with window screen for the bugs to perch on, and with an O-ring sealed center joint. A few green beans and sunflower seeds were added for food, the chamber was fitted with an activated charcoal filter and humidifier (made from a glass tube filled with water-soaked glass wool) on the inlet end, and a volatiles collector fashioned from a glass tube (4 mm ID) with a 1-cm-long bed of activated charcoal (50–200 mesh, precleaned by thermal desorbtion at 200°C under N_2; Fisher Scientific, Pittsburgh, Pennsylvania) and held in place by glass wool plugs. Air was pulled through the apparatus (flow 400 ml/min) for one to five days. The charcoal collector was then eluted with pentane (3 × 0.2 ml). Extracts were concentrated as required under a gentle stream of N_2.

To determine the source of the pheromone components, three sexually mature female bugs were anesthetized with CO_2 and cut into legs, head, thorax, and abdomen sections with a razor blade. The body sections were individually soaked in pentane (~100 μl) for ~15 min, and the extracts were transferred to clean vials and analyzed.

Analyses of Extracts. Extracts were analyzed by gas chromatography (GC) using a Hewlett-Packard (Avondale, Pennsylvania) 5890 instrument and by coupled gas chromatography–mass spectrometry (GC-MS) using an HP 5890 GC coupled to a 5970B mass selective detector. The GC was equipped with a DB-17 column (30 m × 0.25 mm ID; J&W Scientific, Folsom, California), using He carrier gas and a program of 50°C/3 min, 10°/min to 250°C. The GC-MS was equipped with a DB-5MS column (20 m × 0.2 mm ID), using He carrier gas, and a temperature program of 50°C/1 min, 10°/min to 250°C. Injections were made in splitless mode (injector 250°C), and all spectra were obtained in electron impact mode (70 eV). Compounds were identified by comparison of retention times on both columns with those of authentic standards and with mass spectra of standards.

Field Trials. Field tests were carried out in pistachio, plum, and almond orchards not treated with insecticides near Parlier and Caruthers, Fresno County, California. Pherocon-1 sticky traps (Trécé, Salinas, California) were used in the first tests in 1995; Pherocon 1C traps were used in all trials in 1996. Traps were hung in the north quadrant of the lower canopy, ca. 2 m high. Baits consisted of 11-mm gray rubber septa (The West Co., Lititz, Pennsylvania) loaded with methylene chloride solutions of the test compounds. Traps were at least 30 m apart in random order, with traps rotated one position at each count. Treatments were replicated at least five times, with traps being counted every one to four days, depending on weather conditions. The first trials were conducted in October and November 1995, with further trials from April to August 1996. All bugs caught were individually sexed and identified, using the ratio of the length of the first antennal segment to the head capsule width across the eyes as a key male character for identification to species (Stonedahl, 1988).

The components in the volatiles extracts varied considerably in molecular weight and volatility (i.e., C_8 to C_{12} esters). Consequently, in experiments to work out the correct blend of components, and to achieve approximately the same profile of volatiles from a rubber septum bait as was seen in GC traces of the collected bug volatiles, the weights of each compound loaded onto a rubber septum were adjusted for differences in volatility using the following derived approximation. The molar entropy of vaporization at a liquid's boiling point at atmospheric pressure is approximately constant, 21 cal/°mol (Trouton's rule) (Daniels and Alberty, 1975). Expressed as an equation,

$$\Delta S_{vap} = \Delta H_{vap}/T_b \cong 21 \text{ cal/°mol}$$

Rearranged,

$$\Delta H_{vap} = 21 \text{ cal/° mol} \times \text{boiling point of the liquid}$$

Furthermore, the ratio of the vapor pressures (P_1 and P_2) of a liquid at different temperatures T_1 and T_2 can be expressed by the equation:

$$\log(P_2/P_1) = \Delta H_{vap}(T_2 - T_1)/2.303RT_1T_2$$

where R is the gas constant. Substituting for ΔH_{vap}, and the boiling point of a liquid at atmospheric pressure for T_2, with P_2 thus equal to 760 mm Hg, and setting T_1 equal to 293°K (20°C), the equation simplifies to:

$$\log(760/P_1) = 0.0157(\text{bp} - 293°\text{K})$$

Substituting the published boiling points of a series of ester model compounds (ethyl acetate to decyl acetate) (Weast, 1976) into this equation gave estimates of the vapor pressure of each ester at 20°C. Examination of this series of values indicated that each increase in chain length of two carbons resulted in a decrease in vapor pressure at 20°C by a factor of ~4. Thus, the weights of C_8 compounds in baits were held fixed, while the weights of C_{10} compounds were increased fourfold, and the weights of C_{12} compounds were increased 16-fold relative to the proportions found in the insect aeration extracts. Once the key components of the pheromone were determined, a blend ratio trial was run to determine the optimum ratio.

In the final trial to determine lure longevity, the attraction to a set of lures left in traps continuously for the period 29 April–18 June, 1996, was compared to attraction of a set of lures changed at biweekly intervals.

Trap catch data were analyzed by analysis of variance (ANOVA), excluding zero totals with no variance from the analyses. Differences between treatments were determined with the Tukey-Kramer honestly significant differences test, at the $P = 0.05$ significance level. Data sets that were not normally distributed, before or after log $(x + 1)$ transformation, were analyzed using the

nonparametric Kruskal-Wallis ANOVA on ranks test, followed by the Student-Newman-Kuels all pairwise comparison procedure for separation of means.

Chemicals. Authentic standards were obtained from the following sources: Hexyl acetate, (*E*)-2-hexenyl acetate, and octyl butyrate, Aldrich Chemical (Milwaukee, Wisconsin); octyl acetate (Sigma Chemical, St. Louis, Missouri); (*E*)-2-octenyl acetate (Bedoukian Research, Danbury, Connecticut). (*E*)-2-Octenyl butyrate was synthesized by dropwise addition of 1.5 equivalents butyryl chloride to 1 equivalent of (*E*)-2-octenol (Alfa Aesar Organics, Ward Hill, Massachusetts) and 2 equivalents pyridine in ether at 0°C. After warming to room temp and stirring 3 hr, water was added and the mixture was stirred a further 2 hr to destroy excess butyryl chloride. The layers were then separated, and the ether layer was washed successively with dilute aq. $NaHCO_3$, HCl, and brine; dried over Na_2SO_4; and concentrated. The crude product was Kugelrohr distilled (oven temp 75°C, 0.1 mm Hg), producing the desired (*E*)-2-octenyl butyrate contaminated with a trace of the corresponding *Z* isomer (2%). (*Z*)-3-octenyl acetate (bp ~55 at 0.15 mm Hg) was synthesized in analogous fashion from (*Z*)-3-octenol (Lancaster Synthesis, Windham, New Hampshire) and acetyl chloride.

RESULTS

Identification of Compounds. The extracts of volatiles from both males and females consisted of compounds with simple structures that were readily identified by GC and GC-MS. In particular, the mass spectra of the saturated ester components were characterized by small but usually discernible molecular ions, large diagnostic fragments separated by 18 mass units corresponding to RCO^+ and $RCOOH_2^+$, and even-numbered fragments from the C_n alcohol moiety of $C_nH_{2n}^+$ and $C_{n-2}H_{2n-4}^+$. Comparison of retention times with those of standards confirmed that the compounds were formed from a straight-chain acid fragment and an unbranched primary alcohol fragment.

The unsaturated esters were characterized by small molecular ions, by a base peak corresponding to RCO^+, and an even-numbered ion corresponding to C_nH_{2n-2}, derived from the C_n unsaturated alcohol moiety. GC and GC-MS runs with standards determined that the (*E*)-2 and (*Z*)-2 isomers, and other positional isomers, were readily resolved on the DB5-MS column, confirming the (*E*)-2 configuration and position of the double bond. Although the saturated and unsaturated esters were incompletely resolved on that column, they were easily and completely separated on the DB-17 column.

There were major qualitative and quantitative differences in the profiles of volatile compounds collected by aeration of adult male and female bugs (Table 1). Hexyl acetate, hexyl butyrate, and octyl acetate were produced by both sexes, albeit in different ratios, while only females produced (*E*)-2-hexenyl

TABLE 1. VOLATILES COLLECTED BY AERATION OF VIRGIN ADULT *Phytocoris relativus*[a]

Compound	Relative amounts	
	Females	Males
Hexyl acetate	100	22.2
Hexyl butyrate	8.3	100
Octyl acetate	5.9	<1
(*E*)-2-Hexenyl acetate	2.2	
(*Z*)-3-Octenyl acetate	2.1	
(*E*)-2-Octenyl acetate	19.0	
(*E*)-2-Octenyl butyrate	7.8	

[a]Collections from 27 female and 21 male 4- to 5-day-old bugs, aerated for 24 hr.

acetate, (*E*)-2-octenyl acetate, (*E*)-2-octenyl butyrate, and (Z)-3-hexenyl acetate. Trace amounts of several other compounds (<0.5% of major component) were also seen in the female extract.

Analysis of extracts of body sections of adult female bugs determined that the pheromone and other components found in aerations were produced exclusively in the thorax. Attempts to locate and dissect out the pheromone-producing glands were not successful.

Bioassays. The first field trial tested a complete reconstruction of the female blend, the subset of compounds produced by both males and females, the subset of female-specific compounds, and each of the female-specific compounds individually (Table 2). Only the complete blend attracted male bugs, indicating that the pheromone consisted of at least one compound common to both sexes plus at least one female-specific compound. Furthermore, only adult male *P. relativus* were trapped, clearly demonstrating that the blend was a stage- and species-specific sex pheromone.

In the second trial, the complete blend was tested versus various blends with partial deletions made by sequentially deleting one or more of the subset of female-produced compounds or one or more of the subset produced by both sexes (Table 3). This experiment revealed that attractive baits had to contain a minimum of hexyl acetate and (*E*)-2-octenyl butyrate. A final test confirmed that the (2)-component bait was as attractive as the original (7)-component reconstructed blend, with the treatments attracting equivalent numbers of bugs (117 vs 110 bugs; combined totals from three replicates each in plum, pistachio, and almond orchards).

Further tests were run to determine the optimum blend ratio, dose, and

TABLE 2. ATTRACTION OF MALE *Phytocoris relativus* TO PARTIAL AND COMPLETE RECONSTRUCTIONS OF FEMALE-PRODUCED BLEND OF VOLATILES[a]

Blend	Hex Ac	Hex Bu	Oct Ac	*E*2Hex Ac	*Z*3Oct Bu	*E*2Oct Ac	*E*2Oct Bu	Trap catch
1	81	5	19	3.6	4.1	100	38	73
2	81	5	19					0
3				3.6	4.1	100	38	0
4						100		0
5						100		0
6					100			0
7				100				1[b]
8	Blank							2[c]

[a]Blend ratios (weight : weight) are expressed as percentages of the largest component, (*E*)-2-octenyl acetate, which was used in fixed doses of 4 mg. Baits were prepared from gray rubber septa loaded with methylene chloride solutions of test compounds. Each septum was loaded with 4 mg of (*E*)-2-octenyl acetate, and the listed percentages (weight : weight) of the other components. Traps were replicated six times and counted four times, October 19–25, 1995. Kearney Agricultural Center, Parlier, California. Abbreviations: Hex Ac, hexyl acetate: Hex Bu, hexyl butyrate; Oct Ac, octyl acetate; *E*2Hex Ac, (*E*)-2-hexenyl acetate; *Z*3Oct Bu, (*Z*)-3-octenyl butyrate; *E*2Oct Ac, (*E*)-2-octenyl acetate; *E*2Oct Bu, (*E*)-2-octenyl butyrate.

[b]Female.

[c]One male, one female.

TABLE 3. ATTRACTION OF MALE *Phytocoris relativus* TO PARITAL AND COMPLETE RECONSTRUCTIONS OF FEMALE-PRODUCED BLEND OF VOLATILES[a]

Blend	Hex Ac	Hex Bu	Oct Ac	*E*2Hex Ac	*Z*3Oct Bu	*E*2Oct Ac	*E*2Oct Bu	Trap catch
17	81	5	19	3.6	4.1	100	38	69 a
18	81	5	19		4.1	100	38	70 a
19	81	5	19			100	38	59 a
20	81	5	19			100		0
21	81	5	19				38	83 a
22	81		19	3.6	4.1	100	38	68 a
23	81			3.6	4.1	100	38	75 a
24	Blank							0

[a]Blend ratios (weight : weight) are expressed as percentages of the largest component, (*E*)-2-Octenyl acetate, which was used in fixed doses of 4 mg[a]. Baits were prepared from gray rubber septa loaded with methylene chloride solutions of test compounds. Each septum was loaded with 4 mg of (*E*)-2-octenyl acetate, and the listed percentages (weight : weight) of the other components. Traps were replicated six times and counted four times. October 25–30, 1995. Kearney Agricultural Center, Parlier, California. Means followed by the same letter are not significantly different, Tukey-Kramer honestly significant differences test ($P = 0.05$). Abbreviations are as in Table 2.

TABLE 4. TRAP CATCHES OF MALE *Phytocoris relativus* IN TRAPS BAITED WITH DIFFERENT BLENDS OF HEXYL ACETATE (Hex Ac) AND (*E*)-2-OCTENYL BUTYRATE (*E*2Oct Bu)[a]

Blend ratio (w/w) (Hex Ac:*E*2Oct Bu)	Trap catch (Mean ± SD)
97:3	5.6 ± 2.9 e
90:10	12.6 ± 3.2 c
80:20	20.0 ± 3.1 a
66:33	26.2 ± 5.8 a
50:50	20.4 ± 5.5 a
33:66	15.4 ± 3.1 b
20:80	9.8 ± 2.3 d
10:90	4.0 ± 1.0 f
3:97	1.2 ± 0.8 g
Blank	1.4 ± 0.9 g

[a]Lure dose, 4 mg. Mean catches per count period were calculated from five replicates of each treatment, counted twice. The experiment was conducted in an almond orchard near Caruthers, Fresno County, California, June 15–19, 1996. Traps were placed in a randomized complete block design, on a 38-m × 33.5-m spacing. Means followed by the same letter are not significantly different at $P = 0.05$, Kruskal-Wallis ANOVA on ranks, followed by Student-Newman-Keuls all pairwise comparison.

TABLE 5. TRAP CATCHES OF MALE *Phytocoris relativus* IN TRAPS BAITED WITH DIFFERENT DOSES OF 2:1 BLEND OF HEXYL ACETATE AND (*E*)-2-OCTENYL BUTYRATE[a]

Dose (mg)	Trap catch (mean ± SD)
33	93.2 ± 9.6 a
10	59.4 ± 7.7 b
3.3	38.2 ± 6.2 c
1	16.6 ± 3.6 d
0.33	12.8 ± 2.9 e
0.1	6.0 ± 3.0 f
Blank	0.4 ± 0.5 g

[a]Each treatment was replicated five times and counted three times, July 15–22, 1996. Traps were hung in an almond orchard in Caruthers, Fresno County, California. Means followed by the same letter are not significantly different at $P = 0.05$, Kruskal-Wallis ANOVA on ranks, followed by Student-Newman-Keuls all pairwise comparison.

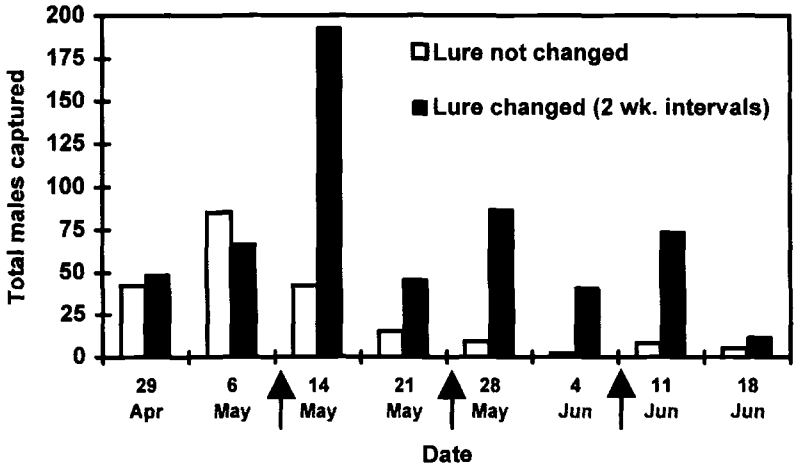


FIG. 1. Collections of *Phytocoris relativus* males in pheromone traps baited with 4 mg pheromone lures, with lures not replaced for eight weeks, or replaced at two-week intervals. Kearney Agricultural Center, Parlier, California; 1996.

longevity. In the blend ratio trial (Table 4), trap catches peaked at the 2:1 ratio of hexyl acetate to (*E*)-2-octenyl butyrate. The 2:1 ratio was very close to the 81:38 ratio used in the first field trial, where adjustments were made to the amounts of compounds added to lures based on their volatilities. In the dose-response trial, trap catch increased with increasing dose over the entire range tested (0.1–33 mg/lure, Table 5). All doses attracted some bugs, including the lowest dose (0.1 mg).

Sequential weekly comparison of the attractiveness of lures aged for a total of eight weeks with lures replaced biweekly indicated that rubber septum lures lost their attractiveness quite rapidly, with attraction to fresh lures being more than four times greater than to two-week-old lures (Figure 1, May 14 trap counts). Furthermore, comparison of trap catches in the week before and after replacement of the lures changed at biweekly intervals showed a clear pattern of increased trap catch in the week following lure replacement (Figure 1), indicating decreased attraction even in the second week of exposure in the field.

DISCUSSION

Despite the importance of a number of true bug species as agricultural pests, relatively few bug pheromones have been identified and developed for use in insect monitoring and management. The chemistry of gland secretions and extracts of volatiles has been the subject of considerable effort, but study

of the function of these chemicals has not kept pace with their identification (Aldrich, 1988). For example, the identification of sex attractant pheromones of only one other mirid bug, the mullein bug *Campylomma verbasci* (Meyer), has been reported (Smith et al., 1991). Females of this species use a blend of butyl butyrate and (*E*)-2-butenyl butyrate [(*E*)-crotyl butyrate] as their sex attractant pheromone, both of which are exclusively female-produced. Analogous to the *P. relativus* pheromone blend, neither component was active alone. However, for *P. relativus*, the pheromone is composed of a saturated ester, which is produced by both sexes, and an unsaturated ester produced exclusively by females. The production of one or more of the components of the female sex pheromone by both sexes has precedent in other bug species. For example, the sex attractant pheromone of the alydid bug *Leptocorisa chinensis* consists of two components, (*E*)-2-octenyl acetate and octanol, which are produced by both sexes. However, the pheromone is reported to be attractive only to males (Leal et al., 1996).

Our strategy of using a reconstruction of the female blend as a starting point for field bioassays proved effective. However, it must be mentioned that this blend was reconstructed from our first large-scale aeration (data not shown), which was discovered after the fact to have been made from a mixed sample of *P. relativus* and its congener, *P. californicus*. These species are difficult to differentiate because of their extremely similar morphology, with the primary distinguishing character for live bugs being a slight difference in the length of the basal antennal segment (Stonedahl, 1988). Further aerations were carried out with subsamples of field collections of bugs, which were separated as best as possible into species. Aeration of each group produced extracts that varied only in the ratios of compounds present; qualitatively, the extracts were very similar. Thus, the ratio of compounds used in the initial reconstruction of the complete female blend may have been skewed, but the blend still contained all of the constituent chemicals. Once field experiments had determined that only two components appeared to be necessary for attraction of *P. relativus*, the blend of the two components was then optimized. Furthermore, the optimized blend was not attractive to males of *P. californicus*.

The strategy of using a reconstructed female blend as the starting point for field bioassays is not universally effective. In ongoing studies, this strategy has been effective with another mirid species, but for two other species, reconstructions containing up to 30 compounds were not attractive to male bugs, despite evidence that virgin females attract males to sticky traps. The reasons for the failure of this strategy in these cases remain to be determined. A similar situation has been reported with the rice bug, *Leptocorisa chinensis*, in which the complete reconstruction of the volatiles blend produced by females was not attractive, but a subset of two of the blend components was highly attractive to males (Leal et al., 1996).

The significance of the apparently redundant compounds in the female-produced volatiles blend is not clear. Some of these compounds, particularly hexyl butyrate, which was also present in males, may be used in defense. One or more of the female-specific compounds also may serve to inhibit attraction of sympatric congeners, such as *P. californicus*, females of which produce volatiles blends with similar constituents but in different ratios. Overall, the production of more compounds than are required for a sex attractant pheromone appears to be common in bug species, with many bugs producing complex bouquets from a variety of different glands (Aldrich, 1988, 1995).

In dose–response trials, trap catches increased steadily with increasing dose, from 0.1 mg/septum up to the highest dose tested (33 mg). Higher doses were not investigated because of the difficulty in absorbing large doses of pheromone into septa. In practical terms, doses of 5–10 mg/septum should represent a good compromise between providing a highly attractive lure for sensitive monitoring, and a lure which is easy to load, that is, which readily absorbs the applied pheromone.

Tolerance for high doses of pheromone has been reported in other bug species. For example, the mullein bug was attracted by lures releasing as much as several milligrams of pheromone per day (McBrien et al., 1994), although it was noted that at release rates of 5 mg/day, trap catches decreased, indicating that there is an upper threshold to acceptable pheromone release rates. In another bug family, doses of pheromone of 100 mg/lure (release rate not reported) were highly attractive to the alydid bug, *Riptortus clavatus* (Leal et al., 1995).

Because the two constituents of *P. relativus* pheromone are different in molecular weight and consequently volatility, the ratio of volatiles emitted by rubber septum dispensers will change quite rapidly as the smaller hexyl acetate evaporates more quickly than the larger, less volatile (*E*)-2-octenyl butyrate. Fortunately, within broad limits, male *P. relativus* do not appear to be particularly sensitive to the blend ratio of the pheromone components because traps baited with ratios of the two components varying from 90:10 to 33:66 caught reasonable numbers of bugs. This relative insensitivity to blend ratio was also indirectly reflected in the fact that lures had some degree of attractiveness to bugs after several weeks in the field, during which time the blend ratio must have changed considerably. Thus, for practical purposes, changes in blend ratio with time should not affect the usefulness of pheromone lures for monitoring bug populations. However, the rapid decline in the overall release rate may need to be addressed, for example, by the release of pheromone through a membrane of fixed size, as opposed to the roughly exponential release from an impregnated solid substrate such as a rubber septum.

In summary, we have identified a female-produced sex pheromone for the mirid bug *P. relativus*. The pheromone is composed of two simple compounds, one of which is produced by both sexes, and the other of which is female-

specific. Male bugs were attracted by a broad range of doses and blend ratios of the pheromone, which should expedite the incorporation of this pheromone into applied insect management programs.

Acknowledgments—We thank Robert Beede and Don Thomas for field collection of insects, J. Steven McElfresh for assistance in the laboratory, Richard Jones for assistance with field tests and data analyses, and T. Dighiera for field trial orchard sites. We also gratefully acknowledge financial support for this project from the California Pistachio Commission.

REFERENCES

ALDRICH, J. A. 1988. Chemical ecology of the Heteroptera. *Annu. Rev. Entomol.* 33:211–238.

ALDRICH, J. A. 1995. Chemical communication in the true bugs and parasitoid exploitation, pp. 318–363, *in* R. T. Cardé and W. J. Bell (eds.). Chemical Ecology of Insects II. Chapman and Hall, New York.

DANIELS, F., and ALBERTY, R. A. 1975. Physical Chemistry, 4th ed. John Wiley & Sons, New York.

LEAL, W. S., IGUCHI, H., MIZUTANI, N., NAKAMORI, H., KADOSAWA, T., and ONO, M. 1995. Multifunctional communication in *Riptortus clavatus* (Heteroptera: Alydidae): conspecific nymphs and egg parasitoid *Ooencyrtus nezarae* use the same adult attractant pheromone as chemical cue. *J. Chem. Ecol.* 21:973–985.

LEAL, W. S., UEDA, Y., and ONO, M. 1996. Attractant pheromone for male rice bug, *Leptocorisa chinensis*: Semiochemicals produced by both males and females. *J. Chem. Ecol.* 22:1429–1437.

MCBRIEN, H. L., JUDD, G. J. R., BORDEN, J. H., and SMITH, R. F. 1994. Development of sex pheromone-baited traps for monitoring *Campylomma verbasci* (Heteroptera: Miridae). *Environ. Entomol.* 23:442–446.

RICE, R. E., BENTLEY, W. J., and BEEDE, R. H. 1988. Insect and Mite Pests of Pistachios in California. University of California, Division of Agriculture and Natural Resources Publication 21452. Oakland, California.

SCHUH, R. T., and SLATER, J. A. 1995. True Bugs of the World (Hemiptera: Heteroptera). Classification and Natural History. Cornell University Press, Ithaca, New York, 336 pp.

SMITH, R. F., PIERCE, H. D., JR., and BORDEN, J. H. 1991. Sex pheromone of the mullein bug, *Campylomma verbasci* (Meyer) (Heteroptera: Miridae). *J. Chem. Ecol.* 17:1437–1447.

STONEDAHL, G. M. 1988. Revision of the mirine genus *Phytocoris* Fallén (Heteroptera: Miridae) for western North America. *Bull. Am. Mus. Nat. Hist.* 188:257 pp.

WEAST, R. C. (ed.). 1976. CRC Handbook of Chemistry and Physics. CRC Press, Cleveland, Ohio.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

PLANT GROWTH AND ARBUSCULAR MYCORRHIZAL FUNGAL COLONIZATION AFFECTED BY EXOGENOUSLY APPLIED PHENOLIC COMPOUNDS

LEADIR L. M. FRIES,[1] RAYMOND S. PACOVSKY,[2] GENE R. SAFIR,* and JOSE OSWALDO SIQUEIRA[3]

Department of Botany and Plant Pathology
Michigan State University
East Lansing, Michigan 48824

(Received September 30, 1996; accepted March 5, 1997)

Abstract—The effects of the application of 0.25 or 1.0 mM *p*-coumaric acid, *p*-hydroxybenzoic acid, or quercetin on growth and colonization of clover (*Trifolium repens* L. cv. Ladino) and sorghum (*Sorghum bicolor* L.) roots by the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* Schenck and Smith were studied. In general, soil application of these compounds at 0.25 mM stimulated plant growth and AM colonization, whereas at 1.0 mM these phenolics were inhibitory to both growth and colonization. Such effects were noted for both clover and sorghum. The results suggest that phenolic compounds, commonly found in many soils, influence the establishment of AM symbioses, and these compounds may have immediate effects on host growth. Studies involving these chemicals and their effects on mycorrhizal associations may provide new insights concerning the importance of the AM symbiosis in agricultural systems. These phenolic compounds may be used as potential soil amendments to enhance AM fungal colonization, and thus, exploit indigenous populations of AM fungi.

Key Words—*Glomus intraradices*, mycorrhizal fungi, phenolics, *p*-coumaric acid, *p*-hydroxybenzoic acid, quercetin, *Sorghum bicolor*, *Trifolium repens*.

INTRODUCTION

Phenolic compounds are an important class of plant secondary metabolites that are released into the soil as leachates or exudates or are provided by decaying

*To whom correspondence should be addressed.
[1]Current address: DTA - UFSM, Santa Maria - R.S. - Brazil.
[2]Current address: EsalQ-USP, Depto De Ciências Do Solo, Caixa Postal 9, 13428-900 Piracicaba-S.P.-Brazil.
[3]Current address: DCS - UFL, Caixa Postal 37, Lavras - MG - 37200 Brazil.

0098-0331/97/0700-1755$12.50/0 © 1997 Plenum Publishing Corporation

plant residues (Siqueira et al., 1991a). Several phenolic compounds act as signal molecules or mediate signal transduction pathways in symbiotic systems (Lynn and Chang, 1990).

Flavonoids, such as chalcones, flavones (luteolin), dihydroxyflavones, flavanones (narigenin), isoflavonoids, flavonols, and anthocyanidins have all been shown to play an essential role in the nodulation of leguminous plants by symbiotic nitrogen-fixing bacteria (Siqueira et al., 1991a). However, the role of flavonoids and other phenolics in the vesicular–arbuscular mycorrhizal association is less well established or understood.

There is increasing evidence that these compounds are capable of influencing plant growth and root colonization by arbuscular mycorrhizal (AM) fungi (Siqueira et al., 1991b), and exogenously applied phenolics may stimulate indigenous populations of AM fungi in the field, resulting in increased plant growth and yields (Siqueira et al., 1992). Concentrations of these compounds in the soil are influenced to a great extent by the native vegetation, soil type, and cropping history (Pederson et al., 1991). Fallow rotations, as well as some perennial or monocropped systems, can either reduce AM root colonization for crops grown in the following season or can cause negative effects on plant growth (Johnson et al., 1992). Unfortunately, there is practically no mechanistic information available regarding the negative effects of phenolics on AM fungi directly. Allelopathic phenolics may affect both AM fungal diversity and the effectiveness of these endophytes in the ecosystem.

Depending on concentration, different flavonoids can either increase or decrease AM fungal spore germination, hyphal growth, and hyphal branching (Gianinazzi-Pearson et al., 1989; Nair et al., 1991; Tsai and Phillips, 1991; Becard et al., 1992; Baptista and Siqueira, 1994). The isoflavones, formononetin and biochanin A, and to a lesser extent the flavone chrysin have been shown to stimulate root colonization and subsequent plant growth in a *Glomus–Trifolium* symbiosis (Siqueira et al., 1991b, c). Formononetin enhanced AM formation and reduced carryover effects following the application of certain herbicides on corn and sorghum (Siqueira et al., 1991c). Since these effects are of practical importance to agriculture, interest in the use of these chemicals is growing.

Other phenolics, such as the phenolic acids, have also been studied and their effects on plant growth have been reported in the literature. They have been commonly found in a variety of soils (Whitehead, 1964), have allelopathic activity (Guenzi and McCalla, 1966), and affect fungal colonization in an AM symbiosis. Ferulic acid, an allelochemical commonly found in asparagus roots, decreased root colonization by AM fungi and reduced the growth of mycorrhizal asparagus (Wacker et al., 1990a). Similarly, the diversity of different AM fungal populations and AM fungal spore density in the soil decreased following asparagus in a crop rotation (Wacker et al., 1990b).

In this study, we chose to work with two different plant species: white

clover (*Trifolium Repens* L.), a legume that produces isoflavinoids, and Sorghum (*Sorghum Bicolor* L. Moench), a grass that does not produce isoflavinoids, but does produce phenolic acids. We wanted to determine if there is a differential response between a legume and a grass to these different phenolic compounds. Prior to this report, the effects of other phenolic acids, such as *p*-coumaric and *p*-hydroxybenzoic acids, on the establishment of AM symbiosis have not been studied. The purpose of this study was to examine the effects of *p*-coumaric acid, *p*-hydroxybenzoic acid, and quercetin applied to the soil on AM formation and growth of white clover and sorghum plants.

METHODS AND MATERIALS

Plant, Substrate and Fungal Material. The effects of three naturally occuring phenolic compounds on mycorrhizae formation and subsequent plant growth were examined using white clover (*Trifolium repens* L. cv. Ladino) and sorghum (*Sorghum bicolor* L. Moench) plants. In all experiments, water-soaked seeds were pregerminated on filter paper for 24 hr and transplanted into plastic pots (4 × 5.5 cm), each containing 80 g of a sterilized sand–soil mix (2 : 1) as the plant-growth substrate. A 9.0-cm-diameter Petri dish was used under each pot to avoid chemical cross-contamination and to facilitate watering. The soil substrate had a pH of 7.1, a cation exchange capacity of 4 meq/100 g soil; and 11.5 μg P, 9 μg K, 720 μg Ca, 47.3 μg Mg, 7 μg Zn, and 34 μg Mn per g of soil (Michigan State University Soil Testing Laboratory). The soil mixture was inoculated with spores of *Glomus intraradices* Schenck and Smith, supplied by Native Plant Inc. (Salt Lake City, Utah). The inoculum was blended with a small amount of soil to release the spores from roots and to facilitate uniform distribution of spores in the soil substrate. Spores (approximately 800 spores per pot) were then incorporated thoroughly into the substrate at rates sufficient to achieve a final spore density of 10 spores/g soil mix in all experiments. Final spore densities were determined by wet-sieving (Gerdemann and Nicolson, 1963), sucrose centrifugation, and then counting spore propagules under a dissecting microscope.

Phenolic Compounds. The phenolic compounds tested, *p*-coumaric acid, *p*-hydroxybenzoic acid, and quercetin (Sigma Chemical Co., St. Louis, Missouri), were first dissolved in 1 ml ethanol, then diluted into a larger volume of distilled water. Solvent controls were prepared using similar volumes of ethanol and water. Concentrations of 0.25 mM and 1.0 mM of the phenolics were used for each compound to treat the clover and sorghum plants. Solutions were freshly prepared for each application. In all experiments, 10 ml of each test solution was applied in the center of each pot (80 g of sand–soil mix) prior to transplanting. We deliberately chose concentrations of 0.25 mM and 1.0 mM with a 10-ml volume for each addition since the total soil substrate volume was

100 cm^3, and the final concentration of the phenolic in the soil would be one tenth of the solution concentration. Therefore, after the first application of the various compounds, the soil concentration would be 25 μM or 100 μM for the 0.25 mM or 1.0 mM solution concentration, respectively. These levels of phenolic acids or isoflavonoids have been commonly found in agricultural soils (Siquiera et al., 1991).

The Experiments. Several growth-chamber experiments were conducted in which we varied the frequency of application of phenolic compounds. All experiments were harvested three weeks after emergence.

The first three experiments involved white clover. In the first experiment, the substrate received 10 ml of solution containing 0.25 mM and 1.0 mM of each compound only once. In the second experiment, 10 ml of each solution of each compound was added to the substrate every seven days. In a third experiment, the frequency of application was increased to every four days.

The fourth and fifth experiments were conducted with sorghum plants. In the fourth experiment, sorghum plants received 10 ml of each compound every seven days. In the fifth experiment, sorghum plants received 10 ml of each compound every four days. The frequency of application was varied to determine the effect of maintaining a more uniform concentration of the phenolic compound in the soil while the experiment progressed.

Growth Conditions. After transplanting, plants were kept in a growth chamber with 300 μmol/m/sec of photosynthetically active light with 14 hr light/10 hr dark, and 28°C or 21°C day or night temperatures, respectively. Plants were watered once a day with distilled water, except when nutrient or phenolic solutions were applied. Half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) was applied (10 ml/pot) three times a week. At three weeks after emergence, plants were harvested and assessed for growth and root colonization. Shoots were separated from roots, weighed, and then dried at 70°C for two days. Roots were washed free of soil, patted dry, fresh weight determined, and then dried at 70°C for two days. A weighed root subsample was removed, cleared, and stained with trypan blue (Phillips and Hayman, 1970). Root colonization, expressed as a percentage of root segments colonized by the AM fungus, was estimated by the line-interest method (Kormanik and McGraw, 1982).

Experimental Design and Statistical Analysis. All experiments were conducted in a completely randomized design with at least 12 plants per treatment, and experiments were repeated at least once, with similar results. Data reported are means from one experiment derived from a complete analysis of variance using the MSTAT statistical package program. Shoot dry weight, root dry weight, and root colonization by *G. intraradices* of white clover and sorghum plants were analyzed and compared by least significant difference (LSD) tests

($P < 0.05$). Experiments involving clover and sorghum plants are presented in a separate table.

RESULTS

Clover. At the lower concentration (0.25 mM), shoot and root dry weights of phenolic-treated mycorrhizal clover plants were significantly greater than that of control plants grown in the absence of the compounds (Table 1, experiment 1). Quercetin also increased clover growth (shoot and root) when the substrate was amended with the 1.0 mM solution. At the 1.0 mM levels, shoot dry weights for plants treated with *p*-coumaric acid and *p*-hydroxybenzoic acid did not differ from control plants, while root growth was greater only in the presence of *p*-coumaric acid.

Percent root colonization for clover was enhanced by all compounds at both concentrations used (Table 1). Application of *p*-hydroxybenzoic acid and quercetin promoted the highest percent root colonization at the 0.25 mM concentration (53 and 57% increase, respectively). Clover plants treated with *p*-coumaric acid had the lowest root colonization when it was applied at 1.0 mM (25%). It might appear that 57%, 53%, and 25% root colonization is low for clover plants, but this is only at 21 days after emergence. Rapid colonization of the root is going on during the early period of the host–endophyte association.

When phenolics were applied every seven days at a concentration of 0.25 mM (Table 1, experiment 2), shoot dry weights for treated plants were not statistically significantly different from that of controls. However, shoot dry weights were reduced for clover plants treated with a 1.0 mM concentration of the phenolics. Plants treated with *p*-hydroxybenzoic acid at 0.25 mM had the greatest relative root growth compared to controls. Relative root growth greatly decreased when plants were treated with 1.0 mM *p*-hydroxybenzoic acid, and treatment with 1.0 mM quercetin reduced root growth, but this reduction was not statistically significant compared to the untreated control. Percent root colonization was higher when *p*-coumaric acid and *p*-hydroxybenzoic acid were applied at 0.25 mM (Table 1, experiment 2) compared to controls that received no phenolics. At 1.0 mM, these compounds stimulated less fungal spread. Root colonization for plants provided with quercetin at 0.25 mM had percent root colonization indistinguishable from control plants, whereas 1.0 mM quercetin significantly inhibited the root colonization by *G. intraradices* (18%).

In a subsequent experiment, phenolic compounds were applied every four days (Table 1, experiment 3). Only *p*-hydroxybenzoic acid-treated clover had a significant increase in terms of shoot dry weight at 0.25 mM (44% over the control), while addition of *p*-coumaric acid and quercetin had no effect on shoot dry weight at this concentration. At 1.0 mM, all compounds applied to clover

TABLE 1. GROWTH AND ROOT COLONIZATION OF *Glomus intraradices*-INOCULATED CLOVER PLANTS AS AFFECTED BY PHENOLIC COMPOUNDS AT DIFFERENT CONCENTRATIONS AND FREQUENCIES OF APPLICATION[a]

Chemicals and solution concentration (mM)	Experiment 1—one application			Experiment 2—three applications			Experiment 3—five applications		
	Shoot dry weight (mg)	Root dry weight (mg)	Root colonization (%)	Shoot dry weight (mg)	Root dry weight (mg)	Root colonization (%)	Shoot dry weight (mg)	Root dry weight (mg)	Root colonization (%)
None	7.86 b	3.64 c	51 d	6.99 a	2.30 abc	59 bcd	4.64 bc	1.95 bc	47 b
p-Coumaric acid—0.25	15.10 a	9.73 a	68 bc	7.52 a	3.04 ab	66 a	5.41 b	2.39 ab	67 a
p-Coumaric acid—1.00	9.84 b	6.09 b	65 c	5.01 b	2.15 bc	61 abc	3.50 de	1.19 c[d]	35 c
p-Hydroxybenzoic acid—0.25	15.30 a	8.85 a	79 a	6.89 a	3.38 a	64 ab	6.67 a	3.06 a	69 a
p-Hydroxybenzoic acid—1.00	9.53 b	4.88 bc	74 ab	4.93 b	1.54 c	53 de	3.15 e	2.25 b	27 c
Quercetin—0.25	15.03 a	8.64 a	81 a	7.15 a	3.08 ab	56 cd	4.17 cd	2.22 b	49 b
Quercetin—1.0	14.70 a	9.19 a	74 ab	4.74 b	1.90 c	48 e	2.67 e	2.01 b	27 c

[a]Means (12 replicates per treatment) followed by the same letter within a column are not significantly different by least significant difference test (LSD < 0.05).

significantly decreased shoot growth compared to nontreated controls. At 0.25 mM, plants treated with *p*-hydroxybenzoic acid had the highest root dry weight, while at 1.0 mM, clover grown in soil amended with *p*-coumaric acid had the lowest root dry weight (39% decreased). *p*-Coumaric and *p*-hydroxybenzoic acids improved clover root colonization (approximately 45% each) at 0.25 mM (Table 1, experiment 3). At this concentration, quercetin-treated clover roots did not differ from control roots. When applied at 1.0 mM, all the exogenously applied compounds significantly reduced root colonization. *p*-Hydroxybenzoic acid-treated and quercetin-treated clover plants caused a 43% decrease in root colonization compared to the control, while *p*-coumaric acid caused a 26% decrease.

Sorghum. Phenolic compounds were exogenously applied to sorghum plants every seven days (Table 2, experiment 4). All compounds at a concentration of 0.25 mM improved sorghum shoot and root dry weights. *p*-Coumaric acid treatment significantly increased sorghum shoot dry weight (19%), followed by *p*-hydroxybenzoic acid (14%) treatment. To a lesser extent, quercetin (7%) treatment resulted in a slight effect, although this was not significantly different from control plants. Root dry weight was significantly increased by *p*-hydroxybenzoic acid (42%), *p*-coumaric acid (19%), and quercetin (11%). At 1.0 mM, the phenolic treatments did not have an effect on the shoot and root dry weights for sorghum.

All compounds significantly stimulated root colonization by *G. intraradices* at 0.25 mM (Table 2, experiment 4). Relative stimulation of root colonization (72%) by 0.25 mM *p*-coumaric acid led to a significant increase in sorghum growth, while treatment with 1.0 mM *p*-coumaric acid had no effect on sorghum colonization. Only *p*-hydroxybenzoic acid application at 1.0 mM increased root colonization, but this increase in colonization of sorghum roots did not result in improved sorghum growth.

When the phenolics were applied every four days (Table 2, experiment 5), shoot dry weight of treated sorghum was not stimulated by any compound. At 0.25 mM, shoot dry weight was similar between controls and *p*-coumaric acid-treated plants. Shoot dry weight was less than controls when either *p*-hydroxybenzoic acid or quercetin was added. Root growth for sorghum that received 0.25 mM of the phenolics was similar to control roots. At 1.0 mM, all compounds were inhibitory for sorghum shoot and root growth relative to the nontreated controls.

p-Hydroxybenzoic acid significantly increased root colonization by *G. intraradices* relative to the control when applied at 0.25 mM (Table 2, experiment 5). Treatment with 0.25 mM *p*-coumaric acid or quercetin did not alter root colonization compared to control roots. At the higher concentration (1.0 mM), a significant decrease in root colonization was observed in all phenolic-treated plants.

TABLE 2. GROWTH AND ROOT COLONIZATION OF *Glomus intraradices*-INOCULATED SORGHUM PLANTS AS AFFECTED BY PHENOLIC COMPOUNDS AT DIFFERENT CONCENTRATIONS AND FREQUENCIES OF APPLICATIONS[a]

Chemicals and solution concentration (mM)	Experiment 4—three applications			Experiment 5—five applications		
	Shoot dry weight (mg)	Root dry weight (mg)	Root colonization (%)	Shoot dry weight (mg)	Root dry weight (mg)	Root colonization (%)
None	82.40 cd	57.80 c	18 e	84.61 a	60.21 a	19 bc
p-Coumaric acid—0.25	97.98 a	68.96 b	31 a	79.79 ab	61.85 a	19 b
p-Coumaric acid—1.00	81.23 cd	55.11 cd	18 e	60.75 d	45.19 cd	13 ef
p-Hydroxybenzoic acid—0.25	94.32 ab	82.29 a	27 b	78.04 bc	61.48 a	24 a
p-Hydroxybenzoic acid—1.00	85.58 c	57.08 cd	23 cd	62.91 d	49.38 bc	14 de
Quercetin—0.25	88.44 bc	64.13 b	26 bc	72.63 c	55.20 ab	18 cd
Quercetin—1.00	76.55 d	51.28 d	20 de	54.84 e	41.71 d	11 f

[a]Means (12 replicates per treatment) followed by the same letter within a column are not significantly different by least significant difference test (LSD < 0.05).

DISCUSSION

The early establishment of AM symbioses and plant growth depended greatly upon the concentration and frequency of application of solutions containing phenolic compounds. All compounds stimulated clover root colonization by *G. intraradices* at three weeks when applied once to the substrate, regardless of concentration. However, positive plant growth responses were observed only when these compounds were applied at the concentration of 0.25 mM, except with quercetin, which also stimulated clover plant growth at a concentration of 1.0 mM. When frequency of application was increased to either every four or seven days, only *p*-coumaric and *p*-hydroxybenzoic acids stimulated the AM colonization at 0.25 mM. Three-week-old sorghum plants were also stimulated by all compounds when applied at 0.25 mM concentration every seven days, but only *p*-coumaric acid and *p*-hydroxybenzoic acid enhanced plant growth in a statistically significant manner. Similarly, appplications of *p*-coumaric acid every four days caused a decrease in root colonization.

Recently, it has been suggested that root flavonoids act as AM-stimulating compounds when present at low concentrations (Siqueira et al., 1991b). Quercetin has been shown to enhance spore germination and hyphal growth of *Glomus* and *Gigaspora* species (Tsai and Phillips, 1991; Becard et al., 1992; Baptista and Siqueira, 1994) when present in low concentrations. Therefore, quercetin was capable of influencing the early stages of mycorrhizal formation and affected root colonization at the onset of mycorrhizal establishment. The timing of the clover and sorghum harvest at 21 days after emergence was intentional. We wished to examine an early point in host–endophyte development when the formation of mycorrhizae was occurring rapidly. However, increasing the number of soil applications impaired the effect of quercetin by causing the soil concentration of quercetin to increase to a point where it was inhibitory (Becard et al., 1992). Quercetin seems to have a very narrow range of concentrations that stimulate AM fungal colonization.

In sorghum, stimulation of root colonization by quercetin at 0.25 mM was less than that achieved by the phenolic acids, and it did not improve plant growth, in spite of the enhanced root colonization. Considering that flavonoids are found primarily in legumes and have been found in low concentrations in soils, their effect on AM symbioses of gramineous plants may not be as relevant as in leguminous plants. Becard and Piche (1989) proposed that certain root exudates regulate the ability of the AM fungus to use its endogenous lipid-rich reserves, although there are different stages throughout the process of interaction between root and mycorrhizal fungus at which the process may be halted (Giovannetti et al., 1994). If quercetin is involved in stimulating AM fungal colonization, similar to the effect achieved with formononetin (Siqueira et al.,

1991b), then there is a possibility that at low concentrations stimulatory effects on growth and root colonization could occur. The mechanisms by which quercetin and flavonoids stimulate spore germination, hyphal growth, branching (Tsai and Phillips, 1991; Becard et al., 1992), and root colonization (as reported here) are unknown and cannot be determined from these experiments.

Phenolic acids, another group of biologically active phenolics, have been isolated from a variety of plants and soils (Siqueira et al., 1991a). *p*-Coumaric and *p*-hydroxybenzoic acids can persist for long periods (Whitehead, 1964) and have been widely studied because of their allelopathic effects. Pederson et al. (1991) indicated that phenolic acids, produced by asparagus plants, could contribute to declines in production in multiyear asparagus plantings. In spite of their negative effects on plant growth (Guenzi and McCalla, 1966), they have never been considered as mycorrhizal regulators.

In our study, *p*-coumaric acid showed a stimulatory effect on AM colonization and growth of sorghum plants when applied at low concentrations. However, at the higher concentration or when applied more frequently, *p*-coumaric acid produced pronounced inhibitory effects. Because it is known that sorghum plants release large quantities of *p*-coumaric acid into the soil (Guenzi and McCalla, 1966), inhibitory effects of *p*-coumaric acid on sorghum growth were unexpected. Nutrient limitation may have contributed to these effects (see Siqueira et al., 1991a for reference); the nutrient solution applied three times a week may not have been sufficient for adequate sorghum growth. *p*-Hydroxybenzoic acid exhibited effects on AM colonization and plant growth similar to those of *p*-coumaric acid.

As AM symbioses are very widespread in the plant kingdom and phenolic compounds are ubiquitous in soils, it is likely that micromolar concentrations of these compounds can stimulate growth of AM fungi and root colonization. However, the plant growth response will depend on factors such as the effectiveness of the AM host at nutrient assimilation, soil nutrient availability, and the efficiency of the specific host-fungal species association. Typically, AM fungi are considered nonspecific to host, but there is increasing evidence that some degree of preference exists between AM fungi and plants (Krishna et al., 1982). Significant differences in colonization potential of 30 genotypes of pearl millet were detected, and it was suggested that phenolics and phytoalexins may be involved. An additional question that we have not addressed, but that is a fruitful area of research, involves the changes in the populations or activities of other soil microorganisms in response to changes in the concentration of soil phenolics.

Giovannetti et al. (1994) speculated that only the perception of the right chemical signals, coming from the roots of host plants, promotes differential morphogenesis of AM hyphae in the rhizosphere and that root cell penetration by the fungus depended on the host genome. Therefore, exogenously applied

phenolic compounds could enhance initial stages of AM establishment, but root penetration and AM development is likely to be regulated by the host plant and subsequent interactions with the fungal partner.

Inhibition of host growth and root colonization were observed when phenolics were applied to the substrate at higher concentrations. In field situations, phenolics can build up in soils and have been shown to selectively influence soil microbial populations (Hartley and Whitehead, 1985). Declines in AM fungal populations in the field can negatively affect the growth of crop plants. In this way, subsequent crops can become more stressed, thus favoring syntheses and accumulation of these phenolic compounds (Siqueira et al., 1991a), as is the case with asparagus (Wacker et al. 1990a, b; Pederson et al., 1991).

It has been suggested that AM fungi are involved in the decline of other monocrop systems and that they account for the beneficial effects of crop rotation for corn and soybeans (Johnson et al., 1992). Changes in AM fungal populations appear to be involved, but there is no reasonable explanation for such shifts in population considering the lack of specificity AM symbioses. As discussed by Siqueira et al. (1991a), monocropping systems allow the build up of phenolic compounds in the soil, which in turn may have an impact on soil microorganisms, including AM fungi. In addition, some soil microorganisms may selectively utilize these phenolics as carbon sources, thus ameliorating their phytotoxic effects (Vaughan et al., 1983). Obviously there is a complex interrelationship involving these phenolics and the ecology of the soil–plant system.

In conclusion, phenolic acids and quercetin can affect AM establishment as well as plant growth. Phenolic acids, commonly found in soils, seem to have less impact on AM symbioses, whereas quercetin may have a greater impact on regulating the AM symbioses, at least in legumes. The build-up of these compounds in the soil also may negatively affect the formation of mycorrhizal symbioses.

Plants and their mycorrhizal fungi have coevolved to form a valuable symbiosis. Phenolics are among the most widespread classes of secondary metabolites produced by plants, and AM fungi can form symbiotic associations with most plant species. Therefore, the development of mycorrhizal associations and the involvement of phenolics as regulatory molecules should not be overlooked. Investigators studying various AM symbioses under monocropped or rotation systems may wish to include a quantification of phenolics present in the soils. Such information would improve our understanding of these symbioses and further improve our potential to use these commonly found fungi more effectively in developing sustainable agricultural systems.

Acknowledgments—The research was supported in part by the Michigan Agricultural Experiment Station and Rhizotech Inc. (Hopewell, New Jersey). In addition, L. L. M. Fries is grateful to CAPES-Brazil for providing a scholarship. Mention of a proprietary product does not constitute an endorsement or recommendation for its use by Michigan State University.

REFERENCES

BAPTISTA, M. J., and SIQUEIRA, J. O. 1994. Efeito de flavonoides na germinação de esporos e do crescimento assimbiótico do fungo micorrízico arbuscular *Gigaspora gigantea*. *Rev. Bras. Fisiol. Veg.* 6:127–134.

BECARD, G., and PICHE, Y. 1989. New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. *New Phytol.* 112:77–83.

BECARD, G., DOUDS, D. D., and PFEFFER, P. E. 1992. Extensive in vitro hyphal growth of vesicular–arbuscular mycorrhizal fungi in the presence of CO_2 and flavonols. *Appl. Environ. Microbiol.* 58:821–825.

GERDEMANN, J. W., and NICOLSON, T. H. 1963. Spores of mycorrhizal endogone species extracted from soil by wet sieving and decanting. *Trans. Br. Mycol. Soc.* 46:235–244.

GIANINAZZI-PEARSON, V., BRANZANTI, B., and GIANINAZZI, S. 1989. In vitro enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. *Symbiosis* 7:243–255.

GIOVANNETTI, M., SBRANA, C., and LOGI, C. 1994. Early processes involved in host recognition by arbuscular mycorrhizal fungi. *New Phytol.* 127:703–709.

GUENZI, W. D., and MCCALLA, T. M. 1966. Phenolic acids in oats, wheat, sorghum, and corn residues and their phytotoxicity. *Agron. J.* 58:303–304.

HARTLEY, R. D., and WHITEHEAD, D. C. 1985. Phenolic acids in soils and their influence on plant growth and soil microbial processes, pp. 109–149, *in* D. Vaughan and R. E. Malcolm (eds.). Soil Organic Matter and Biological Activity. Nijhoff, Boston.

HOAGLAND, D. R., and ARNON, D. I. 1950. The water-culture method of growing plants without soil. California Agricultural Experiment Station Circular 347.

JOHNSON, N. C., COPELAND, P. J., CROOKSTON, R. K., and PFLEGER, F. L. 1992. Mycorrhizae: Possible explanation for yield decline with continuous corn and soybean. *Agron.J.* 84:387–390.

KORMANIK, P. P. and MCGRAW, A. C. 1982. Quantification of vesicular-arbuscular mycorrhizae in plant roots, pp. 37–45, *in* N. C. Schenck (ed.). Methods and Principles of Mycorrhizal Research. American Phytopathological Society, St. Paul, Minnesota.

KRISHNA, K. R., BALAKRISHNA, A. N., and BAGYARAJ, D. J. 1982. Interactions between a vesicular-arbuscular mycorrhizal fungi and *Streptomyces cinnamoneous* and their effects on finger millet. *New Phytol.* 92:401–405.

LYNN, D. G., and CHANG, M. 1990. Phenolic signals in cohabitation: Implications for plant development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 41:497–526.

NAIR, M. G., SAFIR, G. R., and SIQUEIRA, J. O. 1991. Isolation and identification of vesicular-arbuscular mycorrhiza stimulatory compounds from clover (*Trifolium repens*) root. *Appl. Environ. Microbiol.* 57:434–439.

PEDERSON, C. T., SAFIR, G. R., SIQUEIRA, J. O., and PARENT, S. 1991. Effect of phenolic compounds on asparagus mycorrhiza. *Soil Biol. Biochem.* 5:491–494.

PHILLIPS, J. M., and HAYMAN, D. S. 1970. Improved procedure for clearing roots, and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* 55:158–161.

SIQUEIRA, J. O., SAFIR, G. R., and NAIR, M. G. 1991a. Significance of phenolic compounds in plant-soil-microbial systems. *Crit. Rev. Plant Sci.* 10:63–121.

SIQUEIRA, J. O., SAFIR, G. R., and NAIR, M. G. 1991b. Stimulation of vesicular-arbuscular mycorrhizae formation by flavonoid compounds. *New Phytol.* 118:87–93.

SIQUEIRA, J. O., SAFIR, G. R., and NAIR, M. G. 1991c. VA-mycorrhizae and mycorrhiza stimulating isoflavonoid compounds reduce plant herbicide injury. *Plant Soil* 134:233–242.

SIQUEIRA, J. O., BROWN, D. G., SAFIR, G. R., and NAIR, M. G. 1992. Field application of the VAM stimulating isoflavonoid formononetin on corn and soybean in Brazil, p. 132, *in* The International Symposium of Managment of Mycorrhizae. Perth, University of Western Australia.

TSAI, S. M., and PHILLIPS, D. A. 1991. Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores in vitro. *Appl. Environ. Microbiol.* 57:1485–1488.

VAUGHAN, D., SPARLING, G. P., and ORD, B. G. 1983. Ameriolation of the phytotoxicity of phenolic acids by some soil microbes. *Soil Biol. Biochem.* 15:613–614.

WACKER, T. L., SAFIR, G. R., and STEPHENS, C. T. 1990a. Effects of ferulic acid on *Glomus fasciculatum* and associated effects on phosphorus uptake and growth on asparagus (*Asparagus officinalis*). *J. Chem. Ecol.* 16:901–909.

WACKER, T. L., SAFIR, G. R., and STEPHENSON, S. N. 1990b. Evidence for succession of mycorrhizal fungi in Michigan asparagus fields. *Acta Hortic.* 271:273–279.

WHITEHEAD, D. C. 1964. Identification of *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids in soils. *Soil Sci.* 202:417–418.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

PRELIMINARY EVIDENCE SUPPORTING THE ABILITY OF HERMATYPIC CORALS TO AFFECT ADVERSELY LARVAE AND EARLY SETTLEMENT STAGES OF HARD CORAL COMPETITORS

R. J. FEARON* and ANN M. CAMERON

Department of Zoology,
The University of Queensland,
St Lucia 4072, Brisbane, Australia

(Received September 4, 1996; accepted March 9, 1997)

Abstract—Crude, aqueous extracts of five species of hermatypic coral from three scleractinian families were assayed for bioactivity against the early life stages of scleractinian corals. At concentrations between 62.5 and 250 μg/ml, extracts of all species were lethal to at least three of the five species of coral planulae larvae tested. To test for behavioral changes, extracts from four species were tested on planulae of the scleractinian *Pocillopora damicornis*. All four caused immediate but reversible behavioral aberrations in the larvae at concentrations as low as 7.8 μg/ml. To determine the effects on newly settled corals, postmetamorphosis juveniles (spat) of *P. damicornis* were exposed to extracts from four hermatypic species. They were either killed or suffered a reduction in growth rates. If released under natural conditions, substances with lethal or sublethal activities against the early life stages of other scleractinians could allow hermatypic corals to influence patterns of scleractinian recruitment, thus playing a role in ordering coral communities.

Key Words—Larvae, scleractinian, hermatypic, coral, settlement inhibitor, chemical defense, larvotoxin, antifouling, recruitment, community structure.

INTRODUCTION

The possession of chemical defenses against episettlement by fouling organisms is common to many sessile marine invertebrates (see reviews by Davis et al., 1989, and Pawlik, 1992). These animals, which are seldom fouled under natural conditions, possess larvotoxic or noxious allelochemicals that affect the larvae

*To whom correspondence should be addressed.

0098-0331/97/0700-1769$12.50/0 © 1997 Plenum Publishing Corporation

or spores of other marine organisms negatively. Such substances may be lethal or may inhibit larvae from settling on the internal or external surfaces of the animals that produce them. However, many competitors pose a threat not through direct episettlement but by settling near an established organism, growing, and competing with it in the future.

Some benthic organisms living in communities where competition is intense possess toxins effective against larvae of potential sessile competitors other than fouling organisms (e.g., Thompson, 1985; Wahl, 1989; Fearon and Cameron, 1996; but see Bingham and Young, 1991). Maida et al. (1995a,b) found that under natural conditions two species of alcyonacean (soft) corals exuded allelochemicals that deterred settlement of potential competitors at distances of 5 cm. Thus, some sessile animals may influence the levels of recruitment of potential competitors in their immediate area by exuding toxic or deterrent allelochemicals. Such allelopathic interactions have in the past been proposed as a mechanism whereby established sessile organisms might influence succession in benthic communities (Goodbody, 1961; Breitburg, 1985; Bakus et al., 1986).

In coral reef communities where competition can be intense and a large number of organisms possess bioactive natural products (Cameron, 1974; Bakus, 1981), interactions involving allelopathy have been proposed as an ordering mechanism of benthic assemblages (Endean and Cameron, 1983, 1990). Although many reef organisms have been examined for allelopathic substances, little attention has been directed to the scleractinian corals that make up a large component of the living cover and are the chief builders of coral reefs. Reef building (hermatypic) corals compete with each other by a variety of mechanisms and may exude allelochemicals that adversely affect adult corals at a distance (Gunthorpe and Cameron, 1990a; Lang and Chornesky 1990). Their ability to harm adult corals raises the question as to whether hermatypic corals can affect the settlement and recruitment of competitors through allelopathy.

Fearon and Cameron (1996) found that crude extracts of the hermatypic coral *Goniopora tenuidens* caused behavioral modifications in larvae of other hard corals at low concentrations and were lethal to larvae and early post-metamorphic stages at high concentrations. The extracts used were made from tissue suspensions in which the associated nematocysts were intact, having not released their toxins. The authors proposed that the sublethal and lethal activities reflected the possession of allelopathic substances that could inhibit larval settlement if released under natural conditions. However, *G. tenuidens* is unusual among scleractinian corals in possessing relatively potent toxins (see Hashimoto and Ashida, 1973; Gunthorpe and Cameron, 1990b) and consequently has been one of the corals most studied with respect to natural products (see Noda et al., 1985; Qar, 1986). If toxins of *G. tenuidens* are unusually potent, the allelopathic

activity may represent a special case rather than being indicative of toxicity in corals in general.

The aim of this study was to determine if hermatypic corals other than *G. tenuidens* have the potential to inhibit settlement of the larvae of other scleractinian corals, one of their main competitors. In this preliminary study, crude aqueous extracts were favored over purified toxins because the numbers and types of toxins possessed by hard corals may vary between or within species (Gunthorpe and Cameron, 1990b,c) and thus more than one substance may be responsible for toxic effects (see e.g., Gerhart et al., 1988) possibly acting synergistically (see e.g., Hessinger and Lenhoff, 1976; Becerro et al., 1995). The activity of crude extracts represents the minimum potency of actual toxins given that they are diluted with other nontoxic components.

METHODS AND MATERIALS

Collection of Specimens. Hard corals were collected at Heron Island Reef (23°27′S, 151°55′E), Great Barrier Reef (GBR), Australia. Specimens of *Platygyra daedalea* (Ellis and Solander, 1768), *Goniastrea favulus* (Dana, 1846), *Favia matthai* Vaughan, 1918, *Pavona decussata* (Dana, 1846), and *Fungia fungites* (Linné, 1758) were collected between December and March and frozen at −70°C. Thus, all specimens were obtained after the annual mass spawning of corals on the GBR (Harrison et al., 1984). Hard corals used as a source of gametes or larvae were *G. favulus*, *Platygyra daedalea*, *Fungia fungites*, *Oxypora lacera* (Verrill, 1864), and *Pocillopora damicornis* (Linné, 1758), and they were kept in aquaria supplied with a constant flow of seawater. Planulae were obtained using the methods of Fearon and Cameron (1996).

Preparation of Extracts. Extracts were made from pooled groups of corals such that each extract was derived from at least three colonies (or individuals of the solitary coral *F. fungites*). Two extracts were made for each of *G. favulus*, *Platygyra daedalea*, and *Pavona decussata* and one from each of *F. fungites* and *Favia matthai*. The extraction methods of Fearon and Cameron (1996) were used. Briefly, frozen colonies were thawed and the soft tissues were blown off the corallites using a stream of compressed air. The tissues were suspended in 400–500 ml of distilled water, left at 4°C for 72 hr with occasional stirring, and then centrifuged for 10 min at 4080*g*. The supernatants were filtered twice through a double thickness of Whatman No. 1 filter paper under vacuum and the filtrate frozen at −16°C and then freeze dried. This resulted in a crude aqueous extract of all the soft tissues of a coral in the form of a powder that was stored −16°C. The suspensions from which the extracts were made contained nematocysts that were mostly undischarged, having not released their toxins. When required for bioassays, a known mass of the extract was mixed

thoroughly in seawater that had been filtered through a 5-μm sieve (filtered SW). The suspension was centrifuged (2000g, 5 min) to rid it of undissolved particles and the supernatant used as crude extract in bioassays. Controls were exposed to equivalent volumes of filtered SW alone.

Bioassays. Three sets of experiments were performed using the methods developed by Fearon and Cameron (1996). In the first set larvae were placed in 50- or 10-ml containers in filtered SW and subjected to a known concentration of extract. The number of larvae used in each container, the number of replicate containers, and the number of concentrations tested varied depending on availability of larvae (Table 1). Larvae were monitored over seven days. In each experiment, mean numbers of surviving larvae in replicate containers were compared each day with that of the appropriate control using a Kruskal-Wallis one-way analysis of variance (ANOVA) with ranks followed by Dunnett's test (SigmaStat 1.0; Jandel Corporation, 1994). Lethal extracts were those that reduced mean survival to levels significantly lower than that of controls ($P < 0.05$).

In the second set of experiments, the behavior of larvae of *P. damicornis*, immediately after addition of extracts, was tested by placing individual larvae in filtered SW in wells of sterile ELISA Trays (Disposable Products). Extract was added to the wells and larvae monitored continuously for 2 min and then examined at 20 and 60 min. Changes in larval behavior compared with that of control larvae were recorded. Extracts from four species of coral were tested at concentrations of 500, 62.5, and 7.8 μg/ml (Table 2) and each concentration was tested on five larvae.

The third set of experiments tested the effects of extracts on the postsettlement stage (spat) of *Pocillopora damicornis*. Larvae were allowed to settle on the sides and base of plastic containers that were then cut into strips, each with one healthy spat. Five strips were tied together with nylon line, weighted with glass, and placed in 50-ml assay containers such that all spat faced upwards and were further than 5 mm apart. Each extract tested was added to four containers at 62.5 μg/ml, and the spat were monitored for seven days. Controls were treated with filtered SW alone. Each day, spat were measured ($\pm$0.05 mm) along their longest and shortest axes and the mean diameter calculated.

RESULTS

Extracts of all five species were lethal to larvae of at least two species at one or more of the concentrations tested (Table 1). Mean survival of control larvae was never less than 75% and was usually greater than 90%. The number of days required for each extract to exhibit a lethal effect on larvae of a particular species varied between extracts. There was also variation in the effects of each

TABLE 1. LETHAL EFFECTS OF EIGHT EXTRACTS OF HERMATYPIC CORALS TO LARVAE OF CORALS GIVEN BY THE NUMBER OF DAYS TAKEN FOR MEAN NUMBERS OF LARVAE EXPOSED TO EXTRACTS TO BE SIGNIFICANTLY REDUCED COMPARED WITH CONTROLS[a]

	Mortality of five species of coral larvae				
Extract type: Family, species, concentration	*Pocillopora damicornis* $N = 4$ (7)	*Platygyra daedalea*[b] $N = 4$ (12)	*Fungia fungites* $N = 5$ (10–20)	*Oxypora lacera*[b] $N = 4$ (12)	*Goniastrea favulus* $N = 4$ (10)
Faviidae					
Goniastrea favulus (extract 1)					
250 μg/ml	+++	+	+++	−	−
125 μg/ml	++	−	−	n.t.	−
62.5 μg/ml	−	−	−	n.t.	−
G. favulus (extract 2)					
250 μg/ml	++	+	+++	−	n.t.
Platygyra daedalea (extract 1)					
250 μg/ml	++	++	++	−	n.t.
125 μg/ml	++	−	−	n.t.	−
62.5 μg/ml	+	−	−	n.t.	n.t.
P. daedalea (extract 2)					
125 μg/ml	++	−	+	−	n.t.
Favia matthai					
250 μg/ml	+++	+	+++	+	n.t.
125 μg/ml	+++	−	−	n.t.	++
62.5 μg/ml	+	−	−	n.t.	n.t.
Fungiidae					
Fungia fungites					
250 μg/ml	++	+++	+++	++	n.t.
125 μg/ml	++	+	−	n.t.	++
62.5 μg/ml	++	−	−	n.t.	n.t.
Agariciidae					
Pavona decussata (extract 1)					
250 μg/ml	+++	+++	+++	−	n.t.
125 μg/ml	+++	+	−	n.t.	++
62.5 μg/ml	+++	−	−	n.t.	n.t.
P. decussata (extract 2)					
125 μg/ml	++	+	+++	+	n.t.

[a] ($P < 0.05$, Kruskal-Wallis ANOVA on Ranks and Dunnett's test). +, mean number of larvae significantly less than that of control within 7 days ($P < 0.05$); ++, mean number significantly less than that of control in 2–4 days ($P < 0.05$); +++, mean number significantly less than that of control within 2 days ($P < 0.05$); −, not lethal within 7 days; n.t., not tested. N = number of replicate containers with the number of larvae per container in parentheses. Mean survival in controls was always greater than 75%.

[b] No observations made on days 2, 4, and 6.

extract on the different species against which it was tested. However, in the three cases where two extracts were derived from the same species, there was little difference in toxicity. Of the three species tested against larvae of conspecifics, extracts of *Platygyra daedalea* and *Fungia fungites* were toxic at 500 μg/ml, while those of *Goniastrea favulus* did not affect conspecifics, although they were toxic to larvae of three other species.

In the second set of experiments, larvae of *Pocillopora damicornis* responded to all extracts tested by contracting longitudinally, becoming pear-shaped, then spherical, and eventually disc-shaped (Figure 1). When disc-shaped, the larvae ceased coordinated swimming. At the lowest concentrations tested,

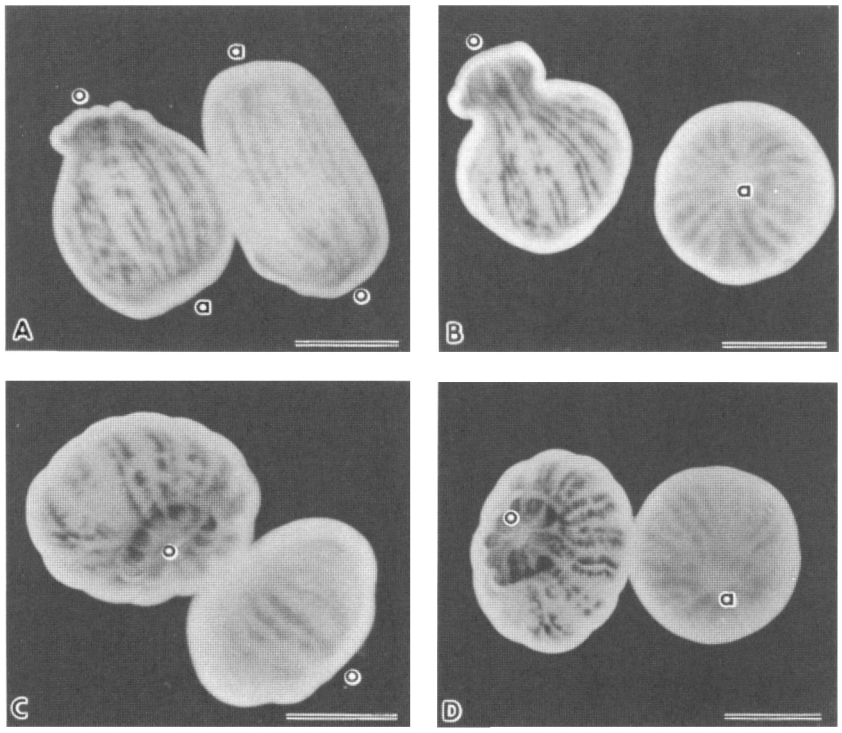


FIG. 1. Typical longitudinal contraction of two larvae of the scleractinian *Pocillopora damicornis* after addition of a crude extract of a hermatypic coral. Two larvae are shown before (A) and then at 20 sec (B), 40 sec (C) and 60 sec (D) after addition of extract. The larva on the left underwent a distinctive puckering at the oral pole in the first 20 sec and then took on a flattened disc shape 60 sec after addition of extract. The contraction of the larva on the right is seen most readily in (A) and (C) where the longitudinal axis of the larva lies in the same plane. a, aboral pole; o, oral pole. Bar = 1 mm.

TABLE 2. NUMBERS OF LARVAE OF *Pocillopora damicornis* THAT CONTRACTED LONGITUDINALLY INTO DISC-SHAPE IN RESPONSE TO EXTRACTS OF FOUR SPECIES OF HARD CORALS

Species	Concentration (μg/ml)	*N*[a]	Number of larvae contracted into disc shape at 2 min	20 min	60 min
Platygyra daedalea	500	5	4	4	4
	62.5	5	5	5	5
	7.8	5	3	5	2
Favia matthai	500	5	5	5	4
	62.5	5	5	5	5
	7.8	5	3	2	0
Fungia fungites	500	5	4	4	4
	62.5	3	3	3	3
	7.8	5	5	4	0
Pavona decussata	500	5	5	—[b]	—
	62.5	5	4	—	—
	7.8	5	0	—	—

[a] *N* = number of larvae tested for each extract.
[b] —, no observation recorded.

the contraction was often reversed within 1 hr, the contracted larvae having resumed their normal shape and swimming behavior (Table 2). Filtered SW alone elicited no change in behavior.

Mortality rates of spat of *P. damicornis* exposed to extracts were high after one day, so data from the four replicate containers of each test were pooled and are summarized in Table 3. Surviving spat initially decreased in diameter by resorbing tissue from their skeletons. These spat were generally larger than the spat killed by the extracts, and they resumed growth two to three days after addition of extracts. There was 100% survival of control spat, and every individual increased in diameter over the seven days.

DISCUSSION

Extracts from all species tested were toxic to both pre- and postsettlement stages of scleractinian corals. All five species tested yielded extracts lethal to scleractinian larvae, although there was variability in the susceptibility of different species. Of the coral extracts tested against larvae of conspecifics, those

TABLE 3. SURVIVAL AND CHANGE IN SIZE OF SPAT OF *Pocillopora damicornis* EXPOSED TO EXTRACTS OF FOUR SPECIES OF CORAL (N = 20)

Extract species	Number of surviving spat (mean diameter in mm ± SD of living spat)							
	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Platygyra daedalea	20 (2.2 ± 0.3)	0	0	0	0	0	0	0
Favia matthai	20 (2.1 ± 0.4)	5 (1.9 ± 0.6)	5 (2.3 ± 0.1)	3 (2.5 ± 0.2)	3 (2.6 ± 0.1)	3 (2.6 ± 0.0)	3 (2.6 ± 0.1)	3 (2.7 ± 0.1)
Fungia fungites	20 (2.2 ± 0.3)	0	0	0	0	0	0	0
Pavona decussata	20 (2.3 ± 0.3)	5 (1.9 ± 0.2)	1 (1.6)	1 (1.6)	1 (1.8)	1 (1.9)	1 (1.8)	1 (1.9)
Control	20 (2.2 ± 0.3)	20 (2.4 ± 0.3)	20 (2.5 ± 0.1)	20 (2.5 ± 0.3)	20 (2.6 ± 0.4)	20 (2.6 ± 0.4)	20 (2.8 ± 0.4)	20 (2.9 ± 0.4)

of two species had lethal effects, while those of the third species, *Goniastrea favulus*, did not. This result is interesting in view of the benthic development of larvae of *G. favulus* that usually occurs close to the parent colony (Kojis and Quinn, 1981; Kojis, 1985).

Larvotoxins have now been demonstrated in species of hermatypic corals from the families Faviidae, Fungiidae, and Agariciidae as well as from *Goniopora tenuidens* of the family Poritidae (Fearon and Cameron, 1996). Together with the report that the ahermatypic coral, *Tubastrea faulkneri* (family Dendrophylliidae), possesses toxins lethal to larvae of other corals (Koh, 1995), these results indicate that possession of larvotoxins may be widespread among the Scleractinia. However, such activity may be variable over space and time (see Gunthorpe and Cameron, 1990c), and not all species may possess larvotoxins. Maida et al. (1995b), for example, found that two scleractinians, *Porites cylindrica* and *Seriatopora hystrix*, had no effect on the nearby settlement of scleractinian coral larvae and used them as controls in their tests for allelopathy in alcyonacean corals.

All four extracts tested against spat of *Pocillopora damicornis* were lethal or retarded growth at 62.5 μg/ml. In contrast, Fearon and Cameron (1996) found that the same concentration of extracts of *G. tenuidens* did not kill spat although they did reduce their rate of growth. Such differences may arise from seasonal variation in the quality of spat but probably result from differing susceptibilities of spat originating from different *P. damicornis* "types" (sensu Richmond, 1984). The spat used in this study, although healthy, were half the size of those used previously. Thus, scleractinian corals possess toxins that affect the early postsettlement stages of corals, but these effects may be variable with respect to the spat tested.

If the substances responsible for the adverse effects on larvae and spat are exuded by corals in a similar manner as are those that affect adult corals (Gunthorpe and Cameron, 1990a; Lang and Chornesky, 1990), they may affect the settlement and the recruitment of potential competitors. Corals releasing such substances could limit establishment of competitors and thus reduce the adverse effects of adult competitors. Even if the exudates were not lethal, they may disrupt the growth or physiology of juveniles allowing the established colony to maintain a competitive advantage.

A similar result could be obtained by sublethal deterrence of larvae before they settled. Larvae of several marine invertebrates have been shown to sense the presence of and avoid organisms that might pose a threat to their postsettlement survival (Grosberg, 1981; Woodin, 1985; Johnson and Strathmann, 1989; Davis et al., 1991). Behavioral modifications elicited by low concentrations of aqueous extracts such as those reported here could lead to a similar reduction in settlement. Larvae encountering the substances at low concentrations contract and cease coordinated swimming. In this state, active searching behavior would

be suspended for a period during which larvae might be transported away from a contaminated area. Such larvae would avoid lethal effects of exudates either from high concentrations or chronic, long-term exposure after they had settled. Even if allelochemicals were present in concentrations that were not toxic, the ability to detect and avoid exudates of competitors would be advantageous to larvae if it allowed them to avoid postsettlement competition (sensu Grosberg, 1981).

Regardless of the mechanism, several species of scleractinian coral possess substances that are deleterious to both larvae and early juvenile stages of other corals. If such substances are exuded under natural conditions, as had been described for soft corals by Maida et al. (1995a,b), then they may act as a subtle mechanism whereby scleractinians influence the recruitment of other corals. Such a mechanism could affect succession in coral communities, thereby influencing the ordering of coral assemblages.

Acknowledgments—We thank the staff of Heron Island Research Station for their help and advice. Thanks also to R. Quin, S. Hobbs, D. Fearon, D. Scheltinga, H. Fox, D. McPhee, J. Keys, and D. Souter for technical assistance and support; and to R. Endean for much helpful advice. This work was funded by The University of Queensland and an Australian Postgraduate Award.

REFERENCES

BAKUS, G. J. 1981. Chemical defense mechanisms on the Great Barrier Reef. *Science*, 211:497–499.

BAKUS, G. J., TARGETT, N. M., and SCHULTE, B. 1986. Chemical ecology of marine organisms: An overview. *J. Chem. Ecol.* 12:951–987.

BECERRO, M. A., TURON, X., and URIZ, M. J. 1995. Natural variation of toxicity in encrusting sponge *Crambe crambe* (Schmidt) in relation to size and environment. *J. Chem. Ecol.* 21:1931–1946.

BINGHAM, B. L., and YOUNG, C. M. 1991. Influence of sponges on invertebrate recruitment. A field test of allelopathy. *Mar. Biol.* 109:19–26.

BREITBURG, D. L. 1985. Development of a subtidal epibenthic community: Factors affecting species composition and the mechanisms of succession. *Oecologia* 65:173–184.

CAMERON, A. M. 1974. Toxicity phenomena in coral reef waters. *Proc. 2nd Int. Coral Reef Symp.* 1:513–518.

DAVIS, A. R., TARGETT, N. M., MCCONNELL, O. J., and YOUNG, C. M. 1989. Epibiosis of marine algae and benthic invertebrates: Natural products chemistry and other mechanisms inhibiting settlement and overgrowth, pp. 85–114, *in* P. J. Scheuer (ed.). *Bioorganic Marine Chemistry*, Vol. 3. Springer, Berlin.

DAVIS, A. R., BUTLER, A. J., and VAN ALTENA, I. 1991. Settlement behaviour of ascidian larvae: Prelininary evidence for inhibition by sponge allelochemicals. *Mar. Ecol. Prog. Ser.* 72:117–123.

ENDEAN, R., and CAMERON, A. M. 1983. Toxins in coral reef organisms. *Toxicon, Suppl.* 3:105–109.

ENDEAN, R., CAMERON, A. M. 1990. Trends and new perspectives in coral-reef ecology, pp. 469–492, *in* Z. Dubinsky (ed.). Ecosystems of the World 25, Coral Reefs. Elsevier, Amsterdam.

FEARON, R. J., and CAMERON, A. M. 1996. Larvotoxic extracts of the hard coral *Goniopora tenuidens*: Allelochemicals that limit settlement of potential competitors? *Toxicon* 34:361–367.

GERHART, D. J., RITTSCHOF, D., and MAYO, S. W. 1988. Chemical ecology and the search for marine anti-foulants. Studies of a predator-prey symbiosis. *J. Chem. Ecol.* 14:1905–1917.

GOODBODY, I. 1961. Inhibition of the development of a marine sessile community. *Nature* 190:282–283.

GROSBERG, R. K. 1981. Competitive ability influences habitat choice in marine invertebrates. *Nature* 290:700–702.

GUNTHORPE, L., and CAMERON, A. M. 1990a. Toxic exudate from the hard coral *Goniopora tenuidens*. *Toxicon* 28:1347–1350.

GUNTHORPE, L., and CAMERON, A. M. 1990b. Widespread but variable toxicity in scleractinian corals. *Toxicon* 28:1199–1219.

GUNTHORPE, L., and CAMERON, A. M. 1990c. Intracolonial variation in toxicity in scleractinian corals. *Toxicon* 28:1221–1227.

HARRISON, P. L., BABCOCK, R. C., BULL, G. D., OLIVER, J. K., WALLACE, C. C., and WILLIS, B. L. 1984. Mass spawning in tropical reef corals. *Science* 223:1186–1189.

HASHIMOTO, Y., and ASHIDA, K. 1973. Screening of toxic corals and isolation of a toxic polypeptide from *Goniopora* spp. The proceedings of the second international symposium on *Cnidaria*. *Publ. Seto Mar. Biol. Lab.*, 20:703–711.

HESSINGER, D. A., and LENHOFF, L. M. 1976. Membrane Structure and function. *Arch. Biochem. Biophys.* 173:603–613.

JOHNSON, L. E., and STRATHMAN, R. R. 1989. Settling barnacle larvae avoid substrata previously occupied by a mobile predator. *J. Exp. Mar. Biol. Ecol.* 128:87–103.

KOH, E. G. L. 1995. Evidence for coral allelopathy. Australian Coral Reef Society Conference, Lismore, July 8–9, 1995, p. 10. Abstract.

KOJIS, B. L. 1985 Reproductive ecology of some hermatypic corals of the west Pacific. PhD dissertation. The University of Queensland.

KOJIS, B. L., and QUINN, N. J. 1981. Aspects of sexual reproduction and larval development in the shallow water hermatypic coral, *Goniastrea australensis* (Edwards and Haime, 1857). *Bull. Mar. Sci.* 31:558–573.

LANG, J. C., and CHORNESKY, E. A. 1990. Competition between scleractinian reef corals—A review of mechanisms and effects, pp. 209–252, *in* Z. Dubinsky (ed.). Ecosystems of the World, 25, Coral Reefs. Elseiver, Amsterdam.

MAIDA, M., SAMMARCO, P. W., and COLL, J. C. 1995a. Preliminary evidence for directional allelopathic effects of the soft coral *Sinularia flexibilis* (Alcyonacea: Octocorallia) on scleractinian coral recruitment. *Bull. Mar. Sci.* 56:303–311.

MAIDA, M., SAMMARCO, P. W., and COLL, J. C. 1995b. Effects of soft corals on scleractinian coral recruitment. I: Directional allelopathy and inhibition of settlement. *Mar. Ecol. Prog. Ser.* 121:191–202.

NODA, M., MURAMATSU, I., FUJIWARA, M., and ASHIDA, K. 1985. Effects of goniopora toxin on bullfrog atrial muscle are frequency-dependent. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 330:59–66.

PAWLIK, J. R. 1992. Chemical ecology of the settlement of benthic marine invertebrates. *Oceanogr. Mar. Biol. A. Rev.* 30:273–335.

QAR, J., SCHWEITZ, H., SCHMID, A., and LAZDUNSKI, M. 1986. A polypeptide from the coral *Goniopora*. Purification and action on Ca^{2+} channels. *FEBS Lett.* 202:331–336.

RICHMOND, R. H., and JOKIEL, P. L. 1984. Lunar periodicity in larva release in the reef coral *Pocillopora damicornis* at Enewetak and Hawaii. *Bull. Mar. Sci.* 34:280–287.

THOMPSON, J. E. 1985. Exudation of biologically active metabolites in the sponge *Aplysina fistularis* I. Biological evidence. *Mar. Biol.* 88:23–26.

WAHL, M. 1989. Marine epibiosis. I. Fouling and antifouling: Some basic aspects. *Mar. Ecol. Prog. Ser.* 58:175–189.

WOODIN, S. A. 1985. Effects of defecation by arenicolid polychaete adults on spionid polychaete juveniles in field experiments: Selective settlement or differential mortality. *J. Exp. Mar. Biol. Ecol.* 87:119–132.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

BIOACTIVE STEROIDS AND TRITERPENES FROM *Melilotus messanensis* AND THEIR ALLELOPATHIC POTENTIAL[1]

FRANCISCO A. MACÍAS,* ANA M. SIMONET, and JUAN C. G. GALINDO

Departamento de Quimica Orgánica
Facultad de Ciencias, Universidad de Cádiz
Apdo. 40, 11510 Puerto Real, Cádiz, Spain

(Received May 17, 1996; accepted March 10, 1997)

Abstract—The aerial parts of *Melilotus messanensis* (sweet clover) afforded, from moderately and polar bioactive fractions, 11 triterpenes and five steroids. A series of aqueous solutions at 10^{-4}–10^{-9} M of five steroids and nine triterpenes was monitored to test their effects on germination and growth of the monocots *Hordeum vulgare* and *Allium cepa*, and the dicots *Lactuca sativa* (var. nigra and var. romana) and *Lycopersicon esculentum*. An important stimulatory effect on *H. vulgare* germination (between 40% and 80% for almost all tested compounds) was observed. Some considerations about the ecological role of triterpenes on *M. messanensis* are made. In addition to known lupane triterpenes (platanic acid and 3β,28,30-lup-20(29)-enetriol), oleanane triterpenes (soyasapogenol B, soyasapogenol G, and messagenolide), a gammacerane triterpene (gammacer-16-en-3-one), five new lupane triterpenes (messagenic acids A–E: (27-*cis*-4-hydroxycinnamoyloxy)-betulinic acid; 27-(*trans*-4-hydroxycinnamoyloxy)betulinic acid; 20(*S*)-3β-hydroxy-29-oxolupan-28-oic acid; 3β,30-dihydroxylup-20(29)-en-28-oic acid; and 3β,20-dihydroxylup-18(19)-en-28-oic acid, respectively), and sterols (β-sitosterol, ergosterol peroxide, 7α-hydroxysitosterol, 7β-hydroxysitosterol, and 7-oxositosterol) were isolated and chemically characterized. Their structures and stereochemistry were elucidated by spectroscopic methods (one- and two-dimensional ^{1}H and ^{13}C NMR, IR, MS).

Key Words—*Melilotus messanensis*, lupane triterpenes, messagenic acids; messagenolide, soyasapogenol G, gammacer-16-en-3-one, oleanane triterpenes, steroids, stigmasterols, ergosterol peroxide, allelopathy, *Lactuca sativa*, *Lycopersicon esculentum*, *Allium cepa*, *Hordeum vulgare*.

*To whom correspondence should be addressed.

[1] Part 5 in the series "Natural Products as Allelochemicals"; for Part 4 see Macías et al. (1996).

0098-0331/97/0700-1781$12.50/0 © 1997 Plenum Publishing Corporation

INTRODUCTION

In continuation of our allelopathic studies in *Melilotus* species (Macías et al., 1994, 1996) we now report the isolation, structure elucidation, and biological activities of five known steroids and 10 triterpenes, five of which are first reported in the literature as natural products. Known steroids are β-sitosterol (**1**) [previously isolated from *M. caspius* (Rasulov and Belyi, 1985) and *M. indica* (Khafagy et al., 1980; Durrani and Ikram 1966)], ergosterol peroxide (**2**), 7β-hydroxysitosterol (**3**), 7α-hydroxysitosterol (**4**), and 7-oxositosterol (**10**) (Della Greca et al., 1990a; Guerriero et al., 1993; Gunatilaka et al., 1981). Known triterpenes are soyasapogenol B (**5**) (Baxter et al., 1990) previously isolated from *M. officinalis* (Kang et al., 1987), platanic acid (**9**) (Aplin et al., 1963; Fijioka et al., 1994), 3β,28,30-lup-20(29)-enetriol (**13**) Macías et al., 1994; González et al., 1992), soyasapogenol G (**14**), and gammacer-16-en-3-one (**15**) (Macías et al., 1996). The new triterpenes are messagenic acids A–E (**6–8**, **11**, **12**; Figure 1). All were identified by spectroscopic techniques (IR, MS, ^{1}H and ^{13}C 1D, 2D NMR experiments). Structure elucidation of the new lupane triterpenes (**6–8**, **11**, **12**) is described below.

As part of our research on bioactive natural products (coumarins, terpenoids, lignans, phenolics), we are conducting a systematic study of their potential activity as allelopathic agents (Macías et al., 1993, 1994). Thus, we are evaluating the regulatory effects of compounds on *Lycopersicon esculentum* L. and *Lactuca sativa* L. (dicotyledon species), and *Hordeum vulgare* L. and *Allium cepa* L. (monocotyledon species), (Castellano, 1997). We have studied the effects of a series of aqueous solutions from 10^{-4} to 10^{-9} M of five steroids (**1–4**, **10**) and seven triterpenes (**5–7**, **9**, **11**, **14**, **15**) on germination, and on root and shoot lengths of the above-mentioned target species.

METHODS AND MATERIALS

Plant Material. Melilotus messanensis (L.) All was collected on April 11, and July 2, 1991, in Trebujena, Cádiz, Spain (voucher specimen is deposited at the University of Seville Herbarium, Spain, SEV-7992) during two different plant development stage periods: (a) plants at the beginning of flowering, and (b) plants with seeds. Period b was selected as it provided the more bioactive aqueous extract in the laboratory bioassay.

Extraction and Isolation. Fresh plants at stage b (382 g) were soaked in deionized H_2O (weight of plant: vol. solvent 1:3), for 24 hr at 25° in the dark. The H_2O extract was extracted 10× [v/v of 0.5 liters of CH_2Cl_2 (DCM) per 1.0 liter of H_2O], and the combined extracts were dried over Na_2SO_4 and evaporated in vacuo to yield 1.2 g of crude extract. Crude extract was separated by

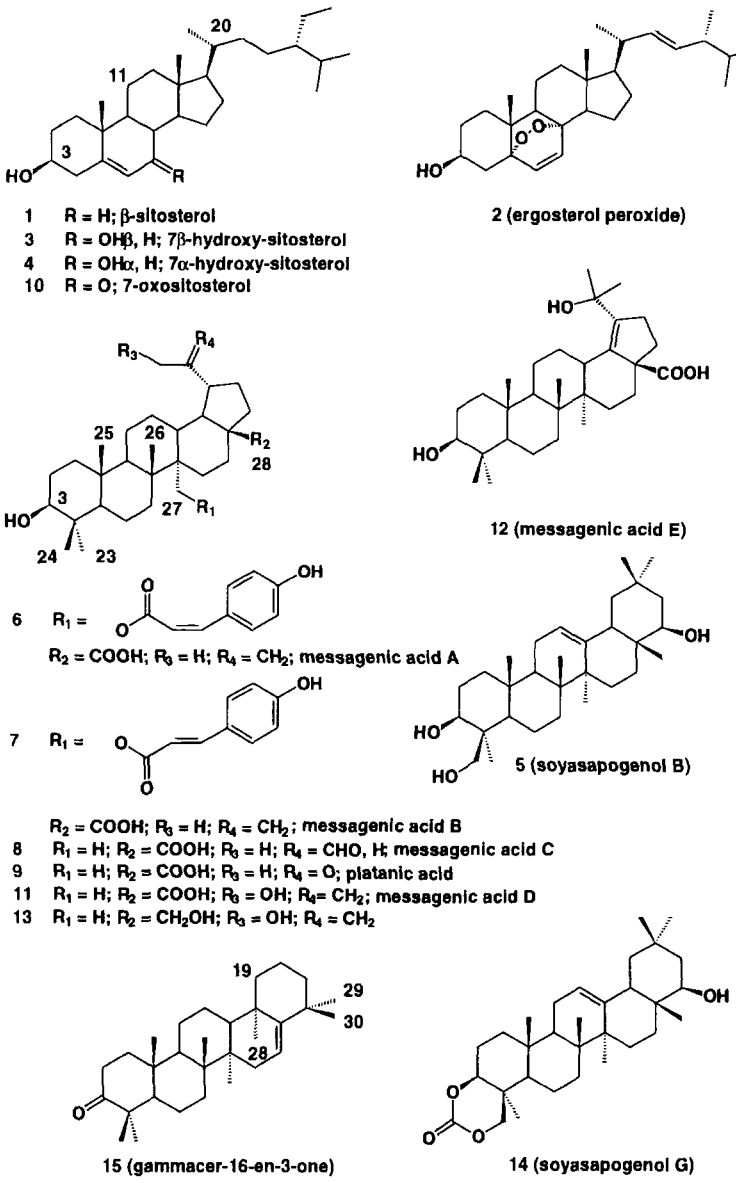


FIG. 1. Isolated compounds from *M. messanensis*.

column chromatography (CC) on silica gel using *n*-hexane–EtOAc mixtures of increasing polarity yielding 214 × 50-ml fractions, which were reduced to 13 fractions (A–M) after comparison by TLC.

Previously, we studied fractions C, D, G, and K (Macías et al., 1994, 1996). The current study of bioactive fractions E, I, L, and M provided 12 compounds. HPLC separation of fraction E (*n*-hexane–EtOAc, 4 : 1) afforded 30 mg of β-sitosterol (**1**). From fraction I, 1 mg of ergosterol peroxide (**2**) was obtained after CC on silica gel (DCM–EtOAc 98 : 2) and HPLC (DCM) purification.

Fraction L was chromatographed with 2-propanol on a Sephadex LH-20 column, providing four fractions. From the second fraction, two steroids were isolated after HPLC (*n*-hexane–EtOAc, 1 : 1) purification: 7β-hydroxysitosterol (**3**) (2 mg) and 7α-hydroxysitosterol (**4**) (5 mg). The third fraction afforded 10 mg of soyasapogenol B (**5**) under the same purification conditions.

Fraction M was chromatographed with 2-propanol on a Sephadex LH-20 column to provide four fractions. The third fraction was separated by CC on silica gel using DCM–EtOAc mixtures of increasing polarity yielding seven fractions (a–g). Fraction c, after HPLC purification (*n*-hexane–EtOAc 7 : 3) provided 2 mg of messagenic acid A (**6**); 1 mg of messagenic acid B (**7**); 5 mg of messagenic acid C (**8**); and 7 mg of platanic acid (**9**). Fraction d (HPLC, *n*-hexane–EtOAc, 3 : 2) afforded 3 mg of 7-oxositosterol (**10**). Chromatography of f by HPLC (*n*-hexane–EtOAc, 11 : 9) gave 4 mg of messagenic acid D (**11**), 3 mg of messagenic acid E (**12**), and 2 mg of 3β,28,30-lup-20(29)-enetriol (**13**).

Identification. Known compounds were identified by comparison of their physical and spectroscopic data (mp, [α], IR, MS, ^{1}H and ^{13}C NMR) with those previously reported in the literature.

Messagenic Acid A (6). $C_{39}H_{54}O_6$, oil; IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3353 (OH), 1722 (α,β-unsaturated ester), 1703 (CO-OH); EM (70 eV) *m/z* (rel. int.): 618 $[M]^+$ (1); 600 $[M\text{-}H_2O]^+$ (2); 557 (2) 454 (24); 439 (16); 436 (10); 393 (10); 278 (8); 233 (8); 207 (24); 189 (25); 147 (100). ^{1}H NMR see Table 1; ^{13}C NMR see Table 2.

Messagenic Acid B (7). $C_{39}H_{54}O_6$, oil; ^{1}H NMR: 7.60 (1H, d, J = 16 Hz, H-7′); 7.45 (2H, dd, J = 8 and 2 Hz, H-2′,6′); 6.84 (2H, dd, J = 8 and 2 Hz, H-3′,5′); 6.30 (1H, d, J = 16 Hz, H-8′); 4.75 (1H, m, H-29); 4.62 (1H, m, H-29); 4.62 (1H, d, J = 13 Hz, H-27); 4.43 (1H, d, J = 13 Hz, H-27); 3.19 (1H, dd, J = 11 and 5 Hz, H-3); 3.01 (1H, ddd, J = 10 Hz, H-19); 1.70 (3H, s, H-30); 0.99 (3H, s, H-26); 0.93 (3H, s, H-23); 0.85 (3H, s, H-25); 0.74 (3H, s, H-24).

Messagenic Acid C (8). $C_{30}H_{48}O_4$, amorphous solid; $[\alpha]_D^{25}$ = +8° ($CHCl_3$, c); IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3452 (OH), 2927, 1717 (COOH); 1684 (CHO); EM (70 eV) *m/z* (rel. int.): 472 $[M]^+$ (2); 454 (8); 439 (6); 414 (14); 207 (100); 189 (65). ^{1}H NMR see Table 1; ^{13}C NMR see Table 2.

TABLE 1. ^{1}H NMR OF TRITERPENES **6**, **8**, **9**, **11**, **12** (399.95 MHz, $CDCl_3$, SIGNAL OF RESIDUAL $CHCl_3$ CENTERED AT δ 7.25 PPM)[a]

H	6[b]	8	9	11	12
1a	0.92	0.92	0.89	0.89	0.91
e	1.73	1.69	1.64	1.65	1.68
2a	1.55	1.54	1.54	1.51	1.55
e	1.59	1.59	1.64	1.60	1.60
3	3.18 *dd*	3.19	3.19	3.18	3.18
5	0.68 *dd*	0.68	0.68	0.67	0.68
6a	1.33	1.37	1.37	1.37	1.37
e	1.46	1.52	1.49	1.51	1.51
7	1.23	1.21	1.54	1.48	
9		0.86			1.19
11a	1.29	1.30	1.25	1.21	1.58
e	1.20	1.21	1.66	1.45	1.34
12a	0.91	1.48	1.07	1.07	1.23
e	1.74	1.71	1.44	1.45	2.13
13	2.37 *ddd*	2.30	2.09	2.16	2.65
15a	1.75	1.48	1.48	1.50	1.44
e	1.22	1.20	1.20	1.20	1.18
16a	1.32	1.37	1.48	1.40	1.36
e	2.22 *ddd*	2.28	2.26	2.27	2.26
18	1.68	1.47	1.49	1.70	—
19	2.98 *ddd*	2.96 *dddd*	3.23 *ddd*	2.87	—
20a	—	2.63 *dq*	—	—	—
e					
21α	1.95	1.69	2.09	2.07	2.09
β	1.40	1.25	1.49	1.40	1.77
22α	1.98	1.92	1.97	1.95	1.95
β	1.41	1.33	1.59	1.51	1.43
23	0.93	0.96	0.95	0.95	0.96
24	0.74	0.75	0.74	0.74	0.75
25	0.83	0.83	0.81	0.81	0.83
26	0.96	0.96	0.90	0.92	0.92
27	4.59 *d*	0.94 *s*	0.99	0.97	0.96
	4.33 *d*				
29a	4.73 *s*	9.63 *s*	2.17	4.96 *s*	1.24 *s*
b	4.61 *s*			4.92 *s*	
30	1.68	1.00 *d*	—	4.12 *sbr*	1.23 *s*

[a]Multiplicities are indicated when coupling constant can be measured and they are not repeated if identical with those in the preceeding column. *J*(Hz): **6**, **8**, **9**, **11**, **12**: 2a,3 = 11; 2e,3 = 5; 5,6a = 10; **6**, **8**, **9**, **11**: 18,19 = 19,21α = 11; 19,21β = 4; 22α,22β = 12; 22α,21β = 8; **6**, **11**, **12**: 12a,13 = 13,18 = 13; 12e,13 = 3; 16a,16e = 13; 16e,15a = 16e,15e = 3; **6**: 27a,27b = 13; 2′,3′ = 9; 2′,5′ = 2; 7′,8′ = 13; **8**: 19,20 = 3; 20,30 = 7.

[b]*cis*-4-Hydroxycinnamoyloxy (**6**): H-2′,6′ = 7.68, *dd*; H-3′,5′ = 6.82, *dd*; H-7′ = 6.85, *d*; H-8′ = 5.84, *d*.

TABLE 2. ^{13}C NMR OF TRITERPENES **6**, **8**, **11**, **12** (100.23 MHz, $CDCl_3$, SIGNAL OF RESIDUAL $CHCl_3$ CENTERED AT δ 77.00 PPM)

H	6[a]	8	11	12
1	38.9	38.7	38.8	38.7
2	27.3	27.3	27.2	27.4
3	78.7	78.9	78.9	79.0
4	38.7	38.8	38.6	39.0
5	55.3	55.2	55.3	55.3
6	18.2	18.2	18.2	18.3
7	35.2	34.3	34.3	34.3
8	41.5	40.6	40.6	40.7
9	51.8	50.1	50.5	50.5
10	37.4	37.1	37.1	37.2
11	20.9	20.7	20.9	20.9
12	25.2	26.6	26.7	26.8
13	38.8	38.2	38.2	49.9
14	45.2	42.6	42.3	42.5
15	30.3	29.5	29.7	32.0
16	32.5	31.9	32.3	32.3[b]
17	55.9	56.3	56.1	56.2
18	49.4	49.9	49.9	106.8
19	46.7	36.8	42.5	151.0
20	151.0	48.1	154.8	65.2
21	30.3	29.7	32.1	38.3
22	36.6	37.4	36.8	36.8
23	27.9	28.0	27.9	28.0
24	15.4	15.3	15.3	15.3
25	16.4	16.1	16.1	16.1
26	16.6	16.0	15.9	16.3
27	63.3	14.5	14.6	14.7
28	181.0	174.7	178.8	178.3
29	110.0	204.7	106.5	29.7[b]
30	19.5	24.3	65.1	29.7[b]

[a]**6**: $C_{1'}$: 131.1; $C_{2'}$–$C_{6'}$: 132.4; $C_{3'}$–$C_{5'}$: 115.0; $C_{4'}$: 157.9; $C_{7'}$: 143.7; $C_{8'}$: 117.4; $C_{9'}$: 166.7.
[b]These assignments may be interchanged.

Messagenic Acid D (11). $C_{30}H_{48}O_4$, white needles; mp: 258–262°C; IR $\nu_{max}^{KBr,neat}$ cm^{-1}: 3442 (OH), 1694 (COOH); EM (70 eV) *m/z* (rel. int.): 472 $[M]^+$ (3); 454 (9); 439 (6); 408 (5); 246 (8); 231 (14); 207 (40); 189 (100). ^1H NMR see Table 1; ^{13}C NMR see Table 2.

Messagenic Acid E (12). $C_{30}H_{48}O_4$, amorphous solid; EM (70 eV) *m/z* (rel. int.): 454 $[M\text{-}H_2O]^+$ (8); 439 (8); 414 (5); 411 (3); 246 (7); 217 (21); 207 (100); 203 (16); 189 (76). ^1H NMR see Table 1; ^{13}C NMR see Table 2.

Seed Germination Bioassays. Seeds of *Lactuca sativa* var. nigra and var. romana, *Lycopersicon esculentum* L., and *Allium cepa* L. were obtained from Fitó, S. L. (Barcelona, Spain). *Hordeum vulgare* L. was obtained from Rancho La Merced, Junta de Andalucía, Jerez, Cádiz, Spain. All undersized and damaged seeds were discarded and the assay seeds were selected for uniformity.

Germination bioassay of *L. sativa* var. nigra consisted of 25 seeds per dish, 5 ml test solution, 60 hr dark, 25°C, and four replicates of each concentration. Germination and growth bioassays for the other species tested were as follows: *L. sativa* var. romana, 25 seeds per dish, 5 ml test solution, four days dark, 25°C, and four replicates of each concentration; *L. esculentum*, 25 seeds per dish, 5 ml test solution, five days dark, 25°C, and four replicates of each concentration; *A. cepa*, 25 seeds per dish, 5 ml test solution, five days dark, 25°C, and four replicates of each concentration; *H. vulgare*, 25 seeds per dish, 10 ml test solution, five days dark, 25°C, and six replicates of each concentration (Castellano, 1997).

Test solutions (10^{-4} M) were prepared using H_2O–MES [2-(*N*-morpholino]ethanesulfonic acid, 10 mM], and test solutions of 10^{-5}–10^{-9} M were obtained by dilution. Parallel controls were performed. All pH values were adjusted to 6.0 before bioassay with MES. Osmotic pressure values were measured on a vapor pressure osmometer (Wescor 5500) and ranged between 30 and 38 mosm.

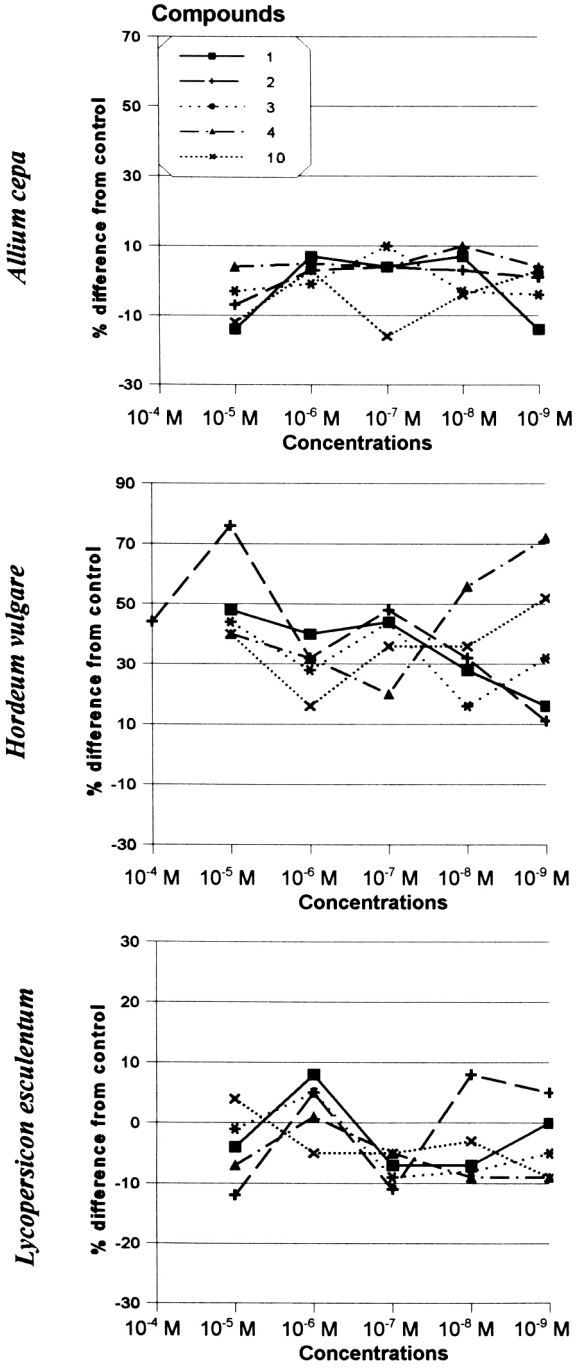
Data are presented as percent differences from control in graphics and tables (Figures 2–6 and Tables 3–6). Thus, zero represents the control; positive values represent stimulation of the studied variable and negative values represent inhibition.

Statistical Treatment. Germination and root and shoot length values were tested by the Mann-Whitney test; differences between experimentals and controls were significant ($P = 0.01$) (Tables 3–6).

RESULTS AND DISCUSSION

Chromatography of moderately bioactive and polar fractions of *M. messanensis* revealed the steroids β-sitosterol (**1**), ergosterol peroxide (**2**), 7β-hydroxysitosterol (**3**), 7α-hydroxysitosterol (**4**), and 7-oxositosterol (**10**); the oleanane triterpenes soyasapogenol B (**5**) and G (**14**); gammacer-16-en-3-one (**15**); the lupanic triterpenes messagenic acids A–E (**6–8**, **11**, **12**); platanic acid (**9**); and 3β,28,30-lup-20(29)-enetriol (**13**). Compounds **1–5**, **9**, **10**, **13–15** have been previously reported.

Chemical Data. Platanic acid (**9**) was characterized by comparison of physical data with those reported in the literature (Aplin et al., 1963; Fijioka et al., 1994) and ^{1}H NMR data are given in Table 1 since they include new and unambiguous assignments.

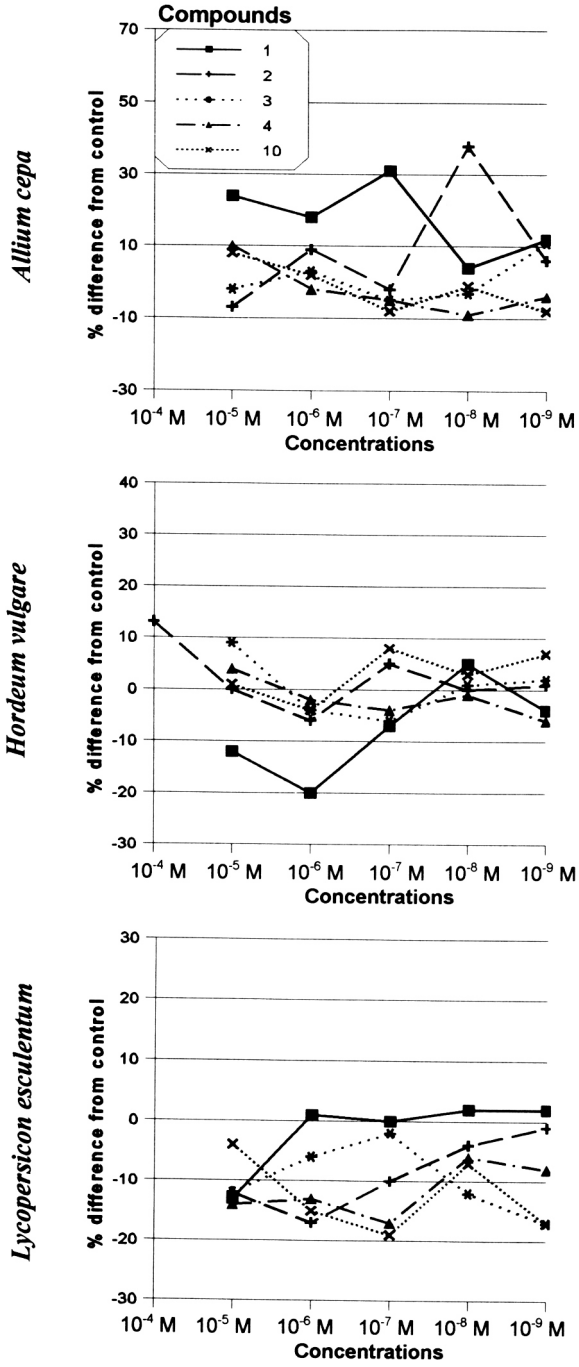

Compounds
1
2
3
4
10
Allium cepa
Hordeum vulgare
Lycopersicon esculentum
% difference from control
Concentrations
10-4 M
10-5 M
10-6 M
10-7 M
10-8 M
10-9 M

The more polar fraction (**M**) afforded, after separation, five new lupane triterpenes of increasing polarity: messagenic acids A–E (**6–8**, **11**, **12**). Messagenic acid A (**6**) and B (**7**) were easily interconverted by light. Only a careful HPLC separation in the dark allowed the isolation of both compounds. Messagenic acid A (**6**) shows a $[M]^+$ peak at *m/z* 618, allowing the molecular formula $C_{39}H_{54}O_6$. The ^1H NMR spectrum (Table 1) of **6** exhibits resonance for two sets of AB doublets at δ 5.84 (1H, d, J = 13 Hz) and 6.85 (1H, d, J = 13 Hz) and δ 6.82 (2H, d, J = 9 Hz) and 7.68 (2H, d, J = 9 Hz), consistent with a *p-cis*-coumaroyloxy substituent (Kashiwada et al., 1993). This is further supported by mass spectral fragments at *m/z* 164 $[C_9H_8O_3]^+$ and 147 $[C_9H_7O_2]^+$. Furthermore, the ^1H NMR (Table 1) spectrum of **6** shows signals of five methyl groups, singlets at δ 0.74, 0.83, 0.93, 0.96, and 1.68; a methylene group attached to the ester side chain [δ 4.59 (1H, d, J = 13 Hz), 4.33 (1H, d, J = 13 Hz)]; a hydroxymethine group [δ 3.18 (1H, dd, J = 11 and 5 Hz)]; and an *exo*-methylene [δ 4.73, 4.61 (bs)]. These data and the presence of fragments at *m/z* 207 $[C_{14}H_{23}O]^+$ and 189 $[C_{14}H_{21}]^+$ (base peak) in the mass spectrum are indicative of pentacyclic lupane triterpenes with a hydroxyl group at C-3 (Macías et al., 1994). The IR spectrum shows two carbonyl signals at 1722 (α,β-unsaturated ester) and 1703 cm^{-1}, corresponding to an additional carboxylic acid. The mass spectrum fragments suggest allocation of the substituents (—COOH and *p-cis*-coumaroyloxy) at C, D, or E rings (Budziekiewicz et al., 1963). The presence of a methyl attached to a double bond at δ 1.68 (C-30) and the peak ions at *m/z* 246 $[C_{16}H_{22}O_2]^+$ and 201 $[C_{15}H_{21}]^+$ are in agreement with two modified methyls at C-14 and C-17 (Siddiqui et al., 1992). The downfield chemical shift showed by H-19 (δ 2.98) indicates the presence of a carboxylic group at C-17 corresponding to betulinic acid (Macías et al., 1994). Consequently, the structure of messagenic acid A (**6**) is established as 27-(*cis*-4-hydroxycinnamoyloxy)betulinic acid.

The complete ^{13}C NMR (Table 2) assignment of messagenic acid A (**6**) was established by comparison with methylated betulinic acid data (Macías et al., 1994) and the diacetylated derivative of 3β,27-dihydroxylup-20(29)-en-28-oic acid (cylicodiscic acid) (Tchivounda et al., 1990).

The mass spectrum of messagenic acid B (**7**) is identical to **6**, and their ^1H NMR spectra showed great similarity. The only difference appeared downfield, where two sets of AB doublets at δ 7.60 (1H, J = 16 Hz), δ 6.30 (1H, J = 16 Hz) and δ 7.45 (2H, J = 8 Hz), δ 6.84 (2H, J = 8 Hz) correspond to a *p-trans*-coumaroyloxy substituent (Wang and Fujimoto, 1993). Thus, the structure of messagenic acid B (**7**) is established as 27-(*trans*-4-hydroxycinnamoyloxy)betulinic acid.

FIG. 2. Germination activity of steroids **1–4**, and **10**. Data are presented as the percentage difference from control (0 value).


Allium cepa
Compounds
1
2
3
4
10
% difference from control
70
50
30
10
-10
-30
10-4 M
10-5 M
10-6 M
10-7 M
10-8 M
10-9 M
Concentrations
Hordeum vulgare
% difference from control
40
30
20
10
0
-10
-20
-30
10-4 M
10-5 M
10-6 M
10-7 M
10-8 M
10-9 M
Concentrations
Lycopersicon esculentum
% difference from control
30
20
10
0
-10
-20
-30
10-4 M
10-5 M
10-6 M
10-7 M
10-8 M
10-9 M
Concentrations

Messagenic acid C (**8**) was the third compound, in increasing order of polarity, isolated from fraction M. Mass spectrum shows a molecular ion at *m/z* 472 ($C_{30}H_{48}O_4$) and fragment ions involving A and B rings at *m/z* 207 and 189, indicative of a lupane triterpene skeleton with a C-3 hydroxyl group. The ^{1}H NMR spectrum (Table 1) presents five singlet methyl signals at δ 0.75, 0.83, 0.94, and 0.96 (6H); a methyl group at δ 1.00 ppm (3H, d, J = 7 Hz); a hydroxymethine group (δ 3.19, dd, J = 11 and 5 Hz, H-3); and an aldehyde group (δ 9.63, s). Its IR shows two absorption signals according to the presence of a carboxylic and an aldehyde group. The absence of olefinic protons and the existence of a methyl doublet suggests that the isopropyl group is modified as CH_3RCH- (δ 2.63, dq, J = 7 and 2 Hz, H-20). All these data suggest a lupane triterpene with a hydroxyl group at the C-3, and an aldehyde and a carboxylic group attached to the C-17 and C-20, or vice versa, based on the chemical shift of H-19.

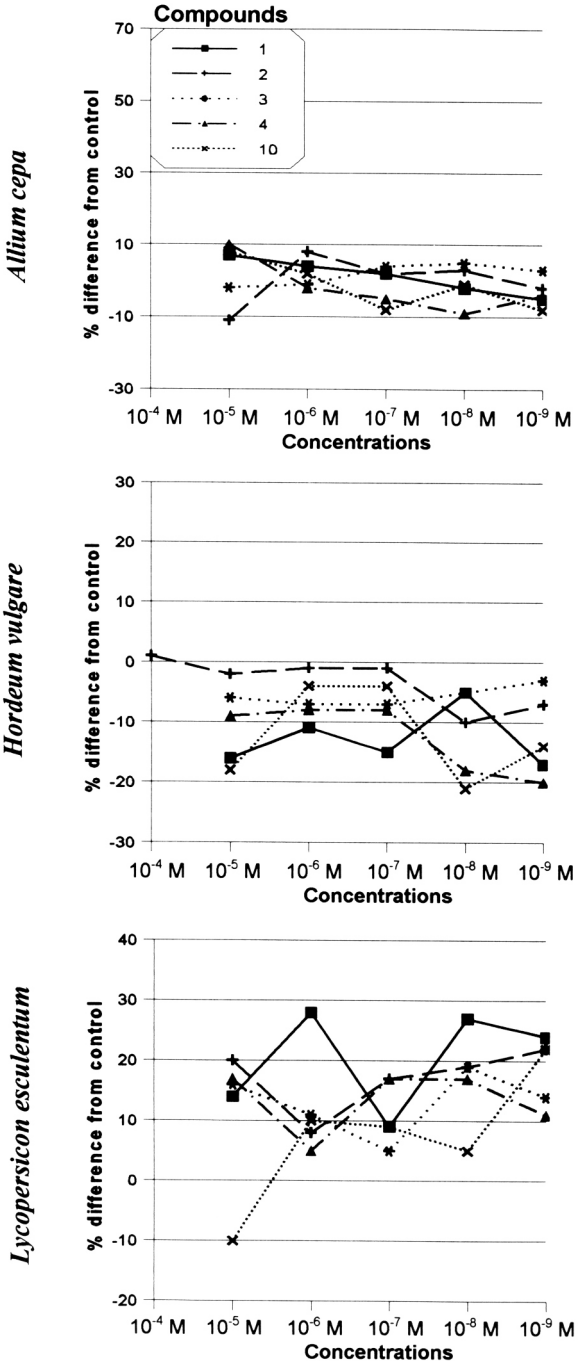
The position of the aldehyde and carboxylic groups at C-17 or C-20 is established by comparison of ^{13}C NMR data of **8** with the acetate derivative of betulinic acid (Macías et al., 1994) and betulinaldehyde (Pathak et al., 1988). The coincidence between C-17 signals of **8** (δ 56.3 ppm) and the acetate derivative of betulinic acid (δ 56.3 ppm), rather than with the corresponding one of betulinaldehyde (δ 59.3 ppm), allowed us to assign the aldehyde group at C-20. Positive NOE effects between H-29, H-19, and H-20 further substantiated the proposed structure. The absolute configuration at C-20 was established by correlation with the 3β,28-acetoxy-29-lupanal epimers (Vystrcil et al., 1973) (20*R*, $[\alpha]^D = -103°$; 20*S*, $[\alpha]^D = +70°$). Compound **8** shows $[\alpha]^D = +8°$, so a structure of (20 *S*)-3β-hydroxy-29-oxolupan-28-oic acid is proposed for messagenic acid C (**8**).

Compound **11** (fraction M) shows a $[M]^+$ at *m/z* 472 and a fragmentation pattern similar to that of **8**, indicative of a lupane triterpene skeleton with a C-3 hydroxyl group. The ^{1}H NMR (Table 1) spectrum is similar to 3β,28,30-lup-20(29)-enetriol (**13**) (Macías et al., 1994). Signals of the H-28 methylene group do not appear, and the H-19 signal appears 0.6 ppm downfield (δ 21.87 ppm) in comparison with the corresponding H-19 signal of **13**. These data confirm the structure of **11** as 3β,30-dihydroxylup-20(29)-en-28-oic acid.

Compound **11** was first isolated as a methyl ester from *Relhania genistifolia* (Tsichritzis and Jakupovic, 1990). This is the first report of the isolation and spectroscopic data of the natural alcohol.

Compound **12** was obtained from a mixture of platanic acid (**9**), which easily degraded. The ^{1}H NMR spectrum shows signals for seven C-Me singlets at δ 1.24, 1.23, 0.96 (6H), 0.92, 0.83, and 0.75; a hydroxymethine group

FIG. 3. Radicle length activity of steroids **1–4**, and **10**. Data are presented as the percentage difference from control (0 value).


Compounds
1
2
3
4
10
Allium cepa
Hordeum vulgare
Lycopersicon esculentum
% difference from control
70
50
30
10
-10
-30
40
30
20
10
0
-20
10-4 M
10-5 M
10-6 M
10-7 M
10-8 M
10-9 M
Concentrations

(δ 3.18, dd, J = 11 and 5 Hz); and a signal at δ 2.65 (dd, J = 11 and 5 Hz). The mass fragmentation pattern shows major peaks at m/z 207 (base peak) and m/z 189. The ^{13}C NMR spectrum (Table 2) shows signals of a carboxylic group, a tetrasubstituted double bond, and two hydroxyl groups. All data suggest a lupane triterpenic structure with a secondary hydroxyl group placed at C-3, a modified methyl group (COOH), a double-bond fully substituted group, and a tertiary hydroxyl group, corresponding to a molecular formula $C_{30}H_{48}O_4$ (mass spectrum, 454 $[M\text{-}H_2O]^+$).

The deshielding effect showed by C-29 and C-30 methyls (δ 1.24 and 1.23 ppm) is in good agreement with the existence of a tertiary hydroxyl group at C-20, as C-20 and C-30 methyl signals in 3β,20-lupanediol appear at δ 1.12 ppm and δ 1.22 ppm (Hui and Li, 1977). The difference between them can be explained by the presence of a tetrasubstituted double bond at C-18(19). The lack of the C-18 proton induces a displacement of the methyl groups, in good agreement with ^1H NMR (Table 1) and ^{13}C NMR data (Table 2) (Wenkert et al., 1978). Thus, the structure is proposed as 3β,20-dihydroxylup-18(19)-en-28-oic acid.

Bioassay Data. Only limited data are available concerning triterpene and steroid activity and growth development. Previous work in our laboratory shows that lupanic triterpenes stimulate germination of *Lactuca sativa* and, in certain cases, *Hordeum vulgare* (Macías et al., 1994). Some steroids, such as chondrillasterol or amasterol, are reported to affect germination (Bradow, 1985; Della Greca et al., 1990b). The only steroids that have been reported with potent plant growth promoter effects at levels as low as 1 ng (Maugh, 1981; Mandava et al., 1981) are the brassinosteroids, acting in many cases in a synergistic manner with auxin (Yopp et al., 1981). Saponins are another group of closely related compounds that have exhibited important inhibitory activities, e.g., several isolated from alfalfa roots belonging to the medicagenic acid, hederagenin, lucernic acid, zhanic acid, and soyasapogenol B types have been tested and proved to be active to some weeds and wheat (Waller et al., 1993). The effects produced by our tested triterpenes and steroids (Figure 1) on germination and growth are presented in Tables 3–6 and Figures 2–6 and show similar patterns. The overall effect on *Lactuca sativa* germination and growth falls below a 10% difference from controls in all cases. It is clear that none of these compounds exerts any significant effect on *L. sativa*. The other dicotyledon tested species, *Lycopersicon esculentum*, is slightly more sensitive than *L. sativa*, especially on growth development. Although the values obtained are low, in all concentrations tested, the inhibitory effect on germination is slightly greater with triterpenes than with steroids.

FIG. 4. Shoot length activity of steroids **1–4**, and **10**. Data are presented as the percentage difference from control (0 value).

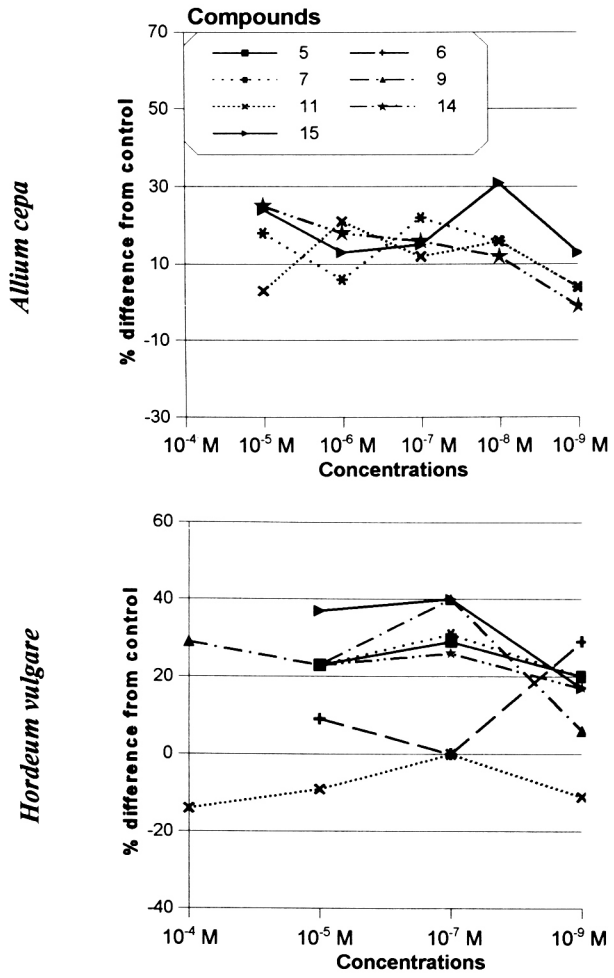


FIG. 5. Germination activity of triterpenes **5**–**7**, **9**, **11**, **14**, and **15**. Data are presented as the percentage difference from control (0 value).

Root growth responds differently, depending upon the substance tested: while triterpenes are clearly stimulatory (with values ranging between 20% and 40%), steroids present a low and homogeneous inhibitory activity profile, pointing out **10** (-15%, 10^{-6} M; -19%, 10^{-7} M; -17%, 10^{-9} M) and **4** (-14%, 10^{-5} M; -17%, 10^{-7} M). Compounds **5** and **11** have a promising profile of activity: from insignificant negative values at 10^{-5} M to a stimulatory effect as the concentration falls (**5**: 29%, 10^{-6} M; 24%, 10^{-8} M; **11**: 27%, 10^{-6} M,

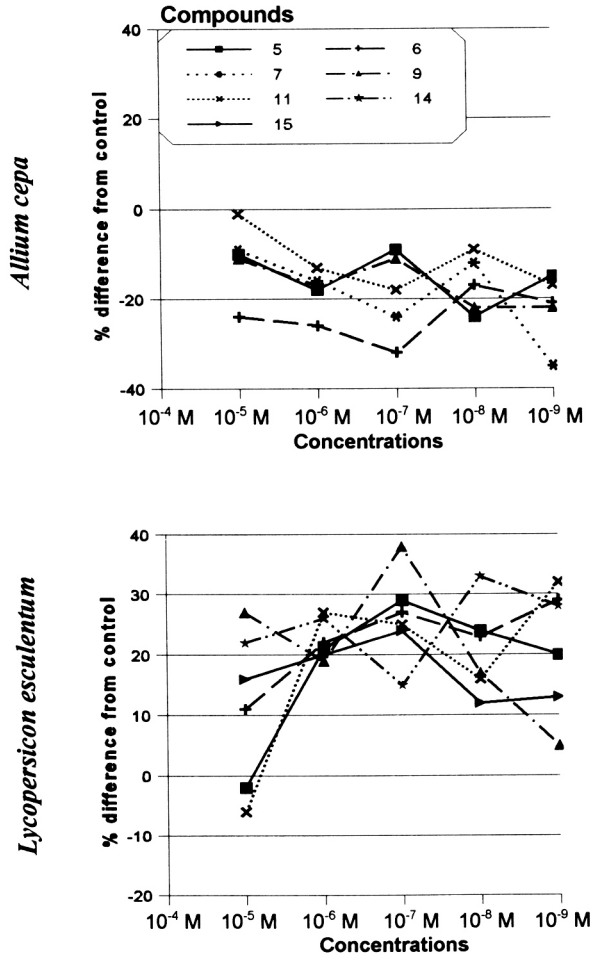


FIG. 6. Radicle length activity of triterpenes **5–7**, **9**, **11**, **14**, and **15**. Data are presented as percentage difference from control (0 value).

32%, 10^{-9} M). The small quantities of product obtained did not allow us to test higher concentrations, but negative effects at higher concentrations would be expected.

Shoot growth behaves in the opposite manner: stimulated by steroids, but showing no general trend with triterpenes. Compound **1** shows stimulatory activity (14%, 10^{-5} M; 28%, 10^{-6} M; 27%, 10^{-8} M; 24%, 10^{-9} M). Among triterpenes, although all values are low, the behavior of **6** and **11** at the highest

TABLE 3. GERMINATION AND GROWTH ACTIVITY OF TRITERPENES **5–7**, **9**, **11**, **14**, **15** ON DICOTYLEDON SPECIES[a]

	Germination (% difference from control)						Root length (% difference from control)						Shoot length (% difference from control)					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
Lactuca sativa (var. nigra and var. romana)																		
5	−6	0	−2	−1	3a	0	12	0	−1	0	6a	−4	10	8	9	5	−1	9
6	—	3	−3	2	−8	−4	—	7	−1	3	5	11	—	4	7a	5	3	−4
7	—	−8	3	4	−11a	−14	—	4	8	−5	7a	5	—	−3	8b	−3	−5b	−6b
9	1b	−8	−1	−2	−4	0	−7b	0	−11	0	2	2	4	13b	−5	−4	15b	−15
11	0	2	−1a	−3	−2	−2	−18	0	10a	−6	−5	−2	−15b	−19a	9	18a	5	26a
14	—	22	−2	−4	−1	0	—	8	7	−9	1	−6b	—	13b	19a	8	11a	7
15	—	0	−2a	−1	−1	2	—	10b	8a	1	−5	8	—	5	11b	8	−7	−1
Lycopersicon esculentum L.																		
5	—	−4a	−6b	−2b	−4a	−5	—	−2	21b	29b	24b	20	—	8	0	7	9	4
6	—	−16	−5	−12a	−15	−16	—	11	22b	27a	23	29	—	14b	23b	16b	7	6
7	—	−11	−19	9	8b	−7b	—	13	22b	2	24b	23b	—	−7	−4	1	−12	−8
9	—	−2b	−5	9a	−2	0	—	27b	19	38a	17	5	—	12	7	−4	−1	−1
11	—	6	−16b	−5b	−4	−8a	—	−6	27b	25b	16	32a	—	−12	−19b	−6	−8	−1
14	—	2b	−5b	−15	−1b	0	—	22b	26b	15	33b	28	—	9	10	8	8	8
15	—	−5	−4	1b	1	−15	—	16	20	24b	12	13	—	10	14	10	4	19

[a] Values are presented as percentage differences from the control (e.g., +16% means 116% compared with the control). Values are significantly different from the control with $P > 0.05$ for the Mann-Whitney test. a: $P < 0.01$; b: $0.01 < P < 0.05$.

TABLE 4. GERMINATION AND GROWTH ACTIVITY OF TRITERPENES **5-7**, **9**, **11**, **14**, **15** ON MONOCOTYLEDON SPECIES[a]

	Germination (% difference from control)						Root length (% difference from control)						Shoot length (% difference from control)					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
Hordeum vulgare L.																		
5	—	23	—	29	—	20	—	−17	—	−4	—	−17b	—	−13	—	−7	—	−23b
6	—	9	—	0	—	29	—	8	—	−3	—	−9	—	−7	—	−11	—	−9
7	—	23	—	31	—	20	—	12	—	12	—	4	—	−5	—	−3	—	−5
9	29	23	—	40b	—	6	−4	−4	—	−6	—	17	8	−4	—	1	—	6
11	−14	−9	—	0	—	−11	−3	2	—	3	—	−6	−3	5	—	5	—	−16
14	—	23	—	26	—	17	—	8	—	0	—	−3	—	−5	—	−23b	—	−11
15	—	37	—	40b	—	17	—	4	—	−9	—	3	—	−9	—	−11	—	5
Allium cepa L.																		
5	—	−9b	−10b	9	16	16	—	−10b	−18b	−9	−24	−15	—	24b	−10	1	16	13
6	—	18a	13b	−22b	3b	15	—	−24a	−26b	−32b	−17b	−21	—	10	14	−3	13	2
7	—	18	6	22	16	4a	—	−9	−16	−24	−12	−35a	—	7	0	−3	13	2
9	—	18	−12	−1	−13	13b	—	−11	−17	−11	−22	−22b	—	19b	16	−3	−1	−7
11	—	3	21b	12	16	4a	—	−1	−13b	−18	−9	−17a	—	12	8	9	11	−3
14	—	25	18	16	12	−1	—	−9	−23	−17	−11	−19	—	13	8	8	14	11
15	—	24b	13	15b	31	13	—	−14	−18	−13b	5	−16	—	4	8	9	11	22b

[a]Values are presented as percentage differences from the control (e.g., +16% means 116% compared with the control). Values are significantly different from the control with $P > 0.05$ for the Mann-Whitney test. a: $P < 0.01$; b: $0.01 < P < 0.05$.

TABLE 5. GERMINATION AND GROWTH ACTIVITY OF STEROIDS **1–4** AND **10** ON DICOTYLEDON SPECIES[a]

	Germination (% difference from control)						Root length (% difference from control)						Shoot length (% difference from control)					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
Lactuca sativa (var. nigra and var. romana)																		
1	−1a	4	0	2	−2	−2	16a	3	3	5	−8	−8	7	10	2	4	−3	−3
2	—	4	0	−1	4	3	1	−3	4	−4	−7	2	4	−2	1	10	8	6
3	—	2	1	−2	−1	2	—	−3	−10	−9	−8	−7	—	13b	6	−2	10	9
4	—	3b	−1	−3	4b	−1	—	−12b	0	−7	−15b	−9	—	19a	8	15	6	4
10	6a	7	−1	6	1	1	20a	0	4	6	−3	−5	5	6	7	5	4	0
Lycopersicon esculentum L.																		
1	—	−4b	8	−7	−7	0	—	−13b	1	0	2	2	—	14a	28a	9	27a	24a
2	—	−12	5	−11	8	5	—	−12	−17b	−10a	−4	−1	—	20a	8	17	19a	22b
3	—	−1	5	−9	−8	−5a	—	−12	−6	−4	−12	−17a	—	16	11b	5	19a	14a
4	—	−7b	1	−5b	−9	−9	—	−14b	−13	−17b	−6	−8	—	17	5	17b	17a	11
10	—	4	−5b	−5b	−3	−9b	—	−4	−15b	−19b	−7	−17b	—	−10b	10b	9	5	22

[a] Values are presented as percentage differences from the control (e.g., + 16% means 116% compared with the control). Values are significantly different from the control with $P > 0.05$ for the Mann-Whitney test. a: $P < 0.01$; b: $0.01 < P < 0.05$.

TABLE 6. GERMINATION AND GROWTH ACTIVITY OF STEROIDS **1–4** AND **10** ON MONOCOTYLEDON SPECIES[a]

	Germination (% difference from control)						Root length (% difference from control)						Shoot length (% difference from control)					
	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M	10^{-4} M	10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
Hordeum vulgare L.																		
1	—	48	40	44	28	16	—	−12b	−20	−7	5	−4	—	−16	−11	−15	−5	−17
2	44	76	32	48	32	11	13	0	−6	5	0	1	1	−2	−1	−1	−10	−7
3	—	44	28	44	16	32	—	9	−4	−6	1	2	—	−6	−7	−7	−5	−3
4	—	40	32	20	56	72	—	4	−2	−4	−1	−6	—	−9	−8	−8	−18	−20
10	—	40	16	36	36	52	—	1	−4	8	3	7	—	−18	−4	−4	−21	−14
Allium cepa L.																		
1	—	−14b	7	4a	7	−14	—	24b	18	31a	4	12	—	7	4	2	−2	−5
2	—	−7	3	4	3b	1	—	−7	9	−2	38b	6	—	−11	8	2	3	−2
3	—	−3	−1	10	−3	−4a	—	−2	3	−5	−3	11	—	−2	−1	4	5	3
4	—	4	5	4	10	4	—	10	−2	−5	−9	−4	—	7	−1	4	−1	14
10	—	−12	3	−16	−4	3	—	8	2	−8	−1	−8	—	5	2	0	−4	4

[a]Values are presented as percentage difference from the control (e.g., +16% means 116% compared with the control). Values are significantly different from the control with $P > 0.05$ for the Mann-Whitney test. a: $P < 0.01$; b: $0.01 < P < 0.05$.

concentrations (**6**: 14%, 10^{-5} M; 23%, 10^{-6} M; 16%, 10^{-7} M; **11**: −12%, 10^{-5} M, −19%, 10^{-6} M) should be pointed out.

H. vulgare is the only bioassay where significant values for the majority of compounds were obtained. Triterpenes, as well as steroids, exert a stimulatory effect on germination, steroids showing higher values (Figures 2 and 5). The most active steroids are **1** and **2**, with similar profiles: high levels of stimulatory activity at higher concentrations (**1**: 48%, 10^{-5} M; 40%, 10^{-6} M, 44%, 10^{-7} M; **2**: 44%, 10^{-4} M; 76%, 10^{-5} M; 48%, 10^{-7} M). Compounds **4** and **10** are the most active at lower dilutions, being less active at higher concentrations (**4**: 32%, 10^{-6} M; 72%, 10^{-9} M; **10**: 16%, 10^{-6} M; 52%, 10^{-9} M). Triterpenes can be divided into two groups: those showing a good level of stimulatory activity within all ranges of concentration (compounds **5**, **7**, **9**, **14**, **15**), and those with no significant activity (compounds **6** and **11**). The only difference in the stereochemistry of compounds **6** and **7** is the double bond in the cinnamoyloxy moiety. The *E* isomer (**7**) behaves as a growth promoter at all concentrations, while the *Z* isomer (**6**) has no activity, except at the lower concentration. No significant effects or general trends were shown on root or shoot length.

Allium cepa germination values are either low or insignificant, e.g., the triterpene **15** has moderate stimulatory activity (24%, 10^{-4} M; 15%, 10^{-7} M; 31%, 10^{-8} M). Root length is clearly inhibited by triterpenes but not by steroids, except **1**, which stimulates root development. The *Z* and *E* isomers **6** and **7** both show inhibition, with the *Z* isomer slightly more active. No significant activity was observed on shoot growth.

In attempting to elucidate roles for these compounds in plants, there are at least three aspects that need further study: (1) The compounds may act as self-germination controllers, since they stimulate germination at low concentrations. Germination data are significant with almost all compounds tested in three of the four target species. (2) They may form micromicelles that facilitate transport of low polar terpenes through cell membranes. Ursolic acid is present in high quantities in the leaves of some allelopathic shrubs of the Florida scrub community (Weidenhamer et al., 1993) and has been proved to enhance monoterpene phytotoxicity. Betulinic acid is a major constituent of *M. messanensis*, and it is the precursor of many other terpenoid acids; thus, it is possible that it or its products may play a role as natural detergents that facilitate transport of low polar compounds towards cell membranes of competitor species. (3) Saponins are known to be growth regulators and have been associated with the allelopathic potential of alfalfa (*Medicago sativa*) (Oleszek, 1993; Waller et al., 1993; Wyman-Simpson et al., 1991). Our previous results (unpublished) revealed a high saponin content in the polar fraction that requires further study to establish the structure–activity relationship. If the saponins exhibit reasonable plant growth regulatory activity, their acidic precursors will also be examined for activity in the same systems.

This paper constitutes a first approach to understanding the roles that triterpenes play in *M. messanensis* L. Further study will establish if any of the above-mentioned hypotheses are true. Whether they are involved in internal regulation of germination or act as plant allelopathic agents, it is clear that they are involved with germination processes. Finally, another point needing further work is whether synergistic effects occur in mixtures. There are many examples where mixtures are much more active than single compounds, e.g., phenolics (Einhellig, 1986, 1987). At present the amounts of isolated compounds available do not allow us to check this possibility.

Acknowledgments—This research was supported by the Dirección General de Investigación Científica y Técnica, Spain (DGICYT; Project No. PB91-0743). We thank Drs. Teodoro Marañón Arana and José Manuel Murillo Carpio, Instituto de Recursos Naturales y Agrobiología (IRNA) C.S.I.C., Sevilla, and Dr. Juan Arroyo Marín, Departamento de Biología Vegetal y Ecología, Universidad de Sevilla, Sevilla, Spain for providing plant material. A.M.S. acknowledges a fellowship from Junta de Andalucía.

REFERENCES

APLIN, R. T., HALSALL, T. G., and NORIN, T. 1963. The chemistry of triterpenes and related compounds (XLIII). The constituents of the bark of *Platanus hybrida* and the structure of platanic acid. *J. Chem. Soc.* 85:3269–3273.

BAXTER, R. L., PRICE, K. R., and FENWICK, R. 1990. Sapogenin structure: analysis of the ^{13}C and ^{1}H NMR spectra of soyasapogenol. *J. Nat. Prod.* 53:298–302.

BRADOW, J. M. 1985. Germination regulation by *Amaranthus palmeri* and *Ambrosia artemisiifolia*, pp. 285–300, *in* A. C. Thompson (ed.). The Chemistry of Allelopathy, Vol 268, ACS Symposium Series. American Chemical Society, Washington, DC.

BUDZIKIEWICZ, H., WILSON, J. M., and DJERASSI, C. 1963. Mass spectrometry in structural and stereochemical problems. XXXII. Pentacyclic triterpenes. *J. Am. Chem. Soc.* 85:3688–3699.

CASTELLANO, D. 1997. In search of a standard laboratory allelopathic bioassay. MS dissertation. University of Cádiz, Puerto Real, Cádiz, Spain.

DELLA GRECA, M., MONACO, P., and PREVITERA, L. 1990a. Stigmasterols from *Typha latifolia*. *J. Nat. Prod.* 53:1430–1435.

DELLA GRECA, M., MANGONI, L., MOLINARO, A., MONACO, P., and PREVITERA, L. 1990b. (20*S*)-4α-methylenecholest-7-en-3β-ol, an allelopathic sterol from *Typha latifolia*. *Phytochemistry* 29:1797–1798.

DURRANI, A. A., and IKRAM, M. 1966. *Melilotus indica*. *Pak. J. Sci. Ind. Res.* 9:173–174.

EINHELLIG, F. A. 1986. Mechanisms and modes of action of allelochemicals, pp. 171–188, *in* A. R. Putnam and C.-S. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

EINHELLIG, F. A. 1987. Interactions among allelochemicals and other stress factors of the plant environment, pp. 343–357, *in* G. R. Waller (ed.). Allelochemicals: Role in Agriculture and Forestry, Vol 330, ACS Symposium Series. American Chemical Society, Washington D.C.

FUJIOKA, T., KASHIWADA, Y., KILKUSKIE, R. E., COSENTINO, L. M., BALLAS, L. M., JIANG, J. B., JANZEN, W. P., CHEN, I., and LEE, K. 1994. Anti-AIDS agents, 11. Betulinic acid and platanic acid as anti-HIV principles from *Syzigium claviflorum*, and the anti-HIV activity of structurally related triterpenoids. *J. Nat. Prod.* 57:243–247.

GONZÁLEZ, A. G., JIMÉNEZ, I. A., and RAVELO, A. G. 1992. Triterpenes from *Maytenus canariensis* and synthesis of a derivative from betulin. *Phytochemistry* 31:2069–2072.

GUERRIERO, A., D'AMBROSIO, M., and PIETRA, F. 1993. Pteridines, sterols, and indole derivatives from the lithistid sponge *Corallistes undulatos* of the coral sea. *J. Nat. Prod.* 56:1962–1970.

GUNATILAKA, A. A. L., GOPICHAND, Y., SCHMITZ, F. J., and DJERASSI, C. 1981. Minor and trace sterols in marine invertebrates. 26. Isolation and structure elucidation of nine new 5α,8α-epidioxy sterols from four marine organisms. *J. Org. Chem.* 46:3860–3866.

HUI, W., and LI, M. 1977. Further triterpenoids from the stems of *Lithocarpus polystachya*. *Phytochemistry* 16:111–112.

KANG, S. S., LIM, C. H., LEE, S. Y., and SOOK, Y. 1987. Soyasapogenols B and E from *Melilotus officinalis*. *Arch. Pharmacol. Res.* 10:9–13.

KASHIWADA, Y., ZHANG, D., CHEN, Y., CHENG, C., CHEN, H., CHANG, H., CHANG, J., and LEE, K. 1993. Antitumor agents, 145. Cytotoxic asprellic acids A and C and asprellic acid B, new *p*-cumaroyl triterpenes, from *Ilex asprella*. *J. Nat. Prod.* 56:2077–2082.

KHAFAGY, S. M., SABRI, N. N., and ABOU DONIA, A. H. 1980. Pharmacognostical study of *Melilotus indicus* (L.) All var. Tommasinii Jord. *Egypt J. Pharm. Sci.* 19:293–300.

MACÍAS, F. A., GALINDO, J. C. G., MASSANET, G. M., RODRÍGUEZ-LUÍS, F., and ZUBÍA, E. 1993. Allelochemicals from *Pilocarpus goudotianus* leaves. *J. Chem. Ecol.* 19:1371–1379.

MACÍAS, F. A., SIMONET, A. M., and ESTEBAN, M. D. 1994. Potential allelopathic lupane triterpenes from bioactive fractions of *Melilotus messanensis*. *Phytochemistry* 36:1369–1379.

MACÍAS, F. A., SIMONET, A. M., ESTEBAN, M. D., and GALINDO, J. C. G. 1996. Triterpenoids from *Melilotus messanensis* L; Soyasapogenol G, the first natural carbonate derivative. *Phytochemistry* 41:1573–1577.

MANDAVA, N. B., SASSE, J. M., and YOPP, J. H. 1981. Brassinolide, a growth-promoting steroidal lactone. II. Activity in selected gibberellin and cytokinin bioassays. *Physiol. Plant.* 53:453–61.

MAUGH, T. H., II. 1981. New chemicals promise larger crops. *Science* 212:33–34.

OLESZEK, W. 1993. Allelopathic potentials of alfalfa (*Medicago sativa*) saponins: Their relation to antifungal and hemolytic activities. *J. Chem. Ecol.* 19:1063–1074.

PATHAK, N. K. R., NEOGI, P., BISWAS, M., TRIPATHI, Y. C., and PAUDEY, V. B. 1988. Betulinaldehyde, an anti-tumor agent from the bark of *Tectona grandis*. *Indian J. Pharm. Sci.* 50:124–125.

RASULOV, F. A., and BELYI, M. B. 1985. Chemical study of *Melilotus caspius* and *Achillea vermicularis*. *Izv. Akad. Nauk Az. SSR, Ser. Biol. Nauk* 2:22–24.

SIDDIQUI, S., SIDDIQUI, B. S., NAEED, A., and BEGUM, S. 1992. Pentacyclic triterpenoids from the leaves of *Plumeria obtusa*. *Phytochemistry* 31:4279–4283.

TCHIVOUNDA, H., KOUDOGBO, P., BESACE, Y., and CASADEVALL, E. 1990. Cylicosidic acid, a dihydroxy pentacyclic triterpene carboxylic acid from *Cylicodiscus gabunensis*. *Phytochemistry* 29:3255–3258.

TSICHRITZIS, F., and JAKUPOVIC, J. 1990. Diterpenes and other constituents from *Relhania* species. *Phytochemistry* 29:3173–3187.

VYSTRCIL, A., POUZAR, V., and KŘEČEK, V. 1973. Triterpenes. XXXII. Absolute configuration at C(20) in 29-substituted lupane derivatives. *Coll. Czech. Chem. Commun.* 38:3902–3911.

WALLER, G. R., JURZYSTA, M., and THORNE, R. L. Z. 1993. Allelopathic activity of root saponins from alfalfa (*Medicago sativa* L.) on weeds and wheat. *Bot. Bull Acad. Sin.* 34:1–11.

WANG, H. and FUJIMOTO, Y. 1993. Triterpene esters from *Eucalyptus fereticornis*. *Phytochemistry* 33:151–153.

WEIDENHAMER, J. D., MACÍAS, F. A., FISCHER, N. H., and WILLIAMSON, G. B. 1990. Just how insoluble are monoterpenes? *J. Chem. Ecol.* 19:1799–1807.

WENKERT, E., BADDELEY, G. V., BURFITT, I. R., and MORENO, L. N. 1978. Carbon-13 nuclear

magnetic resonance spectroscopy of naturally-occurring substances. LVII. Triterpenes related to lupane and hopane. *Org. Mag. Res.* 11:337–343.

WYMAN-SIMPSON, C. L., WALLER, G. R., JURZYSTA, M., MCPHERSON, J. D., and YOUNG, C. C. 1991. Biological activity and chemical isolation of root saponins of six cultivars of alfalfa (*Medicago sativa* L.). *Plant Soil* 135:83–94.

YOPP, J. H., MANDAVA, N. B., and SASSE, J. M. 1981. Brasinolide, a growth-promoting steroidal lactone. I. Activity in selected auxin bioassays. *Physiol. Plant.* 53:445–452.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

DOES SALICYLIC ACID ACT AS A SIGNAL IN COTTON FOR INDUCED RESISTANCE TO *Helicoverpa zea*?

J. L. BI,[1] J. B. MURPHY,[2] and G. W. FELTON[1,*]

[1]*Department of Entomology*
[2]*Department of Horticulture*
University of Arkansas
Fayetteville, Arkansas 72701

(Received September 9, 1996; accepted March 11, 1997)

Abstract—Our previous study indicated that insect herbivory on cotton induced resistance to the cotton bollworm (*Helicoverpa zea*). Here we examine the role of salicylic acid as a signal in cotton for the induced resistance. Abundant evidence has accumulated showing that salicylic acid plays a key role in coordinating the expression of systemic acquired resistance against phytopathogens. We report that herbivory results in significant increases in foliar salicylic acid and H_2O_2, a response frequently observed following pathogenesis. In other well-studied systems (e.g., tobacco), salicylic acid inhibits the enzymatic decomposition of H_2O_2 by catalase and ascorbate peroxidase, but in cotton, salicylic acid has no effect on these enzymes *in vitro*. Furthermore, while herbivory enhances foliar catalase and ascorbate peroxidase activities, the application of salicylic acid or methyl salicylate to cotton plants does not affect foliar resistance to *H. zea*. The possible role of salicylic acid as a signal for induced resistance is discussed in light of these findings.

Key Words—Salicylic acid, systemic acquired resistance, hydrogen peroxide, catalase, ascorbate peroxidase, signaling, induced resistance, herbivory, *Gossypium hirsutum*, *Helicoverpa zea*.

INTRODUCTION

Acquired or induced plant resistance to pests requires the production, systemic mobility, reception, and transduction of signal(s). While the role of signaling pathways in systemic acquired resistance (SAR) to pathogens has been extensively investigated, considerably less is known regarding endogenous signals

*To whom correspondence should be addressed.

0098-0331/97/0700-1805$12.50/0 © 1997 Plenum Publishing Corporation

for induced resistance to insects (Hammerschmidt, 1993). The octadecanoid pathway has been a major focus of recent research on defense signaling against insect herbivores (Doares et al., 1995). Jasmonic acid, the primary active product of the pathway, has been reported to be a signal for induced resistance against insects in several systems (Farmer and Ryan, 1990; Bodnaryk and Rymerson, 1994; Baldwin et al., 1994; Baldwin and Schmelz, 1996). Jasmonic acid is produced within minutes following insect feeding (Blechert et al., 1995), leading to the activation of several defense genes (Doares et al., 1995). Topical application of jasmonic acid also induces the production of pathogenesis-related (PR) proteins and subsequent resistance to phytopathogens in some systems (Cohen et al., 1993; Schweizer et al., 1993).

The role of salicylic acid (SA) as a signal for SAR against pathogens has been a particularly intense focus of investigation during the past several years. A very strong relationship between the appearance of SA and the onset of SAR has been reported for several plant species (Hammerschmidt, 1993; Klessig and Malamy, 1994). Exogenous application of SA to tobacco induced the accumulation of PR proteins, which are strongly correlated with SAR (White, 1979; Ward et al., 1991). SA was also found to induce PR proteins in many other plant species such as tomato, potato, cucumber, rice, and soybean (for review, see Klessig and Malamy, 1994). Increased SA levels in the phloem sap of infected cucumber (Métraux et al., 1990, Rasmussen et al., 1991) and tobacco (Malamy et al., 1990) provides further evidence that SA acts as a signal for SAR (Malamy and Klessig, 1992; Malamy et al., 1992; Raskin, 1992; Métraux, 1994; Ryals et al., 1994). SA in cucumber was also observed to induce other proteins such as peroxidase (POX), which is correlated with pathogen resistance (Rasmussen et al., 1991; Smith et al., 1991).

SA may function as an intermediate signal by altering H_2O_2 metabolism. Chen et al. (1993a,b) purified a soluble foliar protein in tobacco that binds SA and exhibits SA-inhibitable catalase (CAT) activity. Durner and Klessig (1995) demonstrated that SA also inhibits ascorbate peroxidase (APX) activity. Exogenous SA treatment of tobacco leaves resulted in elevated levels of H_2O_2 (Chen et al., 1993b). These data suggest that SA acts to block CAT and APX activities, which elevates normal levels of H_2O_2. The H_2O_2 then acts as a second messenger to activate the defense related genes involved in disease resistance (Chen et al., 1993b, 1995; Klessig and Malamy, 1994; Durner and Klessig, 1995). However, this model has been questioned by several recent experimental results. The SA inhibiting ability on foliar CAT was tested in several different plant species, and the results indicated that SA has similar binding ability to tomato, cucumber, and *Arabidopsis* CAT, but has no binding ability to maize and rice CAT (Sanchez-Casas and Klessig, 1994). Bi et al. (1995) showed that inhibition of wild tobacco foliar CAT activity by SA is weak and requires a SA concentration above 250 μM. Kauss and Jeblick (1996) reported that in cucumber the enhance-

ment of H_2O_2 by SA is due to increased production competence rather than to inhibition of H_2O_2-degrading enzyme activities.

Apparently SA functions differently in various plant species; thus, the precise function of SA in SAR remains unresolved (Chasan, 1995). Malamy et al. (1990) found that SA levels in resistant tobacco increased in both the tobacco mosaic virus (TMV) inoculated and uninoculated leaves of the same plant; the rise in SA levels preceded the induction of PR genes in the uninoculated leaves. Several authors have reported that large enhancement of SA in the phloem exudates of inoculated cumumber leaves preceded the development of SAR and the induction of POX activity in uninoculated leaves (e.g., Métraux et al., 1990; Rasmussen et al., 1991; Smith et al., 1991). However, a grafting experiment involving SA transduction indicated that SA is not the systemic signal but that it is required for the expression of SAR (Vernooij et al., 1994).

There is no report that SA acts as a signal for insect resistance (Hammerschmidt, 1993). Cotton is a particularly relevant system for investigating the role of SA in insect resistance. The application of SA is known to induce higher levels of terpene aldehydes (e.g., gossypol), which have been reported to confer resistance to insects and phytopathogens (Khoshkhoo et al., 1993). Moreover, in cotton, infection with a pathogen confers increased resistance to arthropods and vice versa (Karban et al., 1987). These reciprocal effects suggest the possibility of shared signal pathways. We previously reported that insect feeding induces resistance against the cotton bollworm *Helicoverpa zea* (Boddie) (Bi et al., 1997). The present study was initiated to determine: (1) if herbivory by *H. zea* in cotton elevated foliar levels of SA and (2) if SA were involved in induced foliar resistance to *H. zea*.

METHODS AND MATERIALS

Plants and Insects. Eggs of *H. zea* were obtained from the University of Arkansas Insect Rearing Facility. Larvae were maintained on an artificial diet as formulated by Chippendale (1970) until used in the experiments. Cotton (*Gossypium hirsutum*) seeds (cv. Deltapine 50) were soaked in water for 6 hr and then incubated at 28°C for 24 hr. Germinated seeds were sown in 2-liter plastic pots filled with Redi Earth Peat-Lite Mix soil mixture in a greenhouse. Pots were arranged in a completely randomized experimental design. Plants were watered every two days and fertilized (N:P:K = 20:20:20) weekly. Greenhouse conditions were a 14-hr photophase, using high-pressure sodium light (1000 W) and a day temperature of 33 ± 2°C and a night temperature of 20 ± 2°C. Cotton plants at the four-node stage were used in the experiments.

Induction of Foliar SA and Related Phytochemicals. Forty greenhouse-grown cotton plants were used to determine if feeding by *H. zea* larva affects

levels of foliar H_2O_2, SA, CAT, and APX. At the four-node stage, a single fourth-instar *H. zea* was placed on each of 20 plants individually contained in screen cages to prevent larval escape. Twenty control plants were identically treated except that larvae were excluded. Larvae were starved for 24 hr prior to infesting the plants. The levels of defoliation in the wounded plants were generally less than 30%. After three days, a terminal fully expanded leaf from each damaged plant and the same positioned leaf from each control plant were excised with a single-edge razor blade. The 72-hr period was chosen because preliminary experiments indicated that induced resistance to *H. zea* is not observable 24–48 hr after initial damage (Bi et al., 1997). The terminal fully expanded leaf from each of 10 damaged and 10 control plants was assayed for H_2O_2. The terminal leaves of the other 10 damaged and 10 control plants were immediately placed into plastic bags, which were held on Dry Ice. After becoming fully frozen, the foliage was freeze-dried, ground to powder, and pooled for treatment or control for the test of SA, CAT, and APX.

Effect of Exogenous SA and MeSA on Cotton Foliar Resistance. To determine if the foliar application of exogenous SA induces larval resistance, 40 cotton plants were grown in the greenhouse. At the four-node stage, a terminal fully expanded leaf was excised from each of 20 plants, and the petiole was immersed in 5 ml of 1 mM SA (Sigma, St. Louis, Missouri) as described by Yalpani et al. (1991). The pH of SA solution was adjusted prior to petiole immersion to 7.0 with 0.5 N NaOH. The SA-treated leaves were placed under a combination of UV and cool-white fluorescent light in a growth chamber. The photophase was 14 hr, and the temperature was 25°C. Twenty control leaves from 20 other plants were identically treated except that the petioles were immersed in water (pH 7.0). After being immersed in SA or water for 24 hr, each individual leaf was transferred to a 500-ml clear plastic container (Fabri-Kal Co., Kalamazoo, Michigan) with two layers of the above-described SA or water-soaked filter paper (Whatman No. 1) in the bottom. A newly molted fourth-instar *H. zea* was placed in each container. Containers were randomly placed in the growth chamber. Each larva was weighed at the beginning of the test and again after 24 hr and 48 hr, respectively. The relative growth rate (RGR) of each larva was then calculated. There were no significant differences between the initial weights of larvae used in the treatment or in the control.

To evaluate the effect of exogenous application of methyl salicylate (MeSA) (Aldrich, Milwaukee, Wisconsin) on cotton resistance to *H. zea*, a second test was conducted in the field. MeSA is a volatile metabolite of SA and is also known to induce SAR (Lee et al., 1995). Eighty cotton plants were grown in pots as described above. The plants were fertilized as described in the greenhouse experiment and watered daily. At the four-node stage, the potted plants were divided into two groups separated by a distance of ca. 5 m. Group 1 was sprayed with 100 μM MeSA in 0.1% ethanol, and group 2 was sprayed with

0.1% ethanol as a control. The spraying was continued until runoff on three consecutive evenings. Twenty-four hours following the final application, a single upper-most fully expanded leaf from each plant was excised. Thirty excised leaves from each group were placed individually in a plastic container with two layers of water-moistened filter paper (Whatman No. 1) in the bottom as described above. In each group, a newly molted third instar was placed in each of 20 containers, and seven neonates were placed in each of 10 containers. Containers were randomly placed in a growth chamber at 28°C. Each third instar was weighed at the beginning of the test and again after 48 and 64 hr. The RGR was computed for each interval. The neonates were weighed 96 hr after being placed in the containers. There were no significant differences between the initial weights of larvae used in treatment or the control. Ten individual leaves from the rest of the treatment and control plants were assayed for POX and diamine oxidase (DAO).

Effect of SA on In Vitro Foliar CAT and APX Activities. To test the effect of SA on foliar CAT activity, 0.1-g aliquots of lyophilized powder from *H. zea*-damaged foliage were extracted in 10 ml of 0.05 M ice-cold potassium phosphate buffer (pH 7.0) containing 1% polyvinylpolypyrrolidone (w/v) and 0.5 mM EDTA. The homogenates were centrifuged at −2°C for 20 min at 10,000*g*. The supernatants were immediately mixed with ammonium sulfate to 45% saturation to precipitate proteins. After centrifugation for 20 min at −2°C and 10,000*g*, the protein pellets were dissolved in 6 ml of the above described potassium phosphate buffer with polyvinylpolypyrrolidone and EDTA excluded. One milliliter of 2 mM SA was added to the same volume of the above resulting protein solution and incubated for 3 hr at 4°C as suggested by Chen and Klessig (1991) and Sanchez-Casas and Klessig (1994). The control was treated the same way except that potassium phosphate buffer was added instead of SA. CAT was assayed as described below.

To determine the effect of SA on foliar APX activity, a method was adopted from Durner and Klessig (1995). A 0.1-g aliquot from *H. zea*-damaged foliage was extracted for APX-containing soluble proteins in 10 ml of 0.1 M ice-cold potassium phosphate buffer (pH 7.0) containing 0.25 mM ascorbate and 1% polyvinylpolypyrrolidone (w/v). After centrifugation at 4°C and 14,000*g* for 5 min, the proteins were fractionated with ammonium sulfate between 10% and 85% saturation. The protein fraction was then resuspended in extraction buffer without polyvinylpolypyrrolidone and desalted in a Micron-10 concentrator (Amicon Inc., Beverly, Massachusetts). One milliliter of 2 mM SA was added to the same amount of desalted protein solution and incubated for 3 hr at 4°C. The control was treated the same way except that potassium phosphate buffer was added instead of SA. APX activity was assayed as described below.

Plant Enzyme Assays. Foliar tissue for enzyme assays was prepared following the method of Bi and Felton (1995), unless otherwise stated. The POX

assay used guaiacol as the hydrogen donor according to the procedure of Ridge and Osborne (1970). Fifty microliters of enzyme solution were mixed with 1.0 ml of substrate containing 1 mM H_2O_2 and 2 mM guaiacol in 0.1 M potassium phosphate buffer (pH 7.0). POX activity was measured at 470 nm.

DAO activity was measured with 10 mM putrescine (diaminobutane) in 0.1 M potassium phosphate buffer (pH 7.0). The reaction was initiated with 1 ml of enzyme solution in 3 ml of substrate. After incubation at 37°C for 10 min, the reaction was stopped by adding 0.5 ml of 10% (w/v) TCA, followed by 0.05 ml (10 mg/ml) *o*-aminobenzaldehyde in 95% ethanol. The absorbance of the Δ^1-pyrroline complex was measured at 430 nm following centrifugation at 3000*g* for 20 min (E_0 = 1.86/mM/cm) (Federico et al., 1985).

CAT activity was determined by monitoring the loss of H_2O_2 at 240 nm according to the method of Aebi (1984). The reaction was initiated by adding 100 μl of enzyme solution to 2 ml of 0.03 M H_2O_2 in 0.05 M potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, and the enzyme activity was determined from the linear portion of the rate curve.

APX activity was assayed spectrophotometrically as described by Durner and Klessig (1995). A 100-μl aliquot of enzyme solution was added to 1 ml of 50 mM potassium phosphate (pH 7.0) containing 0.75 mM ascorbate and 0.1 mM H_2O_2. Oxidation of ascorbate was monitored for 3 min at 290 nm. An extinction coefficient of 2.8/mM/cm was used.

Assay of Hydrogen Peroxide. The quantification of H_2O_2 was performed by a chemiluminescence reaction with luminol as described by Warm and Laties (1982) and Chen et al. (1993b) with some modifications. Three grams of quick-frozen foliage was homogenized in 10 ml of ice-cold 5% TCA. The crude extract was centrifuged for 30 min at 10,000*g*. Three milliliters of supernatant was extracted twice with 4% polyvinylpolypyrrolidone (w/v), and each extraction was followed by centrifugation for 10 min at 10,000*g*. The resulting supernatant was then twice bulk-extracted with DEAE Sephadex, followed by centrifugation. The successive extractions were used to remove colored compounds and interfering ions in the extracts. The amount of H_2O_2 in the resulting extract was tested by adding 50 μl of test solution and 50 μl of 0.5 mM luminol (in 0.2 N NH_4OH, pH 9.5) into 0.8 ml of 0.2 N NH_4OH (pH 9.5). Chemiluminescence was initiated by injecting 100 μl of 0.5 mM $K_3Fe(CN)_6$ (in 0.2 N NH_4OH) into the mixture. The emitted photons were counted in a Lumitron ATP photometer for 6 sec at 15 sec after adding the catalyst. An internal standard of H_2O_2 was added to the sample from the beginning of extraction.

Assay of Foliar Salicylic Acid. Foliar salicylic acid was extracted as described by Bi et al. (1997). A sample of 100 mg foliar powder from each treatment was vigorously extracted for 1 min in 1 ml of 50% (v/v) methanol three times, with each extraction followed by a centrifugation for 5 min at 5000*g*. The resulting supernatants were pooled and filtered through a 0.45-μm nylon

filter. The filtrate was added to the same volume of 2.0 N HCl and hydrolyzed for 1 hr in a boiling water bath. After hydrolysis, the samples were extracted with ca. 1 ml of ether three times and dried under nitrogen gas. The resulting sample was resuspended in 0.2 ml of 80% (v/v) methanol and filtered through a 0.45-μm nylon filter. A volume of 10 μl was then injected for the test separation.

Analysis of SA was conducted by reverse-phase high-pressure liquid chromatography (RP-HPLC) using a Nova-Pak C_{18}, 3.8- $\times$ 150-mm stainless steel column. The separation solvents included butanol–methanol–acetic acid–dd water (0.25 : 1.25 : 2 : 96.5) initially, and finally butanol–methanol–acetic acid–dd water (5 : 25 : 2 : 68). Both of the solvents contained 18 mM ammonium acetate (Murphy and Stutte, 1978). The separation was programmed with a linear gradient for 30 min from the initial solvent to 50% of the final solvent, followed by a 20 min linear gradient from 50% to 100% of the final solvent. Mobile-phase flow was 1 ml/min. Absorbance at 254 and 280 nm was monitored by a multiwavelength detector. The peak area, peak height, and retention time were determined on a CR501 Chromatopac (Shimadzu). The sample peak for SA was identified by comparison of the retention time and the 254 : 280 nm absorbance ratio with a salicylic acid standard. Contents were determined by standard curves of salicylic acid and were expressed on a per dry weight basis. The test was replicated three times.

Statistics. A least significant differences (LSD) test in a one-way completely randomized ANOVA in SAS (SAS Institute, 1988) was used to analyze the data in this study, unless otherwise stated.

RESULTS

Induction of Foliar SA and Related Phytochemicals. Feeding by larval *H. zea* significantly enhanced the foliar levels of H_2O_2, SA, CAT, and APX (Table 1). The greatest increase occurred with SA, where levels in the wounded treatment were over threefold higher than in the control. A response of similar magnitude has been observed in plants exhibiting SAR (Yalpani et al., 1991, 1993). The foliar levels of H_2O_2 increased by 62% and APX activity by 73% in comparison to the unwounded controls. CAT activity was slightly elevated in the wounded treatment, with an increase of ca. 9%.

Effect of Exogenous SA and MeSA on Cotton Foliar Resistance. We attempted to induce foliar resistance by applying SA or MeSA to cotton foliage. The concentrations of the compounds used in our study were sufficient to induce SAR in other plant systems (Yalpani et al., 1991). However, treatment of the cotton foliage with these compounds had no effect on larval growth, regardless

TABLE 1. EFFECT OF LARVAL FEEDING BY *H. zea* ON COTTON FOLIAR H_2O_2, SA, CAT, AND APX LEVELS

	Control	Damaged	% change
H_2O_2 (μmol/g fresh weight)[a]	1.49 (0.12)[c]	2.41 (0.35)	+61.7
SA (μg/g dry weight)[b]	6.10 (0.22)	18.58 (1.48)	+204.6
CAT (μmol/min/mg dry weight)[b]	4.77 (0.06)	5.18 (0.12)	+8.6
APX (μmol/min/g dry weight)[b]	1.27 (0.09)	2.20 (0.05)	+73.2

[a] Significantly different as $LSD_{0.05}$.
[b] LSD was not used to analyze the data due to the pooled samples.
[c] Numbers in parentheses are standard errors.

TABLE 2. EFFECT OF APPLICATION OF SA TO COTTON FOLIAGE ON FOURTH-INSTAR *H. zea* GROWTH RATE[a]

Relative growth rate	Control	SA	% change
At 24 hr (mg/day/mg)	0.299 (0.037) a	0.274 (0.045) a	−8.3
At 48 hr (mg/day/mg)	0.439 (0.015) a	0.439 (0.141) a	0

[a] Means in the same row followed by different letter were significantly different at $LSD_{0.05}$. Numbers in parentheses are standard errors.

of the method of SA application or the larval stage tested. Applying exogenous SA to cotton foliage did not significantly ($P > 0.05$) affect fourth-instar *H. zea* growth rate after a 24- or 48-hr feeding period (Table 2). Applying exogenous MeSA did not affect the weight gain or survivorship of neonates after a 96-hr feeding period (Table 3). The effect of MeSA on third-instar *H. zea* growth rate was also insignificant ($P > 0.05$) at both 48- and 64-hr feeding periods (Table 4).

Effect of MeSA and SA on Foliar POX, DAO, CAT, and APX Activities. We tested whether MeSA induced the enzymes POX and DAO. These proteins are correlated with induced resistance (Bi and Felton, 1995; Bi et al., 1997) and SAR (Rasmussen et al., 1991). However, we found that the application of exogenous MeSA had no significant ($P > 0.05$) effect on the foliar POX and DAO levels in comparison with those of the controls (Table 5). Our assays were conducted after the 96-hr treatment because previous studies indicated that POX activity reaches maximal levels 96 hr following wounding (Bi et al., 1997).

TABLE 3. EFFECT OF APPLICATION OF MeSA TO COTTON PLANTS ON NEONATE *H. zea* GROWTH[a]

	Larval weight (mg)	Survivorship (%)
Control	3.52 (0.27) a	79 a
MeSA	3.37 (0.19) a	76 a

[a]Means in the same column followed by the same letter were not significantly different at $LSD_{0.05}$. Larval weight was measured at 96 hr after feeding on excised foliage in the laboratory. Numbers in parentheses are standard errors.

TABLE 4. EFFECT OF APPLICATION OF MeSA TO COTTON ON THIRD-INSTAR *H. zea* GROWTH RATE[a]

	RGR at 48 hr	RGR at 64 hr
Control	0.635 (0.008) a	0.529 (0.007) a
MeSA	0.620 (0.010) a	0.516 (0.010) a

[a]Means in the same column followed by the same letter were not significantly different at $LSD_{0.05}$. RGR (relative growth rate) was expressed as mg/day/mg larva. Numbers in parentheses are standard errors.

TABLE 5. EFFECT OF APPLICATION OF MeSA TO COTTON PLANTS ON FOLIAR POX AND DAO LEVELS[a]

	POX (ΔOD/min/g fresh weight)	DAO (nmol/min/g fresh weight)
Control	42.13 (3.01) a	642.12 (0.04) a
MeSA	43.85 (4.69) a	547.95 (60.71) a

[a]Means in the same column followed by different letter were significantly different at $LSD_{0.50}$. Numbers in parentheses are standard errors.

To determine if SA inhibits CAT or APX in cotton as reported in other plant species, SA was added directly to a foliar protein extract. However, even a relatively high level (1 mM) of SA failed to inhibit either foliar CAT or APX activity *in vitro* (Table 6).

TABLE 6. EFFECT OF SA ON IN VITRO CAT AND APX ACTIVITIES[a]

	CAT (μmol/min/mg dry weight)	APX (μmol/min/g dry weight)
Control	2.90 (0.19) a	1.51 (0.04) a
SA	2.98 (0.07) a	1.57 (0.07) a

[a] Means in columns followed by different letter were significantly different at $LSD_{0.50}$. Numbers in parentheses are standard errors.

DISCUSSION

We previously reported that *H. zea* feeding damage on cotton plants induced resistance to the subsequent infestation of larval *H. zea* (Bi et al., 1997). We proposed that a combination of nutritional and oxidative stress is the primary resistance mechanism (Bi et al., 1997). In addition to inducing a series of oxidases including POX and DAO activities (Bi et al., 1997), *H. zea* herbivory significantly enhanced levels of endogenous H_2O_2 and SA in cotton foliage (Table 1). These results are consistent with plant responses to pathogen attack in many systems (e.g., Chen and Klessig, 1991; Rasmussen et al., 1991; Smith et al., 1991; Yalpani et al., 1993; Meuwly et al., 1995).

Our evidence indicates that SA is not involved as a signal in induced insect resistance in cotton (Tables 2, 3, and 4). We used a technique similar to that used by Yalpani et al. (1991) to demonstrate SAR against phytopathogens, but our results indicate that neither exogenous SA nor MeSA has an effect on cotton foliar resistance to *H. zea* larvae. What role, if any, SA has in insect resistance is unclear. The foliar levels of SA observed in this study (i.e., 18.6 μg/g dry weight) are likely to be innocuous to larvae. Previous work has shown that levels of SA in an artificial diet as high as 2500 μg/g dry weight are nontoxic to *H. zea* larvae (Summers and Felton, 1994). In other insect–plant systems, SA possesses little or no toxicity to the greenbug *Schizaphis graminum* (Todd et al., 1971), but does suppress feeding by the Mexican bean beetle *Epilachna varivestris* at very high concentrations (Fischer et al., 1990).

SA also does not appear to be responsible for the elevated levels of H_2O_2 in cotton (Table 1). In some plant species, SA strongly inhibits the decomposition of H_2O_2 by CAT or APX (Chen et al., 1993b; Durner and Klessig, 1995), but SA does not inhibit cotton foliar CAT or APX even at the relatively high concentrations of 1 mM (Table 6). The rise in H_2O_2 may be partly due to the enhanced activity of DAO (Bi et al., 1997), since DAO catalyzes the oxidation of di- and/or polyamines with the corresponding formation of amino aldehydes, ammonia, and H_2O_2 (Smith and Baker, 1988). H_2O_2 may be directly involved

as a component of induced resistance against *H. zea*. For instance, H_2O_2 in the presence of transitional metals forms the hydroxyl radical. This potent radical easily damages protein and lipid membrane components, triggering cell death (Halliwell and Gutteridge, 1985). Ingestion of damaged cotton foliage causes oxidative damage to the midgut epithelium of *H. zea* and is correlated with high foliar H_2O_2 levels (Bi et al., 1997) (Table 1). In addition, H_2O_2 may act as a substrate, driving the cross-linking of cell wall proteins by POX (Bi and Felton, unpublished data). The resulting cross-linkages may reduce the digestibility of cotton tissues for herbivores.

In conclusion, feeding by larval *H. zea* on cotton foliage induced levels of endogenous H_2O_2, SA, CAT, and APX. Nonetheless, SA does not appear to function as a signal for induced resistance against insects in this system. The role of SA in SAR against phytopathogens in the cotton system may also be questioned because of its lack of effect on CAT and APX activities. Further research is required to determine the significance of induced levels of SA in cotton.

Acknowledgments—The authors wish to thank Herbert Eichenseer, C. Dayton Steelman, Martha Davis, and Reid Baunes for review of the manuscript. This paper is published with the approval of the Director of the Arkansas Agricultural Experiment Station as manuscript 96092. The research was supported by USDA/NRICGP (93-37302-9571) and USDA/CSRS (92-34195-7162)

REFERENCES

AEBI, H. 1984. Catalase in vitro. *Methods Enzymol.* 105:121–126.

BALDWIN, I. T., and SCHMELZ, E. A. 1996. Immunological "memory" in the induced accumulation of nicotine in wild tobacco. *Ecology* 77:236–246.

BALDWIN, I. T., SCHMELZ, E. A., and OHNMEISS, T. E. 1994. Wound-induced changes in root and shoot jasmonic acid pools correlate with induced nicotine synthesis in *Nicotiana sylvestris* Spegazzini and Comes. *J. Chem. Ecol.* 20:2139–2157.

BI, J. L., and FELTON, G. W. 1995. Foliar oxidative stress and insect herbivory: Primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. *J. Chem. Ecol.* 21:1511–1530.

BI, J. L., MURPHY, J. B., and FELTON, G. W. 1997. Antinutritive and oxidative components as mechanisms of induced resistance in cotton to *Helicoverpa zea*. *J. Chem. Ecol.* 23:95–115.

BI, Y. M., KENTON, P., MUR, L., DARBY, R., and DRAPER, J. 1995. Hydrogen peroxide does not function down stream of salicylic acid in the induction of protein expression. *Plant J.* 8:235–245.

BLECHERT, S., BRODSCHELM, W., HOLDER, S., KAMMERER, L., KUTCHAN, T. M., MULLER, M. J., XIA, Z. Q., and ZENK, M. H. 1995. The octadecanoic pathway: Signal molecules for the regulation of secondary pathways. *Proc. Natl. Acad. Sci. U.S.A.* 92:4099–4105.

BODNARYK, R. P., and RMYERSON, R. T. 1994. Effect of wounding and jasmonates on the physicochemical properties and flea beetle defense responses of canola seedlings, *Brassica napus* L. *Can. J. Plant Sci.* 74:899–907.

CHASAN, R. 1995. SA: source or signal for SAR? *Plant Cell* 7:1519–1521.

CHEN, Z. X., and KLESSIG, D. F. 1991. Identification of a soluble salicylic acid-binding protein

that may function in signal transduction in the plant disease-resistance response. *Proc. Natl. Acad. Sci. U.S.A.* 88:8179–8183.

CHEN, Z. X., RICIGLIANO, J. W., and KLESSIG, D. F. 1993a. Purification and characterization of soluble salicylic acid-binding protein from tobacco. *Proc. Natl. Acad. Sci. U.S.A.* 90:9533–9537.

CHEN, Z. X., SILVA, H., and KLESSIG, D. F. 1993b. Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science* 262:1883–1886.

CHEN, Z. X., MALAMY, J. P., HENNING, J., CONRATH, U., SANCHEZ-CASAS, P., SILVA, H., RICIGLIANO, J., and KLESSIG, D. F. 1995. Induction, modification, and transduction of the salicylic acid signal in plant defense responses. *Proc. Natl. Acad. Sci. U.S.A.* 92:4134–4137.

CHIPPENDALE, G. M. 1970. Metamorphic changes in fat body proteins of the southwestern corn borer *Diatraea grandiosella*. *J. Insect Physiol.* 16:1057–1068.

COHEN, Y., GISI, U., and NIDERMAN, T. 1993. Local and systemic protection against *Phytophthora infestans* induced in potato and tomato plants by jasmonic acid and jasmonic methyl ester. *Phytopathology* 83:1054–1062.

DOARES, S. H., SYROVETS, T., WEILER, E. W., and RYAN, C. A. 1995. Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. *Proc. Natl. Acad. Sci. U.S.A.* 92:4095–4098.

DURNER, J., and KLESSIG, D. F. 1995. Inhibition of ascorbate peroxidase by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defense responses. *Proc. Natl. Acad. Sci. U.S.A.* 92:11312–11316.

FARMER, E. E., and RYAN, C. A. 1990. Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proc. Natl. Acad. Sci. USA.* 87:7713–7716.

FEDERICO, R., ANGOLAN, R., CHEST, A., and PIN, C. 1985. Determination of diamine oxidase in lentil seedling by enzymatic activity and immunoreactivity. *Plant Physiol.* 79:62–64.

FISCHER, D. C., KOGAN, M., and PAXTON, J. 1990. Deterency of Mexican bean beetle (Coleoptera: Coccinellidae) feeding by free phenolic acids. *J. Entomol. Sci.* 25:230–238.

HALLIWELL, B., and GUTTERIDGE, J. M. C. 1985. Free Radicals in Biology and Medicine. Clarendon Press, Oxford.

HAMMERSCHMIDT, R. 1993. The nature and generation of systemic signals induced by pathogens, arthropod herbivores, and wounds. *Adv. Plant Pathol.* 10:307–337.

KARBAN, R., ADAMCHAK, R., and SCHNATHORST, W. C. 1987. Induced resistance and interspecific competition between spider mites and a vascular wilt fungus in cotton plants. *Science* 235:678–680.

KAUSS, H., and JEBLICK, W. 1996. Influence of salicylic acid on the induction of competence for H_2O_2 elicitation–comparison of ergosterol with other elicitors. *Plant Physiol.* 111:755–763.

KHOSHKHOO, N., HEDIN, P. A., and MCCARTY, J. C., JR. 1993. Effects of bioregulators on the terpenoid aldehydes in root-knot nematode infected cotton plants. *J. Agric. Food Chem.* 41:2442–2446.

KLESSIG, D. F., and MALAMY, J. 1994. The salicylic acid signal in plants. *Plant Mol. Biol.* 26:1439–1458.

LEE, H., LEON, J., and RASKIN, I. 1995. Biosynthesis and metabolism of salicylic acid. *Proc. Natl. Acad. Sci. U.S.A.* 92:4076–4079.

MALAMY, J., and KLESSIG, D. F. 1992. Salicylic acid and disease resistance. *Plant J.* 2:643–654.

MALAMY, J., CARR, J. P., KLESSIG, D. F., and RASKIN, I. 1990. Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. *Science* 250:1001–1004.

MALAMY, J., HENNIG, J., and KLESSIG, D. F. 1992. Temperature-dependent induction of salicylic acid and its conjugates during the resistance response to tobacco mosaic virus infection. *Plant Cell* 4:359–366.

MÉTRAUX, J. P. 1994. Plants, pp. 1–28, *in* R. J. Turner (ed.). Immunology: A Comparative Approach. John Wiley, London.

MÉTRAUX, J. P., SINGER, H., RYALS, J. A., WARD, E., WYSS-BENZ, M., GAUDIN, J., RASCHDORF, K., SCHMIDT, E., BLUM, W., and INVERARDI, B. 1990. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250:1004–1006.

MEUWLY, P., MOLDERS, W., BUCHALA, A., and MÉTRAUX, J. P. 1995. Local and systemic biosynthesis of salicylic acid in infected cucumber plants. *Plant Physiol.* 109:1107–1114.

MURPHY, J. B., and STUTTE, C. A. 1978. Analysis for substituted benzoic and cinnamic acids using high-pressure liquid chromatography. *Anal. Chem.* 86:220–228.

RASKIN, I., 1992. The role of salicylic acid in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 43:439–463.

RASMUSSEN, J. B., HAMMERSCHMIDT, R., and ZOOK, M. N. 1991. Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv. *syringae*. *Plant Physiol.* 97:1342–1347.

RIDGE, I., and OSBORNE, D. A. 1970. Hydroxyproline and peroxidases in cell walls of *Pisum sativum*: Regulation by ethylene. *J. Exp. Bot.* 21:843–856.

RYALS, J., UKNES, S., and WARD, E. 1994. Systemic acquired resistance. *Plant Physiol.* 104:1109–1112.

SAS Institute. 1988. SAS/STAT User's Guide, Release 6.03 Edition. SAS Institute Inc., Cary, North Carolina.

SANCHEZ-CASAS, P., and KLESSIG, D. F. 1994. A salicylic acid-binding activity and a salicylic acid-inhibitable catalase activity are present in a variety of plant species. *Plant Physiol.* 106:1675–1679.

SCHWEIZER, P., GEES, R., and MOSINGER, E. 1993. Effect of jasmonic acid on the interaction of barley (*Hordeum vulgare* L.) with powdery mildew *Erysiphe graminis* f.sp. *hordei*. *Plant Physiol.* 102:503–511.

SMITH, J. A., HAMMERSCHMIDT, R., and FULBRIGHT, D. W. 1991. Rapid induction of systemic resistance in cucumber by *Pseudomonas syringae* pv. *syringae*. *Physiol. Mol. Plant Pathol.* 38:223–235.

SMITH, T. A., and BAKER, J. H. A. 1988. The di- and polyamine oxidase of plants, pp. 573–587, *in* V. Zappia and A. E. Pegg (eds.). Progress in Polyamine Research. Plenum Press, New York.

SUMMERS, C. B., and FELTON, G. W. 1994. Prooxidant effects of phenolic acids on the generalist herbivore *Helicoverpa zea* (Lepidoptera: Noctuidae): Potential mode of action for phenolic compounds in plant anti-herbivore chemistry. *Insect Biochem. Mol. Biol.* 24:943–953.

TODD, G. W., GETAHUN, A., and CRESS, D. C. 1971. Resistance in barley to the greenbug, *Schizaphis graminum*. 1. Toxicity of phenolic and flavonoid compounds and related substances. *Ann. Entomol. Soc. Am.* 64:718–722.

VERNOOIJ, B., FRIEDRICH, L., MORSE, A., REIST, R., KOLDITZ-JAWHAR, R., WARD, E., UKNES, S., KESSMANN, H., and RYALS, J. 1994. Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell* 6:959–965.

WARD, E. R., UKNES, S. J., WILLIAMS, S. C., DINCHER, S. S., WIEDERHOLD, D. L., ALEXANDER, D. C., AHL-GOY, P., MÉTRAUX, J. P., and RYALS, J. A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. *Plant Cell* 3:1085–1094.

WARM, E., and LATIES, G. G. 1982. Quantification of hydrogen peroxide in plant extracts by the chemiluminescence reaction with luminol. *Phytochemistry* 21:827–831.

WHITE, R. F. 1979. Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus. *Virology* 99:410–412.

YALPANI, N., SILVERMAN, P., WILSON, T. M. A., KLEIER, D. A., and RASKIN, I. 1991. Salicylic

acid is a systemic signal and an inducer of pathogenesis-related protein in virus-induced tobacco. *Plant Cell* 3:809–818.

YALPANI, N., LEÓN, J., LAWTON, M. A., and RASKIN, I. 1993. Pathway of salicylic acid biosynthesis in healthy and virus-inoculated tobacco. *Plant Physiol.* 103:315–321.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

GROWTH INHIBITORY, INSECTICIDAL, AND FEEDING DETERRENT EFFECTS OF (12Z,15Z)-1-ACETOXY-2-HYDROXY-4-OXO-HENEICOSA-12,15-DIENE, A COMPOUND FROM AVOCADO FRUIT, TO *Spodoptera exigua*

CESAR RODRIGUEZ-SAONA,* JOCELYN G. MILLAR, and JOHN T. TRUMBLE

Department of Entomology
University of California
Riverside, California 92521

(Received October 31, 1996; accepted March 11, 1997)

Abstract—We isolated and identified (12Z,15Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (persin) from avocado as a compound toxic to *Spodoptera exigua* larvae. Persin was obtained from oil extracted from the specialized idioblast cells of avocado fruit. When tested for activity using neonates in no-choice artificial diet bioassays, persin at concentrations of 200 μg/g inhibited growth; larvae gained only ~50% of the weight attained by control insects after seven days. At concentrations of 400 μg/g or above, larval growth was reduced by >70% as measured by weight gain, and significant mortality occurred. In choice tests, persin at concentrations of 400 μg/g or higher deterred feeding, with significantly more larvae being found on control diet than on persin-treated diet. Possible applications of persin as a natural insecticide are discussed.

Key Words—*Spodoptera exigua*, *Persea americana*, avocado, idioblast cells, persin, (12Z,15Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene, Lepidoptera.

INTRODUCTION

Public health and environmental safety concerns have contributed to a reduction in the use of synthetic insecticides in commercial agriculture (Trumble, 1990).

*To whom correspondence should be addressed.

0098-0331/97/0700-1819$12.50/0 © 1997 Plenum Publishing Corporation

Consequently, more target-selective and biodegradable compounds are needed to replace the environmentally persistent chemicals with broad-spectrum toxicity that are being phased out (Alkofahi et al., 1989). Alternative sources of potentially suitable insecticides include phytochemicals that may have evolved for plant defense against herbivores (Duke, 1990). One possible source of such chemicals is the unusual idioblast oil cells that occur in the leaves and other organs of dicotyledonous plant species in numerous families (Postek and Tucker, 1983; Baas and Gregory, 1985). These oil cells usually differ from their neighboring mesophyll cells both in morphology and composition. For example, Maron and Fahn (1979) reported that oil cells in *Laurus nobilis* L. are larger, have lower starch contents, and differ in their plastid organization from mesophyll cells. Other reports (Mariani et al., 1989) have shown the presence of insecticidal sesquiterpene lactones in oil cells of *Liriodendron tulipifera* L.

An early study indicated that idioblast oil cells compose approximately 2% of the tissue volume of avocado fruit (*Persea americana* Mill.) (Cummings and Schroeder, 1942). More recently, Platt and Thomson (1992) described avocado idioblast cells in detail and developed a method for their extraction in multigram quantities from ripe fruit. They also reported that these cells are present in the leaf, root, and seed as well as the flesh of fruit. Preliminary analyses indicated the presence of alkaloids, sesquiterpene hydroperoxides, and possibly other terpenes.

Subsequent studies showed that the oil from avocado idioblast cells is biologically active. Kobiler et al. (1993) demonstrated the activity of two compounds against the fungus, *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. More recently, Rodriguez-Saona and Trumble (1996) showed that crude oil extracted from idioblast cells was not only toxic to early [LC_{50} (95% Fiducial Limits (FL) = 0.16% (0.15–0.16%)] and late instars [LC_{50} (95% FL) = 0.51% (0.44–0.61%)] of the generalist herbivore *Spodoptera exigua* (Hübner), but also deterred feeding.

The objectives of the study reported here were: (1) to identify the compound present in the idioblast cell oil most insecticidal to *S. exigua*, and (2) to test the effects of this compound on early-instar *S. exigua* growth, mortality, and feeding behavior.

METHODS AND MATERIALS

Extraction of Oil. Ripe avocado, *P. americana*, fruit were obtained from trees of the Haas cultivar grown at the University of California's South Coast Research and Extension Center, Santa Ana, California. The idioblast cells were separated and the oil was extracted as previously described (Rodriguez-Saona and Trumble, 1996). One kilogram of ripe fruit yielded an average of 7.3 $\pm$ 1.5 g of crude oil.

Insects. S. exigua larvae were reared on an artificial diet modified from Patana (1969), at 28 ± 2°C and a photoperiod of 14L:10D. The colony was originally collected from Orange County, California, in 1982 and had new genetic material added periodically, including within 12 months prior to the study. Cohorts of unfed neonates were used within 12 hr of eclosion. Incubators used in bioassays were maintained at 28 ± 2°C, a relative humidity of ~75%, and a photoperiod of 14L:10D with fluorescent lighting.

Isolation and Identification of Active Compounds. NMR spectra were taken on $CDCl_3$ solutions, using a GE 300 instrument (General Electric, Fremont, California), at 300 MHz for protons, and 75.48 MHz for ^{13}C. Exact mass chemical ionization mass spectra (NH_3) were recorded with a VG 7070 EHF high resolution mass spectrometer (VG Instrument Co., Beverly, Massachusetts).

A portion of the crude oil (4.5 g) was fractionated by flash chromatography on silica gel (230–400 mesh; 5 cm ID × 25 cm) (Aldrich Chemical, St. Louis, Missouri), eluting sequentially with 2 liters each of toluene–ethyl ether–acetic acid at 70:30:1 (v/v/v), 50:50:1, and 10:90:1. The material remaining on the column was then stripped off with ethanol (2 liters). Fractions were checked by thin-layer chromatography (TLC) on silica plates developed with toluene–ethyl ether–acetic acid (70:30:1 v/v/v). Spots on developed plates were visualized using UV (254 nm) followed by spraying with H_2SO_4 and charring with a hot air blower. Subfractions were combined to yield eight fractions that were concentrated under reduced pressure, followed by exposure to ~0.5 mm Hg vacuum to remove traces of solvent. The concentrated fractions were weighed, then diluted with acetone to a final volume of 10 ml, and refrigerated at 4°C until needed.

A portion (~15%) of the most polar active fraction (fraction 3, 0.22 g total weight) was further purified by HPLC, using a 2.25- × 25-cm Econosil silica column (Alltech, Deerfield, Illinois, 10 μm particle size) eluted with 15% acetone in hexane (4 ml/min), monitoring the effluent with a Dynamax RI-2 refractive index detector (Rainin Instrument Co., Emeryville, California). Five fractions were taken, of which the major peak, constituting the active material, eluted at about 32 min. The fractions were concentrated, then taken up in 1.5 ml acetone to maintain the same dilution as was used in the original fraction. Aliquots of the active compound were removed for spectral analysis. Chemical ionization exact mass (direct insertion probe, NH_3 reagent gas): Calcd. for $C_{23}H_{44}NO_4$ ($M+NH_4$): 398.3270; Found: 398.3281. ^1H NMR: 5.45–5.27 (m, 4H), 4.37–4.27 (m, 1H), 4.15–4.02 (m, 2H), 2.78 (br t, 2H, J = 5.6 Hz), 2.63 (d, 2H, J = 6.0 Hz), 2.46 (t, 2H, J = 7.4 Hz), 2.12 (s, 3H), 2.05 (br quartet, 4H, J = 6.6 Hz), 1.4–1.22 (m, ~16H), 0.89 (t, 3H, J = 7 Hz). ^{13}C NMR: 210.8, 171.2, 130.2, 130.0, 128.1, 127.9, 67.2, 66.0, 45.1, 43.6, 31.5, 29.6, 29.3, 29.3, 29.1, 27.2, 27.2, 25.6, 23.5, 22.6, 20.8, 14.1. The ^{13}C and ^1H NMR spectra matched those reported for the known compound

(12Z,15Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (persin) previously isolated from avocado (with the exception that the original report neglected to report the quartet at 2.05, corresponding to the four allylic protons; Prusky et al., 1982).

Bioassays. Bioactivity of the fractions was tested against early-instar *S. exigua* in an artificial diet bioassay, using rates equivalent to 2% crude idioblast cell oil in diet. This rate (20 mg/g) was selected because previous tests using early instars showed high mortality and a significant reduction of larval development at this concentration (Rodriguez-Saona and Trumble, 1996). Treated diets were prepared by transferring 750 μl of acetone solutions of fractions (equivalent to 0.34 g crude oil) into 50-ml polypropylene centrifuge tubes (Fisher, Pittsburgh, Pennsylvania), evaporating the acetone, adding 2 ml of 0.1% Tween-80 solution (Fisher), homogenizing with an ultrasonic homogenizer (Cole-Parmer, Chicago, Illinois), and finally adding prepared diet to produce a final weight of 15 g. The mixture was vortexed for 3 min. Control diet was prepared by mixing 2 ml of Tween solution and 13 g of artificial diet to produce a final weight of 15 g. Control and treated diets were poured into 24-well (15.9 mm diameter and 15.9 mm depth) bioassay trays (C-D International Inc., Pitman, New Jersey). One neonate larva was added per well, and the trays were placed in an incubator under the previously described conditions. Twenty-four neonates were tested for each fraction and the control. Mortality and larval weight were recorded after seven days.

The effect of the purified compound (fraction 3-3) on larval growth and mortality of *S. exigua* was tested. Treated and control diet were prepared as previously described. Eight concentrations were examined: 0 (control), 200, 300, 400, 500, 600, 700, and 800 μg/g. Twenty-four neonates were used per treatment. Bioassays were conducted under standard incubator conditions (see above), and were replicated four times for each treatment (i.e., a total of 96 larvae were tested with each treatment). After seven days, larvae were weighed, and the EC (effective concentration) values were calculated by subtracting the mean larval weight for each treatment from the mean weight of the controls and dividing by the mean weight of the controls. The EC_{50} value (the concentration required to inhibit growth by 50% relative to controls) was estimated by interpolation. Larval mortality was recorded after seven days.

Choice Tests. The ability of early-instar *S. exigua* to discriminate between treated and control diets was assessed in choice tests (as described in detail by Rodriguez-Saona and Trumble, 1996). Briefly, five neonates were placed inside an arena constructed from a 30-ml plastic cup lined with ~1 cm of 4% agar (w/v). Each cup had holes cut in opposite sides where two 1.5-ml polypropylene microcentrifuge tubes were placed. One of the tubes contained the control diet alone and the other contained the treated diet. The tubes were completely filled with the diet. To determine if low concentrations of the active compound

could elicit a larval response, one concentration below the EC_{50} was tested (100 μg/g). In addition, one concentration near the EC_{50} (200 μg/g) and two concentrations above the EC_{50} (400 and 600 μg/g) were tested. Treated and control diet were prepared as previously described. The arenas were held in an incubator under standard conditions (see above). The positions of the larvae were recorded at the beginning of the photophase and again 12 hr later for four days. Each arena was treated as a replicate, and each treatment had a total of 50 replicates.

Data Analysis. In no-choice tests, inhibition of larval growth was related to dose by an exponential function of the general form $Y = 100\,[1 - \exp(-kx)]$ (Smirle and Wei, 1996), where Y is the growth inhibition index expressed as a percent, x is dose, and k is a proportionality constant. Mortality data were analyzed using probit analysis (Finney, 1971). Other comparisons were done using Minitab (1991). Choice test data were analyzed by calculating the number of larvae present in the control diet minus the number of larvae present in the treated diet divided by the total larvae at each observation. We thus tested the hypothesis that the difference was significantly different from zero, using t tests (after Horton, 1995) [the test statistic was approximately t because of the large sample size ($N = 50$)] calculated with Super Anova (1989).

RESULTS

Isolation and Identification of Active Compounds. The first fractionation of the crude oil yielded two fractions (fractions 1 and 3), which produced high levels of larval mortality ($\geq 50\%$) (Figure 1a) and significantly reduced larval weight (Figure 1b, $P < 0.05$) compared to the other fractions. Larvae fed diet mixed with fractions 1 and 3 weighed an average of 4.43 $\pm$ 1.63 (SE) and 5.36 $\pm$ 1.96 (SE) mg, respectively, while control larvae weighed an average of 64.57 $\pm$ 7.38 (SE) mg. In contrast, diet treated with fractions 4 and 5 resulted in significant increases in weight versus the control diet (Figure 1b, $P < 0.05$).

Further efforts were focused on the most insecticidal fraction (fraction 3). This fraction consisted primarily of one compound, as determined by TLC. A portion was further purified by normal-phase HPLC, yielding the major compound in pure form (fraction 3-3). The compound degraded upon attempted coupled GC-MS analysis. However, exact mass measurement using a direct insertion probe and chemical ionization yielded a molecular formula of $C_{23}H_{40}O_4$, corresponding to four sites of unsaturation. The ^{13}C NMR spectrum showed two carbonyl peaks at 210.8 (ketone) and 171.2 ppm (ester), and four peaks (130.2, 130.0, 128.1, 127.9), each with a single attached proton, corresponding to carbons of two nonconjugated 1,2-disubstituted double bonds. These six peaks accounted for all four sites of unsaturation. Two further peaks shifted downfield

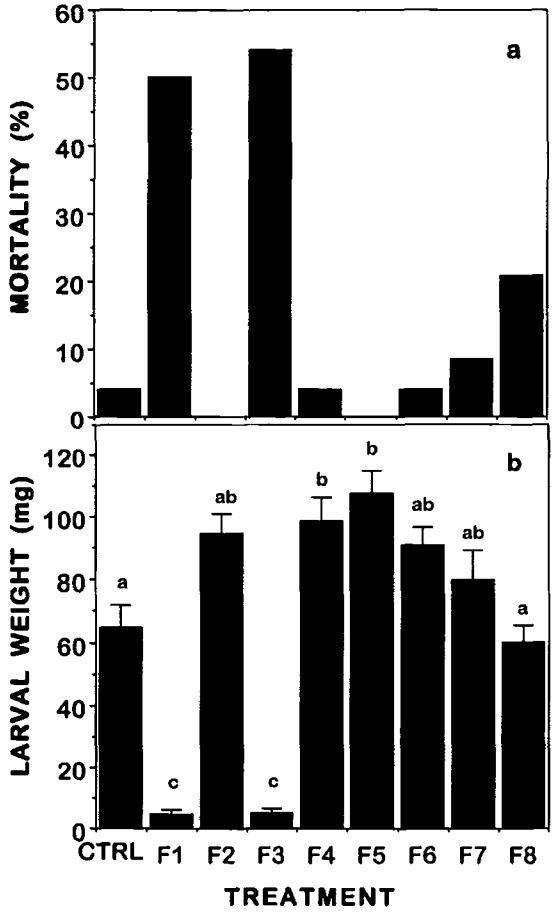
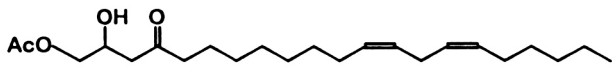


FIG. 1. Percent mortality (a) and weight (b) of *S. exigua* larvae fed control diet and diet containing fractions from avocado idioblast cell oil, from flash chromatographic fractionation. Bars at each data point indicate the standard error. Different letters indicate statistical differences between treatments (Tukey's Pairwise Comparisons, $P < 0.05$).

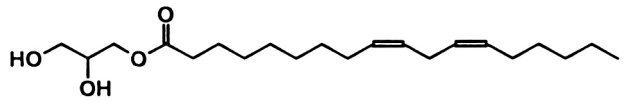
(67.2 ppm, two attached protons; 66.0 ppm, one attached proton) were assigned as methylene and methine carbons bonded to an oxygen, accounting for the two remaining noncarbonyl oxygen atoms. One of these could be attributed to the previously identified ester function, while the other could only belong to an alcohol, since there were no other carbon atoms with a chemical shift indicative of an attached oxygen. There were two methyl groups (20.8 and 14.1 ppm); all other carbons were methylenes.

Further structural details were supplied by the ^1H spectrum. A two-proton triplet (2.46 ppm) and a two-proton doublet (2.63 ppm) corresponded to methylenes on either side of a ketone, with the former being adjacent to another methylene, and the latter, shifted slightly further downfield, being adjacent to a methine. The methine proton was located as a complex multiplet centered at 4.32 ppm, with the downfield shift suggesting an attached oxygen atom. Furthermore, the complexity of the coupling pattern suggested coupling to at least two more protons on an adjacent carbon. These data were corroborated by a complex two-proton multiplet centered at 4.08, indicative of a diastereotopic methylene with an attached oxygen atom. A three-proton singlet (2.12 ppm) was assigned as the methyl portion of an acetate ester. Thus, the "head" end of the molecule was assigned the partial structure shown in the first six atoms of persin (Figure 2, I), with the only uncertainty being the relative positions of the acetate and hydroxyl functions at positions 1 and 2.

The remainder of the structure was assigned as follows. A three-proton triplet (0.89 ppm) indicated a methyl group terminating an alkyl chain. A multiplet consisting of four similar olefinic protons (5.45–5.27 ppm), a broadened quartet consisting of four allylic protons (2.05 ppm), and a broadened triplet (2.78 ppm) consisting of two bisallylic protons suggested a 1,4-diene system isolated in an alkyl chain, typical of an unsaturated fatty acid-type moiety. The only other NMR signal was the large methylene envelope, corresponding to 16 protons on eight methylene units. Assembling all of the above information, the structure was tentatively assigned as a 1-acetoxy-2-hydroxy-4-oxo-heneicosadiene, with only the position and geometry of the 1,4-diene system and the relative positions of the acetoxy and hydroxy groups being in question. A search



(12Z,15Z)-1-Acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene (I)



Monoglyceride of linoleic acid (II)

FIG. 2. Structural relationship between the biologically active compound, (12Z, 15Z)-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene) (persin) (I), and the monoglyceride of linoleic acid (II).

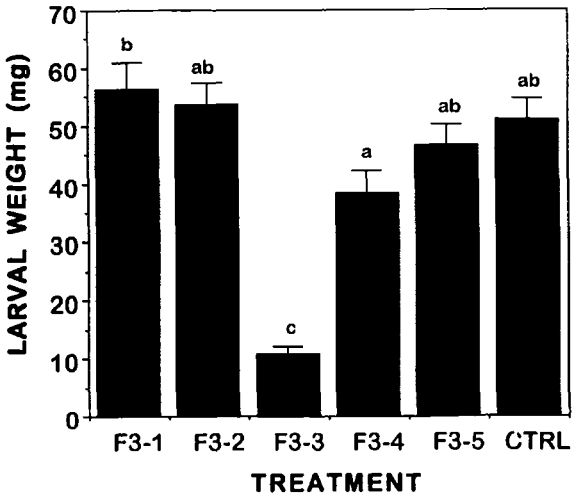


FIG. 3. Mean weights of *S. exigua* larvae fed control diet and diet containing subfractions of avocado idioblast cell oil, fractionated by HPLC. Bars at each data point indicate the standard error. Different letters indicate statistical differences between treatments (Tukey's pairwise comparisons, $P < 0.05$).

of the literature based on the above information turned up persin (12Z,15Z-1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene) (Figure 2, I), which had been isolated previously from avocado leaves (Chang et al., 1975) and fruit (Prusky et al., 1982). The literature ^{1}H and ^{13}C spectra matched those of the isolated compound [with the exception that Prusky et al. (1982) omitted to report the bisallylic triplet at 2.78 ppm], confirming its identity.

In no-choice bioassays, purified persin (fraction 3-3) caused significant reductions in *S. exigua* larval weights (Figure 3, $P < 0.05$) compared to the other subfractions. In further tests with known amounts of the purified compound, persin inhibited *S. exigua* larval growth, with an EC_{50} of 185.6 $\pm$ 4.1 (SE) μg/g (Figure 4). At concentrations higher than 500 μg/g, larval growth was inhibited by over 80% compared to controls, and high levels of mortality (40–50%) occurred (Figure 4). The LC_{50} (95% FL) was 936 μg/g (805–1190 μg/g) of persin in diet, with a log dose–probit regression line slope of 2.71 $\pm$ 0.39.

Choice Tests. In choice tests, persin deterred feeding at concentrations of 400 or 600 μg/g, with more larvae being found on control diet than on treated diet at all observations (Figure 5, t test, $df = 49$, $P < 0.05$; for each time interval). At dietary concentrations of 200 μg/g or less, seven of eight observations showed no significant differences in preference between control and persin-treated diets (Figure 5, t test, $df = 49$, $P > 0.05$; for each time interval).

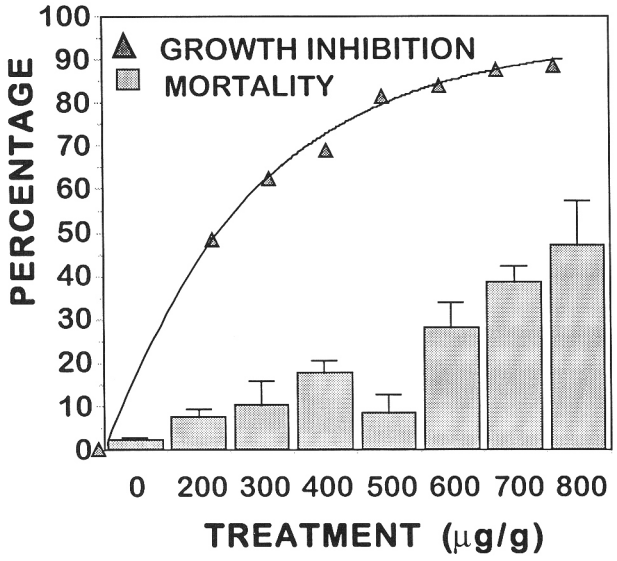


FIG. 4. Percent growth inhibition and mortality of *S. exigua* larvae fed diet containing different concentrations of persin. The equation for growth inhibition is: $Y = 100\,[1 - \exp(-0.0037X)]$. Bars at each data point indicate the standard error.

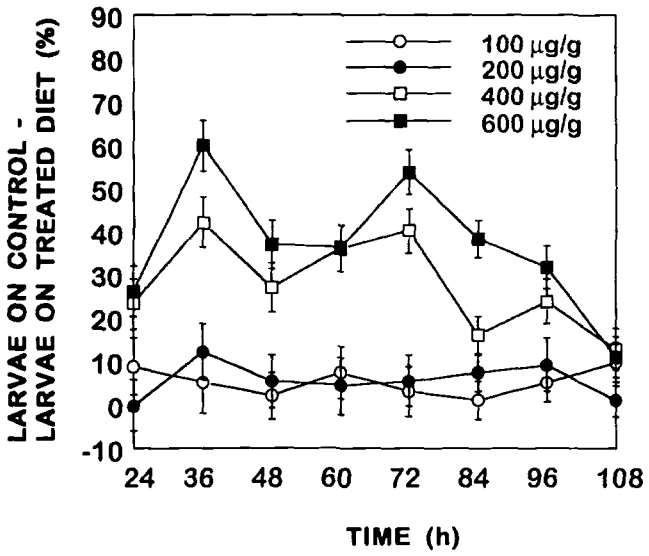


FIG. 5. Feeding deterrency of persin to *S. exigua* larvae in choice tests (first observation recorded 24 hr after initiation of the experiment). Bars at each data point indicate the standard error.

DISCUSSION

Persin, (12*Z*, 15*Z*)-1-acetoxy-2-hydroxy-4-oxo-henecoisa-12,15-diene, was isolated by bioassay-driven fractionation of avocado fruit idioblast cell oil and determined to be both toxic and a feeding deterrent to larvae of *S. exigua*. This compound has been previously isolated, and it apparently has a wide spectrum of biological activity. It was first isolated from avocado leaves and reported to inhibit growth of silkworm larvae, *Bombyx mori* L. (Chang et al., 1975; Murakoshi et al., 1976). Prusky et al. (1982) subsequently isolated persin from the peel of unripe avocado fruit and found that it inhibited in vitro vegetative growth of the avocado fungal pathogen *C. gloeosporioides*. More recently, Kobiler et al. (1993) reported the presence of persin in specialized oil cells of the avocado mesocarp.

At doses of 200 μg/g, persin inhibited growth of fourth-instar *B. mori* by approximately 40% over six days (Murakoshi et al., 1976). We found similar activity at 200 μg/g against early instars of the generalist herbivore *S. exigua*, where growth was inhibited by approximately 50% in seven days. We had anticipated differences between these species because the specialist feeder *B. mori*, with a long history of cultivation exclusively on mulberry, has been protected from most natural or synthetic chemicals. Conversely, *S. exigua* is a generalist herbivore that is constantly exposed to a variety of plant natural products, and it is the frequent target of pesticide applications (Brewer et al., 1990). Apparently, the metabolic enzymes providing substantial resistance for *S. exigua* larvae to pesticides or secondary plant compounds such as the linear furanocoumarins (Brewer et al., 1995) are not as effective against persin.

Although *S. exigua* shows a similar susceptibility to persin as *B. mori*, both insects are affected by concentrations much lower than the levels found in the pericarp and mesocarp of avocado fruit. Kobiler et al. (1993) reported concentrations in avocado fruit pericarp of different ages (starting from 75 days after fruit set until full maturity nearly 170 days later) ranging from 760 to 2280 μg/g of fresh weight. Concentrations in the mesocarp ranged from 1500 to 5800 μg/g. *S. exigua* is not listed as an avocado pest (McKenzie, 1935); this could be explained, at least in part, by the presence of persin in most tissues of the plant.

Our study showed that persin also modifies larval behavior of *S. exigua* by acting as a feeding deterrent at sublethal concentrations. Deterrency was observed on diet treated with persin at concentrations of 400 μg/g or higher during the first 72 hr, after which deterrency declined (Figure 5). Three factors may contribute to this observed decline in activity. First, persin is degraded by exposure to light (Carman and Duffield, 1995). Second, larger larvae may be more capable of tolerating the treated diet. Third, larvae may have habituated. Because only the two highest rates of persin produced avoidance, it is uncertain if these data

represent a threshold effect or a dose–response effect. In a comparison of diets with 400 μg/g and 600 μg/g (Figure 5), significantly more avoidance at the high rate was documented for three of eight observations, suggesting that a dose–response may be occurring.

Choice tests show that early instars of *S. exigua* fed persin at a concentration of 200 μg/g may not avoid treated diet (no behavioral effects, Figure 5), but no-choice tests show physiological effects; larval weights decreased compared to controls (Figure 4), even though larvae suffered low mortality (< 10%). Thus, persin detrimentally affects *S. exigua* larval growth at concentrations in diet of 200 μg/g or higher, but has deterrent effects only at concentrations of 400 μg/g or higher.

The LC_{50} for early-instar *S. exigua* is 936 μg/g persin in diet after seven days, which can be misleading because physiological effects were observed at a much lower concentration (200 μg/g, Figure 4), indicating that persin has strong growth inhibitory effects at a sublethal concentration. In comparison, Prabhaker et al. (1986), in similar diet-incorporation bioassays, found that seed extracts from the neem tree, *Azadirachta indica* A. Juss, at 200 μg/g in artificial diet killed 53% of neonate *S. exigua* larvae in eight days. Similarly, Moar and Trumble (1987) reported an LC_{50} for neem of 130 μg/g to early-instar *S. exigua*. Although the biological activity of persin is in the same range as the published LC_{50}s for neem, the LC_{50} for persin is substantially higher, suggesting that the primary value of this compound may be as a model for more stable and toxic synthetic compounds.

Our results indicate that persin, or more stable analogs, may have potential for insect control. However, persin does have substantial biological activity in mammals. Seawright et al. (1995) reported the experimental use of persin for treatment of mammalian cancers and that persin inhibits or prevents lactation. In particular, persin is a close structural mimic of the monoglyceride of linoleic acid (Figure 2, II), even in the position and the stereochemistry of the two double bonds in the chain. Seawright et al. (1995) speculate that the physiological action of persin in mammals may be due to interference, perhaps irreversibly, with glyceride biosynthesis. Nonetheless, the comparatively high concentrations in avocados have not been reported as a human health concern, even though humans ingest fresh and processed avocados, and avocado oil is widely used in cosmetics (Anonymous, 1980). One possible explanation for this lack of reported toxicity may be due to the nature of the idioblast cells; these cells have a tough cell wall that is probably not degraded by human digestion, so that the cells may pass through the digestive system intact.

Furthermore, substantial information will be required on field rates, mammalian toxicity, photostability, and phytotoxicity before the compound can be considered for commercialization. Should the extraction of persin from relatively expensive avocado fruit prove commercially impractical, total synthesis of the

compound is a possibility (Bull and Carman, 1994; MacLeod and Schäffeler, 1995), and it may also be possible to develop more photostable, more selective, or more potent analogs.

The isolation and identification of the bioactive compounds in fraction 1 (Figure 1) is in progress. This fraction is complex, and ongoing studies have indicated biological activity in at least three subfractions. The identities and biological activities of the purified compounds will be reported in due course.

Acknowledgments—We thank Kristina White, Gregory Kund, Mitchel White, Stephanie Young, and Claudia Trübenbach for assistance in the laboratory. We also thank Carlos Coviella, William Carson, and Drs. Stuart Reitz, J. Daniel Hare, and P. Kirk Visscher for their critical reviews of the manuscript. We acknowledge Dr. Robert Beaver for statistical advice, Drs. Richard Kondrat and Ron New (UC Riverside Analytical Facility) for mass spectra, and the collaborations of Drs. Kathryn Platt and William Thomson. This work was supported by the California Celery Research Advisory Board and the California Tomato Commission.

REFERENCES

ANONYMOUS. 1980. Final report of the safety assessment for avocado oil. *J. Environ. Pathol. Toxicol.* 4:93–103.

ALKOFAHI, A., RUPPRECHT, J. K., ANDERSON, J. E., MCLAUGHLIN, J. L., MIKOLAJCZAK, K. L., and SCOTT, B. A. 1989. Search for new pesticides from higher plants, pp. 25–43, *in* J. T. Arnason, B. J. R. Philogene, and P. Morand (eds.). Insecticides of Plant Origin. ACS Symposium Series 387. American Chemical Society, Washington, DC.

BAAS, P., and GREGORY, M. 1985. A survey of oil cells in the dicotyledons with comments on their replacement by and joint occurrence with mucilage cells. *Isr. J. Bot.* 34:167–186.

BREWER, M. J., TRUMBLE, J. T., ALVARADO-RODRIGUEZ, B., and CHANEY, W. E. 1990. Beet armyworm (Lepidoptera: Noctuidae) adult and larval susceptibility to three insecticides in managed habitats and relationship to laboratory selection for resistance. *J. Econ. Entomol.* 83:2136–2146.

BREWER, M. J., MEADE, T., and TRUMBLE, J. T. 1995. Development of insecticide-resistant and -susceptible *Spodoptera exigua* (Lepidoptera: Noctuidae) exposed to furanocoumarins found in celery. *Environ. Entomol.* 24:392–401.

BULL, S. D., and CARMAN, R. M. 1994. Synthesis of the avocado antifungal, (*Z*, *Z*)-2-hydroxy-4-oxoheneicosa-12,15-dien-1-yl acetate. *Aust. J. Chem.* 47:1661–1672.

CARMAN, R. M., and DUFFIELD, A. R. 1995. The isolation of (*R*)-2-hydroxy-4-oxoheneicosan-1-yl acetate from avocado leaves. *Tetrahedron Lett.* 36:2119–2120.

CHANG, C. F., ISOGAI, A., KAMIKADO, T., MURAKOSHI, S., SAKURAI, A., and TAMURA, S. 1975. Isolation and structure elucidation of growth inhibitors for silkworm larvae from avocado leaves. *Agric. Biol. Chem.* 39:1167–1168.

CUMMINGS, K., and SCHROEDER, C. A. 1942. Anatomy of the avocado fruit. *Yearbook Calif. Avocado Soc.* 1942:56–64.

DUKE, S. O. 1990. Natural pesticides from plants. pp. 511–517, *in* J. Janick, and J. E. Simon (eds.). Advances in New Crops. Proceedings of the First National Symposium on New Crops: Research, Development, Economics. Timber Press, Inc., Portland, OR.

FINNEY, D. J. 1971. Probit Analysis. Cambridge University Press, Cambridge, U.K.

HORTON, D. R. 1995. Statistical consideration in the design and analysis of paired-choice assays. *Environ. Entomol.* 24:179–192.

KOBILER, I., PRUSKY, D., MIDLAND, S., SIMS, J. J., and KEEN, N. T. 1993. Compartmentation of antifungal compounds in oil cells of avocado fruit mesocarp and its effect on susceptibility to *Colletotrichum gloeosporioides*. *Physiol. Mol. Plant Pathol.* 43:319-328.

MACLEOD, J. K., and SCHAFFELER, L. 1995. A short enantioselective synthesis of a biologically active compound from *Persea americana*. *J. Nat. Prod.* 58:1270-1273.

MARIANI, P., CAPPELLETTI, E. M., CAMPOCCIA, D., and BALDAN, B. 1989. Oil cell ultrastructure and development in *Liriodendron tulipifera* L. *Bot. Gaz.* 150:391-396.

MARON, R., and FAHN, A. 1979. Ultrastructure and development of oil cells in *Laurus nobilis* L. leaves. *Bot. J. Linn. Soc.* 78:31-40.

MCKENZIE, H. L. 1935. Biology and Control of Avocado Insects and Mites. University of California, Berkeley Bulletin 592, 48 pp.

MINITAB. 1991. Minitab reference manual, No. 8. Minitab Statistical Software. State College, Pennsylvania.

MOAR, W. J., and TRUMBLE, J. T. 1987. Toxicity, joint action, and mean time of mortality of dipel 2X, avermectin B_1, neem, and thuringiensin against beet armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 80:588-592.

MURAKOSHI, S., ISOGAI, A., CHANG, C. F., KAMIKADO, T., SAKURAI, A., and TAMURA, S. 1976. Effects of two components from the avocado leaves (*Persea americana* Mill.) and the related compounds on the growth of silkworm larvae, *Bombyx mori* L. *Jpn. J. Appl. Entomol. Zool.* 20:87-91.

PATANA, R. 1969. Rearing cotton insects in the laboratory. *U.S. Dep. Agric., Agric. Res. Serv., Prod. Res. Rep.* 108:1-6.

PLATT, K. A., and THOMSON, W. W. 1992. Idioblast oil cells of avocado: distribution, isolation, ultrastructure, histochemistry, and biochemistry. *Int. J. Plant Sci.* 153:301-310.

POSTEK, M. T., and TUCKER, S. C. 1983. Ontogeny and ultrastructure of secretory oil cells in *Magnolia grandiflora* L. *Bot. Gaz.* 144:501-512.

PRABHAKER, N., COUDRIET, D. L., KISHABA, A. N., and MEYERDIRK, D. E. 1986. Laboratory evaluation of neem-seed extract against larvae of the cabbage looper and beet armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 79:39-41.

PRUSKY, D., KEEN, N. T., SIMS, J. J., and MIDLAND, S. L. 1982. Possible involvement of an antifungal diene in the latency of *Colletotrichum gloeosporioides* on unripe avocado fruits. *Phytopathology* 72:1578-1582.

RODRIGUEZ-SAONA, C., and TRUMBLE, J. T. 1996. Toxicity, growth, and behavioral effects of an oil extracted from idioblast cells of the avocado fruit on the generalist herbivore beet armyworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 89:1571-1576.

SEAWRIGHT, A. A., OELRICHS, P. B., NG, J. C., MACLEOD, J., WARD, A., SCHAFFELER, L., and CARMAN, R. M. 1995. Method of treatment of cancer as well as method of inhibition of lactation in mammals. Australian National University, Australia. Patent Coop. Treaty Int. Appl. No. WO 95/22969, 49 pp.

SMIRLE, M. J., and WEI, S. 1996. Effects of neem oil on feeding behavior and development of the pear sawfly, *Caliroa cerasi*. *Entomol. Exp. Appl.* 80:403-407.

SUPER ANOVA. 1989. Abacus Concepts Inc., Berkeley, California.

TRUMBLE, J. T. 1990. Vegetable insect control with minimal use of insecticides. *Hortic. Sci.* 25:159-164.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

INDIVIDUAL VARIATION AND REPEATABILITY OF SEX PHEROMONE EMISSION OF FEMALE TURNIP MOTHS *Agrotis segetum*

MATS G. E. SVENSSON,[1,*] MARIE BENGTSSON,[2] and JAN LÖFQVIST[2]

[1]*Department of Ecology, University of Lund*
Sölvegatan 37, S-223 62 Lund, Sweden

[2]*Department of Chemical Ecology*
Swedish University of Agricultural Sciences
S-230 53 Alnarp, Sweden

(Received July 15, 1996; accepted March 13, 1997)

Abstract—Individual variation of the female sex pheromone signal in *Agrotis segetum* was determined by gas chromatographic analysis. The individuals varied extensively, both in absolute and relative amounts of the three major pheromone components; Z5-10:OAc, Z7-12:OAc and Z9-14:OAc. Within individuals there was a strong correspondence between the sex pheromone gland content and the signal actually emitted. Both pheromone amounts and proportions of components were correlated with effluvia and extracts; with coefficients of 0.55 for total amounts and 0.66 for proportions. In addition, the repeatability of emitted pheromone components by individual females over three calling periods was high, with correlation coefficients between 0.43 and 0.72. Variation among individual females, however, was observed. There were no significant differences between wild-collected and laboratory-reared females in average ratios of the major pheromone components in pheromone gland extracts, only in total amounts. The biological significance of the inter-individual variation among females is discussed in the context of population dynamics and sexual selection.

Key Words—*Agrotis segetum*, Lepidoptera, (Z)-5-decenyl acetate, (Z)-7-dodecenyl acetate, (Z)-9-tetradecenyl acetate, sex pheromone, individual variation, repeatability, effluvia entrainment.

*To whom correspondence should be addressed.

0098-0331/97/0700-1833$12.50/0 © 1997 Plenum Publishing Corporation

INTRODUCTION

Sex pheromone communication is a key factor in moth mating systems. Individual variation of female moth sex pheromone production and release as well as the male pheromone response are essential elements in selection scenarios and in evolutionary pathways. Individual variation of the female sex pheromone in moths has been studied in several species (Miller and Roelofs, 1980; Charlton and Cardé, 1982; Morse et al., 1982; Pope et al., 1982; Haynes et al., 1983; Fitzpatrick et al., 1985; Löfstedt et al., 1985a; Attygalle et al., 1986; Barrer et al., 1987; Schlyter and Birgersson, 1989; Foster et al., 1989; Witzgall and Frérot 1989; Ono et al., 1990; Kou and Chow, 1991).

The sex pheromone of the turnip moth *Agrotis segetum* (Lepidoptera: Noctuidae) (Schiff) is well studied (Arn et al., 1980, 1983; Löfstedt et al., 1982, 1985a,b; Tóth et al., 1992; Wu et al., 1995). It consists of at least three major components: (Z)-5-decenyl acetate (Z5–10:OAc), (Z)-7-dodecenyl acetate (Z7–12:OAc), and (Z)-9-tetradecenyl acetate (Z9–14:OAc) (Arn et al., 1980; Tóth et al., 1980; Löfstedt et al., 1982; Arn et al., 1983). Recently, a fourth behaviorally active component has been reported; (Z)-5-dodecenyl acetate (Z5–12:OAc) (Wu et al., 1995). In addition, female pheromone glands contain at least 15 related compounds (Löfstedt et al., 1982). The absolute and relative amounts of the major components have been reported to vary extensively in individual gland extracts (Löfstedt et al., 1985a). Even females with one of the behaviorally essential components missing or below detectable amounts have been found. It has been hypothesized that due to evolutionary costs of a precise pheromone regulation, females may be unable to regulate the ratio and amount of the individual components (Löfstedt et al., 1985a). Extreme female types also are suggested to be at a disadvantage, perhaps due to delayed mating or increased risk of remaining unmated (the wallflower effect) (De Jong and Sabelis, 1991). Several field tests, however, have shown that pheromone baits with a wide range of ratios of Z5–10:OAc, Z7–12:OAc, and Z9–14:OAc are attractive to male turnip moths (Arn et al., 1983; Löfstedt et al., 1985b). All three components are necessary for attraction in Swedish populations (Löfstedt et al., 1985b; Svensson and Löfqvist, unpublished data).

The repeatability of emitted individual sex pheromone composition has been studied in a few moth species: *Ephestia cautella* (Pyralidae) (Barrer et al., 1987), *Yponomeuta padellus* (Yponomeutidae) (Du et al., 1987), and *Cacoecimorpha pronubana* (Tortricidae) (Witzgall and Frérot, 1989). In all three species, the constancy of individual blend proportions has been found to be remarkably high. Repeatability measurements indicate an upper limit of the heritability of a character (Boake, 1989; Falconer and Mackay, 1995). Heritability, the ratio between the additive genetic variance of a trait and its total phenotypic variance (narrow-sense heritability), is important not only because

it quantifies the resemblance between parent and offspring, but also because it determines the rate of response to selection (Falconer and Mackay, 1995). According to Fisher's fundamental theorem of natural selection (Fisher, 1958), the heritability of a trait should be inversely related to its importance for fitness, i.e., with little heritable variation for important traits. High repeatability may indicate a corresponding high heritability of the pheromone signal, maintained by weak or temporally or spatially varying selection (Charlesworth, 1987).

The major pheromone components of the turnip moth are not geometric isomers, as in the three previously studied moth species, and thus they have different volatilities and the ratios between the components are less easily controlled. The repeatability of such a pheromone signal is not yet known (as pointed out by Linn and Roelofs, 1995).

This paper aims at quantifying female sex pheromone variation and its repeatability in regard to amounts and proportions. The results constitute a basis for studying male pheromone preferences. Field-collected females were analyzed and compared with laboratory-reared females. Pheromone emission and repeatability of the emitted signal were studied in the latter. Variability of the pheromone signal in a wild population of *A. segetum* is yet to be studied.

METHODS AND MATERIALS

Insect Material. Females used for individual analyses of pheromone extract were collected as larvae in the field during October and November, 1991 (Copenhagen, Denmark). Larvae were allowed to pupate in a rearing cabinet at 21°C and transferred to a rearing cabinet at 19°C, 16L:8D, and a relative humidity of 65%.

Females from a laboratory culture were used for individual gland content analyses and for pheromone emission collections. The culture was established from field-collected material (Lund, southern Sweden), maintained for about 15 generations and fortified with field-collected material every year. Insects were reared on a semisynthetic diet based on potato, modified from Hinks and Byers (1976). The culture was maintained at 22°C, 16L:8D and relative humidity of 65%. Larvae were kept under the same conditions.

Chemical Analysis. Gas chromatographic analyses were performed on a 3-m × 0.32-mm ID SE-54 fused silica capillary column (J & W Scientific, Folsom, California) and 5890 Hewlett Packard GC with a FID. Samples were injected in the split-splitless mode with the split opened after 60 sec. Temperature was maintained at 80°C hold for 2 min, then raised to 230°C at a rate of 10°/min. Hydrogen was used as carrier gas with a flow of 2.6/min. Chemicals were purchased from The Institute for Pesticide Research, Wageningen, The

Netherlands, and were >98% pure with respect to geometrical and positional isomers. Solutions were prepared in heptane (Chemicon, redistilled p.a. quality).

Gland Extracts. Females were dissected during their second night after eclosion, 5–6 hr into the scotophase during the peak period of female calling (Löfstedt et al., 1982). Pheromone glands were dissected individually and put in 10 μl redistilled heptane with an internal standard of 10 ng (*Z*)-8-tridecenyl acetate (*Z*8–13:OAc) for 60 min. The solution was transferred to a 50-μl glass capillary that was sealed immediately under open flame. Capillaries were kept frozen at −20°C until analysis.

Detection Limits of Gas Chromatographic Analysis. The detection limits of the pheromone components were determined by linear regression analysis (Miller and Miller, 1984) of a dilution series of the three major components, in concentrations ranging from 100 ng to 10 pg/10 μl solvent with an internal standard (*Z*8–13:OAc). Each concentration was replicated three times. A minimized background signal and high correlation coefficients ($r = 0.978$ or higher) of the calibration curves resulted in low detection limit values. The limits of detection, defined as the analyte concentration giving a signal equal to the blank signal plus two standard deviations of the blank, were estimated at 20 pg for *Z*5–10:OAc, *Z*5–12:OAc, and *Z*7–12:OAc and 30 pg for *Z*9–14:OAc. The limit of quantification, suggested to be defined as the concentration giving a blank signal plus 10 standard deviations of the blank (Miller and Miller, 1984), were estimated at 50 pg for *Z*5–10:OAc, 60 pg for *Z*5–12:OAc, and *Z*7–12:OAc, and 70 pg for *Z*9–14:OAc. Values between the limit of detection and the limit of quantification were compared with values above the limit of detection. These two groups did not differ in their regression coefficients for any of the components (range 10–100 pg, regression coefficients of 0.954, 0.754, 0.601; range 300–3000 pg, regression coefficients 0.910, 0.840, and 0.531 for *Z*5–10:OAc, *Z*7–12:OAc and *Z*9–14:OAc respectively). None were significantly different when compared componentwise for the two ranges with an *F* test, $F_{0.05(1,14)}$ (Sokal and Rohlf, 1995). We therefore decided to include all values down to the limits of detection as defined above.

Effluvia Entrainments. Effluvia were entrained on a glass-adsorption system modified from the one used by Baker et al. (1981) and Charlton and Cardé (1982). Effluvia were adsorbed onto 120-mm-long glass capillaries (5.5 mm OD and 1.2 mm ID) mounted on the outlet of glass chambers (65 mm long, 18 mm ID) with a volume of 17 ml. Glassware was heated at 400°C for 12 hr between collections. The glass chambers and capillaries were connected with a small piece of Teflon tube with the glass surfaces in contact. Glass chambers were mounted with Quickfit onto a glass vessel onto which five chambers were mounted simultaneously. Charcoal-filtered compressed air was used to create an airflow that was kept at 35 ml/min ±2 ml/min. Four samples plus a control

were run simultaneously. Effluvia were collected for 120 min. The glass capillaries were dismounted immediately after collection and kept in aluminum foil at −20°C for no more than 2 hr until GC analysis.

Capillaries were rinsed with 10 μl of redistilled heptane containing 10 ng of Z8–13:OAc added as an internal standard. The remaining rinse volume, 5–6 μl, was directly injected into the GC. The same chamber and capillary were used for each female during the three consecutive collections, as this source of variation is expected to be minimal and the individual variation larger.

The collection efficiency and the breakthrough of the effluvia method were evaluated. Effluvia collections from live females were simulated by a filter paper, 0.7 cm^2, loaded with 2.5 ng Z5–10:OAc + 10 ng Z7–12:OAc + 5 ng Z9–14:OAc, which was placed in the chamber and aerated for 120 min. Collection efficiency, expressed as a percentage, was calculated as the ratio between the amount of pheromone recovered from the capillary and the quantity applied to the filter paper multiplied by 100.

The evaluation of breakthrough was made by connecting two capillaries in series, aerating a rubber septum loaded with 25 μg Z5–10:OAc + 50 μg Z7–12:OAc + 100 μg Z9–14:OAc for 2 hr at 35 ml/min. This mixture has previously been used in field tests (Svensson, unpublished data). The two capillaries were rinsed, and it was established, by GC analysis, whether any pheromone components were present in the second capillary.

Time dependence on effluvia collection was estimated. Effluvia from rubber septa with 1/10 of the mixture above were collected during 3, 10, 30, 100, and 300 min. Three replicates were made for each time period.

The repeatability, the recovery of glass rinses, and the differences in adsorption rates for the pheromone components were controlled. The ratio in a three-component pheromone solution directly injected into the GC was compared with ratios found in glass capillary rinsed 60 min after application of the same solution. Capillaries were rinsed five times and each rinse directly analyzed on GC. This was replicated three times.

Due to considerable differences in vapor pressure Z5–10:OAc, Z7–12:OAc, and Z9–14:OAc, the most effective sampling time was chosen after a study of amounts recovered against sampling time. The greatest amounts of the three major components together were achieved after 100 min of sampling. Three hundred minutes of sampling increased the amount of Z9–14:OAc, while amounts of the two other components began to drop, i.e., the ratio among the three components was affected by sampling time, as expected. For the experimental female effluvia entrainments, a sampling time of 120 min was chosen, and considered optimal for Z7–12:OAc.

Recovery from the glass pipet was highest for Z7–12:OAc, (42%) and slightly lower for the other two components, Z5–10:OAc (39%) and Z9–14:OAc

(33%). No breakthrough was detected when sampling from filter paper sources. However, a smaller breakthrough of Z5-10:OAc was found after 2 hr of sampling from rubber septa loaded with 25 μg Z5-10:OAc + 50 μg Z7-12:OAc + 100 μg Z9-14:OAc, corresponding to a release rate of 1200, 860, and 100 pg/hr of the respective component (Svensson and Löfqvist, unpublished data). An additional 5% of the applied amount was trapped in the second capillary, and a further 15% could be rinsed off the inner walls of the chamber. The other two components were not detected in these rinses. There was no distortion in ratios due to differences in adsorption of the components onto the capillaries. The ratios between the three components were similar in solution, directly injected into the GC, similar to solution applied onto glass capillaries and then rinsed, followed by GC analysis. The coefficient of variation for the injections was estimated to 3% for individual components and less than 1% for ratios.

Female Effluvia. Calling females were taken their second night after eclosion and put in cut pieces of disposable plastic pipet tips (Labsystems, Oy, Finland) with the last segments of the female's abdomen, including the pheromone gland, exposed in the rear opening of the pipet (Baker et al., 1981; Du et al., 1987). A small piece of Kleenex tissue was used to keep the animal in position. Pipet tips containing single females were placed in the glass chambers with the pheromone gland as close as possible to the outlet. Females found with withdrawn glands were discarded.

Repeatability Measurements. Female effluvia collections were also made repetitively from the same individuals during three consecutive nights. Females were taken out of the pipet tips between collections and kept separately in 250-ml plastic jars under the same light and temperature regime. Repeatability, the intraclass correlation coefficients (Sokal and Rohlf, 1995) of the emitted ratios, and amounts of the three main components were calculated using the formula:

$$r = \frac{(MS_A - MS_W)/n_0}{MS_W + [(MS_A - MS_W)/n_0}$$

where MS_A and MS_W is the among-groups variance component and within-group variance component, respectively, and n_0 is the sample size (Lessells and Boag, 1987). This formula provides a satisfying approximation of repeatability, even if the sample sizes are unequal.

Statistical Analyses. All amounts and calculated ratios were checked for normality by Kolmogorov-Smirnov statistics with a Lilliefors significance level, and for homogeneity of variances by Levene's test (Norusis, 1994). In the univariate repeated-measurements ANOVA, a variance–covariance matrix was made to check for circularity (Winer et al., 1991). When the normality condition was

not met, either the variable was transformed to normality or a nonparametric test was used.

RESULTS

Individual Gland Extracts of Wild Females. Of the field-collected material, 32 females (89%) contained amounts above the detection limits of Z5-10:OAc, Z7-12:OAc, and Z9-14:OAc, the three major components. Z5-12:OAc was detected only in 16 of the 32 females. Wild females contained on average 470 pg Z5-10:OAc (31% in average proportion of the three major components), 1 ng Z7-12:OAc (41%), and 640 pg Z9-14:OAc (27%) (Table 1). They contained on average 320 pg Z5-12:OAc, for a total of 2.1 ng.

Z5-10:OAc varied least in terms of amounts but most in terms of proportions (Table 1). All three components showed a coefficient of variance (CV) between 119 and 202% in amounts. The ratios were less variable: 31-41% CV. It is worth noting that few females contained large amounts of pheromone. The highest value was 14 ng and the lowest 210 pg, which is 67 times less

TABLE 1. AVERAGE AMOUNTS OF FOUR MAJOR PHEROMONE COMPONENTS AND RATIO OF THREE (Z5-12:OAc EXCLUDED) IN PHEROMONE GLAND EXTRACTS OF WILD-COLLECTED AND LABORATORY-REARED *A. segetum* FEMALES[a]

	Z5-10:OAc		Z5-12:OAc		Z7-12:OAc		Z9-14:OAc		Total amount (pg) (Z5-12:OAc excluded)	
	Wild	Lab	Wild	Lab	Wild	Lab	Wild	Lab	Wild	Lab
Average amount	470*	120	320*	30	1000*	360	640*	250	2100*	730
SE	±98	±17	±124	±7	±354	±70	±211	±59	±620	±136
N	32	42	16	14	32	42	32	42	32	42
CV (%)	119	90	159	194	202	127	191	151	156	121
Median	310	80	120	0	370	160	230	100	1000	290
Average ratio (%)	31	25			41	45	27	30		
SE	±3	±2			±3	±2	±2	±2		
CV (%)	59	56			36	26	45	36		
Median (%)	35	25			27	27	32	38		

[a] Asterisks denote significant differences in amounts between wild and laboratory-reared females (unpaired two-sided *t* test, $P < 0.01$, log-transformed values). No significant differences were found between the two female groups in component ratios or in variability (Mann-Whitney U test, $P < 0.01$).

(1.8 times on a log-scale). Only three of 32 individuals contained more than 5 ng (Figure 1). The logarithmic value of the total amount of the three components shows a normal distribution (Figure 2A).

Individual Gland Extracts of Laboratory-Reared Females. Laboratory-reared animals contained significantly less pheromone than wild females: 120 pg Z5-10:Ac (25% in average proportion), 360 pg Z7-12:OAc (45%), and 250 pg Z9-14:OAc (30%), on average a total of 730 pg (Table 1).

All components showed a CV of 90–151% in amounts. The ratios were less variable: 25–45% CV. Z5-10:OAc varied most and Z7-12:OAc least in terms of ratios. Z5-12:OAc could only be detected in 14 of 42 females and only in small amounts—30 pg on average. Here as well, the logarithm value of the female gland contents showed an approximately normal distribution (Figure 2B). Only five of 42 contained total amounts above 2 ng. The highest extracted total amount from laboratory-reared females was 3.4 ng and the lowest 90 pg, which is 38 times less (1.6 times on a log-scale).

There was a significant difference between wild and laboratory-reared females with respect to total average pheromone gland contents ($t = 4.32$, $df = 72$, $P < 0.0001$), but not in proportions (Table 1). The two populations were equal in variability.

Female Pheromone Effluvia. Pheromone emissions were entrained from 36 laboratory-reared females. The pheromone gland was extracted from 20 of

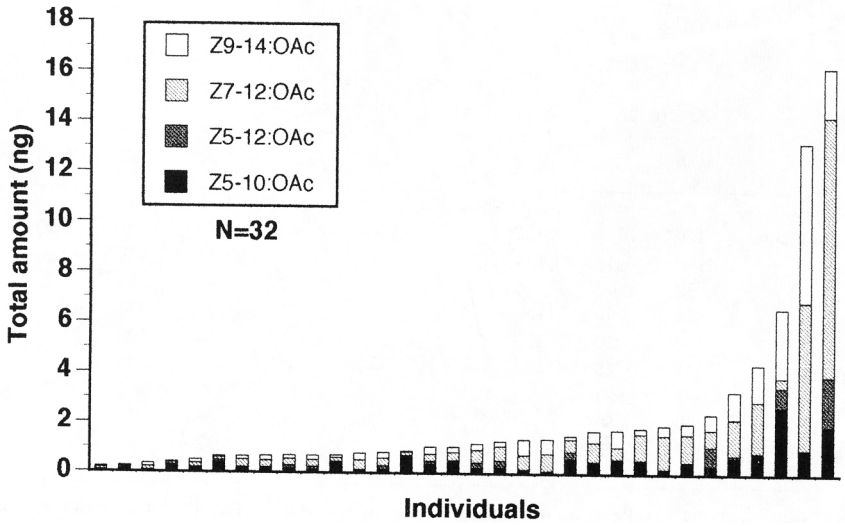


FIG. 1. Individual female *A. segetum* moths sorted in ascending order of total amount of pheromone gland extracts from wild-collected females.

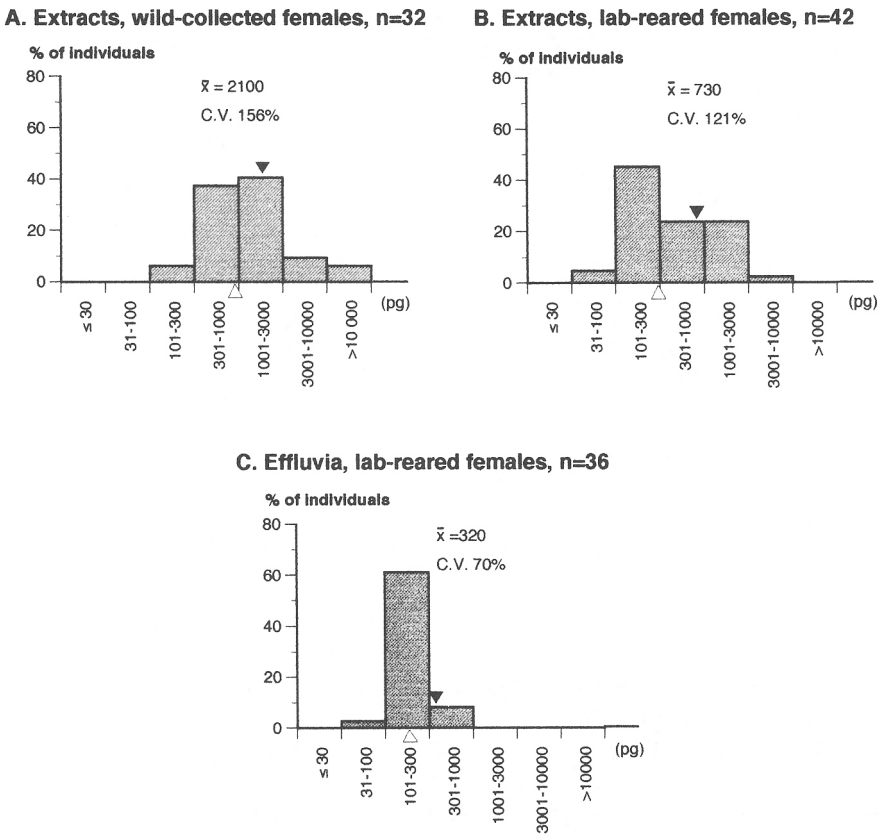


FIG. 2. Categorized total amounts in pheromone gland extracts from individual wild-collected (A) (N = 32) and laboratory-reared *A. segetum* females (B) (N = 42), and in pheromone effluvia (C) (N = 36) on a logarithmic scale. Dark triangles indicate the average values; white triangles indicate the medians.

these after the effluvia had been entrained. All three pheromone components were detected in the pheromone emission of these females. However, the amounts of Z9-14:OAc were low and inconsistent (detection limit: 40 pg, mean amount: 45 ± 4 pg). Z9-14:OAc was therefore excluded from further analyses. The average amounts entrained of Z5-10:OAc and Z7-12:OAc did not differ significantly among the 20 females further investigated and the subset of 16 which were only entrained (t = 1.29, df = 34, p = 0.26). In the group of 20 females a total amount of Z5-10:OAc + Z7-12:OAc of 295 ± 18 pg female effluvia was entrained on average (Table 2). The corresponding value for the sample of

TABLE 2. AVERAGE AMOUNTS AND RATIOS OF THREE MAJOR SEX PHEROMONE COMPONENTS OF LAB-REARED *A. segetum* IN FEMALE GLAND EXTRACTS AND CORRESPONDING ENTRAINED FEMALE EFFLUVIA, $N = 20$[a]

	Z5-10:OAc		Z7-12:OAc		Z9-14:OAc		Total amount (pg)	
	Extract	Effluvia	Extract	Effluvia	Extract	Effluvia	Extract	Effluvia
Average amount	170	170	400*	230	340		930*	400
SE	±27	±31	±60*	±46	±55		±125	±54
CV (%)	73	82	90	68	71		60	61
Median	160	110	420	200	320		960	350
Average ratio (%)	30*	42	70	58				
SE	±3	±4	±2	±4				
CV (%)	57	43	25	38				
Median (%)	16	38	42	50				

[a]Asterisks denote significant differences (two-tailed paired t test, $P < 0.01$, for log-transformed amounts and Wilcoxon's paired ranks test, $P < 0.01$, for ratios). All values, including those under LOQ, were taken into account.

16 females was 234 ± 50 pg. A majority of the individuals had a total effluvium amount of 100–300 pg with a unimodal distribution on a log-scale (Figure 2C). Amounts of Z7-12:OAc differ significantly when extracts and effluvia are compared (Table 2). When Z5-10:OAc and Z7-12:OAc are studied separately, they both show a normal distribution (Lilliefors test statistics, $p < 0.05$).

Correlation Between Individual Female Pheromone Emission and Gland Content. The glands from 20 females were extracted after effluvia entrainment. The amounts of pheromone components extracted show a higher variability than amounts in effluvia (Figures 2 and 3). There were significant correlations between entrained amounts in effluvia and amounts found in gland extracts of Z5-10:OAc and Z7-12:OAc, (Figure 3A and B) and, accordingly, also between the total amounts (Z5-10:OAc + Z7-12:OAc) found in extracts and the corresponding effluvia ($r_s = 0.55$, $P = 0.01$). There was also a significant correlation between the proportion of Z5-10:OAc relative to Z7-12:OAc, found in entrained effluvia and in gland extracts (Figure 3C).

Repeatability of Female Pheromone Emission. The repeatability of amounts of Z5-10:OAc and Z7-12:OAc released were high between nights 1 and 2 (Figure 4), but lower over the three days; $r = 0.36$ and 0.48 for Z5-10:OAc and Z7-12:OAc, respectively (Table 3). There was a significant difference among females in the amounts of Z5-10:OAc and Z7-12:OAc released, but no significant effect of the treatment (repeated-measurements ANOVA; Table

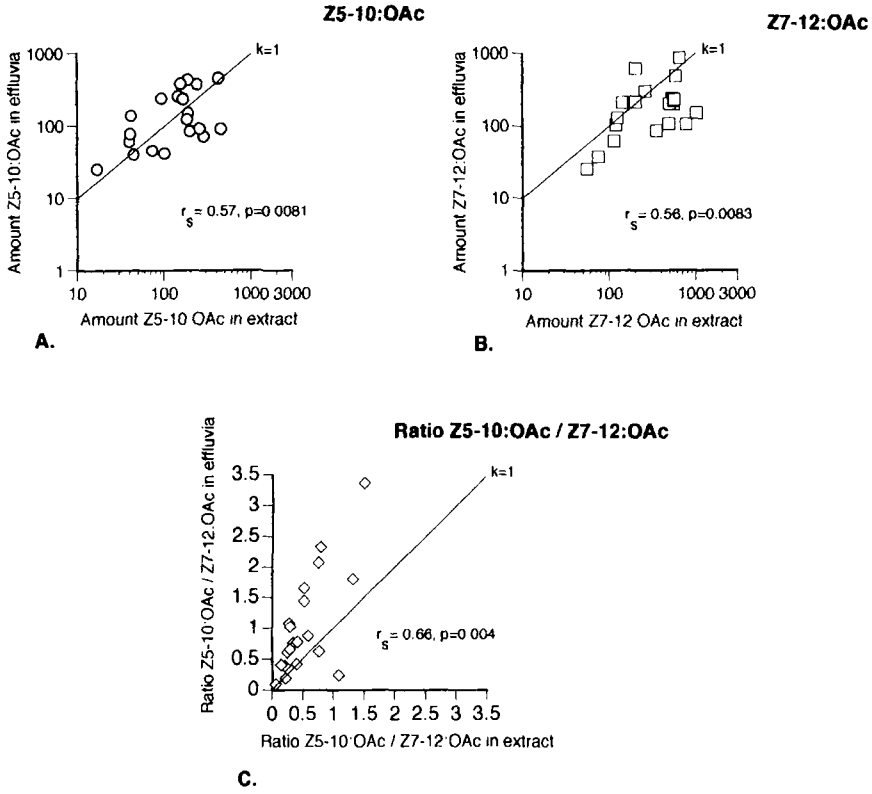


FIG. 3. Correlation between amounts of Z5-10:OAc and Z7-12:OAc (logarithmic values) (A and B) and the Z5-10:OAc/Z7-12:OAc ratio (C) in entrained effluvia and pheromone gland extracts of individual female *A. segetum*, $N = 20$. The corresponding correlation coefficients and their respective significance are given for the amounts and the Spearman's rank-correlation coefficient and the corresponding significance is given for the ratio. The diagonal lines ($k = 1$) show the linear 1 : 1 relationships for comparison only.

3). On average, 150 pg Z5-10:OAc, and 140 pg Z7-12:OAc were recovered over the three days. This is not significantly different from the single individual effluvia ($t = 0.77$, $df = 46$, $P = 0.44$) (Table 2). There is no significant difference between females in total amounts released, and no significant treatment effects (repeated-measurements ANOVA, between-females: 11 *df*, $F = 1.37$, $P = 0.25$; treatment effect: 2 *df*, $F = 2.16$, $P = 0.14$). The total amount of the two components entrained decreased with time of sampling, especially between the first (average 370 pg) and second sampling (average 260

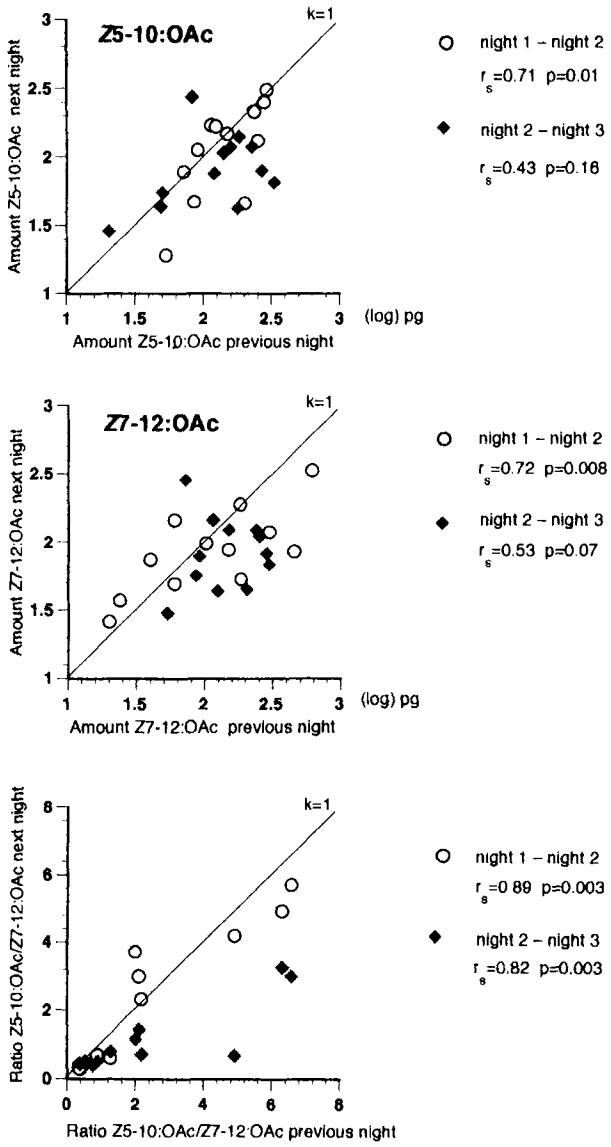


FIG. 4. Correlation of emitted amounts of two major pheromone components of *A. segetum*, Z5–10:OAc (A) and Z7–12:OAc (B) (logarithmic values), and the ratio between these (C), between nights, $N = 12$. The correlation coefficients and their respective significances are given for the amounts and the Spearman's rank-correlation coefficient and the corresponding significance is given for the ratio. The diagonal lines ($k = 1$) show the linear 1:1 relationships for comparison only.

TABLE 3. UNIVARIATE, MIXED-MODELS ANOVA OF REPEATED MEASUREMENTS OF AMOUNTS (LOG-TRANSFORMED VALUES) OF *Z*5–10:OAc AND *Z*7–12:OAc IN PHEROMONE EMISSIONS FROM INDIVIDUAL *A. segetum* FEMALES, $N = 12$

Source	*df*	SS	*F* test	*P*
a. *Z*5–10:OAc amounts				
Among females	11	1.77	2.72	0.02
Within females	24	1.42		
Treatment effects	2	0.32	3.22	0.059
Residual	22	1.10		
Total	35	3.20		
b. *Z*7–12:OAc amounts				
Among females	11	2.90	3.72	0.015
Within females	24	1.70		
Treatment effects	2	0.07	0.51	0.61
Residual	22	1.63		
Total	35	4.60		

pg). If the decrease over time is considered and corrected for, by multiplying with an average decrease factor (amount on night 1 divided by amount on night 2), the individual released amounts will show a higher repeatability, but will not increase to the same level as the repeatability of ratios. The within-individual variation is higher in amounts than in ratios but also relative to the between-individual variation (Table 3).

The repeatability of the ratio of the two pheromone components in female emission is higher than that of the relased amounts; it was estimated as 0.89 and 0.82 between nights 1 and 2 and nights 2 and 3, respectively (Figure 4). There is also a significant correlation over all three nights for the ratio (Kendall's coefficient of concordance, $W = 0.25$, $\chi^2 = 6.0$ $df = 2$, $P < 0.05$). This indicates that the females release individual-specific blends that do not change over time.

DISCUSSION

We conclude that a major part of the documented individual female sex pheromone variation in *A. segetum* is explained by variation between individuals, not within individuals. In addition, we have established the relationship between the content of a female pheromone gland and what is emitted, with respect to two of the major pheromone components. The results also are valid for a wild population of *A. segetum*, by the comparison we have made between wild and laboratory-reared animals.

Individual Female Pheromone Gland Extracts. All gland extracts of wild females contained the three primary pheromone components. A majority of the individuals contained a total amount of less than 2 ng, but a few contained two to eight times more. Very few females contained amounts lower than 300 pg. Z5–12:OAc was found in only half the sampled females and in highly variable amounts, indicating that some individual extracts contained amounts below the detection limit.

Laboratory-reared females contained significantly smaller amounts of pheromone than wild females, between 100 and 300 pg total amount. However, a few lab-reared females contained total amounts up to 3.5 ng. A similar difference in average amounts between wild and laboratory-reared females was found by Löfstedt et al., (1985a) and has its parallel in other species as well (Richerson and Cameron, 1974; Miller and Roelofs, 1980).

The distribution patterns of absolute amounts of the three major components in gland extracts of wild and laboratory-reared females are similar (Figure 3). The proportions are variable among individuals, Z5–10:OAc being the most variable of the three major homologous acetates. Z5–12:OAc was found in only 50% of wild females, and in only 33% of laboratory-reared females. Wu et al. (1995) have, however, reported enhanced pheromone trap catches when this component has been added to a three-component blend in the field. The large individual differences in gland content ratios are manifested in the emitted blends.

In comparison with the study by Löfstedt et al. (1985a), some differences were found; the amounts of Z5–10:OAc are compatible, in both wild and laboratory-reared animals, while the amounts (and therefore also ratios of Z7–12:OAc and Z9–14:OAc are smaller in our study. This may be explained by the use of different analytical methods, but may also be interpreted as a change in the population's mean pheromone mixture over time. The detection limit defined in this study is lower than in the one by Löfstedt et al. (1985a). Therefore, more animals with low amounts of Z7–12:OAc are included here, altering the average ratios.

What Pheromone Signals Are Individual Females Emitting? When considering individual variation, using the appropriate scale is important. If individual pheromone amounts are applied on a linear scale, there is no symmetrical unimodal pattern, while there is one when using a logarithmic scale. A unimodal pattern is expected if there is stabilizing selection acting on pheromone emission. The use of a logarithmic scale is supported by many field and wind-tunnel experiments on pheromones, which, with few exceptions, show clear logarithmic dose–response relationships (e.g., Löfstedt et al., 1985b; Wu et al., 1995).

When absolute amounts in entrained effluvia are studied, a similar pattern is revealed, although with lower variability among females and with a smaller difference between low- and high releasers (Figure 2). The distribution of pheromone amounts follows a unimodal pattern, with some females emitting 2–2.5

times more than the average on a logarithmic scale, and others emitting very low amounts. One interpretation is that the release rate is under weak but stabilizing selection (Danks, 1983). Alternatively, it could be a result of different strategies; a female low-release strategy pays when population densities are high by favoring the most sensitive males, and a high-release strategy pays when population densities are low and finding any mate is of greater importance than finding a "high-quality," sensitive male. The distribution of different female types may then vary according to the previous year's population density. Density is fundamentally a spatial concept. Similar delayed density dependence has been modeled (Gillespie, 1975) and found in vertebrates (e.g., voles; Agrell et al., 1995) as well as in invertebrates (e.g., moths; Ginzburg and Taneyhill, 1994). A comparison of the distribution patterns of wild and laboratory-reared female gland extracts shows a significant difference, with the latter animals containing smaller amounts. It could be argued that low-producing females are not selected against in a laboratory-reared female population, as rearing is made under high densities, which is in line with the above reasoning.

The emission-entrainment method used proved to be efficient for collecting quantities of pheromone in the picogram range for Z5–10:OAc and Z7–12:OAc, for which the results are consistent. The recovery rate for the third component, Z9–14:OAc, was generally low and inconsistent. The large difference in vapor pressure between components presents a problem when using this sampling technique. However, Z9–14:OAc is an essential component in the pheromone blend, and cannot be neglected.

The extracted amounts from the gland tissue and the entrained amounts in effluvia were similar for Z5–10:OAc but significantly different for Z7–12:OAc. This indicates a different turnover rate between the components, with Z5–10:OAc having the highest.

Haynes and Hunt (1990) discussed the assumption that females emit the same pheromone signal whether forced or not. This was verified by comparing free-calling and forced extrusion of the gland. They concluded that the forced-extrusion technique was a valid method for estimating pheromone blend ratio and emission rate.

The repeatability of the amounts was high between nights 1 and 2. However, there was a tendency in these females to release smaller amounts on the second and third entrainment occasions, which may be expected (cf., Barrer et al., 1987; Witzgall and Frérot, 1989). The repeatability measurements were all made at the same temperature and during the same period of the scotophase, so the decrease in amounts between nights 2 and 3 may be a treatment effect. The pheromone extract titer shows no significant age dependence within this time range (1–5 nights; Svensson unpublished data). Another potential source of error is gland tissue only exposed during parts of the sampling period. Female turnip moths exhibit calling bouts (Svensson, unpublished data) and after one

or a few calling bouts may contain less. Temperature effects on vapor-phase composition can be excluded (Olsson et al., 1983; Löfstedt et al., 1985a). The repeatability of the relative proportion of Z5-10:OAc in effluvia from females is high, indicating that there is indeed an interindividual variability in ratios (Figure 4). Sexual signals are often assumed to be stereotyped, but their repeatability is often much lower than that for morphological traits (Boake, 1989). The emitted female pheromone signal of *A. segetum* shows high individual consistency indicated by the repeatability.

Repeatability estimates indicate an individual-specific, fairly precise regulation of ratios of the pheromone components. This suggests that their regulation is genetically determined, as was also suggested for the pink bollworm, *Pectinophora gossypiella* (Collins and Cardé, 1989). Female variation can be explained and maintained if there is no difference in fitness between the different female types in terms of different released ratios. Probably due to an intense male intrasexual selection, all female types will be mated, and very weak selection is at work on "outlier" female types, as long as they contain all components to achieve species specificity in a "noisy" environment. The heritability of *A. segetum* female sex pheromone composition remains to be determined. The preponderance of higher release rates under natural circumstances is obvious, as the wild females contained significantly higher amounts than laboratory-reared females. It is also evident that it is still not known how the release rate in moths influences the communication distance and hence male detection in nature.

Acknowledgments—We thank colleagues in the Pheromone group in Lund for constructive criticism, F. Schlyter for statistical advice, and finally E. Björling for revision of the English text. This study was funded by the Swedish Research Council (NFR).

REFERENCES

AGRELL, J., ERLINGE, S., NELSON, J., NILSSON, C., and PERSSON, I. 1995. Delayed density-dependence in a small-rodent population. *Proc. R. Soc. London Ser. B* 262:65–70.

ARN, H., STÄDLER, E., RAUSCHER, S., BÜSER, H. R., MUSTAPARTA, H., ESBJERG, P., PHILIPSEN, H., ZETHNER, O., STRUBLE, D. I., and BUES, R. 1980. Multicomponent sex pheromone in *Agrotis segetum*: Preliminary analysis and field evaluation. *Z. Naturforsch.* 35C:986–989.

ARN, H. ESBJERG, P., BUES, R., TÓTH, M., SZÖCS, G., GUERIN, P., and RAUSCHER, S. 1983. Field attraction of *Agrotis segetum* males in four European countries to mixtures containing three homologous acetates. *J. Chem. Ecol.* 9:267–276.

ATTYGALLE, A. B., SCHWARZ, J., VOSTROWSKY, O., and BESTMANN, H. J. 1986. Individual variation in the sex pheromone components of the false codling moth *Cryptophlebia leucotreta* Lepidoptera Tortricidae. *Z. Naturforsch.* 41:1077–1081.

BAKER, T. C., GASTON, L. K., POPE, M. M., KUENEN, L. P. S., and VETTER, R. 1981. A high-efficiency collection device for quantifying sex pheromone volatilized from female glands and synthetic sources. *J. Chem. Ecol.* 7:961–968.

BARRER, P. M., LACEY, M. J., and SHANI, A. 1987. Variation in relative quantities of airborne sex

pheromone components from individual female *Ephestia cautella* (Lepidoptera: Pyralidae). *J. Chem. Ecol.* 7:961–968.

BOAKE, C. R. B. 1989. Repeatability: Its role in evolutionary studies of mating behavior *Evol. Ecol.* 3:173–182.

CHARLESWORTH, B. 1987. The heritability of fitness, pp. 21–40 *in* J. W. Bradbury and M. Andersson (eds.). Sexual Selection: Testing the Alternatives. Wiley, Chichester, UK.

CHARLTON, R. E., and CARDÉ, R. T. 1982. Rate and diel periodicity of pheromone emission from female gypsy moths, (*Lymantria dispar*) determined with a glass-adsorption collection system. *J. Insect Physiol.* 28:423–430.

COLLINS, R. D., and CARDÉ, R. T. 1989. Heritable variation in pheromone response of the pink bollworm *Pectinophora gossypiella* Lepidoptera: Gelechiidae. *J. Chem. Ecol.* 15:2647–2660.

DANKS, H. V. 1983. Extreme individuals in natural populations. *Bull. Entomol. Soc. Am.* 29:41–46.

DE JONG, M. C. M., and SABELIS, M. W. 1991. Limits to runaway sexual selection: The wallflower paradox. *J. of Evol. Biol.* 4:637–656.

DU, J.-W., LÖFSTEDT, C., and LÖFQVIST, J. 1987. Repeatability of pheromone emissions from individual female ermine moths *Yponomeuta padellus* and *Yponomeuta rorellus*. *J. Chem. Ecol.* 13:1431–1441.

FALCONER, D. S., and MACKAY, T. F. C. 1995. Introduction to Quantitative Genetics, 4th ed. Longman, Harlow, U.K.

FISHER, R. A. 1958. The Genetical Theory of Natural Selection, 2nd ed. Dover, New York.

FITZPATRICK, S. M., MILLER, D., WEATHERSTON, I., and MCNEIL, J. N. 1985. Determining pheromone content of hairpencils from individual virgin males of *Pseudaletia unipuncta* (Haw.) (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 11:207–215.

FOSTER, S. P., CLEARWATER, J. R., and MUGGLESTON, S. J. 1989. Intraspecific variation of two components in sex pheromone gland of *Planotortrix excessana* and sibling species. *J. Chem. Ecol.* 15:457–466.

GILLESPIE, J. H. 1975. The role of migration in the genetic structure of populations in temporally and spatially varying environments I. Conditions for polymorphism. *Am. Nat.* 109:127–135.

GINZBURG, L. R., and TANEYHILL, D. E. 1994. Population cycles in forest Lepidoptera: a maternal effect hypothesis. *J. Anim. Ecol.* 63:79–92.

HAYNES, K. F., GASTON, L. K., POPE, M. M., and BAKER, T. C. 1983. Rate and periodicity of pheromone release from individual female artichoke moths, *Platyptilia carduictyla* (Lepidoptera: Pterophoridae). *Environ. Entomol.* 12:1597–1660.

HAYNES, K. F., and HUNT, R. E. 1990. Interpopulational variation in emitted pheromone blend of cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol.* 16:509–519.

HINKS, C. F., and BYERS, J. R. 1976. Biosystematics of the genus *Euxoa* (Lepidoptera: Noctuidae) V. Rearing procedures and life cycles of 36 species. *Can. Entomol.* 108:1345–1357.

KOU, R., and CHOW, Y. S. 1991. Individual variation in sex pheromone of smaller Tea tortrix moth *Adoxophyes sp.* Lepidoptera Tortricidae. *J. Chem. Ecol.* 17:1917–1924.

LESSELLS, C. M., and BOAG, P. T. 1987. Unrepeatable repeatabilities: A common mistake. *The Auk* 104:116–121.

LINN, C. E., JR., and ROELOFS, W. L. 1995. Pheromone communication in moths and its role in the speciation process, pp. 263–300, *in* D. M. Lambert and H. G. Spencer (eds.), Speciation and the Recognition Concept. Theory and Application. The John Hopkins University Press, Baltimore.

LÖFSTEDT, C., VAN DER PERS, J. N. C., LÖFQVIST, J., LANNE, B. S., APPELGREN, M., BERGSTRÖM, G., and THELIN, B. 1982. Sex pheromone components of the turnip moth, *Agrotis segetum*: Chemical identification, electrophysiological evaluation and behavioral activity. *J. Chem. Ecol.* 8:1305–1320.

LÖFSTEDT, C., LANNE, B. S., LOFQVIST, J., APPELGREN, M., and BERGSTROM, G. 1985a. Individual variation in the pheromone of the turnip moth, *Agrotis segetum*. *J. Chem. Ecol.* 11:1181–1196.

LÖFSTEDT, C., LINN JR., C. E., and LÖFQVIST, J. 1985b. Behavioral responses of male turnip moths *Agrotis segetum* to sex pheromone in a flight tunnel and in the field. *J. Chem. Ecol.* 11:1209–1221.

MILLER, J. C., and MILLER, J. N. 1984. Statistics for Analytical Chemistry. John Wiley & Sons, New York.

MILLER, J. R., and ROELOFS, W. L. 1980. Individual variation in sex pheromone ratios in two populations of the redbanded leafroller moth, *Argyrotaenia velutinana*. *Environ. Entomol.* 9:359–363.

MORSE, D., SZITTNER, R., GRANT, G. G., and MEIGHEN, E. A. 1982. Rate of pheromone release by individual spruce budworm moths. *J. Insect Physiol.* 10:863–866.

NORUSIS, M. J. 1994. SPSS 6.1 Base System User's Guide. SPSS Inc., Chicago.

OLSSON, A.-M., JÖNSON, J.-Å, THELIN, B., and LILJEFORS, T. 1983. Determination of the vapor pressures of moth sex pheromone components by a gas chromatographic method. *J. Chem. Ecol.* 9:375–385.

ONO, T., CHARLTON, R. E., and CARDÉ, R. T. 1990. Variability in pheromone composition and periodicity of pheromone titer in potato tuberworm moth *Phthorimaea operculella* (Lepidoptera: Gelechiidae). *J. Chem. Ecol.* 16:531–542.

POPE, M. M., GASTON, L. K., and BAKER, T. C. 1982. Composition, quantification and periodicity of sex pheromone gland volatiles from *Heliothis virescens* females. *J. Chem. Ecol.* 8:1043–1055.

RICHERSON, J. V., and CAMERON, E. A. 1974. Differences in pheromone release and sexual behavior between laboratory-reared and wild gypsy moth adults. *Environ. Entomol.* 3:475–481.

SCHLYTER, F., and BIRGERSSON, G. 1989. Individual variation in bark beetle and moth pheromones—a comparison and an evolutionary background. *Holarctic Ecol.* 12:457–465.

SOKAL, R. R., and ROHLF, F. J. 1995. Biometry. 3rd ed. W. H. Freeman & Co, San Franscisco, U.S.A.

TÓTH, M., JAKAB, J., and NOVÁK, L. 1980. Identification of two components from the sex pheromone system of the white-line dart moth, *Scotia segetum* (Schiff.) (Lep., Noctuidae). *Z. Angew. Entomol.* 90:505–510.

TÓTH, M., LÖFSTEDT, C., BLAIR, B. W., CABELLO, T., FARAG, A. I., HANSSON, B. S., KOVALEV, B. G., MAINI, S., NESTEROV, E. A., PAJOR, I., SAZONOV, P., SHAMSHEV, I. V., SUBCHEV, M., and SZOCS, G. 1992. Attraction of male turnip moths *Agrotis segetum* (Lepidoptera: Noctuidae) to sex pheromone components and their mixtures at 11 sites in Europe, Asia, and Africa. *J. Chem. Ecol.* 18:1337–1347.

WINER, B. J., BROWN, D. R., and MICHAELIS, K. M. 1991. Statistical Principles in Experimental Design. McGraw-Hill, New York.

WITZGALL, P., and FRÉROT, B., 1989. Pheromone emission by individual females of carnation tortrix, *Cacoecimorpha pronubana*. *J. Chem. Ecol.* 15:707–717.

WU, W.-Q., HANSSON, B. S., and LÖFSTEDT, C., 1995. Electrophysiological and behavioural evidence for a fourth sex pheromone component in the turnip moth, *Agrotis segetum*. *Physiol. Entomol.* 20:81–92.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

ANTIFEEDANT EFFECTS OF SOME NOVEL TERPENOIDS ON CHRYSOMELIDAE BEETLES: COMPARISONS WITH ALKALOIDS ON AN ALKALOID-ADAPTED AND NONADAPTED SPECIES

CHRISTOPHER A. MULLIN,[1,*] AZUCENA GONZÁLEZ-COLOMA,[2] CARMEN GUTIÉRREZ,[2] MATIAS REINA,[3] HERBERT EICHENSEER,[1,4] BENEDICT HOLLISTER,[1] and SYLWESTER CHYB[1,5]

[1]*Pesticide Research Laboratory, Department of Entomology*
Pennsylvania State University
University Park, Pennsylvania 16802

[2]*Centro de Ciencias Medioambientales, CSIC*
Serrano, 115 dpdo., 28006 Madrid, Spain

[3]*Instituto de Productos Naturales y Agrobiologia, CSIC*
Av. Astrofísico F. Sánchez 3
38206 La Laguna, Tenerife, Spain

(Received October 31, 1996; accepted March 12, 1997)

Abstract—Structure-dose-feeding deterrency relationships were compared between the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), and the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, using 15 alkaloids, terpenoids, and phenolic derivatives. The former species, a specialist herbivore on selected alkaloid-rich Solanaceae species, was on average 100-times less sensitive to the antifeedant effects of alkaloids, but more similarly sensitive to the terpenoids and phenolics than the latter species, a generalist flower herbivore predominantly on Graminae, Cucurbitaceae, and Compositae species. Antifeedant ED_{50} values for the potato beetle and corn rootworm, each from closely related subfamilies of Chrysomelidae, ranged over four orders of dose magnitude among the 15 compounds with major species differences in stereosensitivity to β-hydrastines and analog sensitivity with the silphinenes. Extremes in sensitivity ranged from silphinene, a rare tricyclic sesquiterpene that is 53 times more active on the potato beetle to aconitine, which is 430 times more antifeedant to the corn rootworm. Among

*To whom correspondence should be addressed.
[4]Present address: Department of Entomology, University of Arkansas, Fayettville, Arkansas 72701.
[5]Present address: Department of Anatomy, University of Cambridge, Cambridge CB2 3DY, U.K.

0098-0331/97/0700-1851$12 50/0 © 1997 Plenum Publishing Corporation

silphinene and its two hydrolysis derivatives, there was not a strong correlation between antifeedant potency and injected toxicity for the two beetle species, but there was correlation between behavioral activity and galeal taste cell electrophysiological threshold and frequency responses. That all of the established GABA- and glycinergic compounds tested were antifeedant for both species suggests a shared molecular mechanism for antifeedant taste chemoreception in these divergent Chrysomelidae species. Moreover, the wide differences in antifeedant sensitivities among these and other chrysomelids to a suite of ligand-gated ion channel antagonists implicate a common protein neuroreceptor type with extraordinary heterogeneity in beetle taste.

Key Words—Feeding deterrents, *Diabrotica*, *Leptinotarsa*, indole, isoquinoline, sesquiterpene, diterpene, chemoreception, phagodeterrents, structure–taste relationships, QSAR.

INTRODUCTION

Basic to our understanding of insect–plant interaction is the importance of insect taste on host plant acceptance (Bernays and Chapman, 1994). Do insects have sufficient differences in taste affinities to drive host-plant specialization? For most phytophagous insects, antifeedant compounds appear to play a more important role in host-plant selection than specific phagostimulants (Jermy, 1966; Bernays and Chapman, 1977; Mitchell, 1994). More plant natural products are antifeedant than phagostimulatory to insects, and thus the potential to define species-dependent host-plant affinities via sensitivities to feeding deterrents is greater than through a mere inspection of known feeding stimulants. To explore this, we have been comparing taste responses among Chrysomelidae species to bioactive phytochemicals with known host-plant associations (Mullin et al., 1994; Eichenseer and Mullin, 1997).

In our gustatory studies with western corn rootworm, *Diabrotica virgifera virgifera* LeConte, a direct correlation between antifeedant potency and known antagonistic action at GABA (γ-aminobutyric acid)/glycine amino acid neuroreceptors has been found (Mullin et al., 1992, 1994; Chyb et al., 1995). These neuroreceptor antagonists include isoquinoline (bicuculline, hydrastine) and indole (strychnine) alkaloids and sesquiterpenes (picrotoxinin). By selecting a group of diagnostic antifeedants that combines established and putative GABA/glycinergic agents with other allelochemicals representative of the host-plant range of the insects species under study, we have recently compared structure–feeding deterrency profiles in four species from the subtribe Diabroticina (Eichenseer and Mullin, 1997) to address the relatedness of taste with host plant affinity of the Chrysomelidae beetle.

A recent search for insect control agents from endemic plants of the Canary Islands has led to isolation and characterization of a silphinene sesquiterpene from *Senecio palmensis* (Chr.Sm) with high antifeedant potency on all life stages,

particularly the adult of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (González-Coloma et al., 1995). Interestingly, this unusual tricyclic sesquiterpenoid (Figure 1) has similar structural features to that of the GABA antagonist picrotoxinin, prompting us to screen its activity and that of two structurally related derivatives (González-Coloma et al., 1997) on other chrysomelid beetles.

Relatively few insect studies have compared quantitative structure–taste relationships between species, and even fewer of these have characterized taste sensitivities using a full dose-response curve (Geuskens et al., 1983; van Beek and de Groot, 1986; Simmonds et al., 1989; Eichenseer and Mullin, 1997). Here we show that extraordinary differences in antifeedant ED_{50} and threshold values are apparent between two coexisting species of leaf beetles from different subfamilies, a chrysomeline, the Colorado potato beetle, and a galerucine, the western corn rootworm. This comparison of two related beetles with distinct feeding ecologies demonstrates that species-dependent taste spectrum patterns can be resolved with relatively few diagnostic taste chemicals.

METHODS AND MATERIALS

Beetles. Adult western corn rootworms were collected during August–September of 1994 and 1995 from Blue Hubbard winter squash (*Cucurbita maxima* Duchesne) or Spirit pumpkin (*C. pepo* L.) blossoms grown in an untreated 0.2-ha polyculture with Bodacious sweet corn (*Zea mays* L.) and Giant gray stripe sunflower (*Helianthus annuus* L.) at the Russell E. Larson Field Research Center, Rock Springs, Pennsylvania. Beetles were placed into shoe-sized plastic boxes with store-bought acorn squash fruit and water for at least two days prior to bioassay and maintained at 25°C with a 16:8 light–dark photoperiod. Adult Colorado potato beetles were from a Department of Entomology greenhouse colony reared on Katahdin potatoes (*Solanum tuberosum* L.). The strain used originated from field populations collected at Rock Springs in 1991 (Huang et al., 1995) and subsequently maintained for over 30 generations without selection with insecticides.

Chemicals. The structures of taste chemicals used here are presented in Figure 1. Cucurbitacin B was isolated from etiolated Blue Hubbard squash hypocotyls by a method described in Eichenseer and Mullin (1996). 1(10)-Dehydrosalviarin, a kind gift of Baldomero Esquivel, Instituto de Química, Universidad Nacional Autónoma de México, Coyoacán, México, was isolated previously from *Salvia lineata* (Esquivel et al., 1986). 11β-Acetoxy-5α-angeloyloxy silphinen-3-one (silphinene) was isolated from the aerial parts of *Senecio palmensis* (Chr. Sm.) according to González-Coloma et al. (1995). Hydrolysis of silphinene to give 11β-hydroxy-5α-angeloyloxy silphinen-3-one

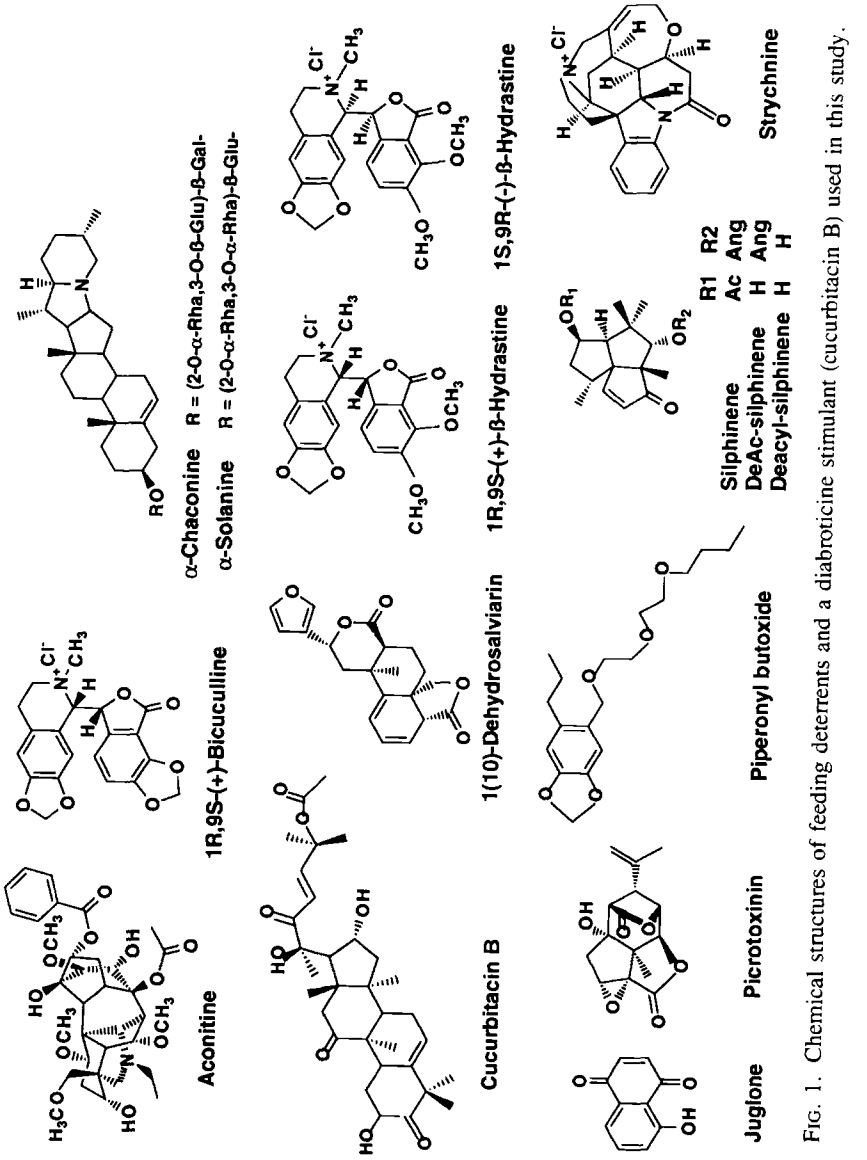


FIG. 1. Chemical structures of feeding deterrents and a diabroticine stimulant (cucurbitacin B) used in this study.

(deAc-silphinene) or the fully hydrolyzed 5α,11β-dihydroxy-silphinen-3-one (deacyl-silphinene) was accomplished in methanolic KOH (González-Coloma et al., 1997). Piperonyl butoxide was obtained from Fluka (Buchs, Switzerland). All other test chemicals were obtained from Sigma (St. Louis, Missouri). Strychnine was purchased as a chloride salt.

Squash Blossom Disk Bioassay. Bioassays were conducted in a growth chamber maintained at 25°C and with a 16:8 light–dark photoperiod. Dose–antifeedant relationships for corn rootworm, unless otherwise noted, were determined using Blue Hubbard floral disks in a choice bioassay (Mullin et al., 1991; Eichenseer and Mullin, 1997). A cork borer was used to cut 1.5-cm-diameter disks (1.7 cm^2; 37.9 $\pm$ 2.3 mg fresh weight, mean $\pm$ SEM, $N = 24$) from staminate flower petals on either side of the midvein. Chemicals were applied to the adaxial surface of disks in eight 1-μl aliquots of methanol or acetone carrier solvents with a Hamilton (Reno, Nevada) syringe repeating dispenser. Two solvent control and two treated disks on top of moist filter paper were placed in a 2- $\times$ 10-cm polystyrene Petri dish along with four beetles. Consumption of each disk was visually rated on a 0 (no feeding) to 10 (complete feeding) scale at 5 and 24 hr. A percentage consumption ratio for each dish was calculated using the sum of treated divided by the sum of control disk scores multiplied by 100. Three to eight concentrations eliciting a percentage consumption between 0 and 100, each dose replicated 4–24 times, were used to determine an antifeedant ED_{50} (concentration giving 50% reduction in feeding) value.

Regenerated Cellulose Disk Bioassay. Subsequent dose–antifeedant studies on corn rootworm with silphinene and its derivatives and with the glycoalkaloids were performed using a regenerated disk choice bioassay (Eichenseer and Mullin, 1996). Beetles were starved 12 hr prior to bioassay. Two randomly selected beetles, sex undetermined, were placed into a 100- $\times$ 20-mm Petri dish consisting of two regenerated cellulose disks (29 mm^2, 0.45-μm pore size; Schleicher and Schuell, Keene, New Hampshire) suspended on opposite sides of the dish on minuten mounting pins 2 mm above a paraffin layer covered with moistened filter paper. The phagostimulant cucurbitacin B (1 nmol) in methanol was applied to all cellulose disks with a Hamilton repeating dispenser in four 1-μl aliquots to ensure complete coverage of the disk. A similar application of either solvent (control) or antifeedant in methanol or acetone was made to paired disks within a dish. Dry disk areas after 24 hr of bioassay were determined to the nearest 0.1 mm^2 using an Optomax V image analyzer (Analytical Measuring Systems, Saffron, Walden, UK) and then subtracted from the original disk area to give the area consumed. Fifteen disks not offered to beetles were used for determination of the uneaten disk area. A percentage consumption ratio for each dish was calculated by dividing the treated area consumed by the control area consumed and multiplying the result by 100. Three to five concentrations eliciting

an average percentage consumption ratio between 10 and 90, each replicated four to eight times, were used in antifeedant ED_{50} calculations.

Potato Leaf Disk Bioassay. Dose–antifeedant relationships for Colorado potato beetle were determined using potato cv. Katahdin leaf disks in a choice bioassay. Chemicals were applied as above to the adaxial surface of 1.5-cm-diameter potato leaf disks (1.7 cm^2; 36.0 $\pm$ 1.4 mg fresh weight, mean $\pm$ SEM, $N = 12$) using eight 1-μl aliquots of methanol or acetone solvents. Two solvent control and two treated disks were placed on top of moist filter paper in a 2- $\times$ 10-cm Petri dish along with two adult beetles. Consumption of each disk was visually rated on a 0 (no feeding) to 10 (complete feeding) scale at 13 and 20 hr. A percentage consumption ratio for each dish was calculated using the sum of treated divided by the sum of control disk scores times 100. Three to five concentrations eliciting a percentage consumption between 0 and 100, each replicated 4–16 times, were used to determine antifeedant ED_{50} values. For some tests with silphinene, its derivatives, and with glycoalkaloids, the method of González-Coloma et al. (1995) was used, which incorporated four each of control and treated leaf disks (1.0 cm^2) and three potato beetles per dish. Disk areas were estimated on an image analyzer and average percentage consumption and antifeedant ED_{50} values were determined as in the cellulose disk bioassay above.

Toxicity Studies. DMSO solutions of silphinene and its two derivatives (10 μg each per insect) were injected through the metepimeron suture of the thorax of adult beetles using a Hamilton repeating syringe. Toxicity symptoms and mortality were recorded up to seven days after injection by maintenance of beetles on their respective squash or potato leaf foods.

Electrophysiological Studies. Tip-recording of galeal taste hairs of the adult western corn rootworm were performed as described previously (Chyb et al., 1995) using 10 mM KCl containing 10% (v/v) ethanol as the reference electrolyte solution. Firing rates were measured for the first second of stimulation.

Data Analysis. Consumption ratios of treated to solvent control were calculated for each replicate and plotted against the log-transformed dose expressed in nanomoles of chemical per square centimeter of disk area. Expressing dose on a nanomole per unit disk area allowed comparisons between beetle species and bioassays that utilized different size disks or where beetles had different consumption rates. Antifeedant ED_{50} (dose at 50% consumption), extrapolated sensitivity threshold (maximum dose for 100% consumption), and their respective confidence intervals were predicted from the slope of the linear regression of log dose versus percentage consumption by the standard least-squares method using JMP version 3.1 (SAS Institute Inc., Cary, North Carolina). At least three but generally five doses giving reduced consumption in the linear range of the dose–response curve were used in the regression to calculate values. A two sample one-way ANOVA of ED_{50} values was used to determine significant differences between species or bioassay methods at the $P < 0.05$ level.

RESULTS

Remarkable differences in sensitivity to 15 phytochemical antifeedants were apparent between the two leaf beetles (Table 1). ED_{50} responses in units of nanomoles per square centimeter of food disk surface were determined from the linear portion of the response curve (usually within 2 log dose units) by standard least-squares regression of log dose versus treatment to control consumption ratios (percent consumption). Insect antifeedant sensitivities among the potato beetle and corn rootworm, each from closely related subfamilies of Chrysomelidae, ranged over four orders of dose magnitude (Figure 2) with up to 430-fold differences in ED_{50} values between species. On average, the corn rootworm adult was about 100 times more sensitive to alkaloid antifeedants than the potato beetle. However, a major reverse in this tendency was found with the terpenoid antifeedants where the Colorado potato beetle was 53 and 14 times, respectively,

TABLE 1. FEEDING DETERRENCY (ED_{50} AND 95% CONFIDENCE LIMITS) OF BIOACTIVE PHYTOCHEMICALS ON TWO CHRYSOMELIDAE BEETLES[a]

Chemical class and compound	Western corn rootworm ED_{50}		Colorado potato beetle ED_{50}	
	nmol/cm^2	95% CI	nmol/cm^2	95% CI
Alkaloid				
Aconitine	0.414	0.104–0.804	178[b]	>39
Strychnine	1.35	0.95–1.86	23.4[b]	17.3–39.8
(+)β-Hydrastine	2.31	1.59–3.45	31.2[b]	17.7–121
(−)β-Hydrastine	2.74	2.20–3.36	320[b]	144–1730
(+)Bicuculline	2.95	2.04–4.07	20.0[b]	>8.5
α-Chaconine	6.58a	1.67–13.1	>100[b]	
α-Solanine	22.7a	9.55–589	>100	
Terpenoid				
1(10)-Dehydrosalviarin	0.541	0.378–0.761	20.4[b]	13.4–48.3
Silphinene	38.2	30.4–51.6	0.72[b]	0.42–1.23
11-Deacetylsilphinene	2.22a	0.80–3.79	21.6[b]	7.42–62.6
5,11-Deacylsilphinene	67.5	40.8–218	4.80[b]	1.51–15.3
Picrotoxinin	171	102–417	>2000[b]	
Cucurbitacin B	>1000[c]		238[b]	>79
Others				
Piperonyl butoxide	13.5	9.0–19.1	61.9[b]	38.2–93.3
Juglone	80.1	51.2–146	59.6	48.5–74.4

[a] All behavioral assays performed with flower (corn rootworm) and leaf (potato beetle) disk choice bioassays except for values followed by the letter "a," for which the cellulose bioassay was used.
[b] Species significantly different at $P < 0.05$.
[c] Cucurbitacin B at doses up to 500 nmol/cm^2 was stimulatory to corn rootworm feeding.

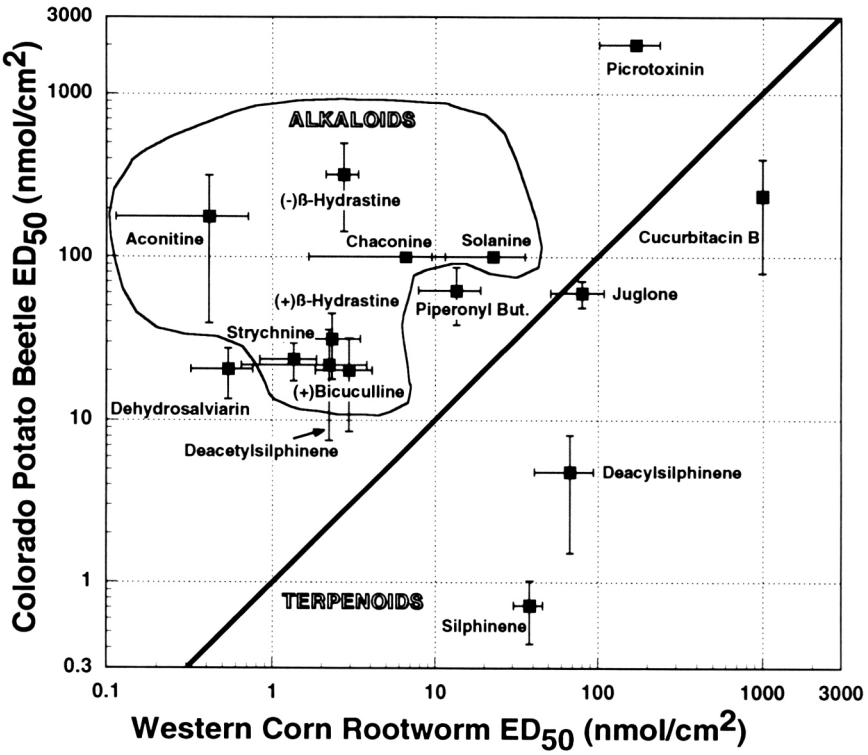


FIG. 2. Comparison of sensitivities (ED_{50} and 95% CI) to antifeedants for the western corn rootworm and the Colorado potato beetle plotted relative to a line of equal sensitivity.

more sensitive to silphinene and its deacylated derivative than the western corn rootworm. Both species had equivalent sensitivities to the phenolic naphthoquinone, juglone (Table 1, Figure 2).

Western corn rootworm was uniformly more sensitive to the feeding deterrency of alkaloids than the Colorado potato beetle, including the Solanaceae steroidal alkaloids, α-chaconine and α-solanine. All comparative alkaloid ED_{50} values were significantly different from and clustered above a line of equal sensitivity (Figure 2), demonstrating the consistently higher potency of alkaloid antifeedants on rootworm versus potato beetle. The range of alkaloids sampled included members from four distinct classes of alkaloids: diterpenoids, steroids, indoles, and isoquinolines.

In addition, dramatic differences in alkaloid selectivity among these two herbivores were noted. The potato beetle, in contrast to the corn rootworm, was

much more deterred by the isoquinolines (+)-β-hydrastine and bicuculline and the indole strychnine than the diterpenoid aconitine. Conversely, (1*R*,9*S*)-(+)-β-hydrastine was 10 times more inhibitory to potato beetle feeding than its (1*S*,9*R*)-(−)-diastereomer (Figure 1), while both isomers were equally effective on the corn rootworm (Table 1, Figure 2).

Much more variability occurred between beetle species response to terpenoids. The Colorado potato beetle was 38 times less sensitive to the diterpenoid 1(10)-dehydrosalviarin than corn rootworm, yet 53 times more susceptible than rootworm to the antifeedant silphinene (Table 1). 11-Deacetylation of the parent sesquiterpene silphinene increased feeding deterrency for rootworm while remarkably decreasing by 30 times the antifeedant effect on the potato beetle. Moreover, total deacylation of silphinene had no significant effect on antifeedant activity in the rootworm, but decreased by seven times its efficacy on the potato beetle (Tables 1 and 2). All silphinene analogs were more potent antifeedants to these beetles than the structurally similar picrotoxinin (Table 1, Figure 3). While the triterpenoid cucurbitacin B was phagostimulatory to rootworm at all tested concentrations, it was a moderate phagodeterrent for the Colorado potato beetle.

Antifeedant efficacy comparisons with silphinene and its hydrolysis products were made between the regenerated cellulose bioassay and the squash flower disk bioassay (Table 2). The latter method introduces, in addition to cucurbitacin B, amino acids and other phagostimulatory modalities (Mullin et al., 1994) intrinsic to this natural plant matrix. ED_{50} values differed between bioassay methods, and the former assay proved to be five- to eightfold more sensitive in detecting antifeedant activity for these sesquiterpenes with 1 nmol cucurbitacin B per disk. No significant differences in slopes of log dose–response lines were noted between the bioassay methods or across compounds (Table 2).

TABLE 2. FEEDING DETERRENCY OF SILPHINENE AND ITS HYDROLYSIS PRODUCTS TO WESTERN CORN ROOTWORM IN TWO BIOASSAYS[a]

Compound	Regenerated cellulose (nmol/cm²)[b]				Squash flower disk (nmol/cm²)[b]			
	ED_{50}	95% CI	Threshold	Slope	ED_{50}	95% CI	Threshold	Slope
Silphinene	7.97	4.9–13.9	2.13	−87.3	38.2[c]	30–52	4.22	−52.3
Deacetylsilphinene	2.22	0.8–3.79	0.40	−67.1				
Deacylsilphinene	7.97	3.2–14.7	0.42	−39.1	67.5[c]	41–218	5.13	−44.7

[a]Choice feeding bioassays were evaluated at 24 hr.
[b]Sensitivity threshold is the dose (nmol/cm²) at 100% consumption predicted from the slope of the linear regression of log dose versus percentage consumption by the standard least squares method.
[c]Method-based ED_{50} differences at $P < 0.05$.

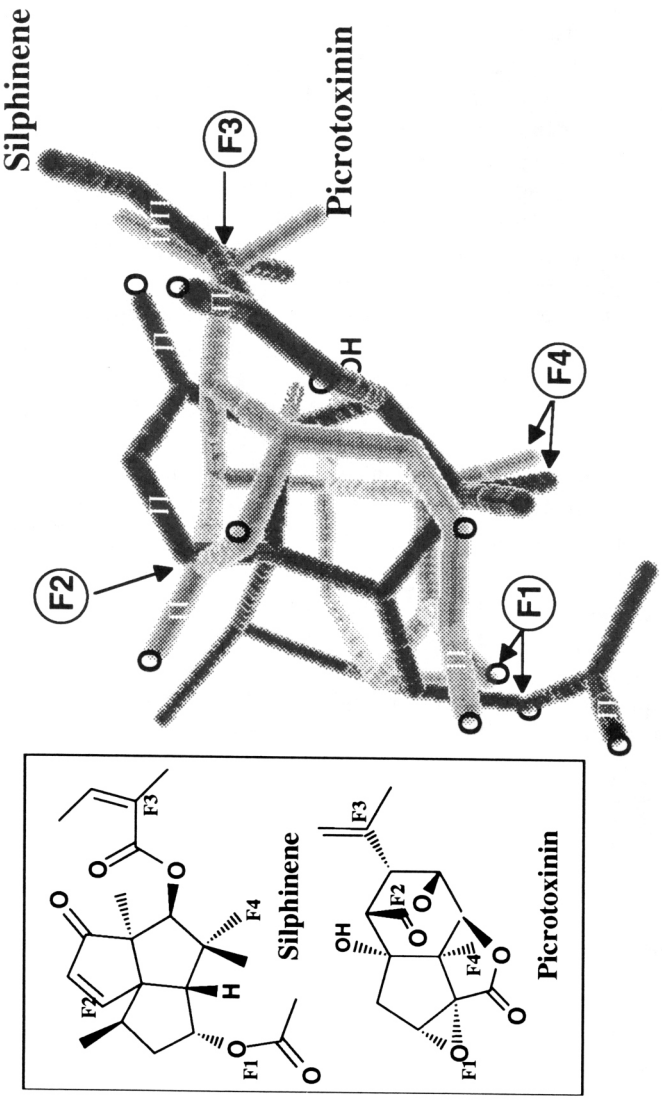


FIG. 3. 3D molecular fit between silphinene and picrotoxinin. Only heteroatoms and π bonds are indicated in these cylinder views of the structures. Average distance between atomic centers F1–F4 (insert) is less than 0.5 Å using Alchemy III (Tripos Associates, St. Louis, Missouri) energy minimization and fit algorithms.

TABLE 3. INJECTED TOXICITY OF SILPHINENE AND ITS HYDROLYSIS PRODUCTS TO WESTERN CORN ROOTWORM AND COLORADO POTATO BEETLE[a]

	Mortality (%)[b]					
	Western Corn Rootworm		Colorado Potato Beetle			
Compound	24 hr	96 hr	24 hr	48 hr	72 hr	7 days
Silphinene	5.3	9.1	0	10	24	40
Deacetylsilphinene	15	39	0	20	29	40
Deacylsilphinene	30	65	0	15	35	87

[a]The rootworm and potato beetle were given a 10 μg dose of compound in 200 nl and 1 μl, respectively, of DMSO. Results are from injections of 20 beetles for each compound and species.
[b]Mortality values were corrected according to Abbott's (1925) formula for control mortality from injected DMSO.

Single-dose toxicity experiments with silphinene and its derivatives (Table 3) did not demonstrate a positive correlation in either beetle species between injected mortality and antifeedant potency (Tables 1 and 2) of the three silphinene analogs. Deacylsilphinene, although less antifeedant (Tables 1 and 2), was more toxic to adult corn rootworm than the other silphinenes. For adult potato beetle, the parent compound, while much more antifeedant than its hydrolysis

TABLE 4. GALEAL CHEMOSENSORY RESPONSES TO SILPHINENE AND ITS HYDROLYSIS PRODUCTS IN ADULT WESTERN CORN ROOTWORM

	Mean firing rate ± SEM (number of samples)[a]		
Concentration (μM)	Silphinene	Deacetylsilphinene	Deacylsilphinene
0	5.8 ± 1.3 (24)	4.6 ± 1.2 (25)	6.0 ± 1.7 (20)
1	17.5 ± 1.3 (32)	16.0 ± 1.8 (25)	14.0 ± 2.2 (30)
10	25.6 ± 1.5 (29)	45.5 ± 3.0 (30)	15.6 ± 1.3 (30)
100	36.4 ± 1.6 (34)	39.7 ± 2.5 (29)	31.3 ± 4.5 (32)
1000	32.4 ± 1.9 (34)	37.4 ± 2.5 (24)	59.7 ± 2.9 (30)
ED_{50}^{b}	3.0 μM	1.4 μM	40 μM

[a]Galeal taste cell output in action potentials during first second of stimulation at indicated dose of compound in a 10 mM KCl solution containing 10% ethanol.
[b]Estimated dose for half-maximal firing rate using regression analysis of log dose versus firing rate (− solvent control) in linear portion of curve.

products (Table 1), was more similarly toxic (Table 3). Mortality was latent and took up to one week to be fully expressed.

A general correlation in western corn rootworm between antifeedant efficacy (mean ED_{50} values across methods) and taste cell firing frequency (estimated from the dose giving half maximal firing rates; Table 4) was found for the three silphinenes ($r^2 = 0.692$). The partial hydrolysis derivative gave the highest frequency of galeal cell response at 10 μM followed by parent silphinene and the fully deacylated derivative (Table 4), in order of their feeding deterrency (Table 1). However, the deacyl-silphinene generated a stronger firing intensity in rootworm taste cells at the high concentration of 1 mM (Table 4). Silphinene and its analogs predominantly elicited a single, high-amplitude spike from each sensilla within the first second of recording, but the background pattern was complicated by low-frequency firing of up to three taste cells per hair with the reference electrolyte plus ethanol.

DISCUSSION

Antifeedant taste sensitivity in the Colorado potato beetle is fundamentally distinct from that of the western corn rootworm. Extraordinary differences in antifeedant ED_{50} and threshold values are apparent between these leaf beetles from related subfamilies that cooccur in the wild but have very distinct feeding ecologies. The Colorado potato beetle, a specialist of some Solanaceae species (Mitchell and Harrison, 1985; Hsiao, 1986; Sinden et al., 1988), was uniformly less sensitive to feeding deterrency of alkaloids, including the *Solanum* glycoalkaloids α-chaconine and α-solanine, than the western corn rootworm, a corn herbivore that additionally feeds on pollen and flowers of many late-season plants, particularly Graminae, Cucurbitaceae, and Compositae (Krysan, 1986; Mullin et al., 1994). Some host range overlap of these species does occur; we have occasionally observed adult corn rootworms feeding on flowers of potato, horse-nettle (*Solanum carolinense*), and smooth ground cherry (*Physalis subglabrata*) (Mullin et al., unpublished). Nevertheless, the western corn rootworm host range is mostly limited to plant organs and families without major alkaloid allelochemicals. Loss of physiological susceptibility to defensive phytochemicals followed by specific tuning of chemoreceptors to these compounds as phagostimulants is a major current hypothesis for evolution of host-plant specialization in insects (Bernays and Chapman, 1994), although a change in the insect's chemoreception system may occur prior to a postingestive adaptation for the allelochemical (Jermy, 1993). The relative role that desensitization of deterrent versus increased affinity at stimulant taste cells has in tuning to taxon-specific phytochemicals needs experimental verification.

Exceptions to a general alkaloid antifeedant insensitivity have been found

in the Colorado potato beetle. This insect is strongly deterred by both a pyrrolizidine alkaloid (acetyltrachelanthamine) and a diterpene alkaloid (cardiopetamine) with ED_{50} values of 4.18 and 3.44 nmol/cm^2, respectively (Reina et al., unpublished data; González-Coloma et al., unpublished data). These compounds were the only active molecules among a family of structural analogs.

In this study, ED_{50} response values were expressed in terms of nmol/cm^2 of food disk surface instead of total dose per disk since the former would allow taste comparison between a smaller corn rootworm relative to the larger Colorado potato beetle. The disk size or amount of disks presented in the bioassay was adjusted to account for the greater consumption rate of the latter species. Taste sensilla are fairly uniform in diameter regardless of insect size (Zacharuk, 1980), and gustatory sampling is primarily dependent on extent of surface contact between compounds and the sensory hair. Moreover, available evidence (Mullin et al., 1994) suggests a ligand–protein receptor interaction is mediating taste transduction, and thus dose units in moles of dose and not by mass will better relate to efficacy.

All of the established GABA- and glycinergic compounds tested, including bicuculline, β-hydrastines, strychnine, and picrotoxinin, were antifeedant for both species. This suggests a shared molecular mechanism for antifeedant taste chemoreception for these divergent Chrysomelidae species. Surprisingly, picrotoxinin had weak activity on the potato beetle while the structurally related tricyclic sesquiterpene silphinene (11β-acetoxy-5α-angeloyloxy-silphinen-3-one, Figure 3) was a potent antifeedant to this species but much less active on the corn rootworm. Similarly large contrasts in sensitivity trends among the hydrolysis products of silphinene were apparent between the two species. The potato beetle in turn had a stereoisomer sensitivity for β-hydrastines (1*R*,9*S* was 10 times more inhibitory than 1*S*,9*R*) more consistent with known action at vertebrate central nervous system GABA receptors (Kardos et al., 1984), whereas both diastereomers were equally potent for the corn rootworm. Significant, but smaller, differences in taste responses were demonstrated by Eichenseer and Mullin (1997) among four species of closely related diabroticine beetles (*D. v. virgifera*, *D. barberi*, *D. u. howardi*, and *Acalymma vittatum*) with a similar set of neuroreceptor antagonists. In that study, all diabroticine species were more sensitive to alkaloids than sesquiterpenoids, the activity spectrum correlated with subgenus status, and a tendency for decreasing antifeedant sensitivity with increasing polyphagy was seen. The wide differences in antifeedant sensitivities among chrysomelids to GABA/glycine-gated ion channel antagonists implicates a common sensory neuroreceptor with numerous subtypes.

Good correlation was found between intensity of the chemosensory response of a single taste neuron (Table 4) and the respective antifeedant potency of the silphinene derivative (Tables 1 and 2) in corn rootworm, arguing for a link between galeal sensillar responses and antifeedant behavior in this beetle as

previously found (Chyb et al., 1995). However, correlations of antifeedant potency with internal toxicity of these phytochemical analogs were poor for both the rootworm and potato beetles (Table 3). These results support the hypothesis that plant secondary chemistry exerts its primary effects at peripheral sensory versus postingestive internal neuroreceptors in driving host-plant associations (Jermy, 1993). Such a lack of behavioral and toxicity relationship has been noted for plant allelochemicals in general (Bernays, 1991). This may reflect the polarity requirements for gustatory versus internal target site interactions where action at a taste cell usually requires more water solubility for a phytochemical than for action at membrane receptor sites in the central nervous or alternative target system of the animal (Mullin et al., 1994). Moreover, silphinene would be expected to be hydrolyzed internally by detoxification esterases, resulting in derivatives that may even be more toxic (Table 3). Sensing the parent compound at a taste chemoreceptor would avoid internal toxicities from either the parent compound or its degradation products.

Silphinene has 3D structural features similar to those of picrotoxinin, a sesquiterpene from *Anamirta* spp. that is an established GABA-gated chloride channel antagonist (Klunk et al., 1983; Ozoe and Matsumura, 1986). Both have an electronegative oxygen (F1), similarly oriented pi bond centers (F2, F3), a hydrophobic edge delimited by a methyl group (F4), and may similarly orient to a GABA-neuroreceptor binding site.

The Colorado potato beetle, in general, is highly sensitive to terpenoid antifeedants including the limonoids (Bentley et al., 1988) and the silphinenes (González-Coloma et al., 1995). Cucurbitacin B, a potent phagostimulatory triterpenoid for all diabroticine beetles (Metcalf et al., 1980), was phagodeterrent to the potato beetle. Exceptions in the potato beetle terpenoid sensitivity have been noted. The diterpenoid 1(10)-dehydrosalviarin, a neoclerodane diterpene from *Salvia lineata* Benth (Esquivel et al., 1994), included here because of its known potency as an antifeedant for *Spodoptera littoralis* (Boisduval) (Simmonds et al., 1996), was poorly active on the potato beetle. Aconitine, a diterpenoid alkaloid antifeedant for insects and a reputed sodium channel antagonist (Wink, 1993) was also strongly active on rootworm but not on the potato beetle.

Compounds such as the phenolic quinone juglone deter feeding in many insect species (Norris, 1986). Piperonyl butoxide, a lignan derivative used as an insecticide synergist in pest control, is strongly antifeedant for rootworm, potato beetle (Mullin et al., 1992) (Table 1), and other beetle species (Nawrot and Harmatha, 1994). Frequently, distinct species selectivities in antifeedant action are evident (Schoonhoven, 1982; van Beek and de Groot, 1986; Bernays and Chapman, 1994; Eichenseer and Mullin, 1997). Certainly species-dependent taste spectrum patterns, illustrated in Figure 2, can be established with relatively few diagnostic compounds. Chemical strategies for selective taste disruption

(Frazier and Chyb, 1995) that are compatible with traditional biological control are of great potential in future pest management of herbivorous insects.

Acknowledgments—We thank Dr. J. L. Frazier for use of his taste electrophysiology lab and C. L. Krebs, J. R. Linderman and C. H. Mason for their technical assistance with some of the rootworm bioassays. This study was supported, in part, by NATO CRG-950822, USDA 95-34050-1484 and 95-37302-1807 grants.

REFERENCES

ABBOTT, W. S. 1925. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18:265–267.

BENTLEY, M. D., RAJAB, M. S., ALFORD, A. R., MENDEL, M. J., and HASSANALI, A. 1988. Structure-activity studies of modified citrus limonoids as antifeedants for Colorado potato beetle larvae, *Leptinotarsa decemlineata. Entomol. Exp. Appl.* 49:189–193.

BERNAYS, E. A. 1991. Relationship between deterrence and toxicity of plant secondary compounds for the grasshopper *Schistocerca americana. J. Chem. Ecol.* 17:2519–2526.

BERNAYS, E. A., and CHAPMAN, R. F. 1977. Deterrent chemicals as a basis of oligophagy in *Locusta migratoria* (L.). *Ecol. Entomol.* 2:1–18.

BERNAYS, E. A., and CHAPMAN, R. F. 1994. Host-Plant Selection by Phytophagous Insects. Chapman and Hall, New York.

CHYB, S., EICHENSEER, H., HOLLISTER, B., MULLIN, C. A., and FRAZIER, J. L. 1995. Identification of sensilla involved in taste mediation in adult western corn rootworm (*Diabrotica virgifera virgifera* LeConte). *J. Chem. Ecol.* 21:313–329.

EICHENSEER, H., and MULLIN, C. A. 1996. Maxillary appendages used by western corn rootworms, *Diabrotica virgifera virgifera*, to discriminate between a phagostimulant and -deterrent. *Entomol. Exp. Appl.* 78:237–242.

EICHENSEER, H., and MULLIN, C. A. 1997. Antifeedant comparisons of GABA/glycinergic antagonists for Diabroticite leaf beetles (Coleoptera: Chrysomelidae). *J. Chem. Ecol.* 23:71–82.

ESQUIVEL, B., CÁRDENAS, J., RAMAMOORTHY, T. P., and RODRÍGUEZ-HAHN, L. 1986. Clerodane diterpenoids of *Salvia lineata. Phytochemistry* 25:2381–2384.

FRAZIER, J. F., and CHYB, S. 1995. Use of feeding inhibitors in insect control, pp. 364–381, *in* R. F. Chapman and G. deBoer (eds.). Regulatory Mechanisms in Insect Feeding, Chapman and Hall, New York.

GEUSKENS, R. B. M., LUTEIJN, J. M., and SCHOONHOVEN, L. M. 1983. Antifeedant activity of some ajugarin derivatives in three lepidopterous species. *Experientia* 39:403–404.

GONZÁLEZ-COLOMA, A., REINA, M., CABRERA, R., CASTAÑERA, P., and GUTIÉRREZ, C. 1995. Antifeedant and toxic effects of sesquiterpenes from *Senecio palmensis* to Colorado potato beetle. *J. Chem. Ecol.* 21:1255–1270.

GONZÁLEZ-COLOMA, A., GUTIÉRREZ, C., CABRERA, R., and REINA, M. 1997. Silphinene derivatives: Their effects and modes of action on Colorado potato beetle. *J. Agric. Food Chem.* 45:946–950.

HSIAO, T. H. 1986. Specificity of certain chrysomelid beetles for Solanaceae, pp. 345–363, *in* W. G. D'Arcy (ed.). Solanaceae: Biology and Systematics. Columbia University Press, New York.

HUANG, H., SMILOWITZ, Z., and SAUNDERS, M. C. 1995. Toxicity and field efficacy of cryolite against Colorado potato beetle (Coleoptera: Chrysomelidae) larvae. *J. Econ. Entomol.* 88:1408–1414.

JERMY, T. 1966. Feeding inhitors and food preference in chewing phytophagous insects. *Entomol. Exp. Appl.* 9:1–12.

JERMY, T. 1993. Evolution of insect–plant relationships—a devil's advocate approach. *Entomol. Exp. Appl.* 66:3–12.

KARDOS, J., BLASKÓ, G., KEREKES, P., KOVÁCS, I., and SIMONYI, M. 1984. Inhibition of [^{3}H]GABA binding to rat brain synaptic membranes by bicuculline related alkaloids. *Biochem. Pharmacol.* 33:3537–3545.

KLUNK, W. E., KALMAN, B. L., FERRENDELLI, J. A., and COVEY, D. F. 1983. Computer-assisted modeling of the picrotoxinin and γ-butyrolactone receptor site. *Mol. Pharmacol.* 23:511–518.

KRYSAN, J. L. 1986. Introduction: Biology, distribution, and identification of pest *Diabrotica*, pp. 1–23, *in* J. L. Krysan and T. A. Miller (eds.). Methods for the Study of Pest *Diabrotica*. Springer, New York.

METCALF, R. L., METCALF, R. A., and RHODES, A. M. 1980. Cucurbitacins as kairomones for diabroticite beetles. *Proc. Nat. Acad. Sci. U.S.A.* 77:3769–3772.

MITCHELL, B.K. 1994. The chemosensory basis of host-plant recognition in Chrysomelidae, pp. 141–151, *in* P. H. Jolivet, M. L. Cox and E. Petitpierre (eds.), Novel Aspects of the Biology of Chrysomelidae, Kluwer, Dordrecht.

MITCHELL, B. K., and HARRISON, G. D. 1985. Effects of *Solanum* glycoalkaloids on chemosensilla in the Colorado potato beetle: A mechanism of feeding deterrence? *J. Chem. Ecol.* 11:73–83.

MULLIN, C. A., ALFATAFTA, A. A., HARMAN, J. L., EVERETT, S. L., and SERINO, A. A. 1991. Feeding and toxic effects of floral sesquiterpene lactones, diterpenes and phenolics from sunflower (*Helianthus annuus* L.) on western corn rootworm. *J. Agric. Food Chem.* 39:2293–2299.

MULLIN, C. A., MASON, C. H., CHOU, J.-C., and LINDERMAN, J. R. 1992. Phytochemical antagonism of γ-aminobutyric acid based resistances in *Diabrotica*, pp. 288–308, *in* C. A. Mullin and J. G. Scott (eds.). Molecular Mechanisms of Insecticide Resistance: Diversity Among Insects. Symposium Series 505. American Chemical Society, Washington, D.C.

MULLIN, C. A., CHYB, S., EICHENSEER, H., HOLLISTER, B., and FRAZIER, J. L. 1994. Neuroreceptor mechanisms in insect gustation: A pharmacological approach. *J. Insect Physiol.* 40:913–931.

NAWROT, J., and HARMATHA, J. 1994. Natural products as antifeedants against stored products insects. *Postharvest News Inf.* 5:17N–21N.

NORRIS, D. M. 1986. Anti-feeding compounds, pp. 97–146, *in* G. Haug and H. Hoffmann (eds.). Sterol Biosynthesis Inhibitors and Anti-Feeding Compounds. Springer, Berlin.

Ozoe, Y., and MATSUMURA, F. 1986. Structural requirements for bridged bicyclic compounds acting on picrotoxinin receptor. *J. Agric. Food Chem.* 34:126–134.

SCHOONHOVEN, L. M. 1982. Biological aspects of antifeedants. *Entomol. Exp. Appl.* 31:57–69.

SIMMONDS, M. S. J., BLANEY, W. M., LEY, S. V., SAVONA, G., BRUNO, M., and RODRIQUEZ, B. 1989. The antifeedant activity of clerodane diterpenoids from *Teucrium*. *Phytochemistry* 28:1069–1071.

SIMMONDS, M. S. J., BLANEY, W. M., ESQUIVEL, B., and RODRIGUEZ-HAHN, L. 1996. Effect of clerodane-type diterpenoids isolated from *Salvia* spp. on the feeding behavior of *Spodoptera* littoralis. Pestic. Sci. 47:17–23.

SINDEN, S. L., SANFORD, L. L., CANTELO, W. W., and DEAHL, K. L. 1988. Bioassays of segregating plants: A strategy for studying chemical defenses. *J. Chem. Ecol.* 14:1941–1950.

VAN BEEK, T. A., and DE GROOT, AE. 1986. Terpenoid antifeedants, part I. An overview of terpenoid antifeedants of natural origin. *Recl. Trav. Chim. Pays-Bas.* 105:513–527.

WINK, M. 1993. Allelochemical properties or the raison d'être of alkaloids, pp. 1–118, *in* G. A. Cordell (ed.). The Alkaloids, Vol. 43. Academic, San Diego.

ZACHARUK, R. Y. 1980. Ultrastructure and function of insect chemosensilla. *Annu. Rev. Entomol.* 25:27–47.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

INFLUENCE OF AGE ON ANTENNAL RESPONSE OF MALE HONEY BEES, *Apis mellifera*, TO QUEEN MANDIBULAR PHEROMONE AND ALARM PHEROMONE COMPONENT

RICHARD S. VETTER* and P. KIRK VISSCHER

Department of Entomology
University of California
Riverside, California 92521

(Received July 2, 1996; accepted March 14, 1997)

Abstract—Using a computer-controlled pheromone-puff delivery system with signal averaging, we investigated the change with age of the electroantennogram (EAG) response of drone (male) honey bees to queen mandibular pheromone (QMP) and an alarm pheromone component, isopentyl acetate (IPA). Drone antennae were significantly more sensitive than worker antennae to QMP, but there was no significant difference in response to IPA. Response to QMP was dose dependent and decreased gradually with the age of the drone. Drone response to IPA did not diminish significantly with age. Drones do not mature sexually or make their first flights until about 1 week old, so the ontogeny of their sensory responsiveness to queen pheromones does not reflect that of their behavior. It is unknown if drones exhibit any behavioral response to IPA. The signal-averaging system we describe could be used to improve resolution of low signal-to-noise ratio signals in other EAG studies.

Key Words—*Apis mellifera* honey bee, drone bees, electroantennogram, queen pheromone, alarm pheromone.

INTRODUCTION

The honey bee, *Apis mellifera* L., is the most thoroughly studied insect species because of its economic value to man since prehistory, extensive intraspecific communication, and relatively complex behavior. However, most honey bee research has concentrated on worker bees, and some on queens, while the biology of drones remains relatively poorly known. Drones' role in the biology of

*To whom correspondence should be addressed.

0098-0331/97/0700-1867$12 50/0 © 1997 Plenum Publishing Corporation

honey bees is relatively simple: they are "sperm with wings" that fly from the colony and mate with virgin queens, usually from other colonies; if successful they immediately die. Drones perform no labor within the hive. In seasons with few mating opportunities and limited resources (usually autumn in temperate climates), the drones are physically driven out of the hive by the workers, prevented from reentering, and left to starve (Morse et al., 1967).

The disparity in research between workers and drones extends to electrophysiological studies. Previous studies have used electroantennograms (EAG) to examine the ability of worker bees to detect plant host odors (Pham-Delègue et al., 1986, 1989, 1990; Patte et al., 1989; Thiery et al., 1990; Henning and Teuber, 1992), queen pheromones (Pham-Delègue et al., 1993), and alarm pheromones (Allan et al., 1987; Robinson 1987; Visscher et al., 1995). Drones' antennae have been shown to respond to queen odors and to be more sensitive to these than are antennae of worker or queen honey bees (Kaissling and Renner, 1968; Adler et al., 1973).

Drones undergo a maturation process in which their behaviors change as they age (Winston, 1987). They initially remain in the brood-rearing area of the hive. Subsequently they move to more peripheral regions (apparently modulated by changing temperature preferences), and finally begin to fly from the hive. These behaviors may be driven by hormonal changes and correlate with the maturation of sperm. Drones first begin making flights from the hive at about 6 to 8 days of age (Howell and Usinger, 1933; Oertel, 1956; Ruttner, 1966; Witherall, 1971). They cannot survive on their own and return to the hive to feed. They initially make orientation flights that last about 5 min, and later shift to flights in search of mates, which last about 25 min (Oertel, 1956) and may take them to distances 2–7 km from the colony (Ruttner, 1966; Ruttner and Ruttner, 1972). The number of spermatozoa in their testes reaches the mature level between 6 and 9 days of age (Jaycox, 1956; Woyke and Jasinski, 1978), and drones are best chosen as sources of sperm for artificial insemination after 12 days of age (Ruttner, 1966; Woyke and Jasinski, 1978). The mean life expectancy of drones is 20–50 days (Ruttner, 1966). In light of this maturation process, in which queen sex attractant pheromones do not become behaviorally relevant to drones until about the second week of life, we investigated whether age might affect the sensitivity of the antennae to queen pheromones. We also examined the response of drone antennae to a honey bee alarm pheromone component, isopentyl acetate (IPA).

METHODS AND MATERIALS

Insects. All drones were reared in and taken from colonies housed at the University of California Agricultural Research Station on the Riverside campus

and were an undetermined mix of European bee subspecies, probably predominantly *A. m. ligustica*. At the time this research was conducted and completed, Africanized honey bees were not yet found in the area.

EAG. The EAG set-up was modified from that used by Visscher et al. (1995). We decapitated a bee, removed an antenna with forceps, and imbedded its base in wax so it stood in a pool of bee saline (Thiery et al., 1990), which also contained the ground electrode. The other electrode was inserted in a Pasteur pipet (filled with saline and covered with grounded aluminum foil), which was lowered onto the antenna so that the distal tip of the antenna was in contact with the saline. We chloridized the silver wire electrodes every day and sometimes twice a day (if the electrodes were exposed more than 5 hr to saline) in order to minimize variation in the sensitivity of the apparatus. Prior to a day's experiment, we gently scraped each electrode with a razor blade to remove discolored chloridization, then placed it as the cathode in 0.1 M HCl and applied a 3-V potential across it and another silver wire anode in the acid for 90 sec to electrolytically clean the wire. We then recoated the electrode with silver chloride by reversing the polarity for 15 sec.

Antennal responses were recorded by amplifying the EAG 100× and passing the signal through a high-pass filter with a cutoff of 1 Hz to keep the slowly changing baseline potential at zero (Bjostadt and Roelofs, 1980). We then digitized the output and displayed and analyzed it on a Macintosh II computer (MacAdios II digitizer and SuperScope II software, GW Instruments, Somerville, Massachusetts).

Pheromone Puff Delivery. Pheromone and clean-air puffs were controlled via computer software through a series of valves and tubing. We passed air from a building air line through a 15-cm × 5-cm-ID steel pipe packed with activated carbon and glass wool, then bubbled it through water in a 500-ml Erlenmeyer flask to humidify it, after which the flow split. One branch flowed continuously over the antenna through a 5.2-mm-ID glass tube at a rate of 2.8 liters/min. The other branch split into two puffer lines, and each of these led to a Pasteur pipet, the tips of which were inserted through a small hole into the continuous-flow tube. Solenoid valves (Dayton 3A440) controlled whether one or the other or neither puffer line flowed, at a rate of 1 liter/min. The solenoids were controlled by solid state relays switched by digital output of the computer software and hardware.

To deliver a pheromone, we loaded a 3-mm × 15-mm piece of Whatman No. 1 filter paper with 2 μl of solution and placed the paper inside the pipet. An unloaded piece of filter paper in the second pipet served as the clean-air-puff control. The puffer lines were checked in preliminary experiments to determine that they were delivering equal puffs, as determined by the response of worker antennae to puffs of alarm pheromone from the two lines.

Chemicals. Queens produce sex pheromones in their mandibular glands.

We used a blend of queen mandibular pheromone components (QMP) as described by Slessor et al. (1988), purchased from Pherotech (Delta, British Columbia, Canada). The blend consisted of 9-oxodecenoic acid, (+) and (−)-9-hydroxydecenoic acid, methyl-*p*-hydroxybenzoate, and homovanillyl alcohol. We prepared an isopropanol solution of 0.5 Qeq (queen equivalents, as defined by the manufacturer) QMP/μl, and made serial decade dilutions to provide solutions with 10^0 through 10^{-6} Qeq/2 μl load. One Qeq of QMP, the amount found in the glands of a typical queen, contains about 150 μg (Slessor et al., 1988) to 260 μg (Winston and Slessor, 1993) of 9-oxodecenoic acid (and corresponding lower concentrations of the other components). We obtained IPA from Aldrich Chemical (Milwaukee, Wisconsin) and applied it in neat form. Preliminary tests showed that there was no antennal response to isopropanol alone, so unloaded filter paper was used in the clean-air control puffs for both pheromones.

Although we loaded a specific amount of QMP and IPA on each paper, we do not know how much actually was carried by a puff of the air surrounding the paper, or how much contacted the antenna. However, in all of the experiments, we were interested in relative comparisons between groups.

Test Series and Signal Averaging. Each test consisted of a series of puffs, controlled automatically by software and hardware switching the puffer line solenoids on and off. In each 1-min series, an 0.025-sec duration air puff was delivered at 1 sec after initiation of the trial and each 10 sec thereafter, and a pheromone puff of the same length was delivered at 6 sec and each 10 sec thereafter, so the antenna received six air and six pheromone puffs in each series (Figure 1). For both the pheromone and control puffs, we then computed a point-by-point average of the digitized response for the 5 sec following the time of initiation of each puff. The effect of this averaging is the reduction of baseline noise in the averaged signals (Figure 1).

Dose–Response Series. We collected mature drones as they returned to their colonies from mating flights between 1300 and 1500 hr. We placed a "queen excluder" grid (4 × 19-mm elliptical holes) in front of the hive entrance; workers readily pass through these holes but drones and queens cannot, because of their larger size. We seized drones as they attempted to go through the excluder and placed them into a 12 × 12 × 12-cm screen cage containing a vial of diluted honey for food. We also collected workers as they left on foraging trips and put them in the cage so they would feed the drones, as well as for a source of antennae for worker EAGs. Most experiments were performed the morning following collection, but some were done on the second day. We tested the relative QMP response of drones and workers with EAGs on 20 drones with decade doses of 10^{-1}–10^{-6} Qeq of QMP and 20 worker honey bees with 10^0–10^{-2} Qeq. In a second series, we were interested in also comparing the IPA response of drones and workers, and we tested 10 drones 13–14 days of

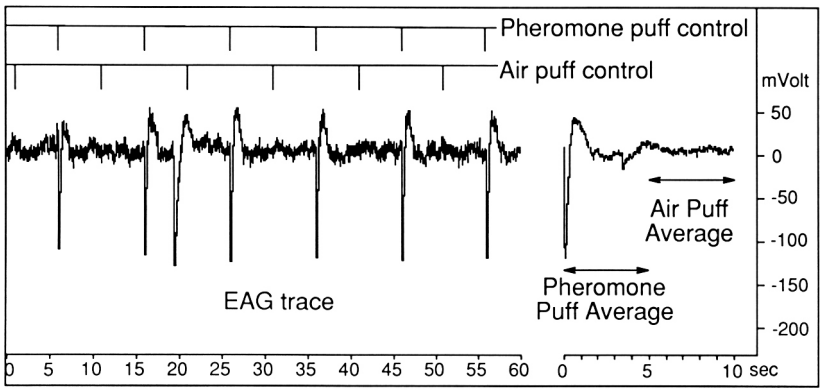


FIG. 1. A test series. The first and second trace at the top show the control signals for the pheromone puffs and air puffs, respectively. The third trace is the EAG response, amplified 100× and filtered. At the right are contiguous averaged 5-sec waveforms for six pheromone-puff responses and six air-puff responses, on an expanded time scale. These show how random baseline noise was reduced about 10 dB (threefold) through signal averaging, as was the spontaneous depolarization at 19.5 sec (which is seen in the 20-dB-smaller blip at 3.5 sec in the averaged trace). This trace is the response of a drone antenna to 10^{-1} Qeq QMP.

age and nine forager workers of unknown age, using 10^{-1} Qeq of QMP and neat IPA.

Drone Age Series. We placed combs containing capped drone brood into a five-frame nucleus hive and then placed this inside an environmental chamber (model I-30; Percival Co., Boone, Indiana) at 33°C with a 14L:10 D light cycle. As frames of brood were depleted by the emerging adult drones, they were removed and replaced with new frames of capped drone brood. We removed newly emerged drones daily and marked them with enamel paint, using a two-dot system on the thorax, so that combinations of seven colors represented 49 consecutive days. We then placed the drones into a queen-excluder-mesh "jail," which contained a frame of comb with uncapped honey, and placed the jail with the marked drones inside a queenright bee colony for a day before releasing the drones into the hive. This step was taken because if drones are simply added to the colony, they are sometimes removed by the workers; after a day of being in contact with workers who enter the jail, the drones are accepted.

We used colonies in pairs to house marked drones such that each colony received drones on alternate days, and the drones in each colony differed in age by two-day steps (although some drifting of marked drones did occur). About every 12 days we switched to a different pair of drone-housing colonies (of three pairs total); this was done to avoid flooding the colonies with drones, which

can result in workers killing excess drones and drone larvae in order to regulate their numbers (Jaycox, 1956).

We began testing after the earliest-marked drones were 30 days of age so that we had a wide age range available for testing on each day. We ran EAGs on drones of even ages of 2–30 days, using drones from each of the three colonies, and choosing drones so ages from 2 to 30 days were about equally represented in the pool for each day's testing (to avoid confounding differences in the sensitivity of our EAG setup from day-to-day with differences in drones of different ages). We tested six drones for each age and no more than three of any one age on any given day. We also collected drones 32–40 days old when we found them (relatively few drones live that long), and ran EAGs on four, five, six, three, and three drones of ages 32, 34, 36, 38, and 40 days, respectively.

The previous experiment showed that 10^{-1}–10^{-3} Qeq gave strongly increasing responses from drone antennae, and we used these for the age series treatments. For each antenna, we presented all three doses of QMP in random order, either before or after the IPA stimulus; the order of QMP and IPA was alternated for each succeeding antenna. The total time required to set up an antenna and run the four pheromone tests was about 15 min.

Drone Flight. We compared the flight ages of drones in our conditions with those reported from areas with different climates. We marked drones on the first day of their emergence (from frames in the incubator) with colored and numbered tags (Opalithplättchen), so that each drone was identifiable and color-coded for age. The first drones were marked May 17, and then every other day we marked drones that emerged that day, for a total of five cohorts with 71, 72, 41, 72, and 61 drones per cohort. We introduced all the marked drones into one colony, employing the jail procedure described above. To facilitate observation of the marked drones, we modified the hive entrance with a 10-cm-long passageway covered with clear plastic. We were able to read the numbers of about 95% of the departing or returning drones as they walked the length of this passageway, so number of drones of an active age cohort was not confounded with multiple flights by the same drone.

Each day from May 26 (when the oldest cohort was 10 days old) to June 3, we counted the number of drones of each cohort flying during a 30-min interval in the afternoon (between 1300 and 1600 hr). By June 3, drones of the youngest cohorts were well represented in the records and observations were terminated. Counts were made by lying on top of the colony above the passageway and dictating the numbers and colors of the drone's tags onto an audio tape, which we later transcribed. We also recorded the temperature and weather conditions during each observation.

Analysis. For each EAG run, we calculated minimum voltage of the average antennal depolarization when exposed to pheromone minus that of the average

when exposed to clean air puffs. These data were statistically analyzed as follows: The dose response data were analyzed with ANOVA of the response to QMP with factors of sex of the bee and dose of QMP. The data set comparing drone and worker antennae to IPA and QMP was analyzed with one-way ANOVA of response to QMP with a factor of sex of the bee and ANCOVA of response to QMP with a factor of sex of the bee, and the response to 10^{-1} Qeq QMP as a covariant (to correct for differences in sensitivity from one antennal preparation to another). The time series data were analyzed by ANCOVA's of response to three doses of QMP with age of the drone as a covariant and a factor of day of observation (to factor out day-to-day variation in the sensitivity of the apparatus or the responsiveness of antennae), and ANCOVA to response to IPA with age of the drone and response to 10^{-1} Qeq QMP as covariants, and a factor of day of observation (the latter two to correct overall sensitivity differences bee-to-bee and day-to-day differences). These analyses were done with SuperAnova software (Abacus Systems, Berkeley, California), utilizing type III sums of squares.

RESULTS

Dose-Response Series. Figure 2 shows the response of worker and drone antennae to a range of doses of QMP. Workers were dramatically less sensitive to QMP than drones, and response varied with dose ($F_{1,1} = 120.25$, $P < 0.0001$ for sex, $F_{6,1} = 42.52$, $P < 0.0001$ for dose). Figure 3 compares the response of workers and drones to IPA and QMP. Here again, the drones were more sensitive than the workers to QMP ($F_{1,1} = 30.64$, $P < 0.0001$). Workers were more sensitive to IPA than drones, but not significantly so ($F_{1,1} = 1.57$, $P = 0.22$ for sex; for covariant of response to 10^{-1} Qeq QMP $F_{1,1} = 0.51$, $P = 0.48$).

Drone Age Series. Throughout the experiment, there was great variation in response. Drone response to QMP was highest when drones were young and decreased with age (Figure 4). The greatest responses occurred when the bees were 4 days old, but this was not significantly higher than at 2 days (t test: $t_{10} = 1.80, 1.21, 1.39$; $P = 0.10, 0.25, 0.20$; for 10^{-1}, 10^{-2}, 10^{-3} Qeq QMP, respectively). Analysis of covariance shows there was a significant reduction of response with age for all three doses, and there were significant differences in sensitivity of the apparatus or response of the antennae with day of observation, but these did not account for the trends shown in the EAG response with age (10^{-1} Qeq QMP: age covariant $F_{1,1} = 22.71$, $P < 0.0001$, day factor $F_{9,1} = 1.20$, $P = 0.31$; 10^{-2} Qeq QMP: age covariant $F_{1,1} = 16.62$, $P < 0.0001$, day factor $F_{9,1} = 4.90$, $P < 0.0001$; 10^{-3} Qeq QMP: age covariant $F_{1,1} = 18.70$, $P < 0.0001$, day factor $F_{9,1} = 5.82$, $P < 0.0001$).

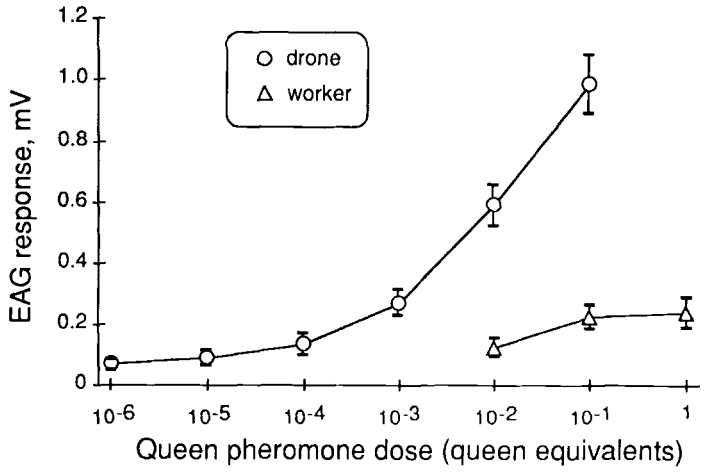


FIG. 2. Mean ± SE of EAG response of drone and worker antennae to a range of doses of a blend of queen pheromone components.

Response to IPA as a function of age of the drone showed a reduction (Figure 5), but this was not significant (covariant of age $F_{1,1} = 0.0003$, $P = 0.98$; covariant of response to 10^{-1} Qeq QMP $F_{1,1} = 7.94$, $P = 0.006$; factor of day $F_{9,1} = 1.12$, $P = 0.36$).

Drone Flight. In the first two days of observations of drone emergence from the hive, we saw only drones >8 days of age that were from the two

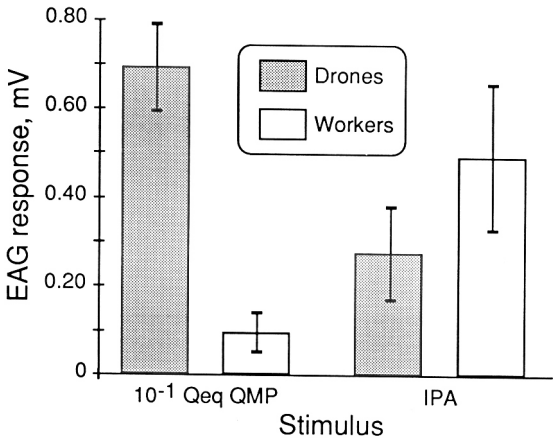


FIG. 3. Response of drone and worker honey bee antennae to one dose of queen pheromones, and to an alarm pheromone component, IPA.

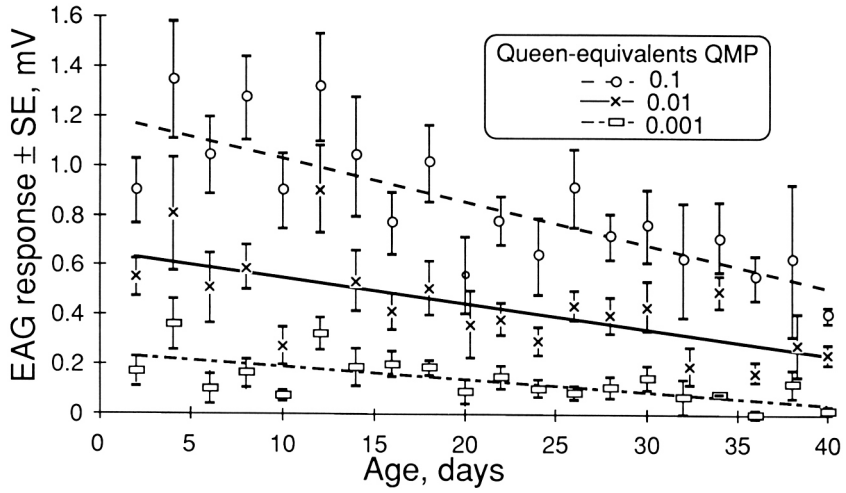


FIG. 4. Change with age of drone honey bee antennal EAG response to queen mandibular pheromones. The lines are regressions of response on age for the three queen pheromone doses.

oldest cohorts (N = 72 and 43 for May 26 and 27, respectively). On May 28, the third (N = 5, 8 days old) and fourth (N = 3, 6 days old) cohorts first were represented, collectively making up 12.5% of all drones observed. The frequency of these two cohorts increased in the next three days, and the oldest two

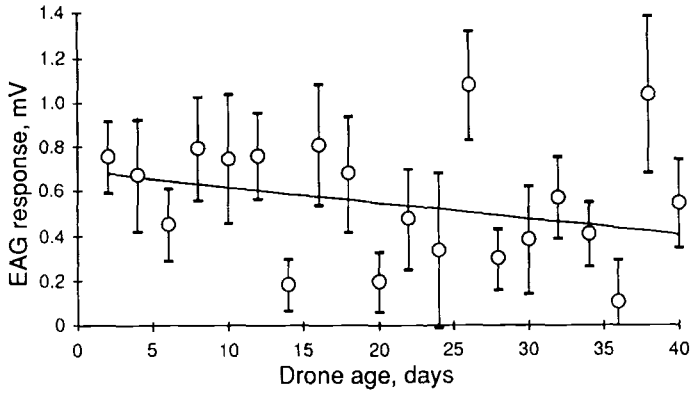


FIG. 5. Effect of age on the EAG response of drone honey bee antennae (mean ± SE) to IPA, an alarm pheromone component. The line is the (nonsignificant) regression fit to the data.

cohorts decreased. One drone from the fifth cohort first emerged when it was 7 days old (1 June), but only seven drones were seen that day and none the next due to inclement weather. Good weather prevailed on June 3, when the youngest cohort was 10 days old and many 10-day-old (N = 59) and 12-day-old (N = 62) drones were seen on this day; the two youngest cohorts made up 70% of the drone force during that observation period.

DISCUSSION

The drone's function in a honey bee colony is solely reproductive. Queens seeking mates will fly to aerial drone congregation areas, where hundreds of drones assemble and where mating takes place. The locations of these drone congregation areas are consistent year to year but their defining features are still unknown (Ruttner, 1966; Loper, 1993). The competition for available queens is immense, and traits with even small effects on a drone's ability to locate queens quickly and orient to them are probably subject to strong selection. Drones have large eyes that provide greater visual acuity, and their antennae are acutely sensitive to queen odor. The drone is much more sensitive to queen odors than is the worker honey bee, as has been previously reported (Adler et al., 1973) and as we found in this study.

Drones undergo sexual and perhaps other physical maturation and begin to fly between 6 and 9 days of age (Ruttner, 1966). The results from our study on flight age agree well with published results from other climates. However, the ontogeny of drones' antennal sensitivity and behavioral response to queen pheromones is strikingly mismatched: drones are most sensitive to these pheromones at the youngest ages, but do not engage in behaviors that involve them until later, when their sensitivity has declined (Figure 4). This indicates that the antennal receptivity of drones to QMP is not related to their sexual maturity and that age-related behavioral differences in drones' response to QMP cannot be explained on the basis of changing input from receptors, but must be controlled by changes in the CNS response to these inputs with maturation.

These patterns can be compared with a considerable body of work on the responses of worker honey bees to pheromones. Decreasing antennal response with age could simply be considered senescence. If so, one might expect a similar pattern in worker honey bees, but this does not occur. In an EAG study on workers (Masson and Arnold, 1984), worker antennal responses to alarm, aggregation and queen recognition pheromones were low one day after emergence, increased by eight days, and then decreased slightly by 15 days, after which they plateaued. Pham-Delégue et al. (1993) showed a similar profile for a queen pheromone blend, as did Allan et al. (1987) and Robinson (1987) for IPA. Thus, in the worker, antennal receptivity is much more correlated with

behavioral use of the pheromones than it is in drones. The CNS differs morphologically between drones and workers (Arnold et al., 1983), and the contrast between drones and worker response ontogeny highlights that maturational effects on both sensory and CNS nervous systems differ as well.

In other well-studied insects, changes in antennal sensitivity are sometimes but not always correlated with behavior changes involving semiochemicals. In moths, the ontogeny of antennal sensitivity has little consistent pattern across species (Ross et al., 1979). In the fall armyworm, *Pseudaletia unipuncta*, the male antennal response is greatest two days after eclosion (Seabrook et al., 1979), which is also when their hairpencils fill with sex pheromone (Grant et al., 1972), yet greatest flight response to female odor in wind tunnels is from 4 to 7 days of age (Turgeon et al., 1983). In the male cabbage looper, *Trichoplusia ni*, EAG response is greatest prior to eclosion and is static from emergence to 4 days of age, in contrast with behavioral response, in which older males respond more strongly to low pheromone concentrations than younger males (Payne et al., 1970). In several Diptera, studies have shown changes in antennal receptor sensitivity based on altered physiological status, such as the shift from host finding to oviposition-site location after a blood meal (Blaney et al., 1986).

A striking result from this study was that drone antennae have the capacity to respond to IPA, an alarm pheromone component (Figures 3 and 5). This electrophysiological response was previously undescribed. Furthermore, behavioral responses of drones to IPA have never been reported. In light of the results here, these should be examined. Drones might respond by moving into the colony to avoid predation. This could also facilitate the movement of soldier bees outside to defend the hive. Alternatively, drones may show no behavioral response to IPA, and antennal response to IPA may have been an aspect of more general antennal chemosensory activity. This latter interpretation might be supported by Lacher's (1964) statement, based on comparison of a variety of drone and worker sensillae, that there was little difference between the two sexes in their sensory ability to various odors. Furthermore, Ruttner (1966) reported that drones responded to floral odors, although much less so than workers, and the biological significance of this capability is not obvious since drones are not known to feed outside of the hive. However, if it is just a general chemosensory function, the sensitivity of the drone's response to IPA is somewhat surprising, since the antennal response of drones is nearly as great as that of workers, which are presumably especially adapted to sense alarm pheromones.

Our study compared the response of drones to a blend of queen pheromone components and a single alarm pheromone component. The queen pheromone blend has been shown to be important in workers' behavioral response (Slessor et al. 1988), but drones can be attracted by just the major component (9-oxo-decenoic acid) and may or may not respond differently to the blend. With IPA,

workers respond behaviorally to the major pheromone component, and nothing was previously known about the response of drones to these pheromones. These considerations probably do not affect the conclusions of this study, but do remain as complicating issues.

The computer-controlled pheromone-puff delivery system described here, coupled with the signal averaging made possible by the precise timing of delivery, provides a very effective means of analyzing even very noisy antennal response signals. This is illustrated by the reduction of the baseline random noise and the averaging out of the potentially confounding spontaneous depolarization in the trace in Figure 1. For the most part, our antennal preparations had moderate noise, relatively little spontaneous activity, and depolarizations that were large compared to noise, so the averaging reduced variation somewhat, but was not essential to the analysis. However, this system could be especially valuable for describing reproducible but low-magnitude responses that would be hidden by noise in EAG recordings.

Acknowledgments—We would like to thank Ann Strawn (UC Davis) for copying and sending to us the difficult-to-obtain Jaycox thesis. Also, Jocelyn Millar, Brandy Darmody, and Alexinna Johns (all from UC Riverside) provided technical assistance.

REFERENCES

ADLER, V. E., DOOLITTLE, R. E., SHIMANUKI, H., and JACOBSON, M. 1973. Electrophysiological screening of queen substance and analogs for attraction to drone, queen and worker honey bees. *J. Econ. Entomol.* 66:33–36.

ALLAN, S. A., SLESSOR, K. N., WINSTON, M. L., and KING, G. G. S. 1987. The influence of age and task specialization on the production and perception of honey bee pheromones. *J. Insect Physiol.* 33:917–922.

ARNOLD, G., MASSON, C., and BUDHARUGSA, S. 1983. Organisation spatiale du systéme nerveux antennaire de l'abeille étudiée au moyen d'une technique de marquage aux ions cobalt. *Apidologie* 14:127–135 (English summary).

BJOSTADT, L. B., and ROELOFS, W. L. 1980. An inexpensive electronic device for measuring electroantennogram responses to sex pheromones with a voltmeter. *Physiol. Entomol.* 5:309–314.

BLANEY, W. M., SCHOONHOVEN, L. M., and SIMMONDS, M. S. J. 1986. Sensitivity variations in insect chemoreceptors; A review. *Experientia* 42:13–19.

GRANT, G. G., BRADY, U. E., and BRAND, J. M. 1972. Male armyworm scent brush secretion: Identification and electroantennogram study of major components. *Ann. Entomol. Soc. Am.* 65:1224–1227.

HENNING, J. A., and TEUBER, L. R. 1992. Combined gas chromatography-electroantennogram characterization of alfalfa floral volatiles recognized by honey bees (Hymenoptera: Apidae). *J. Econ. Entomol.* 85:226–232.

HOWELL, D. E., and USINGER, R. L. 1933. Observations on the flight and length of life of drone bees. *Ann. Entomol. Soc. Am.* 26:239–246.

JAYCOX, E. R. 1956. *Factors affecting the attainment of sexual maturity by the drone honeybee* (*Apis mellifera* L.) PhD dissertation. University of California, Davis, California.

KAISSLING, K-E., and RENNER, M. 1968. Antennale Rezeptoren für Queen Substance und Sterzelduft bei der Honigbeine. *Z. Vergl. Physiol.* 59:357–361.

LACHER, V. 1964. Elektrophysiologische Untersuchungen an einzelnen Rezeptoren für Geruch, Kohlendioxyd, Luftfeuchtigkeit und Temperatur auf den Antennen der Arbeitsbiene und der Drohne (*Apis mellifera* L.) *Z. Vergl. Physiol.* 48:587–623.

LOPER, G. M. 1993. What do we really know about drone flight behavior? *Bee World* 73:198–203.

MASSON, C., and ARNOLD, G. 1984. Ontogeny, maturation and plasticity of the olfactory system in the workerbee. *J. Insect Physiol.* 30:7–14.

MORSE, R. A., STRANG, G. E., and NOWAKOWSKI, J. 1967. Fall death rates of drone honey bees. *J. Econ. Entomol.* 60:1198–1202.

OERTEL, E. 1956. Observations on the flight of drone honey bees. *Ann. Entomol. Soc. Am.* 49:497–500.

PAYNE, T. L., SHOREY, H. H., and GASTON, L. K. 1970. Sex pheromones of noctuid moths: Factors influencing antennal responsiveness in males of *Trichoplusia ni*. *J. Insect Physiol.* 16:1043–1055.

PATTE, F. M., ETCHETO, M., MARFAING, P., and LAFFORT, P. 1989. Electroantennogram stimulus-response curves for 59 odourants in the honey bee, *Apis mellifera*. *J. Insect Physiol.* 35:667–675.

PHAM-DELÈGUE, M. H., MASSON, C., ETIEVANT, P., and AZAR, M. 1986. Selective olfactory choices of the honeybee among sunflower aromas: A study by combined olfactory conditioning and chemical analysis. *J. Chem. Ecol.* 12:781–793.

PHAM-DELÈGUE, M. H., ETIEVANT, P., GUICHARD, E., and MASSON, C. 1989. Sunflower volatiles involved in honey bee discrimination among genotypes and flowering stages. *J. Chem. Ecol.* 15:329–343.

PHAM-DELÈGUE, M. H., ETIEVANT, P., GUICHARD, E., MARILLEAU, R., DOUAULT, P., CHAUFFAILLE, J., and MASSON, C. 1990. Chemicals involved in honey bee–sunflower relationship. *J. Chem. Ecol.* 16:3053–3065.

PHAM-DELÈGUE, M. H., TROUILLER, J., CAILLAUD, C. M., ROGER, R., and MASSON, C. 1993. Effect of queen pheromone on worker bees of different ages: Behavioural and electrophysiological responses. *Apidologie* 24:267–281.

ROBINSON, G. E. 1987. Modulation of alarm pheromone perception in the honey bee: Evidence for division of labor based on hormonally regulated response thresholds. *J. Comp. Physiol.* 160:613–619.

ROSS, R. J., PALANISWAMY, P., and SEABROOK, W. D. 1979. Electroantennograms from spruce budworm moths (*Choristoneura fumiferana*) (Lepidoptera: Tortricidae) of different ages and for various pheromone concentrations. *Can. Entomol.* 111:807–816.

RUTTNER, F. 1966. The life and flight activity of drones. *Bee World* 47:93–100.

RUTTNER, H., and RUTTNER, F. 1972. Untersuchungen über die Flugaktivität und das Paarungsverhalten der Drohnen. V. Drohnensammelplätze und Paarungsdistanz. *Apidologie* 3:203–232.

SEABROOK, W. D., HIRAI, K., SHOREY, H. H., and GASTON, L. K. 1979. Maturation and senescence of an insect chemosensory response. *J. Chem. Ecol.* 5:587–594.

SLESSOR, K. N., KAMINSKI, L. A., KING, G. G. S., BORDEN, J. H., and WINSTON, M. L. 1988. Semiochemical basis of the retinue response to queen honey bees. *Nature* 332:354–356.

THIERY, D., BLUET, J. M., PHAM-DELÈGUE, M. H., ETIEVANT, P., and MASSON, C. 1990. Sunflower aroma detection by the honey bee study by coupling gas chromatography and electroantennography. *J. Chem. Ecol.* 16:701–711.

TURGEON, J. J., MCNEIL, J. N., and ROELOFS, W. L. 1983. Responsiveness of *Pseudaletia unipuncta* males to the female sex pheromone. *Physiol. Entomol.* 8:339–344.

VISSCHER, P. K., VETTER, R. S. and ROBINSON, G. E. 1995. Alarm pheromone perception in honey bees is decreased by smoke (Hymenoptera: Apidae). *J. Insect Behav.* 8:11–18.

WINSTON, M. L. 1987. *The Biology of the Honey Bee.* Harvard University Press, Cambridge, Massachusetts.

WINSTON, M. L., and SLESSOR, K. N. 1993. Applications of queen honey bee mandibular perhomone for beekeeping and crop pollination. *Bee World* 73:111–128.

WITHERALL, P. C. 1971. Duration of flight and of interflight time of drone honey bees, *Apis mellifera*. *Ann. Entomol. Soc. Am.* 64:609–612.

WOYKE, J., and JASINSKI, Z. 1978. Influence of age of drone on the results of instrumental insemination of honeybee queens. *Apidologie* 9:203–212.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

SEX PHEROMONE OF CABBAGE APHID *Brevicoryne brassicae*: IDENTIFICATION AND FIELD TRAPPING OF MALE APHIDS AND PARASITOIDS

B. J. GABRYŚ,[1] H. J. GADOMSKI,[1] Z. KLUKOWSKI,[1] J. A. PICKETT,[2,*] G. T. SOBOTA,[1] L. J. WADHAMS,[2] and C. M. WOODCOCK[2]

[1]*Department of Agricultural Entomology, Agricultural University, Cybulskiego 32, 50-205 Wrocław, Poland*

[2]*IACR-Rothamsted Harpenden, Herts., AL5 2JQ, UK*

(Received May 31, 1995; accepted March 17, 1997)

Abstract—The sex pheromone of the cabbage aphid, *Brevicoryne brassicae*, is shown by GC and GC-MS analysis of pheromone entrained from sexual females, and by electrophysiological studies on single cell preparations from male antennae, to comprise (4a*S*,7*S*,7a*R*)-nepetalactone. The compound proved to be attractive in a laboratory bioassay and release of the pheromone from glass vials placed above water traps in crops of autumn brassicaceous crops increased the catch of males of this species. The specialist parasitoid of *B. brassicae*, *Diaeretiella rapae*, and the more general aphid parasitoid *Praon volucre* were found in significantly larger numbers in pheromone traps as compared to the controls, under certain conditions.

Key Words—Aphid, *Brevicoryne brassicae*, Homoptera, pheromone, attractant, *Diaeretiella rapae*, *Praon volucre*, Hymenoptera, isoprenoid, nepetalactone.

INTRODUCTION

Brevicoryne brassicae (L.) (Homoptera: Aphididae), the cabbage aphid, has a host range restricted to plants in the family Brassicaceae (= Cruciferae), which includes such important crops as oilseed rape, *Brassica napus* L., mustard, *Sinapis* (= *Brassica*) *alba* (L.) and *nigra* (L.) and cabbage vegetables, *Brassica oleracea* L. Under suitable weather conditions in central Europe, *B. brassicae*

*To whom correspondence should be addressed.

0098-0331/97/0700-1881$12.50/0 © 1997 Plenum Publishing Corporation

can build up large colonies covering whole plants, and up to 80% yield of oilseed rape may be lost directly due to feeding by the aphid (Kelm, 1983). In cabbages, *B. brassicae* attacks newly emerging leaves, thereby obstructing head formation. At this stage, intensive pesticide treatment is usually employed. *B. brassicae* does not host-alternate, developing both holocyclic and anholocyclic populations on plants in the Brassicaceae (Blackman and Eastop, 1985). The occurrence of a sexual phase in the life cycle is determined by an established climate pattern of long and severe winters (Blackman, 1975). Although, in Britain, *B. brassicae* is predominantly anholocyclic, in Poland the occurrence of sexual morphs is essential for production of fertilized eggs for survival during winter. *B. brassicae* lays its eggs on head cabbage and Brussels sprouts and on stems of young winter oilseed rape, making these the most important overwintering sites for this species in Poland (Gadmoski, 1992). Males of *B. brassicae* are alate and locate mates by means of a sex pheromone released from glands on the hind tibiae of the apterous sexual females (oviparae). For a number of aphid species, e.g., *Megoura viciae* Buckt., *Aphis fabae* Scop., *Acyrthosiphon pisum* (Harris), *Myzus persicae* (Sulz.), and *Schizaphis graminum* (Rond.), the sex pheromones were identified as comprising one or both of the cyclopentanoid terpenes (4a*S*,7*S*,7a*R*)-nepetalactone and (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (Dawson et al., 1987, 1990). Field studies have shown that the nepetalactone is also attractive to several aphid parasitoids, particularly in the genus *Praon* (Hardie et al., 1994a). Aphid sex pheromones are now being considered for practical application in monitoring aphid populations at their overwintering sites or attracting males to infective sources of aphid-specific fungal pathogens, and in attracting aphid parasitoids and stimulating their searching behaviour (Powell et al., 1993; Pickett et al., 1994).

In the present study, the sex pheromone of *B. brassicae* is identified and its biological activity in the laboratory and in the field investigated.

METHODS AND MATERIALS

Insects. Virginoparous *B. brassicae*, obtained from cabbage in Poland, were maintained on Chinese cabbage (*Brassica campestris* L. var. Chinensis) at 20°C with a 16L:8D light regime. Sexual morphs were obtained by transferring virginoparae from the stock culture to 16°C with an 11L:13D photoperiod.

Pheromone Entrainment. One hundred oviparae of *B. brassicae* on Chinese cabbage were placed in a glass culture vessel (Quickfit FV range, 1 liter) for five days. Air, dried and purified by passage through an activated 5 Å molecular sieve and charcoal, was drawn at 1 liter/min through the vessel. Volatiles were entrained onto Porapak Q, which had been purified by washing with diethyl

ether (5 ml) and heating for 12 hr at 150°C under a stream of nitrogen. Collected volatiles were desorbed from the Porapak by elution with freshly distilled diethyl ether. The resulting extract was concentrated under a stream of nitrogen and stored in sealed glass ampoules at −20°C.

Behavioural Assay. The bioassay was done in a simple choice chamber as described in Dawson et al. (1987), consisting of a 15-cm plastic Petri dish, in the base of which were two 25-mm holes covered with a layer of muslin. Under these holes were placed either the treatment, (4a*S*,7*S*,7a*R*)-nepetalactone at 10^{-7} g, applied to a filter paper square, or an empty pot as a control. Ten male *B. brassicae* were placed in the dish and numbers of aphids positioned over each hole were recorded once a minute for 15 min. The experiment was replicated six times and the cumulative counts were analyzed by Student's *t* test. During the test, the dish was placed in a light-proof box, illuminated from above. The box was aired with an electric fan between tests.

Chemicals. Authentic (4a*S*,7*S*,7a*R*)-nepetalactone was isolated from field-grown catmint, *Nepeta cataria* L. (Lamiaceae = Labiatae), by high-performance liquid chromatography (50 × 1 cm column, 10 μm silica, Partisil Magnum 9 10/50, 5% v/v ether–hexane, monitoring at 208 nm) (Dawson et al., 1987). The compound was purified by liquid chromatography and diluted in hexane for GC and behavioral and electrophysiological studies.

Gas Chromatography (GC). Air entrainment volatiles were separated on a gas chromatograph (AI 93) equipped with a cold on-column injector, a flame ionization detector (FID) and a bonded-phase fused silica capillary column (50 m × 0.32 mm ID, HP-1). The oven temperature was maintained at 40°C for 1 min and then programmed at 5°/min to 100°C and then at 10°/min to 250°C. This system gives good resolution of all diastereomeric nepetalactones.

Coupled Gas Chromatography–Mass Spectrometry (GC-MS). A capillary GC column (50 m × 0.32 mm ID, HP-1) was directly coupled to the MS with an integrated data system (70–250 VG Analytical). Ionization was by electron impact at 70eV and 230°C. The GC was maintained at 30°C for 5 min and then programmed at 5°/min to 180°C. Tentative identifications by GC-MS were confirmed by comparison with authentic samples and then by peak enhancement on GC (Pickett, 1990).

Single Cell Recording (SCR). Electrophysiological recordings from olfactory cells associated with the secondary rhinaria of *B. brassicae* males were obtained using tungsten microelectrodes. The indifferent electrode was positioned in the first or second antennal segment, and the recording electrode was then brought into contact with the rhinaria on the third segment until impulses were recorded. Permanent copies of the action potentials generated by the olfactory cells were obtained by standard methods (Wadhams et al., 1982). The stimulus was delivered into a purified airstream (1 liter/min) flowing continu-

ously over the preparation. The delivery system, employing a filter paper in a disposable Pasteur pipet cartridge, has been described previously (Wadhams et al., 1982). The nepetalactone in hexane (10 μl) was applied to the filter paper and, after evaporation of the solvent, was presented to the preparation. The impulse frequency was determined as the number of impulses elicited during the first 1 sec after stimulus application.

Field Tests. Field experiments were carried out in Poland in 1994 on head cabbage, *Brassica oleracea* L. var. *capitata* f. *alba*, and winter oilseed rape, *B. napus* L. var. *napus* f. *biennis*, at two localities, Pawlowice and Zybiszow near Wrocław (Lower Silesia). The nepetalactone (10 mg) in diethyl ether (50 μl) was placed in borosilicate vials (Chromacol) fitted with polyethylene caps with 1-mm-diameter holes. These lures offered a direct comparison with previous results (e.g., Hardie et al., 1992). Under laboratory conditions at 20°C, the lures were shown to release 200 μg/day over a five-week period, a level comparable to that shown to be released by the related aphid *M. viciae* (Hardie et al., 1990). In the field, lures were replaced after one month. Diethyl ether (50 μl) was used as the control. Vials were suspended 5 cm above water traps comprising clear plastic petri dishes (14 cm diam.), which were positioned 10 cm above the vegetation and adjusted as the plants grew. The traps were placed 2 m apart in a row along the center of the field, the pheromone traps being situated 25 m away from the controls. There were 10 pheromone and 10 control traps per experiment. The experiment started on September 20 and ended on November 20. All traps were emptied every three to four days and the insect material from each trap was identified separately. Statistical analysis was carried out using a generalized linear model (McCullagh and Nelder, 1983) according to Poisson distribution with a link to logarithm function (Genstat for Windows 3.2). The level of significance was determined by the *F* test.

RESULTS

Pheromone Identification. GC analysis of the volatiles entrained from sexual females of *B. brassicae* on Chinese cabbage showed one major peak (Figure 1). GC-MS analysis gave a spectrum for this peak identical to that of (4a*S*,7*S*,7a*R*)-nepetalactone, identified as a sex pheromone component for a number of aphid species, mass spectral details having been published previously (Dawson et al., 1987, 1990). Identification was confirmed by peak enhancement on coinjection with the specific nepetalactone isomer authenticated by nuclear magnetic resonance studies (Dawson et al., 1989). Single ion monitoring at *m/z* 166 (M^+) showed a trace of another isomer of the main nepetalactone, probably *trans* fused at the ring junction from the retention time (Dawson et al., 1989). No nepetalactols could be detected by monitoring at *m/z* 168 (M^+),

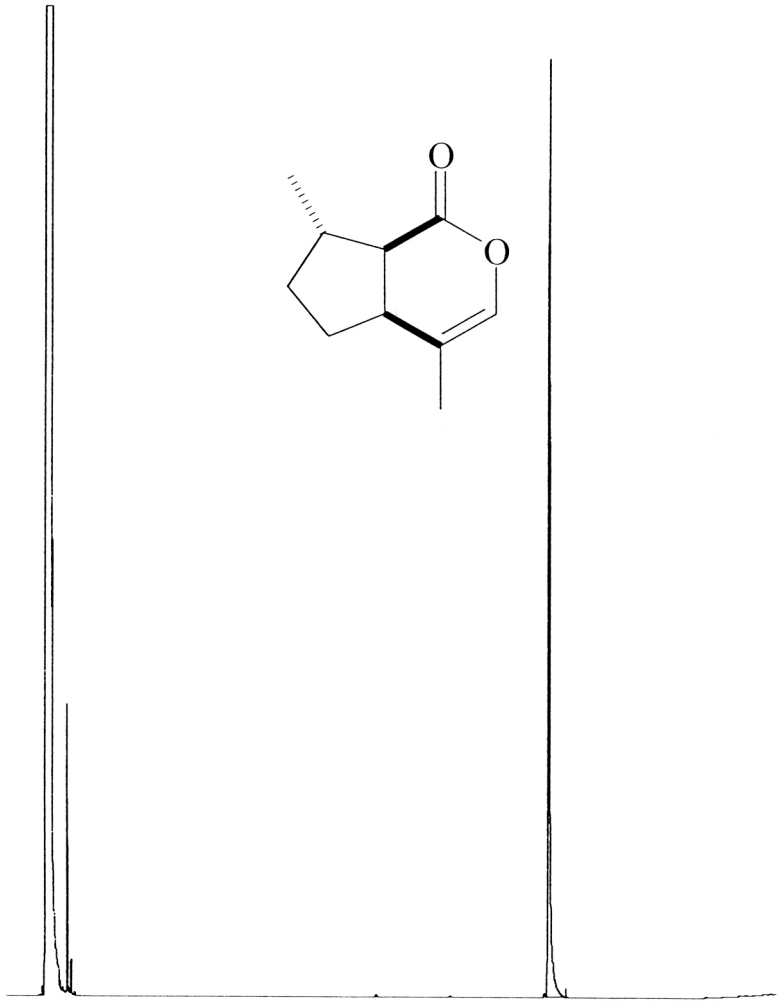


FIG. 1. GC of air entrainment sample of oviparous *Brevicoryne brassicae* on Chinese cabbage. Main peak is from (4a*S*,7*S*,7a*R*)-nepetalactone, structure given (elution time 24.41 min).

although these compounds are often associated with the nepetalactone in other aphid sex pheromones (Dawson et al., 1990). A trace of citronellol (enantiomer not determined) was also identified, but as with other species where this compound occurs in association with the sex pheromone cyclopentanoids (Dawson et al., 1996), there was no comparable electrophysiological activity. For the

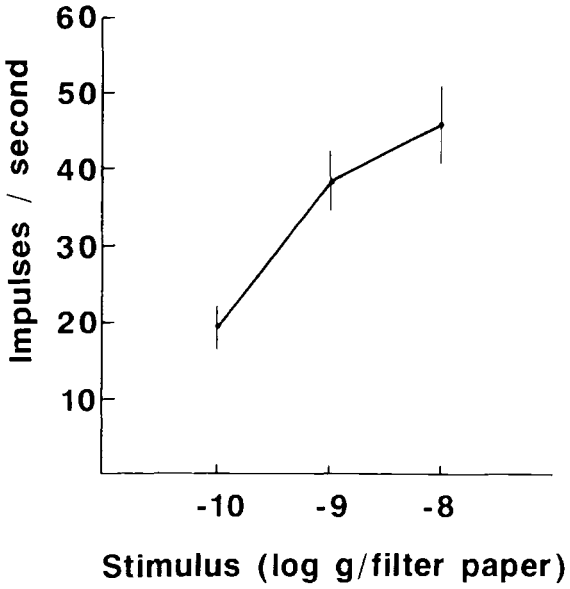


FIG. 2. Dose-response curve of olfactory cells in the secondary rhinaria of *B. brassicae* males (N = 8) to (4a*S*,7*S*,7a*R*)-nepetalactone (bars = SE).

purified nepetalactone, the dose–response curve obtained from olfactory cells in the secondary rhinaria of *B. brassicae* males (Figure 2) showed a similar level of activity to the pheromonal components of other aphids (Dawson et al., 1990). In the behavioral assay, the compound proved to be significantly attractive (treatment 25.0 ± 3.3; control 1.0 ± 0.5; $P < 0.001$).

Field Tests. In both crops and localities, significantly more male *B. brassicae* were caught in pheromone-releasing traps than in the controls (Table 1). Low numbers of *B. brassicae* females (Table 1) and individuals of approximately 30 other aphid species were also found, but there were no significant differences between the pheromone and control traps. However, large numbers of the aphid parasitoid *Diaeretiella rapae* (McIntosh) (Hymenoptera: Aphidiidae) were caught, occurring in significantly higher numbers in pheromone traps in both crops at Pawlowice. These catches comprised both male and female *D. rapae*. Of other Aphidiids, *Praon volucre* (Haliday) was found only in the pheromone traps (Table 1).

DISCUSSION

The results of this study demonstrate that males of *B. brassicae* are strongly attracted to (4a*S*,7*S*,7a*R*)-nepetalactone in the laboratory and to traps releasing

TABLE 1. *Brevicoryne brassicae* AND APHID PARASITOIDS TRAPPED DURING FIELD TRAILS IN POLAND 1994[a]

Species	Oilseed rape, Pawlowice		Cabbage, Pawlowice		Oilseed rape, Zybiszow	
	P	C	P	C	P	C
Brevicoryne brassicae						
Males	18*	5	26**	1	17*	3
Females	0	2	4	2	1	0
Diaeretiella rapae	65**	32	58**	14	140 NS	146
Praon volucre	8	0	6	0	3	0
Aphidius sp.	2	1	0	1	1	1
Toxares sp.	0	0	0	0	1	0
Trioxyx sp.	0	1	0	0	0	0

[a] *F* test: significant differences at $*P < 0.05$ and $**P < 0.01$; NS: not significant. P = pheromone traps, C = control traps.

this compound in the field. The nepetalactone was identified as the major component of the volatiles from oviparous females feeding on Chinese cabbage, previous entrainment experiments having confirmed that brassicaceous species do not produce these cyclopentanoids (Wadhams, unpublished); other trace components showed no detectable neurophysiological role at the levels present. The citronellol may constitute a biochemical precursor (Dawson et al., 1996). (4a*S*,7*S*,7a*R*)-Nepetalactone also comprises the sex pheromone for other aphids, e.g. *Sitobion avenae* (Fab.) (Lilley et al., 1995) and *S. fragariae* (Walk.) (Hardie et al., 1992), but these species were not caught significantly in this trial. Additional factors determining species specificity remain unknown (Guldemond et al., 1993) but may, for some species, involve primary (winter) host components, as has been shown for the damson-hop aphid *Phorodon humuli* (Schrank) (Campbell et al., 1990) and the bird-cherry-oat aphid, *Rhopalosiphum padi* (L.) (Hardie et al., 1994b). Indeed, the brassicaceous ecosystem, inevitably containing a background level of organic isothiocyanates, may cause avoidance by insects unable to feed on these plants, as is the case with the black bean aphid, *A. fabae* (Nottingham et al., 1991). Overwintering eggs are laid by the oviparae of *B. brassicae* on a variety of brassicaceous crops, so it seems likely that the female sex pheromone is the most important cue used by males in mate location and has priority over other cues such as specific plant odors. However, the role of such compounds in habitat location during long-range migratory

flights cannot be excluded (Pickett et al., 1992), especially as *B. brassicae* does not use wild-growing weeds for overwintering (Gadomski, unpublished data).

D. rapae is a specialized parasitoid closely associated with its host, *B. brassicae*, and isothiocyanates, which are volatiles characteristic of the Brassicaceae, are used to locate the habitat and also to find the aphid on the plant (Read et al., 1970; Pickett, et al., 1991). Nonetheless, there is evidence that *D. rapae* readily parasitizes other aphid species on Brassicaceae, e.g., *M. persicae* (Paetzold and Vater, 1967; Chua, 1977; Gabryś and Sobota, 1994), as well as aphids specializing on crops in other families (Stary, 1966). Over 25% of parasitoids reared from cereal aphid mummies on wheat were found to be *D. rapae* when the adjoining crop was yellow mustard, *S. alba*; when it was not, the percentage of *D. rapae* mummies fell to 0–0.7 (Sobota, 1992; Sobota and Gabryś, unpublished data). Maximum parasitization by *D. rapae* in a range of brassicaceous crops coincides with the maximum of the cabbage aphid population (Nawrocka, 1972; Chua, 1977; Gadomski, 1994). However, *D. rapae* overwinters in a prepupal stage inside the aphid mummy (Stary, 1966), and in autumn, when *B. brassicae* is dispersed in small colonies throughout the field, *D. rapae* may use the *B. brassicae* sex pheromone as an additional cue for finding the host. The occurrence of *P. volucre* in the pheromone traps confirms the finding (Hardie et al., 1994a) that the nepetalactone also acts as an attractant for this species. *P. volucre* parasitizes a number of aphid groups including *B. brassicae* and, because of its wide host range, has been considered to be of economic importance by Stary (1966) and may now be more readily exploited by means of aphid sex pheromone components.

Acknowledgments—IACR receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom. Part of this work was supported by the United Kingdom Ministry of Agriculture, Fisheries and Food (MAFF). *B. brassicae* was imported under MAFF Licence no. PHF 1307C/1378/25.

REFERENCES

BLACKMAN, R. L. 1975. Aphids. Ginn & Co., Aylesbury, 175 pp.

BLACKMAN, R. L., and EASTOP, V. F. 1985. Aphids on the World's Crops: An Identification Guide. John Wiley & Sons, Chichester, 467 pp.

CAMPBELL, C. A. M., DAWSON, G. W., GRIFFITHS, D. C., PETTERSSON, J., PICKETT, J. A., WADHAMS L. J., and WOODCOCK, C. M. 1990. Sex attractant pheromone of damson-hop aphid *Phorodon humuli* (Homoptera, Aphididae). *J. Chem. Ecol.* 16:3455–3465.

CHUA, T. H. 1977. Population studies of *Brevicoryne brassicae* (L.), its parasites and hyperparasites in England. *Res. Popul. Ecol.* 19:125–139.

DAWSON, G. W., GRIFFITHS, D. C., JANES, N. F., MUDD, A., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1987. Identification of an aphid sex pheromone. *Nature* 325:614–616.

DAWSON, G. W., JANES, N. R., MUDD, A., PICKETT, J. A., SLAWIN, A. M. Z., WADHAMS, L. J., and WILLIAMS, D. J. 1989. The aphid sex pheromone. *Pure Appl. Chem.* 61:555–558.

DAWSON, G. W., GRIFFITHS, D. C., MERRITT, L. A., MUDD, A., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1990. Aphid semiochemicals—a review, and recent advances on the sex pheromone. *J. Chem. Ecol.* 16:3019–3030.

DAWSON, G. W., PICKETT, J. A., and SMILEY, D. W. M. 1996. The aphid sex pheromone cyclopentanoids: Synthesis in the elucidation of structure and biosynthetic pathways. *Bioorg. Med. Chem.* 4:351–361.

GABRYŚ, B., and SOBOTA, G. 1994. Species spectrum, relative abundance and population dynamics of parasitoids and hyperparasitoids of *Brevicoryne brassicae* (L.) and *Myzus persicae* (Sulz.) (Homoptera, Aphididae) on white mustard (*Sinapis alba* L.). Aphids and other homopterous insects. *PAS, Skierniewice* 4:33–39.

GADOMSKI, H. 1992. Oviposition of *Brevicoryne brassicae* (L.) (Homoptera: Aphididae) on cruciferous plants. Aphids and other homopterous insects. *PAS, Warsaw* 3:33–38.

GADOMSKI, H. 1994. The effectiveness of *Diaeretiella rapae* (McIntosh) in the reduction of the cabbage aphid *Brevicoryne brassicae* L. on cruciferous crops. Aphids and other homopterous insects. *PAS, Skierniewice* 4:41–44.

GULDEMOND, J. A., DIXON, A. F. G., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1993. Specificity of sex pheromones, the role of host plant odour in the olfactory attraction of males, and mate recognition in the aphid *Cryptomyzus*. *Physiol. Entomol* 18:137–143.

HARDIE, J., HOLYOAK, M., NICHOLAS, J., NOTTINGHAM, S. F., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1990. Aphid sex pheromone components: Age-dependent release by females and species-specific male response. *Chemoecology* 1:63–68.

HARDIE, J., NOTTINGHAM, S. F., DAWSON, G. W., HARRINGTON, R., PICKETT, J. A., and WADHAMS, L. J. 1992. Attraction of field-flying aphid males to synthetic sex pheromone. *Chemoecology* 3:113–117.

HARDIE, J., HICK, A. J., HOLLER, C., MANN, J., MERRITT, L. A., NOTTINGHAM, S. F., POWELL, W., WADHAMS, L. J., WITTHINRICH, J., and WRIGHT, A. F. 1994a. The responses of *Praon* spp. parasitoids to aphid sex pheromone components in the field. *Entomol. Exp. Appl.* 71:95–99.

HARDIE, J., STORER, J. R., NOTTINGHAM, S. F., HARRINGTON, J. MERRITT, L. A., WADHAMS, L. J., and WOOD, D. 1994b. The interaction of sex pheromone and plant volatiles for field attraction of male bird cherry aphid, *Rhopalosiphum padi*. *Brighton Crop Protection Conference—Pests and Diseases*. BCPC Publications, Thornton Heath. pp. 1223–1230.

KELM, M. 1983. O zagrozeniu plonow rzepaku ozimego i warzyw krzyzowych przez mszyce kapusciana (*Brevicoryne brassicae*). *Ochr. Rosl.* 9:16–18.

LILLEY, R., HARDIE, J., MERRITT, L. A., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1995. The sex pheromone of the grain aphid, *Sitobion avenae* (Fab.) (Homoptera, Aphididae). *Chemoecology* 5/6(1):43–46.

MCCULLAGH, P., and NELDER, J. A. 1983. Generalized Linear Models. Chapman and Hall, New York.

NAWROCKA, B. 1972. Metoda integracji walki biologicznej z chemiczna przeciwko mszyzy kapuscianej [*Brevicoryne brassicae* (L.)] na kapuscie glowiastej bialej. *Biul. IOR* 52:379–387.

NOTTINGHAM, S. F., HARDIE, J., DAWSON, G. W., HICK, A. J., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1991. Behavioral and electrophysiological responses of aphids to host and non-host plant volatiles. *J. Chem. Ecol.* 17:1231–1242.

PAETZOLD, D., and VATER, G. 1967. Populationsdynamische Untersuchungen an den Parasiten und Hyperparasiten von *Brevicoryne brassicae* (L.) (Homoptera, Aphididae). *Acta Entomol. Bohemoslov.* 64:83–90.

PICKETT, J. A. 1990. Gas chromatography–mass spectrometry in insect pheromone identification: Three extreme case histories, pp. 299–309, *in* A. R. McCaffery and I. D. Wilson (eds.). Chromatography and Isolation of Insect Hormones and Pheromones. Plenum Press, New York.

PICKETT, J. A., POWELL, W., WADHAMS, L. J., WOODCOCK, C. M. and WRIGHT, A. F. 1991.

Biochemical interactions between plant-herbivore-parasitoid. 4th European Workshop on Insect Parasitoids, Perugia, April 3–5, 1991. *Redia* 24:1–14.

PICKETT, J. A., WADHAMS, L. J., WOODCOCK, C. M., and HARDIE, J. 1992. The chemical ecology of aphids. *Annu. Rev. Entomol.* 37:67–90.

PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1994. Attempts to control aphid pests by integrated use of semiochemicals. Proceedings, 1994 Brighton Crop Protection Conference—Pests and Diseases. BCPC Publications, Thornton Heath. pp. 1239–1246.

POWELL, W., HARDIE, J., HICK, A. J., HÖLLER, C., MANN, J., MERRITT, L., NOTTINGHAM, S. F., WADHAMS, L. J., WITTHINRICH, J., and WRIGHT, A. F. 1993. Responses of the parasitoid *Praon volucre* (Hymenoptera: Braconidae) to aphid sex pheromone lures in cereal fields in autumn: Implications for parasitoid manipulation. *Eur. J. Entomol.* 90:435–438.

READ, D. P., FEENY, P. P., and ROOT, R. B. 1970. Habitat selection by the aphid parasite *Diaeretiella rapae* (Hymenoptera:Braconidae) and hyperparasite *Charips brassicae* (Hymenoptera: Cynipidae). *Can. Entomol.* 102:1567–1578.

SOBOTA, G. 1992. Parasitoids of cereal aphids on winter wheat in the vicinity of Wrocław, Poland. Aphids and other homopterous insects. *PAS, Warsaw* 3:83–88.

STARY, P. 1966. Aphid parasites of Czechoslovakia. A review of the Czechoslovak Aphidiidae (Hymenoptera). Academia, Prague, 237 pp.

WADHAMS, L. J., ANGST, M. E. and BLIGHT, M. M. 1982. Responses of the olfactory receptors of *Scolytus scolytus* (F.) (Coleoptera: Scolytidae) to the stereoisometers of 4-methyl-3-heptanol. *J. Chem. Ecol.* 8:477–492.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

VOLATILE, POTENTIAL ATTRACTANTS FROM RIPE COFFEE FRUIT FOR FEMALE MEDITERRANEAN FRUIT FLY

J. D. WARTHEN,[1,*] C.-J. LEE,[1] E. B. JANG,[2] D. R. LANCE,[3] and D. O. McINNIS[4]

[1]*USDA, ARS, Insect Chemical Ecology Laboratory, BARC-W Beltsville, Maryland 20705*

USDA, ARS, Tropical Fruit & Vegetable Research Laboratory
[2]*Hilo, Hawaii 96720*
[4]*Honolulu, Hawaii 96804*

[3]*USDA, APHIS, PPQ, Hawaii Plant Protection Center Waimanalo, Hawaii 96795*

(Received September 11, 1996; accepted March 17, 1997)

Abstract—Twenty-eight volatile compounds from freshly crushed, ripe, dark red coffee fruit, *Coffea arabica*, were identified by dynamic headspace analysis techniques. Identifications were made on the basis of a comparison of Kovats indices and GC-MS spectra for unknowns and authentic samples. Of the compounds identified, 10 were alcohols, nine were aldehydes, five were ketones, and four were monoterpenes. The five most abundant volatiles in decreasing order were hexanal (21%), 2-(*E*)-hexenal (11%), 3-methyl-1-butanol (9.0%), 3-methyl-1-butanal (8.5%), and 1-hexanol (8.4%). The five least abundant volatiles of the 28 identified, in increasing order, were decanal (0.19%), methyl hexanoate (0.33%), pulegone (0.44%), α-isomenthone (0.45%), and 2-nonanone (0.55%). In preliminary tests, many of the identified volatiles attracted more female Mediterranean fruit flies than the control.

Key Words—Coffee fruit, *Coffea arabica*, host, Mediterranean fruit fly, *Ceratitis capitata*, medfly, analysis of volatiles, attractant, lure, Diptera, Tephritidae.

INTRODUCTION

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), is a worldwide agricultural pest of fruits, nuts, and vegetables (Hagen et al., 1981; Jackson

*To whom correspondence should be addressed.

0098-0331/97/0700-1891$12.50/0 © 1997 Plenum Publishing Corporation

and Lee, 1985). The fly's presence in the continental United States is prevented through vigorous detection, quarantine, and eradication in tropical and subtropical areas where hosts exist (Anonymous, 1996).

C. capitata (medfly) oviposit in more than 300 species of fruits and vegetables (Liquido et al., 1991), but the presumed ancestral host in Africa is *Coffea arabica* L. (Vargas et al., 1995). Keiser et al. (1975) indicated that the bark of *C. arabica* and an unspecified part of *C. robusta* Lind. attract medflies. Waikwa (1979) showed that the largest number of medfly eggs are laid in the mature yellow-red coffee fruit and the smallest number in the mature green fruit. The issue of color is addressed by Takana (1965), who demonstrated that yellow and green colored artificial egg-laying receptacles are equally attractive in stimulating oviposition by lab-reared medflies. Although this latter study did not confirm whether the flies use visual or olfactory senses to locate fruits for oviposition, Bateman (1972) suggested that visual senses are involved.

Vargas and Chang (1991) found that the oviposition of medfly eggs in a bottle containing a hot water extract of canned coffee grounds is significantly greater than when orange, guava, or papaya juice is used. Our group (D.R.L., E.B.J., and D.O.M., unpublished data, 1996) found that the odor of crushed ripe coffee fruit is more attractive for female medflies than males in flight-tunnel choice tests; the odor of coffee produced a 2.5-fold increase in the number of female landings on fruit models for oviposition. Traps baited with crushed coffee fruit in release–recapture tests captured 15–30 times greater numbers of flies than did unbaited traps. In preliminary release–recapture tests, bucket-type traps baited with fresh versus previously frozen (−80°C) ripe coffee fruit captured similar numbers of *C. capitata*. In the open field, there was a very quick aggregation of flies on leaves that had been rubbed with the inside of the fresh coffee fruit skins.

Prokopy and Vargas (1996) showed that the odor of intact or crushed coffee fruit is more attractive to both sexes of medflies compared to lower-ranking hosts and nonhosts when exposed to laboratory wind-tunnel and field conditions. The odor of recently crushed ripe coffee fruit is more attractive than that of intact ripe fruit and unripe fruit. Mature females carrying a high egg load are more attracted to the odor of ripe coffee fruit than are immature females. Mature laboratory-cultured female medflies and mature wild-origin female medflies give similar response patterns to coffee fruit.

Candidate female medfly attractants isolated from the odor of freshly crushed, ripe coffee fruit, in conjunction with male medfly produced pheromones (Jang et al., 1994), protein baits, and parapheromones (Beroza et al., 1961; Sivinski and Calkins, 1986; McGovern and Cunningham, 1987, 1988) have potential for monitoring and capturing this economic pest. This paper describes the identification of volatile, potential attractants for female medflies from freshly crushed, ripe, coffee fruit.

METHODS AND MATERIALS

Fruit Collection and Storage. Ripe, dark red coffee fruit, *Coffea arabica* L., Blue Mountain variety, was collected March 7, 1995, from a small commercial grove in Haleiwa Oahu, Hawaii. The fruit was held at −80°C for one week to kill any possible larvae that might have been present so that exportation to the continental United States would be permitted. The fruit was then shipped to Beltsville, Maryland, and stored at −10°C until analyses.

Collection of Volatiles. Coffee fruit (50) were removed from the freezer and allowed to defrost for a few minutes to soften. Each fruit was then crushed with tweezers and immediately placed in a three-neck, round-bottom flask. Prepurified nitrogen (i.e., passed through an activated charcoal bed) was swept at 300 ml/min over the crushed coffee fruit headspace, and volatiles were collected in a glass tube packed with 300 mg of activated charcoal (Darco, 20–40 mesh, Aldrich Chemical Co., Milwaukee, Wisconsin). Activated charcoal was used as the trapping agent because of its high efficiency to adsorb a variety of organic compounds (Heinz et al., 1966). The charcoal used in the collection trap was prepurified by continuous extraction (Soxhlet extractor) with methylene chloride and then benzene (CAUTION: benzene and methylene chloride, suspected cancercausing agents, should be handled with care and with adequate ventilation). After collecting volatiles for 15 hr, the charcoal trap was removed and eluted with ca. 0.3 ml of methylene chloride. The methylene chloride was analyzed without concentration. The efficiency of the charcoal trap to collect volatiles was determined by inserting a second charcoal trap (equal size and load) in the purge stream after the first trap. Gas chromatographic (GC) analysis of the eluate from the second trap showed complete absence of any volatiles associated with the coffee fruit. The collection of volatiles was replicated three times using freshly crushed coffee fruit each time.

Gas Chromatography (GC). A Shimadzu model GC-9 (Shimadzu, Columbia, Maryland) equipped with a flame ionization detector (FID) and a bonded DB-1 (J&W Scientific, Folsom, California) fused silica capillary column (60 m × 0.248 mm ID, 0.25-μm film thickness) was used to analyze volatile components from each replicate collection. GC peak areas were quantified using a Shimadzu CR-4A Integrator. GC operating conditions: injector/detector temperature, 280°C; helium carrier, ca. 1 ml/min (4 kg/cm^2 head pressure), injector operated in split mode, 175:1; temperature program, 50°C to 250°C at 5°C/min.

Gas Chromatography–Mass Spectrometry (GC-MS). GC-MS was performed on a Hewlett-Packard 5890A-GC-MS equipped with a 5971A MSD and an HP5 (Hewlett-Packard, Avondale, Pennsylvania) bonded fused-silica capillary column (25 m × 0.2 mm ID, 0.11-μm film thickness) for each replicate collection. GC conditions were the same as those described for GC analysis on

the Shimadzu instrument except that the injection port was operated in the splitless mode. MS conditions (EI mode) were: ionization voltage, 70 eV; mass range, *m/z* 30–550; ion source temperature, 180°C. The mass spectra of the unknown compounds were compared with those in the Wiley/NBS spectral data base. Identifications were also made by comparing Kovats indices (KI) of unknowns with those determined for authentic samples (Kovats, 1966).

Insect Rearing, Bioassay, and Statistics. Laboratory-reared medfly pupae were obtained from the USDA-ARS, Tropical Fruit & Vegetable Research Laboratory rearing facility, Honolulu, Hawai. Flies were allowed to emerge in a screen cage containing sugar, water, and hydrolyzed protein. They were kept at 24–26°C, 60–70% relative humidity, and 12L:12D cycle. At five to eight days after emergence, cages were placed in a cold room where females were put into groups of 50 females in plastic containers (11.5 cm in diameter, 7.5 cm deep) with nylon mesh covers and containing sugar, water, and hydrolyzed protein. Flies used for testing were held for 24 hr at room temperature.

Bioassays were conducted in a flight tunnel (Jang and Light, 1991), 0.9 m × 0.9 m × 2.8 m. Fifty previously frozen coffee fruit were crushed and placed in a one-quart mason jar outside of the tunnel. A second one-quart mason jar was left empty. Compressed air was pumped into the jars, and the headspace odor from the jars was pumped via Teflon tubing into the tunnel. The Teflon tubing from each jar was attached to a panel trap. The panel trap was made out of a dark green file folder (14 cm × 23 cm) with a 6-cm hole cut 1 cm from the bottom. The front and back of the panel were coated with Tanglefoot sticky glue (Tanglefoot Co., Grand Rapids, Michigan). The odor chamber was made using a 250-ml plastic cylindrical bottle (Nalgene), with the bottom third cut off and a hole drilled into the cap to insert the Teflon tubing. The odor chamber was placed through the hole in the panel. The flow rate of the air into the cylindrical bottle was 200 ml/min.

For each assay, 50 mated females (5–8 days old) were released at the back of the tunnel and allowed to fly within the tunnel for 1 hr. The number of females captured on the sticky panel was recorded. Data were analyzed using PROC TTEST (SAS Institute, 1988).

RESULTS AND DISCUSSION

The constituents of the presumed ancestral host, *Coffea arabica* L., (Vargas et al., 1995) are of interest in understanding the complex life cycle of the medfly. In preliminary release–recapture tests, bucket-type traps baited with fresh versus previously frozen (−80°C) ripe coffee fruit captured similar numbers of *C. capitata* (D.R.L., E.B.J., and D.O.M., unpublished data, 1996). The same observations with female medflies were made for fresh versus previously frozen

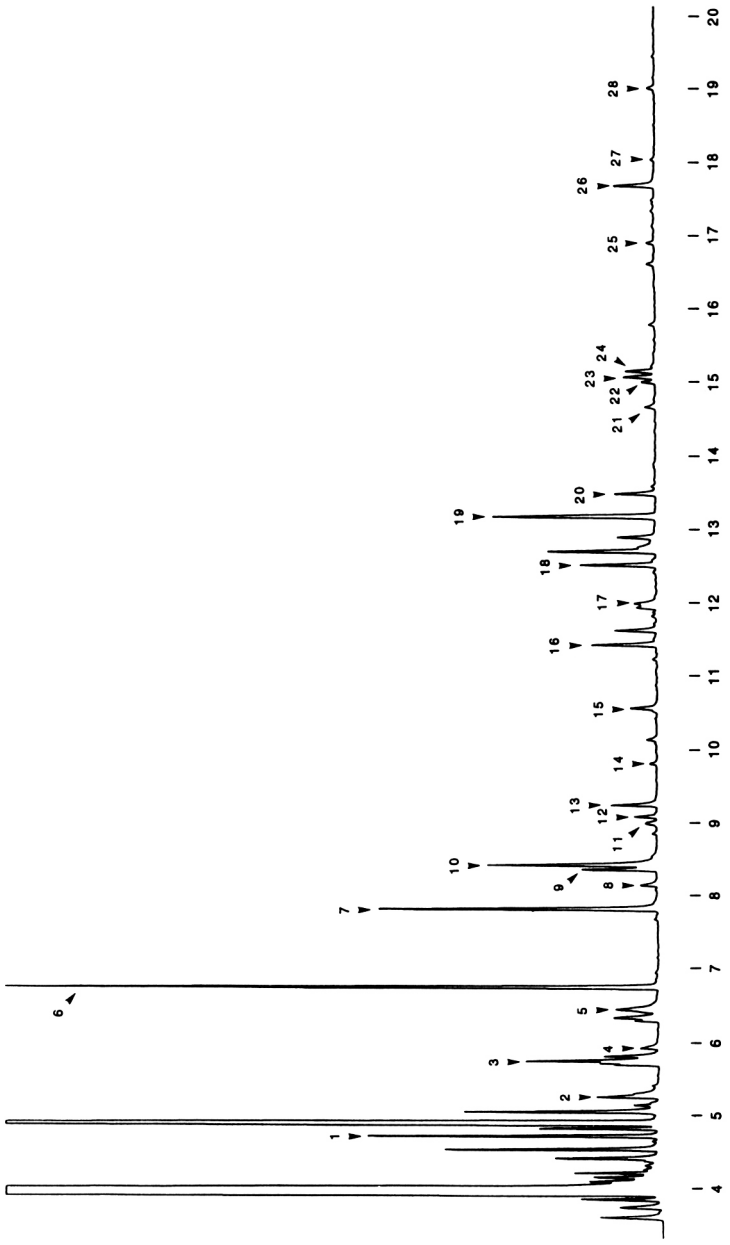


FIG. 1. Gas chromatogram from a temperature programmed analysis of the volatile components from 50 freshly crushed, ripe, dark red coffee fruit, *Coffea arabica*. Peak numbers correspond to peak numbers in Table 1. See Table 1 for MS data, Kovats indices, relative area, and flight tunnel tests.

TABLE 1. VOLATILES IDENTIFIED FROM 50 CRUSHED, RIPE DARK RED COFFEE FRUITS, *Coffea arabica* BLUE MOUNTAIN VAR.

GC peak[a]	Compound	Mass spectral ions, *m/z*		Ref. KI[b]	KI[c]	Relative area (%)	Female medflies (*N*)[d]	Control female medflies (*N*)
		M^+ (intensity)	Base peak					
1	3-methyl-1-butanal benzene[e]	86 (8)	44	633	631	8.5	12	1
2	pentanal	86 (1)	44	674	674	4.0	0	0
3	3-methyl-1-butanol	ND[f]	55	724	719	9.0	9	2
4	2-(*E*)-pentenal	84 (56)	55	727	730	0.88	1	1
5	2-(*Z*)-pentenol	86 (13)	57	760	760	3.6	10	1
6	hexanal	100 (1)	44	776	776	21	3	3
7	2-(*E*)-hexenal	98 (16)	41	827	827	11	0	1
8	3-(*Z*)-hexenol	100 (3)	41	839	841	0.95	9	0
9	2-(*E*)-hexenol	100 (3)	57	849	850	3.2	12	0
10	1-hexanol	ND	56	852	852	8.4	1	0
11	2-heptanone	114 (5)	43	874	876	0.70	5	1
12	4-(*Z*)-heptenal	112 (4)	41	879	880	0.92	0	0
13	2-heptanol	ND	45	886	886	2.1	7	0
14	methyl hexanoate	130 (0.2)	74	908	908	0.33	3	0
15	benzaldehyde	106 (95)	77	933	934	1.5	1	3
16	1-octen-3-ol	ND	57	964	964	3.2	2	0
17	3-octanol	ND	59	982	985	1.7	6	1

18	1-methoxy-4-methylbenzene	122 (53)	91	1003	1003	3.3	1	0
19	2-cyclohepten-1-one	110 (21)	81	1020	1024	7.7	1	2
20	2-(*E*)-octenal	126 (1)	55	1035	1035	1.9	0	4
21	2-nonanone	142 (7)	58	1073	1074	0.55	3	3
22	α-terpinolene	136 (68)	71	1083	1085	0.55	3	1
23	linalool	ND	71	1087	1087	1.3	1	0
24	phenethyl alcohol	122 (33)	91	1089	1089	1.2	3	4
25	α-isomenthone	154 (25)	112	1148	1148	0.45	1	0
26	methyl 2-hydroxybenzoate	152 (48)	120	1175	1175	2.1	3	1
27	decanal	ND	43	1187	1187	0.19	8	3
28	pulegone	152 (56)	81	1221	1221	0.44	4	5

[a] Peak numbers correspond to those in Figure 1.
[b] Ref. Kovats indices calculated from retention time data of authentic sample obtained on a DB-1 capillary column.
[c] Kovats indices calculated from retention time data on a DB-1 capillary column.
[d] Flight-tunnel tests with 5 μl volatile compound on 4.25 cm filter paper in half-size Jackson traps; 100 female flies (5–8 days old) were released and trap captures were recorded after 2 hr.
[e] A contaminant from the prepurification of the charcoal.
[f] ND = not detected.

guava fruits, *Psidium guajava* L., another host of medfly. Flight-tunnel responses of female medflies to the odor of crushed coffee fruit that had been previously frozen showed a clear preference to the coffee over clean air in dual choice assays. The response of the flies to coffee volatiles [13.7 ($\pm$1.8) flies] was significantly greater than to the clean air control [2.8 ($\pm$0.8) flies] [$P < 0.05$]. The presence of volatile compounds attractive to gravid female medflies in the flight-tunnel assays suggested that one or more of the compounds identified in this study may be responsible. The response noted in this study was comparable to the results of flight tunnel responses of female flies to the odor of freshly crushed (non-frozen) fruit (D.R.L., E.B.J., and D.O.M., unpublished data, 1996).

In preliminary tests, several identified compounds appear more attractive to female medflies than other compounds when compared to a control (Table 1). Further testing of the individual compounds is currently being carried out to try to determine the most attractive compounds.

A typical GC profile of the volatiles purged from 50 freshly crushed, ripe, dark red coffee fruit, *C. arabica*, is shown in Figure 1. Chemical identities are shown in Figure 1 and listed in Table 1 with the Kovats indices (KI) and relative concentrations. Excluding benzene (a contaminant from the prepurification of the charcoal), a total of 28 peaks appearing in the chromatogram of the volatiles were identified on the basis of a comparison of the KI values and GC-MS spectra with those obtained from authentic samples. Four chemical classes are represented by the 28 identified compounds. The class and number of compounds corresponding to that class were: alcohols (10), aldehydes (9), ketones (5), and monoterpenes (4). The five most abundant volatiles in decreasing order were hexanal (21%), 2-(*E*)-hexenal (11%), 3-methyl-1-butanol (9.0%), 3-methyl-1-butanal (8.5%), and 1-hexanol (8.4%). The five least abundant volatiles of the 28 identified, in increasing order, were decanal (0.19%), methyl hexanoate (0.33%), pulegone (0.44%), α-isomenthone (0.45%), and 2-nonanone (0.55%).

Of the 28 compounds identified in freshly crushed, ripe, dark red coffee fruit, 57% are reported as volatiles in fresh, ripe guava fruits, which also attract medflies. Pentanal, 2-(*E*)-pentenal, 2-(*Z*)-pentenol, 1-octen-3-ol, terpinolene, and decanal (Idstein and Schreier, 1985); hexanal and 3-(*Z*)-hexenol (Chyau et al., 1992; Idstein and Schreier, 1985; Nishimura et al., 1989; MacLeod and Gonzalez De Troconis, 1982); 2-(*E*)-hexenal, 2-(*E*)-hexenol, and 1-hexanol (Chyau et al., 1992; Idstein and Schreier, 1985; Nishimura et al., 1989); methyl hexanoate (Idstein and Schreier, 1985; Macleod and Gonzalez De Troconis, 1982); benzaldehyde (Idstein and Schreier, 1985; Nishimura et al., 1989; MacLeod and Gonzalez De Troconis, 1982); phenyethyl alcohol (Idstein and Schreier, 1985; Nishimura et al., 1989); 3-methyl-1-butanol (MacLeod and Gonzalez De Troconis, 1982); and linalool (Nishimura et al., 1989) are also observed as constituents of fresh, ripe guava fruit.

The latter compound, linalool, is one of the volatiles produced in the male medfly that elicits responses from female medfly antennae (Cossé et al., 1995). Baker et al. (1990) also tested this compound as a component of the male medfly sex pheromone and found that it caught 23 times more flies than controls.

The presence of the commonly reported members of the green leaf volatile complex [hexanol, 2-(*E*)-hexenol, 3-(*Z*)-hexenol, hexanal, and 2-(*E*)-hexenal], except for 3-(*Z*)-hexenal (Dickens et al., 1990), among the 28 identified volatile components of freshly crushed, red, ripe coffee fruit is of considerable interest because this complex enhances the pheromone response of the medfly. Green leaf volatiles increase the number of landings made by female flies on the odor source relative to male odor alone. The single green leaf volatile alone, 2-(*E*)-hexenal, also enhances response of female medflies to male odor.

The isolation and identification of headspace volatiles of the primary host of medflies has added more data to the complex problem of developing an effective lure for the female Mediterranean fruit fly.

CONCLUSIONS

In summary, we have identified 28 volatile compounds present in freshly crushed, ripe, dark red coffee fruit, *C. arabica*, the primary host of Mediterranean fruit flies. Four classes of compounds were identified with most volatiles being either alcohols or aldehydes. Five of the six components of the green leaf volatile mixture (Dickens et al., 1990) were present in the volatiles, and one of the reported constituents of the male medfly sex pheromone complex (Cossé et al., 1995) was present. In preliminary tests, several identified compounds appear more attractive to female medflies than other compounds when compared to a control. Further testing of the individual compounds is currently being carried out to determine the most attractive compounds. We hope that information from this study and future studies will contribute to the development of effective lures for the female Mediterranean fruit fly.

REFERENCES

ANONYMOUS. 1996. http://www.aphis.usda.gov/oa/medfly.html

BAKER, P. S., HOWSE, P. E., ONDARZA, R. N., and REYES, J. 1990. Field trials of synthetic sex pheromone components of the male Mediterranean fruit fly (Diptera: Tephritidae) in southern Mexico. *J. Econ. Entomol.* 83:2235–2245.

BATEMAN, M. A. 1972. Ecology of fruitflies. *Annu. Rev. Entomol.* 17:494–518.

BEROZA, M. GREEN, N., GERTLER, S. I., STEINER, L. F., and MIYASHITA, D. M. 1961. New attractants for the Mediterranean fruit fly. *J. Agric. Food Chem.* 9:361–365.

CHYAU, C.-C., CHEN, S.-Y., and WU, C.-M. 1992. Differences of volatile and nonvolatile constituents between mature and ripe guava (*Psidium guajava* Linn) fruits. *J. Agric. Food Chem.* 40:846–849.

COSSÉ, A. A., TODD, J. L., MILLAR, J. G., MARTÍNEZ, L. A., and BAKER, T. C. 1995. Electroantennographic and coupled gas chromatographic-electroantennographic responses of the Mediterranean fruit fly, *Ceratitis capitata*, to male-produced volatiles and mango odor. *J. Chem. Ecol.* 21:1823-1836.

DICKENS, J. C., JANG, E. B., LIGHT, D. M., and ALFORD, A. R. 1990. Enhancement of insect pheromone responses by green leaf volatiles. *Naturwissenschaften* 77:29-31.

HAGEN, K. S., ALLEN, W. W., and TASSAN, R. L. 1981. Mediterranean fruit fly: The worst may be yet to come. *Calif. Agric.* 35:5-7.

HEINZ, D. E., SEVENANTS, M. R., JENNINGS, W. G. 1966. Preparation of fruit essences for gas chromatography. *J. Food Sci.* 31:63-68.

IDSTEIN, H., and SCHREIER, P. 1985. Volatile constituents from guava (*Psidium guajava*, L.) fruit. *J. Agric. Food Chem.* 33:138-143.

JACKSON, D. S., and LEE, B. B. 1985. Medfly in California 1980-1982. *Bull. Entomol. Soc. Am.* 31:29-37.

JANG, E. B., and LIGHT, D. M. 1991. Behavioral responses of female oriental fruit flies to the odor of papayas at three different ripeness stages in a laboratory flight tunnel (Diptera: Tephritidae). *J. Insect Behav.* 4:751-762.

JANG, E. B., LIGHT, D. M., BINDER, R. G., FLATH, R. A., and CARVALHO. 1994. Attraction of female Mediterranean fruit flies to the five major components of male-produced pheromone in a laboratory flight tunnel. *J. Chem. Ecol.* 20:9-20.

KEISER, I., HARRIS, E. J., MIYASHITA, D. H., JACOBSON, M., and PERDUE, R. E., JR. 1975. Attraction of ethyl ether extracts of 232 botanicals to oriental fruit flies, melon flies and Mediterranean fruit flies. *Lloydia* 38:141-152.

KOVATS, E. 1966. Gas chromatographic characterization of organic substances in the retention index system, *in* J. C. Giddings and R. A. Keller (eds.). Advances in Chromatography, Vol. 1. Marcel Dekker, New York.

LIQUIDO, N. J., SHINODA, L. A., and CUNNINGHAM, R. T. 1991. Host plants of the Mediterranean fruit Fly: An annotated world review. *Misc. Publ. Entomol. Soc. Am.* 77:1-52.

MACLEOD, A. J., and GONZALEZ DE TROCONIS, N. 1982. Volatile flavour components of guava. *Phytochemistry* 21:1339-1342.

MCGOVERN, T. P., and CUNNINGHAM, R. T. 1987. New medfly attractants: Halogen analogs of trimedlure. National Conference of the Entomological Society of America, Boston, Massachusetts, Paper #1283. November 29-December 3.

MCGOVERN, T. P., and CUNNINGHAM, R. T. 1988. Persistent attractants for the Mediterranean fruit fly, the method of preparation and method of use. US Patent 4,764,366. Issued August 16.

NISHIMURA, O. YAMAGUCHI, K., MIHARA, S., and SHIBAMOTO, T. 1989. Volatile constituents of guava fruits (*Psidium guajava* L.) and canned puree. *J. Agric. Food Chem.* 37:139-142.

PROKOPY, R. J., and VARGAS, R. I. 1996. Attraction of *Ceratitis capitata* (Diptera: Tephritidae) flies to odor of coffee fruit. *J. Chem. Ecol.* 22:807-820.

SAS Institute. 1988. SAS/STAT User's Guide. Release 6.03 edition. SAS Institute, Cary, North Carolina.

SIVINSKI, J. M., and CALKINS, C. 1986. Pheromones and parapheromones in the control of Tephritids. *Fla. Entomol.* 69:157-168.

TAKANA, N. 1965. Artificial egg laying receptacles for three species of Tephritid flies. *J. Econ. Entomol.* 58:177-178.

VARGAS, R. I., and CHANG, H. B. 1991. Evaluation of oviposition stimulants for mass production of melon fly, oriental fruit fly, and Mediterranean fruit fly. *J. Econ. Entomol.* 84:1695-1698.

VARGAS, R. I., WALSH, W. A., and NISHIDA, T. 1995. Colonization of newly planted coffee fields: Dominance of Mediterranean fruit fly over oriental fruit fly. *J. Econ. Entomol.* 88:620-627.

WAIKWA, J. W. 1979. Ovipositional behaviour of *C. capitata* with reference to coffee berry age. *Kenya Coffee* 44:23-27.

Journal of Chemical Ecology, Vol. 23, No. 7, 1997

ANTIFUNGAL XANTHONES FROM *Calophyllum brasiliensis* HEARTWOOD[1]

RICARDO REYES-CHILPA,*[,1] MANUEL JIMENEZ-ESTRADA, and ELIZABETH ESTRADA-MUÑIZ

Instituto de Química, Universidad Nacional Autónoma de México
Ciudad Universitaria
Coyoacán, 04510 México, DF

(Received October 14, 1996; accepted March 17, 1997)

Abstract—The heartwood of the tropical tree *Calophyllum brasiliensis* is known to be highly resistant to fungi and termites. To determine whether resistance to wood-rotting fungi could be caused by bioactive secondary metabolites, a chemical and biological study was carried out. Hexane, acetone, methanol, and water extracts were prepared. The yield of the extracts ranged from 0.04% (hexane) to 4.81% (acetone). Methanol, acetone, and water extracts (5 mg/ml = 0.5%) inhibited the mycelial growth of the brown rot fungus *Postia placenta* by 83%, 59%, and 21%, respectively. Chromatographic separation of the acetone and methanol extracts afforded five prenylated xanthones: 6-desoxyjacareubin (**I**), 1,5-dihydroxy-2-(3,3-dimethylallyl)-3-methoxy-xanthone (**II**), jacareubin (**III**) and 1,3,5-trihydroxy-2-(3,3-dimethylallyl)-xanthone (**IV**) and 1,3,5,6-tetrahydroxy-2-(3,3-dimethylallyl)-xanthone (**V**). Xanthones **III**, **IV**, and especially **V**, were the most abundant constituents of both extracts and inhibited at 0.25 mg/ml the mycelial growth of *P. placenta*. Inhibitory activity ranged from 55.5% (**V**) to 68.8% (**III** and **IV** mixture). Acetylation of xanthones did not induce a sharp change in the extent of fungistasis compared with parent compounds. The above results suggest that *C. brasiliensis* xanthones actually play a defensive role against wood decay fungi

Key Words—Xanthones, *Calophyllum brasiliensis*, heartwood, antifungal activity, *Postia placenta*, Guttiferae, wood, fungi, extractives.

*To whom correspondence should be addressed.

[1]Contribution 1264 from Instituto de Química, UNAM. Presented in part at the 22nd International Symposium on Natural Products Chemistry, Mexico City, May 6–7, 1996.

0098-0331/97/0700-1901$12.50/0 © 1997 Plenum Publishing Corporation

INTRODUCTION

The genus *Calophyllum* (Guttiferae) is composed of about 130 species confined to the warm humid tropics of the world. In the Western Hemisphere, one of the most widely distributed species is *Calophyllum brasiliensis* Camb. This large tree is found in tropical forests from southern Mexico to Brazil. It can reach 40 m in height and 1.3 m diameter at breast height. The timber is used for construction, flooring, and furniture (Chudnoff, 1984, Ortega-Escalona et al., 1991). Experimental tests indicate that *C. brasiliensis* heartwood is highly resistant to the brown rot fungus *Lenzites trabea* (Torelli, 1982) and to the subterranean termites *Reticulitermes flavipes* and *Coptotermes formosanus* (Carter and Camargo, 1983); it has also been rated as resistant to the white rot fungus *Coriolus versicolor* (Torelli, 1982).

Natural resistance to fungi and termites is primarily attributed to the content of secondary metabolites present in heartwood, since these compounds frequently exhibit antifungal (Gómez-Garibay et al., 1990; Scheffer and Cowling, 1966) and antitermitic (McDaniel 1992; Scheffrahn, 1991; Reyes-Chilpa et al., 1995) properties. In addition, extraction of secondary metabolites with organic solvents and water renders heartwood susceptible to wood-destroying organisms (Déon 1983; Reyes-Chilpa et al., 1987). In this paper we report the isolation of five xanthones (**I**–**V**) from *Calophyllum brasiliensis* heartwood. We also report the antifungal activity of heartwood extracts and compounds **III**, **IV**, and **V** against the brown rot fungus *Postia placenta.*

METHODS AND MATERIALS

Biological Material. Calophyllum brasiliensis heartwood was obtained from a tree collected in the Lacandona Rain Forest, State of Chiapas, Mexico (Bárcenas-Pazos, 1995). Vouchers and wood samples are deposited at the Institute of Ecology A.C. Herbarium (XAL) in Xalapa, Mexico. *Postia placenta* fungus, strain Mad 698, was obtained from the Forest Products Laboratory, Madison, Wisconsin.

Isolation of Compounds. Heartwood shavings (711.5 g) were extracted at room temperature with hexane, acetone, methanol, and water. Extracts were concentrated under reduced pressure. After preparative thin-layer chromatography (pTLC), the hexane extract yielded β-sitosterol. Part of the acetone extract (28 g) was subjected to column chromatography (silica gel) eluting with hexane, acetone, and mixtures of these solvents. Fractions 9–16 eluted with hexane–acetone (9:1) yielded a yellow powder (5 mg) that was identified as 6-desoxyjacareubin (**I**) (Figure 1). Fractions 17–20, eluted with hexane–acetone (9:1), yielded a yellow powder (12 mg), identified as 1,5-dihydroxy-2-(3,3-dimeth-

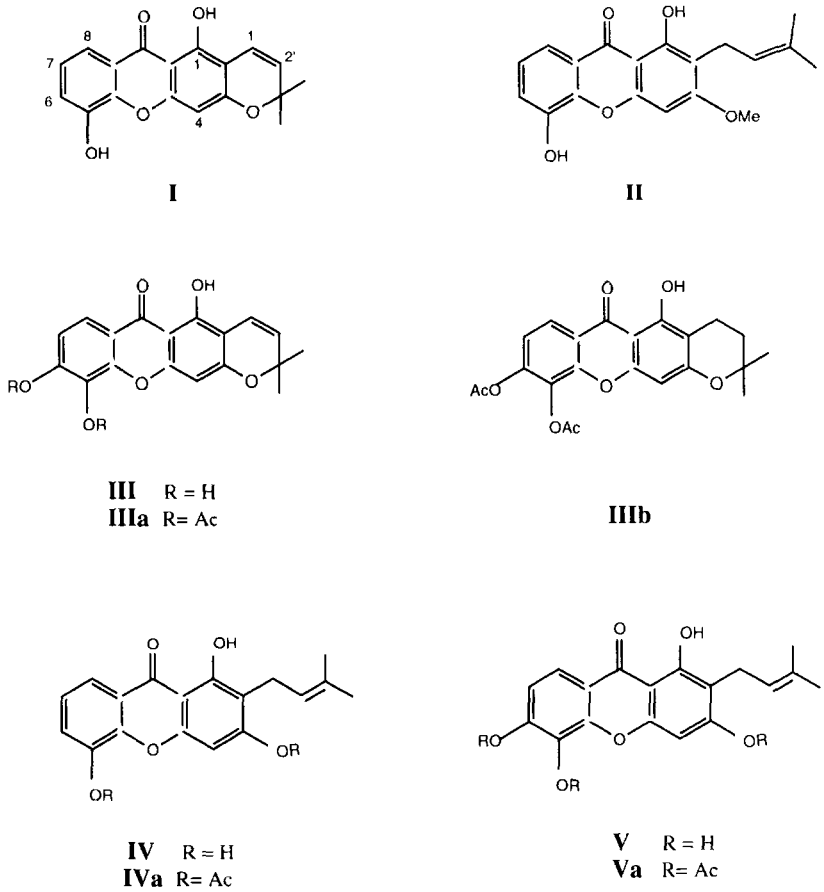


FIG. 1. Natural and transformed xanthones from *Calophyllum brasiliensis* heartwood.

ylallyl)-3-methoxyxanthone (**II**). Fractions 21–24, eluted with hexane–acetone (8:2), yielded a yellow powder (790 mg) that was further identified as a mixture of jacareubin (**III**) and 2-(3,3-dimethyllallyl)-1,3,5-trihydroxyxanthone (**IV**). Preparative TLC of this mixture yielded a pure sample of **IV**. Fractions 30–43, eluted with hexane–acetone (7.5:2.5), yielded 2-(3,3-dimethylallyl)-1,3,5,6-tetrahydroxyxanthone (**V**) as a yellow powder (3.3 g).

Part of the methanol extract (2.5 g) was subjected to column chromatography (silica gel) and eluted with hexane, ethyl acetate, and mixtures of these solvents. Fractions 12–18, eluted with a 9:1 mixture, yielded a mixture of compounds **III** and **IV** (86 mg). Fractions 26–39, eluted with 8:2 mixture,

afforded **V** (516 mg). Structures of compounds were elucidated from their IR, UV, ^{1}H NMR and MS spectroscopic data.

Transformation of Compounds. Compound **V** (500 mg) was acetylated with anhydrous acetic in pyridine at room temperature for 24 hr. The reaction was stopped with the addition of water giving a solid that was filtered and washed with 10% HCl and water. The solid (391 mg, yield 56%) was crystallized from CH_2Cl_2/MeOH and identified as 2-(3,3-dimethylallyl)-1-hydroxy-3,5,6-triacetylxanthone (**Va**). A mixture of **III** and **IV** (150 mg) was also acetylated as previously described, and the reaction products were separated by CC. First fractions yielded 1,3,5-triacetyl-2-(3,3-dimethylallyl)-xanthone (**IVa**) (8 mg), while the latter fractions yielded 6 mg of a mixture of triacetyljacareubin (**IIIa**) and 1′,2′-dihydro-5,6-diacetyljacareubin (**IIIb**).

Bioassays. The effects of extracts (5 mg/ml = 0.5% w/v) and isolated compounds (0.25 mg/m = 0.025%) on the mycelial growth of the fungus *Postia placenta* were examined as described by Reyes-Chilpa et al. (1997). The extracts were dissolved in acetone, methanol, or water and incorporated into the growth medium (malt-agar 1.5%). The isolated compounds were dissolved in acetone. Controls containing each solvent were run simultaneously. Phenol (Sigma) was also tested in the same way for comparison. Finally, to test whether fungal metabolism could modify xanthones in vitro, we redissolved agar from plates with compound **V** and extracted the solution several times with ethyl acetate. The organic phase was then subjected to pTLC.

RESULTS

Effects of Extracts

The highest yield of soluble metabolites was obtained with acetone (4.81%), while the poorest was achieved with hexane (0.04%) (Table 1). At the concentration tested (5 mg/ml = 0.5% w/v), three extracts showed fungistatic activity against *Postia placenta* (Table 1). The methanol extract was the most active, inhibiting the mycelial growth by 83.6%. Acetone and water extracts were less active, inhibiting mycelial growth by 59% and 21.9%, respectively. Differences between extracts were statistically significant. The hexane extract was not tested because of its low yield.

Compounds Isolated

Chromatographic separation of the acetone and methanol extracts yielded five prenylated xanthones: 6-desoxyjacareubin (**I**), 1,6-dihydroxy-2(3,3-dimethylallyl)-3-methoxyxanthone (**II**), jacareubin (**III**), 1,3,5-trihydroxy-2-(3,3-dimethylallyl)-xanthone (**IV**), and 1,3,5,6-tetrahydroxy-2-(3,3-dimethylallyl)-

TABLE 1. INHIBITION OF *Postia placenta* MYCELIAL GROWTH BY *C. brasiliensis* HEARTWOOD EXTRACTS (5 mg/ml)

Extract	Yield (%)	Growth (cm)[a]	Inhibition (%)
Control		4.88 ± 0.16[a]	0.0 ± 3.2
Hexane	0.04	nt	nt
Acetone	4.81	2.00 ± 0.00[b]	59.0 ± 0.0
Methanol	0.92	0.80 ± 0.14[c]	83.6 ± 2.8
Water	0.13	3.81 ± 0.06[d]	21.9 ± 1.2

[a]Mean ± standard deviation of three replicates five days after innoculation. Least significant difference > 0.26; values followed by a different letter are significantly different at $P = 0.05$ (Tukey's t test). nt: not tested.

xanthone (**V**). Their ^{1}H NMR data are shown in Table 2. Besides these compounds, spectroscopic evidence also suggested the presence of 1',2'-dihydro-5,6-diacetyljacareubin (**IIIb**) along with the diacetyl derivative of jacareubin (**IIIa**). The mass spectrum of **IIIa** showed surplus peaks at 412 *m/z* (19%), 370 (15%), and 328 (31%), which accounted for the molecular ion $[C_{22}H_{20}O_8]^+$, and the loss of one and two C_2H_2O fragments, respectively. ^{1}H NMR of **IIIa** also suggested residual **IIIb**, considering two small triplets at 2.7 and 1.85 ppm assigned to methylene protons at the 1' and 2' positions. The origin of compound **IIIa** as a natural product or as an artifact was not determined.

Xanthones **III**, **IV**, and especially **V** were the most common constituents of both the acetone and methanol extracts. The yields of compound **V** were 11.7% and 20.6%, respectively; while the mixture of compounds **III** and **IV** accounted for 2.8% and 3.4%, respectively.

6-Desoxyjacareubin (I). Yellow powder, mp 214–215°C (reported 212–214°C; Jackson et al., 1967, 1969). EMIE 70 eV (*m/z*): 310 M^+ (21.3%) $[C_{18}H_{14}O_5]$, 295 (100%) $[M^+-CH_3]$, 257 (4.1%) $[M^+-C_4H_7]$, 147 (11.5%).

1,5-Dihydroxy-2-(3,3-dimethylallyl)-3-methoxyxanthone (II). Yellow powder, mp 254–255°C (reported 242–244°C; Sen et al., 1981). EMIE 70 eV (*m/z*): 326 M^+ (53.3%) $[C_{19}H_{18}O_5]$, 311 (41.6%) $[M^+-CH_3]$, 283 (73.3%) $[M^+-C_3H_7]$, 271 (100%) $[M^+-C_4H_7]$, 258 (10%), 241 (11.6%).

1,3,5-Triacetyljacaraubein (IIIa). Yellow powder, mp 169–170°C. UV λ_{max} [MeOH], nm (ϵ): 239 (3496), 292 (3584), 327 (1493). EMIE 70 eV (*m/z*): 410 M^+ (50%) $[C_{22}H_{18}O_8]$, 395 (93%) $[M^+-CH_3]$ = A, 353 (97%) $[A-C_2H_2O]^+$, 311 (100%) $[A-2C_2H_2O]^+$, 43 (42%) $[C_2H_3O]^+$.

1,3,5-Trihydroxy-2-(3,3-dimethylallyl)-xanthone (IV). Yellow powder, mp 288–290°C (reported 280–281°C; Gunasekera et al., 1977). EMIE 70 eV

TABLE 2. ^{1}H NMR DATA (δ, ppm) OF XANTHONES FROM *C. brasiliensis*[a]

	I	II	IIIa
OH-1	13.32 s (D_2O exch.)	13.08 s (D_2O exch.)	12.99 s (D_2O exch.)
H-4	6.38 s	6.63 s	6.33 s
H-6	7.35 dd (J = 1.8 and 7.8 Hz)	7.34 m	
H-7	7.28 t (J = 7.8 Hz)	7.27 t (J = 8.1 Hz)	7.20 d (J = 8.8 Hz)
H-8	7.67 dd (J = 1.8 and 7.8 Hz)	7.67 d (J = 7.8 Hz)	8.13 d (J = 8.8 Hz)
H-1′	6.69 d (J = 10.2 Hz)	3.33 d (J = 7.2 Hz) [2H]	6.72 dd (J = 0.6 and 10.0 Hz)
H-2′	5.76 d (J = 9.9 Hz)	5.20 tt (J = 1.5 and 7.2 Hz)	5.61 d (J = 10.1 Hz)
CH_3	1.47 s	1.77 s	1.55 s
CH_3	1.47 s	1.63 s	1.47 s
Ar-OH	9.15 s (D_2O exch.)	9.4 s (D_2O exch.)	
Ar-OH			
Ar-OH			
OCH_3		4.0 s	
OAc			2.42 s
OAc			2.36 s
	300 MHz/acetone-D6	300 MHz/acetone-D6	200 MHz/$CDCl_3$

[a]s = singlet, d = doublet, t = triplet, m = multiplet, w = wide.

(*m/z*): 312 M^+ (55%) $[C_{18}H_{16}O_5]$, 297 (35%) $[M^+-CH_3]$, 269 (60.8%) $[M^+-C_3H_7]$, 257 (100%) $[M^+-C_4H_7]$, 244 (10.8%).

*1-Hydroxy-2-(3,3-dimethylallyl)-3,5,6-triacetylxanthone (**IVa**).* Yellow powder, mp 121–123°C. UV λ_{max} [MeOH], nm (ϵ): 360 (6879), 304(17894), 239(60534). IR ν_{max} (KBr): 2925, 1774, 1641, 1614 (C=C), 1436. EMIE 70 eV (*m/z*): 396 M^+ (80.3%) $[C_{22}H_{20}O_7]^+$, 353 (51.7%) $[M^+-C_2H_3O]$ = A, 341 (34.8%) $[M^+-C_4H_7]$ = B, 311 (69.6%) $[A-C_2H_3O]^+$, 299 (100%) $[B-C_2H_3O]^+$, 269 (37.5%) $[C_{15}H_8O_4]^+$, 257 (66%) $[C_{14}H_8O_4]^+$, 55 (20%) $[C_4H_7]^+$, 43 (25%) $[C_2H_3O]^+$.

*1,3,5,6-Tetrahydroxy-2-(3,3-dimethylallyl)-xanthone (**V**).* Yellow powder, mp 260–262°C (reported 255–257°C; Jackson et al., 1966). EMIE 70 eV (*m/z*): 328 M^+ (54%) $[C_{18}H_{16}O_6]^+$, 313 (30.3%) $[M^+-CH_3]$, 285 (66.6%) $[M^+-C_3H_6]$, 273 (100%) $[M^+-C_4H_7]$, 260 (13.5%)

*1-Hydroxy-2-(3,3-dimethylallyl)-3,5,6-triacetylxanthone (**Va**).* Rectangular yellow prisms, mp 193–196°C. IR ν_{max} ($CHCl_3$): 2917, 1785, 1649, 1611, 1452, 1373, 1260, 1161, 116, 1087. EMIE 70 eV (*m/z*): 454 M^+ (100%) $[C_{24}H_{22}O_9]^+$, 411 (32.2%) $[M^+-C_2H_3O]$ = A, 399 (44%) $[M^+-C_4H_7]^+$ = B, 368 (60%) $[A-C_2H_3O]^+$, 357 (68.6%) $[B-C_2H_2O]^+$, 355 (10.1%), 327 (62.7%), 315 (61.8%), 273 (50%), 272 (32.2%), 69 (15.2%), 43 (33%).

Effects of Xanthones

The natural xanthones **III**, **IV**, and **V** showed fungistatic properties against *P. placenta* when tested at 0.25 mg/ml. Phenol showed fungicidal activity at the same concentration. Differences among the control, xanthones, and phenol were statistically significant by ANOVA tests (Table 3). Under our conditions, the natural xanthones exhibit similar inhibitory activity, ranging from 55.8% (**V**) to 67.3% (**III** and **IV**). Acetylation of xanthones did not induce a sharp change in the extent of fungistasis compared with parent compounds. At best, the derivatives showed a 8% increase (**Va**) or decrease (**IIIa** and **IVa**) in activity. Significant differences among natural and acetylated xanthones could not be detected (Table 3).

The effect of increasing concentrations of xanthone **V** on *P. placenta* growth was also examined. Concentrations of 0.5 and 1.0 mg/ml inhibited the mycelial growth by 64.4 and 74.6%, respectively. This last value is significantly different from the 0.25 mg/ml treatment (Table 3). Inhibition caused by xanthone **V** at the highest concentration was not significantly different from phenol at 0.25 mg/ml. Therefore it seems that **V** is about four times less potent than phenol. At the end of the experiments, all the agar plates treated with **V** were pooled and extracted to examine if this compound had suffered any transformation. Only one compound could be reisolated and purified in good yield (71%); it was identified as **V** according to ^1H NMR data.

TABLE 3. INHIBITION OF *Postia placenta* MYCELIAL GROWTH BY *C. brasiliensis* XANTHONES AND PHENOL[a]

Compound (mg/ml)	Growth (cm)	Inhibition (%)
Control	4.53 ± 0.33[a]	00.0 ± 7.2
V (1.00)	1.15 ± 0.41[bd]	74.6 ± 9.0
V (0.50)	1.60 ± 0.14[b]	64.6 ± 3.0
V (0.25)	2.00 ± 0.42[bc]	55.8 ± 9.2
IV (0.25)	1.95 ± 0.40[bc]	56.9 ± 8.8
IIIa and IVa (0.25)	1.85 ± 0.07[bc]	59.1 ± 1.5
Va (0.25)	1.68 ± 0.16[b]	62.9 ± 3.5
III and IV (0.25)	1.48 ± 0.28[b]	67.3 ± 6.1
Phenol (0.25)	0.60 ± 0.00[cd]	100.0 ± 0.0

[a]Mean of three replicates ± standard deviation six days after innoculation. Least significant difference >0.59; values followed by a different letter are significantly different at $P = 0.05$ (Tukey's t test).

DISCUSSION

The heartwood of *Calophyllum* species contains xanthones and neoflavonoids, while the leaves possess coumarins, benzopyrans, and triterpenes (Ampofo and Waterman, 1986; Patil et al., 1993). Xanthones isolated from this genus can be simple or modified, especially with prenyl (3,3-dimethylallyl) -derived substituents. Our results indicate that 2-prenylated xanthones (**I**, **II**, **III**, **IV**, and **V**) are the main constituents of *C. brasiliensis* heartwood. All of these compounds exhibit an 1,3,5-trioxygenated substitution pattern. In addition, compound **V** has an extra hydroxyl on C-6. While compounds **I**, **III**, **IV**, and **V** have been previously isolated from other *Calophyllum* species, compound **II** has only been obtained from *Garcinia mangosta* hulls (Sen et al., 1981). Compound **IIIb** has not been reported as a natural product; so it is possible that it could be an artifact produced during the acetylation procedure.

The natural xanthones **III, IV**, and **V** showed fungistatic activity against the brown rot fungus *Postia placenta* (Table 3). Xanthones **III**, **IV**, and especially **V**, were the most abundant constituents of both the acetone and methanol extracts. For the heartwood sample here analyzed, these compounds represent at least 0.65% (w/w). Compound **V** alone accounts for 0.53%. *C. brasiliensis* heartwood resistance against wood decay fungi thus appears to depend mostly on these compounds, especially **V**. It was previously reported that 1,3,5,6-tetrahydroxyxanthone isolated from *Maclura pomifera* heartwood inhibits the

growth of the wood rotting fungi *Gleophyllum trabeum* and *Trametes versicolor* (Schultz et al., 1995). This compound is the biogenetic precursor of **V** and showed an IC_{50} (50% inhibition of radial mycelial growth) greater than 200 ppm with both fungi (Schultz et al., 1995). In our case, compound **V**, at a similar concentration (0.25 mg/ml = 250 ppm), inhibited the growth of *P. placenta* by 55.8% (Table 3). It is noteworthy that at the same concentration the antifungal activity of *C. brasiliensis* heartwood xanthones was lower than that exhibited by synthetic phenol. Schultz et al. (1995) also observed that *M. pomifera* heartwood compounds were less active than commercial fungicides.

Some other xanthones have been shown to be inhibitory to phytopathogenic fungi. For instance 1,5-dihydroxyxanthone, 6-desoxyjacareubin, 5-hydroxy-1-methoxyxanthone (Rocha et al., 1994), and 1,3,5-trihydroxy-2-methoxyxanthone (Pinto et al., 1994) inhibited the growth of *Cladosporium curcumerinum*. The former compound was also active against *Trichophyton mentagrophytes* (Pinto et al., 1994). On the other hand, four 3-OMe substituted xanthones were inactive against *C. curcumerinum* (Rodriguez et al., 1995; Pinto et al., 1994), suggesting that free hydrogen or hydroxyl at this position might be essential for antifungal activity. Nevertheless, blocking of hydroxyls (including that on C-3) by acetylation of **V** or the mixture of **III** and **IV** did not induce a significant change in fungistasis as compared with parent compounds (Table 3).

Several xanthones have been recently reported as antioxidants and free radical scavengers (Minami et al., 1994, 1995). These properties are important considering that wood degradation by brown rot fungi involves secretion of fungal H_2O_2 and its interactions with wood Fe^{2+} ions (Kirk, 1983). From this perspective, it is possible to hypothesize that during fungal attack xanthones could first be oxidized, thus delaying degradation of structural polymers. Preliminary evidence indicates that compound **V** was not oxidized in vitro by *P. placenta*, but the presence of Fe^{2+} ions was not assured in this system. We are currently studying oxidative metabolism of **V** under controlled conditions.

Acknowledgments—Research was supported by grant NI214996 DGAPA-UNAM. The authors are grateful to Fernando Ortega-Escalona and Guadalupe Bárcenas Pazos for providing *C. brasilienis* wood, to Dr. Terry Highley for donation of the fungus, and to Dr. Ana Luisa Anaya Lang for her facilities for culturing it.

REFERENCES

AMPOFO, S. A., and WATERMAN, R. G. 1986. Xanthones and neoflavonoids from two Asian species of *Calophyllum*. *Phytochemistry* 25:2611–2620

BÁRCENAS-PAZOS, M. G. 1995. Características tecnológicas de veinte especies maderables de la Selva Lacandona. *Madera y Bosques (México)* 1:9–38.

CARTER, F. L., and CAMARGO, C. R. R. 1983. Testing antitermitic properties of Brazilian woods and their extracts. *Wood Fiber Sci.* 15:350–357.

CHUDNOFF, M. 1994. Tropical Timbers of the World. Agriculture Handbook No. 607. United States Department of Agriculture. Washington, DC, 466 pp.

DÉON, G. 1983. Les composés flavoniques du dabema et leur role dans la résistance de ce bois a la pourriture. Cahiers Scientifiques du Centre Technique Forestier Tropical, Supplément au 6. Nogent su Mer, France, 16 pp.

GÓMEZ-GARIBAY, F., REYES-CHILPA, R., QUIJANO, L., Calderon-Pardo, J. S., and RIOS-CASTILLO, T. 1990. Methoxy furan auronols with fungistactic activity from *Lonchocarpus castilloi*. *Phytochemistry* 29:459–463.

GUNASEKERA, S. P., JAYATILAKE, G. S., SELLIAH, S. S. and SULTANBAWA, M.U.S. 1977. Chemical investigation of Ceylanose plants. Part 27. Extractives of *Calophyllum cuneifolium* Thw. and *Calophyllum soulattri* Burm. F. (Guttiferae). *J. Chem Soc. Perkin I* 1977:1505–1511.

JACKSON, B., LOCKSLEY, H. D., and SCHEINMANN, F. 1966. Extractives from Guttiferae. Part I. Extractives of *Calophyllum sclerophyllum* Vesq. *J. Chem. Soc. (C)* 1966:178–181.

JACKSON, B., LOCKSLEY, H. D., and SCHEINMANN, F. 1967. Extractives from Guttiferae. Part. VII. The isolation and structure of seven xanthones from *Calophyllum scriblitifolium* Henderson and Wyatt-Smith. *J. Chem. Soc. C* 1967:2500–2507.

JACKSON, B., LOCKSLEY, H. D., and SCHEINMANN, F. 1969. The isolation of 6-desoxyjacareubin, 2-(3,3-dimethylallyl)-1,3,5,6-tetrahydroxyxanthone and jacareubin from *Calophyllum inophyllum*. *Phytochemistry* 8:927–929.

KIRK, T. K., 1983. Degradation and conversion of lignocelluloses, *in* J. E. Smith, D. R. Berry, and B. Kristiansen (eds.). *Filamentous Fungi*, Vol. 4, Fungal Technology. Edward Arnold, London, 122 pp.

MCDANIEL, C. A. 1992. Major antitermitic components of the heartwood of southern catalpa. *J. Chem. Ecol.* 18:359–369.

MINAMI, H., KINOSHITA, M., FUKUYAMA, Y., KODAMA, M., YOSHIZAWA, T., SIGIURA, M., NAK AGAWA, K., and TAGO, H. 1994. Antioxidant xanthones from *Garcinia subelliptica*. *Phytochemistry* 36:501–506.

MINAMI, H., TAKAHASHI, E., FUKUYAMA, Y., KODAMA, M., YOSHIZAWA, T., and NAKAGAWA, K. 1995. Novel xanthones with superoxide scavenging activity from *Garcinia subelliptica*. *Chem. Pharmacol. Bull.* 43:347–349.

ORTEGA-ESCALONA, F., CASTILLO-MORALES, I., and CARMONA-VALDOVINOS, T. 1991. *Anatomía de la Madera de Veintiseis Especies de la Selva Lacandona, Chiapas*. Angiospermas Arboreas de México No. 3. La Madera y su Uso 26. Instituto de Ecología A.C. and Universidad Autónoma Metropolitana, México, 200 pp.

PATIL, A. D., FREYER, A. J., EGGLESTON, D. S., HALTIWANGER, R. C., BEAN, M. F., TAYLOR, P. B., CARANFA, M. J., BREEN, A. L., BARTUS, H. R., JOHNSON, R. K., HERTZBERG, R. P., and WESTLEY, J. W. 1993. The inophyllums, novel inhibitors of HIV-1 reverse transcriptase isolated from the Malaysian tree, *Calophyllum inophyllum* Linn. *J. Med. Chem.* 36:(26) 4131–4138.

PINTO, D. G., FUZZATI, N., PAZMINO, X. C., and HOSTETTMANN, K. 1994. Xanthone and antifungal constituents from *Monnina obtusifolia*. *Phytochemistry* 37:875–878.

REYES-CHILPA, R., PÉREZ-MORALES, V., and DEL ANGEL-BLANCO, S. 1987. Influencia de los extractivos en la resistencia natural de seis maderas tropicales al hongo de pudrición morena *Lenzites trabea*. *Biotica (Mexico)* 12:7–13.

REYES-CHILPA, R., VIVEROS-RODRÍGUEZ, N., GÓMEZ-GARIBAY, F., and ALAVEZ-SOLANO, D. 1995. Antitermitic activity of *Lonchocarpus castilloi* flavonoids and heartwood extracts. *J. Chem. Ecol.* 21:455–463.

REYES-CHILPA, R., QUIROZ-VÁSQUEZ, R. I., JÍMENEZ-ESTRADA, M., NAVARRO-OCAÑA, A., and CASSANI-HERNÁNDEZ, J. 1997. Antifungal activity of selected plant secondary metabolites against *Coriolus versicolor*. *J. Trop. For. Prod.* In press.

ROCHA, L., MARSTON, A., KAPLAN, M. A. C., STOEKLI-EVANS, H., THULL, U., TESTA, B., and HOSTETMANN, K. 1994. An antifungal γ-pyrone and xanthones with monoamine oxidase inhibitory activity from *Hypericum brasiliense*. *Phytochemistry* 36(6):1381–1385.

RODRIGUEZ, S., WOLFENDER, J. L., HAKIZAMUNGU, E., and HOSTETTMANN, K. 1995. An antifungal naphthoquinone, xanthones and secoiridoids from *Swertia calycina*. *Planta Med.* 61:362–364.

SCHEFFRAHN, R. H. 1991. Allelochemical resistance of wood to termites. *Sociobiology* 19:257–281.

SCHEFFER, T. C., and COWLING, E. B. 1966. Natural resistance of wood to microbial deterioration. *Ann. Rev. Phytopathol.* 4:147–170.

SCHULTZ, T. P., HARMS, W. B., FISHER, T. H., MCMURTREY, K. D., MINN, J., and NICHOLAS, D. D. 1995. Durability of angiosperm heartwood: The importance of extractives. *Holzforschung* 49:29–34.

SEN, A. K., SARKAR, K. K., MAJUMDER, P. C., and BASEERJI, N. 1981. Minor xanthones of *Garcinia mangosta*. *Phytochemistry* 20:183–185.

TORELLI, N. 1982. Estudio promocional de 43 especies forestales tropicales mexicanas. Reporte Técnico Programa de Cooperación Científica y Técnica México-Yugoslavia. Secretaria de Agricultura y Recursos Hidraúlicos. México.

Journal of Chemical Ecology, Vol. 23, No. 8, 1997

BIPIPERIDYL AND QUINOLIZIDINE ALKALOIDS IN FRUITS OF *Viscum cruciatum* HEMIPARASITIC ON *Retama sphaerocarpa*

C. MARTÍN-CORDERO,[1] M. A. PEDRAZA,[1] A. M. GIL,[2] and M. J. AYUSO[1,*]

[1]*Laboratorio de Farmacognosia, Facultad de Farmacia*
Profesor García González s/n.
41012 Sevilla, Spain

[2]*Departamento de Química Orgánica, Facultad de Química*
41012 Sevilla, Spain

(Received August 19, 1996; accepted March 24, 1997)

Abstract—Fruits (berries) of *Viscum cruciatum* accumulate bipiperidyl (ammodendrine) and quinolizidine (retamine and lupanine) alkaloids. *Viscum* obtains the alkaloids by root parasitism on the host plant *Retama sphaerocarpa*. The presence of these alkaloids in the fruits of *Viscum cruciatum* may represent some competitive disadvantage to mistletoe itself because the seeds may not be so well dispersed by blackcaps (*Sylvia atricapilla*).

Key Words—*Viscum cruciatum*, Loranthaceae, hemiparasites, quinolizidine alkaloids, *Retama*.

INTRODUCTION

Viscum cruciatum Sieber (Loranthaceae) is one of the two recognized species of *Viscum* in the European flora. It is found in both the eastern and western areas of the Mediterranean coast (Post and Dismore, 1932; Sennen and Mauricio, 1933; Ball, 1964; Amaral Franco, 1971). In Spain, *V. cruciatum*, known as "muerdago colorado" due to its red berries, is a hemiparasitic species of lowland evergreen Mediterranean forests. This mistletoe has a wide range of angiosperm hosts, i.e., olive (*Olea europaea*), almond (*Prunus amygdalus*), hawthorn (*Crataegus monogyna*), and retama (*Retama sphaerocarpa*). The consumption of fruits by blackcaps (*Sylvia atricapilla*) in southern Spain appears to be essential for the dissemination of *Viscum cruciatum* (Herrera, 1986).

*To whom correspondence should be addressed.

0098-0331/97/0800-1913$12.50/0 © 1997 Plenum Publishing Corporation

Martín-Cordero et al. (1989, 1993) showed that root parasitic *Viscum cruciatum* acquires bipiperidyl and quinolizidine alkaloids from its host *Retama sphaerocarpa*. Wink (1985, 1987, 1988,1992, 1993a,b) has studied in detail quinolizidine alkaloids of the Fabaceae, and the toxicity of these alkaloids to a range animals is well known. They are general feeding deterrents for herbivorous mammals, insects, and microorganisms. The present work indicates the presence of alkaloids in fruits of *V. cruciatum*, which may be an ecological disadvantage for *Viscum* dispersion.

METHODS AND MATERIALS

Viscum cruciatum was collected in Grazalema (Cadiz, Spain) in January 1995 and the identity confirmed. A preliminary and rapid qualitative analysis of the fruits of *Viscum cruciatum* showed the presence of alkaloids. The unripe and ripe fruits from the air-dried plants were separated and made alkaline by addition of NH_4OH and extracted with $CHCl_3$ in a Soxhlet apparatus. The alkaloid mixture was further purified by acid–base treatment (HCl, 2%, followed by $NH_4OH/CHCl_3$). Quantitative determination of alkaloids was done gravimetrically (Paris and Moyse, 1976).

Qualitative analysis was by gas chromatography–mass spectrometry and performed using a Kratos MS80RFA mass spectrometer combined with a Carlo Erba/Kratos MFC 500 gas chromatograph using a CP-Sil 5 capillary column, 25 m × 0.32 mm. The temperature program was 100–280°C at 10°C/min. The detector and injector heaters were set at 250°C. The carrier gas was helium, at a flow rate of 1 ml/min.

Alkaloids were identified by comparing their mass spectra with those we had previously isolated (Martín Cordero et al., 1989) and with data reported in the literature (Wink, 1993a).

Gas chromatographic quantitative analysis was performed by the relative proportions method (areas), under the same conditions specified in the qualitative analysis described above.

RESULTS AND DISCUSSION

The current study reveals that the fruits (unripe and ripe) of *Viscum cruciatum* accumulate alkaloids and the percentage present was 0.02–0.03%. The qualitative analysis of alkaloid extracts from fruits showed the presence of the bipiperidyl alkaloid, ammodendrine (3.76%), in ripe fruits and the quinolizidine alkaloids, retamine (73.88 and 55.76%) and lupanine (26.12 and 40.48%), in both unripe and ripe fruits, respectively. Retamine was the main alkaloid present during the fruiting period.

Previous reports (Martín Cordero et al. 1993) demonstrated the presence of ammodendrine, lupanine, 5,6-dehydrolupanine, retamine, anagyrine, cytisine, and *N*-methylcytisine in stems and leaves of *V. cruciatum*. *N*-Methylcytisine is the major component. In fruits, however, the most toxic alkaloids (α-pyridone type) such as anagyrine, cytisine, and *N*-methylcytisine were not identified. These qualitative and quantitative differences could be explained by a selective transport and sequestration of the alkaloids in the red berries following their absorption through root parasitism by *V. cruciatum*.

In the host plant, *Retama sphaerocarpa*, we have observed the presence of sparteine, ammodendrine, lupanine, 5,6-dehydrolupanine, retamine, 17-oxo-retamine, anagyrine, cytisine, and *N*-methylcytisine. As we found in *V. cruciatum*, the *N*-methylcytisine represents the major component of stems and leaves of *R. sphaerocarpa* (Martín-Cordero et al., 1993). The quinolizidine alkaloids formed in the host are metabolically dynamic compounds. They are formed in leaves and translocated via phloem to the other plant organs (Wink, 1992, 1993a,b; El-Shazly, 1996). The hemiparasitic *Viscum* also appears to acquire the alkaloids via phloem.

V. cruciatum fruits are consumed by blackcaps (*Sylvia atricapilla*). Seeds are defecated 15–20 min after feeding (Herrera, 1981, 1986), and this is essential for the dissemination of mistletoe. At first sight the accumulation of bipiperidyl and quinolizidine alkaloids in fruits seems to be disadvantageous for *V. cruciatum* hemiparasitic on *R. sphaerocarpa*. However, the selective transport and sequestration of alkaloids and the observation that α-pyridone-type alkaloids were not stored in the fruits may be considered as strategies both for the protection of seeds and for their dissemination.

Furthermore, as quinolizidine alkaloids contain two atoms of nitrogen per molecule, they might also constitute nitrogen sources for germination and growth of the seedlings (Wink, 1992). This could be important for survival of the seedlings while establishing the association with the host (i.e., the nonparasitic period between the ripe berry stage and nutritional contact of the young plant with the living cells of a suitable host) (Salle, 1983)

Thus these results have important implications for *V. cruciatum* ecology and for the study of birds and other herbivores that are *Viscum* specialists.

Acknowledgments—We are very grateful to the Mass Spectrometry Service of the University of Seville for the provision of the spectroscopic equipment used in this investigation.

REFERENCES

AMARAL FRANCO, J. 1971. Nova flora de Portugal (Continente et Açores), Sociedade Astoria, Lisboa.

BALL, P. W. 1964. *Viscum* L., p. 72, *in* V. H. Heywood, T. G. Tutin, N. A. Burges, D. H. Valentine, and S. M. Walters (eds.). Flora Europaea, Cambridge University Press, Cambridge.

EL-SHAZLY, A., ATEYA, A. M., WITTE, L., and WINK, M. 1996. Quinolizidine alkaloids profiles of *Retama raetam*, *R. sphaerocarpa* and *R. monosperma*. *Z. Naturforsch.* 51c:301–308.

HERRERA, C. M. 1981. Are tropical fruits more rewarding to dispersers than temperate ones? *Am. Nat.* 118:896–907.

HERRERA, C. M. 1986. Vertebrate-dispersed plants: why they don't behave the way they should, pp. 5–18, *in* A. Estrada and T. H. Fleming (eds.). Frugivores and Seed Dispersal. Dr. W. Junk Publishers, Dordrecht.

MARTÍN-CORDERO, C., AYUSO, M. J., RICHOMME, P., and BRUNETON, J. 1989. Quinolizidine alkaloids from *Viscum cruciatum*, hemiparasitic shrub of *Lygos sphaerocarpa*. *Planta Med.* 55:196.

MARTÍN-CORDERO, GIL SERRANO, A. M., and AYUSO GONZALEZ, M. J. 1993. Transfer of bipiperidyl and quinolizidine alkaloids to *Viscum cruciatum* Sieber (Loranthaceae) hemiparasitic on *Retama sphaerocarpa*. *J. Chem. Ecol.* 19:2389–2393.

PARIS, R. R., and MOYSE, H. 1976. Precis de Matière Médicale. Masson, Paris.

POST, E. G., and DISMORE, J. E. 1932. Flora of Syria, Palestine & Sinaí. American Press, Beirut.

SALLE, G. 1983. Germination and Establishment of *Viscum album* L., p. 145, *in* M. Calder and P. Bernhardt (eds.). The Biology of Mistletoes. Academic Press, Sydney.

SENNEN, F., and MAURICIO, H. 1933. Catálogo de la flora del Rif Oriental. Gráficas, Ibérica, Melilla.

WINK, M. 1985. Chemische Verteidigung der Lupinen: Zur biologischen Bedentung der Chinolizidinalkaloide. *Plant Syst. Evol.* 150:65–81.

WINK, M. 1987. Chemical ecology of quinolizidine alkaloids. *ACS Symp. Ser.* 330:524–533.

WINK, M. 1988. Plant breeding: Importance of plant secondary metabolites for protection against pathogns and herbivores. *Theor. Appl. Genet.* 75:225–233.

WINK, M. 1992. The role of quinolizidine alkaloids in plant–insect interactions, pp. 131–166, *in* E. A. Bernays (ed.). Insect-plant Interactions. CRC Press, Boca Raton, Florida.

WINK, M. 1993a. Quinolizidine alkaloids, pp. 197–239, *in* P. G. Waterman (ed.). Methods in Plant Biochemistry, Volume 8. Academic Press, London.

WINK, M. 1993b. Allelochemical properties or the raison d'etre of alkaloids, pp. 1–118, *in* G. Cordell (ed.). The Alkaloids Academic Press, San Diego.

Journal of Chemical Ecology, Vol. 23, No. 8, 1997

MONOTERPENE COMPOSITION OF *Pinus sylvestris* VARIETIES RESISTANT AND SUSCEPTIBLE TO *Dioryctria zimmermani*

C. S. SADOF[1,*] and G. G. GRANT[2]

[1]*Department of Entomology, Purdue University*
West Lafayette, Indiana 47907-1158
[2]*Canadian Forest Service, Department of Natural Resources*
P.O. Box 490
Sault Ste. Marie, Ontario P6A 5M7, Canada

(Received September 24, 1996; accepted April 16, 1997)

Abstract—The proportion of monoterpenes and enantiomeric ratios of chiral monoterpenes were determined in two susceptible and two resistant varieties of Scotch pine, *Pinus sylvestris*, by capillary GC analysis of headspace volatiles emitted by 9-year-old trees. Half the trees sampled in each variety had been wounded by *Dioryctria zimmermani*, while the other half were unwounded. The presence of wounds had no significant ($P < 0.05$) effect on the proportion of monoterpenes or the ratio of enantiomers of each of the optically resolved monoterpenes in the four varieties. In contrast, monoterpene composition varied significantly ($P < 0.05$) among varieties. However, the results do not entirely support the putative role of car-3-ene and terpinolene as an oviposition stimulant for *D. zimmermani* in resistant varieties of Scotch pine. Although the resistant Lake Superior Blue virtually lacked these two monoterpenes, the resistant Belgian had a relatively high concentration of car-3-ene and terpinolene. Both resistant varieties, however, emitted relatively high levels of limonene, a monoterpene associated with increased oviposition in three other *Dioryctria* species. There was no evidence linking enantiomeric composition of chirally resolved monoterpenes with varietal resistance. The relative importance of monoterpenes as resistance factors and oviposition stimulants is discussed.

Key Words—Headspace volatiles, enantiomeric composition, host-plant resistance, phytochemical induction, oviposition, plant chemical defense.

INTRODUCTION

Zimmerman pine moth, *Dioryctria zimmermani* (Grote) (Lepidoptera: Pyralidae), is an important univoltine pest of Scotch pine, *Pinus sylvestris* L. Resis-

0098-0331/97/0800-1917$12.50/0 © 1997 Plenum Publishing Corporation

tance to this pest varies widely among European geographic varieties (Wright et al., 1975). Cortical monoterpene compositions of susceptible varieties tend to be higher in car-3-ene and terpinolene (Tobolski and Hanover, 1971; Ruby and Wright, 1976). Wright et al. (1975) suggested that these monoterpenes were highly volatile and could attract females during their summer oviposition flight.

Host plant volatiles emitted from pruning and mechanical injury, rust galls, and damage from other boring insects attract and stimulate oviposition by *Dioryctria* species (Heinrich, 1956; Fatzinger and Merkel, 1985; Hanula et al., 1985; Yonker and Schuder, 1987; Jactel et al., 1994, 1996a,b; Valterova et al., 1995; Shu et al., 1997). As major constituents of pine oleoresin and emitted pine volatiles, monoterpenes appear to be the critical cues for host finding and oviposition by female *Dioryctria*. Individual monoterpenes and their blends stimulate oviposition by the southern pine coneworm, *D. amatella* (Hulst) (Fatzinger and Merkel, 1985; Hanula et al., 1985), and fir coneworm, *D. abietivorella* (Grote) (Shu et al., 1997). The latter species is a shoot and cone borer that occasionally infests wounds produced by *D. zimmermani* on *P. sylvestris* (Carlson and Butcher, 1967). Several monoterpenes in pines are chiral, and specific enantiomers may be responsible for oviposition activity of *Dioryctria* and for host resistance (Valterová et al., 1995; Shu et al., 1997).

We report the identification and enantiomeric composition of monoterpene volatiles emitted by attacked and unattacked *P. sylvestris* varieties with widely different levels of resistance to *D. zimmermani*, and we evaluate the resistance hypothesis of Wright et al. (1975).

METHODS AND MATERIALS

Selection of Trees and Collection of Pine Volatiles. Nine-year-old Scotch pine trees exposed to attack by *D. zimmermani* were selected from plots near Idaville, Indiana. These plots contained three replicate blocks of nine varieties of Scotch pine from a range of European sources (Sadof, 1997). Each of the nine plots in each block contained a single variety of 36–42 trees. The relative resistance of each variety, based on the percent of trees in each plot that were wounded by *D. zimmermani*, has been reported separately (Sadof, 1997). Trees from the two most resistant varieties, Belgian and Lake Superior Blue, and the two most susceptible varieties, Hensler Orchard and Clonal East Anglia, were used in this study. Percentages of wounded trees were substantially lower for both Belgian (13.0 $\pm$ 3.6%) and Lake Superior Blue (10.7 $\pm$ 1.4%) than for Hensler Orchard (74.8 $\pm$ 0.7%) and Clonal East Anglia (61.2 $\pm$ 7.8%) varieties (Sadof, 1997). Seed of the Belgian trees was imported from the Campaign region of Belgium, Lake Superior Blue from France, and Hensler Orchard and Clonal East Anglia from the East Anglia area of England (Sadof 1997).

The hypothesis of Wright et al. (1975) implies that ovipositing *D. zimmermani* females select host trees by olfactory perception of volatile monoterpenes. We collected and analyzed volatile emissions rather than oleoresin collected from trees, to examine only those compounds likely to be used by ovipositing females. Volatiles emitted by Scotch pine branch sections were collected in the field under ambient conditions over a three-day period (August 28–30, 1995). We verified adult flight during this time by collecting adult females during the evening at our study site.

Four trees from each of the two susceptible Scotch pine varieties and from the Lake Superior Blue resistant variety and six trees from the Belgian resistant variety were sampled. Half the trees from each variety had at least one whorl that was wounded by *D. zimmermani*. Five sections (10 cm × ~1 cm diameter) were cut from a single branch from one of the resistant varieties and at the same time five sections were cut from a branch from one of the susceptible varieties, except for the final sample in which branch sections from wounded and unwounded Belgian trees were sampled at the same time. Both sets of branch sections were placed immediately into separate, clean, 0.5-liter glass Mason jars. The metal lid of each jar was fitted with an inlet glass tube (~6 mm ID) filled with 900 g activated charcoal to filter incoming air, and an outlet tube (~6 mm ID) filled with 100 mg Porapak Q (PQ) (held in place with silanized glass wool plugs) to collect the tree volatiles. The inlet and outlet tubes, which had been prepared in the laboratory before going to the field, were fastened to the lids with Swagelok GC fittings and Teflon ferules. The outlet tubes of both jars were connected by tubing to flowmeters and then to a Y-tube connected to a vacuum pump. Power for the pump was provided by a gas-powered generator. Airflow through each jar was adjusted to 200 ml/min by means of needle valves on the flowmeters.

Volatiles were collected from the branch sections for 1.5 hr. Jars were held in the shade and ambient air temperature (27–31.5°C) was monitored with a shaded thermometer. A clean jar was used for each sample of branch sections. Controls to check for breakthrough of atmospheric contaminants consisted of blank jars fitted with charcoal filter and Porapak Q. These jars were also sampled for 1.5 hr. At the end of a sampling period, each glass tube containing the Porapak Q and silanized glass wool was removed from the jars and quickly stored in a clean glass culture tube (13 × 100 mm), which was then stored on ice in a cooler. At the end of the field experiments, the Porapak Q samples were shipped on ice in the cooler to Sault Ste. Marie and stored in a freezer at −20°C until analyzed.

Monoterpene Analysis. Each sample of Porapak Q and associated glass wool was extracted with three 1-ml aliquots of double distilled *n*-hexane containing (−)-fenchone (0.20 μg/μl) as an internal standard. The aliquots were pooled and filtered through silanized glass wool (prewashed with acetone and

double-distilled *n*-hexane) to form a stock solution. The stock solutions were diluted 10-fold, and analyzed for monoterpene composition on two capillary GC columns: a nonpolar HP-1 (25 m × 0.2 mm ID × 0.33-μm film thickness; Hewlett Packard, Mississauga, Ontario) housed in a Varian 3500 gas chromatograph, and, to determine the enantiomeric composition of optically active monoterpenes, on a chiral b-DEX-120 column (30 m × 0.25 mm ID × 0.25-μm film thickness; Supelco, Mississauga, Ontario), housed in a Varian 3700 gas chromatograph. The GC conditions for analysis on the HP-1 column were as follows: the detector temperature was 300°C; injector temperature was 250°C; the column temperature was programmed as follows: the initial temperature of 40°C was held for 2 min, then increased at a rate of 2.5°C/min to 175°C, which was held for 10 min, and then increased at 30°C/min to a final temperature of 210°C, which was held for 8 min. For the chiral b-DEX-120 column, the detector temperature was 300°C and injector temperature was 250°C; the column temperature was programmed as follows: an initial temperature of 35°C held for 1.0 min, then increased at a rate of 1°C/min up to 160°C, which was held for 30 min. Identification of monoterpenes was confirmed by comparison of GC retention times with authentic monoterpenes.

Statistical Analysis. The contribution of each monoterpene component to headspace volatiles was analyzed in a two-way analysis of variance to determine if it varied significantly among pine varieties and between wounded and unwounded trees (PROC GLM; SAS Institute, 1985). To correct for nonnormality, arcsin square root transformations on the proportional contribution of each monoterpene were conducted prior to analysis. Type III sums of squares were used in the analysis to correct for the unbalanced experimental design. Fisher's protected least significant difference test was conducted on each of the significant ($P < 0.05$) sources of variation in the analysis of variance. The pattern of significantly different means was examined to determine if they were similar within and between the two resistant and susceptible varieties.

The potential importance of enantiomers of chiral monoterpenes was determined by examining their variation among varieties. The effects of variety and wounding by *D. zimmermani* on the percent (−)-enantiomers of each chirally resolved monoterpene was determined in a two-way analysis of variance. The proportion of (−)-enantiomers of each chirally resolved terpene was transformed by arcsin square root prior to statistical analysis.

RESULTS

Effects of Wounding and Tree Variety on Monoterpene Composition. There was no effect of wounding on the monoterpene composition (Table 1) or the ratio of monoterpene enantiomers (Table 2) in the headspace volatiles collected

from trees used in this study. There was, however, a significant ($P < 0.05$) effect of tree variety on the proportions of seven monoterpenes in the headspace volatiles (Table 1).

Monoterpene composition of headspace volatiles emanating from susceptible varieties was more homogeneous than that of the resistant varieties. No significant differences ($P < 0.05$) between the susceptible varieties were found for any of the monoterpenes. In contrast, six monoterpenes varied significantly ($P < 0.05$) between the two resistant varieties, Lake Superior Blue and Belgian. Only limonene was homogeneous within susceptible and resistant varieties. Subsequent pooling of data within resistance classes in an unbalanced one-way analysis of variance indicated that limonene emission levels were significantly greater ($F = 12.56$; $df = 1,16$; $0.01 > P > 0.001$) in resistant varieties than in those of the susceptible varieties.

Of the five chiral monoterpenes detected in the Scotch pine trees sampled, only car-3-ene could not be resolved on the chiral GC column. With the exception of α-pinene, the (−)-enantiomers predominated (>60%) (Table 2). Although there was a trend to higher levels of some of the (−)-enantiomers in the resistance varieties, their proportions did not vary significantly among tree varieties and were not affected by the presence of *D. zimmermani* wounds.

DISCUSSION

Our data do not entirely support hypotheses associating susceptibility of pines to attack by *D. zimmermani* with high levels of car-3-ene or terpinolene in pine oleoresin (Tobolski and Hanover, 1971; Wright et al., 1975; Ruby and Wright, 1976). Although the proportions of these two monoterpenes were high in the two susceptible varieties, they were also equally high in Belgian, one of the resistant varieties in this study. In contrast, as predicted by this hypothesis, both of these monoterpenes, were virtually lacking in the resistant Lake Superior Blue variety. Car-3-ene has been established as an oviposition stimulant for *D. abietivorella* (Shu et al., 1997), a species that coinfests Scotch pine with *D. zimmermani* (Carlson and Butcher, 1967). A similar behavioral effect for car-3-ene on *D. zimmermani* would be consistent with the hypothesis (Wright et al., 1975) that low levels of this monoterpene are associated with resistance. Lake Superior Blue also had significantly lower levels of four additional quantitatively minor monoterpenes (sabinene, α-phellandrene, α-terpinolene, and *p*-cymene) than were present in the Belgian and the two susceptible varieties, and these low levels could also contribute to resistance.

Limonene was the only monoterpene that was consistently higher in both resistant varieties. Its has been identified as a resistance factor against conifer-feeding scolytids and curculionids in *Abies*, *Picea*, and *Pinus* due to its action

TABLE 1. EFFECTS OF WOUNDING AND VARIETY ON MONOTERPENE COMPOSITION OF HEADSPACE VOLATILES OF *Pinus sylvestris* ON TREES WITH AND WITHOUT WOUNDS FROM *Dioryctria zimmermani:* SUMMARY OF ANALYSIS OF VARIANCE AND VARIETAL MEANS[a]

	Effect of wounding [df = (1,10)]		Effect of variety [df = (3,10)]		Composition (%) in each variety			
					Susceptible		Resistant	
Monoterpene	F	P	F	P	Clonal East Anglia (N = 4)	Hensler Orchard (N = 4)	Belgian (N = 6)	Lake Superior Blue (N = 4)
Major monoterpenes								
α-Pinene	1.54	0.24	2.76	0.097	11.8 a	17.0 a	21.5 a	30.0 a
β-Pinene	0.09	0.77	2.18	0.153	9.5 a	17.1 a	4.7 a	31.0 a
Myrcene	0.66	0.44	0.42	0.74	16.1 a	18.0 a	9.1 a	14.2 a
Car-3-ene	1.75	0.22	4.58	0.028*	37.3 a	32.4 a	27.3 a	0.0 b
β-Phellandrene	0.19	0.67	0.90	0.47	11.7 a	7.9 a	17.1 a	7.9 a
Limonene	0.01	0.9	4.26	0.035*	2.7 a	1.9 a	11.3 ab	15.8 b
Terpinolene	0.26	0.62	4.60	0.029*	7.8 a	5.0 a	6.0 a	0.4 b

Minor monoterpenes								
Tricyclene	1.90	0.2	2.80	0.095	0.02 a	0.02 a	0.02 a	0.03 a
α-Thujene	0.15	0.7	0.89	0.48	0.05 a	0.06 a	0.06 a	0.04 a
Camphene	1.74	0.22	2.35	0.13	0.16 a	0.23 a	0.32 a	0.43 a
Sabinene	1.60	0.24	7.94	0.005*	1.23 a	1.83 a	0.97 a	0.12 b
α-Phellandrene	0.22	0.65	4.00	0.041*	0.12 a	0.10 a	0.15 a	0.06 b
α-Terpinolene	1.40	0.26	5.07	0.022*	0.20 a	0.20 a	0.20 a	0.01 b
p-Cymene	0.06	0.81	5.67	0.016*	0.20 a	0.13 a	0.17 a	0.04 b
γ-Terpinene	0.17	0.68	2.81	0.09	0.69 a	0.49 a	0.58 a	0.03 a
Camphor	1.52	0.25	1.95	0.19	0.02 a	0.01 a	0.00 a	0.00 a
Borneol	0.15	0.7	0.68	0.58	0.00 a	0.01 a	0.00 a	0.05 a
Bornyl acetate	0.18	0.68	2.56	0.11	0.18 a	0.03 a	0.11 a	0.02 a
Terpinyl acetate	0.01	0.9	0.38	0.76	0.01 a	0.00 a	0.02 a	0.01 a

[a] Means followed by the same letter are not significantly different within a row according to a Fishers protected least significant difference test ($P < 0.05$).
*Significant effect of variety ($P < 0.05$).

TABLE 2. EFFECTS OF WOUNDING AND VARIETY ON PERCENT (−)-ENANTIOMERIC COMPOSITION OF FOUR CHIRAL MONOTERPENES IN HEADSPACE VOLATILES OF *Pinus sylvestris* ON TREES WITH AND WITHOUT WOUNDS FROM *Diorycrtria zimmermani:* SUMMARY OF ANALYSIS OF VARIANCE AND VARIETAL MEANS[a]

	Effect of wounding [df = (1,10)]		Effect of variety [df = (3,10)]		(−)-Enantiomer (%) in each variety			
					Susceptible		Resistant	
Monoterpene	F	P	F	P	Clonal East Anglia (N = 4)	Hensler Orchard (N = 4)	Belgian (N = 6)	Lake Superior Blue (N = 4)
(−)-α-Pinene	0.00	0.9	0.59	0.6	43.5 a	48.7 a	60.0 a	55.6 a
(−)-β-Pinene	0.08	0.78	0.33	0.8	93.7 a	94.4 a	88.0 a	94.8 a
(−)-β-Phellandrene	3.61	0.09	0.07	0.9	98.9 a	99.3 a	98.6 a	99.0 a
(−)-Limonene	0.15	0.71	1.59	0.25	59.9 a	62.4 a	76.1 a	86.4 a

[a]Means followed by the same letter are not significantly different within a row according to a Fishers protected least significant difference test ($P < 0.05$).

as a toxicant and oviposition deterrent (Coyne and Lott, 1976; Sturgeon, 1979; Alfaro et al., 1980; Raffa et al., 1985, Nordlander, 1990). It is unlikely, however, to be involved in resistance against *D. zimmermani* because of its function as an oviposition stimulant for three other species in this genus (Hanula et al., 1985; Fatzinger and Merkel, 1985; Shu et al., 1997; Jactel et al., 1996b). Still, limonene remains as the only monoterpene that sets Belgian apart from the susceptible varieties. The volatile fraction of limonene in the present study is well within the range reported in studies demonstrating its stimulatory effect on *Dioryctria* (5–15% of total). There was no evidence that specific enantiomers of any of the chiral monoterpenes play a role in resistance.

We were unable to detect ($P < 0.05$) effects of wounding by *D. zimmermani* on the monoterpene composition of host tree volatiles or the enantiomeric ratio of the chiral monoterpenes in a tree. Studies that report differences between monoterpene content of pines infested and uninfested by *Dioryctria* spp. involve plantings of conspecifics with a high degree of intertree variability (Valterová et al., 1995; Jactel et al., 1996b). These differences reflect the influence of monoterpenes on oviposition and are not evidence for an induced response. Trees in the present study, however, were likely to exhibit less intertree variability because they are varieties of a single species selected for uniformity in growth and appearance. Our inability to find an effect of wounding by *D. zimmermani* is, therefore, consistent with studies of *Pinus pinaster*, *P. ponderosa*, *P. resinosa*, and *P. banksiana*, which indicate that simple mechanical injury fails to change monoterpene composition (Raffa, 1991; Klepzig et al., 1995; Raffa and Smalley, 1995; Jactel et al., 1996b). Our practice of excising stems from wounded and unwounded trees during our sampling process was not, by itself, likely to have obscured real differences in monoterpene composition. Total concentration of monoterpenes, however, can increase in response to mechanical injury, albeit lesser in magnitude than that induced by fungal inoculum (Raffa and Smalley, 1995; Popp et al., 1995).

The overwhelming dissimilarity of the monoterpene profiles between Lake Superior Blue and Belgian suggests that the resistance mechanisms for these two resistant varieties are different. Low levels of car-3-ene (and possibly low levels of some quantitatively minor monoterpenes) in Lake Superior Blue could reduce oviposition by *D. zimmermani* females, as suggested by Wright et al. (1975). In contrast, the overwhelming similarity between the monoterpene composition of Belgian and two susceptible varieties suggests oviposition rates on these three varieties could be similar. Therefore, resistance in the Belgian variety may occur by some other mechanism, such as differences in oviposition chemical cues other than monoterpenes or differences in host chemicals that kill larvae after eggs hatch (Raffa and Smalley, 1995). Alternatively, resistance mechanisms could include deterrent physical factors for either larval or adult stages acting alone or in combination with chemical cues. It is important to acknowledge that

our relatively small sample size limits our ability to make inferences about specific resistance mechanisms. Further studies on the behavioral cues that control host finding and oviposition by *D. zimmermani* on these varieties are needed to evaluate these possibilities.

Acknowledgments—This research was supported in part by a grant from the Indiana Christmas Tree Growers Association to C.S. and funds from the Canadian Forest Service Green Plan to G.G.G. We thank Mr. Daryl Miller for allowing us to conduct this research on his farm. We also thank D. Lombardo for assistance with GC analysis of the monoterpenes. This is scientific paper number 15218 of the Indiana Agricultural Research Program.

REFERENCES

ALFARO, R. I., PIERCE, H. D., Jr., BORDEN, J. H., and OEHLSCHLAGER, A. C. 1980. Role of volatile and nonvolatile components of Sitka spruce bark as feeding stimulants for *Pissodes strobi* Peck (Coleoptera: Curculionidae). *Can. J. Zool.* 58:626–632.

CARLSON, R. B., and BUTCHER, J. W. 1967. Biology and behavior of Zimmerman pine moth, *Dioryctria zimmermani*, in Michigan. *Can. Entomol.* 99:529–436.

COYNE, K. F., and LOTT, L. H. 1976. Toxicity of substances in pine oleoresin to southern pine beetles. *J. Ga. Entomol. Soc.* 11:301–305.

FATZINGER, C. W., and MERKEL, E. P. 1985. Oviposition and feeding preferences of the southern pine coneworm (Lepidoptera: Pyralidae) for different host-plant materials and observations on monoterpenes as an oviposition stimulant. *J. Chem. Ecol.* 11:689–699.

HANULA, J. L., BERISFORD, C. W., and DEBARR, G. L. 1985. Monoterpene oviposition stimulants of *Dioryctria amatella* in volatiles from fusiform rust galls and second year loblolly pine cones. *J. Chem. Ecol.* 11:943–952.

HEINRICH, C. 1956. American moths of the subfamily Phycitinae. USNM Bul. 207, Smithsonian Institution, Washington, D.C.

JACTEL, H., MENASSIEU, P., and RAISE, G. 1994. Infestation dynamics of *Dioryctria sylvestrella* (Ratz.) (Lepidoptera: Pyralidae) in pruned maritime pine (*Pinus pinaster* Ait.). *For. Ecol. Manage.* 67:11–22.

JACTEL, H., MENASSIEU, P., RAISE, G., and BURBAN, C. 1996a. Sensitivity of pruned maritime pine (*Pinus pinaster* Ait) to *Dioryctria sylvestrella* Ratz. (Lep., Pyralidae) in relation to tree vigour and date of pruning. *J. Appl. Entomol.* 120:147–153.

JACTEL, H., KLEINHENTZ, M., MARPEAU-BEZARD, A., MARION-POLL, F., MENASSIEU, P., and BURBAN, C. 1996b. Terpene variations in maritime pine constitutive oleoresin related to host tree selection by *Dioryctria sylvestrella* Ratz. (Lepidoptera: Pyralidae). *J. Chem. Ecol.* 22:1037–1050.

KLEPZIG, K. D., KRUGER, E. L., SMALLEY, E. B., and RAFFA, K. F. 1995. Effects of biotic and abiotic stress on induced accumulation of terpenes and phenolics in red pines inoculated with bark beetle-vectored fungus. *J. Chem. Ecol.* 21:601–626.

NORDLANDER, G. 1990. Limonene inhibits attraction to α-pinene in the pine weevils *Hylobies abietis* and *H. pinastri*. *J. Chem. Ecol.* 16:1307–1320.

POPP, M. P., JOHNSON, J. D., and LESNEY, M. S. 1995. Characterization of the induced response of slash pine to inoculation with bark beetle vectored fungi. *Tree Physiol.* 15:619–623.

RAFFA, K. F. 1991. Induced defensive reactions in conifer-bark beetle systems, pp. 245–276, *in* D. W. Tallamy and M. J. Raupp (eds.). Phytochemical Induction by Herbivores. John Wiley & Sons, New York.

RAFFA, K. F., and SMALLEY, E. B. 1995. Interaction of pre-attack and induced monoterpene concentrations in host conifer defense against bark beetle-fungal complexes. *Oecologia* 102:285–295.

RAFFA, K. F., BERRYMAN, A. A., SIMASKO, J., TEAL, W., and WONG, B. L. 1985. Effects of grand fir monoterpenes on the fir engraver, *Scolytus ventralis* (Coleoptera: Scolytidae), and its symbiotic fungus. *Environ. Entomol.* 14:552–556.

RUBY, J. L., and WRIGHT, J. W. 1976. A revised classification of geographic varieties in Scots pine. *Silvae Genet.* 25:169–175.

SADOF, C. S. 1996. Resistance of Scotch pine varieties to Zimmerman pine moth (Lepidoptera: Pyralidae) and its impact on sales in a choose and cut Christmas tree plantation. *Great Lakes Entomol.* 29:183–190.

SAS INSTITUTE. 1985. SAS User's Guide: Statistics, Version 5 Edition. SAS Institute Inc., Cary, North Carolina, 956 pp.

SHU, S., GRANT, G. G., LANGEVIN, D., LOMBARDO, D., and MACDONALD, L. 1997. Oviposition and electroantennogram responses of *Dioryctria abietivorella* (Lepidoptera: Pyralidae) elicited by host monoterpenes and their enantiomers. *J. Chem. Ecol.* 23:35–50.

STURGEON, K. B. 1979. Monoterpene variation in ponderosa pine xylem resin related to western pine beetle predation. *Evolution* 33:803–814.

TOBOLSKI, J. J., and HANOVER, J. W. 1971. Genetic variation of the monoterpenes of Scotch pine. *For. Sci.* 17:293–299.

VALTEROVÁ, I., SJÖDIN, K., VROC, J., and NORIN, T. 1995. Contents and enantiomeric composition of monoterpene hydrocarbons in xylem oleoresins from four *Pinus* species growing in Cuba. Comparison of trees unattacked and attacked by *Dioryctria horneana*. *Biochem. Syst. Ecol.* 23:1–15.

WRIGHT, J. W., WILSON, L. F., and BRIGHT, J. N. 1975. Genetic variation in resistance of Scotch pine to Zimmerman pine moth. *Great Lakes Entomol.* 8:231–236.

YONKER, J. W., and SCHUDER, D. L. 1987. Appearance of damage symptoms and reinfestation rates for Christmas trees attacked by the Zimmerman pine moth, *Dioryctria zimmermani* (Lepidoptera: Pyralidae). *Great Lakes Entomol.* 20:25–29.

Journal of Chemical Ecology, Vol. 23, No. 8, 1997

(Z)-11-EICOSEN-1-OL, A MAJOR COMPONENT OF *Apis cerana* VENOM

J. O. SCHMIDT,[1,*] E. D. MORGAN,[2] N. J. OLDHAM,[2,3] R. R. DO NASCIMENTO,[2,4] and F. R. DANI[2]

[1]*Carl Hayden Bee Research Center, USDA-ARS*
2000 East Allen Road
Tucson, Arizona 85719

[2]*Department of Chemistry, Keele University*
Staffordshire ST5 5BG, England

(Received December 4, 1996; accepted March 24, 1997)

Abstract—The unusual venom of *Apis cerana* contains large oily droplets within an otherwise aqueous secretion. Chemical analysis (GC-MS) revealed that the venom oil consists of (Z)-11-eicosen-1-ol (81.2%), other linear alcohols (7.7%), and linear hydrocarbons (11.1%). The eicosenol is present in extremely large quantities, averaging over 250 μg per insect, and is absent, or present in small quantities, in other parts of the sting apparatus. An investigation of the site of eicosenol storage in *A. mellifera* showed it to be absent from the venom and to be associated with the setose area where the more volatile components of the alarm pheromone are stored, as previously shown by others. A third honeybee species, *A. dorsata*, does not to contain the alcohol. The function of eicosenol in *A. cerana* in not clear, but may serve to mark stung intruders with pheromone or to attract foragers to marked floral resources.

Key Words—(Z)-11-Eicosen-1-ol, octadecanol, eicosanol, docosenol, alarm pheromone, venom, *Apis cerana*, *Apis koschevnikovi*, *Apis dorsata*, Apidae, Hymenoptera.

INTRODUCTION

Honeybees (*Apis mellifera* L.) are well known for their defensive behavior and multiple stinging attacks. The crucial role played by odor emanating from the

*To whom correspondence should be addressed.
[3]Present address: Department of Zoology, AMS, University of Reading, Whiteknights, Reading RG1 5RN, England.
[4]Present address: Departmento de Quimica, Universidade Federal de Alagoas, 57000 Maceió AL, Brazil.

0098-0331/97/0800-1929$12.50/0 © 1997 Plenum Publishing Corporation

sting apparatus in enhancing and stimulating these attacks was first recorded by Huber (1814), who surmised that the attack was induced by the odor of the venom. More than a century later, the source of that alarm odor was shown not to be the venom itself, nor was it associated with the venom glands and sac (Ghent and Gary, 1962; Maschwitz, 1964; Free and Simpson, 1968; Lensky et al., 1995). Instead, it arose from elsewhere in the venom apparatus and collected in an absorbent membranous area, called the setose area, that surrounded the sting base (Ghent and Gary, 1962; Maschwitz, 1964; Lensky et al., 1995). Isopentyl acetate (isoamyl acetate) was shown to be an important component of the alarm pheromone, although it did not reproduce the effect of a whole poison apparatus (Boch et al., 1962; Free and Simpson, 1968). Meanwhile, many other alarm pheromone components were being identified from the sting chamber (Blum et al., 1978; Blum and Fales, 1988). From a list of more than two dozen identified sting compounds, at least 15 were shown active by bioassay (Collins and Blum, 1982, 1983; Free et al., 1989). All of the original active compounds were oxygenated, low-molecular-weight volatiles containing 4–11 carbons. Pickett et al. (1982) identified the first active high-molecular-weight pheromonal component, (Z)-11-eicosen-1-ol, and showed it to be an important component of the alarm pheromone from the sting. Large quantities were present (approximately 5 μg per worker) and the source, along with isopentyl acetate, was traced to the "dorsal anterior parts" of the sting apparatus "associated with the quadrate plates" (Pickett et al., 1982), i.e., presumably the setose area.

All four tested species of Apis were shown to possess sting-based alarm pheromones, and all contained isopentyl acetate in their pheromonal blend (Morse et al., 1967), but differences in the alarm pheromone were evident: *Apis dorsata* and *A. florea* reacted longer to their alarm pheromones than those of *A. mellifera* or *A. cerana*, and their pheromonal blends contained 2-decen-1-yl acetate, which was not present in the blends of *A. mellifera* or *A. cerana* (Koeniger et al., 1979). The longer lasting activities of the pheromones of *A. dorsata* and *A. florea* were attributed to this unsaturated C_{10} acetate, which also appeared to serve as a long term olfactory marker placed in stung attackers (Veith et al., 1978).

Despite 35 years of effort, the exact glandular or biosynthetic source(s) of the sting alarm pheromone has eluded discovery. The one fact that seems certain, at least in the case of *Apis mellifera*, is that the pheromone collects and is stored in the setose area surrounding the sting base (Ghent and Gary, 1962; Maschwitz, 1964; Gunnison and Morse, 1968; Lensky et al., 1995). The setose area is thin, membranous, and contains no secretary cells (Ghent and Gary, 1962; Lensky et al., 1995). This had led to the assumption that the pheromones were synthesized somewhere else in glands and flowed to the setose area. Of the four known glands in the sting apparatus of *A. mellifera*, the Dufour's gland and the venom

gland reservoir were ruled out as pheromone synthesis or storage sites (Maschwitz, 1964). The remaining two glandular complexes, the Koschevnikov gland and the sting sheath gland, have been variously suggested as the pheromone sources (Ghent and Gary, 1962; Cassier et al., 1994; Lensky et al., 1995). Nevertheless, none of these glands appear to contain meaningful quantities of alarm pheromone, and none alone, or combined in the absence of the setose area, are capable of eliciting near normal levels of response (Maschwitz, 1964; Gunnison and Morse, 1968; Cassier et al., 1994). The Koschevnikov gland appears to be too distant from the setose area to be the source (but see Tel-Zur and Lensky, 1995). The sting sheaths appear in some studies to be viable sources of pheromone (Lensky et al., 1995), but conflicting conclusions are reported by Maschwitz (1964), who discovered that newly emerged bees whose sting sheaths with glands were ablated could, nevertheless, produce full alarm pheromone activity at age 7 days.

In dissecting the venom apparatus of *A. cerana*, we observed oily droplets in the aqueous venom, which were not seen in *Apis mellifera*, *A. dorsata*, or *A. florea*, and which suggested a possible new pheromone source. Described here is the identification of the composition of these oily droplets and, in an effort to locate sources of alarm pheromones in honeybees, a comparison of the sting chemistry and tissue location of eicosenol in *Apis cerana* and two other species of honeybees.

METHODS AND MATERIALS

Initial samples of *Apis cerana* were obtained in 1994 from frozen laboratory bees collected in Hong Kong. Further samples of *A. cerana* were collected March 10, 1995, from laboratory colonies maintained at the University of Malaysia located at Serdan, Selangor. *Apis dorsata* were collected March 12, 1995, from wild colonies located in a tree near Pedu Lake, Kedah, Malaysia. *Apis mellifera* (both European and Africanized) were obtained in October 1994 from laboratory (European) and feral (Africanized) colonies located in Tucson, Arizona, USA.

Bees were collected by net and placed in jars that were held either on ice until dissected, or frozen first in the case of the Hong Kong samples. Pooled venom samples from *A. cerana* (47 from Hong Kong and 21 from Malaysia) were collected according to the method of Schmidt (1986). Briefly, the sting apparatus was quickly removed from the bee by pulling the sting shaft with forceps, it was placed in a droplet of distilled water, the duct leading from the venom sac to the sting base pinched closed, and the venom sac removed. The removed sac was then rinsed quickly in water and placed in a fresh drop of

water along with the other pooled sacs. The sacs were then ruptured, the venom drained into the water, and the empty sacs discarded. The aqueous portion was removed with a 50-μl chromatography syringe (Hamilton Corp., Reno, Nevada), and the remaining oily liquid that adhered to the spot plate was dissolved in methylene chloride and placed in a vial. Sting apparatuses from *A. cerana*, *A. dorsata*, and *A. mellifera* were also dissected without water and isolated glands or parts were flame sealed in small glass tubes according to the method of Morgan (1990). For controls, entire sting apparatuses were also sealed in capillaries.

Solid samples were subjected to solventless GC-MS analysis (Morgan and Wadhams, 1972; Morgan, 1990), and oil samples were analyzed by liquid injection. GC-MS analysis was conducted on a Hewlett Packard 5890 gas chromatograph, equipped with a BPX5 capillary column (12 m $\times$ 0.32 mm $\times$ 0.25 μm, SGE, Milton Keynes, UK), coupled to a HP 5970B mass selective detector. The oven temperature was programmed from either 35°C or 100°C to 300°C at 8°C/min, and helium was used as the carrier gas at a flow of 1 ml/min. (*Z*)-11-Eicosen-1-ol was quantified by comparison of peak area with that of a standard solution of oleyl alcohol. Hydrocarbons were similarly quantified using a solution of *n*-alkanes (C_{10}–C_{20}). Reference standards of (*Z*)- and (*E*)-11-eicosen-1-ol were obtained from Nu Chek Prep (Elysian, Minnesota).

Dimethylthiolation was carried out on an aliquot of the venom oil solution (30 μl) as described previously (Billen et al., 1986) except that the reaction was carried out at 80°C overnight and under a nitrogen atmosphere with complete exclusion of oxygen, which gave almost quantitative yield of the addition product. The products were analyzed by GC-MS using the condition listed above, except that the oven temperature was programmed from 150°C to 270°C at 5°C/min.

RESULTS

Dissections of stings of *Apis mellifera* (European, Africanized, and Cape honeybees), *A. dorsata*, *A. florea*, and *A. andreniformis* revealed all to contain venom sacs filled with clear, transparent liquid that readily dissolved in water when the sacs were ruptured. This water-soluble fraction contains on average 27, 150, and 218 μg/sac of proteinaceous material for *Apis florea*, *A. mellifera*, and *A. dorsata*, respectively (Schmidt, 1995). In contrast, venom sacs of *Apis cerana* collected in 1985 in Sorsogon, Philippines as well as the analyzed sacs from *A. cerana* from Hong Kong and Malaysia contained very large and conspicuous oily droplets within the otherwise aqueous contents of the venom sac. Similar lipoidal droplets were observed in the venom sacs of *Apis koschevnikovi*, a sister species of *A. cerana* (which until recently was considered a subspecies

of *A. cerana*), collected in Sabah, Borneo, Malaysia. In all ruptured sacs of *A. cerana* or *A. koschevnikovi*, the aqueous portion was freely miscible with water and the oily droplets floated on the surface. The sacs from Sorsogon contained 43 μg of proteinaceous material (Schmidt, 1995), and those from Hong Kong and Malaysia contained over 300 μg of oil.

Analysis of the oily droplets from *A. cerana* venom, by GC-MS, revealed the presence of one major substance and seven identifiable minor components (Figure 1 and Table 1). Compounds 2, 4, 6, and 8, which combined represented 11.15% of the total, were identified as the linear, saturated hydrocarbons with odd carbon numbers ranging from C_{21} to C_{27} by their characteristic mass spectra. Compounds 1, 4, and 5, representing 7.68% of the total secretion, were identified as linear, primary alcohols with even carbon numbers ranging from C_{18} to C_{22}. They were identified on the basis of their parent ion being the molecular ion less 18 amu resulting from the loss of water, plus their retention times, which were typical of alcohols, rather than hydrocarbons. Traces of linear alkanes with an even number of carbon atoms ranging from C_{22} to C_{26} were found in more concentrated samples. The molecular ions, and relative retention times allowed the identity of each compound to be established (see Table 1). Compound 3 possessed a somewhat different mass spectrum, reminiscent of those exhibited by dienes. Indeed, the highest visible ion, at *m/z* 278, would have been consistent with eicosadiene ($C_{20}H_{38}$). However, the retention time of compound 3 (almost coeluting with peak 4, tricosane), and the fact that linear

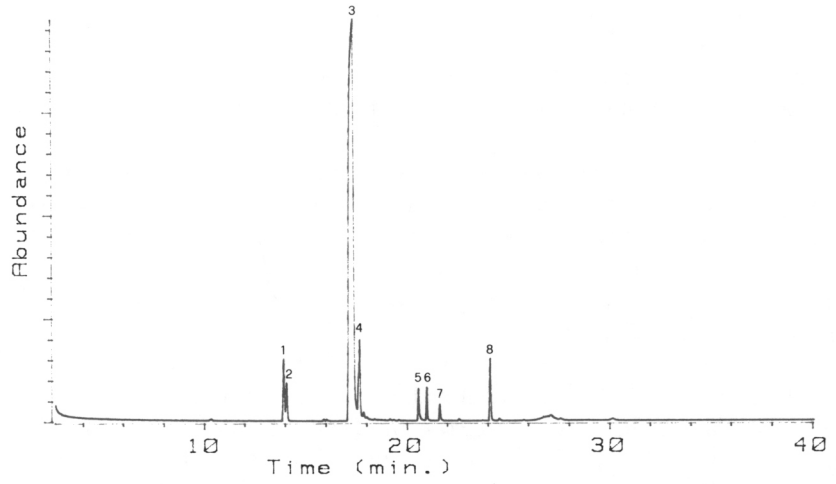


FIG. 1. Gas chromatogram of the venom oil of *A. cerana*. Numbering of the peaks corresponds to that in Table 1.

TABLE 1. PERCENTAGE COMPOSITION OF VENOM OIL OF *A. cerana*

Compound	Number in Figure 1	Amount (%)
Octadecanol	1	3.80
Heneicosane	2	2.40
(Z)-11-Eicosen-1-ol	3	81.18
Eicosanol and tricosane[a]	4	2.07
		3.54
Docosenol	5	1.81
Pentacosane	6	1.73
Phthalate plasticizer (impurity)	7	
Heptacosane	8	3.48

[a]The percentages of the two compounds were estimated on the basis of the proportion between the area given by two diagnostic ions.

hydrocarbons, with an even number of carbon atoms, seldom occur as major components of insect secretions, made this structure unlikely. A much more likely possibility was that compound 3 was eicosenol, with the ion at *m/z* 278 occurring due to loss of water from the absent molecular ion at *m/z* 296. A C_{20} alcohol was expected to have such a retention time and was also deemed more feasible than the diene on biogenetic grounds. Furthermore, (Z)-11-eicosen-1-ol had already been identified in *Apis*, as a component of the alarm pheromone of *A. mellifera* (Pickett et al., 1982). Comparison of the GC retention time and mass spectrum of eicosenol, from *A. cerana*, with that from *A. mellifera* confirmed the identification. The double bond position in eicosenol from *A. cerana* was established by GC-MS analysis of the dimethylthiolation reaction mixture, which showed a disappearance of the eicosenol peak and the appearance of a new peak with a molecular ion at *m/z* 390, corresponding to the adduct of eicosenol and dimethyldisulfide. The mass spectrum showed two ions due to characteristic alpha-cleavage about the two thio ether groups, one at *m/z* 173 and one at *m/z* 217, which were consistent with a double-bond position at C_{11} in the eicosenol. The stereochemistry of the double bond was determined as *Z* by comparison of *A. cerana* eicosenol with synthetic standards of (*Z*)- and (*E*)-11-eicosenol. The bee material had an identical relative retention time and mass spectrum to that of (Z)-11-eicosenol. There was no evidence of traces of (*E*)-11-eicosenol in the venom or intact sting material. Octadecanol, eicosanol (unresolved from tricosane), and docosenol were also identified by mass spectra and retention times. Heneicosenol was found in more concentrated samples.

Venom oils from colonies from Hong Kong and from Malaysia gave the

same composition. Interestingly, none of the volatile components associated with the *A. cerana* alarm pheromone, such as isoamyl acetate, octyl acetate, and 2-nonanol were detected in the venom oil.

Although the venom oil was quite clearly visible as droplets within the venom sac, we decided to demonstrate unambiguously that the venom gland was the actual source of the material. To facilitate this, a number of *A. cerana* workers were dissected to yield either the whole sting apparatus (including venom gland and sac, Dufour's gland, Koschevnikov glands, sting sheath glands, and the setose area), the venom sac alone, or the sting apparatus minus the venom sac. The dissected tissues individually sealed in small glass capillaries and analyzed by solventless GC-MS revealed that eicosenol was present in the whole sting apparatus and venom sac samples in large amounts (184 $\pm$ 49 μg and 265 $\pm$ 139 μg per insect respectively). However, samples of the sting apparatus minus the venom sac contained relatively little eicosenol (8.1 $\pm$ 7.3 μg per insect), which could result from the venom left over in the duct base as the venom sac was torn from the sting. Thus, the venom was clearly identified as the source of eicosenol in *A. cerana*.

While volatile alarm pheromone components (isopentyl acetate, octyl acetate etc.) were visible in variable amounts in whole sting apparatus samples and in sting apparatuses minus their venom sacs, they were absent from the cleanly dissected venom sacs. This supported our earlier finding that the volatiles did not form part of the venom oil.

In light of these results, we decided to reexamine the site of eicosenol storage in both European and Africanized *A. mellifera*. As with *A. cerana*, the sting apparatus of *A. mellifera* was dissected to give samples of the isolated clean venom sac and of the sting apparatus minus the venom sac. The amounts of eicosenol found in each tissue (Table 2) demonstrated that the venom sac was not the source of the alcohol, since venom sacs of Africanized bees contained only 49 ng and sacs of European bees 8.7 ng of eicosenol. In contrast, the rest of the sting apparatus contained 899 ng and 565 ng, respectively.

In order to locate the storage site of the alcohol, the sting apparatus of *A. mellifera* (Africanized form) was further dissected to yield the setose area, the Koschevnikov glands, the sting sheath glands, and the Dufour's gland. The amounts of eicosenol in each of these tissues revealed that the setose area contained 800 ng of eicosenol [together with (*Z*)-9-nonadecene in similar quantity, and the volatile components of the alarm pheromone], while the other glands contained only 10–30 ng. This finding confirmed the earlier work of Pickett et al. (1982), which showed the dorsal anterior quadrate plate area as the location of eicosenol in *A. mellifera*, since the setose area is associated with that area. We also examined the sting apparatus of *A. dorsata* for the presence of eicosenol and could not detect the alcohol in whole stings, or in any of the sting apparatus tissues.

TABLE 2. DISTRIBUTION OF (Z)-11-EICOSEN-1-OL IN STING APPARATUS OF *A. mellifera*

Gland/tissue	(Z)-11-Eicosen-1-ol (ng, mean ± SD) (N = 4)
Africanized	
Venom sac	49 ± 37
Sting apparatus minus venom sac	899 ± 663
European	
Venom sac	9 ± 10
Sting apparatus minus venom sac	565 ± 342
Setose area	800 ± 300
Koschevnikov glands	10 ± 10
Sting sheath glands	30 ± 40
Dufour gland	20 ± 40

DISCUSSION

The presence of oily droplets is highly unusual in the venom of bees and other Hymenoptera (Schmidt, 1986; and unpublished observations). Further evidence of the unusual nature of the venom of *Apis cerana* is seen in the gross composition of the venom: only 43 μg of protein is present along with 300 μg oil, as compared to the slightly larger *A. mellifera*, which contains 150 μg protein (Schmidt, 1995) and no oil. The oil composition of 81% eicosenol plus smaller quantities of long-chain alcohols and hydrocarbons suggests a pheromonal function. Although eicosenol is present in large quantities in the alarm pheromone of the common honeybee, *A. mellifera*, the amount in the Asian hive bee, *A. cerana*, is 50–100 times higher. Such enormous quantities of eicosenol in *A. cerana*, plus the fact that it is in association with the sting apparatus, suggests a major role as an alarm pheromone.

All investigated species of *Apis* possess alarm pheromones originating in the sting area. All also contain isopentyl acetate as a component of their alarm pheromone cocktail, and all respond to it. However 2-decen-1-yl acetate, a major component of the alarm pheromones of *A. dorsata* and *A. florea* (Veith et al., 1978), is not present in the pheromone of *A. mellifera* or *A. cerana* (this study), and (Z)-11-eicosen-1-ol is present only in *A. mellifera* and *A. cerana* and not in the other two species or in the giant Himalayan honeybee, *A. laboriosa* (M. S. Blum, personal communication). The functions of decenyl acetate and eicosenol in their respective species is uncertain, although they may serve in part as carriers to prolong the release time of the more volatile alarm com-

ponents, as suggested by Veith et al. (1978) and Pickett et al. (1982). Alternatively, these components might function as active alarm components in their own right as suggested by Pickett et al. (1982). However, in the case of eicosenol in *A. mellifera* mixed results from assays complicate interpretations. Pickett et al. (1982) found eicosenol stimulated stinging attacks on moving targets, but did not attract guard bees at the hive entrance (as did isopentyl acetate). In contrast, Free et al. (1989) reported that eicosenol was inactive in eight of their nine assay categories (including releasing stinging) and was only active in inhibiting the release of Nasonov pheromone by scenting workers.

All previous reports of honeybee venom itself have revealed that the venom is lacking in pheromones, including eicosenol, and that the pheromonal repository is the setose area of the sting base (Ghent and Gary, 1962; Maschwitz, 1964; Pickett et al., 1982; Lensky et al., 1995). Since the setose area is not glandular, it is unlikely that it is produced there and may be synthesized and secreted elsewhere and later transferred to the setose area; however, the source(s) has not yet been found. For this reason we investigated the potential anatomical source of eicosenol in the sting apparatus of *Apis cerana* and reinvestigated the sources in two subspecies of *A. mellifera*. Our results confirm those of Pickett et al. (1982) that eicosenol is present in meaningful quantities only in the setose area. In *A. cerana* virtually all the eicosenol was present in the venom itself and relatively little, if any, appeared present in the setose area. This leads to the question of the function of eicosenol in *A. cerana* and why it is present in the venom is such large quantities. Although we do not yet have definitive evidence, three potential roles seem possible. Firstly, as demonstrated by Free et al. (1982), eicosenol is highly attractive to foragers of *A. mellifera*, and it is conceivable that *A. cerana* uses venom-based eicosenol to mark particularly rich floral or other resources so that other foragers can locate them. A second possible role of eicosenol is that of a spreading factor, which aids in the spreading and penetration of aqueous venom toxins that are topically applied to the surface of an assailant. Thirdly, eicosenol could be a "tagging" pheromone to mark a potential assailant and then to alert other nestmate defenders of the location of the assailant. This could result from the small quantities of venom (containing eicosenol) that usually leak during the venom delivery process onto the skin around the sting puncture. Alternatively, the attacking bee might deposit eicosenol on the surface fur or feathers of the assailant, especially if the worker cannot actually penetrate deeply enough to sting the skin.

Acknowledgments—We thank M. Mardan and M. Crosland, respectively, for making it possible to collect bee samples in Malaysia and Hong Kong, G. Otis for gifts of frozen *Apis koschevnikovi* and *A. andreniformis*, W. S. Sheppard for the gift of frozen *Apis florea*, and G. R. Jones for advice on the dimethylthiolation reaction and other helpful advice.

REFERENCES

BILLEN, J., EVERSHED, R. P., ATTYGALE, A. B., MORGAN, E. D., and OLLETT, D. G. 1986. Contents of the Dufour glands of workers of three species of *Tetramorium* (Hymenoptera: Formicidae). *J. Chem. Ecol.* 17:669–685.

BLUM, M. S., and FALES, H. M. 1988. Chemical releasers of alarm behavior in the honeybee: Informational "plethora" of the sting apparatus signal, pp. 141–148, *in* G. R. Needham, R. E. Page, Jr., M. Delfinado-Baker, and C. E. Bowman (eds.). Africanised Honey Bees and Mites. Wiley, New York.

BLUM, M. S., FALES, H. M., TUCKER, K. W., and COLLINS, A. M. 1978. Chemistry of the sting apparatus of the worker honeybee. *J. Apic. Res.* 17:218–221.

BOCH, R., SHEARER, D. A., and STONE, B. C. 1962. Identification of iso-amyl acetate as an active component in the sting pheromone of the honey bee. *Nature (London)* 195:1018–1020.

CASSIER, P., TEL-ZUR, D., and LENSKY, Y. 1994. The sting sheaths of honey bee workers (*Apis mellifera* L.): Structure and alarm pheromone secretion. *J. Insect Physiol.* 40:23–32.

COLLINS, A. M., and BLUM, M. S. 1982. Bioassay of compounds derived from the honeybee sting. *J. Chem. Ecol.* 8:463–470.

COLLINS, A. M., and BLUM, M. S. 1983. Alarm responses caused by newly identified compounds derived from the honeybee sting. *J. Chem. Ecol.* 9:57–65.

FREE, J. B., and SIMPSON, J. 1968. The alerting pheromones of the honeybee. *Z. Vergl. Physiol.* 61:361–365.

FREE, J. B., WILLIAMS, I. H., PICKETT, J. A., FERGUSON, A. W., and MARTIN, A. P. 1982. Attractiveness of (Z)-11-eicosen-1-ol to foraging honeybees. *J. Apic. Res.* 21:151–156.

FREE, J. B., FERGUSON, A. W., and SIMPKINS, J. R. 1989. Honeybee responses to chemical components from the worker sting apparatus and mandibular glands in field tests. *J. Apic. Res.* 28:7–21.

GHENT, R. L., and GARY, N. E. 1962. A chemical alarm releaser in honeybee stings (*Apis mellifera* L.). *Psyche* 69:1–6.

GUNNISON, A. F., and MORSE, R. A. 1968. Source of the ether-soluble organics of stings of the honeybee, *Apis mellifera* (Hymenoptera: Apidae). *Ann. Entomol. Soc. Am.* 61:5–8.

HUBER, F. 1814. Nouvelles Observations sur les Abeilles. Paschoud, Paris.

KOENIGER, N., WEIS, J., and MASCHWITZ, U. 1979. Alarm responses of the sting in the genus *Apis*. *J. Insect Physiol.* 25:467–475.

LENSKY, Y., CASSIER, P., and TEL-ZUR, D. 1995. The setaceous membrane of honeybee (*Apis mellifera* L.) workers' sting apparatus: Structure and alarm pheromone distribution. *J. Insect Physiol.* 41:589–595.

MASCHWITZ, U. 1964. Gefahrenalarmstoffe und Gefarenalarmierung bei sozialen Hymenopteran. *Z. Vergl. Physiol.* 47:596–655.

MORGAN, E. D. 1990. Preparation of small scale samples from insects for chromatography. *Anal. Chim. Acta* 236:227–235.

MORGAN, E. D., and WADHAMS, L. J. 1972. Gas chromatography of volatile compounds in small samples of biological materials. *J. Chromatogr. Sci.* 10:528–529.

MORSE, R. A., SHEARER, D. A., BOCH, R., and BENTON, A. W. 1967. Observations on alarm substances in the genus *Apis J. Apic. Res.* 6:113–118.

PICKETT, J. A., WILLIAMS, I. H., and MARTIN, A. P. 1982. (Z)-11-Eicosen-1-ol, an important new pheromonal component from the sting of the honeybee *Apis mellifera* L. (Hymenoptera, Apidae). *J. Chem. Ecol.* 8:163–175.

SCHMIDT, J. O. 1986. Chemistry, pharmacology, and chemical ecology of ant venoms, pp. 425–508 *in* T. Piek (ed.). Venoms of the Hymenoptera. Academic, London.

SCHMIDT, J. O. 1995. Toxinology of the venoms from the honeybee genus *Apis*. *Toxicon* 33:917–927.

TEL-ZUR, D., and LENSKY, Y. 1995. Bioassay and apparatus for measuring the stinging response of an isolated worker honeybee (*Apis mellifera* L. var. *lingustica* Spin.). *Comp. Biochem Physiol.* 110A:281–288.

VEITH, J., WEISS, J., and KOENIGER, N. 1978. A new alarm pheromone (2-decen-1-yl acetate) isolated from the stings of *Apis dorsata* and *Apis florea* (Hymenoptera: Apidae). *Experientia* 34:423.

Journal of Chemical Ecology, Vol. 23, No. 8, 1997

ENTERIC AND PLANT DERIVED DETERRENTS IN REGURGITATE OF AMERICAN BIRD GRASSHOPPER, *Schistocerca americana*

F. ORTEGO,[1,*] P. H. EVANS, and W. S. BOWERS

Department of Entomology
University of Arizona
Tucson, Arizona 85721

(Received May 28, 1996; accepted April 2, 1997)

Abstract—Regurgitates of the grasshopper *Schistocerca americana* were deterrent to *Crematogaster* ants. Regurgitates obtained from insects fed upon wheat seedlings were more strongly deterrent than those obtained from insects fed on romaine lettuce leaves. After extraction of the regurgitates with ethyl acetate, deterrence could be demonstrated in both the organic and aqueous phases. Wheat seedlings, lettuce, and feces from wheat- or lettuce-fed insects yielded extracts with lower deterrency. Our results suggest that deterrents in the organic phase of the regurgitate are primarily due to the presence of plant natural products, whereas the deterrency of the aqueous phase is probably due to digestive enzymes from enteric components, probably digestive enzymes.

Key Words—*Schistocerca americana*, Orthoptera, grasshopper regurgitate, deterrent.

INTRODUCTION

When disturbed, many grasshoppers regurgitate a viscous, dark brown fluid composed of their gut contents, which may often spread over their bodies (Edmunds, 1974, p. 199; Matthews and Matthews, 1978, p. 335; Whitman et al., 1990). There is considerable evidence suggesting grasshopper regurgitates are defensive against both vertebrate and invertebrate predators. Oral administration of grasshopper regurgitate produces vomiting and contractions of the diaphragm in guinea pigs, sheep, and goats (Curasson, 1934), and topical appli-

*To whom correspondence should be addressed.
[1] Present address: Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Velázquez 144, Madrid 28006, Spain.

0098-0331/97/0800-1941$12.50/0 © 1997 Plenum Publishing Corporation

cation to the eyes of birds elicits immediate distress behavior and irritation of the cornea (Freeman, 1968). When the grasshoppers *Romalea microptera* and *Brachystola magna* were immobilized near *Pogonomyrmex* colonies, the aggressive ants quickly attacked but were repelled following grasshopper regurgitation (Eisner, 1970). The Australian acridid *Goniaea* sp. produces a regurgitate repellent to a diversity of small predators, including skinks, spiders, and ants (Lymbery and Bailey, 1980). Steiner (1981) observed that digger wasps were reluctant to attack acridid grasshoppers after they spread the regurgitate over their bodies, and even accidental contact triggered vigorous body grooming in an attempt to eliminate the viscous fluid. Regurgitates of *Locusta migratoria* cause hemorrhaging, paralysis, and/or death when injected into mice, guinea pigs, sheep, and goats (Curasson, 1934; Freeman, 1968). However, regurgitates are seldom introduced into predators subcutaneously except by certain grasshoppers with particularly powerful raptorial mandibles, such as *B. magna*, that bites and regurgitates simultaneously (Whitman, 1990).

Grasshopper regurgitates contain recently eaten plant materials combined with digestive and salivary secretions (Freeman, 1967, 1968; Knecht et al., 1974; Lymbery and Bailey, 1980). Repellency could derive from several sources including acidic pH, enzymatic activity, or ingested plant secondary compounds (Whitman, 1990). The efficacy of grasshopper regurgitates may also be due to their mechanical entangling properties, since Lymbery and Bailey (1980) noted that regurgitates harden following the evaporation of water and adhere to the mouthparts of ants and skinks. Although, there is scant evidence for the presence of plant natural products in regurgitates, Eisner (1970) observed that the repellency of regurgitates from the grasshopper *R. microptera* varied depending on the diet of the insect. Moreover, Whitman (1990) suggested that ingested plant secondary compounds might be important, since few predators are adapted to deal with plant allelochemicals.

The study examined the possible role for enteric and plant derived compounds as deterrents in the regurgitate of the American bird grasshopper, *Schistocerca americana*.

METHODS AND MATERIALS

Insects. A stock population of the American bird grasshopper was reared in the laboratory under crowded conditions (Bowers and Ortego, 1991). Colonies of *Crematogaster opuntia* ants were maintained as described by Wheeler and Buck (1995).

Collection and Extraction of Regurgitates. Nymphs in the last instar or adults of *S. americana* were held by the thorax while the mouthparts were gently pressed against the rim of a 6-ml glass vial to collect the regurgitate. Fluid collected from several specimens was pooled and frozen for subsequent study.

Six milliliters of regurgitate from grasshoppers feeding on wheat were collected. One milliliter was lyophilized to calculate the weight of material in each milliliter of regurgitate (214 mg/ml). The remaining 5 ml were extracted three times with 15 ml of ethyl acetate. Removal of the solvent on the rotary evaporator in vacuo at ~40°C yielded the organic extract (9 mg/ml of regurgitate), whereas the aqueous phase was lyophilized to yield 166 mg/ml of regurgitate. Similarly, from 6 ml of regurgitate of grasshoppers feeding on lettuce, 1 ml was lyophilized to calculate the weight of material in each milliliter of regurgitate (218 mg/ml), and the other 5 ml were extracted to obtain an organic phase (5.6 mg/ml of regurgitate) and an aqueous phase (186 mg/ml of regurgitate).

Extraction of Wheat and Lettuce. Fresh wheat seedlings (121 g; 14.3 g estimated dry weight) were homogenized in 1 liter of distilled water in a Sorvall Omnimixer for 5 min and held at room temperature for 24 hr. The homogenate was filtered through Whatman No. 1 paper and extracted with 1 liter of ethyl acetate (three times). Removal of the ethyl acetate in vacuo gave the organic phase (130 mg). The aqueous phase was lyophilized and yielded 6 g. In an identical procedure, we extracted 43 g of fresh romaine lettuce leaves (4.6 g estimated dry weight) to obtain an ethyl acetate extract (43 mg) and an aqueous phase (516 mg).

Extraction of Feces. Ten grams of dry feces from grasshoppers fed on wheat seedlings were homogenized with 200 ml of water in a Sorvall Omnimixer and held at room temperature overnight. The homogenate was filtered through Whatman No. 1 paper and extracted with 200 ml of ethyl acetate (three times). The ethyl acetate was evaporated in vacuo to give the organic phase (83.4 mg), whereas the aqueous phase was lyophilized (965 mg). Similarly, from 5 g of dry feces from grasshoppers fed on romaine lettuce leaves we obtained an ethyl acetate extract (38.7 mg) and an aqueous phase (451 mg).

Tests for Deterrence Activity with Ants. Samples were dissolved in water (lyophilized regurgitates, homogenates, and aqueous phases) or methanol (organic extracts), and 10 μl was added to 20 μl of a 40% sucrose solution on a glass microscope slide. A 20-μl portion of the 40% sucrose solution plus 10 μl of water or methanol dispensed as a single drop on the same slide served as control. The slide was placed inside a colony of *Crematogaster* ants, and the number of ants feeding on the control (C) and treated (T) sucrose solutions were recorded over a period of 10 min. A deterrent index (DI) was then calculated as: $DI = 100[(C - T)/C]\%$. Each concentration was replicated four times using four different ant colonies. The concentration that deterred 80% (DI_{80}) of the ants from feeding on the sucrose solution containing the experimental sample was calculated for each extract or fraction (Bowers et al., 1993).

HPLC of Organic Extracts. The ethyl acetate extracts from plants, regurgitates, and feces were dried in a nitrogen stream and redissolved in acetonitrile to give a final concentration of 10 mg/ml. The samples were then filtered with HV-13 filter units. Reverse-phase HPLC was performed on a 10-cm × 0.8-cm

C8 column. Samples of 10 μl (0.1 mg) were injected and separated isocratically with 20% acetonitrile/water at a flow of 1 ml/min. The eluent was monitored with UV detection at 254 nm (0.1 AUFS). Fractions from three injections (0.3 mg) were collected to test deterrency. Fractions were dried under a stream of nitrogen to eliminate completely all traces of the acetonitrile. The residual aqueous suspension was extracted with 1 ml of ethyl acetate (three times). The ethyl acetate phase was dried under nitrogen and dissolved in 40 μl of methanol to use in four assays (10 μl each). Fractions taken 1 ml from the baseline were submitted to the same extraction process and used as controls in the deterrency assays.

RESULTS AND DISCUSSION

Deterrent Activity of Whole Regurgitates, Wheat Seedlings, Lettuce, and Feces. Regurgitates from *S. americana* fed on wheat seedlings or romaine lettuce were tested for their deterrent activity against *C. opuntiae*. Ants immediately approached the experimental set on introduction into the colony and examined the control- and regurgitate-treated sucrose solutions with their antenna. Within a few minutes a group of 20–30 ants gathered around the control sucrose solution and rapidly consumed it. However, when ants touched the regurgitate-treated solution, they were immediately repelled and displayed vigorous cleaning of their antenna. Complete deterrence was obtained when the lyophilized regurgitates were tested at the same concentration as when produced by the grasshoppers, i.e., 214 μg/μl for the grasshoppers fed on wheat and 218 μg/μl when fed on lettuce. Moreover, the regurgitate from grasshoppers feeding on wheat has a deterrent index (DI_{80}) of 38 μg/μl (Figure 1). This demonstrates that the regurgitate was able to deter 80% of the ants from feeding on the sucrose solution even when diluted to one fifth of its normal concentration. The regurgitate from grasshoppers feeding on lettuce has a DI_{80} of 69 μg/μl (Figure 2), which represents about one third of its original concentration. The deterrent indices show that deterrent activity of the regurgitates depends on what the grasshoppers were fed. Regurgitates after a diet of wheat showed higher deterrency than regurgitates from lettuce fed grasshoppers. Eisner (1970) observed that the regurgitates of *R. microptera* were very repellent to ants after the grasshoppers had fed on *Eupatorium capillifolium* or *Salix nigra*, whereas regurgitates derived from grasshoppers, which had eaten lettuce or *Myrica cerifera* were not repellent.

Wheat seedlings yielded extracts with higher deterrency (DI_{80} = 10 μg/μl) than lettuce extracts (DI_{80} = 84 μg/μl) (Figures 1 and 2). On the other hand, feces from wheat-fed (DI_{80} = 143 μg/μl) and lettuce-fed (DI_{80} = 121 μg/μl) insects yielded extracts of similar deterrency (Figures 1 and 2).

Effect of Organic Phases and HPLC Fractions. Deterrence was found in both the organic and aqueous phases of the regurgitates (Figures 1 and 2).

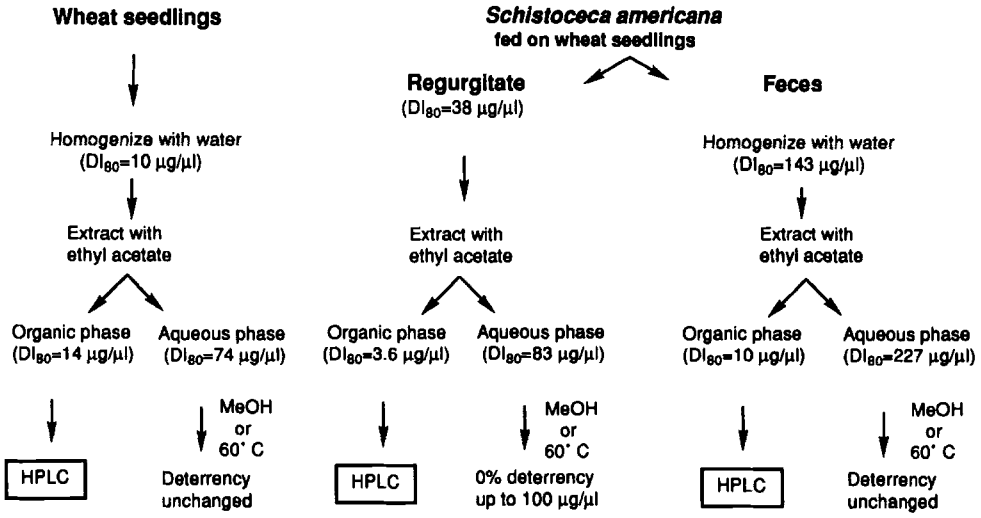


FIG. 1. Ethyl acetate extraction and deterrent activity against *Crematogaster* ants of wheat seedlings, regurgitates and feces from *Schistocerca americana* feeding on wheat seedlings. DI_{80} denotes the concentration that deterred 80% of the ants from feeding on a sucrose solution.

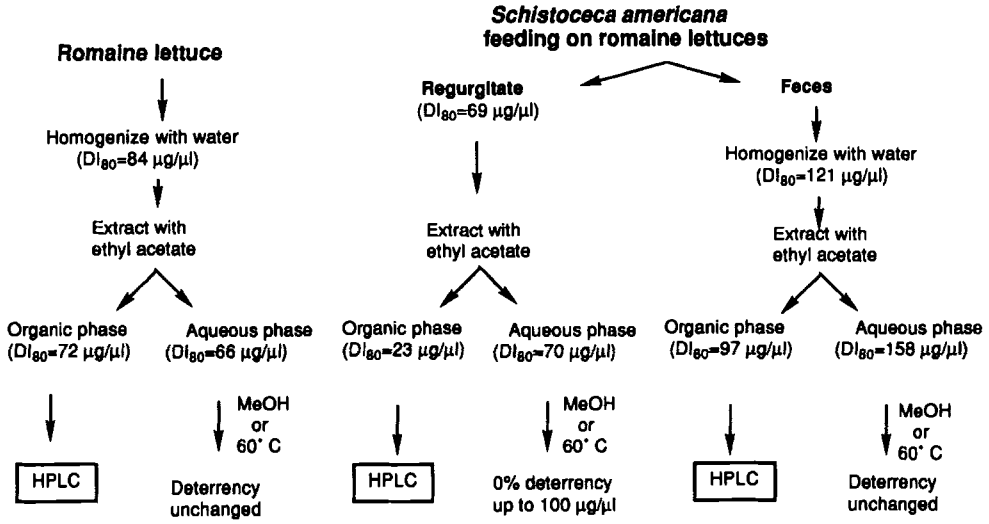


FIG. 2. Ethyl acetate extraction and deterrent activity against *Crematogaster* ants of lettuce and of regurgitates and feces from *Schistocerca americana* feeding on lettuce. DI_{80} as in Figure 1.

Deterrence of the aqueous phases were similar in grasshoppers fed on either wheat (DI_{80} = 83 $\mu g/\mu l$) or lettuce (DI_{80} = 70 $\mu g/\mu l$). The concentration of the aqueous phases was 166 and 186 $\mu g/\mu l$, respectively, showing that they were able to deter 80% of the ants at half their original concentration in the regurgitate. The organic phase from grasshoppers fed on wheat gave a DI_{80} of 3.6 $\mu g/\mu l$, less than half its original concentration in the regurgitate (9 $\mu g/\mu l$). The organic phase from grasshoppers fed on lettuce required four times its original concentration in the regurgitate (5.6 $\mu g/\mu l$) to produce the same deterrency (DI_{80} = 23 $\mu g/\mu l$). These results suggest that the difference found in deterrency of regurgitates from grasshoppers feeding on wheat or lettuce is due to different concentrations of deterrents in the organic phases.

The organic phase of wheat seedlings yielded a level of deterrent activity (DI_{80} = 14 $\mu g/\mu l$) lower than that of the organic phase from regurgitates of grasshoppers fed on wheat (DI_{80} = 3.6 $\mu g/\mu l$) and similar to that of feces (DI_{80} = 10 $\mu g/\mu l$) (Figure 1). Fractionation by HPLC of the organic phase from regurgitate of grasshoppers fed on wheat, followed by deterrency assay of the resulting fractions revealed two peaks (4.4 and 11.4 min) with deterrent activity (Figure 3). These HPLC peaks from the regurgitate coincided in retention time with HPLC peaks from the wheat seedlings and feces. Both peaks from the wheat seedlings were deterrent, but only the last one from the feces was active. Our results suggest that the deterrence of the organic phase of regurgitates is due primarily to the presence of plant natural products. Compounds with deterrent activity from the wheat seedlings may not have been metabolized in the digestive tract of the grasshoppers and are likely more concentrated in the regurgitates. Similar results were obtained with the organic phases from lettuce and from regurgitates and feces from grasshoppers fed on lettuce (Figure 2). However, none of the fractions obtained by HPLC showed deterrent activity (Figure 4), probably because the activity of the organic phase from lettuce was 5–10 times lower than that obtained from wheat. Additionally, the biological activity may be too small to be detected after losses encountered during fractionation. It has been suggested that insects may have evolved the capacity to concentrate ingested plant compounds or even alter them to more toxic products, which could then serve as potent repellents when liberated by regurgitation or defecation (Blum, 1981). Furthermore, Eisner (1970) suggested that it may be adaptive for these insects to select food plants whose repellent natural products are not detoxified in the digestive tract.

Effect of Aqueous Phases. The aqueous phase of the wheat seedling extract has a deterrent activity (DI_{80} = 74 $\mu g/\mu l$) similar to that of the aqueous phase from regurgitates of grasshoppers feeding on wheat (DI_{80} = 83 $\mu g/\mu l$) (Figure 1). However, incubation at 60°C for 30 min or treatment with methanol inactivates the deterrent activity of the aqueous phase from regurgitate, but not that from wheat seedlings. The aqueous phase from feces of grasshoppers fed on

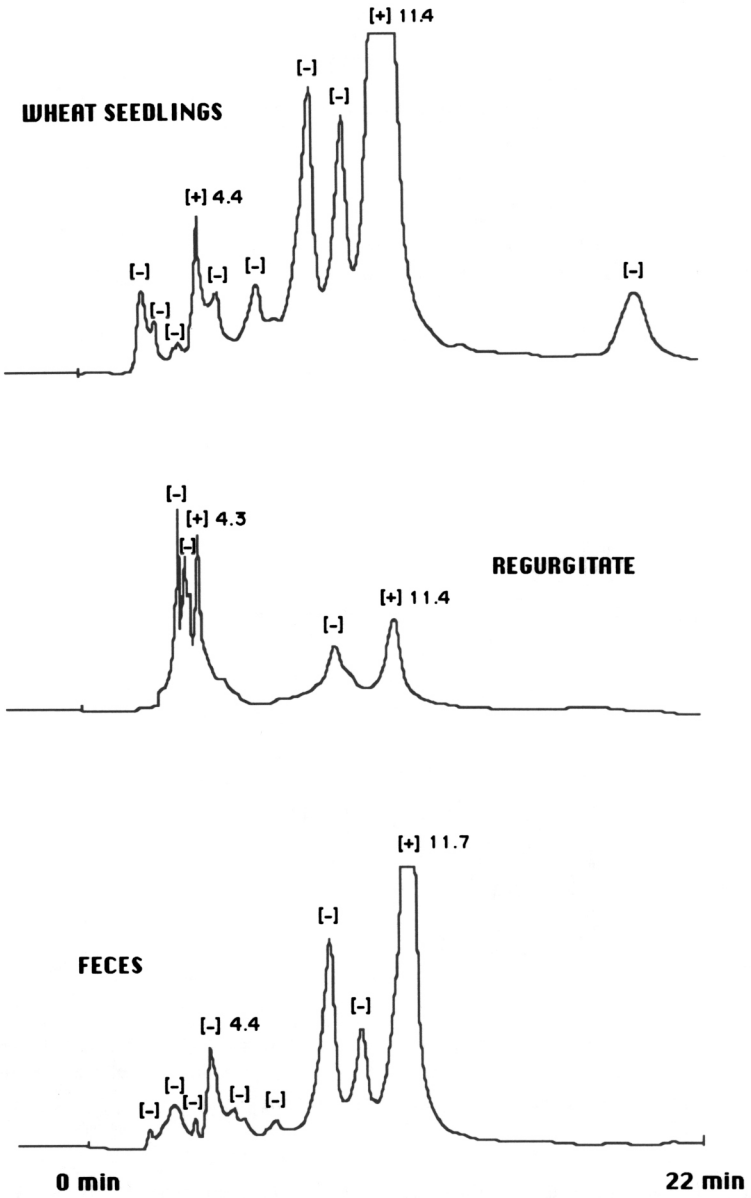


FIG. 3. HPLC of the ethyl acetate extracts from wheat seedlings and regurgitates and feces from *Schistocerca americana* feeding on wheat seedlings. [+] and [−] denote deterrent activity or lack of deterrent activity, respectively, against *Crematogaster* ants.

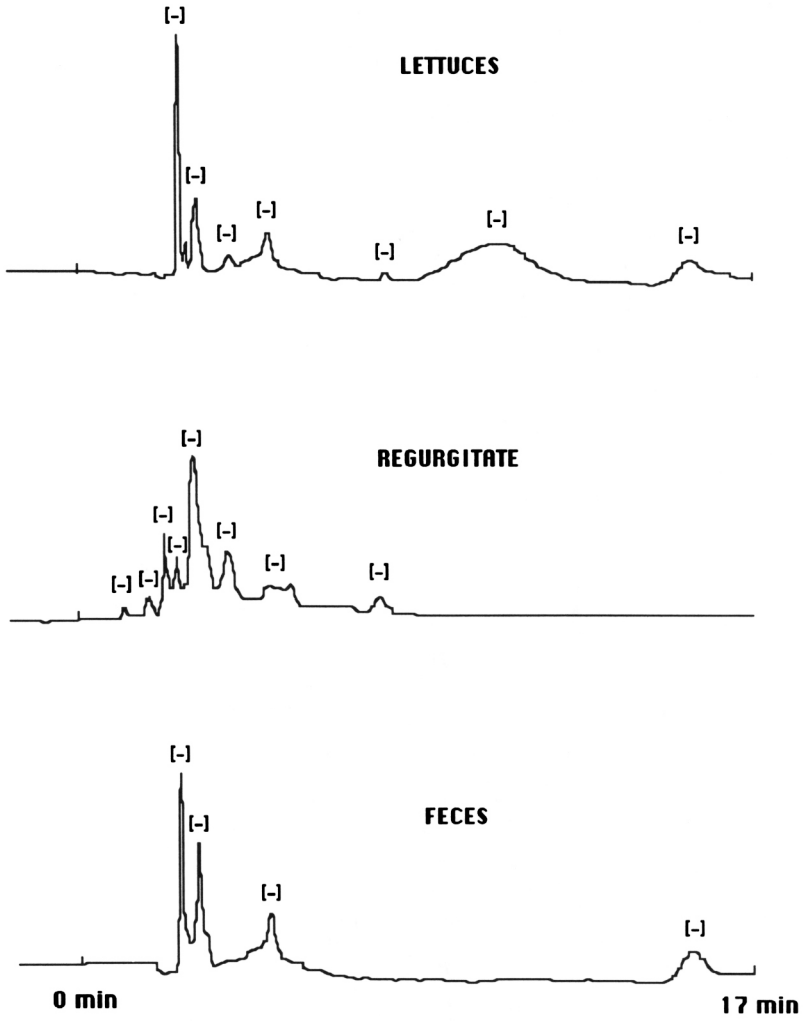


FIG. 4. HPLC of the ethyl acetate extracts from lettuce, regurgitates and feces from *Schistocerca americana* feeding on lettuces. [+] and [−] denote deterrent activity or lack of deterrent activity, respectively, against *Crematogaster* ants.

wheat had less deterrent activity (DI_{80} = 227 $\mu g/\mu l$) but was unaffected by heat or methanol. Similar results were obtained from grasshoppers fed on lettuce. These results, taken together with the heat and methanol lability of the deterrency activity in the aqueous portions of the extracts, suggest that the biological activity in the regurgitates is composed of enteric components, possibly digestive

enzymes, whereas the compounds with deterrent activity in the aqueous extracts of wheat are probably metabolized, as indicated by low activity in the aqueous phase from the feces. Serine proteinases (Freeman, 1967; Knecht et al., 1974; Sakal et al., 1988, 1989, 1992; Hinks and Erlandson, 1995) and carbohydrases (Anstee and Charnley, 1977) have previously been identified in the digestive fluids of several species of grasshoppers.

Acknowledgments—We thank Dr. Diana Wheeler for providing the colonies of *Crematogaster* ants.

REFERENCES

ANSTEE, J. H., and CHARNLEY, A. K. 1977. Effects of frontal ganglion removal and starvation on activity and distribution of six gut enzymes in *Locusta*. *J. Insect Physiol.* 23:965–974.

BLUM, M. S. 1981. Chemical Defenses of Arthropods. Academic Press, New York.

BOWERS, W. S., and ORTEGO, F. 1991. Evaluation of juvenoid insect growth regulators on *Schistocerca americana*. *Insect Sci. Appl.* 12:71–75.

BOWERS, W. S., ORTEGO, F., YOU, X., and EVANS, P. H. 1993. Insect repellents from the Chinese prickly ash *Zanthoxylum bungeanum*. *J. Nat. Prod.* 56:935–938.

CURASSON, G. 1934. Sur le toxicité de la sécrétion buccale des Sauterelles. *Bull. Acad. Vet. Fr.* 17:377–382.

EDMUNDS, M. 1974. Defence in Animals. A Survey of Anti-predator Defenses. Longman, New York.

EISNER, T. 1970. Chemical defense against predation in Arthropods, pp. 157–217, *in* E. Sondheimer and J. B. Simeone (eds.). Chemical Ecology, Academic Press, New York.

FREEMAN, M. A. 1967. Proteolytic enzymes of the crop fluid from *Locusta migratoria* L. *Comp. Biochem. Physiol.* 20:1013–1015.

FREEMAN, M. A. 1968. Pharmacological properties of the regurgitated crop fluid of the African migratory locust, *Locusta migratoria* L. *Comp. Biochem. Physiol.* 26:1041–1049.

HINKS, C. F., and ERLANDSON, M. A. 1995. The accumulation of haemolymph proteins and activity of digestive proteinases of grasshoppers (*Melanoplus sanguinipes*) fed wheat, oats or kochia. *J. Insect Physiol.* 41:425–433.

KNECHT, M., HAGENMAIER, H. E., and ZEBE, E. 1974. The proteases in the gut of the locust, *Locusta migratoria*. *J. Insect Physiol.* 20:461–470.

LYMBERY, A., and BAILEY, W. 1980. Regurgitation as a possible antipredator defensive mechanism in the grasshopper *Goniaea* sp. (Acrididae, Orthoptera). *J. Aust. Entomol. Soc.* 19:129–130.

MATTHEWS, R. W., and MATTHEWS, J. R. 1978. Insect Behavior. Wiley, New York, 507 pp.

SAKAL, E., APPLEBAUM, S. W., and BIRK, Y. 1988. Purification and characterization of *Locusta migratoria* chymotrypsin. *Int. J. Pept. Protein Res.* 32:590–598.

SAKAL, E., APPLEBAUM, S. W., and BIRK, Y. 1989. Purification and characterization of trypsins from the digestive tract of *Locusta migratoria*. *Int. J. Pept. Protein Res.* 34:498–505.

SAKAL, E., APPLEBAUM, S. W., and BIRK, Y. 1992. Detection and determination of *Locusta migratoria* trypsin by radioimmunoassay. *Arch. Insect Biochem. Physiol.* 20:157–164.

STEINER, A. L. 1981. Anti-predator strategies. II. Grasshoppers (Orthoptera, Acrididae) attacked by *Prionyx parkeri* and some *Tachysphex* wasps (Hymenoptera, Sphecinae and Larrinae): A descriptive study. *Psyche* 88:1–24.

WHEELER, D. E., and BUCK, N. A. 1995. Storage proteins in ants during development and colony founding. *J. Insect Physiol.* 41:885–894.

WHITMAN, D. W. 1990. Grasshopper chemical communication, pp. 357–391, *in* R. F. Chapman and A. Joem (eds.). Biology of Grasshoppers. Wiley-Interscience, New York.

WHITMAN, D. W., BLUM, M. S., and ALSOP, D. W. 1990. Allomones: Chemicals for defense, pp. 289–351, *in* D. L. Evans and J. O. Schmidt (eds.). Insect Defenses. Adaptive Mechanisms and Strategies of Prey and Predators. State University of New York Press, Albany.

Journal of Chemical Ecology, Vol. 23, No. 8, 1997

CHEMISTRY AND DEFENSIVE EFFICACY OF SECRETION OF BURROWING BUG (*Sehirus cinctus cinctus*)

BRYAN S. KRALL,[1,*] BRUCE W. ZILKOWSKI,[2] SCOTT L. KIGHT,[3] ROBERT J. BARTELT,[2] and DOUGLAS W. WHITMAN[1]

[1]*4120 Biological Sciences, Illinois State University Normal, Illinois 61790*

[2]*USDA, ARS National Center for Agricultural Utilization Research Bioactive Agents Research Unit 1815 N. University, Peoria, Illinois 61604*

[3]*Department of Biology and Center for the Integrative Study of Animal Behavior Indiana University Bloomington, Indiana 47405*

(Received September 16, 1996; accepted April 2, 1997)

Abstract—Adult *Sehirus cinctus cinctus* emit a volatile secretion from their metathoracic scent glands when tactually stimulated. We identified the volatile components by gas chromatography, high-performance liquid chromatography, and mass spectrometry. The secretion of both sexes contained (1*R*)-(+)-α-pinene, (1*S*)-(−)-β-pinene, β-myrcene, (*R*)-(+)-limonene, and α-terpinolene. Two additional compounds were found in only female secretions: (*E*)-2-hexenyl acetate and (*E*)-2-octenal. We also tested the defensive capability of this insect by offering it to various predators. Anoles, starlings, and a killdeer rejected *S. c. cinctus* after an initial sampling. These findings suggest that the secretion plays a defensive role.

Key Words—Terpenes, chemical defense, Heteroptera, Cydnidae, *Sehirus cinctus cinctus*, (1*R*)-(+)-α-pinene, (1*S*)-(−)-β-pinene, β-myrcene, (*R*)-(+)-limonene, α-terpinolene, (*E*)-2-hexenyl acetate, (*E*)-2-octenal.

INTRODUCTION

Hemipterans are well known for their use of repellent chemicals to defend themselves against predators (Staddon, 1979; Blum, 1981; Aldrich, 1988).

*To whom correspondence should be addressed.

0098-0331/97/0800-1951$12.50/0 © 1997 Plenum Publishing Corporation

However, not much is known about the defensive chemistry of cydnids, a cosmopolitan family closely related to the Pentatomidae (Miller, 1971). Even less is known about chemical defense in the cydnid subfamily Sehirinae. In the United States, the Sehirinae are represented by one genus containing one species and three subspecies. The most common subspecies, *Sehirus cinctus cinctus* (Palisot de Beauvois), ranges from Massachusetts to Florida and as far west and south as Iowa, New Mexico, and Mexico (Froeschner, 1960).

Cydnids in general, and *S. c. cinctus* in particular, are unique in that they practice brood care in underground chambers (Sites and McPherson, 1982). Adult *S. c. cinctus* overwinter and emerge in late March to April to feed in aggregations on several species of mint. By the end of April, adults mate and move to the ground (Sites and McPherson, 1982), where the females find or dig a small (ca. 0.5 cm^3) chamber approximately 2 cm below the soil surface and lay a batch of 120–150 eggs. Females remain with the eggs and early-instar larvae for several weeks, guarding them against ants and other predators and feeding them by carrying mint seeds from the surface to the chamber (Southwood and Hine, 1950; McDonald, 1968).

The nymphs leave the brood chamber when they reach the third instar and travel to the surface to feed on mint foliage (Sites and McPherson, 1982). They pass through five instars before reaching adulthood and can be found between early May and mid-August, while adults can be found from early Spring to late Summer. *S. c. cinctus* nymphs are aposematically colored. They are black with reddish orange markings on the head, thorax, and abdomen. Adults may also be aposematic when viewed against green vegetation. They are small (ca. 5 mm long), black insects with a thin cream-colored stripe along the lateral margins of each wing beginning at the head and extending down to the anterior of the wings. When stimulated by lightly pinching the legs or antennae, the adults emit a volatile secretion. This suggests that the secretion functions in chemical defense against predators.

METHODS AND MATERIALS

Insect Collection and Care

Adult *S. c. cinctus* were collected on wild mints near Bloomington, Indiana, in early April 1995. The bugs were then kept in a cold chamber for three months at 7°C under a 10L:14D photoperiod to preserve them until needed. After retrieval from the cold chamber, individuals were maintained according to Sites and McPherson (1982) with the following modifications: no plastic wrap was placed over the Petri dish, as this induced harmfully high humidities. Instead, a 1-cm^2 screened ventilation hole was placed in the center of each plastic Petri

dish lid. Vial caps containing dried fruits of *Prunella vulgaris* (Labiatae) allowed the bugs to feed ad libitum.

Chemical Analysis

Chemicals. (1*S*)-(−)-α-Pinene (98%), *dl*-α-pinene (99%), (1*R*)-(+)-β-pinene (98%), (1*S*)-(−)-β-pinene (99%), (*R*)-(+)-limonene (97%), (*S*)-(−)-limonene (96%), α-terpinolene (99%), and (*E*)-2-octenal (94%) were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. β-Myrcene (90%) and (*E*)-2-hexenyl acetate (97%) were obtained from Sigma Chemical Co., St. Louis, Missouri.

Volatile Collection and Analysis. The sex of ca. 25 *S. c. cinctus* adults was determined using a microscope, and the insects were placed individually into Hewlett-Packard autosampler vials (glass, 12 mm × 32 mm, with an 11-mm Teflon-lined aluminum crimp cap). A 500-μl gas-tight syringe was used to draw 200 μl of headspace volatiles out of each vial. Volatiles were then immediately injected into a GC for analysis (Hewlett-Packard 5890, equipped with splitless injector, and flame ionization detector). The DB-1 capillary column (J & W Scientific, Folsom, California) was 15 m × 0.25 mm and had a 1.0-μm film thickness. The temperature program was 50–150°C at 10°C/min. Following analysis of the first gas sample, the cap was removed from the vial, and the insect was agitated by either grabbing a leg with forceps or holding the individual against the sides or bottom of the vial until the characteristic odor was obvious to the investigator. (In some cases merely attempting to grab an insect without actually restraining it was enough to elicit release of the secretion). At the first hint of chemical release, a new cap was tightly crimped on to the vial and a second headspace volatile sample (200 μl) was removed and injected into the GC.

A second method of volatile collection was used for ca. 50 unsexed dead adult *S. c. cinctus*. Dead adults (deceased adults stored at −5°C within 24 hr of death and kept up to one month before extraction) were covered with 5 ml of hexane and then crushed with a glass rod. The sample was filtered, reduced in volume under N_2, and then fractionated isocratically with high-performance liquid chromatography (HPLC) using a Waters Associates 6000 pump and a Waters Associates R401 refractometer detector. A silica column (Lichrosorb Si60, 25 cm × 0.46 cm, 5-μm particle size) was eluted with hexane (flow 1 ml/min) and effluent was collected as consecutive 1-ml fractions. These fractions were analyzed by GC. The fractions provided a larger amount of material than from single headspace collections, making it possible to obtain mass spectra for the insect-derived compounds and to determine chirality for some compounds. Mass spectra were obtained on a Hewlett-Packard 5970 MSD instrument, with sample introduction through a DB-1 capillary column (15 m × 0.25

mm ID with 1.0-μm film thickness, temperature program 50–250°C increased at 10°C/min). Chirality was determined on a chiral GC column by comparison to authentic standards. A 30-m × 0.25-mm-ID CDX-B fused silica capillary column (J & W Scientific) with a 0.25-μm film thickness was used (temperature program 40–75°C at 10°C/min, then holding at 75°C for 30 min).

Seed Extraction. Prunella vulgaris seeds were cleaned of debris and 0.5 g was placed into a glass vial with 2 ml of hexane; the seeds were crushed with a glass rod. The extract was filtered and analyzed by GC (Hewlett-Packard 5890, 15-m × 0.25-mm-ID DB-1 capillary column with 0.25-μm film thickness, temperature program 50–250°C increased at 10°C/min).

Predator Trials

Anolis carolensis carolensis. Six adult anole lizards, obtained from a local pet shop, were maintained for two weeks at 27°C with surplus food (various arthropods) and water. The anoles were isolated and starved for two days prior to testing. Each anole was tested individually in its home container by gently introducing a series of adult *S. c. cinctus*. Houseflies (*Musca domestica*) and small, second- to fourth-instar *Acheta domesticus* crickets served as control prey.

To begin each test, an anole was initially offered a control insect to verify hunger. Next, *S. c. cinctus* adults were sequentially offered until the anole refused to attack them. This two-step process was repeated until the anole continually rejected *S. c. cinctus*, yet continually ate controls. This process was repeated two or three days later.

Onychomys leucogaster. Two laboratory-reared northern grasshopper mice were obtained from the Animal Care Facility at Illinois State University. Mice were starved for 4 hr prior to testing and were tested in their home containers. Third- and fourth-instar *A. domesticus* crickets were used as controls. Each mouse was offered a control cricket followed by five *S. c. cinctus* in sequence.

Charadrius vociferus. A 1-week-old killdeer was reared in the lab on a diet of arthropods, earthworms, snails, fish and fruit. At about 4 weeks of age, when the bird could feed by itself and discriminate among prey (i.e., began to prefer some prey items over others), it was tested in the following method: The bird was given a control third-instar *A. domesticus* cricket to demonstrate hunger. Then *S. c. cinctus* individuals were sequentially offered until the bird no longer consumed the bugs. When this occurred, the bird was then offered another control cricket to verify that the bird was still hungry. If the control was eaten, then another bug was offered. The experiment ended when the bird continued to eat the controls, but repeatedly rejected the bugs.

Sturnus vulgaris. A 3-week-old European starling was reared in captivity on a diet of arthropods, fruit, and cat food. When the bird showed strong food

discrimination patterns (i.e., began to prefer some foods over others), it was tested. The feeding trials were conducted in the same way as for *S. vociferus*.

Source of Secretion

Three adult males and three adult females were chilled to 10°C and then glued ventral side up onto cardstock. They were then warmed to room temperature, viewed under a dissecting microscope, and gently pinched with tweezers to elicit expulsion of the secretion. Slivers of filter paper (3 mm^2) were then placed on the metathoracic gland opening, beak, or anus to absorb any ejected fluids.

RESULTS

Chemical Analysis

Headspace Analysis by GC. No volatiles were detected from nonagitated adult male or adult female *S. c. cinctus*. However, both male and female *S. c. cinctus* produced a blend of at least seven compounds when agitated (Figure 1). Five of the compounds were abundant enough for good-quality mass spectra to be obtained and were tentatively identified by matching to library spectra. These identifications were confirmed when authentic standards gave identical mass spectra and GC retention times (Table 1, Figure 2).

Of the five terpenes identified, α-pinene, β-pinene, and limonene have asymmetric centers. The CDX-B capillary column separated all three pairs of enantiomers, based on the commercial standards: (1*S*)-(−)- and (1*R*)-(+)-α-pinene eluted at 13.5 and 13.8 min, respectively; retentions of (1*S*)-(−)- and (1*R*)-(+)-β-pinene were 18.0 and 18.2 min, respectively; and (*S*)-(−)- and (*R*)-(+)-limonene eluted at 21.4 and 21.7 min, respectively. The insect-derived samples had GC peaks for these terpenes only at 13.8, 18.0, and 21.7 min. Therefore, (1*R*)-(+)-α-pinene, (1*S*)-(−)-β-pinene, and (*R*)-(+)-limonene were the only detectable enantiomers in the *S. c. cinctus* volatiles. Subsequently, coinjection with selected commercial terpene enantiomers confirmed that GC retention times were stable and that chirality assignments were correct.

Two additional peaks (Figure 1B) were detected in six of 12 female collections but were never seen in any of the nine male collections. Averaged over the females actually showing these peaks, the two compounds represented 21% of the female secretion. The compounds were tentatively identified as (*E*)-2-hexenyl acetate and (*E*)-2-octenal from the mass spectra (Table 1), and these identifications were then confirmed when authentic standards produced identical spectra and GC retentions.

Ignoring the (*E*)-2-octenal and (*E*)-2-hexenyl acetate present in some female

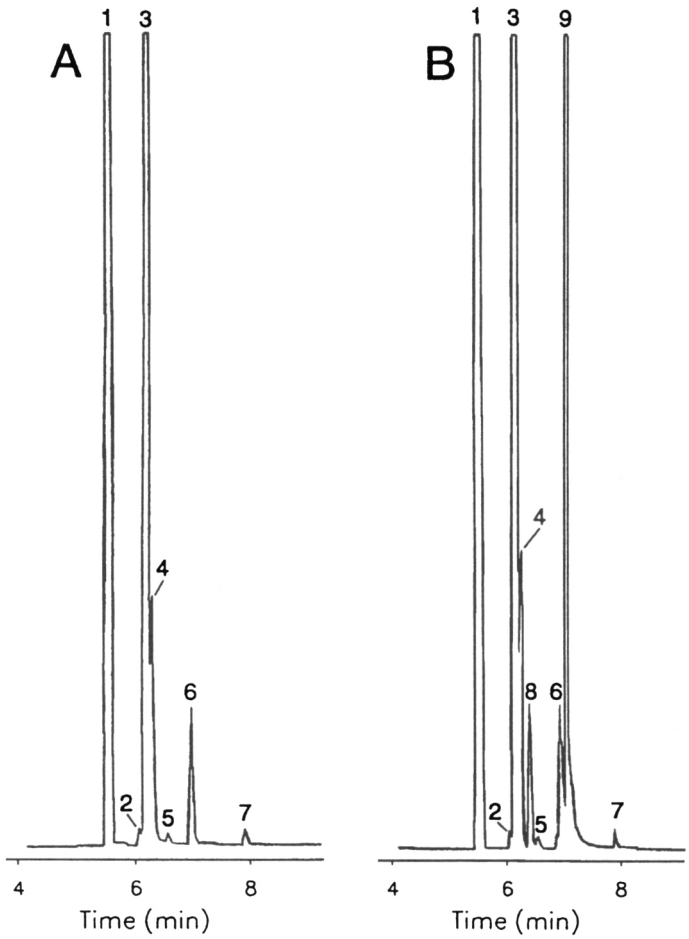


FIG. 1. Gas chromatograms of volatile emissions from adult (A) male and (B) female *S. c. cinctus*. Peak numbers correspond to those in Table 1 and Figure 2.

samples, the relative compositions of volatile blends were not significantly different (two-sample t test) between males and females. Pooled over both sexes ($N = 10$), the mean percentages for terpenes were: (1*R*)-(+)-α-pinene, 46.6% (SE ± 0.46); (1*S*)-(−)-β-pinene, 43.7% (SE ± 0.49); β-myrcene, 6.2% (SE ± 0.16); (*R*)-(+)-limonene, 3.2% (SE ± 0.07); α-terpinolene, 0.4% (SE ± 0.02).

Seed Extraction. Analysis of *P. vulgaris* seeds yielded no trace of the identified compounds by GC analysis.

TABLE 1. CHEMICALS IDENTIFIED IN *S. c. cinctus* SECRETIONS

GC peak	Identification[a]	Major MS ions[b] and (percentages)
1	(1*R*)-(+)-α-pinene	136 (M^+, 7), 93 (100), 91 (48), 79 (28), 77 (39), 41 (26)
2	(unidentified)	not available
3	(1*S*)-(−)-β-pinene	136 (M^+, 8), 93 (100), 91 (31), 77 (31), 69 (35), 41 (60)
4	β-myrcene	136 (M^+, 2), 93 (63), 91 (16), 79 (13), 69 (56), 41 (100)
5	(unidentified)	not available
6	(*R*)-(+)-limonene	136 (M^+, 17), 93 (83), 77 (33), 68 (100), 67 (82), 41 (40)
7	Terpinolene	136 (M^+, 57), 121 (76), 93 (100), 91 (55), 79 (52)
8[c]	(*E*)-2-hexenyl acetate	142 (M^+, 0.4), 100 (12), 82 (22), 67 (36), 57 (13), 55 (16), 43 (100), 41 (23)
9[c]	(*E*)-2-octenal	126 (M^+, 0.1), 83 (53), 82 (27), 58 (14), 57 (46), 55 (84), 43 (16), 42 (36), 41 (100)

[a] Chirality determined on chiral GC column; set text.
[b] Major MS ions (*m/z*), in order of decreasing abundance.
[c] Compounds detected only from some female samples; see text.

Predator Trials

Anolis carolensis carolensis *vs.* Sehirus cinctus cinctus. Of the six anoles tested, two were eliminated because they did not attack the initial control insect. The remaining four anoles developed strong predator avoidance towards *S. c. cinctus* (Table 2).

During the feeding trials, all four anoles behaved in a similar fashion. Each anole quickly attacked and consumed the first control and one or more bugs. When attacked, the bugs emitted a strong odor. Within 7 min of the beginning of the trials, the anoles became more hesitant and eventually ignored *S. c. cinctus* but readily attacked and consumed controls. The anoles clearly discriminated between controls and *S. c. cinctus*; they quickly approached, attacked, and consumed controls, but ignored *S. c. cinctus*, even when the bugs crawled under the head or over the feet of the lizard.

In a second feeding trial two to three days after the first, two of the anoles refused to attack, and two ate one *S. c. cinctus* each, then refused to attack subsequent bugs; yet, all anoles eagerly attacked and consumed numerous crickets.

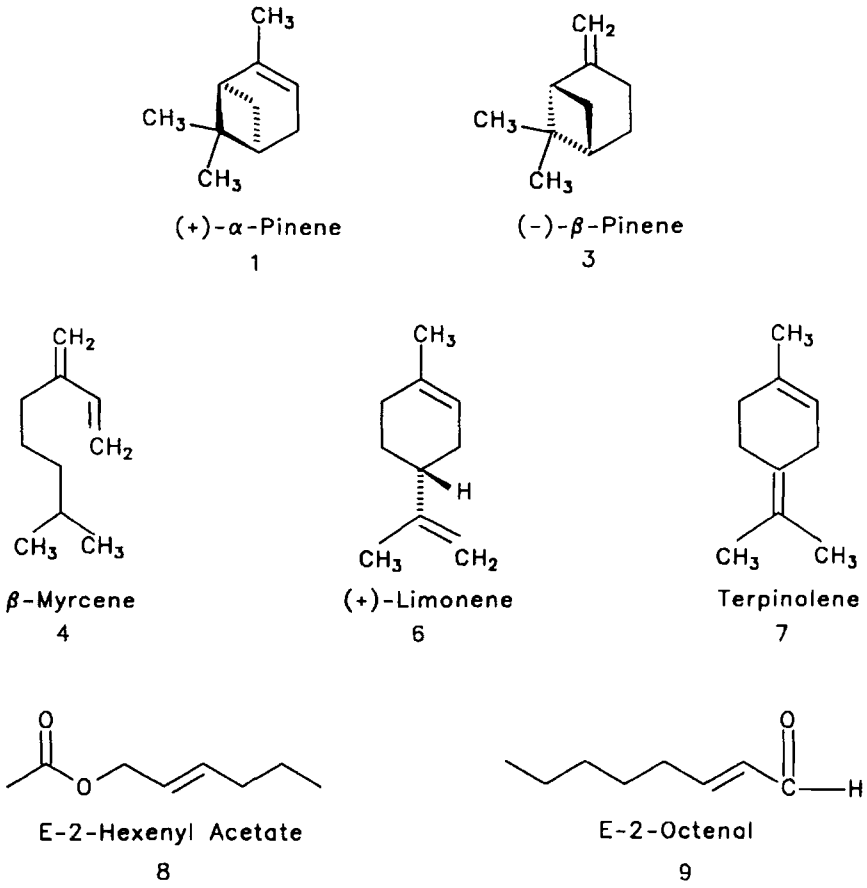


FIG. 2. Major components from adult *S. c. cinctus* volatile secretions. Components 1, 3, 4, 6, and 7 are found in both sexes; components 8 and 9 are found only in females.

TABLE 2. NUMBER OF ADULT BURROWING BUGS CONSUMED BY LIZARDS DURING TWO TRIALS SEPARATED BY TWO OR THREE DAYS

Anole	First trial	Second trial
1	14	1
2	6	0
3	6	1
4	1	0

Charadrius vociferus *and* Sturnus vulgaris *vs.* S. c. cinctus. Both the killdeer and starling showed similar responses. Each readily attacked and consumed the first few (4–6) bugs offered. Then, all subsequent bugs offered (4–12) were either ignored (in spite of the bugs crawling over the bird's feet) or picked up and immediately ejected out of the beak, whereas control insects were readily eaten.

Onychomys leucogaster *vs.* S. c. cinctus. Both mice behaved in a similar manner. The first two bugs offered were hesitantly grabbed, bitten into, released, recaptured, and then eaten. The next three bugs offered were quickly grabbed and eaten. Throughout these feeding trials the bug's secretion was readily noticeable.

Source of Secretion

Upon pertubation of adults, secretion was observed originating from the metathoracic gland opening for both sexes. Furthermore, the characteristic odor was evident on the filter paper sliver placed on the metathoracic gland opening but not on the filter paper squares placed on the mouth or anus.

DISCUSSION

Our results suggest that the volatile secretion produced by *Sehirus cinctus cinctus* functions in antipredator defense: (1) The chemicals are secreted in response to disturbance. (2) Both sexes produce the major constituents, suggesting that they are not used as sexual pheromones. (3) The primary constituents (α-pinene, β-pinene, and limonene) are found in other arthropod defensive secretions and are known to have deterrent, irritating, or toxic qualities (Baker and Walmsley, 1982; Smith, 1963). (4) The bugs exhibit features of the chemical defense syndrome (Whitman et al., 1985) such as aposematic coloration, gregariousness, and poor locomotory capability. For example, when attacked, they do not readily fly or run away; their primary escape defense is to drop to the ground. (5) Anoles and birds rejected them after consuming only a few. (6) Anoles developed strong food aversion conditioning after only one feeding trial.

The only predators that did not show an aversion to feeding on *S. c. cinctus* were grasshopper mice, which each consumed the five bugs offered. The large body mass of the mice and their eagerness to consume the bugs (the speed of consumption increased with subsequent prey) led us to believe that they could probably consume many dozens of bugs during one feeding (a limited supply of bugs prevented us from testing this). In contrast, the anoles and birds clearly exhibited increasing signs of aversion with each bug consumed. It is common for insect defenses to function differently against different predator taxa (Whitman, 1988; Whitman et al., 1990). In addition, *Onychomys* species are known

to be indiscriminate insectivores, attacking a wide range of toxic or otherwise defended prey including scorpions, centipedes, darkling beetles, lubber grasshoppers, etc. (McCarty, 1978; Whitman et al., 1985).

Five compounds were found in the secretion from both sexes [(1*R*)-(+)-α-pinene, (1*S*)-(−)-β-pinene, β-myrcene, (*R*)-(+)-limonene, and terpinolene]. α-Pinene and β-pinene together comprised the major portion of the secretion volatiles (91% of GC peak area). These two compounds have been found in other hemipteran groups such as Scutelleridae (Hamilton et al., 1985), Lygaeidae (Olagbemiro and Staddon, 1983), and Rhopalidae (Aldrich et al., 1990). However, the combined concentration of these two compounds typically comprises no more than 10–20% of the total secretion (Hamilton et al., 1985; Olagbemiro and Staddon, 1983). These two compounds have also been found in termites (Baker and Walmsley, 1982).

Terpenes, as a group, are fairly common in the secretions of Hemiptera (Aldrich, 1988). In addition, a reduviid bug, *Apiomerus flaviventris*, uses terpenoids to protect its eggs (Eisner and Meinwald, 1987). The insect gathers resins from plants and coats its eggs with the resins, which contain repellent terpenoids (borneol, camphor, 1,8-cineole, etc.).

The roles of two additional compounds [(*E*)-2-octenal, and (*E*)-2-hexenyl acetate] that were present only in females are unknown. Female-specific compounds are found in other heteropterans (Scales, 1968; Boivin and Stewart, 1982) where they are thought to serve as sexual pheromones. Indeed, in *Sehirus bicolor* (L.), the secretion is reported to be expelled during courtship (Southwood and Hine, 1950). Furthermore, since *S. c. cinctus* aggregate, one or both of these compounds may function as an aggregation pheromone.

It is not known how the various secretion components of *S. c. cinctus* function in defense. Many chemically defended insects possess two allomonal systems that serve interactive, but divergent roles: (1) distasteful, toxic, or emesis-producing compounds are held in the body; and (2) volatile compounds are secreted (Whitman et al., 1990). The chemical composition of these two systems can be quite different. Internal allomones are often large, nonvolatile compounds, whereas defensive secretions are usually mixtures of highly volatile, reactive, low-molecular-weight compounds (Blum, 1981). In such insects, the internal compounds produce toxicosis and elicit food aversion conditioning in predators, whereas the volatile secretion acts to startle or deter some predators, or functions in chemical aposematism (Eisner and Grant, 1981). For some predators, the defensive secretion is not deterrent by itself, but becomes deterrent when it becomes associated with the internal toxin via classical Pavlovian conditioning (Brower, 1984). Some aspects of our predator tests suggest that a scenario similar to the latter might be the case in *S. c. cinctus*. First, no predators rejected the first *S. c. cinctus* they encountered, suggesting that the odor or taste of the defensive secretion was not enough, by itself, to deter consumption. It

was only after consumption that aversion developed, suggesting the possible presence of a slower-acting internal toxin or alternatively, an internal response to the secretion.

Mint plants are notoriously rich in terpenoid compounds, and hence, *S. c. cinctus* may have obtained its secretion components from the seeds they feed on. However, none of the five major terpenes found in the secretions of *S. c. cinctus* was found in the *P. vulgaris* seeds. This suggests that *S. c. cinctus* synthesizes these chemicals de novo, modifies related precursors, or sequesters the terpenes from the mint leaves, not seeds.

The life cycle of burrowing bugs is predominantly spent on or near the ground (Sites and McPherson, 1982) where these bugs may frequently encounter ants that may pose a threat to individuals or females guarding their brood. Eisner et al. (1976) have shown that α- and β-pinene act as irritants to ants, causing scratching and grooming reflexes that subsequently spread these compounds over the surface of the ants. We are in the process of examining the potential effectiveness of the defensive secretion of *S. c. cinctus* against ant predators.

Acknowledgments—We wish to thank Dr. Jeffrey Aldrich; USDA-ARS, for helpful comments.

REFERENCES

ALDRICH, J. R. 1988. Chemical ecology of the Heteroptera. *Annu. Rev. Entomol.* 33:211-238.

ALDRICH, J. R., CARROLL, S. P., OLIVER, J. E., LUSBY, W. R., RUDMANN, A. A., WATERS, R. M. 1990. Exocrine secretions of scentless plant bugs: *Jadera*, *Boisea* and *Niesthrea* (Hemiptera: Heteroptera: Rhopalidae). *Biochem. Syst. Ecol.* 18:369-376.

BAKER, R., and WALMSLEY, W. 1982. Soldier defense secretions of the South American termites *Cortaritermes silvestri*, *Nasutitermes* species N.D. and *Nasutitermes kemneri*. *Tetrahedron* 38:1899-1910.

BLUM, M. S. 1981. Chemical Defenses in Arthropods. Academis Press, New York.

BOIVIN, G., and STEWART, R. K. 1982. Attraction of male green apple bugs, *Lygocoris communis* (Hemiptera: Miridae), to caged females. *Can. Entomol.* 114:765-766.

BROWER, L. P. 1984. Chemical defense in butterflies, pp. 109-137, *in* R. I. Vane-Wright and P. R. Ackery (eds.). The Biology of Butterflies. Academic Press, London.

EISNER, T., and GRANT, R. P. 1981. Toxicity, odor aversion, and "olfactory aposematism." *Science* 213:476.

EISNER, T., and MEINWALD, J. 1987. Pheromones and sexual selection, pp. 251-269, *in* G. D. Prestwich and G. J. Blomquist (eds.). Pheromone Biochemistry. Academic Press, Orlando, Florida.

EISNER, T., KRISTON, I., and ANESHANSLEY, D. J. 1976. Defensive behavior of a termite (*Nasutitermes exitiosus*). *Behav. Ecol. Sociobiol.* 1:83-125.

FROESCHNER, R. C. 1960. Cydnidae of the Western Hemisphere. *Proc. U.S. Natl. Mus.* 111:337-680.

HAMILTON, J. G. C., GOUGH, A. J. E., STADDON, B. W., and GAMES, D. E. 1985. Multichemical defense of plant bug *Hotea gambiae* (Westwood) (Heteroptera: Scutelleridae): (*E*)-2-Hexenol from abdominal gland in adults. *J. Chem. Ecol.* 11:1399-1409.

MILLER, N. C. E. 1971. The Biology of the Heteroptera. E. W. Classey, Hampton, Middlesex, UK.

MCCARTY, R. 1978. *Onychomys leucogaster*. *Mamm. Species* 87:1–6.

MCDONALD, F. J. D. 1968. Some observations on *Sehirus cinctus* (Palisot de Beauvois) (Heteroptera: Cydnidae). *Can. J. Zool.* 46:855–858.

OLAGBEMIRO, T. O., and STADDON, B. W. 1983. Isoprenoids from metathoracic scent gland of cotton seed bug, *Oxycarenus hyalinipennis* (Costa) (Heteroptera: Lygaeidae). *J. Chem. Ecol.* 9:1397–1412.

SCALES, A. L. 1968. Female tarnished plant bugs attract males. *J. Econ. Entomol.* 61:1466–1467.

SITES, R. W., and MCPHERSON, J. E. 1982. Life history and laboratory rearing of *Sehirus cinctus cinctus* (Hemiptera: Cydnidae), with descriptions of immature stages. *Ann. Entomol. Soc. Am.* 75:210–215.

SMITH, R. H. 1963. Toxicity of pine resin vapors to three species of *Dendroctonus* bark beetles. *J. Econ. Entomol.* 56:827–831.

SOUTHWOOD, T. R. E., and HINE, D. J. 1950. Further notes on the biology of *Sehirus bicolor* (L.) (Hemiptera, Cydnidae). *Entomol. Mon. Mag.* 86:299–301.

STADDON, B. W. 1979. The scent glands of Heteroptera. *Adv. Insect Physiol.* 14:351–418.

WHITMAN, D. W. 1988. Allelochemical interactions among plants, herbivores, and their predators, pp. 11–64, *in* P. Barbosa and D. Letourneau (eds.). Novel Aspects of Insect–Plant Interactions. John Wiley & Sons, New York.

WHITMAN, D. W., BLUM, M. S., and JONES, C. G. 1985. Chemical defense in *Taeniopoda eques* (Orthoptera: Acrididae): Role of the metathoracic secretion. *Ann. Entomol. Soc. Am.* 78:451–455.

WHITMAN, D. W., BLUM, M. S., and ALSOP, D. W. 1990. Allomones: Chemicals for defense, pp. 289–351, *in* D. L. Evans and J. O. Schmidt (eds.). Insect Defenses. State University of New York Press, Albany.

Journal of Chemical Ecology, Vol. 23, No. 8, 1997

BALSAM FIR FOLIAR CHEMISTRY IN MIDDLE AND LOWER CROWNS AND SPRUCE BUDWORM GROWTH, DEVELOPMENT, FOOD AND NITROGEN UTILIZATION

NATHALIE CARISEY* and ERIC BAUCE

Centre de Recherche en Biologie Forestière
Département des Sciences du Bois et de la Forêt
Faculté de Foresterie et de Géomatique
Université Laval
Ste-Foy, Québec, Canada G1K 7P4

(Received August 23, 1996; accepted April 5,1997)

Abstract—The impact of current-year foliage from the middle or lower crown section of balsam fir trees on larval growth, development, nitrogen, and food utilization was studied with laboratory rearing experiments, gravimetric analyses, and foliage chemical analyses. Pupal dry weight, pupal nitrogen weight, and total development times were not affected by feeding on current-year foliage from either the middle or the lower crown section. However, the nutritive and, particularly, allelochemical profiles were significantly different between crown levels and influenced spruce budworm food utilization, depending on development stage. Newly molted fifth instars feeding since post-diapause on middle crown foliage had lower dry weight and nitrogen weight than those fed lower crown foliage. This apparently resulted from the high monoterpene content in the mid-crown foliage. At the end of the fifth instar, however, insects from both crown levels had similar larval dry weights and nitrogen weights. Larvae fed middle crown foliage compensated with several mechanisms that led to increased relative nitrogen accumulation rate (RNAR) including increased efficiencies of conversion of ingested and digested nitrogen (ECIN, ECDN). Sixth-instar development time was nearly two days shorter for larvae fed middle crown foliage compared to those fed lower crown foliage. A higher relative consumption rate (RCR) due to the high content of two phagostimulatory components (soluble sugars and β-pinene) may account for reduced sixth-instar development time. Larvae from the middle crown section had higher relative growth rates (RGR) and RNAR than those from the lower crown. Pupal development time was also longer in the lower crown. The existence of spruce budworm flexibility to adapt to foliar biochemistry variations by developing compensatory mechanisms enables larvae to exploit the whole tree crown.

*To whom correspondence should be addressed.

0098-0331/97/0800-1963$12.50/0 © 1997 Plenum Publishing Corporation

Key Words—*Choristoneura fumiferana*, Spruce budworm, *Abies balsamea*, balsam fir tree, intratree variations, nutritional indices, nitrogen budget, foliage chemistry.

INTRODUCTION

The distribution of spruce budworm larvae (SBL), *Choristoneura fumiferana* (Clem.), within balsam fir trees, *Abies balsamea* L. (Mill.), has been studied several times during investigations of sampling methods (Morris, 1955; Sanders, 1980; Régnière et al., 1989). Depending on population density, tree crown structure, and the unit used to express density, the distribution of SBL in different instars varies, but it appears they can grow anywhere within the live tree crown. Piene (1996) mentioned that SBL could cause defoliation anywhere throughout the crown of young balsam fir trees. In old balsam fir stands, small quantities of current-year foliage are available in the upper crown level compared to the middle and lower crown sections. Thus, SBL mostly feed in these latter two levels. SBL primarily occur in the middle crown when larval population density is low, and at high population density they usually feed on current-year foliage throughout the crown (unpublished data).

Light availability decreases with depth in a large-scale photosynthetic organization (canopy) (Wu, 1993; Pearcy and Sims, 1994), and that in turn affects photosynthetic capacity in leaves throughout the plant canopy. Ginn et al. (1991) reported that in unthinned loblolly pine stands, both light-saturated photosynthesis and needle conductance were significantly decreased in the lower crown. The production of monoterpenes is closely related to the intensity of sunlight radiation and to photosynthesis (Gleizes et al., 1980; Gershenzon and Croteau, 1991; Schürmann et al., 1993). In this study we hypothesized that nutritional and allelochemical profiles of current-year foliage from different crown levels influence the biological performance of SBL. To test this hypothesis, we reared female SBL under laboratory conditions and provided them with foliage from either the middle or lower crown section of trees located in an old balsam fir stand. Using the gravimetric method of Waldbauer (1968), we studied growth, development, and food and nitrogen utilization of SBL during the fifth and sixth instars as related to the nutritional and allelochemical profiles of current-year foliage within balsam fir trees.

METHODS AND MATERIALS

Description of Study Area

The research took place in a 60-year-old balsam fir stand located in compartment 20 of the Forêt Montmorency (47°19′N, 71°09′W), an experimental

forest of Université Laval. The stand fits Grandtner's (1966) description of the balsam fir–white birch association and was classified as a site 1 quality stand with good drainage (Bélanger et al., 1983), 15% slope, deep uncompacted glacial till, and 65% crown cover. We divided the live crown of trees into lower, middle, and higher crown sections of equal length.

Chemical Analysis of Foliage

A total of 52 trees were randomly selected for chemical analyses. At three- or four-day intervals, three branches in the lower crown section and three branches in the middle crown section were harvested on four trees. Different trees were used at each collection date. Current-year foliage from the three branches collected in each section were pooled. Thus, foliage analyses were performed on four tree samples per crown section per collection date. One-year-old foliage was harvested and processed in a similar manner.

The first harvest was on May 24, eight days before budbreak, and when 100 degree-days were reached in the field using 2.6°C as the threshold of temperature for budworm development. The last harvest was on July 5, when all budworms in each crown section had reached pupal stage.

Two grams of each fresh sample were freeze-dried, ground in a 20-mesh Wiley mill, and kept at −20°C prior to chemical analysis. Foliar contents of minerals (N, P, K, Ca, Mg) and total soluble sugars, tannins, phenolics, and monoterpenes were determined according to Bauce et al. (1994) and Bauce (1996). The nitrogen–tannins ratio was used as an index of protein availability (Feeny, 1969). A fresh subsample of each foliage sample was placed in a crimped-seal vial and kept at −20°C prior to monoterpene analysis (Bauce et al., 1994).

Laboratory Insect Rearing

To obtain food for larvae reared in the laboratory, 30 balsam fir trees were randomly selected in the field, and at three- or four-day intervals, 12 branches in the lower and middle crown sections were harvested on two trees. Foliage collected in the same section was randomly distributed to larvae according to treatments: (1) feeding on current-year foliage from the middle section, and (2) feeding on current-year foliage from the lower section. Different trees were used at each collection date.

SBL obtained from the Forest Pest Management Institute, Forestry Canada, Sault Saint-Marie, Ontario, were maintained in a rearing cabinet at 12°C, 65% relative humidity, and 16L:8D photoperiod. These conditions corresponded to the average conditions prevailing at the Forêt Montmorency during the feeding period of spruce budworm in summer 1992. Postdiapausing larvae emerged in rearing containers, similar to those described by Bauce et al. (1994),

and were provided with fresh 1-year-old balsam fir twigs harvested on May 24. Four to six days later, some larvae in both treatments began feeding on new buds and spinning webs, while others were still mining 1-year-old needles as reported by Atwood (1944) and McGugan (1954). On June 5, 11 days after beginning the experiment, larvae in both treatments were provided with only current-year foliage. Insect development was monitored every 6 hr for 24 hr/day, and every 15 min during molting. When SBL reached the fifth instar, they were sexed (Robertson, 1985), and 40–50 female larvae per treatment were transferred to individual rearing containers. Fifth and sixth instars with exuviae were weighed 1 hr after molting. Pupae were weighed 8 hr after pupation. In each treatment, 40 newly molted fifth instars, 20 newly molted sixth instars, and 10 pupae were weighed, freeze-dried, and weighed again in order to determine percentage of insect dry matter. These insects were also used to obtain larval nitrogen content. Nitrogen content in insects and feces was determined with the Kjeldahl method (Bremner and Mulvaney, 1982). The data on mortality during the fifth and sixth instars and pupal stages were analyzed using chi-square analysis to determine if mortality was dependent on crown section.

Data were analyzed with the Statistical Analysis System (SAS Institute, 1988). Tests of normality and homogeneity of variance were performed, and data were subjected to analysis of variance (ANOVA).

Larvae and Foliage Manipulations

Postdiapause Emergence to Fifth Instar. Development time from second to fifth instar, and fifth-instar dry weight and nitrogen weight were subjected to ANOVA in a complete randomized design with each individual insect as the experimental unit. Foliar analyses corresponded to the day insects began feeding on new buds until they reached the fifth instar. Thus, chemical analyses were performed on current-year foliage that was collected in each crown section on May 29, June 1, and on June 5. Data were subjected to ANOVA with a two-stage crossed nested design with tree samples ($N = 4$) nested within date ($N = 3$) (error term for the date effect), and the crown section ($N = 2$) factor crossed with trees nested within the date factor (error term for the crown section effect and the date × crown section interaction).

Fifth Instar. Fifth-instar development time was recorded, and foliage ingested and feces excreted were quantified as described by Bauce et al. (1994). Nutritional indices were calculated according to Waldbauer (1968) and Montgomery (1983) on a dry-weight and a nitrogen-content basis. The formulas for nutritional indices and nitrogen nutritional indices are the following:

$$RGR \text{ (relative growth rate)} = G/[MW \times \text{hours (development time)}]$$

$$RCR \text{ (relative consumption rate)} = I/[MW \times \text{hours (development time)}]$$

AD (approximate digestibility) $= (I - F) \times 100/I$

ECI (efficiency of conversion of ingested food) $= G \times 100/I$

ECD (efficiency of conversion of digested food) $= G \times 100/(I - F)$

where G is gained weight = (final weight − initial weight), MW is mean larval weight = G/log (final weight/initial weight), I is ingested food, and F is feces.

$RNAR$ (relative nitrogen accumulation rate)

$= GN/[MNW \times$ hours (development time)]

$RNCR$ (relative nitrogen consumption rate)

$= IN/[MNW \times$ hours (development time)]

ADN (approximate digestibility of nitrogen) $= (IN - EN) \times 100/IN$

$ECIN$ (efficiency of conversion of ingested nitrogen) $= GN \times 100/IN$

$ECDN$ (efficiency of conversion of digested nitrogen)

$= GN \times 100/(IN - EN)$

where GN is gained larval nitrogen weight = (nitrogen final weight − nitrogen initial weight), MNW is mean larval nitrogen weight = GN/log (nitrogen final weight/nitrogen initial weight), IN is ingested nitrogen, and EN is excreted nitrogen.

A linear or nonlinear equation of regression was computed for each ratio or nutritional index in order to determine an adjusted ratio and allow meaningful comparisons of indices (Bauce et al., 1994). Adjusted ratios, development times, larval dry weight, and nitrogen weights were subjected to analysis of variance in a complete randomized design with insect as the experimental unit. A multivariate ANOVA in the same design was carried out on each group of parameters: (1) insect performance (i.e., development time, dry weight, and nitrogen weight); (2) insect food utilization (i.e., nutritional indices); and (3) insect nitrogen utilization (i.e., nitrogen nutritional indices). The traditional P value of 0.05 was adjusted by the number of univariate tests performed in each group of parameters (Bonferonni adjustment). Thus, we only considered P values less than or equal to the adjusted P value as significant. Chemical data from current-year foliage harvested from June 9 to 17 were subjected to ANOVA with a two-stage crossed nested design with tree samples ($N = 4$) nested within date ($N = 3$) (error term for the date effect), and the crown section ($N = 2$) factor crossed with trees nested within the date factor (error term for the crown section effect and the date × crown section interaction). A multivariate analysis was carried out on data corresponding to foliage nutritional compounds (N, P, K, Ca, Mg, total soluble sugars).

Sixth Instar. The same parameters as described for fifth instars were determined for sixth instars and analyzed using the same methods. Pupal and total development times were also recorded. Chemical data from current-year foliage harvested from June 17 to July 5 were subjected to ANOVA with the same two-stage crossed nested design as used for data from fifth instars. A multivariate ANOVA was carried out on each group of parameters: (1) nutritional compounds (N, P, K, Ca, Mg, total soluble sugars); and (2) allelochemical compounds (total phenolics, tannins, monoterpenes). As the most important factor deals with the nutritional and allelochemical quality between foliage from the two crown sections during the feeding period of SBL, we only reported results of the crown section effect from ANOVA in the two-stage crossed nested design.

RESULTS

Data for performance of the fifth and sixth instars are presented in Tables 1 and 2, respectively. With regards to general parameters, such as total development time, pupal dry weight, and nitrogen weight, feeding on current-year foliage from the middle or lower crown section had no significant effect on SBL (Table 2). However, the allelochemical profiles of current-year foliage, from budbreak to the fifth instar and during the fifth instar, were significantly different between crown levels (Tables 3 and 4). During the sixth instar, both foliar nutritive and allelochemical profiles were significantly different between crown levels (Table 4), and this influenced spruce budworm food and nitrogen utilization, depending on development stage.

Postdiapausing Emergence to Fifth Instar. SBL that were fed lower crown foliage reached the fifth instar nearly two days before those that were fed middle crown foliage. Newly molted fifth instars from the lower crown had heavier dry weights and nitrogen weights (Table 1). During this period, within-tree current-year foliage had equal nutritive value. However, foliage from the middle crown contained, respectively, 110 and 133% more δ-3-carene and terpinolene (Table 3).

Fifth Instar. SBL that fed in either middle or lower crown section had similar fifth-instar development times, larval dry weights, and nitrogen weights as newly molted sixth instars, and nutritional indices, except for the approximate digestibility (AD), which was 13% higher for insects fed in middle crown (Table 1). However, nutritional nitrogen indices were totally different. Insects fed middle crown foliage showed higher RNAR and efficiencies of nitrogen conversion (ECDN, ECIN). Mortality during the fifth instar was similar in both treatments, 5.7% for lower crown and 0% for middle crown sections (likelihood ratio chi-square: $\chi^2 = 3.17$; $df = 1$; $P = 0.08$).

Results of the crossed nested multivariate ANOVA indicated that within-

TABLE 1. WEIGHT, DEVELOPMENT, NUTRITIONAL INDICES, AND NITROGEN NUTRITIONAL INDICES (MEAN ± 2 SE) OF FIFTH-INSTAR SPRUCE BUDWORM FED ON CURRENT-YEAR FOLIAGE FROM MIDDLE OR LOWER CROWN SECTION

	Middle crown	Lower crown	P^a
Insect performances			
Development time (2–5 instars) (hr)	445 ± 16 (39)[b]	400 ± 11 (32)	0.0001
5-instar development time (hr) (mg)	169 ± 23 (39)	174 ± 12 (32)	0.70
Newly molted 5th-instar dry weight (mg)	0.90 ± 0.06 (39)	1.04 ± 0.07 (32)	0.002
Newly molted 6th-instar dry weight (mg)	3.63 ± 0.22 (39)	3.91 ± 0.24 (32)	0.09
Newly molted 5th-instar N weight (mg)	0.12 ± 0.01 (39)	0.17 ± 0.01 (32)	0.0001
Newly molted 6th-instar N weight (mg)	0.47 ± 0.03 (39)	0.44 ± 0.03 (32)	0.10
	Wilk's λ^c = 0.02	$F(6, 64)$ = 462	P = 0.0001
Nutritional indices[d]			
RGR (mg/mg/hr)	0.0089 ± 0.0005 (39)	0.0082 ± 0.0008 (32)	0.10
RCR (mg/mg/hr)	0.0923 ± 0.0083 (39)	0.0895 ± 0.0080 (32)	0.60
AD (%)	58.9 ± 3.6 (39)	52.0 ± 3.8 (32)	0.01
ECD (%)	20.9 ± 4.1 (39)	21.3 ± 4.5 (32)	0.90
ECI (%)	10.0 ± 1.0 (39)	9.6 ± 1.2 (32)	0.60
	Wilk's λ = 0.84	$F(5, 65)$ = 2.5	P = 0.04
Nitrogen nutritional indices[e]			
RNAR (mg/mg/hr)	0.0090 ± 0.0005 (39)	0.0057 ± 0.0005 (32)	0.0001
RNCR (mg/mg/hr)	0.0226 ± 0.0026 (39)	0.0242 ± 0.0022 (32)	0.40
ADN (%)	65.5 ± 3.0 (39)	68.2 ± 2.6 (32)	0.20
ECDN (%)	57.4 ± 7.0 (33)	39.8 ± 6.5 (32)	0.0005
ECIN (%)	42.7 ± 4.3 (39)	25.1 ± 3.3 (32)	0.0001
	Wilk's λ = 0.40	$F(5, 59)$ = 17.4	P = 0.0001

[a] P value is for univariate analysis of variance. Significant P values are less than or equal to the adjusted P value, which corresponds to the traditional P value of 0.05 divided by the number of univariate tests carried out on each class of parameters ($P \leq 0.008$ for insect performances; $P \leq 0.01$ for nutritional indices and nitrogen nutritional indices).

[b] Values in parentheses are numbers of larvae per treatment.

[c] Wilk's λ tests the hypothesis of no overall "crown section effect."

[d] See Methods and materials for abbreviations of nutritional indices expressed in dry weight.

[e] See Methods and materials for abbreviations of nutritional indices expressed in amounts of nitrogen.

TABLE 2. WEIGHT, DEVELOPMENT, NUTRITIONAL INDICES, AND NITROGEN NUTRITIONAL INDICES (MEAN ± 2 SE) OF SIXTH-INSTAR SPRUCE BUDWORM FED ON CURRENT-YEAR FOLIAGE FROM MIDDLE OR LOWER CROWN SECTION

	Middle crown	Lower crown	P^a
Insect performances			
Development time (2–6 instars) (hr)	602 ± 17 (33)[b]	574 ± 13 (31)	0.01
6-instar development time (hr)	421 ± 19 (34)	463 ± 13 (31)	0.0008
Pupal development time (hr)	518 ± 12 (20)	553 ± 10 (19)	0.0001
Total development time (2nd instar to imago) (hr)	1547 ± 53 (20)	1583 ± 34 (19)	0.30
Newly molted 6-instar dry weight[d] (mg)	3.57 ± 0.24 (33)	3.91 ± 0.25 (31)	0.06
Pupal dry weight (mg)	31.89 ± 2.74 (34)	34.81 ± 2.47 (31)	0.10
Newly molted 6-instar N weight (mg)	0.46 ± 0.03 (33)	0.44 ± 0.03 (31)	0.20
Pupal N weight (mg)	2.30 ± 0.20 (34)	2.26 ± 0.16 (31)	0.80
	Wilk's $\lambda^c = 0.03$	$F(7, 31) = 157$	$P = 0.0001$
Nutritional indices[d]			
RGR (mg/mg/hr)	0.0055 ± 0.0002 (33)	0.0049 ± 0.0001 (31)	0.0001
RCR (mg/mg/hr)	0.0776 ± 0.0072 (33)	0.0591 ± 0.0049 (31)	0.0001
AD (%)	38.4 ± 4.0 (32)	38.5 ± 5.2 (30)	0.97
ECD (%)	23.7 ± 6.2 (33)	28.7 ± 7.5 (30)	0.30
ECI (%)	7.5 ± 0.5 (33)	8.3 ± 0.5 (31)	0.02
	Wilk's $\lambda = 0.63$	$F(5, 54) = 6.3$	$P = 0.0001$
Nitrogen nutritional indices[e]			
RNAR (mg/mg/hr)	0.0041 ± 0.0002 (33)	0.0037 ± 0.0001 (31)	0.002
RNCR (mg/mg/hr)	0.0186 ± 0.0018 (33)	0.0173 ± 0.0014 (31)	0.30
ADN (%)	42.3 ± 4.3 (34)	45.3 ± 4.6 (30)	0.30
ECDN (%)	48.3 ± 8.0 (30)	48.4 ± 7.4 (28)	0.97
ECIN (%)	22.8 ± 1.4 (33)	22.3 ± 1.3 (31)	0.60
	Wilk's $\lambda = 0.87$	$F(5, 51) = 1.5$	$P = 0.20$

[a] P value is for univariate ANOVA. Significant P values are less than or equal to the adjusted P value, which corresponds to the traditional P value of 0.05 divided by the number of univariate tests carried out on each class of parameters ($P \leq 0.006$ for insect performances; $P \leq 0.01$ for nutritional indices and nitrogen nutritional indices).

[b] Values in parentheses are numbers of larvae per treatment.

[c] Wilk's λ tests the hypothesis of no overall "crown section effect."

[d] See Methods and materials for abbreviations of nutritional indices expressed in dry weight.

[e] See Methods and materials for abbreviations of nutritional indices expressed in amounts of nitrogen.

TABLE 3. CHEMICAL ANALYSES (MEAN ± 2 SE) OF CURRENT-YEAR BALSAM FIR FOLIAGE ACCORDING TO CROWN SECTION, FROM BUDBREAK TO BEGINNING OF FIFTH INSTAR

Foliar chemicals	Middle crown	Lower crown	P^a
Nutritional compounds			
N[b]	3.08 ± 0.54	3.41 ± 0.34	0.40
P (ppm)	4538 ± 916	5432 ± 601	0.20
K (ppm)	10042 ± 1620	10868 ± 1529	0.70
Ca (ppm)	1814 ± 575	1689 ± 342	0.70
Mg (ppm)	961 ± 88	991 ± 44	0.90
Total soluble sugars[b]	7.05 ± 1.56	5.61 ± 1.01	0.10
Allelochemical compounds			
Total phenolics[b]	18.40 ± 7.84	18.06 ± 8.38	0.60
Total tannins (cm^2/mg)	0.92 ± 0.28	0.80 ± 0.21	0.40
N:total tannins	4.18 ± 1.38	4.71 ± 0.90	0.60
Total monoterpenes[b]	1.36 ± 0.67	0.96 ± 0.32	0.40
Tricyclene	0.004 ± 0.001	0.004 ± 0.002	0.80
α-pinene	0.40 ± 0.15	0.36 ± 0.09	0.20
Camphene	0.018 ± 0.004	0.014 ± 0.002	0.30
β-pinene	0.51 ± 0.41	0.43 ± 0.29	0.60
Myrcene	0.03 ± 0.01	0.02 ± 0.01	0.30
δ-3-carene	0.21 ± 0.04	0.10 ± 0.03	0.04
Limonene-δ-phellandrene	0.08 ± 0.05	0.08 ± 0.04	0.60
Terpinolene	0.007 ± 0.002	0.003 ± 0.003	0.005
Bornyl acetate	0.029 ± 0.007	0.023 ± 0.003	0.20

[a] P value is for univariate ANOVA. Significant P value are less than or equal to the P value of 0.05.
[b] % dry weight.

tree nutritive value of foliage was similar (Table 4). Multivariate analysis was not performed on variables corresponding to the allelochemical compounds because of the high number of parameters (13) relative to that of degrees of freedom from the error term (Table 4). Results from univariate ANOVA indicated that foliage from the middle crown had 34% more total monoterpenes than foliage from the lower crown and contained, respectively, 50, 20, 65, and 43% more tricyclene, camphene, β-pinene, and bornyl acetate.

Sixth Instar. In the lower crown, sixth instar and pupal development times lasted, respectively, nearly 2 and 1.5 days longer than in the middle crown (Table 2). Results of the multivariate ANOVA performed on nutritional indices indicated there were significant differences between the two treatments. Indeed, RGR and RCR were higher for insects fed on foliage from the middle crown.

TABLE 4. CHEMICAL ANALYSES (MEAN ± 2 SE) OF CURRENT-YEAR BALSAM FIR FOLIAGE ACCORDING TO CROWN SECTION DURING FIFTH AND SIXTH INSTARS

Foliar chemicals	Fifth instar			Sixth instar		
	Middle crown	Lower crown	P^a	Middle crown	Lower crown	P^a
Nutritional compounds						
N[b]	3.23 ± 0.39	3.38 ± 0.39	0.20	2.16 ± 0.13	2.19 ± 0.16	0.50
P (ppm)	4773 ± 673	5206 ± 643	0.30	3385 ± 214	3312 ± 257	0.20
K (ppm)	11397 ± 1261	13020 ± 809	0.052	8512 ± 576	9767 ± 607	0.0001
Ca (ppm)	849 ± 126	797 ± 110	0.40	1433 ± 193	1336 ± 205	0.06
Mg (ppm)	947 ± 69	940 ± 67	0.80	815 ± 32	789 ± 34	0.04
Total soluble sugars[b]	10.43 ± 1.72	8.33 ± 1.17	0.03	13.69 ± 1.06	9.27 ± 095	0.0001
	Wilk's λ^c = 0.14,	$F(6, 3)$ = 3.0,	P = 0.2	Wilk's λ = 0.10,	$F(6, 16)$ = 24.3,	P = 0.0001
Allelochemical compounds						
Total phenolics[b]	25.11 ± 5.64	28.64 ± 8.56	0.40	18.39 ± 1.32	23.33 ± 4.27	0.049
Total tannins (cm^2/mg)	1.17 ± 0.25	1.16 ± 0.20	0.90	0.85 ± 0.08	1.11 ± 0.10	0.0001
N: total tannins	3.02 ± 0.53	3.26 ± 0.80	0.80	2.65 ± 0.23	2.13 ± 0.32	0.0008
Total monoterpenes[b]	1.33 ± 0.45	0.99 ± 0.23	0.04	2.27 ± 0.24	1.75 ± 0.27	0.0001
Tricyclene	0.012 ± 0.005	0.008 ± 0.002	0.03	0.021 ± 0.002	0.017 ± 0.003	0.0004
α-pinene	0.40 ± 0.13	0.34 ± 0.10	0.20	0.37 ± 0.05	0.31 ± 0.07	0.10
Camphene	0.06 ± 0.02	0.05 ± 0.01	0.04	0.11 ± 0.01	0.09 ± 0.02	0.001
β-pinene	0.51 ± 0.25	0.31 ± 0.09	0.04	0.92 ± 0.13	0.71 ± 0.14	0.0006
Myrcene	0.05 ± 0.03	0.03 ± 0.01	0.06	0.08 ± 0.02	0.04 ± 0.01	0.0001
δ-3-carene	0.044 ± 0.040	0.05 ± 0.03	0.90	0.16 ± 0.07	0.12 ± 0.05	0.052
Limonene-δ-phellandrene	0.14 ± 0.06	0.12 ± 0.05	0.70	0.40 ± 0.10	0.30 ± 0.08	0.0007
Terpinolene	0.004 ± 0.003	0.004 ± 0.002	0.50	0.014 ± 0.003	0.011 ± 0.003	0.002
Bornyl acetate	0.10 ± 0.04	0.07 ± 0.02	0.003	0.20 ± 0.02	0.16 ± 0.03	0.002
				Wilk's λ = 0.05,	$F(12, 9)$ = 15.4,	P = 0.0002

[a] P value is for univariate ANOVA. Significant P values are less than or equal to the adjusted P value that corresponds to the traditional P value of 0.05 divided by the number of univariate tests carried out on each class of parameters ($P \leq 0.008$ for nutritional compounds; $P \leq 0.004$ for allelochemical compounds).

[b] % dry weight.

[c] Wilk's λ tests the hypothesis of no overall "crown section" effect.

Otherwise, AD and ECD and ECI were similar within trees. However, results of the multivariate ANOVA performed on nutritional nitrogen indices indicated no significant differences between the two crown sections. Thus, although the ANOVA performed on RNAR indicated that it was higher for insects that fed on foliage from the middle crown compared to those that fed on foliage from the lower crown, this result should be interpreted with caution. Mortality during the sixth instar was similar in both treatments, 3.1% for lower and 4.9% for middle crown sections (likelihood ratio chi-square: $\chi^2 = 0.14$; $df = 1$; $P = 0.71$). As for mortality during the pupal stage, no significant differences could be detected; these results were, respectively, 5.0% and 11.0% for the lower and middle crown sections (likelihood ratio chi-square: $\chi^2 = 0.58$; $df = 1$; $P = 0.45$).

Results of the crossed nested multivariate ANOVA showed that the foliar chemistry of the two sections was significantly different (Table 4). Lower crown foliage contained 15% more potassium and 32% less total soluble sugars than middle crown foliage. Foliage from the lower crown also had more total tannins and 23% less total monoterpenes. Except for α-pinene and δ-3-carene, all other monoterpenes were higher in content in middle crown foliage than lower.

DISCUSSION

Under laboratory rearing conditions, the general biological performance (total development time, pupal dry weight, and pupal nitrogen weight) of SBL fed on current-year foliage from either middle or lower crown section was similar. These results are consistent with those from field experiments reported by Bauce and Carisey (1996). In addition to foliar chemical analysis, studies on food and nitrogen utilization revealed that, although insects had similar biological performance, they exhibited differences in their food utilization.

SBL that were fed lower crown foliage reached the fifth instar nearly two days earlier and had higher dry weight and nitrogen weight compared to larvae fed middle crown foliage. Two factors might explain this. First, 1-year-old foliage that was mined by newly emerged second instars before budbreak may contain more total monoterpenes in the middle crown than lower. Current-year middle crown foliage had more total monoterpenes than lower during the feeding period, and thus, this tendency may remain in aging foliage. As many monoterpenes were found detrimental to western spruce budworm (Redak and Cates, 1984; Cates and Redack, 1986; Cates et al., 1987; Clancy et al., 1992) and to spruce budworm (Mattson et al., 1983, 1991), high total monoterpene content in 1-year-old middle crown foliage may have negatively affected spruce budworm growth. Second, new buds from the middle crown contained more δ-3-carene and terpinolene than those from the lower crown. High concentrations

of these monoterpenes have been associated with a decrease in growth and consumption rates for sixth-instar SBL fed current-year foliage from young balsam fir trees compared to larvae fed foliage from old trees (Bauce et al., 1994). Although no information is available on the effects of monoterpenes on early stage larvae, the poor biological performance of early stage larvae fed in the middle crown may result from lower consumption rates and/or lower food and nitrogen utilization due to repellency and/or postingestional feedback from high monoterpene foliar contents.

During the fifth instar, similar RGRs were observed for SBL reared on foliage from either section. This was sufficient for insects from the middle crown section to compensate for the deficiency in weight at the beginning of the instar. Indeed, although newly emerged fifth instars that were fed as second–fourth instars on middle crown foliage had 13% lower dry weight than those fed lower crown foliage, no significant differences in larval weights between treatments could be detected at the end of the fifth instar. Although middle crown foliage had higher total monoterpenes, particularly tricyclene, camphene, β-pinene, and bornyl acetate than lower crown, RCR, ECD, and ECI were similar in both treatments. This indicated that at concentrations found in foliage, monoterpenes had no particular effect on nutritional indices during the fifth instar. Although foliage from both sections had statistically similar total soluble sugar content, middle crown foliage showed a greater sugar content. This might account for the observed increase in AD. Indeed, Bidon (1993) reported a positive relationship between total soluble sugar in artificial diets and AD of fifth-instar SBL. At the beginning of the fifth instar, SBL from the middle crown contained 29% less nitrogen than those from the lower, but at the end of the instar similar nitrogen weights were observed for larvae from both sections. SBL from the middle crown developed several mechanisms to increase their RNAR and compensated for the deficiency in larval nitrogen amounts. The availability of higher quantities of carbohydrates in middle crown foliage may have enabled insects to increase ECDN and ECIN. To our knowledge, the ability of larvae to compensate for nitrogen deficiencies from one instar to another has not yet been shown in the literature. Further investigations should be carried out on this particular phenomenon.

Pupal dry weights and nitrogen weights were similar in both crown sections. However, sixth instars that were fed middle crown foliage reached the pupal stage nearly two days before those fed lower crown foliage. This explains the higher RGR and RNAR that were observed in these former larvae. Current-year middle crown foliage contained 48% more total soluble sugars than lower crown. Sugar, particularly sucrose, is known to be a phagostimulant for spruce budworm (Heron, 1965; Albert et al., 1982). Thus, high total soluble sugars in foliage might have led to an increase in food ingestion (RCR) by middle crown

larvae, which in turn may have resulted in reduced development times. Cates et al. (1987) reported that β-pinene may act as a feeding stimulant for western spruce budworm. High β-pinene in middle crown foliage may have improved larval ingestion, acting in combination with sugar.

Otherwise, during the sixth instar, insects used food and nitrogen similarly for both treatments. Although the allelochemical profile of current-year foliage was different between the middle and lower crown sections, the detrimental effect of high monoterpene contents (Redak and Cates, 1984; Cates and Redack, 1986; Cates et al., 1987; Clancy et al., 1992; Mattson et al., 1983, 1991; Bauce et al., 1994) in middle crown foliage may have been compensated for by the negative effect of high total tannins and lower nitrogen–tannins ration in lower crown foliage (Bernays, 1981; Bryant et al., 1987; Bernays et al., 1989; Bauce et al., 1994). With regard to the foliar nutritive profile, potassium content was higher from the lower crown relative to middle crown. However, no information could be found on the potential effects of mineral concentration on insect physiology. Foliar chemistry was different between the middle and lower crown sections and undoubtedly reflected the impact of sunlight irradiation. Current-year middle crown foliage contained more soluble sugars and monoterpenes than that of the lower crown. Their production depends upon light (Gleizes et al., 1980; Schürmann et al., 1993). Nevertheless, in the field, neither sunlight nor foliar chemistry appeared to have a particular effect on SBL that were reared at different crown levels (Bauce and Carisey, 1996). This illustrates the flexibility of spruce budworm in adapting to foliar chemistry variations by developing compensatory mechanisms. These mechanisms appear to be efficient only when foliar concentrations of nutritive and allelochemical compounds do not deviate from a given, but not yet determined range of concentrations. For example, spruce budworm from young trees were able to increase their ECD, but were unable to reach the same pupal dry weight as those from old trees. This is presumably because of higher concentrations of tannins, terpinolene, δ-3-carene, and bornyl acetate as well as the lower nitrogen–tannins ratio in foliage of young trees compared to old trees (Bauce et al., 1994).

Aside from foliar suitability, the within-tree availability of current-year foliage does not appear to be a problem for SBL, as the production of new foliage was similar between the middle and lower crowns (Bauce and Carisey, 1996). Nevertheless, since insects had different development patterns depending on crown level, the impact of natural enemies on population dynamics might also be different. Loader and Damman (1991) reported that *Pieris rapae* (L.) larvae reared on high-nitrogen food developed faster and were less susceptible to predators but more susceptible to parasitism than those fed on low-nitrogen food. Such a phenomenon might occur for SBL from the lower crown, which, until the beginning of the fifth instar, developed faster and contained more

nitrogen compared to middle crown larvae. In contrast, due to longer development times during the sixth and pupal stages, lower crown insects might be more susceptible to predators than insects from the middle crown. Further investigations on the effects of natural enemies on SBL relative to crown position and larval instar should be conducted.

Finally, the fact that at low population density, SBL are located more specifically in the middle crown than in the lower does not seem to be related to nutritional considerations. This phenomenon may depend upon other factors. The middle crown may be preferred by female adults for oviposition. Branches from the middle crown usually bear staminate flower scars (Bauce and Carisey 1996) that are the most suitable sites for spruce budworm diapause (Dr. J. Régnière, personal communication). At high population density, because of the lower availability of current-year foliage, SBL tend to disperse themselves within the crown (Blais, 1952).

In conclusion, allelochemical and nutritive values of current-year foliage from the lower and middle crown sections are similar in terms of nutritional requirements of larvae. Because of their ability to utilize compensatory mechanisms, SBL can effectively exploit the whole tree crown. This flexibility may enable spruce budworm populations to reduce phenotypic and physiological variations among individuals and provides insect populations with more opportunity to adapt to changing environments.

Acknowledgments—We thank M. Charest, M. Crépin, G. Moreau, D. Verge, I. Coté, J. F. Demers, J. Forest, M. Marin, S. Perreault, C. Paquet, and L. Roy for their technical assistance during insect rearing. This work was supported by the Natural Sciences and Engineering Research Council of Canada, by the Canada Green Plan Integrated Forest Pest Management and Forestry Practices programs, and by the "Direction de la recherche du Ministère des Ressources Naturelles du Québec".

REFERENCES

ALBERT, P. J., CEARLEY, C., HANSON, F., and PARISELLA, S. 1982. Feeding responses of eastern spruce budworm larvae to sucrose and other carbohydrates. *J. Chem. Ecol.* 8:233–239.

ATWOOD, C. E. 1944. The feeding habits of young spruce budworm larvae. *Can. Entomol.* 76:64–66.

BAUCE, E. 1996. One and two years impact of commercial thinning on spruce budworm feeding ecology and host tree foliage production and chemistry. *For. Chron.* 72:393–398.

BAUCE, E., and CARISEY, N. 1996. Larval feeding behaviour affects the impact of staminate flower production on the suitability of balsam fir trees for spruce budworm. *Oecologia* 105:126–131.

BAUCE, E., CRÉPIN, M., and CARISEY, N. 1994. Spruce budworm growth, development and food utilization on young and old balsam fir trees. *Oecologia* 97:499–507.

BÉLANGER, L., DUCRUC, J. P., and PINEAU, M. 1983. Analyses et commentaires. Proposition d'une méthodologie d'inventaire écologique adaptée au territoire forestier périurbain. *Nat. Can.* 110:459–476.

BERNAYS, E. A. 1981. Plant tannins and insect herbivores: an appraisal. *Ecol. Entomol.* 6:353–360.

BERNAYS, E. A., COOPER DRIVER, G., and BILGENER, M. 1989. Herbivores and plant tannins. *Adv. Ecol. Res.* 19:262–302.

BIDON, Y. 1993. Influence des sucres solubles et de l'azote sur la croissance, le développement et l'utilisation de la nourriture par la tordeuse des bourgeons de l'épinette [*Choristoneura fumiferana* (Clem.)]. MS thesis. Université Laval, Ste-Foy, Québec, Canada, 60 pp.

BLAIS, J. R. 1952. The relationship of the spruce budworm to the flowering condition of balsam fir. *Can. J. Zool.* 30:1-29.

BREMNER, J. M., and MULVANEY, C. S. 1982. Nitrogen-total, pp. 595–624, *in* A. L. Page (ed.). Methods of Soil Analysis, Part 2, 2nd ed. Agronomy Monograph 9. ASA and SSSA, Madison, Wisconsin.

BRYANT, J. P., CLAUSEN, T. P., REICHARDT, P. B., MCCARTHY, M. C., and WERNER, R. A. 1987. Effect of nitrogen fertilization upon the secondary chemistry and nutritional value of quaking aspen (*Populus tremuloides* Michx.) leaves for the large aspen tortrix [*Choristoneura conflictana* (Walker)]. *Oecologia* 73:513-517.

CATES, R. G., and REDACK, R. A. 1986. Between-year population variation in resistance of Douglas-fir to the western spruce budworm, pp. 3–19, *in* M. B. Green and P. A. Hedin (eds.). Natural Resistance of Plants to Insects, Roles of Allelochemicals. Symposium Series 208. American Chemical Society, Washington, D.C.

CATES, R.G., HENDERSON, C. B., and REDACK, R. A. 1987. Responses of western spruce budworm to varying levels of nitrogen and terpenes. *Oecologia* 73:312-316.

CLANCY, K. M., FOUST, R. D., HUNTSBERGER, T. G., WHITAKER, J. G., and WHITAKER, D. M. 1992. Technique for using microencapsulated terpenes in lepidopteran artificial diets. *J. Chem. Ecol.* 18:543–560.

FEENY, P. P. 1969. Inhibitory effect of oak tannins on the hydrolysis of proteins by trypsin. *Phytochemistry* 8:2119–2126.

GERSHENZON, J., and CROTEAU, R. 1991. Terpenoids, pp. 165–219, *in* G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites, 2E, Vol. I: The Chemical Participants. Academic Press, London.

GINN, S. E., SEILER, J. R., CAZELL, B. H., and KREH, R. E. 1991. Physiological and growth responses of eight-year-old loblolly pine stands to thinning. *For. Sci.* 37:1030–1040.

GLEIZES, M., PAULY, G., BERNARD-DAGAN, C., and JACQUES, R. 1980. Effects of light on terpene hydrocarbon synthesis in *Pinus pinaster*. *Physiol. Plant.* 50:16–20.

GRANDTNER, M. M. 1966. La végétation forestière du Québec méridional. Université Laval, Ste-Foy, Québec, Canada.

HERON, R. J. 1965. The role of chemotactic stimuli in the feeding behavior of spruce budworm larvae on white spruce. *Can. J. Zool.* 43:247-269.

LOADER, C., and DAMMAN, H. 1991. Nitrogen content of food plants and vulnerability of *Pieris rapae* to natural enemies. *Ecology* 72:1586–1590.

MATTSON, W. J., SLOCUM, S. S., and KOLLER, C. N. 1983. Spruce budworm (*Choristoneura fumiferana*) performance in relation to foliar chemistry of its host-plants, pp. 55-66, *in* R. L. Talerico and M. Montgomery (eds.). CANUSA Workshop on Forest Defoliators–Host Interaction: A Comparison Between Gypsy Moth and Spruce Budworm. New Haven, Connecticut, April 5–7, 1983. General Technical Report NE 85. USDA Forest Service, Broomall, Pennsylvania.

MATTSON, W. J., HAACK, A. R., LAWRENCE, R. K., and SLOCUM, S. S. 1991. Considering the nutritional ecology of the spruce budworm in its management. *For. Ecol. Manage.* 39:183-210.

MCGUGAN, B. M. 1954. Needle-mining habits and larval instars of spruce budworm. *Can. Entomol.* 86:439–454.

MORRIS, R. F. 1955. The development of sampling techniques for forest insect defoliators with particular reference to the spruce budworm. *Can. J. Zool.* 33:225–294.

MONTGOMERY, M. E. 1983. Biomass and nitrogen budgets during larval development of *Lymantria dispar* and *Christoneura fumiferana*, pp. 133–140, *in* R. L. Talerico and M. Montgomery (eds.). CANUSA Workshop on Forest Defoliators-host-interaction: A Comparison Between Gypsy Moth and Spruce Budworm. New Haven, Connecticut, April 5–7, 1983. General Technical Report NE 85. USDA Forest Service, Broomall, Pennsylvania.

PEARCY, R. W., and SIMS, D. A. 1994. Photosynthetic acclimation to changing light environments: scaling from the leaf to the whole plant, pp. 145–174, *in* M. M. Caldwell and R. W. Pearcy (eds.). Exploitation of Environmental Heterogeneity by Plants. Ecophysiological Processes Above- and Belowground.

PIENE, H. 1996. Changes in spruce budworm defoliation with crown level. *Can. Entomol.* 128:1109–1113.

REDAK, R. A., and CATES, R. G. 1984. Douglas fir (*Pseudotsuga menziesii*)-spruce budworm (*Choristoneura occidentalis*) interactions: The effect of nutrition, chemical defenses, tissue phenology, and tree physical parameters on budworm success. *Oecologia* 62:61–67.

RÉGNIÈRE, J., LYSYK, T. J., and AUGER, M. 1989. Population density estimation of spruce budworm, *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae) on balsam fir and white spruce from 45-cm mid-crown branch tips. *Can. Entomol.* 121:267–281.

ROBERTSON, J. L. 1985. *Christoneura occidentalis* and *Choristoneura fumiferana*, pp. 227–236, *in* P. Singh, and R. F. Moore (eds.). Handbook of Insect Rearing, Vol. II. Elsevier, New York.

SANDERS, C. J. 1980. A Summary of Current Techniques Used for Sampling Spruce Budworm Populations and Estimating Defoliation in Eastern Canada. Inf. Rep. O-X-306. Great Lakes Forestry Centre, Canadian Forest Service, Sault Ste Marie, Ontario.

SAS Institute. 1988. SAS User's Guide; Statistics 1988. SAS Institute, Cary, North Carolina.

SCHÜRMANN, W., ZIEGLER, H., KOTZIAS, D., SCHÖNWITZ, R., and STEINBRECHER, R. 1993. Emission of biosynthesized monoterpenes from needles of Norway spruce. Naturwissenschaften 80:276–278.

WALDBAUER, G. P. 1968. The consumption and utilization of food by insects. *Adv. Insect Physiol.* 5:229–288.

WU, R. L. 1993. Simulated optimal structure of a photosynthetic system: Implication for the breeding of forest crop ideotype. *Can. J. For. Res.* 23:1631–1638.

Journal of Chemical Ecology, Vol. 23, No. 8, 1997

PHYTOTOXICITY OF VULPIA RESIDUES: I. INVESTIGATION OF AQUEOUS EXTRACTS

MIN AN,[1,*] J. E. PRATLEY,[2] and T. HAIG[2]

[1]*Environmental and Analytical Laboratories*
[2]*Farrer Centre for Conservation Farming*
Charles Sturt University
Wagga Wagga, New South Wales 2678, Australia

(Received August 19, 1996; accepted April 6, 1997)

Abstract—Phytotoxic properties of vulpia (*V. myuros*) residue extracts on wheat (*Triticum aestivum* L. cv. Vulcan) were examined in the laboratory. Vulpia residues contained water-soluble materials that were toxic to germination and to coleoptile and root growth of wheat. There were strong correlations between extract concentration and toxicity, between extraction times and toxicity, and between extract pH and phytotoxicity. The phytotoxicity was residue rate dependent. There was no significant correlation between the phytotoxicity of residue extracts and the electrical conductivity of aqueous extracts. Milled residues were more toxic than chopped residues. The toxicity of vulpia residues increased as their decomposition proceeded, reaching a peak phytotoxicity after decomposition for 40 days and remaining potent for up to 60 days, gradually declining thereafter. Radicle elongation of wheat was the most sensitive indicator, germination was the least sensitive, and coleoptile growth was intermediate. The phytoxic effects of residue extracts on seed germination had two aspects: germination delay and inhibition. The relative magnitude of each aspect depended upon the potency of the extracts. With a strong phytotoxic potential, inhibition of germination was dominant over seed germination, whereas with a weak toxic level, delay was dominant over seed germination.

Key Words—Phytotoxicity, vulpia, silvergrass, toxicity, weed control, allelochemical, residue

INTRODUCTION

Plant residues of numerous weeds have allelopathic effects on the germination and growth of following crops (Toai and Linscott, 1979; Wilson, 1981; Mersie

*To whom correspondence should be addressed.

0098-0331/97/0800-1979$12.50/0 © 1997 Plenum Publishing Corporation

and Singh, 1987). Such effects are attributed to chemical compounds, called allelochemicals (Weston et al., 1987; Buta and Spaulding, 1989), which may be leached directly from the residues or may result from microbial activity during decomposition (Lynch and Cannell, 1980, Mason-Sedun and Jessop, 1988).

Vulpia (*Vulpia* spp.), also known as silvergrass, is a naturalized winter-growing annual grass that originated in the Mediterranean region. It has become an increasingly important weed in pastures and cereal crops in southern Australia (McIntyre and Whalley, 1990). We have investigated vulpia in an attempt to understand why this weed is so successful in competition with crop plants.

Vulpia competes with other plants for environmental resources, in many cases completely dominating subterranean clover pastures and severely debilitating cereal crops, particularly wheat. It is a low-value pasture species due to its low winter production and to its unpalatability. Its awned seeds can seriously injure livestock, reducing the value of the carcasses, and infesting fleeces. During the pasture phase, vulpia is a host for a wide range of pathogens causing root diseases in cereal crops. It has been estimated that vulpia costs Australian farmers in excess of $220 million annually (Leys et al., 1991a, 1993).

The proliferation and spread of vulpia are attributed to a number of factors, including direct drilling of crops, strong competitiveness, poor pastures and crops, high production of seeds and large quantities of residues being carried over on the soil surface from one season to the next (Forcella, 1984; Leys et al., 1991b; Jones et al., 1992).

Previous work showed that vulpia residues have strong potential to adversely affect crop and pasture species (Pratley, 1989; Pratley and Ingrey, 1990). Experiments conducted under glasshouse conditions showed that germination and root and top growth of wheat, lucerne, phalaris, and canola were significantly affected by the presence of vulpia residues (Pratley and Ingrey, 1990). Recent work in the laboratory indicated differential phytotoxicity of vulpia residues from two *Vulpia* species on germination and growth of wheat seedlings; variable phytotoxicity among seeds, inflorescence, shoot, root, and whole plant parts of vulpia (An et al., 1996c); and differential susceptibility between cultivars of wheat and subterranean clover (An et al., 1996b). The degree of phytotoxicity of vulpia residues appears to depend on factors such as the extent of weathering of residues, including ultraviolet light and moisture pretreatment of the residue (Pratley, 1989; Pratley and Ingrey, 1990), and the ratio of residues to soil (An et al., 1993b).

The following objectives were explored to investigate further the phytotoxic properties of aqueous extracts of vulpia residues under controlled conditions, as well as to examine the potential for separation, identification, and characterization of vulpia allelochemicals: (1) determine the relative phytotoxicity of water extracts from differently treated residues; (2) assess the effects of extrac-

tion times on extract phytotoxicity; and (3) evaluate the persistence of toxicity of vulpia residues without amending any soil.

METHODS AND MATERIALS

Plant Material

Material obtained from pure mature stands of *V. bromoides* and *V. myuros* in December 1991, was provided by Dr. A. R. Leys, Agricultural Research Institute, Wagga Wagga, NSW. Residue material was either cut 1 cm above the soil surface, or whole plants, including roots, were harvested by hand. All material was oven dried at 40°C for 72 hr and stored under dry conditions until initiation of the experiment. Since *V. myuros* is the most widely spread *Vulpia* species in southern Australia (Forcella, 1984), its residues were used as the standard material throughout the project. *V. bromoides* was only used in phytotoxicity differentiation studies between the two species.

Preparation of Aqueous Extracts

Vulpia residues were cut into 1- to 2-cm lengths and, in lots of 10 g, soaked in a 250-ml Erlenmeyer flask with 100 ml of distilled water for the desired time in the dark at 20°C. Flasks were stoppered with nonabsorbent cotton wool, sealed in laboratory film, and completely enclosed in aluminum foil to prevent photodecomposition. The liquid was filtered through one layer of Whatman No. 1 filter paper and centrifuged for 30 min at 3900 rpm. The supernatant was decanted and used for bioassays. The volume of aqueous extract recovered from this procedure was about 60% of the total original volume.

Petri Dish Bioassays

A Petri dish with filter paper was chosen as the medium for the bioassay system. Such factors as the volume of extracts applied in each Petri dish, seed numbers per dish, osmotic pressures of extracts, and pH have been well established (Putnam and Duke, 1978; Mason-Sedun, 1986; Leather and Einhellig, 1986) and were adopted in this project with little modification after preliminary testing (data not presented).

Wheat (cv. Vulcan) is one of the main crop species grown in vulpia-infested areas and was chosen as the test species. The use of wheat also had the advantages of ease in handling growth measurement and higher homogeneity.

Seed Germination Bioassay. Germination was carried out in 9-cm plastic Petri dishes lined with one Whatman No. 1 filter paper. Twenty seeds of wheat (*Triticum aestivum* cv. Vulcan) were used in each dish and 5 ml of extract

added. Control treatments received 5 ml of distilled water instead of extract. Dishes were placed in an incubator, in the dark, at 24°C. Germination of seeds was recorded every 24 hr for four consecutive days. Both control and extract germination were expressed as the percentage of 20 seeds germinating. Germinated seeds were removed when each count was made. Both accumulated germination rates over the whole germinating period (i.e., 96 hr) and after 48 hr were used for analysis. Seeds were considered germinated if the radicle had emerged 2-mm from the seed coat. Three replications were utilized.

After 48 hr or at the time when 90–95% of control seeds had germinated (generally at this time an accumulated germination rate of the control is not significantly different from those following), the negative impact on seed germination affected by extract is defined as a "germination reduction." From this time to the end of the germinating period, some recovery may take place, this being defined as "germination delay." The persistent reduction at the end of the full germinating period is defined as the "germination inhibition" and is often permanent (Figure 1). Both germination delay and inhibition were expressed as the percentage of 20 seeds germinating. Thus, as indicated in Figure 1,

$$\text{germination reduction (a)} = \text{germination inhibition (b)} + \text{germination delay (c)}$$

Seedling Elongation Bioassay. Seeds were germinated as for the control before imposition of treatments. Then, 15 germinated seeds, radicle length 3–5

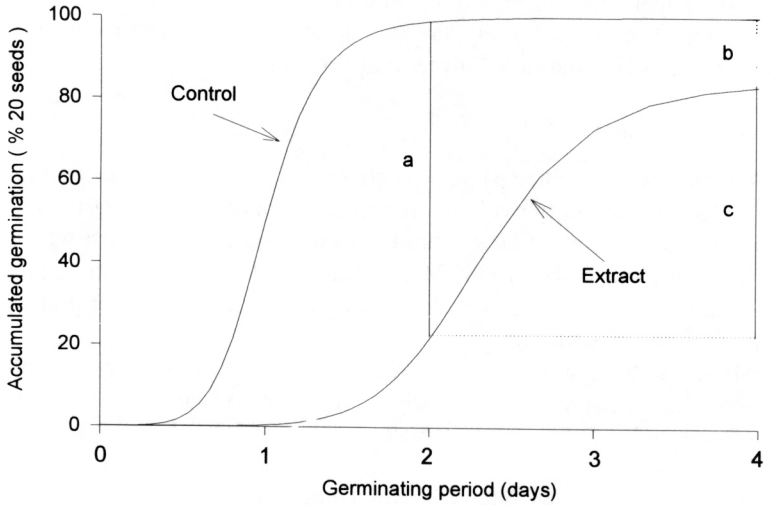


FIG. 1. Representation of definitions for dynamic germination where a is germination reduction, b is germination inhibition, and c is germination delay.

mm, were transplanted into each dish containing extract or distilled water. Other conditions were as for the germination bioassay. After 48 hr, the lengths of the longest seminal root (millimeters) and coleoptile (millimeters) were measured. The average length per Petri dish was used for statistical analysis. The bioassay was arranged in a randomized complete block design with three replicates.

Data Analyses

The data were subjected to analysis of variance, and significant treatment differences were tested at the 5% level using the least significant difference test. Where necessary, regression between measured parameters was made, and the significance of the correlation coefficient was tested. An arcsin transformation was used when the percentages fell outside the 30–70% range.

Effect of Extraction Time and Decomposition Time on Phytotoxicity

Aqueous extracts were made following the general methods. Treatment of decomposing residue for 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, 60, 80, and 110 days was applied. At each incubation, the aqueous portion of each treatment was filtered as the "first extract." The equivalent volume of distilled water was then added to the residues, which were allowed 1 hr further decomposition under the same conditions, before again filtering the aqueous component out as the second extract. Equal volumes of each extract were taken and mixed for the mixture treatment. The pH and electrical conductivity (EC) of the extracts were determined using a Watson Victor model 5005 digital pH meter and a Philips PW9505 conductivity meter. Experiments were repeated twice, and data presented as the results of one experiment.

Effect of Chopped and Milled Treatments on Phytotoxicity

Whole plants of *V. myuros* were chopped into 2- to 3-cm lengths as a chopped treatment or ground in a mill to pass a 1.0-mm^2 screen as a milled treatment. Preparation of extracts followed the general method with the residues being soaked in distilled water for two days. There were three replications.

Rate Study with Residue Extracts

An aqueous extract was prepared by soaking 100 g of chopped vulpia residues in 1 liter of distilled water for 10 days. The liquid was decanted, filtered, and centrifuged. The recovered volume was 580 ml. Residues were reextracted by adding 450 ml distilled water and decomposing for 1 hr. The supernatant was recovered following the above procedure, then added to the first filtrate and made up to 1 liter. A series of aqueous concentrations was

made: full strength, 0.001, 1, 5, 10, and 50% dilutions. Only the seedling elongation bioassay was used.

RESULTS

Effect of Extraction Time and Decomposition Time

Germination. All aqueous extracts significantly affected seed germination of wheat. The extent of the effect was dependent upon decomposition, seed germinating period, and extraction times (Figures 2 and 3). As decomposition proceeded, phytotoxicities of residue extracts rapidly increased in terms of depression on germination (Figure 2). The first extract reached peak phytotoxicity after 10 days of residue decomposition, maintained that level subsequently until 60 days, and then started to decline.

The longer germinating period alleviated the depression from aqueous extracts on germination, prolonged time reaching peak phytotoxicity, and shortened the potency period. This indicated that part of the toxicity effect upon germination was germination delay. Thus, in comparison with 90–95% germination rates for most controls within a 48-hr germinating period, aqueous extracts not only inhibited germination, but also delayed it. The percentage of germination delay in overall germinated seeds could be more than 50%, even 100%. A large proportion of extract treatments suffered a one-day delay. The germi-

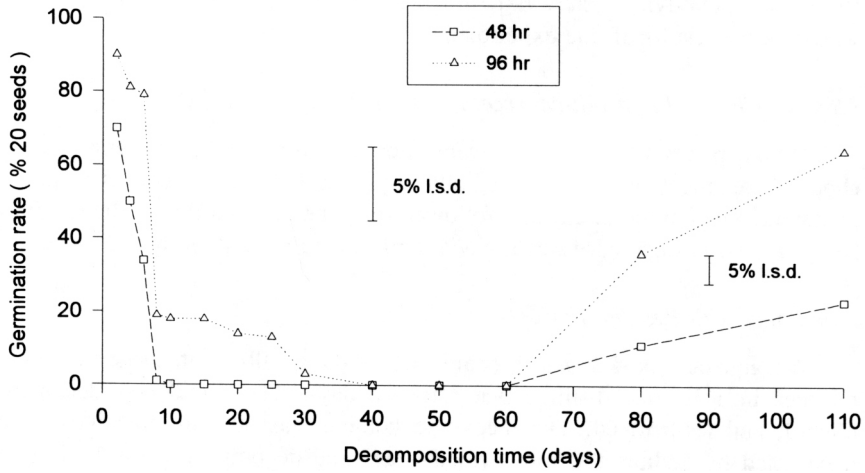


FIG. 2. Phytotoxicity time course of vulpia residue on seed germination as affected by germinating time.

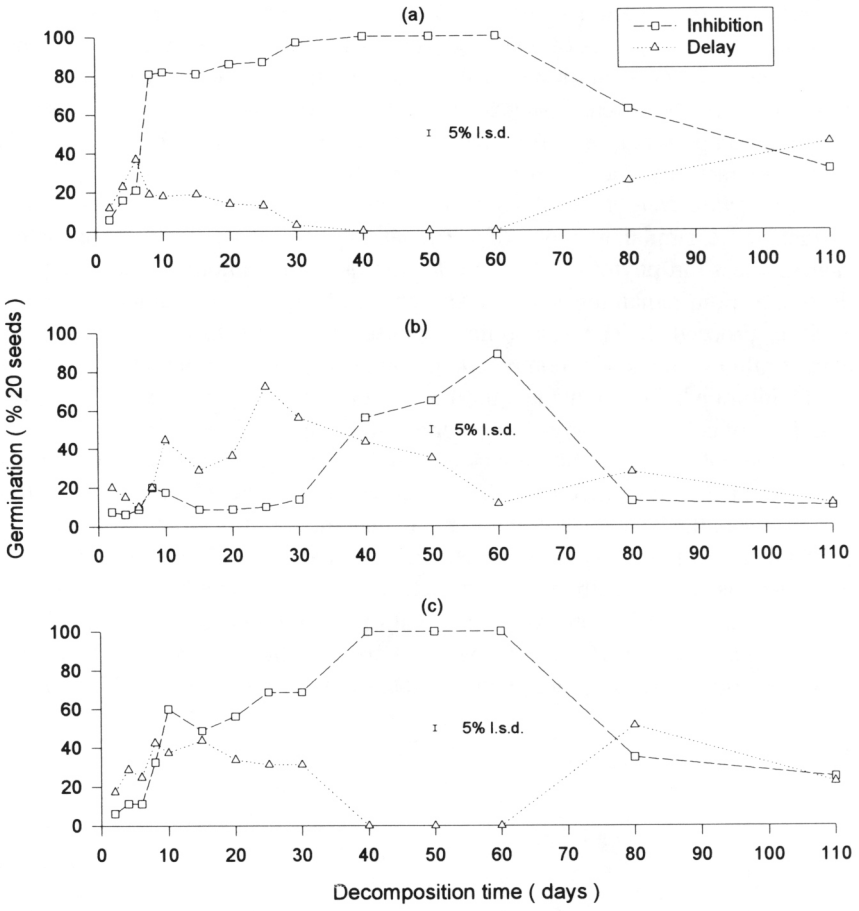


FIG. 3. Dynamics of inhibition and delay effects of vulpia residue extracts on seed germination as affected by extraction times: (a) the first extract, (b) the second extract, (c) the mixture.

nation delay and the inhibition interacted, and their relative magnitudes with respect to effects on germination changed as decomposition of residues proceeded, being affected by extraction times (Figure 3). At a very early stage of residue decomposition, the delay of germination was dominant while the inhibition was less significant. As decomposition proceeded, inhibition rapidly dominated germination, until complete inhibition was reached and subsequently remained across a range of decomposition periods. At the fifth day of the germinating course, ungerminated seeds in extract dishes had rotted. Thus complete

inhibition of germination at the end of the germinating course was likely to be permanent. At late stages of residue decomposition, the dominance of delay and inhibition over germination returned (Figure 3), more or less reflecting the pattern that occurred at early stages of residue decomposition. The most severe inhibition on germination was associated with the first extract, the least with the second extract, and the mixture was intermediate.

Coleoptile Height and Root Length. Root and coleoptile responses to all extracts were similar to those of germination (Figure 4). All demonstrated a sharp increase in phytotoxicity, with a subsequent maximum at early stages of decomposition, remaining at peak level for a while, and then declining as decomposition proceeded. The coleoptile response was less sensitive. The aqueous extract, after 40 days of decomposition, completely inhibited radicle elongation, such inhibition being maintained until 60 days of decomposition, while complete inhibition of coleoptile elongation never occurred, although inhibition was severe. Maximum coleoptile inhibition was eventually reached after 60 days and thereafter diminished. As extraction times increased further, the inhibition on seedling growth was alleviated. The three extract types demonstrated significant differentiation in phytotoxicity, but similar dynamic patterns. The most severe inhibition was associated with the first extract, less with the second, and intermediate with the mixture. Differences declined with increased decomposition time.

pH and Electrical Conductivity. Dynamic changes in pH throughout decomposition were correlated with phytotoxicity. After 10 days, pH markedly

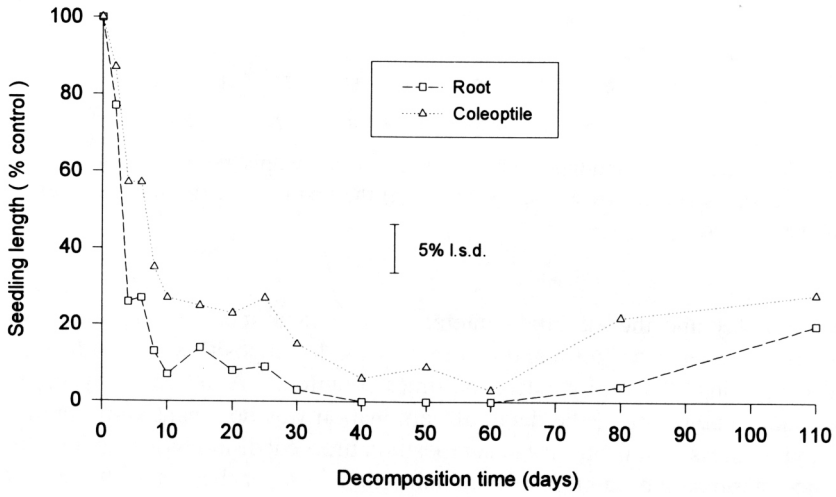


FIG. 4. Phytotoxicity dynamics of vulpia residue extracts as measured by seedling lengths of test species (wheat cv. Vulcan). Only the first extract results are shown.

decreased by 0.98–1.16 units, with corresponding root inhibition of 54–93%. During the next 50-day decomposition, pH changed 0.53–0.54, and phytotoxicity was 6.8–44%. In the late period, root inhibition was alleviated 19.5–56%, and correspondingly the pH of extracts increased 0.25–1.0 units (Figures 4 and 5). There was a significant correlation between pH change and phytotoxicities of residue extracts ($r = 0.817^{**}$ for root, $r = 0.816^{**}$ for coleoptile, $r = 0.704^{**}$ for germination, regardless of extraction times; $r = 0.845^{**}$ for the first extract, $r = 0.844^{**}$ for the mixture, $r = 0.695^{**}$ for the second extract, regardless of test species parameters measured; **, significant correlation at 1% level of probability). The order of such correlations was consistent with phytotoxicities of the extracts and sensitivity of test species parameters measured, i.e., the first extract > the mixture > the second extract; root > coleoptile > germination. No significant differences in pH of the three residue extract types could be detected (Figure 5).

Figure 6 shows the change in conductivity over a decomposition of 110 days. The first extract increased only 1.1 mmho/cm over 40 days, the second extract 0.23, and the mixture 0.4. Conductivity increased a little at later stages of decomposition. The dynamic changes in electrical conductivity differed from those of phytotoxicity and pH and were generally flat over the whole decomposition period. Differences among the three extract types were detectable, the first having the highest conductivity, the second least, and the mixtures intermediate. There was a significant correlation between phytotoxicity and electrical conductivity ($r = -0.774^{**}$ for germination, $r = -0.740^{**}$ for coleoptile, r

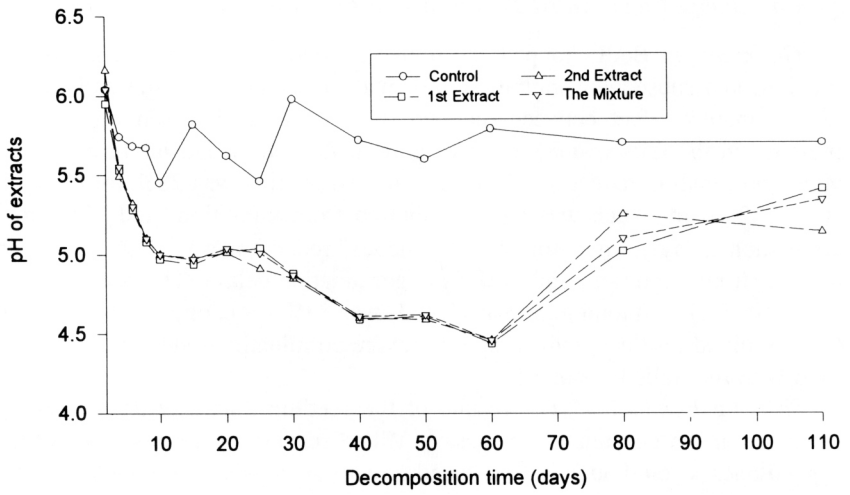


FIG. 5. Dynamics of pH of three residue extracts with decomposition period. The 5% l.s.d. is 0.15 for extraction time, 0.28 for decomposition time.

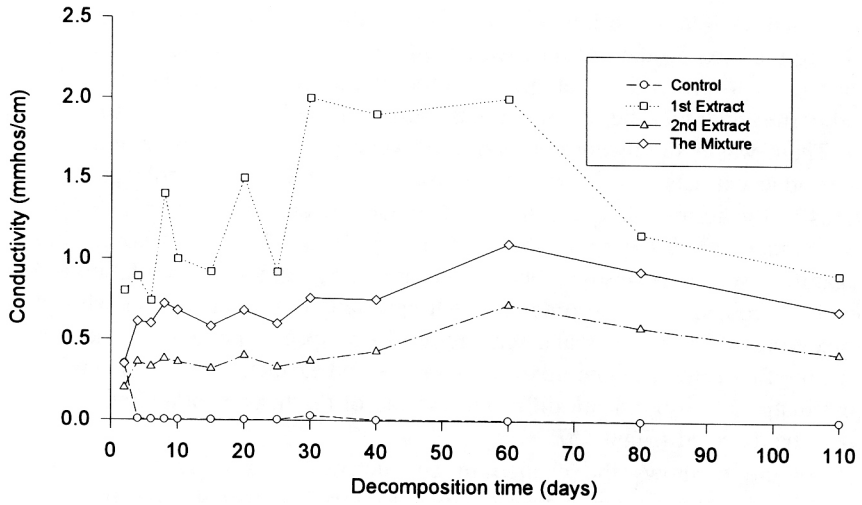


FIG. 6. Dynamics of electrical conductivity of three residue extracts with decomposition period. The 5% l.s.d. is 0.19 for extraction time, 0.35 for decomposition time.

= −0.661** for root, regardless of extraction times; r = −0.663** for the second extract, r = −0.662** for the first extract, r = −0.581** for the mixture, regardless of test species parameters measured).

Effect of Chopped and Milled Treatments on Phytotoxicity

Germination. Both chopped and milled residues significantly reduced germination, and subsequent inhibition was evident (Figure 7). Differentiation in the germination effect between the two treatments can be seen through the difference in the relative magnitude of germination delay and inhibition to the overall germination reduction. Reduction in germination was 28% for chopped residues (25% of which was the contribution from inhibition, and 75% from germination delay), while for milled residues, reduction was 58% (of which 38% was from inhibition, and 62% from germination delay). The proportion of germination delay in total germinated seeds was 23% for chopped residues, and 46% for milled residues, indicating that severe germination reduction was associated with the milled residues.

Seedling Elongation. The results of the seedling bioassay are consistent with those of the germination bioassay. Milled residues were more toxic than chopped ones, even though both reduced growth of roots and coleoptiles (Table 1). The milled residues depressed the radicle length and coleoptile height to different extents, 81% and 69% of inhibition, respectively, and the chopped residues only depressed then 19% and 17%, respectively.

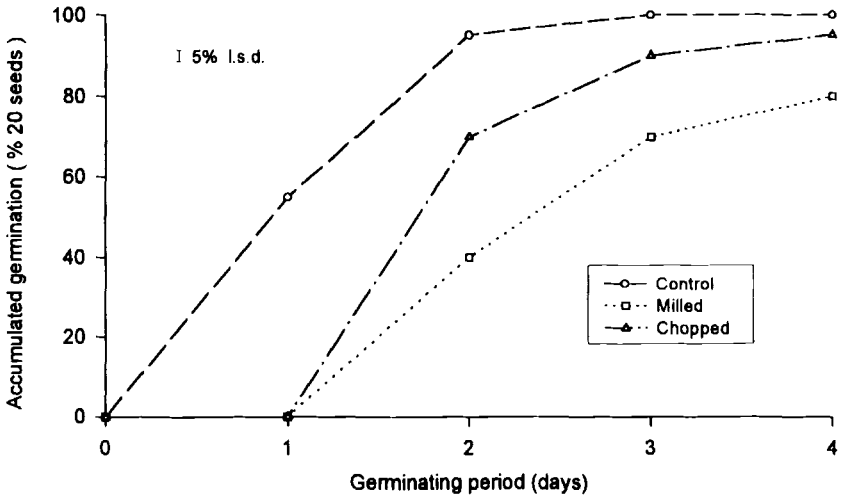


FIG. 7. Relative phytotoxicity of chopped and milled residue extracts on dynamics of seed germination.

Rate Study

The responses of seedlings to different concentrations of extract varied (Figure 8). At high concentrations, both root and coleoptile lengths were inhibited, such inhibition decreasing as concentration levels decreased. The root responded more sensitively than the coleoptile. From concentrations of 1–100 (% v/v), the root had a 95% inhibition, while coleoptile had only 73% inhibition. The coleoptile tolerance range to varying concentrations is wider than that

TABLE 1. EFFECT OF CHOPPED AND MILLED RESIDUE EXTRACTS ON SEEDLING GROWTH OF WHEAT

Treatment	Seedling length (mm)	
	Coleoptile	Root
Control	33.0	48.4
Chopped	27.5	39.4
Milled	10.4	9.2
F value	[a]	[a]
LSD (0.05)	1.07	3.11

[a] 1% level of probability.

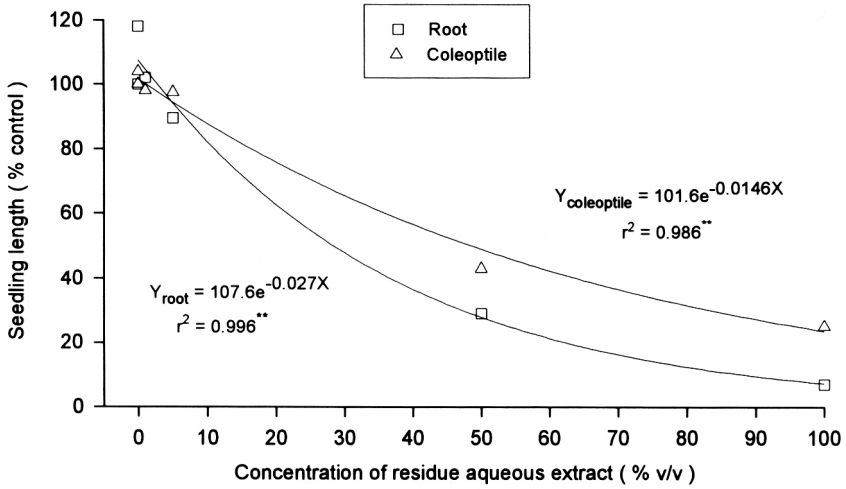


FIG. 8. The effect of various concentrations of aqueous extracts of vulpia residues on their phytotoxicity as measured by seedling length of test species (wheat cv. Vulcan).

of the root. Over the concentration range of 0.001–10 (% v/v), coleoptile responses were quite stable, whereas the root responded dramatically, from 26% inhibition to 18% stimulation. In general, at biologically realistic concentrations (1–100% v/v), wheat seedlings were inhibited, and such inhibition sharply increased as concentrations increased. The concentrations at which no response occurred were low, the range being 1.0–0.001% v/v. Stimulation occurred only at very low concentrations.

DISCUSSION

The evidence presented demonstrates the phytotoxic potential of aqueous extracts of vulpia residues to significantly delay and inhibit the germination and early growth of wheat. Water is the extraction solvent in nature and residue decomposition requires water for microbial growth and for the movement of materials from the residues. Although the experiments reported here were carried out in the laboratory, the use of aqueous extraction simulated the natural release of water-soluble phytotoxins over time.

In research on allelopathy, the germination response of seed is the most widely measured parameter, emphasis often being on inhibition (or sometimes stimulation) of germination (Leather and Einhellig, 1986). Williams and Hoagland (1982) found that many compounds delayed rather than inhibited germi-

nation. They further stressed the need to consider the kinetics of germination. Germination bioassay results presented here reveal a more holistic view of the germination process as affected by plant residue extracts. The data show that the effects on seed germination proceed through two aspects, germination delay and inhibition. Generally, with strong phytotoxic potential, the inhibition effect was strong; with a weak toxic level, the effect on the seed was a delay in germination time whereas with the intermediate phytotoxic potential, both delay and inhibition of germination were significant. Decomposition of residues and extraction times affected the phytotoxic potential of aqueous residue extracts. As decomposition increased, the period of delay in germination was extended, inhibition was enhanced, and recovery in germination decreased.

The importance of considering germination dynamics when investigating effects of plant residues on germination is an often overlooked factor. The data demonstrate that a nonsignificant germination inhibition response at the end of a germination period does not necessarily guarantee the exclusion of germination delay or temporary inhibition over the germinating course. Given the often harsh environment seedlings face, such delays and temporary inhibition in germination could be significant in terms of survival and subsequent productivity. McCalla and Norstadt (1974) indicated that germination is one of the most susceptible periods of wheat to phytotoxins. A brief period of inhibition or stimulation at this stage could affect its ability to compete with other plants, and often such effects are permanent (McCalla, 1971; Rice, 1984).

That the phytotoxicity of residues was extraction time-dependent was also reported by Chou and Patrick (1976), who used residues of corn. Those results reported and the ones here showed that whether subsequent extractions occurred from the same decomposition time or from periods less than the first, phytotoxicities were still less than the first extraction. This indicates that the first extraction did not completely extract the phytotoxic agents but did extract a substantial amount. The production of phytotoxins is proportional to the amount of endogenous toxins and other convertable components for microbial activity within the residues.

The finding that phytotoxicity of aqueous extracts increased over time and then declined was generally in agreement with the literature (Patrick et al., 1963; Kimber, 1973; Toai and Linscott, 1979; Mersie and Singh, 1987). However, the observation that phytotoxicity increased up to 60 days of decomposition before decline may indicate that phytotoxicity could sometimes increase over weeks or months of decomposition prior to declining and that the decline may take months of decomposition. This is evidenced by the work of Welbank (1963), Wilson (1981), and Horowitz and Friedman (1971). Thus, early or late decomposition as a relative concept should be considered in a broad scope. The environmental factors in which residues undergo decomposition and the plant species from which residues are derived play an important role. In an experiment

with residues of canola (*Brassica campestris* cv. Barossa) under similar conditions to that of vulpia residues, the phytotoxicity of aqueous extracts on wheat seedling growth and germination reached a maximum after 20 days of decomposition, and then declined. In this research on plant residues, various decomposition times were employed to obtain residue extracts (Weston and Putnam, 1986; Read and Jensen, 1989; Habib and Rahman, 1988). The data presented here indicate that caution must be exercised when interpreting experimental results, and when comparing results obtained from different sources.

The high correlation obtained between the relative phytotoxicity of residue extracts and their pH is in agreement with the literature (Patrick and Koch, 1958). Differential phytotoxicity among three residue extracts can not be detected by their pH values, indicating that the bioassay as a means of phytotoxicity measurement is more sensitive than pH value. In addition, the concentrations of phytotoxins in residue extracts are often detected at micromole or microgram levels (Chou and Patrick, 1976; Buta and Spaulding, 1989). Small changes in such concentrations are unlikely to cause a significant change of pH but can cause dramatic responses in test plants (Alsaadawi et al., 1985; Stevens and Molyneux, 1988; Hartung et al., 1990). This may explain the observation that the pH range of plant residue extracts is within the tolerance range of test species, but test plants show inhibition (Mason-Sedun, 1986; Kimber, 1967).

The osmotic pressure of aqueous extracts is occasionally considered a problem in influencing seed germination (Anderson and Loucks, 1966). The correlation between phytotoxicity of residue extracts and their electrical conductivity seems to support such a consideration. However, the order of correlation values was not consistent with the phytotoxicities of the extracts and the sensitivity of the test species parameters measured. The highest correlation was associated with the second extract and germination rate, which possessed the least phytotoxicity and showed the least sensitivity to residue extracts, respectively. This suggests that, although the electrical conductivity of extracts has some correlation with phytotoxicity of residue extracts, the osmotic effect of extracts is not sufficient to account for the observed phytotoxicity. Indeed, numerous investigations have demonstrated that the osmotic effect of extracts are negligible (Del Moral and Cates, 1971; Mason-Sedun, 1986), or do not contribute to extract toxicity (Kimber, 1967; Buta and Spaulding, 1989).

Milling of residues resulted in greater phytotoxicity than chopping. The phytotoxicity of an aqueous extract of milled residues decomposed for two days is equivalent to that of chopped residues decomposed for six to eight days. Mason-Sedun (1986) also found that milled residues of *Brassica* were more toxic than threshed residues, which were more toxic than chopped. Milling of residues may accelerate the release of free phytotoxins inherent in the material owing to an increase in surface area contact with water and may also help compounds bound in cell walls (Harborne, 1980) overcome any mechanical

impediment to release or provide microorganisms with increased surface area activity. This, in turn, would accelerate the decomposition.

One of the most consistent features found in studies of phytotoxicity has been the recognition of the characteristic responses of an organism to an allelochemical, i.e., stimulation or attraction at low concentrations and inhibition or repellence as the concentration increases (Lovett, 1982; An et al., 1993a). The data presented here show the existence of such phenomena in vulpia residue extracts. The fact that the coleoptile is less sensitive than the root to changes in aqueous rates may indicate that under field conditions allelopathy may be less readily identified because effects are not easily observed initially in aboveground plant parts even though the impact on root growth may show up above ground eventually.

The measurement of when phytotoxins affect a plant and when peak phytotoxicity occurs and diminishes is important to integrating residue allelopathy into residue management strategies. As An et al. (1996a) indicated, by analyzing risk situations of residue retention, the inhibitory period of decaying residues may be avoided, thus minimizing the negative effects of residues, while bringing stimulatory effects to crop plants and enhancing the benefits on the soil of residue retention. By extending the inhibitory period of decaying residues and increasing phytoxin concentration, some measure of control over weed plants could be obtained.

Acknowledgments—The financial support of the Grains Research & Development Corporation, Australia, is gratefully acknowledged. Vulpia residues and wheat seeds were provided by Dr. A. Leys and Mr. Phillip Morrison, Agricultural Research Institute of NSW, Wagga Wagga, Australia.

REFERENCES

ALSAADAWI, I. S., ARIF, M. B., and ALRUBEAA, A. J. 1985. Allelopathic effects of *Citrus aurantium* L. II. Isolation, characterization, and biological activities of phytotoxins. *J. Chem. Ecol.* 11:1527–1534.

AN, M., JOHNSON, I., and LOVETT, J. 1993a. Mathematical modelling of allelopathy: Biological response to allelochemicals and its interpretation. *J. Chem. Ecol.* 19:2379–2388.

AN, M., PRATLEY, J. E., and HAIG, T. 1993b. The effect of soil on the phytotoxicity of residues of *Vulpia myoros*, pp. 162–164, *in* Proceedings of the 7th Australian Agronomy Conference, Adelaide, South Australia.

AN, M., JOHNSON, I., and LOVETT, J. 1996a. Mathematical modelling of allelopathy: I. Phytotoxicity caused by plant residues during decomposition. *Allelopathy J.* 3:33–42.

AN, M., PRATLEY, J. E., and HAIG, T. 1996b. Variation of wheat and subterranean clover cultivars in susceptibility to vulpia residue phytotoxicity, pp. 60–63, *in* Proceedings of the 8th Australian Agronomy Conference, Toowoomba, Queensland.

AN, M., PRATLEY, J. E., and HAIG, T. 1996c. Differential phytotoxicity of *Vulpia* species and their plant parts. *Allelopathy J.* 3:187–194.

ANDERSON, R. C., and LOUCKS, O. L. 1966. Osmotic pressure influence in germination tests for antibiosis. *Science* 152:771–773.

BUTA, J. G., and SPAULDING, D. W. 1989. Allelochemicals in tall fescue—abscisic and phenolic acids. *J. Chem. Ecol.* 15:1629–1636.

CHOU, C. H., and PATRICK, Z. A. 1976. Identification and phytotoxic activity of compounds produced during decomposition of corn and rye residues in soil. *J. Chem. Ecol.* 2:369–387.

DEL MORAL, R., and CATES, R. G. 1971. Allelopathic potential of the dominant vegetation of western Washington. *Ecology* 52:1030–1037.

FORCELLA, F. 1984. Size structure of silvergrass (*Vulpia* spp.) populations in direct drilled wheat. *Aust. Weeds* 3:3–5.

HABIB, S. A., and RAHMAN, A. A. A. 1988. Evaluation of some weed extracts against field dodder on alfalfa (*Medicago sativa*). *J. Chem. Ecol.* 14:443–452.

HARBORNE, J. B. 1980. Plant phenolics, *in* E. A. Bell and B. V. Charlwood (eds). Secondary Plant Products. Encyclopedia of Plant Physiology, New Series, Volume 8. Springer-Verlag, New York.

HARTUNG, A. C., NAIR, M. G., and PUTNAM, A. R. 1990. Isolation and characterization of phytotoxic compounds from asparagus (*Asparagus officinalis* L.) roots. *J. Chem. Ecol.* 16:1707–1718.

HOROWITZ, M., and FRIEDMAN, T. 1971. Biological activity of subterranean residues of *Cynodon dactylon* L., *Sorghum halepense* L., and *Cyperus rotundus* L. *Weed Res.* 11:88–93.

JONES, C. E., WHALLEY, R. D. B., LOVETT, J. V., and MCINTYRE, S. 1992. Seed bank dynamics of *Vulpia* spp. in pastures, p. 532, *in* Proceedings of the 6th Australian Agronomy Conference, Armidale, New South Wales.

KIMBER, R. W. L. 1967. Phytotoxicity from plant residues. I. The influence of rotted wheat straw on seedling growth. *Aust. J. Agric. Res.* 18:361–374.

KIMBER, R. W. L. 1973. Phytotoxicity from plant residues. II. The effect of time of rotting of straw from some grasses and legumes on the growth of wheat seedlings. *Plant Soil* 38:347–361.

LEATHER, G. R., and EINHELLIG, F. A. 1986. Bioassays in the study of allelopathy, pp. 133–146, *in* A. R. Putnam and C. S. Tang (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

LEYS, A. R., CULLIS, B. R., and PLATER, B. 1991a. Effect of spraytopping applications of paraquat and glyphosate on the nutritive value and regeneration of vulpia [*Vulpia bromoides* (L.) S. F. Gray]. *Aust. J. Agric. Res.* 42:1405–1415.

LEYS, A. R., PLATER, B., and LILL, W. J. 1991b. Response of vulpia [*Vulpia bromoides* (L.) S. F. Gray and *V. myuros* (L.) C. C. Gmelin] and subterranean clover to rate and time of application of simazine. *Aust. J. Exp. Agric.* 31:785–791.

LEYS, A. R., DOWLING, P. M., and KEYS, M. J. 1993. Vulpia. *AGFACTS*, NSW Agriculture, 6 pp.

LOVETT, J. V. 1982. Allelopathy and self-defence in plants. *Aust. Weeds* 2:33–35.

LYNCH, J. M., and CANNELL, R. Q. 1980. Plant residues, pp. 26–37, *in* Crop Seed and Soil Environment. Agricultural Development and Advisory Service, U.K. Ref. Book No. 321.

MASON-SEDUN, W. 1986. Differential phytotoxicity of residues from the genus *Brassica*. Ph.D. thesis. University of New England, Armidale.

MASON-SEDUN, W., and JESSOP, R. S. 1988. Differential phytotoxicity among species and cultivars of the genus *Brassica* to wheat. II. Activity and persistence of water-soluble phytotoxins from residues of the genus *Brassica*. *Plant Soil* 107:69–80.

MCCALLA, T. M. 1971. Studies on phytotoxic substances from soil microorganisms and crop residues at Lincoln, Nebrasca, pp. 39–43, *in* Biochemical Interactions Among Plants. National Academy of Science, Washington, D.C.

MCCALLA, T. M., and NORSTADT, F. A. 1974. Toxicity problems in mulch tillage. *Agric. Environ.* 1:153–174.

McINTYRE, S., and WHALLEY, R. D. B. 1990. Co-occurrence of *Vulpia* species on the northern tablelands of New South Wales. *Aust. J. Bot.* 38:445–450.

MERSIE, W., and SINGH, M. 1987. Allelopathic effect of parthenium (*Parthenium hysterophorus* L.) extract and residue on some agronomic crops and weeds. *J. Chem. Ecol.* 13:1739–1747.

PATRICK, Z. A., and KOCH, L. W. 1958. Inhibition of respiration, germination, and growth of substances arising during the decomposition of certain plant residues in soil. *Can. J. Bot.* 36:621–647.

PATRICK, Z. A., TOUSSOUN, T. A., and SNYDER, W. C. 1963. Phytotoxic substances in arable soils associated with decomposition of plant residues. *Phytopathology* 53:152–161.

PRATLEY, J. E. 1989. Silvergrass residue effects on wheat, p. 72, *in* Proceedings of the 5th Australian Agronomy Conference, Perth, Western Australia.

PRATLEY, J. E., and INGREY, J. D. 1990. Silvergrass allelopathy on crop and pastures, pp. 436–439, *in* Proceedings of the 9th Australian Weeds Conference, Adelaide, South Australia.

PUTNAM, A. R., and DUKE, W. B. 1978. Allelopathy in agroecosystems. *Annu. Rev. Phytopathol.* 16:413–451.

READ, J. J., and JENSEN, E. H. 1989. Phytotoxicity of water-soluble substances from alfalfa and barley soil extracts on four crop species. *J. Chem. Ecol.* 15:619–628.

RICE, E. L. 1984. Allelopathy, 2nd ed. Academic Press, New York.

STEVENS, K. L., and MOLYNEUX, R. J. 1988. Castanospermine—a plant growth regulator. *J. Chem. Ecol.* 14:1467–1473.

TOAI, T. V., and LINSCOTT, D. L. 1979. Phytotoxic effect of decaying quackgrass (*Agropyron repens*) residues. *Weed Sci.* 27:595–598.

WELBANK, P. J. 1963. Toxin production during decay of *Agropyron repens* (couchgrass) and other species. *Weed Res.* 3:205–214.

WESTON, L. A., and PUTNAM, A. R. 1986. Inhibition of legumes seedling growth by residues and extracts of quackgrass (*Agropyron repens*). *Weed Sci.* 34:366–372.

WESTON, L. A., BURKE, B. A., and PUTNAM, A. R. 1987. Isolation, characterization and activity of phytotoxic compounds from quackgrass [*Agropyron repens* (L.) Beauv.]. *J. Chem. Ecol.* 13:403–421.

WILLIAMS, R. D., and HOAGLAND, R. E. 1982. The effects of naturally occurring phenolic compounds on seed germination. *Weed Sci.* 30:206–212.

WILSON, R. G., JR. 1981. Effect of Canada thistle (*Cirsium arvense*) residue on growth of some crops. *Weed Sci.* 29:159–164.

Journal of Chemical Ecology, Vol. 23, No. 8, 1997

PHYTOALEXINS, RESISTANCE TRAITS, AND DOMESTICATION STATUS IN *Phaseolus coccineus* AND *Phaseolus lunatus*

ROBERTO LINDIG-CISNEROS, BETTY BENREY, and FRANCISCO J. ESPINOSA-GARCÍA

Instituto de Ecología, Universidad Nacional Autónoma de México
A.P. 70-275, 04510, México, D. F., México

(Received November 22, 1996; accepted April 6, 1997)

Abstract—Resistance to pathogenic fungi and the isoflavonoids that confer such resistance capabilities were studied in two systems of wild and cultivated *Phaseolus* spp. seedlings. Results for *P. coccineus* show a gradient in isoflavonoid diversity with pathogen resistance high in wild varieties and low in cultivated ones. *P. lunatus* varieties showed the same trend in resistance and cyanogenic capacity, but not in isoflavonoid diversity. It is suggested that the effectiveness of the phytoalexin natural mixture depends more on its diversity than its total levels in seedlings.

Key Words—*Phaseolus coccineus*, *Phaseolus lunatus*, phytoalexins, isoflavonoids, domestication, wild, cultivated, fungal infections, phytoalexin diversity.

INTRODUCTION

As a result of domestication, plants have higher yields, particularly those parts that are of agronomic interest (Tivy, 1990). In many cases, domesticated plants also have lower levels of natural products in those same parts compared to their wild relatives (Roddick, 1986; Sotelo et al., 1995). Although this reduction in defensive chemicals in harvested parts can be explained as a consequence of selection for palatability, it cannot explain the reduction in defensive traits in nonharvested parts (Krischik and Denno, 1983). Low levels of defenses in nonharvested parts of cultivated plants are considered to be a consequence of reallocation patterns within the plant. Because no net increases in photosynthetic

*To whom correspondence should be addressed.

0098-0331/97/0800-1997$12.50/0 © 1997 Plenum Publishing Corporation

rates have been documented for cultivated plants (Evans, 1984), increased yield is possible only if resources are diverted from other plant functions. These observations support a model by Rosenthal and Dirzo (1996), which predicts a gradient of defense according to resource allocation. In this model, wild cultivars have the highest defense and lowest yield, whereas modern cultivars have lower defense levels but higher yields, because their selection has been carried out under conditions of low pressures from herbivores and pathogens, thus allowing greater reallocation to yield. Because land races have been selected under local conditions, in the presence of moderate pressure from herbivores and pathogens, they represent intermediate levels of defense and yield. Finally, wild relatives, as a consequence of high selective pressure, should allocate more to defense and less to yield. Although an assumed costly constitutive defense is accounted for by the resource allocation model, induced defense is not clearly explained because this mode of defense has been assumed to be both costly (da Cunha, 1987) and noncostly (Clark and Harvell, 1992; Herms and Mattson, 1992).

An induced response is elicited in *Phaseolus* beans when tissues are challenged with fungal hyphae (for review see Sequeira, 1983). The response consists of the production of isoflavonoid phytoalexins, compounds that have been isolated from many artificially infected *Phaseolus* species (Adesanya et al., 1985; O'Neill et al., 1984; Stössel, 1985; Anderson, 1978; García-Arenal et al., 1978; Bandara, 1979; Zavala et al., 1989). The fungitoxicity of several isoflavonoid phytoalexins has been established, and the degree of toxicity is species-specific (Skipp and Bailey, 1977).

We selected wild and cultivated populations of two species of *Phaseolus* beans, to explore the relationship between their induced defenses, their resistance, and their domestication status.

METHODS AND MATERIALS

Phaseolus coccineus and *P. lunatus* have been cultivated for several thousand years in Mesoamerica (Delgado-Salinas, 1988), although *P. coccineus* cultivation is now restricted to its centers of origin, mainly in the Mexican highlands (Sauer, 1993). *Phaseolus coccineus* grows wild, sometimes at the edge of cultivated bean plots (Delgado-Salinas, 1988). *P. lunatus* is found in the coastal zones of the Pacific ocean, particularly in the states of Guerrero, Michoacán, and Jalisco (Baudoin, 1988). Cultivated forms (land races) are found in several Mexican locations and are mainly grown for local use.

Wild seeds of *Phaseolus coccineus* were collected from two different populations, one located in the Bosque de Tlalpan National Park in Mexico City, and the second at Huitzilac (State of Morelos). Seeds from a land race of this species (ayocote variety) were obtained from the state of Puebla. *Phaseolus*

lunatus wild seeds were collected in the Chamela region on the Pacific coast of the Mexican State of Jalisco. Land-race seeds were obtained from the State of Guerrero, and seeds from a commercial cultivar were purchased from a NK Lawn and Garden Co. distributor.

Infection Rates in Field and Environmental Chamber Experiments. To assess seedling resistance to pathogenic fungi, we conducted two field and four environmental chamber experiments with *P. coccineus* seedlings and two environmental chamber experiments with *P. lunatus* seedlings. One field experiment with *Phaseolus coccineus* took place in an agricultural field in the Xochimilco region of the Valley of Mexico. The site was cleared, after being fallow for five years, reducing possible effects of chemical pesticides and fertilizers normally used in this region. For this experiment, seeds from the Tlalpan population and the ayocote land race were randomly chosen and scarified by puncturing with a needle, superficially sterilized with 1% sodium hypochloride solution for 5 min and rinsed with sterilized water. The seeds were randomly distributed in two 15 × 10 grids (75 seeds per variety in each grid), spaced 10 cm apart to avoid cross contamination, individually covered with plastic mesh to avoid animal predation and allow inspection with minimal disturbance, and covered with a thin layer of soil. Field plots were watered daily. Six days after planting, seedlings were chosen to form cohorts if the radicle was clearly visible. The experiment ended when the noninfected seedlings presented the first true leaves.

In the population from Bosque de Tlalpan, three different zones in which wild *P. coccineus* grow can be characterized. These vegetational zones correspond to a disturbed oak forest, a severely disturbed oak forest with planted eucalyptus trees, and the pedregal zone characterized by plants that grow on old lava flows. To take into account this environmental heterogeneity, three experiments were conducted, one for each zone.

Seed treatment and cohort selection were carried out as previously described. In each experiment, 80 seeds of each variety were randomly distributed, covered with mesh, and litter added to cover the entire plot. Water was supplied by natural rain.

For growth chamber experiments, soil was collected from locations where wild populations of both species grow, from agricultural fields in the Chapingo region of the State of Mexico, and from Xochimilco in Mexico, D.F. Independent experiments for each species were carried out in the different soil types. For each experiment the procedure described below was followed. The Xochimilco soil was used with *P. coccineus* to compare field and laboratory results for this species.

Each soil sample was sieved through a No. 10 mesh and then stirred. Saturation water capacity (SWC) for each sample was estimated by adding distilled water to a sample of 50 g soil in a well-drained container and, after several minutes, weighing the wetted soil.

Soil was placed in polyethylene cups (200 ml) up to approximately one fifth of the cup's capacity. Sterilized distilled water was added to each cup to SWC, in order to have equal conditions in all replicates. Seeds were treated in the same manner as described for the field experiments. One seed of each variety was placed in each cup under sterile conditions and covered with a translucent plastic cover. Soil and water quantities for each experiment varied with the nature of the different soils used. For all experiments 65 seeds per variety were planted, and cohorts selected from among those that germinated.

Experiments were carried out in an environmental chamber (Coenviron, model E15) 11:13 L:D. In order to obtain a constant temperature of 29°C inside the cups, it was necessary to set the temperature in the chamber at 26°C during the light period, and 29°C during the dark.

Each cup was inspected daily to select seedlings for cohorts and later to follow the infection process. For *P. coccineus*, cohorts were selected 48 hr after planting, and for *P. lunatus* after 36 hr; only seedlings showing the radicle were chosen. Cohort sizes for all treatments were matched to the variety with the smaller cohort size to avoid unbalanced experiments. Each experiment was considered completed when the first true leaves of the noninfected seedlings appeared.

Results were statistically analyzed, assuming the result was a consequence of a binomial trial with a sample size 1 (Crawley, 1993), since the response variable was either infected or noninfected and no interaction between seedlings was possible. The analysis was carried out with GLIM 3.77 (Royal Statistical Society, 1985) using a logit link function and a binomial probability distribution. The probabilities were calculated with TAB 1.3 (Ezcurra, i995).

Chemical Analysis. To calculate chemical diversity values and levels of chemicals for the varieties under study, several analyses were performed. After superficial sterilization, 180 seeds of each variety of *P. coccineus* and 130 of *P. lunatus* varieties were allowed to imbibe distilled and sterilized water for 8 hr, and then germinate under sterile conditions at 25°C. Cohorts were selected 48 hr after germination. Selected seedlings were treated with 5 mM aqueous $CuCl_2$ for 2 hr and incubated another 48 hr in an incubator at 25°C. After incubation, cotyledons were removed and isoflavonoids extracted 20× with chromatographic-grade ethyl acetate.

For diversity analysis, high-performance liquid chromatography (HPLC) was performed on 20 samples per variety (Waters HPLC system model 600 MS). Isoflavonoid separation was carried out on a Lichrosphere 10μ 250- × 3.2-mm column (Phenomenex), using a solvent gradient of ethyl acetate–hexane (85:15) to ethyl acetate–hexane–chloroform (85:6:9) with a flow gradient of 0.7 to 1.2 ml/min. (curve 6 of the Waters protocol). UV spectra were obtained for all compounds using a photodiode-array detector (Waters, model 990).

Using peak parameters, chemical diversity was calculated using the Shannon index. Diversity estimates were used to compare varieties; statistical analysis was carried out with Kruskal-Wallis ANOVA by ranks due to inequalities of diversity variances. Quantitative analysis of total and individual levels of isoflavonoids was carried out by evaporating under reduced pressure the ethyl acetate from extracts and weighing the remaining solids. Total level was calculated as grams of isoflavonoid mixture per gram of fresh seedling tissue, and the results analyzed by Kruskal-Wallis ANOVA by ranks (StatSoft Inc., 1993).

Phaseolus lunatus is unique among *Phaseolus* species because its seeds contain cyanogenic glucosides. Chemical and physiological aspects of the synthesis and accumulation of these compounds have been well studied (Frehner et al., 1990; Conn, 1980). Levels in wild *P. lunatus* seeds are higher than in cultivated varieties, often almost 150 times greater (Lucas and Sotelo, 1984). An analysis of cyanogenic capacity was performed with *P. lunatus* seeds. The standard curve was prepared with KCN (Lucas and Sotelo, 1984), but without corn starch because we were only interested in quantifying the liberated HCN from the seeds. HCN release from the glucosides was elicited by acid hydrolysis (HCl, 0.5 N). The released HCN reacted with sodium picrate in paper strips (2 × 0.5 cm) and the reaction product, isopurpurin, was redisolved in distilled water (3 ml) and read with a spectrophotometer at 510 nm.

In vitro Assay of Phytoalexin Activity. To assess the inhibitory properties of phytoalexin mixtures, an in vitro randomized two-block experiment with eight seedling extracts per variety of *Phaseolus coccineus* was performed. An isolate of *Aspergillus* sp., previously obtained from a *P. coccineus* infected seedling, was used. Two cultures were prepared on malt extract agar media.

Ethyl acetate was evaporated from phytoalexin extracts and the remaining solids dissolved in two times the fresh weight of the extracted seedling distilled sterilized water. An equal amount of malt extract agar was added. Controls were prepared following the previous procedure, but without phytoalexins. Sterilized capillary tubes (0.4 × 100 mm) were filled with 0.040 ml of each of the phytoalexin agar solutions, and the open ends sealed with paraffin wax.

To prepare the assay, two Petri dish covers were concentrically perforated (16 holes per cover at 1.0 cm from the edge). Covers were placed on top of the *Aspergillus* cultures and capillary filled tubes were randomly placed in the perforations in a such way that the medium in the tubes was in contact with the growing hyphae in the cultures. The spaces between the edge of the holes and the capillary tubes were sealed with epoxy resin to avoid contamination and loose tubes. Each of two *Aspergillus* cultures was a complete experiment with four replicates per treatment (wild, land race, cultivar, and control). Experiments were incubated at 25°C for six days.

Growth of hyphae inside the capillary tubes was measured as the linear distance (in millimeters) between the surface of the agar in the Petri dish and

the growing end of the mycelia inside the capillary tube. For statistical analysis, three categorical variables were established: treatment (with four levels, one for each variety and the control), block (two, one for each Petri dish experiment), and replicates (four for each treatment), with growth being the dependent variable. Data were analyzed by means of an ANOVA (GLM procedure, SAS Institute, 1988). Differences among individual treatments were established with a Sidak T test (GLM procedure; SAS Institute, 1988).

RESULTS

All field and environmental chamber experiments show that wild seedlings are more resistant to fungi than cultivated ones. Analysis of data from field experiments showed a highly significant difference in infection rate (Table 1), the domesticated plants being more susceptible. The data for all blocks (grids) were combined because blocks were not statistically different (for wild seedlings; change in deviance = 0.9, $df = 1$, $P = 0.343$, and for land-race seedlings; change in deviance = 0.08, $df = 1$, $P = 0.777$). Experiments conducted in three different parts of the Bosque de Tlalpan show a similar trend, although the differences for the oak forest were not significant (Table 2).

The environmental chamber experiments also show that wild varieties of both species are more resistant (Table 3). The resistance of the *P. coccineus* Huitzilac wild seedlings falls between that of Tlalpan and land-race, a trend that is also observed in chemical and in vitro assays. *Phaseolus lunatus* varieties show the expected resistance gradient, with the highest resistance in wild seedlings, followed by land race and the modern cultivars.

Results of isoflavonoid chemical diversity are shown in Figure 1. For

TABLE 1. *Phaseolus coccineus* SEEDLINGS INFECTED AFTER GERMINATION IN AGRICULTURAL FIELD EXPERIMENT[a]

	Wild seedlings		Cultivar seedlings	
	Total	Infected	Total	Infected
Block A	48	2	59	15
Block B	71	6	65	18

[a]Blocks were pooled into one statistical analysis. $P = 0.0002$.

TABLE 2. PROPORTION OF NONINFECTED SEEDLINGS OF *Phaseolus coccineus* WILD AND LAND-RACE SEEDLINGS AFTER GERMINATION IN THREE LOCALITIES OF BOSQUE DE TLALPAN[a]

	Noninfected seedling (proportions)		Statistical analysis results	
	Wild	Land-race	Change in deviance	P (df = 1)
Oak forest	0.904	0.770	2.7	0.100
Eucalyptus-oak forest	0.914	0.718	6.1	0.013*
Pedregal	0.851	0.639	5.73	0.016*

[a]See text for details.
*Statistically significant.

Phaseolus coccineus the differences between wild Tlalpan and land-race seedlings are statistically significant [Kruskal-Wallis ANOVA by ranks: H (2, N = 56) = 20.39, P = 0.001]. *Phaseolus lunatus* isoflavonoid chemical diversities show no trend between varieties [Kruskal-Wallis ANOVA by ranks: H (2, N = 59) = 1.491, P = 0.474]. The comparison between total concentrations for both species shows significant differences among some varieties (Figure 2). Interestingly, *Phaseolus lunatus* land-race seedlings have the lowest concentrations [Kruskal-Wallis ANOVA by ranks: H (2, N = 30) = 13.52, P = 0.0012], whereas in *P. coccineus* varieties, the Huitzilac (wild) seedlings have the lowest concentrations [Kruskal-Wallis ANOVA by ranks: H (2, N = 41) = 9.699, P = 0.0073].

Results of cyanogenic capacity tests for *Phaseolus lunatus* seedlings show the expected gradient (Figure 3). Wild seedlings produced more HCN than the other varieties, although the value range is far lower than some reported previously for this species (Lucas and Sotelo, 1984; Frehner et al., 1990). Differences between varieties for this trait are highly significant ($F_{2,12}$ = 25.654, $P < 0.0001$).

Results of the in vitro assay of activity of isoflavonoid extracts are shown in Figure 4; a significant effect of treatment was obtained ($F_{3,16}$ = 12.79, $P < 0.0001$). All treatments differed from the control and, although no statistical differences were detected among them (α = 0.05), an apparent gradient in hyphae growth was observed.

TABLE 3. ENVIRONMENTAL CHAMBER RESULTS FROM EXPERIMENTS IN SOILS OF DIFFERENT ORIGIN FOR BOTH SPECIES STUDIED[a]

	Cohort size	Noninfected seedlings proportion		
		Tlalpan	Huitzilac	Land race
A. *Phaseolus coccineus*				
Tlalpan[b]	40	0.975	0.800	0.650
Huitzilac[b]	38	0.974	0.868	0.658
Chapingo	31	0.840	0.700	0.600
Xochimilco	39	0.945	0.897	0.692

Multiple comparisons for *P. coccineus* varieties [deviance, *df* (*P*)]

Tlalpan vs. land race	Tlalpan vs. Huitzilac	Huitzilac vs. land race
2.885, 78 (0.005)	**2.115**, 78 (0.037)	**1.487**, 78 (0.141)
2.789, 74 (0.007)	**1.549**, 74 (0.126)	**2.092**, 74 (0.040)
2.606, 68 (0.010)	**1.646**, 68 (0.103)	**1.048**, 68 (0.297)
2.662, 77 (0.009)	**0.845**, 77 (0.401)	**2.194**, 77 (0.031)

	Cohort size	Noninfected seedlings proportion		
		Wild	Land race	Cultivar
B. *Phaseolus lunatus*				
Chamela[b]	40	0.925	0.950	0.800
Chapingo	40	0.950	0.850	0.575

Multiple comparisons for *P. lunatus* varieties [deviance, *df* (*P*)]

Wild vs. Land race	Wild vs. cultivar	Land race vs. cultivar
1.917, 68 (0.059)	**1.572**, 68 (0.126)	**0.471**, 68 (0.638)
1.446, 78 (0.152)	**3.395**, 78 (0.001)	**2.622**, 78 (0.010)

[a]Both sets of results show gradients of resistance between the varieties under study, for *Phaseolus coccineus* (a) Tlalpan (wild) seedlings being more resistant, followed by Huitzilac (wild) and land race being the least resistant. For *P. lunatus*, a similar gradient from wild to cultivar seedlings was found.

[b]Wild population soil samples.

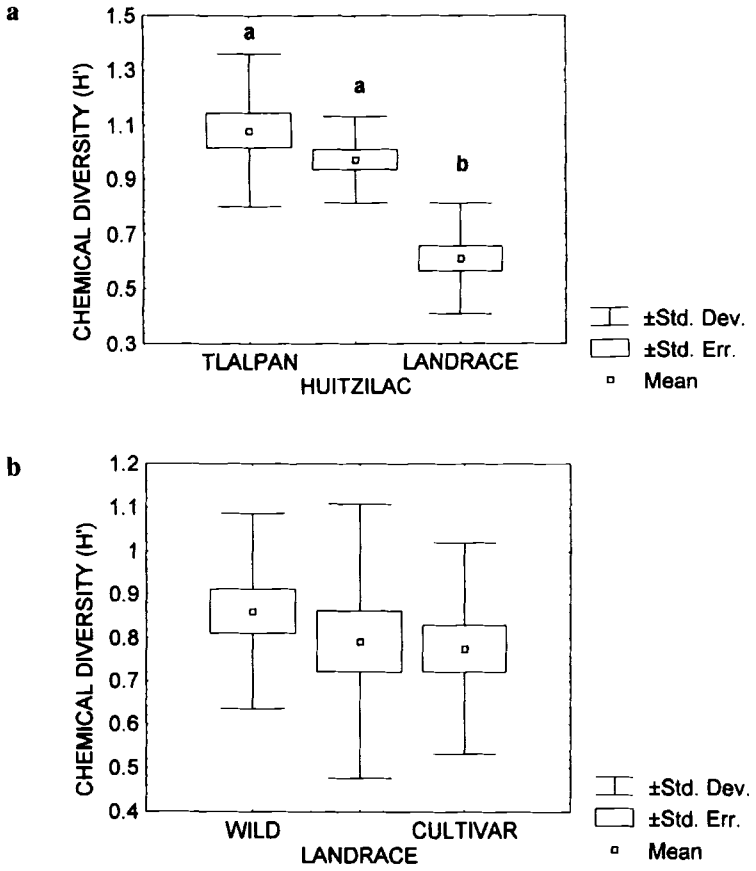


FIG. 1. Chemical diversity of *Phaseolus coccineus* and *P. lunatus* calculated with the Shannon index. *P. coccineus* varieties show a gradient in diversity (a), differences are statistically significant because of Tlalpan and land-race values ($N = 20$ per variety, $P = 0.001$). *Phaseolus lunatus* shows no differences for this same parameter (b) ($N = 20$ per variety, $P = 0.474$). Means ($\pm$SD and SE) marked with different letters are significantly different (Mann-Whitney U test; $P < 0.01$).

DISCUSSION

Overall, wild seedlings of *Phaseolus coccineus* and *P. lunatus* showed higher resistance to fungal infections than cultivated ones. The experiments carried out in the Bosque de Tlalpan wild population site also revealed that the proportions of noninfected seedlings for both varieties in forested sites were

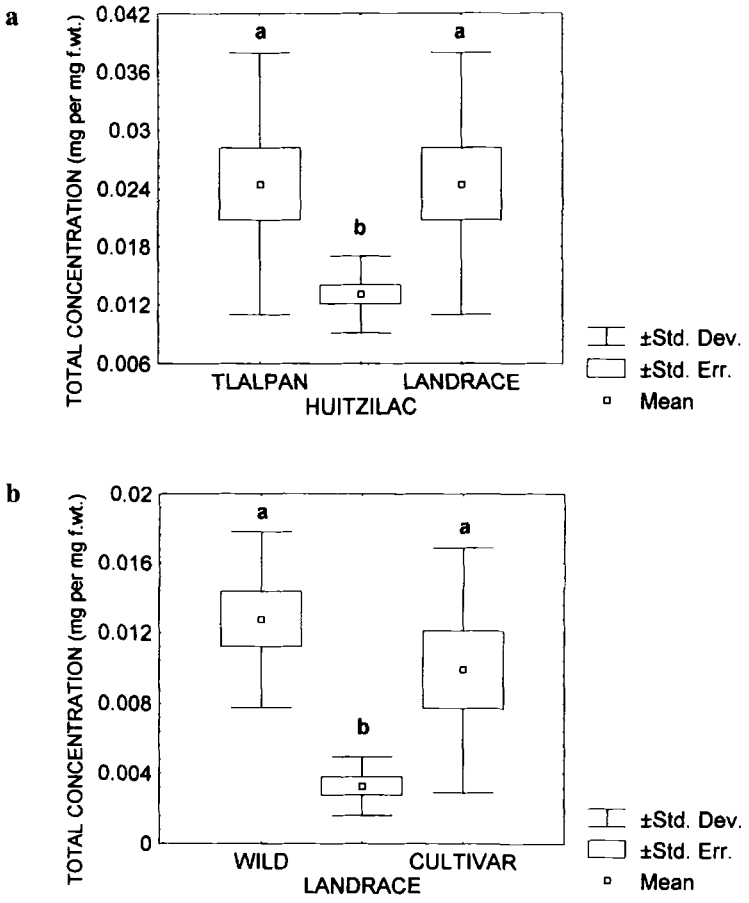


FIG. 2. Total levels of isoflavonoids in the species studied. In both species one variety had lower concentrations than the others. *Phaseolus coccineus* variety results show lower levels for Huitzilac seedlings (a) and for *P. lunatus* the variety with lower concentrations is the land race. Overall, *P. lunatus* concentrations tend to be lower than those of *P. coccineus*. Means (±SD and SE) marked with different letters are significantly different (Mann-Whitney U test; $P < 0.05$).

higher than the corresponding proportions in the pedregal site. This could be a consequence of greater stress imposed on the seedlings in the more open pedregal site, resulting from longer desiccation periods between rain events and greater temperature changes during day and night than in forested sites. This added stress may also have resulted in a proportional decrease in healthy seedlings for both varieties.

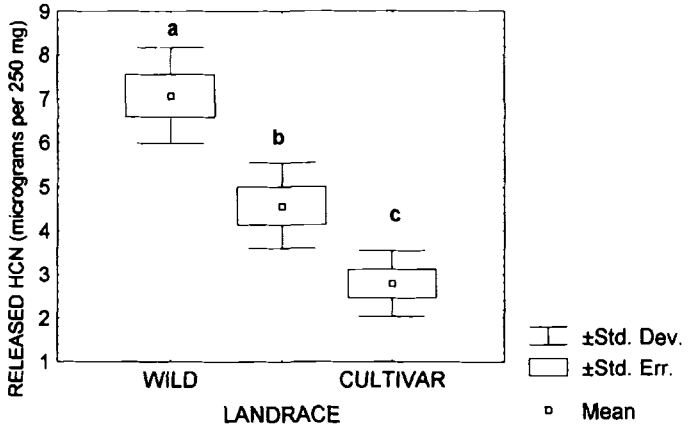


FIG. 3. Released HCN (in micrograms) for *Phaseolus lunatus* varieties. Differences among all three varieties are significant (for details see text). Means (±SD and SE) marked with different letters are significantly different (Mann-Whitney U test; $P < 0.05$).

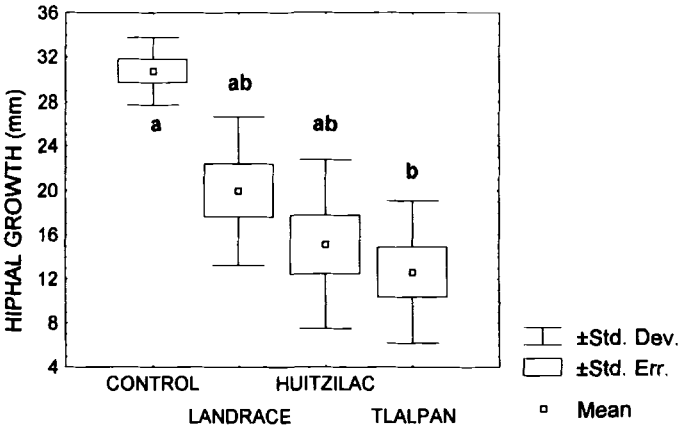


FIG. 4. Inhibitory properties of isoflavonoid mixtures produced by three varieties of *Phaseolus coccineus*. *Aspergillus* sp. mycelia growth inside the capillary tubes (see text) was inhibited in all seedling treatments. Although no significant differences were detected between the varieties, there appears to be a trend from less inhibition in land-race seedlings to more inhibition in Tlalpan ones. Means (±SD and SE) marked with different letters are significantly different ($P < 0.01$; t tests adjusted with Sidak's inequality).

The experiments conducted in the environmental chamber confirmed the trend observed in field experiments for *P. coccineus*. The Huitzilac seedlings (wild) presented an intermediate resistance in all the experiments, Tlalpan seedlings (wild) had the highest resistance and the land-race seedlings the lowest. This pattern was observed in the diversity of isoflavonoids and in the in vitro inhibitory properties of the extracts. *Phaseolus lunatus* environmental chamber experiments showed the highest susceptibility for the commercial cultivar, although, interestingly, no differences were found between the land-race and wild seedling resistance.

It is possible that Huitzilac seedlings were less resistant than Tlalpan ones because more introgression can occur in this population that grows near *P. coccineus* agricultural fields. High outcrossing rates between wild and cultivated forms have been observed for this species (Escalante et al., 1994).

Opportunistic infections cannot be ruled out in some cases, for they can reflect the susceptibility of infected seedlings, since mechanically damaged tissues also produce isoflavonoids and because herbivores such as nematodes (Stadler et al. 1994; for a review see Mateille, 1994), loopers (Liu et al., 1992), or phytophagous beetles (Fishcer et al., 1990) are susceptible to isoflavonoid phytoalexins.

For both species, wild seedlings had greater chemical diversity than cultivated ones, but no differences in total levels were found. *P. coccineus* data show interesting chemical diversity trends with wild Tlalpan seedlings having greater diversity than the other varieties. Furthermore, the mean chemical diversity shows a gradient that corresponds to that of the resistance trend (Tlalpan > Huitzilac > land race). For several seedlings fewer compounds were detected or one compound was the overwhelmingly predominant component—the latter was particularly frequent in the land-race seedlings—thus reducing the chemical diversity parameter. The situation for *P. lunatus* is more complicated. In this species, two defenses are effective against pathogens (cyanide and isoflavonoids). Loss of cyanogenic compounds is certainly the result of human selection for palatability and perhaps increased yields, and it is possible that selection pressure by pathogens prevents further losses of chemical isoflavonoid diversity.

For total concentration of flavonoids in *Phaseolus coccineus*, the lowest values were obtained for the Huitzilac wild seedlings. Higher, and statistically equal ranges, between wild Tlalpan and land-race varieties were obtained. These results suggest that this characteristic may not be a determinant of resistance to fungi, because the more resistant Tlalpan seedlings and the more susceptible land race had high and equal total phytoalexin concentrations. The same trend can be seen in *P. lunatus*, with lower total levels for the more resistant land race variety than those for the commercial cultivar, although for this species cyanogenic capacity should also be considered.

Results from the *Phaseolus coccineus* in vitro assay suggest a possible role of phytoalexins in the resistance of beans. Although differences between varieties are not statistically significant, a trend toward less infection for the extracts of wild plants was observed (Figure 4). This, together with the results of the chemical analyses, shows the importance of phytoalexin diversity in the resistance against fungal infection in beans.

The induced response appears to be nonspecific because the same compounds are synthesized in plant tissues in response to a wide range of pathogens (Skipp and Bailey, 1977). Such biosynthesis of a set of phytoalexins may be beneficial to the plant because of the increased probability that pathogens would be confronted with at least one or several toxic compounds. It is also possible that diversity itself provides plants with greater resistance capabilities due to synergistic effects in secondary metabolite mixtures. Thus the lack of differences in total flavonoid levels does not necessarily reflect equal defense allocation among varieties. Differences in chemical diversity could be the result of different defense allocations if the associated costs of producing groups of chemicals varies for certain secondary metabolites. A reduction in chemical diversity may imply a reduction in defense allocation because fewer precursors are diverted from primary metabolism to the final steps of isoflavonoid synthesis. Moreover, resources allocated to defense may decrease even more if fewer resources are allocated to the production of isoflavonoid catalyzing enzymes and no precursor diversion occurs from primary metabolism to the absent isoflavonoid pathway branches.

Our results for *P. coccineus* are in accordance with the model of Rosenthal and Dirzo (1996), which predicts that induced defenses can be somewhat costly and can follow the same trends constitutive defenses suffer under domestication. By providing useful empirical evidence, these results also illustrate the usefulness of wild–cultivated plant systems to test ecological theory.

Acknowledgments—We wish to thank Dr. Rodolfo Dirzo for this comments and suggestions on the research and this manuscript, Santiago López for lending part of his agricultural field to us and all his useful help, and also Dr. Alfonso Delgado and Dr. María del Carmen Mandujano for their comments on different aspects of this work.

R. Lindig also wishes to acknowledge the support received from CONACyT through scholarship no. 89253.

REFERENCES

ADESANYA, A., O'NEILL, M., and ROBERTS, M. 1985. Isoflavonoids from *Phaseolus coccineus*. *Phytochemistry* **24**(11):2699-2702.

ANDERSON, A. 1978. Isolation from three species of *Colletotricum* of glucan-containing polysaccharides that elicit browning and phytoalexin production in bean. *Phytopathology* **68**(2);189-194.

BANDARA, J. 1979. Accumulation of phytoalexins in bean cotyledons infected with *Fusarium solani* f. species *phaseoli*. *J. Agric. Nat. Soc. Ceylon* **16**:69-80.

BAUDOIN, J. 1988. Genetic Resources, domestication and evolution of lima bean, *Phaseolus lunatus*, pp. 393-407, *in* P. Gepts (ed.). Genetic Resources of *Phaseolus* Beans. Kluwer Academic Publishers, Dordrecht.

CLARK, C., and HARVELL, D. 1992. Inducible defenses and the allocation of resources: A minimal model, *Am. Nat.* **139**:521-539.

CONN, E. 1980. Cyanogenic compounds. *Annu. Rev. Plant Physiol.* **31**:433-344.

CRAWLEY, M. 1993. GLIM for Ecologists. Blackwell Scientific Publications, London, pp. 291-300.

DA CUNHA, A. 1987. The estimation of L-phenylalanine ammonia-lyase shows phenylpropanoid biosynthesis to be regulated by L-phenylalanine supply and availability. *Phytochemistry* **26**:2723-2727.

DELGADO-SALINAS, 1988. Variation taxonomy, domestication, and germplasm potentialities in *Phaseolus coccineus*, pp. 441-463, *in* P. Gepts (ed.). Genetic Resources of *Phaseolus* Beans. Kluwer Academic Publishers, Dordrecht.

ESCALANTE, A., COELLO, G., EGUIARTE, L., and PIÑERO, D. 1994. Genetic structure and mating systems in wild and cultivated populations of *Phaseolus coccineus* and *P. vulgaris* (Fabaceae). *Am. J. Bot.* **8**(9):1096-1103.

EVANS, L. 1984. Physiological aspects of varietal improvement, pp. 121-146, *in* J. Gustafson (ed.). Gene Manipulation in Plant Improvement. Plenum Publishing, New York.

EZCURRA E. 1995. TAB 1.3 Centro de Ecología. UNAM. México.

FISCHER, D., KOGAN, M., and PAXTON, J. 1990. Effect of glyceollin, a soybean phytoalexin, on feeding by three phytophagous beetles (Coleoptera: Coccinellidae and Chrysomelidae): Dose versus response. *Environ. Entomol.* **19**(5):1278-1282.

FREHNER, M., SCALET, M., and CONN, E. 1990. Pattern of the cyanide-potential in developing fruits. *Plant Physiol.* **94**:28-34.

GARCÍA-ARENAL, F., FRAILE, A., and SAGASTA, E. 1978. The multiple phytoalexin response of bean (*Phaseolus vulgaris*) to infection by *Botrytis cinerea*. *Physiol. Plant Pathol.* **13**(2):151-156.

HERMS, D., and MATTSON, W. 1992. The dilemma of plants: To grow or to defend. *Q. Rev. Biol.* **67**(3):283-335.

KRISCHIK, V. and DENNO, R. 1983. Individual, population, and geographic patterns in plant defense, pp. 463-512, *in* R. Denno and M. McClure (eds.). Variable Plants and Hervibores in Natural and Managed Systems. Academic Press, New York.

LIU, S., NORRIS, D., HARTWIG, E., and XU, M. 1992. Inducible phytoalexins in juvenile soybean geotypes predict soybean looper resistance in the fully developed plants. *Plant Physiol.* **100**(3):1479-1485.

LUCAS, B., and SOTELO, A. 1984. A simplified test for the quantification of cyanogenic glucosides in wild and cultivated seeds. *Nut. Rep. Int.* **29**(3):711-719.

MATEILLE, T. 1994. Biology of the plant-nematode relationship: Physiological changes and the defense mechanism of plants. *Nematologica* **40**(2):276-311.

O'NEILL, M., ADESANYA, S., and ROBERTS, M. 1984. Isosojagol, A coumestan from *Phaseolus coccineus*. *Phytochemistry* **23**(11):2704-2705.

RODDICK, J. 1986. Steroidal alkaloids of the Solanaceae, pp. 65-84, *in* W. G. D'Arcy (ed.). Solanaceae: Biology and Systematics. Columbia University Press, New York.

ROSENTHAL, J., and DIRZO, R. 1996. Effects of life history, domestication and agronomic selection on plant defense against insects: Evidence from maizes and wild relatives. *Evol. Ecol.* In press.

Royal Statistical Society. 1985. GLIM 3.77. Royal Statistical Society, London.

SAS Institute. 1988. SAS7STAT User's Guide, Release 6.03 Edition. SAS Institute Inc., Cary, North Carolina.

SAUER, J. 1993. Historical Geography of Crop Plants, A Select Roster. CRC Press, Boca Raton, Florida, pp. 145-217.

SEQUEIRA, L. 1983. Mechanisms of induced resistance in plants. *Annu. Rev. Microbiol.* **37**:51-79.

SKIPP, R., and BAILEY, J. 1977. The fungitoxicity of isoflavonoid phytoalexins measured using different types of bioassay. *Physiol. Plant Pathol.* **11**:101–112.

SOTELO, A., SOUSA, H., and SANCHEZ, M. 1995. comparative study of the chemical composition of wild and cultivated beans. *Plant Foods Hum. Nutr.* **47**(2):93–100.

STADLER, M., DANGE, E., and ANKE, H. 1994. Nematicidal activities of two phytoalexins from *Tavernaria abyssinica*. *Planta Med.* **60**(6):550–552.

StatSoft Inc. 1993. Statistica for Windows 4.3.

STÖSSEL, P. 1985. Structure-activity relationship of some bean phytoalexins and related isoflavonoids. *Physiol. Plant Pathol.* **26**:269–277.

TIVY, J. 1990. Agricultural Ecology. Longman Scientific and Technical Publications, London, pp. 7–20.

ZAVALA, G., CANO, H., and SORIANO, E. 1989. Phytoalexin accumulation in *Phaseolus vulgaris* in response to elicitor treatment to different bean tissues. *Biol. Plant.* **31**(3):221–226.