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PHYTOTOXICITY OF SORGOLEONE FOUND IN GRAIN SORGHUM ROOT EXUDATES

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Abstract—Root exudates of *Sorghum bicolor* consist primarily of a dihydroquinone that is quickly oxidized to a *p*-benzoquinone named sorgoleone. The aim of this investigation was to determine the potential activity of sorgoleone as an inhibitor of weed growth. Bioassays showed 125 μM sorgoleone reduced radicle elongation of *Eragrostis tef*. In liquid culture, 50-μM sorgoleone treatments stunted the growth of *Lemna minor*. Over a 10-day treatment period, 10 μM sorgoleone in the nutrient medium reduced the growth of all weed seedlings tested: *Abutilon theophrasti*, *Datura stramonium*, *Amaranthus retroflexus*, *Setaria viridis*, *Digitaria sanguinalis*, and *Echinochloa crus-galli*. These data show sorgoleone has biological activity at extremely low concentrations, suggesting a strong contribution to *Sorghum* allelopathy.

Key Words—Sorgoleone, phytotoxin, allelochemical, allelopathy, root exudate, *Sorghum bicolor*, Sorghums, weed inhibition.

INTRODUCTION

Several *Sorghum* species, including *Sorghum halepense*, *S. vulgare*, *S. sudanense*, and *S. bicolor*, have been shown to cause allelopathic interference (Breazeale, 1924; Abdul-Wahab and Rice, 1967; Hussain and Gadoon, 1981; Putnam and DeFrank, 1983; Putnam et al., 1983; Alsaadawi et al., 1986). Both autotoxicity and interspecific allelopathic effects have been noted in sorghums (Rice, 1984). They produce and release cyanogenic glycosides and an array of

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phenolic acids that can contribute to suppression of plant growth (Martin et al., 1938; Guenzi and McCalla, 1966; Nicollier et al., 1983; Lehle and Putnam, 1983; Rice, 1984; Weston et al., 1989).

Work in Michigan demonstrated that *S. bicolor* (grain sorghum) could be used as an allelopathic cover crop to control weeds in vegetable crops and orchards (Putnam and DeFrank, 1983; Putnam et al., 1983). Weeds were suppressed by either fall-planted–winter-killed or spring-planted–herbicide-desiccated grain sorghum. Forney et al. (1985) found a sorghum–sudangrass hybrid (*S. bicolor* × *S. sudanense*) was effective in suppressing weeds prior to late-summer, no-till planting of alfalfa (*Medicago sativa*). Purvis (1990) reported that wild oat (*Avena sterilis*) germination was suppressed in *S. bicolor* stubble when compared to its germination in the stubble of four other crops. Einhellig and Rasmussen (1989) showed that inhibitory effects of grain sorghum on weeds carried over from one growing season to the next in a field situation in northeastern Nebraska. It is not likely that this carryover is due entirely to inhibitory phenolic acids that are released from the residue, since their activity seems to diminish substantially in a few months (Guenzi et al., 1967).

Netzly and Butler (1986) isolated several hydrophobic *p*-benzoquinones in exudates from grain sorghum roots. Subsequently, these compounds were identified and named sorgoleones (Netzly et al., 1988). The initial exudate consists of related dihydroquinones, which appear to be natural stimulants of witchweed (*Striga asiatica*) germination (Chang et al., 1986; Netzly et al., 1988; Fate et al., 1990). The dihydroquinones readily oxidize to the more stable quinones. Netzly et al. (1988) reported these quinones may have herbicidal activity. They also found that soil containing sorghum roots had methylene chloride-extractable sorgoleone equivalent to 10^{-4} to 10^{-5} mol/liter in the soil (Netzly et al., 1988). In personal communication, Dr. Larry Butler (Purdue University) stated they recovered sorgoleone from the soil in the spring where a sorghum plant was grown the year before. Hence, sorgoleone may be an integral part of the explanation for some of the allelopathic effects of grain sorghum.

Our objectives in these investigations were to determine the relative toxicity of sorgoleone as an allelopathic chemical and to investigate this compound as a possible inhibitor of weed growth under laboratory conditions.

METHODS AND MATERIALS

Exudate Collection. Grain sorghum [*Sorghum bicolor* (L.) Moench; Dekalb Hybrid DK28] root exudate was collected, as needed, by germinating seeds in 9-cm-diameter Petri dishes on Whatman No. 1 filter paper moistened with deionized water. Ten seeds were placed in each of 40 Petri dishes and germinated for five days under room light (day: 10 $\mu E/m^2/sec$) and temperature (23

± 2°C) conditions. Seedling roots were dipped in 20 ml of methylene chloride containing 50 μl of concentrated acetic acid (Netzly et al., 1988). The methylene chloride solution was evaporated to dryness and the residue recovered. Fate et al. (1990) indicated that the methylene chloride-soluble hydrophobic exudate collected in this way contains only sorgoleone and its dihydroquinone (Figure 1). The exudate collected will be referred to as sorgoleone, since the dihydroquinone readily oxidizes to the quinone (Netzly et al., 1988). Analysis of the exudate components by high-pressure liquid chromatography (HPLC: Spectra Physics, model SP8800) using a Spherisorb 5 μM ODS2 column (4.6 × 250 mm) showed approximately 90% of the exudate corresponded to the sorgoleone peak. HPLC methods followed procedures of Netzly and Butler (1986) and Netzly et al. (1988) with Dr. Larry Butler (Purdue University) providing the initial sample of sorgoleone for comparison. In the numerous repetitions of exudate collection, the quantity of sorgoleone obtained ranged from 1.3 to 1.8 mg/100 roots dipped. Sorgoleone was stored at −4°C until used.

Growth Bioassays. Initial investigations of the effects of sorgoleone on plant growth employed a bioassay with *Eragrostis tef* (Zucc) Trotter, commonly known as teff. *E. tef* is a small-seeded cereal crop common to northern Africa that is being tested in South Dakota and other areas of the northern Great Plains as a forage crop (Boe et al., 1986). This bioassay was developed in our laboratory to test the biological activity of a natural product when only a small quantity of test compound is available. *E. tef* germination approaches 100%, radicle elongation is rapid, and many seeds can be germinated in a small volume of test solution.

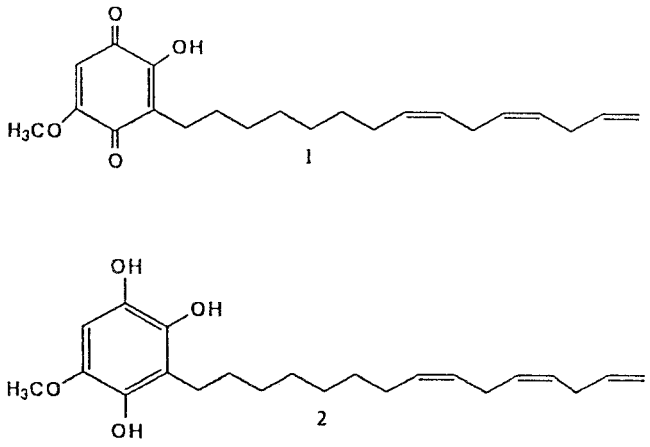

FIG. 1. Structures of (1) sorgoleone and (2) dihydroquinone of sorgoleone (Chang et al., 1986; Netzly et al., 1988; Fate et al., 1990).

Seed germination and radicle elongation studies were conducted in 4.5-cm-diameter Petri dishes with three replicates per treatment. Twenty-five seeds were germinated in a Petri dish on Whatman No. 1 paper moistened with 700 μl of test solution. Sorgoleone was first added to each Petri dish in 50 μl of ethanol, the ethanol evaporated by air drying, and deionized water was then added. The same protocol was used for controls, except they received no sorgoleone. Tests were conducted with 0, 125, and 250 μM sorgoleone. All references to sorgoleone concentrations used in these treatments and subsequent work assume that the compound dissolved in the aqueous test solutions. This is probably an overestimation of true concentration due to the relative hydrophobicity of sorgoleone. Petri dishes were placed in the dark at 25 ± 1°C and percentage germination and radicle lengths were obtained after 48 hr.

A *Lemna minor* L. bioassay was used to determine the effects of sorgoleone on whole plant growth and chlorophyll content (Einhellig et al., 1985). *L. minor* was grown in 24-well tissue culture cluster dishes. The culture media was E medium (Cleland and Briggs, 1967) modified to contain no sucrose or tartaric acid. Each culture dish well contained 1.5 ml of sterile medium and sorgoleone was added in 5 μl of methylene chloride; the control group also received 5 μl of methylene chloride. Treatment solutions were 0, 10, 50, and 100 μM sorgoleone. Efforts to enhance the solubility of sorgoleone by utilizing Tween 20 or Triton X were abandoned because *L. minor* was very sensitive to these surfactants. Each well was inoculated with three fronds, and six replications were used per sorgoleone treatment. *L. minor* was grown under continuous light (200 $\mu E/m^2/sec$) and 28°C. Frond condition, number, and dry weight (24 hr, 80°C) were obtained after seven days. In other experiments with the same design, the chlorophyll content of fronds was determined at the end of the treatment. Fronds were soaked in ethanol for two 24-hr periods, and the absorbance of the chlorophyll extract was read at 665 and 649 nm for quantification using standard equations (Wintermans and DeMots, 1965; Ramirez-Toro et al., 1988; Einhellig and Rasmussen, 1979).

Sorgoleone was tested against six commonly occurring field weeds (three broadleaf and three grass weed species) using an aqueous growth system. Seedling growth of velvetleaf (*Abutilon theophrasti* Medic.), jimsonweed (*Datura stramonium* L.), redroot pigweed (*Amaranthus retroflexus* L.), green foxtail [*Setaria viridis* (L.) Beauv.], crabgrass [*Digitaria sanguinalis* (L.) Scop.], and barnyard grass [*Echinocholoa crus-galli* (L.) Beauv.] was studied. Seeds were germinated in vermiculite in the greenhouse for seven to 10 days; then seedlings were individually transplanted into opaque vials containing 70 ml of one-half strength Hoagland's solution that contained two and one-half times the normal iron (Hoagland and Arnon, 1950). After two days acclimatization, seedlings were selected for uniformity and transferred to fresh nutrient solution, which contained either 0, 10, 50, or 100 μM sorgoleone. Eight plants were utilized

for each treatment and the control. Since sorgoleone was not fully soluble in the growth media, each treatment concentration was prepared by direct addition of sorgoleone rather than by a dilution process from the most concentrated solution. Seedlings were grown in the greenhouse for 10 days and harvested. Shoot and root dry weights were obtained after drying for 48 hr at 105°C. Leaf area and leaf weight were also obtained from the broadleaf weeds with the exception that dried and dead leaves in pigweed treatments did not allow for collection of leaf area measurements. Shoot–root ratios and the specific leaf weight (SLW = leaf weight/leaf area) were computed for *A. theophrasti* and *D. stramonium*.

Data Analyses. Comparisons among treatments in an experiment were analyzed using analysis of variance (ANOVA) and Duncan's multiple-range test.

RESULTS

The small volume of solution required for a test fostered the use of *E. tef* as the first bioassay for potential inhibitory effects of sorgoleone. In repeated trials, *E. tef* seed had a germination rate above 95%, with the radicle first appearing by 24 hr. No sorgoleone concentration tested inhibited or delayed this initial evidence of germination. However, 48-hr radicle elongation was almost completely stopped by 250 μM sorgoleone, and radicle lengths of seeds germinated in 125 μM sorgoleone were about half those of controls (Table 1).

Tests with *L. minor* showed that sorgoleone was inhibitory to the total growth of this plant. After seven days of treatment, *L. minor* grown with a 100-μM concentration of sorgoleone had only 73% of the frond count and 78% of the dry weight of controls (Table 2). *L. minor* fronds growing in this treatment also exhibited less total chlorophyll, but there was no difference from control plants in the ratio of chlorophyll a to b (data not shown). The treatment of 50 μM sorgoleone significantly inhibited frond number. Although not statistically significant, the 10-μM sorgoleone treatment resulted in a slight increase in frond number and dry weight.

TABLE 1. EFFECTS OF SORGOLEONE ON RADICLE ELONGATION OF *Eragrostis tef*[a]

	Sorgoleone treatment (μM)		
	0	125	250
Radicle Length after 48 hr (mm)	11.4 a	6.1 b	0.6 c

[a] Each value is the mean of 75 seedlings. Those not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test.

TABLE 2. EFFECTS OF SORGOLEONE ON *Lemna minor* GROWTH AND CHLOROPHYLL CONTENT[a]

Plant parameter	Sorgoleone treatment (μM)			
	0	10	50	100
Frond number	45.5 a	52.2 a	41.7 b	33.0 c
Dry weight (mg)	5.0 a	5.3 a	4.9 a	3.9 b
Total chlorophyll (mg chl/frond)	2.07 a	2.09 a	2.06 a	1.87 b

[a] Values ($N = 6$) in a row not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test.

The growth of each of the broadleaf weeds tested was markedly inhibited by 10 μM and higher concentrations of sorgoleone in the nutrient medium (Table 3). Inhibition was concentration-dependent, with the most severe effects occurring from 100 μM sorgoleone. In the case of *A. theophrasti* and *A. retroflexus*, plants grown with 100 μM sorgoleone appeared slightly smaller than controls at the end of three days of treatment. Differences in growth between the treated plants and controls became more apparent as the experiment progressed, and by the time of harvest several 100-μM treated *A. retroflexus* seedlings had wilted or dried leaves and appeared dead. The visible effects of sorgoleone on *Datura stramonium* seedlings were not as severe as for the other two species.

Leaf area and shoot, root, and leaf weights of all weed species were inhibited by sorgoleone even at 10-μM treatments (Table 3). However, the shoots of *A. theophrasti* and *D. stramonium* seedlings were more affected than the roots, as shown by the lower shoot–root ratios among treated plants. The reduction in shoot–root ratio was greatest in plants grown with 100 μM sorgoleone. A similar reduction in the shoot–root ratio occurred in sorgoleone-treated *A. retroflexus* plants except at the highest treatment level. This exception was probably due to early cessation of growth and/or death of *A. retroflexus* seedlings grown in 100-μM sorgoleone solutions. Calculations of SLW (specific leaf weight) for *A. theophrasti* and *D. stramonium* did not show any clear trends across treatment levels.

Similar to effects found with broadleaf weeds, growth of the three grass weeds evaluated was suppressed by each of the sorgoleone concentrations tested, and the extent of the inhibition was concentration-dependent (Table 4). *D. sanguinalis* seedlings showed the greatest reductions in plant weight with the 10-, 50-, and 100-μM sorgoleone treatments having 34%, 23%, and 22%, respectively, of the weight of untreated plants at harvest. Most of the 100-μM treated *D. sanguinalis* seedlings were dead by the time of harvest. Because of the strong

TABLE 3. EFFECTS OF SORGOLEONE ON GROWTH OF BROADLEAF WEEDS[a]

	Sorgoleone treatment (μM)			
	0	10	50	100
Abutilon theophrasti				
Total plant wt (mg)	79.0 a	48.6 b	38.4 b	22.4 c
Shoot wt[b] (mg)	66.4 a	39.9 b	30.5 b	16.9 c
Root wt (mg)	12.6 a	8.7 b	7.9 b	5.5 c
Shoot–root ratio	5.4 a	4.6 ab	3.9 bc	3.3 c
Leaf wt (mg)	48.6 a	27.8 b	20.3 b	10.9 c
Leaf area (cm^2)	20.9 a	12.6 b	7.9 c	4.1 d
SLW[c] (mg/cm^2)	2.3 a	2.2 a	2.6 a	2.7 a
Datura stramonium				
Total plant wt (mg)	220.5 a	131.6 b	116.3 bc	99.3 c
Shoot wt (mg)	191.5 a	109.3 b	96.6 bc	80.4 c
Root wt (mg)	29.0 a	22.3 ab	19.7 b	19.0 b
Shoot–root ratio	7.5 a	5.4 ab	5.7 ab	4.7 b
Leaf wt (mg)	140.4 a	76.4 b	66.6 b	57.2 b
Leaf area (cm^2)	23.9 a	15.1 b	12.0 c	10.9 c
SLW (mg/cm^2)	5.9 a	5.1 b	5.6 ab	5.2 ab
Amaranthus retroflexus				
Total plant wt (mg)	73.1 a	39.8 b	24.2 b	5.7 c
Shoot wt (mg)	65.2 a	35.4 b	20.7 bc	5.2 c
Root wt (mg)	7.9 a	4.4 b	3.5 b	0.5 c
Shoot–root ratio	8.3 b	7.9 bc	6.1 c	11.0 a
Leaf wt (mg)	49.0 a	26.4 b	14.6 c	2.9 d

[a] Each value is the mean of eight plants. Row values not followed by the same letters are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test.
[b] Shoot weight included leaf and stem weights.
[c] SLW (specific leaf weight) = leaf weight/leaf area.

sorgoleone effects found in other species, *E. crus-galli* seedlings were not tested at the 100-μM level. In the experiments with each of the grass weeds, shoots and roots were about equally inhibited and no significant differences in the shoot–root ratio were evident.

DISCUSSION

This research clearly shows that sorgoleone is a potent inhibitor of plant growth that may likely be involved in allelopathic interference from *Sorghum* species. Sorgoleone addition at low concentrations resulted in suppressions of radicle elongation in the *E. tef* bioassay and reduction in total plant growth in

TABLE 4. EFFECTS OF SORGOLEONE ON GROWTH OF GRASS WEEDS[a]

	Sorgoleone treatment (μM)			
	0	10	50	100
Setaria viridis				
Total plant wt (mg)	42.9 a	29.4 b	15.8 c	13.4 c
Shoot wt (mg)	32.8 a	22.0 b	11.8 c	10.5 c
Root wt (mg)	10.1 a	7.4 b	4.0 c	2.9 c
Shoot–root ratio	3.3 a	3.2 a	3.6 a	3.6 a
Digitaria sanguinalis				
Total plant wt (mg)	29.1 a	9.9 b	6.8 b	6.5 b
Shoot wt (mg)	24.3 a	7.9 b	5.7 b	4.8 b
Root wt (mg)	4.8 a	2.0 b	1.1 b	1.7 b
Shoot–root ratio	5.2 a	7.3 a	5.9 a	3.2 a
Echinochloa crus-galli				
Total plant wt (mg)	134.8 a	87.4 b	40.6 c	NT[b]
Shoot wt (mg)	105.0 a	65.7 b	29.9 b	NT
Root wt (mg)	29.8 a	21.7 a	10.7 b	NT
Shoot–root ratio	3.5 a	3.1 a	2.9 a	

[a] Each value is the mean of eight plants. Row values not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test.
[b] NT = no test.

L. minor. Growth suppression was also pronounced in the six weed species tested. Even the 10-μM treatments of sorgoleone significantly reduced growth of each of the weed seedlings over the 10-day treatment. In general, growth reductions were nonselective, as sorgoleone affected both broadleaf and grass weeds. Tests of the effects of sorgoleone on a greater diversity of weed species are needed to confirm this lack of selectivity. However, it appears that the action of sorgoleone alone cannot explain the findings of Einhellig and Rasmussen (1989) that broadleaf weed abundance and biomass were more reduced after a crop of grain sorghum than were grass weeds.

It is important to recognize that the treatment concentrations that reduced seedling growth were very low in comparison to many allelopathic chemicals that have been tested in similar experimental designs. Inhibition of seedling growth by 10 μM sorgoleone in the nutrient medium is one to two orders of magnitude lower than the concentration required for growth reduction by many phenolic acids, which is often in the 100–1000 μM range (Einhellig, 1986, 1989). Allelopathic coumarins, flavonoids, and sesquiterpene lactones are typically inhibitory in a concentration range similar to phenolic acids (Einhellig et al., 1970; Scholes, 1987; Haar, 1990). We did not test sorgoleone concentrations below 10 μM, but the data suggest the threshold for growth inhibition of

some seedlings, such as *D. sanguinalis*, may be below this level. Inhibition by sorgoleone at micromolar concentrations is even more surprising when it is recognized that sorgoleone was not completely soluble in the aqueous nutrient medium. Hence, the treatment concentrations cited for the tests must be considered somewhat higher than what was available in the treatment solutions.

Relatively few quinones have been implicated in allelopathy, and even fewer as allelochemicals from higher plants (Rice, 1984). Juglone (5-hydroxy-1,4-naphthoquinone) is well known as the principle chemical responsible for walnut (*Juglans nigra*) allelopathy. Its potency is similar to the effects of sorgoleone. Micromolar levels of juglone have been reported to suppress the growth of several herbaceous species even though no visible signs of injury occurred (Rietveld, 1983).

We speculate that sorgoleone interferes with membrane functions. Investigations are now in progress to elucidate its possible physiological effects. Regardless of the mechanism(s) of sorgoleone action, it is a powerful inhibitor of plant growth. The occurrence of sorgoleone as a relatively pure compound in root exudates indicates it must be considered a part of the explanation for the allelopathic activity of the *Sorghum* crops in conjunction with the action of phenolic acids and other allelopathic compounds known to be released from *Sorghum* species (Guenzi and McCalla, 1966; Nicollier et al., 1983; Lehle and Putnam, 1983; Panasiuk et al., 1986; Weston et al., 1989). In summary, sorgoleone is a novel allelochemical that has biological activity at extremely low concentrations.

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Abraxas grossulariata L. (LEPIDOPTERA: GEOMETRIDAE): IDENTIFICATION OF (3Z,6Z,9Z)-3,6,9-HEPTADECATRIENE AND (6Z,9Z)-6,9-*cis*-3,4-EPOXYHEPTADECADIENE IN THE FEMALE SEX PHEROMONE

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Abstract—(3Z,6Z,9Z)-3,6,9-Heptadecatriene and (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene have been identified in female sex pheromone gland extracts of *Abraxas grossulariata* L. (Lepidoptera: Geometridae). The compounds were detected by gas chromatography with electroantennographic detection (GC-EAD) and identified by GC-mass spectrometry (GC-MS). The amounts of hydrocarbon and epoxide were 0.13 and 0.42 ng/female, respectively. These identifications were confirmed by synthesis; the absolute configuration of the epoxide remains to be determined. Another isomeric epoxide, (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene, was tentatively identified in small amounts (0.04 ng/female). Racemic (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene elicited the largest electroantennogram response within a series of heptadecadiene and nonadecadiene monoepoxides and the corresponding trienic hydrocarbons and, in field tests, attracted male *A. grossulariata* into traps. Addition of 10% (3Z,6Z,9Z)-3,6,9-heptadecatriene to this epoxide enhanced attractiveness; addition of (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene had no effect at low proportions while higher proportions caused a reduction in the numbers of males caught.

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Key Words—Pheromone identification, (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene, (3Z,6Z,9Z)-3,6,9-heptadecatriene, *Abraxas grossulariata*, Lepidoptera, Geometridae.

INTRODUCTION

The currant moth, *Abraxas grossulariata* L., is a beautiful geometrid with black and brown dots on its white wings. The species is rather polyphagous and can cause significant damage in gooseberries, currants, and similar berries (Alford, 1983; Bognár, 1978; Vereschagina and Vereschagin, 1969). In the early 1980s, when a severe outbreak was observed in the northern part of Hungary, we started analyzing pheromone extracts prepared from insects collected in the wild. Further work was hampered by a sudden decrease of the insect population and, for a successful continuation of the project, a rearing method capable of producing hundreds of insects was required. The insect overwinters as small larvae on the plants, and all efforts to break this diapause were unsuccessful. Rearing of one generation took one year in each case. Here we report the identification of sex pheromone components of *Abraxas grossulariata*.

METHODS AND MATERIALS

Insects. The insect culture derived from eggs of female moths collected from the wild. The larvae were reared in an outdoor cage, where they were left to overwinter. They were fed with fresh leaves of black currant until pupation. The pupae were removed from their pupal cocoons, sexed, and the sexes were held separately under a reversed 18:6 light–dark photoregime and 25°C in the laboratory for bioassays and observations on calling behavior. Emerging adults were collected daily and were transferred to 1-liter glass jars where they were supplied with water on cotton wool.

Extracts. Females were observed calling from the second day of their adult life, after the onset of the dark period. For preparation of pheromone extracts, the terminal segments of the abdomina of unmated calling females were excised and extracted in small volumes (5 μl/female) of *n*-pentane or *n*-hexane (Merck, analytical grade) for 15 min.

Analysis. Female gland extracts were analyzed by gas chromatography with flame ionization and electroantennographic detection (GC-FID/EAD) (Arn et al., 1975) using a male antenna of *A. grossulariata*. A Carlo Erba 5360 gas chromatograph equipped with a homemade 25-m Silar 10c high-resolution (HRGC) glass capillary (0.32 mm ID) column was used. This column was tem-

perature programmed as follows: 70°C, 1 min isothermal, 10°C/min to 100°C, then at 3°C/min to 180°C, followed by an isothermal hold at this temperature. Splitless injections (injector temperature: 220°C) were made of 1 female equivalent (1 FE) in 2 μl of solvent.

Analysis by GC-mass spectrometry (GC-MS) was carried out initially with a Finnigan 4023 quadruple mass spectrometer and the Silar 10c HRGC column. Final GC-MS analysis was made on a VG Tribrid double-focusing magnetic sector mass spectrometer (VG Analytical Ltd., Manchester, England) operating in the electron ionization (El, 70 eV, 180°C) mode. El mass spectra (*m/z* 35–485, 1.16 sec/scan) were recorded at unit mass resolution. In this case a 25-m SE 54 HRGC fused silica column (0.32 mm ID; Mega, Legnano, Italy) was used and temperature programmed as follows: 50°C, 2 min isothermal, 20°C/min to 140°C, then at 5°C/min to 280°C, followed by an isothermal hold at this temperature. The samples (2 μl in *n*-hexane) were on-column injected at 50°C. Extracts of up to 10 female glands of 1 to 3-day-old females (40 μl carefully concentrated to 2 μl) were injected. Data acquisition was started at 60°C and retention times were measured from this point. Retention indices (RI) were calculated relative to *n*-alkanes (C_{12}–C_{31}; RI 1200–3100) present in the samples or coinjected; linear interpolation was used in the temperature programmed runs.

Synthesis. The synthesis and characterization of (3*Z*,6*Z*,9*Z*)-3,6,9-heptadecatriene and the preparation of the racemic 3,4-, 6,7-, and 9,10-epoxides by random epoxidation and separation of the three isomers by liquid chromatography was described by Tóth et al. (1991).

Electroantennograms. Electroantennograms (EAG) were recorded from excised antennae of 2- to 3-day-old male moths, applying platinum electrodes to the base and tip of the antenna. Connection between the electrodes and the insect tissues was accomplished with an electrically conducting gel (Valleylab, Boulder, Colorado). Responses were amplified by a high-impedance amplifier (Rumbo, 1981) and displayed on an OH850 chart recorder (Radelkis, Budapest, Hungary). One microgram of the test compound applied to a 10 × 10-mm piece of filter paper inside a Pasteur pipet was used as the odor source. Stimuli were provided by injecting 1 ml of air through the Pasteur pipet into a continuous airstream (1300 ml/min) flowing over the antenna. The interval between stimuli was ca. 60 sec. Responses were normalized to the compound with highest activity.

Field Trapping. Field tests were conducted at several locations in Hungary. Traps similar to those described by Arn et al. (1986) made from transparent polyethylene sheets were used. Traps were hung at the height of 1.5 m from branches of bushes and small trees. Traps containing different baits were set up in rectangular blocks. The distance between traps within a block was 4–5 m. There were six to eight traps in a block; the distance between blocks

ranged between 100 and 1000 m. At every inspection, trap positions were rotated and sticky inserts renewed. In statistical analysis, catches recorded at each trap inspection were regarded as replicates; data were transformed to log $(x + 1)$ and differences between means were tested for significance by Duncan's new multiple-range test (NMRT). In case of treatments with zero catches, the Wilcoxon-Mann-Whitney test was used for paired comparisons of the remaining treatments.

For preparing the baits, the compounds were applied to 1 × 1-cm pieces of rubber tubing (Taurus, Budapest, Hungary, No. MSZ-9691/6; extracted three times in boiling 70% ethanol for 10 min, then three times in methylene chloride overnight, prior to usage) in *n*-hexane solutions. Dispensers were individually wrapped in aluminum foil and were stored at −65°C until use.

RESULTS AND DISCUSSION

Analysis. GC-EAD analysis on a Silar 10c column showed the presence of two biologically active components, an earlier-eluting component I (RI = 1731) and a later-eluting component II of very high EAD activity (RI = 2091). Both components gave very weak mass spectra (6 FE injected) using the quadrupole mass spectrometer. Full identification of the two compounds was not possible; nevertheless, characteristic ions (*m/z* 79, 80, 91, 93, 95, 107 or 108, and 178) were indicative of polyunsaturated aliphatic compounds for both components. A further extract (10 FE) was then analyzed on the VG Tribrid mass spectrometer using an SE 54 column. In this case the mass spectra indicated the presence of a series of *n*-alkanes (C_{23}–C_{31}; odd members C_{23}–C_{31} predominating), as well as some branched species (2- and 3-methylalkanes, C_{27}–C_{31}) and later-eluting steroids (major component cholesterol). Also present was a series of carboxylic acids (C_{16}–C_{20}; saturated, and mono-, di-, and triunsaturated). On this column, components I and II showed RI values of 1672 and 1845, respectively. In Figure 1, a partial chromatogram of this extract shows the elution of components I and II; also indicated is the elution of some *n*-alkanes present in this sample. Mass spectra of the biologically active components I and II are given in Figure 2A,B.

The mass spectrum of component I (RI_{SE54} = 1672; see Figure 2A) showed the characteristic ions previously observed for this component (see above), and new ions at *m/z* 205 and 234. The small difference between the retention indices on the two columns ($\Delta RI = RI_{Silar} - RI_{SE54} = 1731 - 1672 = 59$) suggested component I to be a hydrocarbon. The signals in the lower mass range (*m/z* 35–150) were very similar to those from (3*Z*,6*Z*,9*Z*)-3,6,9-nonadecatriene (M^{+} = 262; RI = 1940 and 1870 on Silar and SE 30, respectively), previously iden-

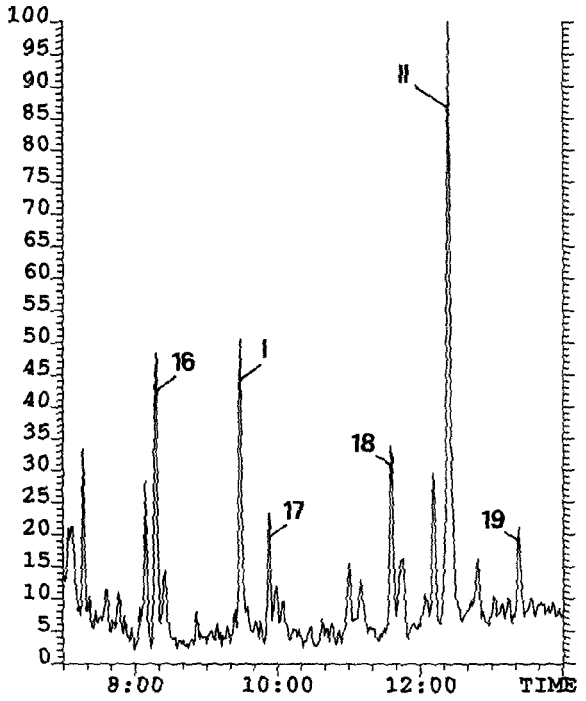

FIG. 1. Reconstructed total ion chromatogram (retention time 7–14 min, 25-m SE 54 HRGC column, VG Tribrid) of a female gland extract of *A. grossulariata* (10 FE injected) showing elution of the biologically active components I (RI_{SE54} = 1672) and II (RI_{SE54} = 1845). Elution of *n*-alkanes (C_{16}–C_{19}) present in the sample as indicated.

tified as a female sex pheromone component of *Peribatodes rhomboidaria* Schiff. (Buser et al., 1985). The signals at *m/z* 234 and 178 for component I and its retention indices suggested it to be a lower homolog, presumably a 3,6,9-heptadecatriene (M^+ = 234; M^+-C_4H_8, *m/z* 178). As methylene-interrupted unsaturations in sex pheromones of moths have been *Z* in all cases reported (see Buser et al., 1985; Millar et al., 1987; Li et al., 1988; Tóth et al., 1989; among others), we decided to synthesize (3*Z*,6*Z*9*Z*)-3,6,9-heptadecatriene according to known methods (Tóth et al., 1991); its EI mass spectrum and retention index proved to be identical to that of component I.

The mass spectrum of component II (see Figure 2B) showed signals at *m/z* 79, 80, 91, 93, 107 and 178, and very weak signals at *m/z* 232 and 250. The ions in the lower mass range also suggested this compound to be polyunsaturated. This and the characteristic ion at *m/z* 178 indicate a possible relationship to component I. The difference between the retention indices (ΔRI =

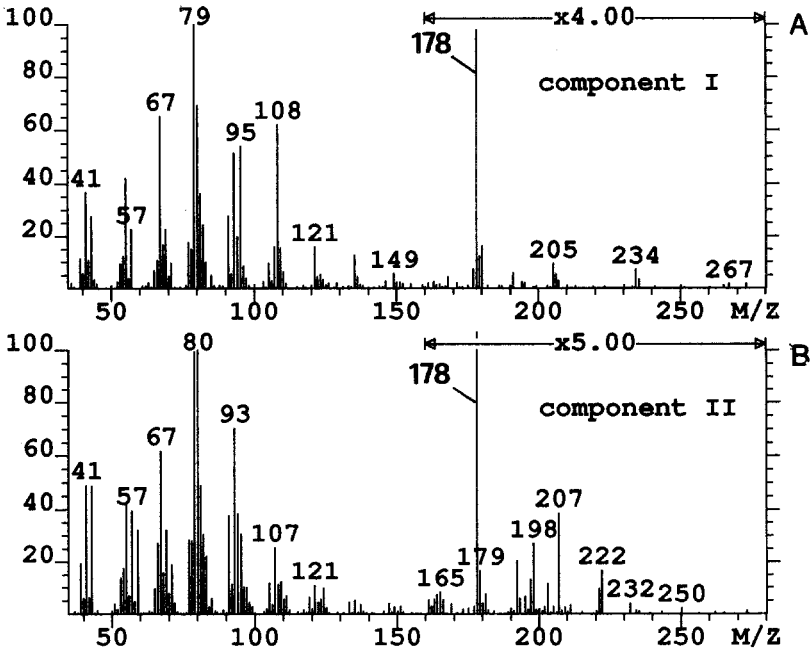

FIG. 2. (A) El mass spectrum of component I (RI_{SE54} = 1672), identified as (3Z,6Z,9Z)-3,6,9-heptadecatriene (M^+ = 234); (B) El mass spectrum of component II (RI_{SE54} = 1845), identified as (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene (M^+ = 250; note presence of signals *m/z* 198, 207, 222 from coeluting compound). Note vertical expansion in mass range *m/z* 160–280.

$RI_{Silar} - RI_{SE54}$ = 2091 − 1845 = 246) suggested component II to be considerably more polar than a hydrocarbon, presumably an oxygenated analog of component I. Furthermore, the retention indices (RI_{SE54}) of components I and II showed a difference (ΔRI = 171), similar to the differences previously observed for a series of monoepoxy analogs of (3Z,6Z,9Z)-3,6,9-heneicosatriene, (3Z,6Z,9Z)-1,3,6,9-heneicosatetraene, (3Z,6Z,9Z)-3,6,9-nonadecatriene and the parent hydrocarbons (Buser et al., 1985; Tóth et al., 1989). The signal at *m/z* 178 would indicate an oxygen function (epoxide) within the first four carbons. A 3,4-epoxide rather than 6,7- and 9,10-epoxides was also indicated by the presence of a *m/z* 59 ion ($C_3H_7O^+$). This information led us to conclude that component II could be a monoepoxy analog of component I, presumably the (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene (M^+ = 250).

The three positional monoepoxy analogs of the (3Z,6Z,9Z)-3,6,9-heptadecatriene were then synthesized as racemates (Tóth et al., 1991). The mass spectra of the three closely eluting epoxides (RI_{SE54} = 1845, 1842, and 1850

for the 3,4-, 6,7-, and 9,10-epoxy analogs, respectively) are reported in Figure 3A–C, and show characteristic differences. The mass spectra were very similar to the spectra previously reported for these compounds (Millar et al., 1987). The mass spectrum of (6*Z*,9*Z*)-6,9-*cis*-3,4-epoxyheptadecadiene (RI_{SE54} = 1845) showed the characteristic signals (particularly *m/z* 59, 79, 80, 107, 178, and others) previously observed and matched that of component II. The retention index of this epoxide and component II also matched. However, the absolute configuration of the epoxide remained unknown. From mass chromatograms (*m/z* 178) the amounts of (3*Z*,6*Z*,9*Z*)-3,6,9-heptadecatriene (component I) and

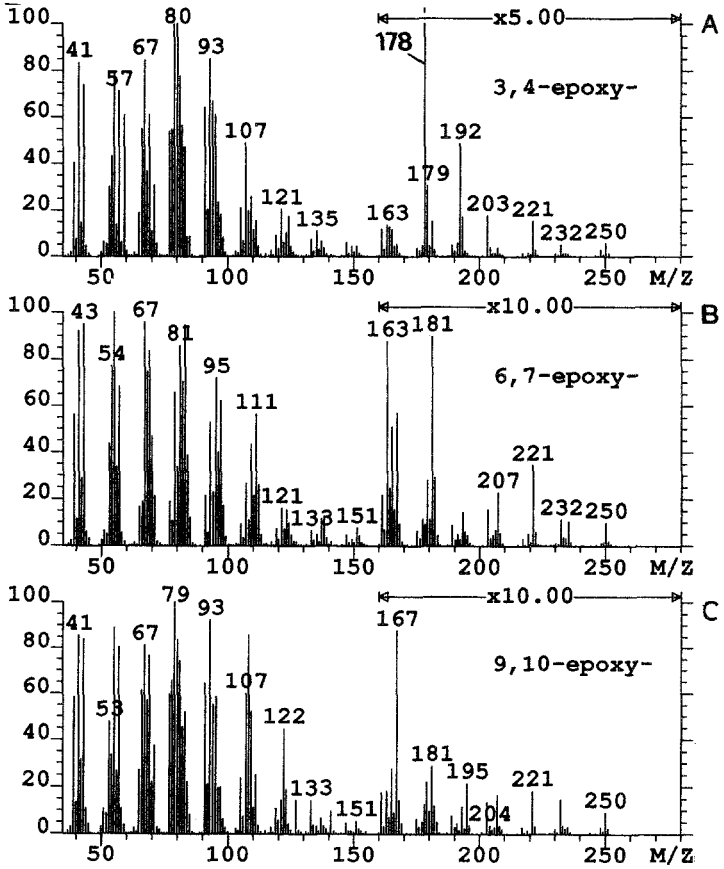

FIG. 3. EI mass spectra of three isomeric monoepoxy analogs (M^+ = 250) of (3*Z*,6*Z*,9*Z*)-3,6,9-heptadecatriene: (A) (6*Z*,9*Z*)-6,9-*cis*-3,4-epoxyheptadecadiene (RI_{SE54} = 1845); (B) (3*Z*,9*Z*)-3,9-*cis*-6,7-epoxyheptadecadiene (RI_{SE54} = 1842); (C) (3*Z*,6*Z*)-3,6-*cis*-9,10-epoxyheptadecadiene (RI_{SE54} = 1850).

(6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene (component II) were determined to be 0.13 and 0.42 ng/FE, respectively.

The mass spectra of the female *A. grossulariata* extract were searched for the presence of the other two monoepoxy analogs. Mass chromatograms showed the elution of a third polyunsaturated component (III) as a trailing shoulder to component II with the correct retention index (RI_{SE54} = 1849) of the (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene. The presence of this epoxide was supported from signals at *m/z* 79, 80, 93, 107, and particularly at *m/z* 108 (see Figure 4); characteristic is the changed intensity ratio for ions *m/z* 107 and 108 of the 3,4- and the 9,10-epoxides (see also Figure 3A,C). The amount of (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene possibly present was estimated at 0.04 ng/FE. This amount was too low to allow confirmation of its presence by higher mass ions (*m/z* 167 and others), and its identification therefore remained tentative. No indication for the presence of (3Z,9Z)-3,9-*cis*-6,7-epoxyheptadecadiene (RI_{SE54} = 1842) was obtained (limit of detection, 0.04 ng/FE).

Electrophysiology. In the EAG tests racemic (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene was found to be highly active on the male antenna (Figure 5). Among the other C_{17} compounds, responses in decreasing order were elicited by racemic (3Z,9Z)-3,9-*cis*-6,7-epoxyheptadecadiene, (3Z,6Z,9Z)-3,6,9-heptadecatriene, and racemic (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene. Responses to the C_{19} compounds showed a parallel trend, with highest response to the 3,4-epoxide and lower responses to the other compounds.

Field Trapping. When the three positional C_{17} epoxides were screened in traps, (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene caught a total of 148, 4, 4, 2, 13, and 8 male *A. grossulariata* at Adony (Fejér County, mixed poplar forest), Aszód (Heves County, mixed oak forest), Budakeszi (Pest County, mixed oak forest), Gyöngyös (Heves County, row of mixed bushes along the edge of an alfalfa field), Gyöngyössolymos (Heves County, mixed oak forest), and Ladánybene (Bács-Kiskun County, juniper and poplar vegetation), respectively, even in habitats where the species was not expected in high numbers (e.g., alfalfa field at Gyöngyös). The highest catches were observed at the flood area of Danube (Adony), so that all other comparative tests were set up at this site.

Racemic (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene was combined with (3Z,6Z,9Z)-3,6,9-heptadecatriene and racemic (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene at the approximate proportions present in the gland extract. At doses of 100 and 10 μg, this mixture caught less than (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene on its own (Figure 6). At lower dosages catches were generally very low but showed a similar trend.

Since the proportions determined in female gland extracts may not reflect optimal proportions for attraction, (3Z,6Z,9Z)-3,6,9-heptadecatriene and (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene were added in varying amounts to

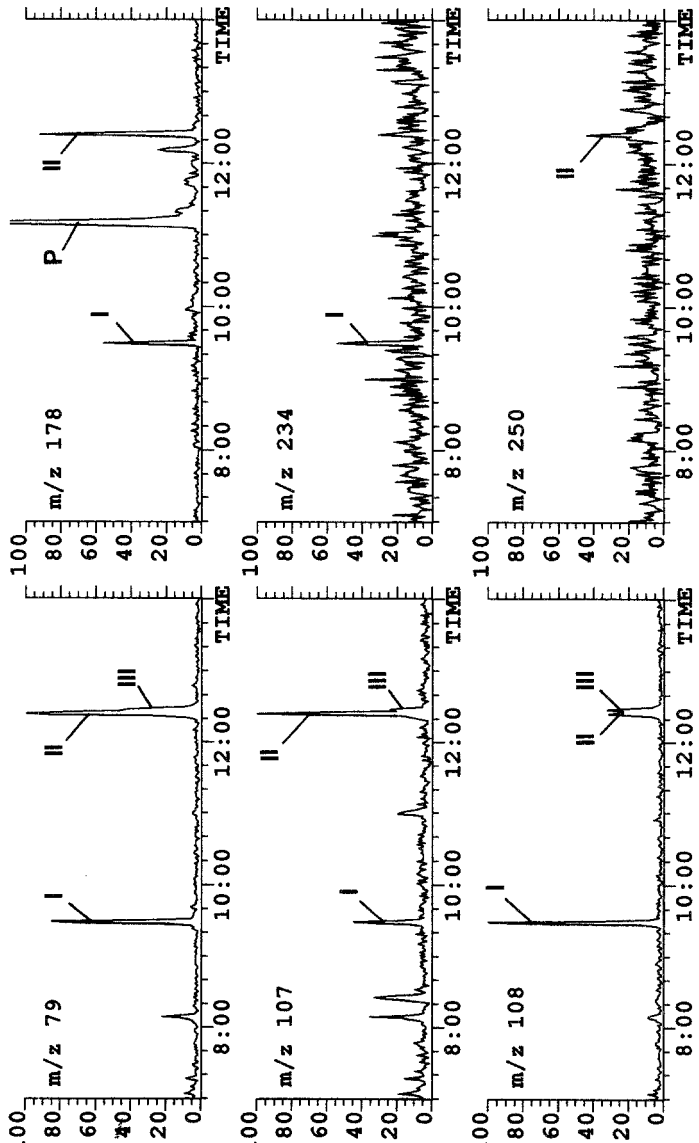

FIG. 4. Mass chromatograms (retention time range 7–14 min) of a female gland extract of *A. grossulariata* showing presence of (3Z,6Z,9Z)-3,6,9-heptadecatriene (I), (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene (II), and a third component, tentatively identified as (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene (III). Also indicated is elution of phenanthrene (P; $M^+ = 178$).

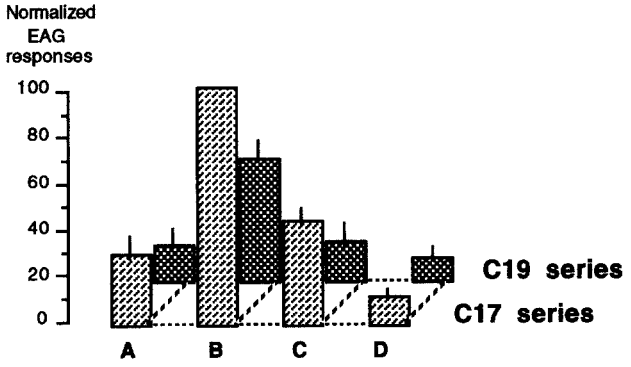

FIG. 5. Average EAG responses of five male *A. grossulariata* antennae to (3Z,6Z,9Z)-3,6,9-heptadeca- and -nonadecatriene, and to their monoepoxy analogs: A = (3Z,6Z,9Z)-3,6,9-hepta- or -nonadecatriene, respectively, B = (6Z,9Z)-6,9-*cis*-3,4-epoxy-, C = (3Z,9Z)-3,9-*cis*-6,7-epoxy-, and D = (3Z,6Z)-3,6-*cis*-9,10-epoxyhepta- and -nonadecadiene, respectively; bars show SD; responses normalized against response to (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene.

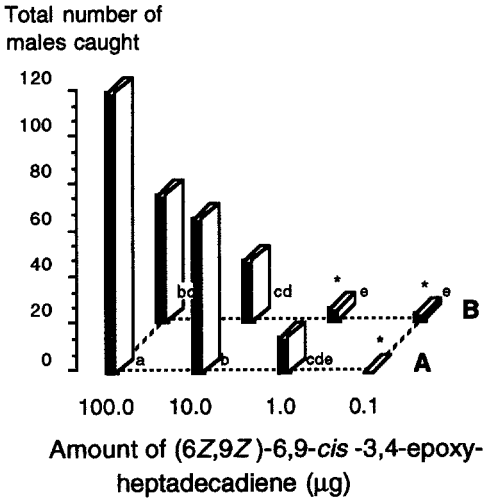

FIG. 6. Total number of male *A. grossulariata* caught in traps baited with racemic (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene alone (A), or in combination (100:10:30) with racemic (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene and (3Z,6Z,9Z)-3,6,9-heptadecatriene (B) at different dose levels. (Adony, Hungary, June 15–25, 1990; 5 traps/bait combination; traps inspected every second day; captures with same letter are not significantly different at $P = 5\%$ by Duncan's NMRT; captures labeled by an asterisk are not significantly different from zero capture by the Wilcoxon-Mann-Whitney test).

(6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene (Figure 7A). The only lure that was slightly more attractive than (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene was a mixture of this compound with 10% of (3Z,6Z,9Z)-3,6,9-heptadecatriene added. A mixture with 30% of hydrocarbon added caught similar numbers of males, whereas the mixture with 100% of hydrocarbon added was almost inactive, indicating a possible antagonism of the hydrocarbon at such a ratio. A similar trend was observed when 1% of (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene was

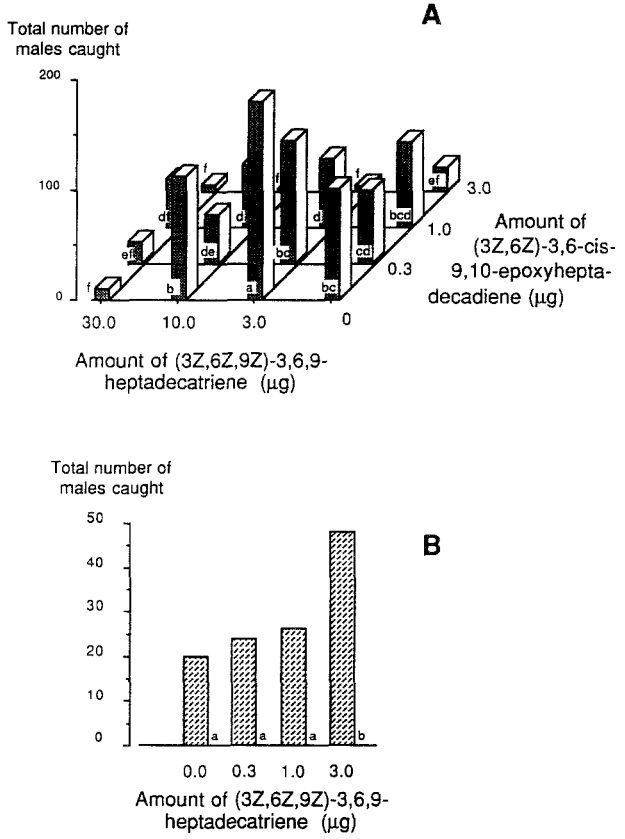

FIG. 7. Total number of male *A. grossulariata* caught at Adony, Hungary, 1990, in traps baited with racemic (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene or with its binary and ternary mixtures with racemic (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene and (3Z,6Z,9Z)-3,6,9-heptadecatriene at different ratios. The basic attractant was 30 μg of (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene in each case. (A) June 21–29; 8 traps/bait combination; (B) June 30–July 13; 10 traps/bait combination; in both tests traps were inspected every second day. Captures with same letter within one test are not significantly different at $P = 5\%$ by Duncan's NMRT.

added to the other two compounds, although here the catch of the blend with 10% of the hydrocarbon was not significantly different from that of the epoxide mixture without the hydrocarbon. At lures with 3–10% of (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene, catches decreased to low values, indicating a possible inhibitory effect of this compound. This effect could also be responsible for the low catches of the mixtures (10% of this epoxide added) observed in the first test (Figure 6).

The increased activity of the mixture of (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene and (3Z,6Z,9Z)-3,6,9-heptadecatriene was confirmed in another test (Figure 7B); the addition of (3Z,6Z,9Z)-3,6,9-heptadecatriene at less than 10% had no effect.

Based on the above chemical data and the biological activity, we conclude that (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene is the main pheromone component of *A. grossulariata*; the absolute configuration of this epoxide remains unknown. The racemic synthetic epoxide is highly active on the male antenna and acts as an attractant in the field. Addition of 10% of (3Z,6Z,9Z)-3,6,9-heptadecatriene, also identified in pheromone gland extracts, enhances the catch. Since the synergistic activity of the triene is not dramatic, the seasonal flight pattern of *A. grossulariata* can be monitored adequately with traps containing racemic (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene on its own. Another epoxide tentatively identified in the female gland, (3Z,6Z)-3,6-*cis*-9,10-epoxyheptadecadiene, did not augment trap catches when added to the main component; at higher amounts added it seemed to be inhibitory.

Unsaturated hydrocarbons with an odd number of carbons in their chain and their oxygenated derivatives form an important group of sex pheromone components of Geometridae. (6Z,9Z)-6,9-*cis*-3,4-Epoxyheptadecadiene and (3Z,6Z,9Z)-3,6,9-heptadecatriene were previously reported as sex pheromone components of *Semiothisa signaria dispuncta* Wlk. (Millar et al., 1987). Their presence was inferred from GC-FID/EAD and field trapping; the presence of the hydrocarbon was confirmed by GC-MS, but the amount of the epoxide ($\sim$ 1 ng/FE) was too low for this confirmation. The same compounds have been identified in the sex pheromone of *Tephrina arenacearia* Hbn. and *Chiasma clathrata* L. (Tóth et al., 1991), and a Chinese *Semiothisa* species (Li et al., 1988). Enantiomers of (6Z,9Z)-6,9-*cis*-3,4-epoxyheptadecadiene were found to be attractive to two *Itame* species and *Epelis truncataria* Wlk. (Millar et al., 1990b). *Lomographa semiclarata* Wlk. was reported to be attracted to (3Z,6Z,9Z)-3,6,9-heptadecatriene (Millar et al., 1990a), and the same compound was tentatively identified in *S. bicolorata* Fabr. and *S. neptaria* Guenée (Millar et al., 1987).

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MANDIBULAR GLAND SECRETIONS OF THE MALE BEEWOLVES *Philanthus crabroniformis*, *P. barbatus*, AND *P. pulcher* (HYMENOPTERA: SPHECIDAE)

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Abstract—The composition of the territorial marking pheromones from mandibular glands of males of the beewolves *Philanthus crabroniformis*, *P. barbatus*, and *P. pulcher* have been determined. The structures of the components were elucidated by gas chromatography–mass spectrometry and gas chromatography–Fourier transform infrared spectroscopy. The major compound of *P. crabroniformis* is isopropyl tetradecanoate, with somewhat lesser amounts of 2-tridecanone, 3-methyl-3-butenyl tetradecanoate, and 92:8 (*Z*):(*E*)-11-eicosen-1-ol. The major compounds of *P. barbatus* are ethyl tetradecanoate and hexadecanal, which are present in approximately a 60:40 ratio. These two compounds comprise over 95% of the neutral lipids. Also present in lesser amounts are ethyl dodecanoate, tetradecanal, hexadecan-1-ol, a Δ^x-octadecen-1-ol, and octadecan-1-ol. The major compounds of *P. pulcher* are ethyl (*Z*)-7-hexadecenoate and geranylgeraniol acetate, which comprise nearly 90% of the neutral lipid fraction, with smaller amounts of tetradecanal, pentadecanal, and ethyl hexadecanoate; trace amounts of Δ^x-hexadecenal, hexadecanal, and octadecanal are also present.

Key Words—*Philanthus crabroniformis*, *Philanthus barbatus*, *Philanthus pulcher*, Hymenoptera, Sphecidae, beewolf, mandibular glands, pheromones, semiochemicals, mass spectrometry, infrared spectroscopy.

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INTRODUCTION

Males of many species of philanthine wasps (Hymenoptera: Sphecidae: Philanthinae) exhibit the unusual wasp behavior of scent-marking territories. These territories are usually in open, sunny areas, often near female nesting sites. After they are scent-marked with mandibular gland pheromones, they are actively defended against conspecific males and other intruders (Simon Thomas and Poorter, 1972; Alcock, 1974, 1975; Gwynne, 1978; O'Neill, 1979, 1983; Borg-Karlson and Tengo, 1980; Evans, 1982; Schmidt et al., 1985). Philanthini scent-marking and territorial behavior is best characterized for the beewolves of the genus *Philanthus*, a large, nearly cosmopolitan group that contains over 130 species. In North America, all but one of the 18 species for which male behavior has been studied scent-mark (Evans and O'Neill, 1988). Scent-marking appears to play a crucial role in the reproductive biology of beewolves and apparently functions both to bring the sexes together and to provide species recognition cues (Schmidt et al., 1985; Evans and O'Neill, 1988).

In our previous investigations of male *Philanthus* mandibular gland chemistry, we reported that two North American species, *P. bicinctus* and *P. basilaris* (both members of the *zebratus* group), possessed idiosyncratic blends of 13- to 20-carbon ketones, alcohols, aldehydes, esters, and fatty acids (Schmidt et al., 1985, McDaniel et al., 1987). The European species, *P. triangulum*, contained a mixture of 18- to 20-carbon lipids that included an aldehyde, a ketone, an alcohol, and an acid (Schmidt et al., 1990). We have now investigated the chemistry of three more *Philanthus* species—*P. crabroniformis* and *P. barbatus* in the *gibbosus* group and *P. pulcher* in the *pacificus* group—in an effort to elucidate further the diversity of mandibular gland compounds synthesized by *Philanthus*.

METHODS AND MATERIALS

Specimens of *Philanthus crabroniformis* were collected July 11, 1989; those of *P. barbatus* were collected July 25, 1988, both in an area approximately 56 km northwest of Fort Collins in Larimer County, Colorado. *Philanthus pulcher* wasps were collected in late June 1989 in the same area. Male wasps were collected while they were on their territories, and three replicates of 8–10 pooled heads and thorax–abdomens of each species were separately placed in methylene chloride (Burdick and Jackson Laboratories, Inc., Muskegon, Michigan[4]). These extracts were separated into hydrocarbon, carboxylic

[4]Mention of a company, trade name, or commercial product is for identification purposes only and does not imply endorsement by the USDA.

acid, and neutral fractions as previously described (Schmidt et al., 1985, 1990; McDaniel et al., 1987).

Most derivatizations were conducted in 5-ml Reactivials (Pierce Chemical Company, Rockford, Illinois) and heated when necessary in a Thermdyne Dri-Bath (Fisher Scientific, Pittsburgh, Pennsylvania). *N,N*-Dimethylhydrazones were prepared as previously described (McDaniel and Howard, 1985).

Double bond positions were determined by mass spectral analysis of dimethyl disulfide (DMDS) derivatives (Scribe et al., 1988, Buser et al., 1983, Dunkelblum et al., 1985, Francis and Veland, 1981).

Pentenyl esters of tetradecanoic (myristic) acid were prepared by reacting ca. 50 mg of myristoyl chloride (Pfaltz and Bauer, Inc., Waterbury, Connecticut) with approximately 200 μl of the appropriate pentenol for ca. 8 hr at 40°C. After reaction, approximately 2 ml of hexane were added, and the mixture was shaken with a 1 M solution of $NaHCO_3$. Microhydrogenation of pentenyl esters of myristic acid was accomplished by placing the esters onto melting-point capillary columns packed with palladium chloride (Pfaltz and Bauer, St. Louis, Missouri) on Celite 545 (Johns-Manville Co., Baltimore, Maryland) and passing a stream of hydrogen through the capillary (Schwartz et al., 1972). The hydrogenated products were then eluted with ca. 20 μl of CS_2.

Acetate esters were prepared by placing microgram amounts of the alcohols in a vial and reacting them with acetic anhydride and pyridine, followed by the customary work-up of the reaction mixture.

Authentic samples of (*E*)- and (*Z*)-11-eicosen-1-ol and (*E*)- and (*Z*)-9-hexadecenoic acid ethyl esters were obtained from Nu-Chek-Prep, Inc., (Elysian, Minnesota).

Gas chromatography–mass spectrometry (GC-MS) analyses were performed on a Hewlett-Packard 5890 gas chromatograph interfaced to a 5970 Mass Selective Detector. The system was connected to a Series 9000-216 computer. The column employed was a 30-m × 0.25-mm DB-5 (J&W Scientific, Inc., Folsom, California) with the following chromatographic parameters: injection port temperature 250°C; transfer line 280°C; oven temperature 50°C initially, then programmed to 150°C at 25°C/min, then programmed to 325°C at 4°C/min. The carrier gas was chromatography-grade helium at a head pressure of 105 kPa. All spectra were obtained at 70 eV.

Relative abundances and Kovats indices of the neutral lipids were obtained from a Shimadzu GC-6AM gas chromatograph equipped with a flame ionization detector and a 15-m × 0.53-mm Megabore DB-1 column (J&W Scientific, Inc.) and a Hewlett-Packard integrator. The chromatographic parameters were: injection port and detector temperatures 275°C; oven temperature 130°C initially, programmed to 275°C at 5°C/min. The carrier gas was chromatography-grade nitrogen at a flow rate of 5 ml/min.

Fourier-transform infrared (FTIR) vapor-phase spectra were obtained on a

Hewlett-Packard 5890 GC with a 5965A FTIR detector and a 7958 data system. The column employed was a 0.32-mm × 30-m DB-5 (J&W Scientific, Inc.) with chromatographic parameters identical to those previously given.

RESULTS

Philanthus crabroniformis males had the least complex mandibular gland composition of any North American species yet examined (Table 1). 2-Tridecanone was readily characterized by mass spectrometry, with a characteristic McLafferty rearrangement ion at *m/z* 58 as the base peak and a molecular ion at *m/z* 198. FTIR showed a carbonyl absorption at 1731 cm^{-1}. The *N,N*-dimethylhydrazone had a mass spectrum consistent with the assigned identity: the ion at *m/z* 100 had a relative abundance of 83%, and the molecular ion at *m/z* 240 had a relative abundance of 42%. Isopropyl tetradecanoate (isopropyl myristate) was the most abundant compound in the mixture. The mass spectrum had a base peak at *m/z* 228 and a small molecular ion (1%) at *m/z* 270. The ion at *m/z* 228 may be interpreted as the molecular ion of myristic acid that arises from proton transfer from the isopropyl group with subsequent loss of neutral propene. FTIR data showed a characteristic C=O at 1747 cm^{-1}, a C—O—C absorption at 1113 cm^{-1}, and other diagnostic bands at 1177 and 1244 cm^{-1}. A computer search of the FTIR library yielded a 99% match with isopropyl myristate. Likewise, the compound had a retention time and mass spectrum identical to that of an authentic sample synthesized as previously described. The 3-methyl-3-buten-1-ol ester of myristic acid, 3-methyl-3-butenyl tetradecanoate, had a base peak at *m/z* 68 and a small molecular ion (2%) at *m/z* 296. There were two other small (<5%) ions at *m/z* 228 (M−68), and at *m/z* 211 (M−85). The *m/z* 68 ion is a C_5 diene fragment that arises through proton transfer to form myristic acid (*m/z* 228). FTIR data included the C=O absorption at 1752 cm^{-1}, bands at 3050 cm^{-1} (=C—H stretch), 897 cm^{-1} (C—H out of plane deformation), and 1653 cm^{-1} (C=C stretch). The compound had a retention time and mass spectrum identical to that of the synthesized ester. In

TABLE 1. MANDIBULAR GLAND NEUTRAL LIPIDS, THEIR RELATIVE ABUNDANCES, AND KOVATS INDICES IN *Philanthus crabroniformis* MALES

Component	Relative abundance (%)	Kovats index
2-Tridecanone	20	1481
Isopropyl tetradecanoate	55	1814
3-Methyl-3-butenyl tetradecanoate	16	2125
11-Eicosen-1-ol	9	2250

addition, microhydrogenation of the compound yielded 3-methylbutyl tetradecanoate (isoamyl myristate), which was identified by comparing the mass spectrum and retention time with that of an authentic synthesized sample. The two 11-eicosen-1-ol isomers had mass spectra consistent with those of alcohols. There is no molecular ion at *m/z* 296, but there is a significant ion (6%) at *m/z* 278 (M−18). FTIR data of the alcohols contained an absorption at 3739 cm^{-1} (OH), 3012 cm^{-1} (=C−H), 1458 cm^{-1} (OH), and at 1039 cm^{-1} (C−OH prim). A computer search of the FTIR library yielded alcohols as the three best matches (98%). The double bond positions were determined by GC-MS of the vicinal dithiomethyl ether derivatives of the acetates. Large α-cleavage fragments at *m/z* 173 and at *m/z* 259 established the double bond at the Δ^{11} position. Similarly, comparison of the mass spectra and retention times of the acetates with the spectra of the synthesized acetates of (*E*)-11-eicosen-1-ol and (*Z*)-11-eicosen-1-ol indicated that the *P. crabroniformis* alcohols consisted of 92% *Z* isomer and 8% *E* isomer.

In *P. barbatus* (Table 2), the neutral lipids of the mandibular gland extract were composed of three compound types: ethyl esters, aldehydes, and alcohols. The ethyl esters, ethyl dodecanoate and ethyl tetradecanoate, were characterized by rearrangement ions at *m/z* 88, which were base peaks; by M−45 ions that corresponded to the loss of an ethoxy group from the parent compound at approximately 10% relative abundance; and by molecular ions that had relative abundances of 8–10%. The mass spectra of the 1,1-dimethylhydrazones of the aldehydes had the rearrangement ions at *m/z* 86 as base peaks and molecular ions at relative abundances of approximately 20%. The alcohols, hexadecan-1-ol, Δ^x-octadecen-1-ol, and octadecan-1-ol, which were minor constituents of the lipid mixture, were identified by the mass spectra of their acetate derivatives. The presence of the *m/z* 61 ion characterized them as acetates, and their

TABLE 2. MANDIBULAR GLAND NEUTRAL LIPIDS, THEIR RELATIVE ABUNDANCES, AND KOVATS INDICES IN *Philanthus barbatus* MALES

Component	Relative abundance (%)	Kovats index
Ethyl dodecanoate	0.2	1573
Tetradecanal	1.5	1584
Pentadecanal	tr[a]	1687
Ethyl tetradecanoate	56.7	1777
Hexadecanal	38.9	1791
Hexadecan-1-ol	0.6	1856
Δ^x-Octadecen-1-ol	0.7	2028
Octadecan-1-ol	0.6	2061

[a]tr = <0.1%.

molecular ions and M−60 ions further established their identities. The octadecen-1-ol was not present in sufficient abundance for the double bond position to be determined. The two compounds ethyl tetradecanoate (ethyl myristate) and hexadecanal, present in approximately a 60:40 ratio, comprised over 95% of the total neutral lipids of the mandibular gland of *P. barbatus*.

In *P. pulcher* (Table 3), as in *P. barbatus*, ethyl esters and aldehydes were found, but the compounds present were different, and they were further differentiated by the presence of the acetate of the diterpene alcohol, geranylgeraniol. The ethyl ester of (*Z*)-7-hexadecenoic acid and geranylgeraniol acetate comprised nearly 90% of the total neutral lipids of the mandibular glands. Mass spectral analysis of the vicinal dithiomethyl ether of ethyl (*Z*)-7-hexadecenoate yielded fragments at *m/z* 173 and at *m/z* 203 that show the double bond position to be at the 7 position. Our evidence for the stereochemistry is somewhat indirect, since it is based on comparison of the retention times of similar compounds, the ethyl esters of (*E*)- and (*Z*)-9-hexadecenoic acid. The ethyl ester from *P. pulcher* eluted very slightly (KI = 1978) before the authentic sample of *Z* ethyl ester (KI = 1987) and appreciably (KI = 2009) before the *E* ethyl ester. We therefore concluded that the configuration is most likely *Z*. The mass spectrum of geranylgeraniol acetate had a base peak at *m/z* 69, which corresponds to the isoprene group and is characteristic for many acyclic terpenoids. FTIR data showed a strong C=O absorption at 1763 cm^{-1} and bands at 1234 cm^{-1} (C−O−C str. vib.) and at 1026 cm^{-1} (*trans* C=C). A computer search of the FTIR library yielded geranyl acetate as the closest match, and a visual comparison of the spectra also showed a very good match. A comparison of the Kovats index of the compound and its hydrolysis product with published values (Ahlquist et al., 1978) yielded acceptable matches. In addition, the mass spec-

TABLE 3. MANDIBULAR GLAND NEUTRAL LIPIDS, THEIR RELATIVE ABUNDANCES, AND KOVATS INDICES IN *Philanthus pulcher* MALES

Component	Relative abundance (%)	Kovats index
Tetradecanal	1.1	1584
Pentadecanal	10.2	1687
Δ^x-Hexadecenal	tr[a]	ND[b]
Hexadecanal	tr	ND
Ethyl 7-hexadecenoate	51.0	1978
Ethyl hexadecanoate	0.8	1983
Octadecanal	tr	ND
Geranylgeraniol acetate	36.5	2282

[a] tr = <0.1%.
[b] ND = not determined; components detected as their dimethylhydrazones.

trum of the hydrolysis product of the compound was quite similar to the published spectrum of geranylgeraniol (Ahlquist et al., 1978). As with *P. barbatus*, the aldehydes were identified by mass spectra of their *N,N*-dimethylhydrazones. The C_{16} aldehydes, in fact, were present in such small amounts that their presence was unnoticed until the mixture was treated with DMH. Their identities were established by the definitive rearrangement ion at *m/z* 86 as their base peaks and their parent ions at *m/z* 280 and *m/z* 282.

DISCUSSION

In this study, we assumed that the lipids of the head extract would be identical to those found when excised mandibular glands were extracted, as had been found with *P. basilaris* and *P. bicinctus* (Schmidt et al., 1985). The territorial marking pheromone of *P. crabroniformis* consists of oxygenated compounds of moderate to low volatility. The most volatile compound, 2-tridecanone is a common insect allomone or pheromone found in termites (*Schedorhinotermes putorius*), notodontid caterpillars (*Heterocampa manteo*), and Hymenoptera, where it is found in a variety of stingless bees, formicine ants, and *Philanthus* wasps (see Blum, 1981, for specific references; Schmidt et al., 1985). Isopropyl tetradecanoate is a minor component distilled from ants, *Iridomyrmex humilis* (Cavill and Houghton, 1974), in the pheromone blend of the dermestid beetle *Dermestes maculatus* (Francke et al., 1979), has been found in the defensive secretion of rove beetles, *Coprophilus striatulus* and *Deleaster dichrous* (Dettner, 1984), and is a minor active component of the southwestern corn borer moth, *Diatraea grandiosella*, sex pheromone (Hedin et al., 1986).

The compound 3-methyl-3-butenyl tetradecanoate is an unusual natural product that previously has been reported only in bees. It made up less than 1% of the total volatiles from the Dufour's gland secretion of the sweat bee *Nomia nevadensis* (Duffield et al., 1982). In the sweat bee this compound functions as part of the macrocyclic lactone-rich blend used to line and waterproof the underground cells in which the larvae are reared. The long-chain fatty alcohol 11-eicosen-1-ol was known previously only from the honey bee *Apis mellifera* (Free et al., 1982) and its predator, the beewolf *P. triangulum* (Schmidt et al., 1990). In *P. crabroniformis*, however, this alcohol is found in not only the *Z* configuration, as in the other two species, but also in the *E* configuration, although in lesser amounts.

The major components (aldehydes and ethyl esters) found in *P. barbatus* are similar to those found in *P. bicinctus*, but the compounds themselves are different (Schmidt et al., 1985). In addition, there is a series of fatty alcohols present as minor components.

The diterpene alcohol ester geranylgeraniol acetate that was found in *P.*

TABLE 4. COMPARISON OF NEUTRAL LIPID COMPONENTS OF MALE MANDIBULAR GLANDS OF *Philanthus* SPECIES

Chemical classifications and compounds	Composition (%)					
	P. barbatus	*P. basilaris*	*P. bicinctus*	*P. crabroniformis*	*P. pulcher*	*P. triangulum*
Alcohols						
Hexadecan-1-ol	0.6					
Δ^{x}-Octadecen-1-ol	0.7					
Octadecan-1-ol	0.6					
(*E*)-11-Eicosen-1-ol				0.7		
(Z)-11-Eicosen-1-ol				8.3		89.5
Aldehydes						
Tetradecanal	1.5				1.1	
Pentadecanal	tr[a]				10.2	
Δ^{x}-Hexadecenal					tr	
Hexadecanal	38.9				tr	
(Z)-9-Heptadecenal		0.1–0.3				
(Z)-9-Octadecenal						
Octadecenal					tr	
(Z)-9-Nonadecenal			0.2–0.7			
Δ^{x}-Eicosenal						tr
Esters						
Ethyl dodecanoate	0.2					
Ethyl tetradecanoate	56.7					
Ethyl (Z)-7-hexadecenoate					51.0	
Ethyl (Z)-9-hexadecenoate			10–33			
Ethyl hexadecanoate			1–11		0.8	
Ethyl $\Delta^{x,y}$-octadecadienoate			3–14			
(+)-Ethyl (Z)-9-octadecenoate						
Isopropyl-tetradecanoate				55.0		
3-Methyl-3-butenyl tetradecanoate				16.0		
Geranylgeraniol acetate					36.5	
Ketones						
2-Tridecanone		0.4	0–0.1	20.0		
2-Pentadecanone		53–54	24–53			
2-Heptadecanone		0.2–0.4	0–3			
10-Nonadecen-2-one						5.2

[a] tr = <0.1%.

pulcher has been identified previously in Dufour's glands of *Formica nigricans*, *F. rufa*, and *F. polyciena*. It also has been found in the male labial gland secretion of bumblebees of the genera *Bombus* and *Psithyrus* (Ahlquist et al., 1978), where it was interpreted to function as a marking and species-isolation pheromone. The other major compound, ethyl 7-hexadecenoate, differs from the ethyl hexadecenoate previously reported in *P. bicinctus* (McDaniel et al., 1987) in the double bond position. Both compounds, however, have the *Z* stereochemical configuration.

The particular components in the pheromone blends of male *P. crabroniformis*, *P. barbatus*, and *P. pulcher* appear to have physical properties well suited to territorial marking pheromones. The fatty alcohols have low ability to wet surfaces and spread, as do the aldehydes and ketones. The esters, however, particularly isopropyl tetradecanoate, possess excellent wetting, solubilizing, and spreading properties, hence their widespread use (50,000 kg annually) in the pharmaceutical, cosmetic, and toiletry industries (Opdyke, 1976). The combined physical properties of the components in these pheromone blends make wetting and spreadable solutions that can be applied to waxy plant surfaces, where they can volatilize.

These species of *Philanthus*, as do several other *Philanthus* species, frequently nest sympatrically in similar habitats at a given location (Evans and O'Neill, 1988). The nearby presence of other related species raises the potential for interbreeding with the usual concomitant deleterious effects. A territorial marking pheromone that contains a species-specific blend can function to maintain species isolation. Our previous investigations of *Philanthus* marking pheromones, in conjunction with those reported here, reveal that for the six analyzed species, each clearly possesses a unique blend of 13- to 20-carbon compounds (Schmidt et al., 1985, 1990; McDaniel et al., 1987). Not only do the individual components vary among the species, but the classes of compounds also vary among the species (Table 4). Specifically, *P. barbatus* contains esters and aldehydes while lacking ketones; *P. basilaris* contains mainly ketones and lacks esters; *P. bicinctus* contains predominantly esters and ketones and lacks alcohols; *P. crabroniformis* contains all three of those classes and lacks aldehydes; *P. pulcher* contains mainly esters and lacks both ketones and alcohols; and, finally, *P. triangulum* contains primarily a single alcohol and lacks esters. Whether these differences are true reflections of evolutionary selection pressure for species isolation remains to be seen.

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ALLELOPATHIC EFFECTS OF WATER EXTRACTS OF *Artemisia princeps* var. *orientalis* ON SELECTED PLANT SPECIES

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Abstract—The allelopathic effects of wormwood plants (*Artemisia princeps* var. *orientalis*) and their possible phytotoxicity on receptor species were investigated. The aqueous extracts of mature leaf, stem, and root of wormwood plants caused significant inhibition in germination and decreased seedling elongation of receptor plants, whereas germination of some species was not inhibited by extracts of stems and roots. Dry weight growth was slightly increased at lower concentrations of the extract, whereas it was proportionally inhibited at higher concentrations. The calorie value of the organic matter in receptor plants measured by bomb calorimeter was reduced proportionally to the extract concentration. However, results with extracts of juvenile leaf did not correlate with inhibition or promotion of elongation and dry weight.

Key Words—*Artemisia princeps* var. *orientalis*, allelopathy, water extract, concentration, germination, growth, juvenile leaf extract.

INTRODUCTION

Plants in the genus *Artemisia* (Anthemideae, Asteraceae) are widely distributed (Mata et al., 1985) and are known to exhibit allelopathic effects. It is a Korean

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custom to uproot wormwood plants in old tomb areas because wormwood retards turf growth. Volatile substances emitted from wormwood plants have special fragrances and the burning smoke of the plants has been used as a mosquito repellent during summer nights. Thus, it may be postulated that wormwood contains phytotoxic substances.

The purpose of the present study was to find evidence of allelopathic characteristics of wormwood plants by evaluating seed germination and seedling growth in various concentrations of water extracts.

METHODS AND MATERIALS

Donor and Receptor Plants. To study the allelopathic potential of wormwood [*Artemisia princeps* var. *orientalis* (Pampan.) Hara], nine receptor plants were selected. The receptor species included *Lactuca sativa* L., *Achyranthes japonica* (Miq.) Nakai, *Artemisia princeps* var. *orientalis* (Pampan.) Hara, *Oenothera odorata* Jacq., *Plantago asiatica* L., *Zoysia japonica* Steud., *Echinochloa crus-galli* (L.) Beauv., *Hordeum vulgare* var. *hexastichon* Aschers., and *Chrysanthemum boreale* Makino.

Germination and Growth Test. Aqueous extracts were made from leaf, stem, and root of the wormwood plant. One liter of distilled water was added to 200 g of each type of explant at 20°C. After 24 hr, each aqueous extract was filtered through a 0.5-mm sieve, and the filtrates were either diluted to 10, 30, 50, and 70% extracts or used as an undiluted 100% extract.

The germination test was carried out in glass Petri dishes (12 cm) on one layer of filter paper wetted with the extracts. Fifty seeds were evenly dispersed in each dish. The control was treated with distilled water instead of an aqueous extract. The Petri dishes were held at 20°C by day and 15°C at night. The experiment extended over a period of 10 days to allow maximum seed germination. The results were determined by counting the number of germinated seeds and measuring the length of seedlings in millimeters.

Seeds of each type of receptor plant were sown in four plastic pots (10 cm) containing vermiculite. After germination was completed and small plants were eliminated, the 10 largest seedlings were grown for four to five weeks. Various concentration extracts (40 ml) were added to each pot every two to three days. The results included determination of the total length of each seedling and its dry weight. After the samples were oven-dried at 80°C to constant weight, dry weight of the seedlings was measured and the caloric content was determined by using a calorimeter (Shimadzu, CA-4). Ratio of germination, elongation, dry weight, and caloric ratios were calculated as suggested by Rho and Kil (1986):

$$\text{Relative germination ratio (RGR)} = \frac{\text{Germination ratio of tested plant}}{\text{Germination ratio of control}} \times 100$$

$$\text{Relative elongation ratio (RER)} = \frac{\text{Mean length of tested plant}}{\text{Mean length of control}} \times 100$$

$$\text{Relative dry weight ratio (RWR)} = \frac{\text{Dry weight of tested plant}}{\text{Dry weight of control}} \times 100$$

$$\text{Relative caloric ratio (RCR)} = \frac{\text{Calorie of tested plant}}{\text{Calorie of control}} \times 100$$

To test allelopathic effects of juvenile leaf of wormwood, sprouting wormwood leaves were collected in April 1990 and used in experiments as described above.

Osmotic potentials below 143 mosm/kg had no influence (Elakovich and Wooten, 1991); the extracts in this study were diluted to osmotic potentials no greater than 100 mosm.

The results of the bioassays were based on three or four replicates. Comparisons between treatments were made at the 5% level using Duncan's multiple-range test.

RESULTS

The effects of wormwood aqueous extract on receptor plants are shown in Tables 1–6. The germination percentages of *L. sativa*, *A. japonica*, and *A. princeps* var. *orientalis* were reduced at increasing concentrations of wormwood leaf extract. Germination of *P. asiatica* and *Z. japonica* also was inhibited at lower concentrations but germination did not occur at concentrations higher than 70%. RGR of *O. odorata* was little influenced at concentrations below 70% extract (RGR > 90%), but RGR was 48.3% at 100% extract. RGR of *E. crus-galli* was above 96.0% at all concentrations. In the case of *H. vulgare* var. *hexastichon*, germination percentage was similar to the control at 10, 30, and 50% extract but inhibition occurred at 70% and 100%.

Germination reduction of receptor plants by the stem extract was divided into three types. First, the higher the concentration of extract, the lower the germination percentage in *H. vulgare* var. *hexastichon*, *A. japonica*, and *Z. japonica*. Second, the germination percentage decreased in the test treatments compared to the control, and it was concentration-independent. Third, germination percentage was almost the same in all the test treatments.

In germination tests with root aqueous extracts, the germination percentage of *A. japonica* and *C. boreale* was lowered by increasing the extract concentration and showed significant differences between treatments. However, germination percentage of *L. sativa*, *H. vulgare* var. *hexastichon*, and *Z. japonica*

TABLE 1. GERMINATION PERCENTAGE OF RECEPTOR PLANTS SOWN IN PETRI DISHES AT DIFFERENT CONCENTRATIONS OF WORMWOOD LEAF EXTRACT[a]

Species	Control	Concentration (%)				
		10	30	50	70	100
Lactuca sativa	87.5 a	81.5 ab	67.5 bc	41.5 de	55.5 cd	38.5 e
Hordeum vulgare var. *hexastichon*	55.5 a	51.5 a	52.0 a	53.0 a	38.0 b	22.5 c
Achyranthes japonica	69.0 a	45.0 b	18.8 c	21.0 c	10.5 c	11.0 c
Oenothera odorata	74.5 a	73.5 a	71.5 a	77.5 a	72.5 a	36.0 b
Chrysanthemum boreale	80.0 a	65.5 a	36.0 b	32.5 b	19.5 bc	8.0 c
Echinochloa crus-galli	88.5 a	87.5 a	89.5 a	85.5 a	85.0 a	86.5 a
Plantago asiatica	95.3 a	90.5 a	83.5 a	66.5 b	0.0 c	0.0 c
Artemisia princeps var. *orientalis*	93.5 a	76.0 b	60.5 c	39.5 d	36.5 d	34.5 d
Zoysia japonica	31.5 a	16.0 b	3.0 c	3.0 c	0.0 c	0.0 c

[a] Values in rows having the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

TABLE 2. GERMINATION PERCENTAGE RECEPTOR PLANTS SOWN IN PETRI DISHES AT DIFFERENT CONCENTRATIONS OF WORMWOOD STEM EXTRACT[a]

Species	Control	Concentration (%)				
		10	30	50	70	100
Lactuca sativa	94.5 a	80.5 b	81.3 b	72.0 b	81.3 b	79.0 b
Hordeum vulgare var. *hexastichon*	55.5 a	50.0 a	42.0 ab	33.0 bc	23.0 c	21.0 c
Achyranthes japonica	71.5 a	56.5 b	55.0 b	37.0 c	24.0 d	24.0 d
Oenothera odorata	78.0 a	74.0 a	79.0 a	71.5 a	71.5 a	72.0 a
Chrysanthemum boreale	70.0 a	75.5 a	70.5 ab	59.0 b	40.5 c	28.5 c
Echinochloa crus-galli	90.0 a	84.5 a	88.5 a	89.3 a	84.8 a	87.3 a
Plantago asiatica	83.0 a	76.0 a	74.5 a	40.5 b	58.0 ab	36.0 b
Artemisia princeps var. *orientalis*	80.5 a	81.5 a	77.5 a	75.5 a	68.0 a	70.0 a
Zoysia japonica	28.5 a	22.0 abc	23.5 ab	15.0 bc	14.0 bc	13.0 c

[a] Values in rows having the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

TABLE 3. GERMINATION PERCENTAGE OF RECEPTOR PLANTS SOWN IN PETRI DISHES AT DIFFERENT CONCENTRATIONS OF WORMWOOD ROOT EXTRACT[a]

Species	Control	Concentration (%)				
		10	30	50	70	100
Lactuca sativa	87.5 a	81.5 a	80.5 a	83.0 a	82.0 a	77.5 a
Hordeum vulgare var. *hexastichon*	57.5 a	48.0 a	48.5 a	50.5 a	52.0 a	50.0 a
Achyranthes japonica	71.5 a	56.5 b	55.0 b	37.0 c	24.0 d	24.0 d
Oenothera adorata	71.5 ab	68.0 abc	62.5 bc	72.0 a	63.5 abc	60.0 c
Chrysanthemum boreale	84.5 a	69.0 ab	63.0 bc	48.0 c	52.0 bc	52.0 bc
Echinochloa crus-galli	85.0 a	86.5 a	87.0 a	84.5 a	91.0 a	86.5 a
Plantago asiatica	90.5 a	76.5 ab	47.0 a	48.5 b	61.5 ab	50.5 b
Artemisia princeps var. *orientalis*	94.0 a	88.0 ab	87.5 ab	80.5 ab	82.5 ab	78.5 b
Zoysia japonica	27.5 a	18.5 a	19.3 a	22.0 a	25.5 a	22.8 a

[a] Values in rows having the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

TABLE 4. SEEDLING ELONGATION (mm) OF RECPTOR PLANTS GROWN IN PETRI DISHES AT DIFFERENT CONCENTRATIONS OF WORMWOOD LEAF EXTRACT[a]

Species	Control	Concentration (%)				
		10	30	50	70	100
Lactuca sativa	82.0 a	88.8 a	67.1 b	37.3 b	58.2 b	39.4 c
Hordeum vulgare var. *hexastichon*	57.9 a	54.7 ab	39.9 abc	30.4 bc	29.9 bc	18.6 c
Achyranthes japonica	55.2 a	34.7 b	34.9 b	35.7 b	30.0 bc	18.0 c
Oenothera odorata	23.8 a	23.0 a	20.9 a	18.0 ab	11.7 bc	10.2 c
Chrysanthemum boreale	23.0 a	17.3 b	13.7 bc	10.6 c	9.8 c	9.7 c
Echinochloa crus-galli	55.4 a	67.4 a	60.6 a	53.9 a	48.8 ab	28.0 b
Plantago asiatica	32.1 a	27.2 ab	23.0 bc	17.9 c	0.0 d	0.0 d
Artemisia princeps var. *orientalis*	14.2 a	11.8 b	8.0 c	7.0 c	5.6 d	5.3 d
Zoysia japonica	50.3 a	13.5 b	4.8 c	4.8 c	0.0 c	0.0 c

[a] Values in rows having the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

TABLE 5. SEEDLING ELONGATION (mm) OF RECEPTOR PLANTS GROWN IN PETRI DISHES AT DIFFERENT CONCENTRATIONS OF WORMWOOD STEM EXTRACT[a]

Species	Control	Concentration (%)				
		10	30	50	70	100
Lactuca sativa	76.4 a	85.0 a	79.8 a	76.5 a	83.5 a	67.3 b
Hordeum vulgare var. *hexastichon*	67.6 a	57.9 a	43.8 bcd	38.1 bcd	26.1 cd	18.8 d
Achyranthes japonica	47.8 a	44.7 a	34.9 bc	35.9 bc	31.3 c	28.5 c
Oenothera odorata	25.9 a	27.5 a	27.0 a	24.3 a	22.6 a	20.0 a
Chrysanthemum boreale	23.5 a	19.1 b	17.7 bc	15.0 bcd	13.9 cd	11.3 d
Echinochloa crus-galli	58.9 ab	73.6 a	61.3 ab	48.8 b	50.5 b	49.6 b
Plantago asiatica	15.8 b	22.4 a	17.2 ab	11.0 b	16.1 b	14.5 b
Artemisia princeps var. *orientalis*	13.5 a	13.5 a	12.3 a	12.4 a	11.5 a	9.1 b
Zoysia japonica	50.6 a	38.4 b	15.0 c	13.4 c	10.3 c	11.7 c

[a] Values in rows having the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

TABLE 6. SEEDLING ELONGATION (mm) OF RECEPTOR PLANTS GROWN IN PETRI DISHES AT DIFFERENT CONCENTRATIONS OF WORMWOOD ROOT EXTRACT[a]

Species	Control	Concentration (%)				
		10	30	50	70	100
Lactuca sativa	80.3 b	88.7 ab	92.8 a	93.3 a	95.6 a	89.0 ab
Hordeum vulgare var. *hexastichon*	64.0 a	65.1 a	55.0 a	53.0 a	48.1 a	43.2 a
Achyranthes japonica	49.0 a	49.1 a	44.2 a	42.8 a	32.0 b	35.7 b
Oenothera odorata	23.8 a	27.8 a	27.0 a	28.1 a	28.1 a	24.9 a
Chrysanthemum boreale	20.8 a	18.2 a	14.4 b	13.0 b	14.6 b	13.5 b
Echinochloa crus-galli	60.9 ab	67.0 ab	69.2 a	63.8 ab	58.9 ab	54.4 b
Plantago asiatica	18.3 ab	23.7 a	18.2 ab	14.7 b	14.6 b	16.9 b
Artemisia princeps var. *orientalis*	14.3 a	13.6 ab	14.6 a	13.8 ab	12.7 bc	11.4 c
Zoysia japonica	44.9 a	41.8 a	33.0 a	30.9 a	32.2 a	33.8 a

[a] Values in rows having the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

were similar to that of the control, and *E. crus-galli* and *P. asiatica* showed uneven germination percentage with increased extract concentration.

Seedling elongation of *L. sativa* was promoted slightly at 10% leaf extract but inhibited at concentrations higher than 30%. RER of *E. crus-galli* was more than doubled at 100% than in the 30% extract, whereas higher reduction in elongation at other concentrations was remarkable. The elongation of other plants was inversely proportional to concentration of the leaf extract at the 5% level.

The seedling elongation of plants such as *L. sativa*, *O. odorata*, *E. crus-galli*, and *P. asiatica* was promoted at low concentrations of the stem extracts, whereas the others were inhibited at high concentrations. *H. vulgare* var. *hexastichon*, *A. japonica*, *C. boreale*, *A. princeps* var. *orientalis*, and *Z. japonica* were inhibited evenly as the extract concentration was increased.

As shown in Figure 1, elongation of *L. sativa* by leaf extract was by far lower in the test treatment than in the control, and there was no growth at the

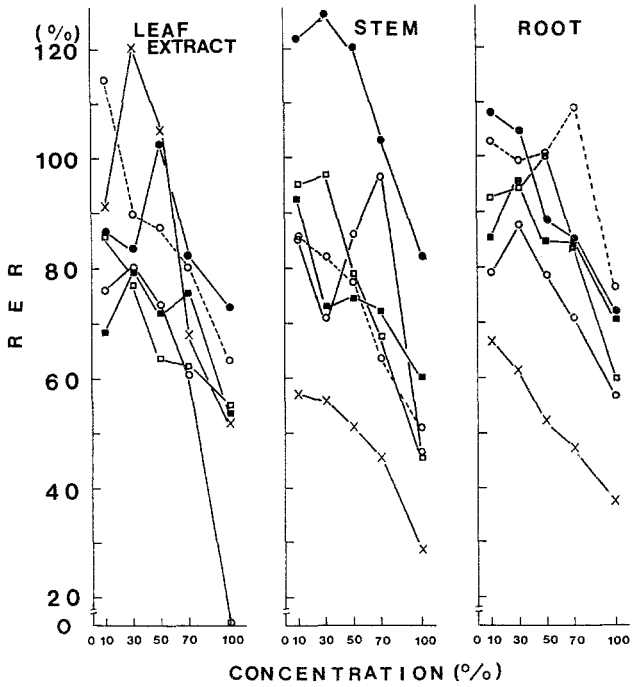

FIG. 1. Relative elongation ratio (RER) of receptor plants grown in pots at different concentrations of wormwood extracts. ○ —— ○, *Lactuca sativa;* × —— ×, *Achyranthes japonica;* □ —— □, *Artemisia princeps* var. *orientalis;* ● —— ●, *Oenothera odorata;* ○ ---- ○, *Plantago asiatica;* ■ —— ■, *Echinochloa crus-galli.*

100% extract. In the case of *A. japonica*, RER were 120.8% and 105.2% in 30% and 50% extracts, respectively, but decreased at 70% and higher extracts. RER of the wormwood plant itself was comparatively low regardless of the extract concentration and was 55.2% at 100% extract. The elongation rate of receptor plants, except in some species, was in inverse proportion to the extract concentration.

RWR of seedlings grown with extracts of leaf, stem, and root are shown in Figure 2. Dry weight reduction of *L. sativa*, *A. princeps* var. *orientalis*, and *P. asiatica*, which was grown with leaf extract, was inversely proportional to the extract concentration, but the dry weight of *A. japonica* was somewhat increased at 10%, 30%, and 50% extracts and reduced at concentrations over 70%. This tendency of dry weight changes was found in other receptor species.

The extracts of wormwood stem and root also induced lower dry weights in receptor plants.

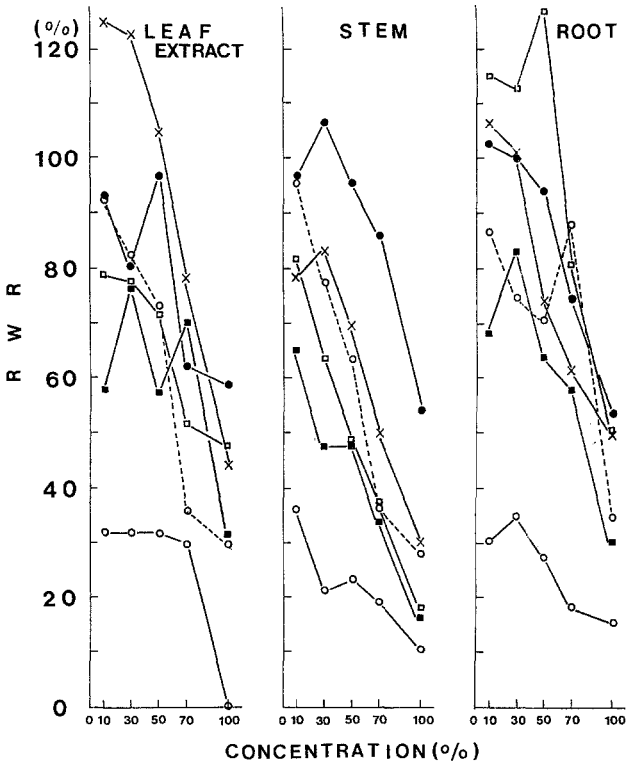

FIG. 2. Relative dry weight ratio (RWR) of receptor plants grown in pots at different concentrations of wormwood extracts. Key to species as in Figure 1.

The calorie measurement of *A. japonica* grown at different concentrations of wormwood leaf, stem, and root extracts demonstrated that remarkable growth inhibition occurred at higher concentrations of the three extracts, but there was a slight growth promotion at lower concentrations of root extract (Figure 3).

In Table 7 are shown the germination rates of receptor species by extracts of juvenile wormwood leaf, which were quite different from extracts of mature wormwood leaf; germination of *L. sativa* was somewhat promoted at 50% and 70% extracts over the control, and the germination percentages at 10%, 30%,

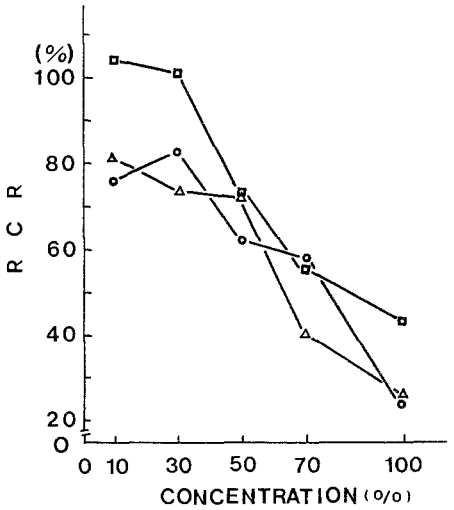

FIG. 3. Relative calorie ratio (RCR) of *Achyranthes japonica* grown in pots at different concentrations of wormwood extracts. ○ —— ○, leaf extract; △ —— △, stem extract; □ —— □, root extract.

TABLE 7. GERMINATION PERCENTAGE OF RECEPTOR PLANTS SOWN IN PETRI DISHES AT DIFFERENT CONCENTRATIONS OF JUVENILE WORMWOOD LEAF EXTRACT[a]

Species	Control	Concentration (%)				
		10	30	50	70	100
Lactuca sativa	59.5 a	59.5 a	56.0 a	64.0 a	67.0 a	58.5 a
Achyranthes japonic	94.5 a	87.5 a	72.0 b	58.0 c	50.0 c	27.5 c
Artemisia princeps var. *orientalis*	76.0 a	59.0 b	67.0 b	65.0 b	63.5 b	66.0 b

[a] Values in rows having the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

and 100% extracts were similar to those of the control. In the case of *A. japonica*, germination percentage was considerably decreased with increasing concentration. As to *A. princeps* var. *orientalis*, germination percentage was the lowest at 10% extracts and almost the same at extracts over 30%. In contrast, seedling length of receptor plants grown in Petri dishes with juvenile leaf extracts was less inhibited, in general, than those with mature leaf extract (Table 8).

Seedling growth of all the receptor plants with extract of juvenile leaf was far greater in the test treatment than in the control. The higher the extract concentration, the greater the elongation rate of the receptor plant (Figure 4). All

TABLE 8. SEEDLING ELONGATION (mm) OF RECEPTOR PLANTS GROWN IN PETRI DISHES AT DIFFERENT CONCENTRATIONS OF JUVENILE WORMWOOD LEAF EXTRACT[a]

Species	Control	Concentration (%)				
		10	30	50	70	100
Lactuca sativa	63.1 b	72.7 a	62.7 b	58.0 bc	51.7 c	44.1 d
Achyranthes japonica	63.8 a	64.0 a	55.2 b	41.3 c	43.8 c	26.8 d
Artemisia princeps var. *orientalis*	13.9 a	13.0 a	13.0 a	10.5 b	10.4 b	9.9 b

[a]Values in rows having the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

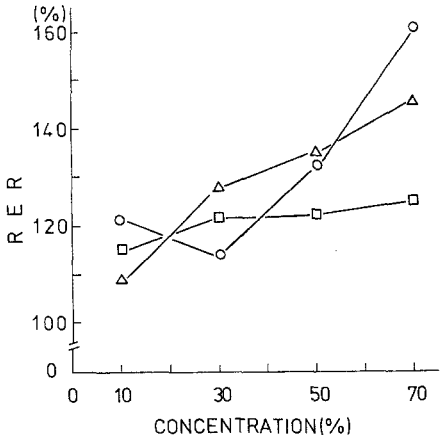

FIG. 4. Relative elongation ratio (RER) of receptor plants grown in pots at different concentrations of juvenile wormwood leaf extract. ○ —— ○, *Lactuca sativa;* △ —— △, *Achyranthes japonica;* □ —— □, *Artemisia princeps* var. *orientalis*.

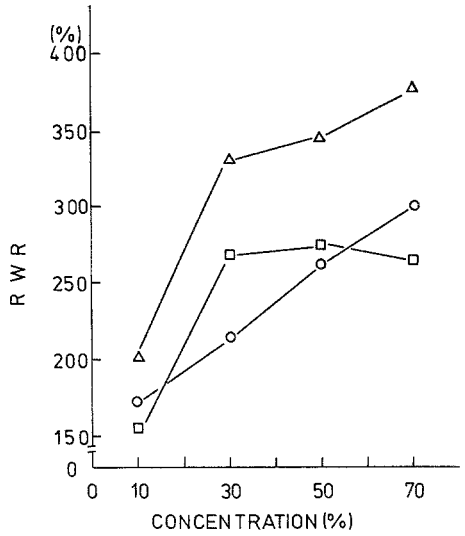

FIG. 5. Relative dry weight ratio (RWR) of receptor plants grown in pots at different concentrations of juvenile wormwood leaf extract. Key to species as in Figure 4.

receptor plants also showed a rapid increase in dry weight: *L. sativa* and *A. japonica* at 70% extract showed more than three times that of the control (Figure 5).

DISCUSSION

Allelopathy has been claimed to exist between numerous species. Data presented here suggest the toxic potential of *Artemisia princeps* var. *orientalis* against some species.

Phytotoxic extracts from leaf, stem, and root of wormwood were studied using 10 receptor plants. Germination and seedling elongation were significantly inhibited by the extracts. Rates of inhibition were increased with increasing extract concentration. Jobidon (1986) described extract solutions prepared by soaking 10 g of leaves in 100 ml of distilled water for 24 hr at 20°C; the initial solution was termed 10%. In this study, water extracts were prepared by soaking 200 g of leaf, stem, and root of wormwood in 1000 ml of distilled water for 24 hr at 20°C and the undiluted solution was termed 100%.

Grant (1964) pointed out that the extracts of four legumes and four grasses affected the germination, root growth, and shoot growth of the same species and found that extracts of aerial portions of the plants had greater inhibitory effects than root extracts. Bieber and Hoveland (1968) found that extracts from aerial portions of six crop and four weed species were more inhibitory than

those from roots. Ballester *et al.* (1979) noted that the strongest effect of the *Erica* plants was produced by flower extracts, followed by stem and leaf extracts. Smith (1989) stated that leaf extracts of bitter sneezeweed were more phytotoxic than either stem or root extracts, and seedling growth of alfalfa and Italian ryegrass was reduced more than seed germination, results that are very similar to those of the present studies. Based on the effects of aqueous extract, wormwood plant tissues inhibited the germination and growth of tested species, and the response was dependent upon the source of extract, the extract concentration, and the species tested.

There seemed to be species specificity in RGR and RER of receptor plants tested in leaf, stem, and root extracts of the wormwood plant. At 10% leaf extract (Tables 1 and 4) several species were slightly affected by the extracts but *Z. japonica* was heavily inhibitory, while many receptor species, except *E. crus-galli*, were almost suppressed in 100% leaf extracts.

It is likely that the tested species could be classified into three types according to the sensitivity of receptor plants to the wormwood toxicity. The first group, the susceptible species, showed low germination and seedling growth in wormwood extracts. The second group, the nonsusceptible species, showed comparatively high germination and seedling growth in the extracts. The third group, the intermediate species, showed moderate germination and seedling growth in the extracts. The susceptible species, for example, *A. japonica*, *Z. japonica*, *P. asiatica*, *H. vulgare* var. *hexastichon*, and *C. boreale*, grow well outside wormwood stands, including farm land, but are so sensitive to the toxic substances emitted from the wormwood plants that they are rarely observed in wormwood fields. The nonsusceptible species, such as *O. odorata*, *E. crus-galli*, *L. sativa*, and *A. princeps* var. *orientalis*, have grown well either in or outside wormwood stands, and they are less influenced than the susceptible species by the toxic substances. Rice (1984) indicated that some pioneer weed stages produce substances inhibitory to their own seedlings and seedlings of other species in that stage, but not to next stage species, *Aristida oligantha*. He hypothesized, therefore, that several species of pioneer stage eliminate the species of that next stage and that *A. oligantha* invades after that because it is not inhibited by the substances that are toxic to the pioneer species.

Park (1966) has described that the early stage of grassland succession in Korea would be composed of *P. asiatica*, *A. priceps* var. *orientalis*, *O. odorata*, and *Z. japonica* community, in that order. However, seed germination and seedling growth of *Z. japonica* was inhibited greatly by the wormwood extracts in this study. These contrasting views could result from differences between laboratory work and field observations.

Wormwood plants showed autotoxicity in bioassays with water extracts. Many plants exhibit such autotoxicity: *Kochia scoparia* (Lodhi, 1979), cuc-

umber seed (Lockerman and Putnam, 1981), *Parthenium hysterophorus* (Picman and Picman, 1984; Kumari and Kohli, 1987).

Unlike the mature leaf extract, juvenile leaf extract inhibited germination but promoted the elongation and dry weight of lettuce and wormwood. The growth of these species was proportional to the extract concentration. Edward et al. (1988) reported that juvenile leaves of the pokeweed plant seemed to lack extractable compounds inhibitory to seed germination, unlike mature leaves.

We plan to continue our search for other factors for the allelopathic potential of *Artemisia princeps* var. *orientalis*.

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(Z)-10-HEPTADECEN-2-ONE AND OTHER 2-KETONES IN THE AGGREGATION PHEROMONE BLEND OF *Drosophila martensis*, *D. buzzatii*, AND *D. serido*

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Abstract—(Z)-10-Heptadecen-2-one was identified as the major aggregation pheromone component in *Drosophila martensis*, *D. buzzatii*, and *D. serido*. Aliphatic 2-ketones were not detected in newly eclosed males or in virgin females of any age. The amount of (Z)-10-heptadecen-2-one in the ejaculatory bulb and on the cuticle of males increased with age. Only trace quantities of (Z)-10-heptadecen-2-one were transferred to the female during mating. 2-Tridecanone was found in the ejaculatory bulb and on the cuticle of male *D. martensis* and *D. buzzatii*, but not *D. serido*. The amount of 2-tridecanone increased with age. Unexpectedly, 2-tridecanone inhibited the aggregation activity of (Z)-10-heptadecen-2-one in *D. buzzatii*. In *D. martensis*, 2-tridecanone was significantly less attractive than controls but did not decrease the attractiveness of one fly equivalent of (Z)-10-heptadecen-2-one. *D. serido* males did not have any 2-tridecanone and the activity of (Z)-10-heptadecen-2-one was not diminished in *D. serido* by the presence of 2-tridecanone.

Key Words—*Drosophila martensis*, *D. buzzatii*, *D. serido*, Diptera, Drosophilidae, (Z)-10-heptadecen-2-one, 2-tridecanone, aggregation pheromone.

INTRODUCTION

Aliphatic unsaturated and saturated 2-ketones in combination with a variety of other compounds compose the aggregation pheromone blend in a number of *Drosophila* species. In the *repleta* group, *Drosophila hydei* (*hydei* subgroup) (Moats et al., 1987) and *D. repleta* (*repleta* subgroup) (Schaner and Jackson,

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unpublished) produce 2-tridecanone that is highly synergistic with a mixture of tiglate esters to make the aggregation pheromone blend. *D. mulleri* (*mulleri* subgroup) were attracted to (*Z*)-10-heptadecen-2-one [abbreviated (*Z*)-10-H], which was synergistic with the coattractant (*S*)-2-tridecanol acetate (Bartelt et al., 1989). *D. busckii* (*Dorsilopha* subgenus) employed 2-pentadecanone, which was synergistic with (*S*)-2-pentadecanol acetate (Schaner et al., 1989d).

Here we identify and characterize similar, but slightly different blends of unsaturated and saturated 2-ketones as aggregation pheromone components of *D. martensis*, *D. buzzatii*, and *D. serido*.

METHODS AND MATERIALS

Flies, Extraction, Chromatography, and Bioassay. Drosophila martensis (strain 15081-1321), *D. buzzatii* (strain 15081-1291), and *D. serido* (strain 15081-1431) were obtained from the National *Drosophila* Species Resource Center at Bowling Green, Ohio. *D. buzzatii* were reared on Instant *Drosophila* Medium Formula 4-24 (Carolina Biological Supply Co., Burlington, North Carolina) and *D. martensis* and *D. serido* were reared on banana–cactus medium (Yoon, 1985). Details of rearing, extraction, chromatography, and bioassay of the flies have been described previously (Bartelt and Jackson, 1984). Briefly, flies were separated by sex when 0–12 hr old, aged in 3.5-cm-diameter × 10-cm food vials to 6–8 days, and extracted at that time by soaking the flies in hexane at room temperature for 24 hr. The hexane extracts of males were fractionated on open columns of silica gel (100–200 mesh; Bio-Rad), eluted with hexane, 5% ether in hexane, 10% ether in hexane, 50% ether in hexane, and 10% methanol in methylene chloride.

Gas chromatography (GC) was conducted on a 15-m × 0.53-mm-ID Megabore DB-1 (J & W Scientific) capillary column, and on a 50-m × 0.2-mm ID HP-1 narrowbore capillary column (Hewlett-Packard) in a Varian 3700 gas chromatograph. Electron impact mass spectra were obtained on a VG-MM16 mass spectrometer using a 30-m × 0.25-mm-ID DB-5 capillary GC column for introduction of the sample.

Bioassays were conducted in a wind-tunnel olfactometer containing ca. 1000 flies (1–4 days old) that had been without food and water for approximately 16 hr. An extract, fraction, synthetic compound, or control solvent was applied to a filter paper strip inserted around the lip of a glass vial. Each bioassay test consisted of placing two differently treated vials, to be compared, into the olfactometer for 3 min. After 3 min, the vials were capped and removed, and the captured flies were sexed and counted. Tests were separated by 7–10 min, and as many as 18 tests could be run in a single day after stocking the olfactometer once. All experiments consisted of more than two treatments, and

these were tested in pairs, in all possible combinations (a randomized, balanced incomplete block design). The bioassay data were transformed to the log $(X + 1)$ scale before analysis to stabilize variance, and analysis was done by the method of Yates (1940).

Inclusion of some "food odor" in all treatments in the bioassay does not greatly increase the fly catch in the control, but does increase the fly catch in the pheromone-containing assays (Schaner et al., 1989a). For *D. martensis* assays, 10 μl of 0.1% acetic acid in hexane were added to all treatments as a coattractant. Since *D. serido* and *D. buzzatii* utilize *Opuntia* for feeding and breeding sites (Richardson, 1982), an extract of the fruit from *Opuntia* was prepared by extracting 100 ml of blended fruit with 50 ml of methylene chloride for 2 hr, and 10 μl of *Opuntia* fruit extract were added to all treatments for *D. serido* and *D. buzzatii*.

Synthetic 2-ketones were prepared as previously described (Moats et al., 1987; Bartelt et al., 1989).

Production, Transfer, and Dispersal of Pheromone Components. The methods were fully described previously (Schaner et al., 1989a,d). Briefly, ejaculatory bulbs were removed from males at various ages and extracted in hexane for 2 hr ($N = 3$ sets of 3). A cuticular extract of the remainder of the fly was obtained by soaking the fly in 40 μl of hexane for 5 min. Both the ejaculatory bulbs and cuticular extracts were analyzed by GC relative to an internal standard of nonadecane. The transfer of pheromone component during mating was quantitated by removing reproductive tracts of females and ejaculatory bulbs of males immediately after mating, followed by a 5-min cuticular soak of the fly ($N = 3$ sets of 5). To determine if males were capable of emitting the pheromone component, virgin males (8–9 days old) were placed for 18 hr in 8-ml vials fitted with wire mesh caps ($N = 3$ sets of 5). The vials were then extracted with 300 μl of hexane and analyzed by GC relative to an internal standard of nonadecane.

RESULTS

Bioassay of Crude Extracts and Fractions. For all three species, the crude extract of mature males was clearly active in bioassay (Table 1) and attracted flies of both sexes. The crude extract of mature females has little if any activity. After fractionation of the male-derived crude extracts on silica gel, the 10% ether–hexane fractions were substantially more active than controls (Table 1). GC of the 10% ether–hexane fractions from all three species revealed one common peak with the retention times on GC of synthetic (*Z*)-10-H (Bartelt et al., 1989).

Ketone Components. The common GC peak to all three male-derived 10%

TABLE 1. BIOASSAY RESPONSE TO FRACTIONS OF MALE EXTRACTS AND FEMALE CRUDE EXTRACTS FOR THREE SPECIES OF *D. martensis* COMPLEX

Extracts or fractions[a]	Relative response[b] ($N \geq 8$)		
	D. martensis	*D. buzzatii*	*D. serido*
Crude extract of males	100***[c]	100***	100***
Hexane eluant	7	−3	0
	(1.8, 2.4, 10.0)[d]	(0.9, 0.7, 8.8)	(0.6, 0.6, 11.8)
5% ether–hexane	16	0	22*
	(1.7, 3.8, 15.1)	(1.7, 1.5, 11.5)	(1.2, 4.0, 14.0)
10% ether–hexane	101***	47***	65***
	(2.3, 9.7, 9.6)	(1.5, 7.2, 13.6)	(1.2, 5.5, 7.8)
50% ether–hexane	6	2	−16
	(1.9, 2.7, 15.5)	(0.9, 1.2, 19.3)	(2.2, 0.6, 12.5)
10% MeOH–CH_2Cl_2	11	8	8
	(4.0, 9.2, 14.8)	(1.3, 7.5, 16.4)	(0.9, 1.9, 13.0)
Crude extract of females	18	14	5
	(1.0, 2.8, 11.1)	(1.3, 2.8, 12.2)	(0.5, 1.0, 11.5)

[a] All extracts and fractions were used at one hexane extractable fly equivalent per test.
[b] Relative response = (fraction − control)/(crude extract of males − control) × 100.
[c] * and *** differ from control at $P < 0.05$ and $P < 0.001$ (LSD).
[d] Numbers in parentheses are mean bioassay catches for solvent control, treatment, and crude extract of males, respectively.

ether–hexane fractions had the same retention time on two GC columns (KI = 1855 and 1870 on HP-1 and DB-5, respectively) and a $AgNO_3$ HPLC column (Bartelt et al., 1986) as (*Z*)-10-H. (*E*)-10-Heptadecen-2-one eluted from the $AgNO_3$ HPLC column ca. 2 min earlier than (Z)-10-H. The GC-MS of the peak and of the dimethyl disulfide adduct (Nichols et al., 1986) with M^+ ion at *m/z* 345 (18%) and with peaks at *m/z* 201 (100%), 145 (50%), and 43 (35%) were identical to synthetic (Z)-10-H (Bartelt et al., 1989).

The male-derived 10% ether–hexane fractions from *D. martensis* and *D. buzzatii* (but not *D. serido*) also contained 2-tridecanone, (Z)-10-pentadecen-2-one, 2-pentadecanone, and 2-heptadecanone (Table 2).

Bioassay of Synthetic Ketones. A quantity of synthetic (Z)-10-H, equal to that in the male-derived 10% ether–hexane fractions (Table 3A) accounted for all the activity of the male-derived 10% ether–hexane fractions for *D. serido* and *D. martensis*. A quantity of synthetic (Z)-10-H equal to that in the male-derived 10% ether–hexane fraction of *D. buzzatii* was more attractive than the male-derived 10% ether–hexane fraction (Table 3A). Bioassay of a synthetic mixture of all the ketones equal in quantity to the male-derived 10% ether–hexane fraction (Table 2) was also not as active as pure (Z)-10-H (Table 3A).

TABLE 2. KETONE CONTENT OF MALE EXTRACTS AND RESPONSE OF FLIES TO KETONES RELATIVE TO (Z)-10-HEPTADECEN-2-ONE

Synthetic ketone	Relative response[a] (Amount per male in ng) (N = 8)		
	D. martensis at 200 ng/assay	*D. buzzatii* at 700 ng/assay	*D. serido* at 100 ng/assay
2-Tridecanone	−19*[b] (400 ng)	−83*** (240 ng)	−5 (0 ng)
(Z)-10-Pentadecen-2-one	31** (2 ng)	75** (25 ng)	50** (0 ng)
2-Pentadecanone	2 (9 ng)	5 (55 ng)	−10 (0 ng)
(Z)-10-Heptadecen-2-one	100*** (200 ng)	100*** (690 ng)	100*** (100 ng)
2-Heptadecanone	61*** (10 ng)	−13 (65 ng)	−9 (0 ng)

[a]Relative response (test ketone − control)/((Z)-10-heptadecen-2-one − control) × 100.
[b]*, **, and *** differ from control at $P < 0.05$, $P < 0.01$ and $P < 0.001$ (LSD).

TABLE 3. BIOASSAY OF 10% ETHER–HEXANE FRACTION OF MALES FOR THREE SPECIES IN *D. martensis* COMPLEX

Treatment	Mean bioassay catch (N = 8)		
	D. martensis	*D. buzzatii*	*D. serido*
A. Comparison of 10% ether–hexane fraction of males and synthetic (Z)-10-heptadecen-2-one at levels equivalent to that in one male fly			
Control	2.2 a[a]	4.0 a	4.9 a
(Z)-10-Heptadecen-2-one	8.7 b	23.7 b	15.7 b
Male 10% ether–hexane fraction	9.1 b	8.6 c	14.9 b
All synthetic ketones	ND[b]	10.5 c	ND
B. Inhibition of (Z)-10-heptadecen-2-one with one fly equivalent of 2-tridecanone			
Control	1.6 a	6.8 a	2.3 a
(Z)-10-Heptadecen-2-one + 2-tridecanone	8.8 b	12.9 b	8.1 b
(Z)-10-Heptadecen-2-one	8.6 b	38.2 c	8.7 b

[a]Means followed by the same letter were not significant at the 0.05 level (LSD). Comparisons of means between columns are not meaningful because the experiments were done on different days, with different species of flies.
[b]Not determined.

The activity of (Z)-10-H in the male-derived 10% ether–hexane fraction of *D. buzzatii* appeared to be inhibited by one or more of the other ketones present in the fraction.

To determine which, if any, of the male-produced ketones other than (Z)-10-H had activity, a control and each ketone, equal in quantity to the amount of (Z)-10-H extracted per male fly (Table 2), were bioassayed. Synthetic (Z)-

10-pentadecen-2-one was the only ketone other than (Z)-10-heptadecen-2-one that was preferred over controls by all three species (Table 2). However, the quantity of (Z)-10-pentadecen-2-one used for this bioassay was much higher than is present in the flies (22 times, 13 times, and 100 times for *D. martensis*, *D. buzzatii* and *D. serido*, respectively). In *D. buzzatii*, (Z)-10-pentadecen-2-one in combination with (Z)-10-H at the dose of one fly equivalent (Table 2) was not significantly different from (Z)-10-H alone. In *D. martensis*, 2-heptadecanone was preferred over controls when tested at 20 times one fly equivalent (Table 2).

2-Tridecanone [at a concentration equal to the (Z)-10-H concentration] was not attractive to any of the three species. For *D. martensis* and *D. buzzatii*, synthetic 2-tridecanone was significantly less attractive than controls (Table 2). 2-Tridecanone inhibited the response of *D. buzzatii* more than the response of *D. martensis* ($P < 0.001$ and $P = 0.02$, respectively). When tested at one fly equivalent, synthetic 2-tridecanone inhibited the activity of (Z)-10-H for *D. buzzatii* (Table 3B). 2-Tridecanone had no inhibitory effect on (Z)-10-H for *D. serido* and *D. martensis* (Table 3B). The quantity of 2-tridecanone relative to (Z)-10-H differs markedly in the last two species. In *D. martensis*, the quantity of 2-tridecanone is twofold greater than the quantity of (Z)-10-H (Table 2 and Figure 1), and in *D. serido* there is no 2-tridecanone.

Location of Pheromone Components. For all three species, the primary location of both unsaturated and saturated 2-ketones was the ejaculatory bulb,

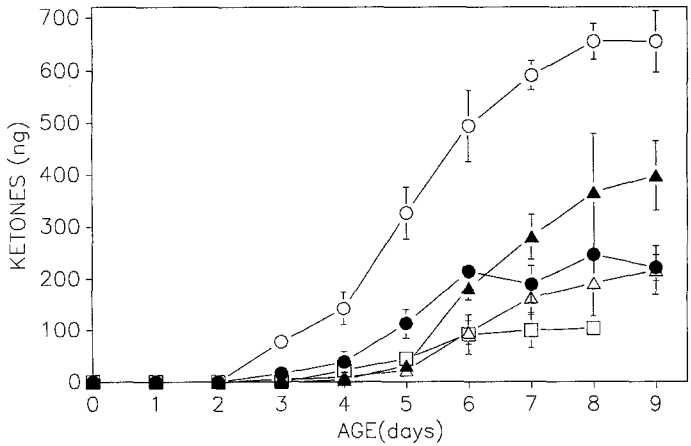

FIG. 1. Content (mean ± SD, $N = 3$ sets of 5) of (Z)-10-heptadecen-2-one and 2-tridecanone in the ejaculatory bulb of male flies as they age past eclosion. Open symbols are for (Z)-10-heptadecen-2-one and closed symbols are for 2-tridecanone. Circles, triangles, and squares are for *D. buzzatii*, *D. martensis*, and *D. serido*, respectively.

and there was a dramatic increase in 2-ketone content in the ejaculatory bulb as the flies mature posteclosion (Figure 1). *D. martensis* and *D. buzzatii* started to produce the 2-ketones at three days and *D. serido* at four days. With increasing age, there was an increase in (*Z*)-10-H in the bulb up to a plateau at six days for *D. serido* and eight days for *D. martensis* and *D. buzzatii*. The plateau level of (*Z*)-10-H was ca. 200, 690, and 100 ng/ejaculatory bulb for *D. martensis*, *D. buzzatii*, and *D. serido*, respectively. The amount of 2-tridecanone also increased with age to a plateau of 230 ng/ejaculatory bulb at six days for *D. buzzatii* and 400 ng/ejaculatory bulb at eight days for *D. martensis*.

Cuticles of male *D. martensis*, *D. buzzatii*, and *D. serido* contained appreciable quantities of (*Z*)-10-H representing 85 $\pm$ 21 (mean $\pm$ SD, $N = 3$ sets of 5), 125 $\pm$ 39 and 20 $\pm$ 9 ng/cuticle, respectively. Cuticles of *D. martensis* and *D. buzzatii* males also contained 12 $\pm$ 4 and 17 $\pm$ 12 ng of 2-tridecanone, respectively.

Release of Pheromone Compounds. In 18 hr, males released a small quantity of unsaturated and saturated 2-ketone to the surrounding vial. *D. serido* released 24 $\pm$ 8 ng/fly (mean $\pm$ SD) of (*Z*)-10-H, while *D. martensis* and *D. buzzatii* released only 14 $\pm$ 6 ng/fly of (*Z*)-10-H and even less 2-tridecanone (8 $\pm$ 2 ng/fly and 5 $\pm$ 2 ng/fly, respectively).

Transfer of Pheromone Components. Virgin females of all three species did not possess 2-ketones at any age. During mating, about 3% of the (*Z*)-10-H from the male in each species was transferred to the female reproductive tract, and only trace quantities were transferred to the cuticle of the female.

In an attempt to understand the role of the apparent "antiaggregation" activity of 2-tridecanone in *D. buzzatii*, a preliminary study of activity and production of 2-tridecanone was undertaken. In the assay of the male-derived 10% ether–hexane fractions and of the individual and mixed synthetic 2-ketone preparations (Tables 2 and 3), there was no clear preference for any of the 2-ketones by either sex of the responding flies (42–57% males in each assay). The density of male flies in the rearing vials (1, 10, 50, or 100 flies/vial) did not affect the production of either (*Z*)-10-H (626 $\pm$ 30 ng/bulb) or 2-tridecanone (214 $\pm$ 18 ng/bulb) at 8–9 days of age. Attempts were made to provide the males with controlled quality food sources to determine if especially 2-tridecanone production depended on food-source quality. We were unsuccessful in controlling food-source quality for the 8 to 9-day maturation process. We did determine that decreases in food-source quality delayed maturation. However, when delayed flies were put on a high-quality food source, the maturation was rapid and rapidly reached full production of (*Z*)-10-H and 2-tridecanone. When we attempted to determine whether sex ratio in the rearing vial (5:35, 20:20, 35:5) had any affect on 2-ketone production, we found that the males appeared to mature and produce 2-ketones quicker (1–2 days) when there

was a majority of females present. In all but one case ($N = 5$), the apparent quality of the food source was better (not as dry, better yeast growth) when more female flies were present in the rearing vials.

DISCUSSION

Although they have very different 2-ketone profiles in their ejaculatory bulbs, *D. martensis*, *D. buzzatii*, and *D. serido* are all similar in that they produce and perceive (Z)-10-H as a major component of the aggregation pheromone blend. When tested at a dose much higher than found in the flies, all three species also responded to (Z)-10-pentadecen-2-one, and *D. martensis* was attracted to 2-heptadecanone. This suggests that either the receptors for (Z)-10-heptadecen-2-one lack specificity and can detect (Z)-10-pentadecen-2-one and 2-heptadecanone or that the specific receptors for detection of (Z)-10-pentadecen-2-one and 2-heptadecanone require higher levels of compound than are present in individual flies. Since species of both the *martensis* and *mulleri* (Bartelt et al., 1989) complexes of the *mulleri* subgroup produce and respond to (Z)-10-H, we suggest that production and perception of (Z)-10-H is a primitive trait. More species of the *hydei* and *repleta* subgroups need to be investigated to determine if (Z)-10-H production and perception are present in other species of these subgroups, but was subsequently lost during speciation to *D. hydei* and *D. repleta*. These investigations also would be an attempt to determine whether the proposed "primitive I" form (Wasserman, 1982) might have produced and perceived (Z)-10-H.

2-Tridecanone is the first compound identified in *Drosophila* that inhibits the aggregation activity of another compound. A similar reduction in the bioassay activity of the sex pheromone blend in *Heliothis* species was observed with the presence of (Z)-10-hexadecenol [a precursor to the pheromone (Z)-10-hexadecenal] (Kehat et al., 1980; Teal et al., 1981, 1984). It is not known if 2-tridecanone is a precursor to (Z)-10-H since the biosynthetic pathways to both saturated and unsaturated 2-ketones are unknown. The inhibitory activity in *D. buzzatii* was also surprising because 2-tridecanone has been identified as an aggregation pheromone component in two other members of the *repleta* group, *D. hydei* (Moats et al., 1987) and *D. repleta* (Schaner and Jackson, unpublished). Production of 2-tridecanone also appears to be a primitive trait, which is lost in *D. serido*. The perception of 2-tridecanone appears to have undergone considerable change somewhere in the evolutionary pathway to the *martensis* cluster, such that it is perceived as an inhibitor of aggregation in *D. buzzatii* but not in *D. martensis* or *D. serido*.

In most *Drosophila* species of the *melanogaster* group, the aggregation pheromone components were transferred from the male ejaculatory bulb to the

female reproductive tract and/or cuticle during mating (Bartelt et al., 1985; Schaner et al., 1987, 1989a–d). Large amounts of pheromone were transferred (ca. 30–60% of the ejaculatory bulb content), and the female would then release the aggregation pheromone to the substrate within a few hours after mating where the pheromone along with fermenting food odors could attract additional flies. However, in *D. martensis*, *D. buzzatii*, and *D. serido*, the amount of (*Z*)-10-H transferred to the female was about 3% of that possessed by the male. The small quantity that was transferred to the female reproductive tract was directly proportional to the male ejaculatory bulb content rather than the cuticle content. Although mated females are capable of releasing a minor amount of (*Z*)-10-H, the major sources of (*Z*)-10-H vapor are either directly from the male cuticle or from the substrate where the male deposited the (*Z*)-10-H. Whether (*Z*)-10-H on the cuticle of mature males plays a role in sexual selection is yet to be determined.

Unlike members of the *melanogaster* group that had either (*Z*)-11-octadecenyl acetate, (*Z*)-11-eicosenyl acetate, or a combination of both in their ejaculatory bulb (Bartelt et al., 1985; Schaner et al., 1987, 1989a–c), the lipid contents of the ejaculatory bulb of the *repleta* group species are more diverse, and the variation in composition of the ejaculatory bulb lipids seems to follow the phylogenetic lines of Wasserman (1982). In the *repleta* group, 2-tridecanone is a primary component of the aggregation pheromone of *D. hydei* (Moats et al., 1987) and *D. repleta* (Schaner and Jackson, unpublished) as well as a postulated precursor to the 2-tridecanol acetate pheromone component of *D. mulleri* (Bartelt et al., 1989). In *D. buzzatii*, 2-tridecanone inhibits the aggregation activity of (*Z*)-10-H, and although present in *D. martensis*, 2-tridecanone has no effect on aggregation of that species. *D. serido* does not make 2-tridecanone nor do they show any attractive or inhibitory activity to the compound. (*Z*)-10-H is a minor component of the aggregation pheromone of *D. mulleri* (Bartelt et al., 1989) and is the major aggregation pheromone component for *D. martensis*, *D. buzzatii*, and *D. serido*. The involvement of (*Z*)-10-pentadecen-2-one, 2-pentadecanone, and 2-heptadecanone is less clear, but their presence in the ejaculatory bulb of some species and their absence in others is important information contributing to the remarkable evolutionary story that is developing around the aggregation pheromone components from species of the *repleta* group.

In addition to the aggregation pheromone activity in *Drosophila*, medium chain-length saturated methylketones (2-tridecanone, 2-pentadecanone, and 2-heptadecanone) are common insect allomone or pheromone components found in termites (*Schedorhinotermes putorius*), notodontid caterpillars (*Heterocampa manteo*), and Hymenoptera, where they are found in many formicine ants, a variety of stingless bees (Luby et al., 1973; Franke et al., 1983), two species of beewolfs (Schmidt et al., 1985; McDaniel et al., 1987), and *Philanthus* wasps

(reviewed by Blum, 1981). The methyl-branched-methylketone 10-methyl-2-tridecanone is the sex pheromone of the southern corn rootworm, *Diabrotica undecimpunctata howardi* (Guss et al., 1983).

Unsaturated methylketones (10-heptadecen-2-one, 10-nonadecen-2-one, etc.) are usually considered to be more novel arthropod chemicals but have, in addition to *Drosophila*, been isolated from the scent-marking pheromone of a species of beewolf (Schmidt et al., 1990), the Argentine ant (*I. humilis*) (Cavill et al., 1980) and, along with saturated methylketones, from the defensive secretions of *Uloma tenebrionoides* (Gnanasunderam et al., 1985).

Resistance of the wild tomato *Lycopersicon hirsutum* f. *glabratum* to attack by important insect pests including the tobacco hornworm, *Manduca sexta* (L.), the tomato fruitworm, *Heliothis zea*, the tobacco budworm, *H. virescen*, and the Colorado potato beetle, *Leptiotarsa decemlineata* (Say) is due, in part, to the presence of toxic 2-tridecanone in the glandular tricomes (Kauffman and Kennedy, 1989, and references cited therein).

It is clear that medium chain-length methylketones are produced by a variety of species to provide signals and protection. In a majority of the insect species that produce methylketones, the methylketones are involved in chemical warfare in the form of defensive secretions to repel intruders; yet in some species, methylketones are functioning in scent-marking, aggregation, and sex attraction. Methylketones are clearly unique compounds used to transmit attractive signals in some cases and repulsive signals in other cases.

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CHEMISTRY OF PHEROMONAL AND DEFENSIVE SECRETIONS IN THE NYMPHS AND THE ADULTS OF *Dysdercus cingulatus* FABR. (HETEROPTERA, PYRRHOCORIDAE)

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Abstract—The exocrine secretions from the nymphs and the adults of both sexes in *D. cingulatus* were chemically investigated. Seven compounds were identified in the anterior glands, and eleven in the median dorsoabdominal glands of nymphs and adults of both sexes. Fifty-five compounds were identified in the defensive glands (34 in posterior dorsoabdominal glands of nymphs and 21 in metathoracic glands of adults), and six in the sternal glandular epithelium of the males. The biological function of the glandular secretions and of their identified compounds were investigated and are discussed.

Key Words—*Dysdercus cingulatus*, Heteroptera, Pyrrhocoridae, exocrine glands, pheromones, bug, aggregation, allomones, cotton stainer, defensive secretions.

INTRODUCTION

Most adults and nymphs of terrestrial Heteroptera emit volatile substances from their scent glands when they are disturbed or attacked by predators. The morphology of scent glands and the chemistry of their secretions have been studied extensively in many heteropteran species (Dazzini and Pavan, 1978; Staddon, 1979, 1986; Blum, 1981; Aldrich, 1988a). These glands are usually involved

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in alarm and/or defense and produce repulsive and/or toxic chemicals; however, in some true bugs, scent glands could exhibit a strong sexual dimorphism and are involved in the sexual behavior (Aldrich et al., 1978, 1984; Hamilton et al., 1985). In other species, chemicals act as command signals, both for aggregation and alarm, according to the concentration at which they are emitted (Ishiwatari, 1974; Lockwood and Story, 1985, 1987; Kou et al., 1989).

D. cingulatus is an aposematic species that lives in dense and conspicuous aggregation on cotton plants (Fuseini and Kumar, 1975; Duviard, 1981). Preliminary studies (Farine and Lobreau, 1984) indicated that the complex behavior observed in this species required a complete survey of the glandular system and chemicals produced in the nymphs and the adults of both sexes. The morphology of the glandular system was studied exhaustively (Farine, 1987, 1988). This paper deals with the chemistry of the secretions of the three dorsoabdominal glands of the nymphs, of the two functional dorsoabdominal glands and the metathoracic glands of the adults in both sexes, and of the sternal glandular epithelium (sternal glands) in adult males. The chemical composition, with special attention to minor compounds, is reported for the secretion of each gland and discussed in regard to its possible function as a chemical signal in alarm, defense, aggregation, and sexual behavior.

METHODS AND MATERIALS

Insects. A laboratory culture of *D. cingulatus* was developed from specimens obtained from Rhône-Poulenc in Lyon. The bugs were reared on cotton seeds and water-soaked cotton pads at 27°C and 70% relative humidity under a 14 : 10 hr day–night period.

Preparation of Nymph Extracts. All the nymphs dissected were at the fifth instar. Two thousand anterior dorsoabdominal glands (DAGs 1) and 2000 median dorsoabdominal glands (DAGs 2) were quickly dissected from chilled insects and dipped into 1 ml of pure distilled pentane for 24 hr at −20°C. Then, samples were concentrated to 100 μl under gentle nitrogen flow.

Preparation of Adult Extracts. Two thousand of each of the two functional dorsoabdominal glands (DAG 1 and DAG 2) of 3-day-old adults from both sexes, and 5000 sternal glands (STGs) (sterna 2–6) of 3-day-old males were dissected and extracted as described above.

Preparation of Extracts of Defensive Glands of Nymphs and Adults. The secretions of the defensive glands (posterior dorsoabdominal gland of fifth instar nymphs (DAG 3) and metathoracic glands (MTGs) of adults) were collected by immersing about 10,000 living insects in distilled pentane for 30 sec. The extracts were concentrated to 500 μl by microdistillation and stored at −20°C. Our conditions of GC analysis showed no differences between the extracts

obtained by immersion of living insects in pentane and those from dissected glands. In addition, due to the very brief extraction time, the cuticular hydrocarbons were not extracted and no carbon chains between C_{19} and C_{40} were detected in the insects' extracts, so only the former method was used.

Chemical Analysis. Gland extracts were analyzed by gas-chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). GC analyses were performed on a Packard 429 with a flame ionization detector. Two fused silica capillary columns of different polarity were used: a DB 5 (30 m × 0.32 mm ID, J & W Scientific) and a CP Wax 58 CB (30 m × 0.22 mm ID, Chrompack). Injector and detector temperatures were, respectively, 250 and 270°C. One microliter of each sample was injected in split-splitless mode with the oven at 50°C for 5 min, then programmed at 2°C/min to 250°C using helium as carrier gas at a flow rate of 5 ml/min. Reported compound percentages were based on millivolt output from the detector using a CR 3 A Shimadzu recorder (Kyoto, Japan).

GC–MS was carried out on a Nermag R 10-10 C quadrupole mass spectrometer coupled with a Girdel 31 gas chromatograph. Fused silica capillary column (J & W Scientific) coated with DB 5 (30 m × 0.32 mm ID) was used for analysis. The GC conditions were those described above. Electron impact mass spectra were obtained at 70 eV.

The compounds were tentatively identified by comparison of their spectra to those of various library data (Heller and Milne, 1980; mass spectra from the library of the Laboratoire de Recherches sur les Arômes de l'I.N.R.A., Dijon, France). As mass spectra are subject to variability caused by experimental conditions, correct identification is confirmed if the GC characteristics of the identified compound are the same as those of the reference compound analyzed in the same conditions on DB 5 and CP Wax 58 CB columns. For spectral confirmations, commercial compounds were purchased (Interchim, Montluçon, France) or were obtained as gifts (I.N.R.A., Dijon). Biological extracts and pure compounds were coinjected on the polar and apolar columns. (*E*)-4-Oxo-2-hexenal and (*E*)-4-oxo-2-octenal were identified by comparing their mass spectra to those published by Gilby and Waterhouse (1965) and MacLeod et al. (1975), respectively.

RESULTS

Anterior Dorsoabdominal Glands of Nymphs and Adults. These sac-shaped glands open between terga 3 and 4. Morphological studies (Farine, 1987, 1988) indicated that the DAGs 1 of males and females are similar to their counterparts in nymphs.

The seven compounds present in the nymph, male, and female secretions

are listed in Table 1. Besides three hydrocarbons, two terpenes, and one aromatic compound, the major compound, 1-decyne, represents more than 80% of the secretion.

Median Dorsoabdominal Glands (DAGs 2) of Nymphs and Adults. A little more enlarged than DAGs 1, these glands open between terga 4 and 5. They are morphologically similar in nymphs, males, and females (Farine, 1987, 1988).

The GC analysis reveals the presence of 11 compounds (Table 1): five terpenes, four saturated hydrocarbons, one aromatic compound, and one alkyne. In nymphs, 70% of the secretion is composed of tridecane, 1-decyne, and *m*-cymene, whereas in males tridecane and *m*-cymene are the major components. In females, *m*-cymene alone represents 50% of the secretion.

Posterior Dorsoabdominal Gland (DAG 3) of Nymphs. Only active in nymphs, this gland opens between terga 5 and 6 and is about five times as large as the other glands (Farine, 1987).

The 34 compounds present in the secretion are listed in Table 2. Ninety-six percent of the secretion is composed of four aldehydes [(*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-4-oxo-2-hexenal, and (*E*)-4-oxo-2-octenal] and one hydrocarbon (tridecane). Twenty-nine minor components were identified: 11 aldehydes, four saturated hydrocarbons, four alcohols, three terpenes, two alkenes,

TABLE 1. *Dysdercus cingulatus:* VOLATILE COMPOUNDS IDENTIFIED BY GC–MS IN SECRETIONS OF ANTERIOR DORSOABDOMINAL GLAND (DAG 1) AND MEDIAN DORSOABDOMINAL GLAND (DAG 2) FROM NYMPHS (N), MALES (M), AND FEMALES (F)[a]

		DAG 1 (%)			DAG 2 (%)		
Compound	M.W.	N	M	F	N	M	F
Methylbenzene	92	1.5	2.2	3.0	1.4	0.8	0.6
Decane	142	2.5	2.0	1.0	2.3	1.1	1.5
1-Decyne	138	87.8	82.8	83.2	21.1	3.3	10.5
m-Cymene	134	1.3	1.1	2.6	19.0	45.5	50.7
Undecane	156	1.0	5.0	6.2	11.1	0.4	0.5
(*cis*)-Verbenol	152				0.4	1.5	5.0
(*trans*)-Verbenol	152				0.9	2.2	4.5
Terpinen-4-ol	154	2.1	3.6	1.1	2.2	0.7	0.6
p-Cymen-8-ol	150				2.6	8.8	7.5
Dodecane	170				7.0	9.9	9.6
Tridecane	184	3.6	3.1	2.1	30.5	25.6	8.9

[a] The compounds were listed in order of elution on DB5 capillary column. The percentages (%) of each compound were calculated as indicated in material and methods. M.W.: molecular weight.

TABLE 2. *Dysdercus cingulatus*: VOLATILE COMPOUNDS IDENTIFIED BY *GC–MS* IN SECRETION OF POSTERIOR DORSOABDOMINAL GLAND (DAG 3) FROM NYMPHS (N), AND IN METATHORACIC GLANDS (MTG) FROM MALES (M) AND FEMALES (F)[a]

Compound	M.W.	%		
		N	M	F
1-Pentanol	88	0.02		
Methylbenzene	92	0.03	0.12	0.05
Hexanal	100	0.26	0.11	0.28
Methyl pentenal[b]	98	0.01	0.06	0.10
(*E*)-2-Hexenal	98	6.65	88.53	86.87
(*E*)-2-Hexenol	100	0.01	0.06	0.09
(*E*)-2-Isopentenyl acetate	128		0.83	0.83
(*cis*)-Dimethyl-1,2-cyclohexane	112	0.04	0.12	0.20
(*Z*)-2-Heptenal	112	trace		
(*E*)-4-Oxo-2-hexenal	112	58.00	0.10	0.05
(*Z*)-4-Oxo-2-hexenal	112	0.11		
Octanal	128	0.09		
Hexanoic acid	116	0.07		
(*E*)-2-Hexenyl acetate	142		3.22	6.26
m-Cymene	134	0.06	1.80	0.81
5-ethyl-2(5H)-furanone	112	0.09		
(*E*)-2-Octenal	126	13.77	2.86	2.38
(*E*)-2-Octenol	128	0.04	0.01	0.01
1-Octanol	130	trace		
(*E*)-3-Heptenyl acetate	156		0.08	0.08
Undecane	156	0.01		
Linalool	154		1.29	1.39
(*E*)-2-Hexenyl propanoate	156		0.07	0.11
(*E*)-4-Oxo-2-octenal	140	5.74	0.04	0.03
2-Ethylcyclohexanone	126	trace		
(*E*)-2-Nonenal	140	trace		
(*Z*)-4-Oxo-2-octenal	140	0.06	0.11	0.06
Terpinen-4-ol	154	0.01	0.16	0.08
p-cymen-8-ol	150	0.01	0.27	0.14
(*E*)-3-Octenyl acetate	170		0.04	0.08
Dodecane	170	0.21		
Decanal	156	trace		
(*E*)-4-Oxo-2-nonenal	154	trace		
(*E*)-2-Decenal	154	0.06		
1-tridecene	182	0.03		
Tridecane	184	14.37	0.09	0.09
(*E*)-4-Oxo-2-decenal	168	0.02		
Tetradecane	198	0.01		
1-Pentadecene	210	trace		
Pentadecane	212	0.08		

[a] For further information refer to caption in Table 1.
[b] Methyl-branched pentenal, configuration not determined.

and five other compounds. Identification of (*E*)-4-oxo-2-nonenal and (*E*)-4-oxo-2-decenal was made by comparison of their spectra with those of (*E*)-4-oxo-2-hexenal and (*E*)-4-oxo-2-octenal. In addition, the variations between the retention time of the four identified (*E*)-4-oxo-2-alkenals are identical when using a polar or an apolar column. All these observations confirm the presence of a series of homologous compounds.

Metathoracic Glands (MTGs) of Males and Females. These paired glands open at the base of the hind legs. No morphological differences were found between sexes (Farine, 1988).

No remarkable differences were observed between males and females (Table 2). Among the 21 identified compounds, five represent 97% of the male secretion: (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-hexenyl acetate, *m*-cymene, and linalool. In females, the four major compounds are (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-hexenyl acetate, and linalool. The remainder of the secretion is a mixture of 16 compounds only present in small or trace amounts: five aldehydes, three acetates, two alcohols, two terpenes, and four other products.

Sternal Glands (STGs) of Males. In *D. cingulatus* we noticed a conspicuous sexual dimorphism characterized by the secretory activity of a sternal glandular epithelium in the male (sterna 2–6); this activity is correlated with their sexual maturity (Farine, 1988).

The GC analysis reveals that 88% of the secretion is composed of three major components (Table 3): methyl (*Z*)-4-decenoate, β-caryophyllene, and α-humulene. 8-Ethoxy-*p*-cymene, β-cadinene, and β-caryophyllene oxide are present in small amounts. Several other compounds, only present in trace amounts, were not identifiable by their mass spectra and additional analytical techniques were not usable.

DISCUSSION

In Hemiptera, the existence of an alarm pheromone in *D. intermedius* was first reported by Calam and Youdeowei (1968). When they are attacked by predators (e.g., ants), nymphs and adults of *D. cingulatus* straighten their legs, raise their body, and try to orient their glandular orifices toward the attacker (Farine, 1989). The chemicals produced by the MTGs of adults and DAG 3 of nymphs deter predators and elicit an alarm response, warning conspecifics and inducing their dispersion. The secretion may also be considered as defensive, the ants not only being repelled but also paralyzed (Farine, 1989).

As in *D. intermedius* (Calam and Youdeowei, 1968) and *Pyrrhocoris apterus* (Staddon and Daroogheh, 1982; Schmuck, 1987), the major components of the DAG 3 secretion in nymph *D. cingulatus* are (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-4-oxo-2-hexenal, (*E*)-4-oxo-2-octenal, and tridecane. Carayon (1971)

TABLE 3. *Dysderscus cingulatus:* VOLATILE COMPOUNDS IDENTIFIED BY GC–MS IN SECRETIONS OF STERNAL GLANDS (STG) FROM MALES[a]

Compound	M.W.	%
Methyl (*Z*)-4-decenoate	184	26
β-Caryophyllene	204	50
α-Humulene	204	12
8-Ethoxy-*p*-cymene	178	1
β-Cadinene	204	4
β-Caryophyllene oxide	220	3

[a]For further information refer to caption in Table 1.

reported that adult MTGs in Heteroptera are morphologically and functionally analogous to the nymphal DAG 3, so the presence in *D. cingulatus* of 15 identical components in both nymphs and adults is not surprising. Nevertheless, our observations reveal the chemical complexity of the secretions and the existence of numerous components that are characteristic of nymphs or adults.

Various authors emphasized the possible function of the chemical compounds found in the defensive glands. Aldehydes are able to act as nonspecific irritants and intoxicants (Blum, 1981; Pasteels et al., 1983). Because saturated hydrocarbons and alkenes are frequently present along with more polar components, their primary function was thought to be as wetting and spreading agents that could promote the penetration of the most polar compounds into the cuticle of the predators (Blum and Brand, 1972; Eisner, 1970).

It is well known that *Dysdercus* species form aposematic aggregations (Giles, 1958; Youdeowei, 1967; Gupta, 1979; Farine and Lobreau, 1984) and have smaller scent apparati than the non-warningly colored families (Schaeffer, 1972). In *D. intermedius*, Youdeowei (1967, 1969) pointed out the fact that visual and tactile stimuli are necessary together with semiochemicals to maintain aggregation. In this species, the aggregation of nymphs and adults is induced and maintained by DAG 1 and DAG 2 secretions (Youdeowei, 1967). In *D. cingulatus* (Farine, 1987, 1988), DAGs 1 and DAGs 2 of nymphs and adults are morphologically identical to their counterparts in *D. intermedius* (Youdeowei, 1967). Regarding the qualitative variations of composition between DAG 1 and DAG 2 secretions, an exclusive aggregative function for these glands is questionable and needs further investigation.

In the field, *Dysdercus* species are attracted to specific volatile chemicals from their host plants (Saxena, 1965; Duviard, 1981). Numerous chemicals identified in the exocrine secretions of nymphs and adults in *D. cingulatus* [i.e., (*E*)-2-hexenal, (*E*)-2-octenal, hexanal, (*E*)-2-hexenol, linalool, 1-decyne, ter-

pinen-4-ol, decane, undecane] are also found in the cotton plant (Hedin et al., 1971; Thompson et al., 1971; Hedin, 1976; Berni and Stanley, 1982). The major compound, 1-decyne, which had been identified in *D. cingulatus*, was never noted previously in animal semiochemicals. 1-Decyne was found among the volatiles from the cotton plant (Berni and Stanley, 1982) and was also identified as a degradative product of cotton seed oil (Fan et al., 1983).

Sun (1986) showed that various volatiles from cotton plant attract nymphs and adults of *D. cingulatus*. Linalool elicits feeding reactions in *D. intermedius* (Everton et al., 1979). The function of the aldehydes and hydrocarbons found in the defensive glands of the Heteroptera varies according to their concentration or according to the way they are emitted. Such a dual function of the aldehydes was first reported in *Cimex lectularius* (Cimicidae) (Levinson and Bar Ilan, 1971) and *Eurydema rugosa* (Pentatomidae) (Ishiwatari, 1974, 1976). (*E*)-2-Hexenal is an alarm pheromone when it is sprayed suddenly, but it acts as an aggregation pheromone when exuded gradually and in small amounts. In the first instars of *Nezara viridula* (Pentatomidae), tridecane also functions as a bimodal pheromone causing dispersal at high concentrations (1–10 individual equivalents) and aggregation at low concentrations (0.01 individual equivalent) (Lockwood and Story, 1985, 1986). Similar observations were reported for *D. intermedius* (Calam and Youdeowei, 1968) and *P. apterus* (Schmuck, 1987), and the first studies in *D. cingulatus* and *D. fasciatus* (Farine, 1989) are in keeping with these results. Consequently, production and release by nymph and adult *D. cingulatus* of chemicals found in the cotton plant aroma may enhance the potential attractivity of the food plant towards conspecifics, thus increasing gregariousness.

As suggested by Aller and Caldwell (1979), aggregation pheromones may serve as sex pheromones by promoting the encounters between males and females. Release of sex pheromones by females has been demonstrated in various heteropteran families (Aldrich, 1988b and references therein) and several species of Pyrrhocoridae (Osmani and Naidu, 1966; Zdarek, 1970, Gupta, 1979; Hebbalkar and Sharma, 1982; Siddigui and Khan, 1982). In *Dysdercus koenigii* (Hebbalkar and Sharma, 1982) and *D. cingulatus* (Siddiqui and Khan, 1982), the sealing of the female MTG openings causes about a 50% decline in the sexual responses of the males, but there is no sexual dimorphism in the morphology (Farine, 1988) or in the chemistry of the MTGs in adult *D. cingulatus*. However, females of this species are about twice as big as males. Consequently, the amount of exudate female secretion is larger than in males. If we consider that one of the functions of adult MTG secretions is to be aggregative, a larger amount of female secretions may increase their long-range attraction on the males.

In the STGs of the males of *D. cingulatus*, we identified three major compounds: methyl (*Z*)-4-decenoate, β-caryophyllene, and α-humulene. The ses-

quiterpenoids had been found in the DAG secretions of *Hotea gambiae* (Scutelliridae) (Gough et al., 1985). β-Caryophyllene and β-caryophyllene oxide are present in the cotton volatiles (Thompson et al., 1971). β-Caryophyllene preferentially increases the attraction of female *D. cingulatus*, but only at close range (Sun, 1986). Methyl (*Z*)-4-decenoate has never been reported previously in insect exocrine secretions but is very similar in structure to many well-known lepidopteran sex pheromones. Several authors have reported that the sternal glandular areas of adult male Pentatomoidea (Carayon, 1981, 1984; Aldrich, 1988b; Staddon, 1990), Scutelleridae (Knight et al., 1985), and *D. fasciatus* (Lawrence and Staddon, 1975) are likely sources of sex pheromones or of aphrodisiacs. However that may be, the greatest care must be taken in the interpretation of such behavioral observations. For example, in the field, the major components of the sternal glandular epitelia of some male pentatomids do not attract only females, but also conspecific males and nymphs (Aldrich et al., 1987; Kochansky et al., 1989). It is well known that reproductive females, but not males, of *Dysdercus* species histolyzed their flight muscles (Duviard, 1981), so that female reproduction is physiologically limited to the host plants. In contrast, males retain the potential to fly to feeding clusters among host plants. All these observations postulated an intricate role of the male sternal glands in close-range attraction and/or aggregation rather than a sexual attraction sensu stricto.

The present chemical data emphasized the complexity of the chemical communication in *D. cingulatus*. Although the composition of the glandular secretion has been identified and quantified in many Heteroptera, the biological function of each compound in the scent secretion still needs further study. Using *D. cingulatus* as a model, we will attempt an overview of the chemical communication in this species.

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ULTRASTRUCTURAL AND CHEMICAL EXAMINATION OF PAPER AND PEDICEL FROM LABORATORY AND FIELD NESTS OF THE SOCIAL WASP *Polistes metricus* SAY

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Abstract—The ultrastructure and chemical composition of paper and pedicel from nests of *Polistes metricus* that were constructed in the laboratory from known building material were compared to paper and pedicel from nests constructed in the field. Scanning electron micrographs showed the addition of a gluelike secretion from the wasp to the construction material. Solid-state [^{13}C]NMR, elemental analyses, and amino acid analyses indicate that this secretion is a silklike protein with serine, glycine, alanine, and proline comprising 65–73% of the identified residues.

Key Words—Scanning electron microscopy, CP/MAS [^{13}C]NMR, Hymenoptera, Vespidae, social wasps, *Polistes metricus*, nest paper, nest pedicel.

INTRODUCTION

Nests of social wasps serve as a site to rear brood and are clearly the center of colony activity (Starr, 1991). Social wasps construct nests that have a variety of architectural designs and that vary in the building materials utilized (Jeanne,

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1975; Edwards, 1980; Akre, 1982; Hansell, 1984; Wenzel, 1991). Many wasps use primarily woody fibers, which they masticate and mix with an oral secretion in order to produce their nest paper. The salivary secretion used in nest construction by *Pseudochartergus chartergoides* has been characterized as chitin (Schremmer et al., 1985). However, chemical examination of the nest paper of *Dolichovespula maculata* (McGovern et al., 1988) and *Ropalidia opifex* (Maschwitz et al., 1990) and of the paper and pedicel of *Polistes annularis* nests (Espelie and Himmelsbach, 1990) indicates that for several genera of social wasps this secretion is composed primarily of protein.

In the present paper we examine the ultrastructure and chemical composition of the oral secretion utilized by *Polistes metricus* in nest construction. We compare the secretion found on the paper of field-made nests to that found on nests constructed in the laboratory where the wasps were provided with a uniform building material of known chemical composition.

METHODS AND MATERIALS

Insect Collecting. Thirty colonies of *Polistes metricus* Say were collected from various locations in Clarke County, Georgia, in May and June 1990. Most colonies had a single adult and a nest with fewer than 10 cells. The colonies were immediately returned to the laboratory where they were maintained separately for continued nest building and colony growth (Matthews and Ross, 1985; Downing and Jeanne, 1988). Portions of each nest that were constructed in the laboratory from filter paper were white and, therefore, they were distinguishable from the portions of each nest (grey/brown) that were made in the field. Field-made nests were collected later in the summer (August 1990).

Insect Rearing. Nests brought into the laboratory were attached to thin wooden strips that previously had been glued to the ceiling of Plexiglas chambers (20 × 20 × 20 cm). The top of each chamber had a ventilation opening (11 cm diameter) that was covered with plastic mesh. The chambers, which were open on the bottom so that food and materials could be easily offered to the wasps, were kept on glass plates (20 × 20 cm) (Singer and Espelie, 1992). Whatman No. 1 filter paper (15 cm diameter) was cut into pieces (approximately 20 cm^2) that were placed into the chambers to provide nest construction material for the wasps. Wasps were provided with water and with 20% honey–water. Once wasp larvae were present in the nest, the wasps were provided daily with mature caterpillars (either *Spodoptera frugiperda* or *Helicoverpa zea*).

Laboratory-made nests were removed from their chamber when two or more workers had emerged and at least four pupae were present. The wasps that were left in the chamber were utilized in kin recognition bioassays (Singer and Espelie, 1992). In the chambers where the wasps constructed a new nest,

the pedicel was used for ultrastructural and chemical analysis of laboratory-made pedicels.

Microscopy. Nest paper samples (approximately 5 mm^2) were cut from two laboratory nests and from two field nests. The paper samples were cut from nest cells that had not been used as pupal cells and, therefore, did not contain larval silk. A sample (approximately 5 mm^2) also was cut from the Whatman No. 1 filter paper that had been provided to the wasps for lab nest construction. The samples were mounted on aluminum stubs and adhered with sticky tabs and silver paint so that the outer surface of the nest paper could be observed. Nest pedicels were removed carefully from laboratory-made nests and from field-made nests and then mounted onto aluminum stubs with sticky tabs and silver paste. A razor blade was utilized to cut pedicel samples into cross-sections. A 50-nm layer of gold–palladium was applied to the samples with a Hummer x Sputter Coater (Anatech). A Phillips 505 scanning electron microscope operated at 20 keV and a Jeol 35 scanning electron microscope operated at 15 keV were utilized for observing and photographing samples.

Amino Acid Analyses. Samples of nest paper and pedicel from laboratory-made and field-made nests (10–20 mg) were hydrolyzed in 6 N HCl at 110°C for 24 hr in sealed, evacuated tubes. Amino acids were analyzed with a Microsystems HPLC (Espelie and Himmelsbach, 1990).

Elemental Analysis. Carbon, hydrogen, and nitrogen analyses were performed on nest paper and pedicels from laboratory-made and field-made nests and Whatman No. 1 filter paper by Atlantic Microlab, Inc. (Atlanta, Georgia). Samples of pedicels contained material from several nests.

Solid-State NMR Spectroscopy. Solid-state NMR spectra of paper from laboratory-made and field-made nests, of pedicels from field-made nests, and of Whatman No. 1 filter paper were obtained by employing the CP/MAS technique. A Bruker MSL 300 NMR spectrometer operating at 75.47 MHz for ^{13}C and equipped with high-power amplifiers and a medium-bore (89 mm) high-speed probe was utilized for this purpose. The samples were packed in 4-mm-diameter zirconia rotors and were spun at approximately 6.0 kHz. Data were collected by taking 8K scans using a 3.0-μsec 90° 1H pulse, a 1.5-msec contact time, and a 5-sec delay time. All spectra were smoothed with Lorentzian broadening (LB = 20) and externally referenced to the benzene ring signal in hexamethylbenzene at 132.3 ppm with respect to tetramethylsilane.

RESULTS

The paper of nests that were constructed in the laboratory by *P. metricus* wasps had a very distinct appearance, with that portion of the nest made from filter paper easily recognized (Figure 1A). Scanning electron micrographs of

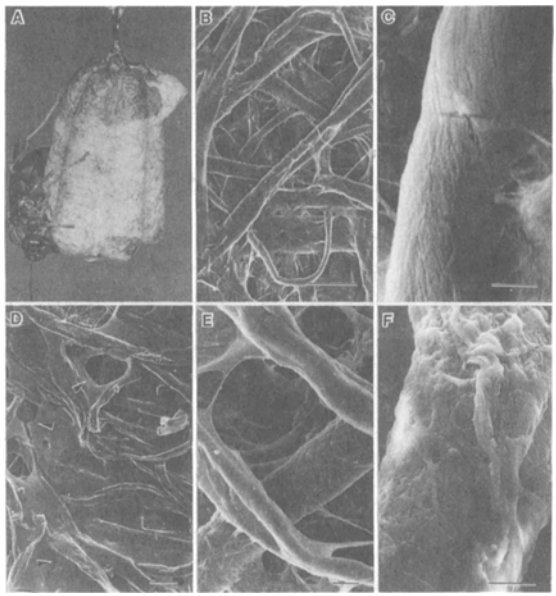

FIG. 1. (A) *Polistes metricus* and nest that was started in the field and continued in the laboratory. Scanning electron micrographs of: (B and C) filter paper; and (D–F) paper from *P. metricus* laboratory-made nests. Scale bars = 40 μm in B and D; 5 μm in C and F; and 10 μm in E.

the filter paper that the wasps utilized in the laboratory for nest construction (Figure 1B) show fine threads that bind together larger fibers of the paper. Longitudinal striations that form the texture of individual fibers of the filter paper are seen at higher magnification (Figure 1C).

Micrographs of the laboratory-made nest paper (Figure 1D) show a smooth, amorphous layer covering the fibers of the filter paper. This layer covers and connects most of the filter paper fibers, although some regions contain cracks or openings (Figure 1D, arrows). At higher magnification, this layer appears thick and gluelike and seems to serve as a binding between fibers (Figure 1E). The striations that were seen in the fibers of filter paper (Figure 1C) are no longer apparent in individual fibers of the laboratory-made nest paper (Figure 1F), presumably because they are covered by the gluelike material.

Low magnification scanning electron micrographs of nest paper from field-made nests of *P. metricus* (Figure 2A) reveal a surface that is rougher and has less of the gluelike coating than the laboratory-made nest paper. The layer of coating material, however, is still evident at higher magnification (Figure 2B). Examination of an individual fiber from a field-made nest shows a coating layer on the fiber as well as pollen grains scattered on the nest paper (Figure 2C).

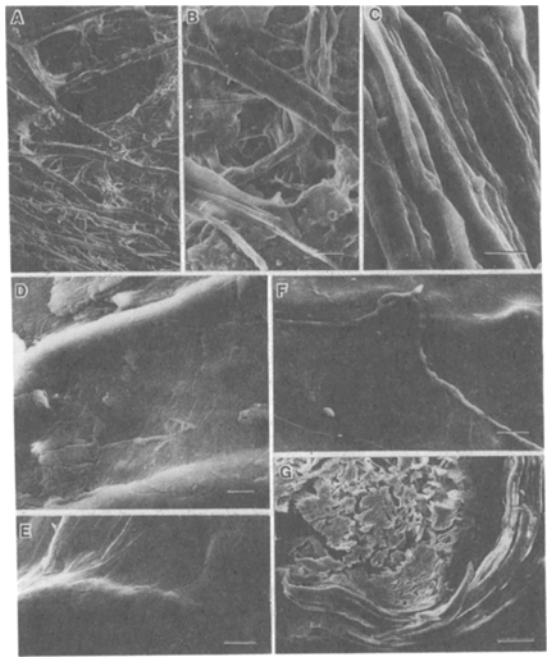

FIG. 2. Scanning electron micrographs of: (A–C) the paper of a *Polistes metricus* field-made nest; (D and E) surface of the pedicel of a *P. metricus* laboratory-made nest; (F) surface of the pedicel of a *P. metricus* field-made nest; and (G) cross-section of pedicel of *P. metricus* field-made nest. Scale bars = 40 μm in A and G; 10 μm in B and E; 7 μm in C and D; and 20 μm in F.

Scanning electron micrographs of the surface of laboratory-made pedicels of *P. metricus* nests show fibers that appear to be from the filter paper and a covering of an amorphous material laid down in random arrays (Figure 2D). At higher magnification, this material has a very smooth appearance (Figure 2E). Pedicels from field-made nests have a very similar surface with a random array of fibers and a smooth, amorphous matrix (Figure 2F). A cross-sectional view of a field pedicel shows that the outer portion consists of a series of concentric rings with fibrous material in the center (Figure 2G).

Elemental analyses indicated that nitrogen was present in both the paper and pedicel of laboratory- and field-made nests of *P. metricus* (Table 1), but there was no nitrogen detectable in the filter paper that was utilized by the wasps for the nests made in the laboratory. The nitrogen content of pedicels from field-made nests was much higher (8.4%) than it was in the pedicels of laboratory-made nests (3.2%).

CP/MAS [^{13}C]NMR spectroscopy indicated that the paper of laboratory-

TABLE 1. ELEMENTAL ANALYSIS (% COMPOSITION) OF PAPER AND PEDICEL FROM FIELD AND LABORATORY NESTS OF *Polistes metricus* AND OF FILTER PAPER

	Carbon	Hydrogen	Nitrogen
Field paper	44.1	6.6	1.4
Field pedicel	44.0	7.0	8.4
Laboratory paper	43.3	6.3	1.9
Laboratory pedicel	48.3	7.6	3.2
Filter paper	42.5	6.2	0.0

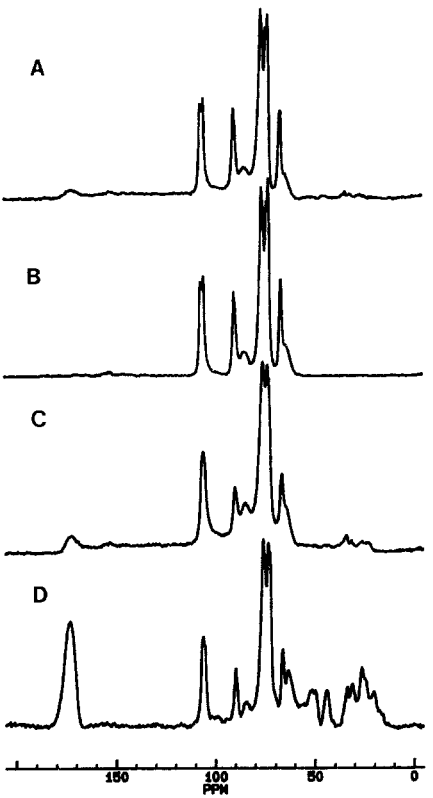

FIG. 3. CP/MAS [^{13}C]NMR spectra of: (A) paper from a laboratory-made *Polistes metricus* nest; (B) Whatman No. 1 filter paper; (C) paper from a field-made *P. metricus* nest; and (D) pedicel from a field-made *P. metricus* nest.

made nests is composed primarily of cellulose (Figure 3A). The narrow resonance bands for C_1 (104–106 ppm), and the small signals for C_4 and C_6 (84 and 63 ppm, respectively) from amorphous cellulose, indicate that the cellulose is highly crystalline (Atalla, 1984). The spectrum shows that there is also some protein present in the nest paper. There is a small carbonyl band at 175 ppm and aliphatic carbon resonances between 20 and 60 ppm that are indicative of protein (Figure 3A). The filter paper that the wasps utilized for nest construction also consists of highly crystalline cellulose, as shown by the splitting of C-1 in the spectrum of the filter paper (Figure 3B). The cellulose in the laboratory-made nest paper had the same degree of crystallinity as the filter paper, indicating that the cellulose portion of the filter paper had not been modified during nest construction. The paper from field-made nests also contains cellulose and protein. Here the resonance bands of the carbohydrate carbons are much broader, and the signals for C_4 and C_6 from amorphous cellulose are larger, thus indicating that this cellulose is noncrystalline (Figure 3C). The spectrum of the pedicel from field-made nests (Figure 3D) also contains the less crystalline cellulose and a much higher proportion of protein than the nest paper from either field- or laboratory-made nests.

Amino acid analysis of the hydrolysate of paper from laboratory-made nests gave a composition that was dominated by glycine (32%), serine (16%), alanine (14%), and proline (11%) (Table 2). Laboratory-made pedicels and the paper and pedicels from field-made nests contained protein with essentially the same amino acid composition.

DISCUSSION

The scanning electron micrographs of *Polistes metricus* nest paper clearly show that these wasps utilize secretions to construct and fortify their nests. Comparison of the scanning electron micrographs of the paper from nests made in the laboratory (Figure 1D–F) with those of the filter paper that the wasps utilized (Figure 1B and 1C) indicates that the adult wasps use a gluelike salivary secretion to bind together the paper fibers. This secretion, detectable as strands or plates interconnecting the construction fibers, is also seen on the paper of nests made in the field (Figure 2A–C). The secretion is not as evident in the micrographs of the field-made nests, possibly because there is less secretion in these nests. The diversity of building materials used by the wasps may also cause the secretion to be more difficult to discern in the micrographs of the field-made nests than it is in the nests that are made from filter paper.

The strands and plates of secretion seen in the scanning electron micrographs of the laboratory nests of *P. metricus* are very similar in appearance to those seen in the nests of the Oriental hornet, *Vespa orientalis* (Ishay and Ganor,

TABLE 2. AMINO ACID COMPOSITION[a] OF PROTEIN FROM PAPER AND PEDICEL OF LABORATORY AND FIELD NESTS OF *Polistes metricus*

	Laboratory nest		Field nest	
Amino acid	Paper	Pedicel	Paper	Pedicel
Asx	1.8	5.2	3.7	5.2
Glx	4.4	5.9	6.6	6.1
Ser	16.0	16.4	15.1	16.6
Gly	31.9	25.6	26.3	24.5
His			0.6	0.6
Arg			1.5	1.0
Thr	4.1	3.6	3.6	3.7
Ala	14.2	14.1	12.6	13.5
Pro	10.6	12.5	11.3	11.5
Tyr				0.2
Val	7.2	6.8	6.9	6.6
Ile	3.9	3.5	3.8	3.1
Leu	3.9	3.9	5.0	4.2
Phe			0.9	0.3
Lys	1.9	2.5	2.2	2.8

[a] Number of residues per 100 identified amino acids.

1990). The cracks in the plates of secretion (Figure 1D and 1E, arrows) are also similar to those seen in *V. orientalis* nests. These cracks may be a result of the hardening of the secretion, and they may serve to provide ventilation in the nest (Ishay and Ganor, 1990).

Previous chemical analyses of social wasp field-made nests have shown the nests to be composed of cellulose and protein (McGovern et al., 1988; Espelie and Himmelsbach, 1990; Maschwitz et al., 1990). The CP/MAS [^{13}C] NMR spectra in this study also show cellulose and protein to be the major components of the laboratory- and field-made nest paper (Figure 3). The protein found in both the nest papers and pedicels (Table 2) is similar in amino acid composition to that found in several insect silks (Lucas and Rudall, 1968a,b; Crewe and Thompson, 1979) with very high proportions of glycine, alanine, and serine. The relatively large amount of proline (10.6–12.5%) found in both the paper and pedicel proteins (Table 2) is comparable to that found in the nest pedicel of *Polistes annularis* (Espelie and Himmelsbach, 1990) and in the nest paper of *Dolichovespula maculata* (McGovern et al., 1988). Proline also has been found to comprise 10–15% of the protein in the nest paper of several species of South American and Asian social wasps (Wenzel and Espelie, in preparation). Proline is frequently a dominant residue in structural proteins

(Bursell, 1981; Chen, 1985), and its presence in the protein secreted by social wasps may give added strength to their nests.

Several species of social wasps rub onto the pedicel of the nest a sternal gland secretion that serves to deter predators (Jeanne, 1970; Turillazzi and Ugolini, 1979; Post and Jeanne, 1981; Kojima, 1983; Jeanne et al., 1983). The sternal gland of *Polistes fuscatus* has been shown to contain methyl hexadecanoate (Post et al., 1984), which is applied to the pedicel and functions as an ant deterrent (Henderson and Jeanne, 1989). Hexadecanoic acid and octadecenoic acid have been found on the nest pedicel of *P. annularis*, and these components may also deter potential predators (Espelie and Hermann, 1990). Cross-sections of the pedicel of the *P. metricus* field-made nests show concentric rings of hardened, crisp material encircling a fibrous core (Figure 2G). The outer layers may be a combination of material applied from both oral and sternal gland secretions of the wasps. The [^{13}C]NMR spectrum (Figure 3D) and elemental analysis (Table 1) indicate that the pedicel has a high protein content. This protein may be primarily on the outer region of the pedicel, while the central fibrous region is mainly cellulose. Chemical and ultrastructural examination of the thickening of the pedicel of *P. metricus* nests throughout the colony season should yield additional evidence regarding the origin and function of this outer layer of material.

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THE ROLE OF CHEMOSENSORY CUES IN DISCRIMINATION OF PREY ODORS BY THE AMPHISBAENIAN *Blanus cinereus*

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Abstract—Responses of amphisbaenians (*Blanus cinereus*) to deionized water, a control for pungency (cologne), and integumental prey odors (coleopteran larvae and adult ants) on cotton swabs were studied in experiments with a randomized blocks design to discover whether amphisbaenians use chemical cues to detect and identify prey. No individual bit the applicators. Amphisbaenians tongue-flicked at lower rates than epigean saurians, which are active foragers. Tongue-flick rate differed among treatments, but responses to prey odors were not significantly different from those to cologne. The number of directed tongue-flicks emitted during the 60-sec trials was, however, lower in response to deionized water than in response to cologne or prey odors. Response details, the low rate of tongue-flick, and absence of biting are discussed in relation to the foraging behavior and fossoriality of amphisbaenians. Evidence from this study indicates that the vomeronasal sense is used by amphisbaenians to identify odors, but our experiments failed to demonstrate that amphisbaenians discriminate between prey and nonprey odors.

Key Words—Lacertilia, Amphisbaenidae, *Blanus cinereus*, prey odor, tongue-flicking, fossoriality.

INTRODUCTION

Epigean saurians use visual and olfactory senses to locate prey. The prevalence of one of these senses seems to be related to foraging mode. Active foragers

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appear to use chemosensory senses. They regularly tongue-flick during foraging and can detect and recognize prey by chemical cues. Sit-and-wait predators, however, do not tongue-flick at high rates and detect prey visually while foraging (Bissinger and Simon, 1979; Huey and Pianka, 1981; Cooper, 1989, 1990). There have been few studies on senses used in prey detection by subterranean saurians (Hetherington, 1989).

Amphisbaenians are squamate reptiles showing many adaptations to a burrowing life (Gans, 1978). Their foraging behavior is unknown, specially in relation to prey detection. Vision is reduced. Hearing and olfactory capabilities, however, are enhanced (Gans and Wever, 1975; Gans, 1978), indicating an important role in predation. Nevertheless, no evidence is available of a chemosensory role in any amphisbaenian species.

Blanus cinereus is an amphisbaenian endemic to the Iberian Peninsula (Busack, 1988). Its diet mainly consists of insect larvae and ants, which are the most abundant invertebrates in their habitat, but insect larvae of large size are selected (López et al., 1991). The use of chemosensory cues by *B. cinereus* in the discrimination of prey was tested using responses to prey chemicals presented on cotton-tipped applicators (Cooper and Burghardt, 1990). We tested the ability of *B. cinereus* to detect prey odors and to discriminate between prey odors and nonprey odors. We also address the way fossoriality influences the foraging behavior of a burrowing reptile, in comparison to epigean saurians.

METHODS AND MATERIALS

Eighteen *Blanus cinereus* (six adult males, three adult females and nine immatures; snout-vent length: $\bar{X} \pm SE = 147 \pm 8$ mm), were captured in March 1990 near Torrelodones (40°35′N, 3°56′W; Madrid Province, Spain). They were housed at "El Ventorrillo" Field Station laboratory (Navacerrada, Madrid Province). The photoperiod was that of the surrounding region, but ambient temperature was maintained at a constant 20°C. Individuals were maintained in 5-liter glass jars containing sand substrate from the capture area. They were fed twice weekly on coleopteran larvae, adult ants, ant eggs, and earthworms, which they readily consumed. Humidity was raised daily with a spray. All animals were acclimatized to laboratory conditions and the experimenter's presence for at least one month before testing. Amphisbaenians were not fed for five days prior to the study.

In a first experiment, each of 18 amphisbaenians was tested with coleopteran larvae odors, deionized water, and cologne (Eau Jeune, L'Oréal). The water served as an odorless control. Cologne was used to determine response to an odorous but nonprey stimulus. Every amphisbaenian was tested with each stimulus once in a randomized block design. Order of presentation was coun-

terbalanced. One trial was conducted per day for each animal. We prepared stimuli by dipping the cotton tip (1 cm) of a wooden applicator (10 cm) in deionized water. Other stimuli were added by dipping the wetted cotton in diluted cologne or by rolling it over the body surface of coleopteran larvae. A new stimulus was used in each trial. The experiment was conducted April 23–28, 1990, between 0930 and 1900 hr. A second experiment was made using ant odor (*Pheidole pallidulla*), deionized water, and cologne. This experiment was conducted May 2–10, 1990, between 0930 and 1900 hr., using the procedures described for experiment 1.

Because the amphisbaenians showed defensive and escape behavior on the soil surface, we simulated fossorial conditions to perform experiments by using transparent plastic tubes (one for each individual). Inside each tube, the lower half of the surface was covered with sand using adhesive. Individuals were kept in their tubes 6 hr per day during the acclimatization period, during which they showed normal behavior and were fed with larvae and ants. Individuals were kept in their tubes 10 min before trials, closing both sides with cotton. Between trials, we opened both sides of the tube for 10 min to avoid odor mixture. During experiments the laboratory was darkened and observations were made using a 50-W red light.

To begin a trial, we slowly approached a tube, removed the cotton from one side and slowly put the cotton-tipped applicator to a position 2 cm anterior to the amphisbaenian's snout. We counted total number of tongue-flicks (TFs) for 60 sec beginning with the first TF. We also noted the number of TFs directed to the swabs during this period. Latency to the first TF was computed as the period elapsed between closing the tube and the first TF. If an individual did not tongue-flick in 30 sec, we touched its snout with the swab. If it still did not tongue-flick, the data were discarded. To demonstrate that lack of responsiveness to stimuli was not caused by satiation, food was given to the amphisbaenians at the conclusion of trials.

To examine differences in number of TFs between trials we used nonparametric Friedman two-way ANOVA (Siegel, 1956). Individual comparisons were made using Wilcoxon signed rank-matched pairs test. Spearman rank correlations were conducted between latency to first TF and number of TFs (Sokal and Rohlf, 1981).

RESULTS

Experiment 1: Using Coleopteran Larvae as Prey Odor. All of 18 individuals tongue-flicked. Fifteen tongue-flicked in all conditions, and three in only two conditions. Analyses were conducted only on those 15 individuals. No amphisbaenians bit an applicator. There were significant differences ($\chi^2 = 7.58$;

$df = 2,28$; $P = 0.001$) in total number of TFs in responses to different odor conditions ($\bar{X} \pm$ SE: prey = 4.9 ± 0.7; cologne = 7.7 ± 1.0; water = 3.9 ± 0.5). The tongue-flick rate in response to prey odor was significantly higher (Wilcoxon test, $T = 2.0$, $P < 0.05$) than to deionized water but did not significantly differ ($T = 1.88$, $P = 0.06$) from those to cologne odor. Response to cologne was significantly higher ($T = 3.0$, $P = 0.002$) than to water. Number of TFs directed to the cotton-tip were also significantly different between treatments ($\chi^2 = 7.96$; $df = 2,42$; $P = 0.019$) (Figure 1).

Latency to first TF and total number of TFs in 60 sec in all trials had a negative Spearman rank correlation ($r_S = -0.40$; $P = 0.008$), and latency to first TF was negatively, but not significantly, correlated with the number of TFs directed to the cotton-tip ($r_S = -0.21$; $P = 0.17$). There was a significant treatment effect on time of latency to first TF ($\chi^2 = 14.53$; $df = 2,28$; $P < 0.001$). Latency to first TF in response to distilled water ($\bar{X} \pm$ SE = 29.2 ± 6.8 sec) was significantly higher than to either prey ($\bar{X} \pm$ SE = 13.1 ± 2.5 sec; $T = 2.87$, $P = 0.004$) or cologne odor ($\bar{X} \pm$ SE = 10.2 ± 1.9 sec; $T = 2.73$, $P = 0.006$). Latency in response to prey odor and cologne did not differ significantly ($T = 0.68$, $P = 0.49$).

Experiment 2: Using Ants as Prey Odor. This experiment was conducted on 16 animals. All 16 individuals tongue-flicked. Fifteen tongue-flicked in all treatments, and one in only two treatments. Analyses were conducted only on those 15 individuals. No amphisbaenians bit the applicator. The total number of TFs emitted in responses to different odor conditions ($\bar{X} \pm$ SE: prey = 7.5 ± 1.1; cologne = 7.8 ± 1.1; water = 3.7 ± 0.5) were significantly different ($\chi^2 = 21.83$; $df = 2,28$; $P < 0.0001$). The tongue-flick rate in response to water odor was significantly lower than to either prey ($T = 3.38$, $P = 0.0007$) or cologne ($T = 3.26$, $P = 0.001$). Responses to prey and to cologne did not

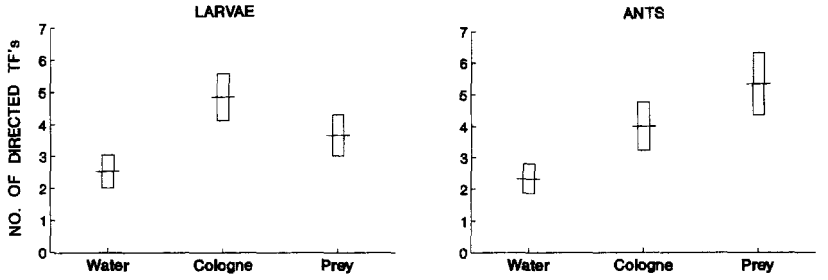

FIG. 1. Number of directed tongue-flicks (TFs) emitted in 60 sec in response to odor stimuli presented on cotton-tipped applicators. Responses shown are for *Blanus cinereus* to larval prey (experiment 1) and to ant prey (experiment 2). Data are means (horizontal lines) ± 1 SE.

differ significantly ($T = 0.44$, $P = 0.66$). The number of TFs directed at the cotton-tip were also significantly different between treatments ($\chi^2 = 9.25$; $df = 2,42$; $P = 0.0098$) (Figure 1).

Latency to first TF was negatively, but not significantly, correlated with the total number of TFs in 60 sec ($r_S = -0.24$; $P = 0.11$), and with the number of TFs directed at the cotton-tip ($r_S = -0.16$; $P = 0.29$). Latency to first TF in response to distilled water ($\bar{X} \pm$ SE $= 15.8 \pm 2.6$ sec) was not significantly different ($\chi^2 = 4.28$; $df = 2,28$; $P = 0.12$) from either cologne ($\bar{X} \pm$ SE $= 9.5 \pm 1.9$ sec) or prey odor ($\bar{X} \pm$ SE $= 11.8 \pm 2.7$ sec).

DISCUSSION

Blanus cinereus can detect odors but did not demonstrate an ability to discriminate prey odors from nonprey odors as tested in the present experiment. The greater number of tongue-flicks was in response to cologne compared to water, but does not rule out recognition of food odor. However, their failure to do so does not necessarily indicate an inability to recognize food stimuli by tongue-flicking. Critical tactile or hearing stimuli needed to release attack may have been absent. Lizards need not rely exclusively on one sensory cue to detect prey (Cooper, 1990). *B. cinereus* may use many cues to locate prey, and the ability to detect odors by chemical cues could be useful to confirm identification of potential prey (Graves and Halpern, 1990).

The tongue-flick rates of *B. cinereus* are close to those produced by epigean saurians, which are unable to detect prey odors. Nevertheless, the iguanid lizard *Dipsosaurus dorsalis*, although emitting a relatively low number of TFs, discriminates food odors in opposition to cologne (Cooper and Alberts, 1991). Amphisbaenians may discriminate quickly, on the basis of a few or even a single tongue-flick. Low rates of tongue-flicking could also reflect low metabolic requirements related to fossoriality (Withers, 1981; Kamel and Gatten, 1983).

Compacted particles of substrate that separate amphisbaenians from their potential prey probably complicate detection of invertebrates by the vomeronasal system. Cues associated with moving prey could be much more effective, as they can be used at greater distances (Hetherington, 1989). Olfactory cues may help to identify nearby prey items whose odor can be detected through particles of substrate. The absence of attacks on applicators may indicate that amphisbaenians were not previously stimulated by hearing cues.

The construction of a permanent system of tunnels is unknown in *B. cinereus*; however, we observed permanent galleries under stones that allowed access for heating (Martín et al., 1990). Vomerolfaction may aid detection of potential predators or prey in galleries. Both sexes of *B. cinereus* possess femoral pores,

and their secretion may impregnate the walls of tunnels, in which case the vomeronasal system may be used in individual and sex recognition (Alberts, 1991; Cooper and Vitt, 1986).

The experimental design used in this paper was able to demonstrate that *B. cinereus* can detect odors using its tongue, probably via vomerolfaction. Nevertheless, it did not demonstrate discrimination between prey odors and nonprey odors. Alternative experimental techniques, such as sealing of vomeronasal ducts (Graves and Halpern, 1990) or teaching the animals to respond to a particular chemical stimulus to be rewarded with food could be useful to determine the chemosensory abilities of amphisbaenians.

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REPLACEMENT OF CARCINOGENIC ALKYLATING AGENT ETHYLENE OXIDE IN THE SYNTHESIS OF (*Z*)-3-DODECEN-1-YL (*E*)-2-BUTENOATE, SEX PHEROMONE OF SWEET-POTATO WEEVIL, *Cylas formicarius elegantulus* (SUMMERS) AND *Cylas formicarius formicarius* (F.)

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Abstract—An alternative synthesis of (*Z*)-3-dodecen-1-yl (*E*)-2-butenoate without use of carcinogenic ethylene oxide and HMPA is described. By coupling of the tetrahydropyranyl (THP) ether of 3-butyn-1-ol with 1-bromooctane with sodamide in liquid ammonia, 12-(2-tetrahydropyranyloxy)-9-dodecyne was obtained; subsequent hydrolysis and semihydrogenation afforded (*Z*)-3-dodecen-1-ol. The alcohol was then reacted with crotonyl chloride to give the desired crotonate with a total yield of 29.8%. Males of sweet-potato weevil, *Cylas formicarius* were strongly attracted to the synthetic sex pheromone. The attraction from the dispenser of polyethylene tube was better than the attraction from the dispenser of rubber septum in the field.

Key Words—Sweet-potato weevil, Coleoptera, Curculionidae, *Cylas formicarius*, sex pheromone, (*Z*)-3-dodecen-1-yl (*E*)-2-butenoate, ethylene oxide, hexamethylphosphoric triamide, 3-butyn-1-ol, liquid ammonia.

INTRODUCTION

The sex pheromone of (*Z*)-3-dodecen-1-yl (*E*)-2-butenoate (I) was attractive to the male sweet-potato weevil *Cylas formicarius elegantulus* (Summers) (Heath

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et al., 1986; Hwang et al., 1989), and also to *Cylas formicarius formicarius* (F.) (Talekar and Lee, 1989). The compound was first synthesized by Heath et al. in 1986, and the key step in the synthesis of (*Z*)-3-dodecen-1-yl (*E*)-2-butenoate was the synthesis of 3-dodecen-1-ol (III) by coupling 1-decyne (II) with ethylene oxide (EO), using *n*-butyllithium (BuLi in dry THF) as a base, and hexamethylphosphoric triamide (HMPA) as a cation complexing agent for metalation (Scheme 1).

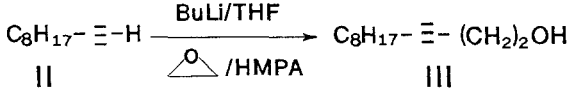
Traditionally, EO is a good alkylation agent and commonly has been used in many alkylation reactions (Henrick, 1977), such as in the synthesis of key intermediates for the sex pheromones (*Z*)-13-octadecen-3-yn-1-ol and (*E*)-13-octadecen-3-yn-1-ol (Voerman, 1979), or 3,13-octadecadiyne-1-ol and (*Z*)-13-octadecen-3-yn-1-ol (Doolittle et al., 1980). HMPA also is used extensively as a solvent for many alkylation reactions due to its ability to form cation ligand complexes (Normant, 1967; Henrick, 1977; Lo and Chao, 1990).

However, EO should be considered a carcinogenic chemical (Kolman et al., 1986), and the U.S. National Institute for Occupational Safety and Health (NIOSH) recommended that the EO exposure level should be reduced to 0.1 ppm (NIOSH, 1981). HMPA has also been shown to be carcinogenic in rats, and a permissible airborne exposure limit of 0.5 ppb was suggested (Schmutz, 1978).

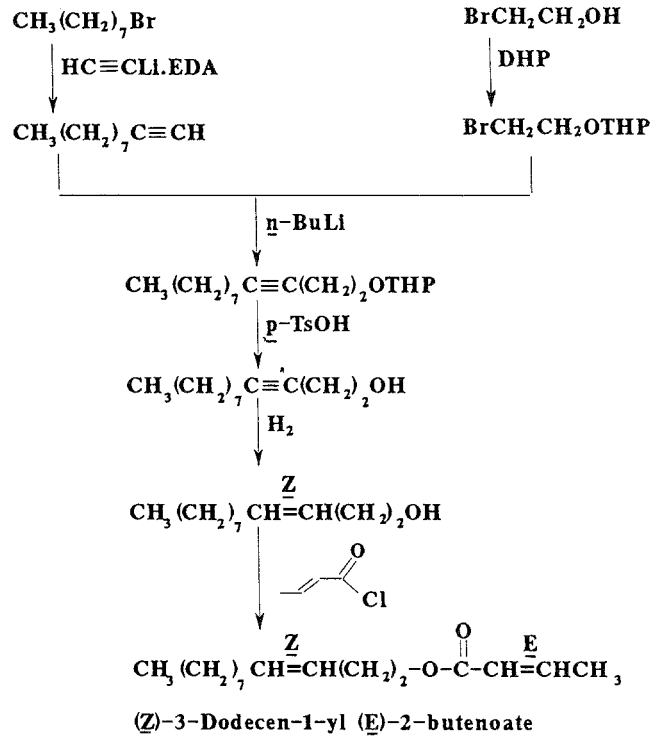
Thus, a safe substitute for carcinogenic agents EO and HMPA in the synthesis of the key intermediate 3-dodecyn-1-ol of (*Z*)-3-dodecen-1-yl (*E*)-2-butenoate is necessary, especially when large-scale synthesis of this sex pheromone is required for the possible application in the sweet-potato fields of 20,000 hectares in Taiwan.

A possible route is to synthesize 12-(2-tetrahydropyranyloxy)-9-dodecyne[$CH_3(CH_2)_7C{\equiv}C(CH_2)_2OTHP$], a precursor of 3-dodecyn-1-ol, without EO and HMPA. The synthesis shown in Scheme 2 was considered. However, 2-bromoethanol($BrCH_2CH_2OH$) induced mutagenic response in the histidine-dependent *Salmonella typhimurium* strains TA155 and TA100 (Pfeiffer and Dunkelbery, 1980). Further evidence suggested that 2-bromoethanol might be converted into ethylene oxide *in vivo* (Jones and Wells, 1981). Thus, use of 2-bromoethanol or other halohydrins should be avoided.

The alkylation agent 3-butyn-1-ol seems to be noncarcinogenic, its toxicity being attributed to the inactivation of alcohol dehydrogenase in horse liver (Alston et al., 1979), and this hepatoxicity is typical for alcohols. DMPU (1,3-dimethyl-



SCHEME 1.



SCHEME 2.

2-oxo-hexahydropyrimidine) has been reported to be a safe substitute for HMPA in many reactions (Mukhopadhyay and Seebach, 1982; Bengtsson and Liljefors, 1988), although our review has indicated that a potential toxicological risk to man should be considered (Lo and Chao, 1990). DMI (1,3-dimethyl-2-imidazolidinone) is also recommended as a safe substitute for HMPA (Barker et al., 1979), and if an alkylation reaction can be conducted at temperature higher than 0°C, use of DMI should be safer than use of DMPU or HMPA (Lo and Chao, 1990).

We have worked out an alternative procedure of synthesizing the sex pheromone (*Z*)-3-dodecen-1-yl (*E*)-2-butenoate (I), which allows the synthesis of the key intermediate 3-dodecen-1-ol (III) without carcinogenic risk (Figure 1). Starting with 3-butyn-1-ol (IV), the alcohol was first protected by 3,4-dihydro-2*H*-pyran (DHP), then ω-tetrahydropyranyloxy-1-butyne (V) was alkylated with 1-bromooctane and sodamide in liquid ammonia, followed by treatment with *p*-toluenesulfonic acid (TsOH) in methanol to give the corresponding alcohol (III). The remaining procedures were basically the same as those of Heath et

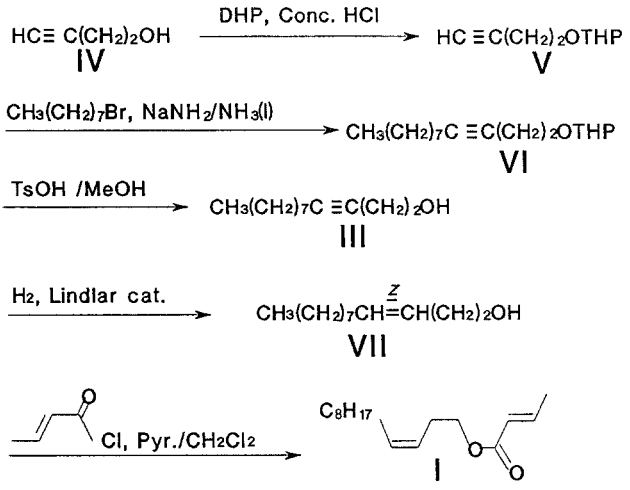


FIG. 1. Synthesis of (*Z*)-3-dodecen-1-yl (*E*)-2-butenoate.

al. (1986), except that the final work-up procedures were modified because of a serious emulsion that occurred when methylene chloride was used to extract the sex pheromone (I); there was no emulsion when ethyl acetate was used as extraction solvent.

METHODS AND MATERIALS

Gas–liquid chromatography (GLC) was performed on the synthetic materials with a Hewlett-Packard 5890 equipped with a flame ionization detector (FID). A glass column of 3% OV-1 on 80–100 Chromosorb W HP, 2 mm × 1.8 m, was used to analyze the synthetic intermediates with the initial oven temperature at 100°C (1 min), then programmed to 230°C (8°C/min). A glass column of 3% OV-17 on 100–120 Chromosorb W HP was used to analyze the final synthetic pheromone; the initial oven temperature was set at 180°C (1 min), then programmed to 230°C (8°C/min). Nitrogen was used as carrier gas at the rate of 29.9 ml/min, and air and hydrogen flows were set as recommended for FID.

GC-MS measurements were recorded on a Hewlett-Packard 5890A GLC combined with a 5970B mass selective detector (EI, 70 eV). Infrared data were obtained with a Perkin-Elmer 882 infrared spectrophotometer, and [^{1}H] nuclear magnetic resonance data were obtained with a Varian VXR-300 spectrometer.

ω-Tetrahydropyranyloxy-1-butyne (V). For the synthesis of ω-tetrahydropyranyloxy-1-butyne, we added 5.30 g (0.076 mol) of 3-butyn-1-ol (IV) in a

three-neck flask, cooled the contents of the flask to 0°C in an ice bath, added five drops of concentrated HCl (35.0–37.0%) and then 3,4-dihydro-2*H*-pyran (DHP) (9.54 g, 0.11 mol) dropwise. The cold solution was stirred for 3 hr at 0°C, 2 g of sodium bicarbonate ($NaHCO_3$) was added, and the solution was stirred for 1 hr. The precipitate was filtered and washed with ether. The solvent was removed, and the distillate was collected at 73–75°C, 5 mm. Yield 9.40 g (80.6%) of compound V; IR (cm^{-1}film) 3294 (terminal≡C−H), 2120 (C≡C), 1200–1036 (tetrahydropyranyl), and 639 (≡C−H).

12-(2-Tetrahydropyranyloxy)-9-dodecyne (VI). A three-neck flask was precooled below −50°C (Dry Ice–acetone), liquid NH_3 (300 ml) was added via a Dry Ice–acetone-filled, modified Dewar condenser (with a release vent for excess gaseous NH_3). Sodium amide (2.84 g, 0.073 mol) was added, and compound V (9.25 g, 0.06 mol) was injected from a syringe. The contents of the flask were cooled below −50°C and stirred for 1.5 hr. 1-Bromooctane (22.66 g, 0.12 mol) was injected, the mixture was stirred for 3 hr, and the mixture was allowed to rise to room temperature overnight for NH_3 evaporation. Ice was added and the mixture was extracted with ether three times. The ether extract was washed with water and dried over Na_2SO_4. The residue after removal of the solvent was purified by column chromatography through silica gel (eluent: *n*-hexane: EtOAc = 200:3, v/v). After removing the solvent with a rotary evaporator, 10.23 g of compound VI was obtained (yield 64.0%). The IR spectrum of the product showed no absorption at 3294 cm^{-1} (terminal≡C−H).

3-Dodecyn-1-ol (III). Compound VI (9.89 g, 0.037 mol) was converted to the alcohol by treatment with *p*-toluenesulfonic acid (TsOH, 19.2 g) in methanol (100 ml) for 3 hr. After removing the solvent with a rotary evaporator, the residue was extracted with ether, then the extract was washed with saturated aq. $NaHCO_3$, saturated aq. NaCl, and dried over Na_2SO_4. The ether was removed by rotary evaporation to afford a yellow oily crude compound. The oily compound was further purified by silica gel column chromatography (eluent: *n*-hexane: ether = 20:1, v/v) to obtain compound III (5.53 g, 81.7%; purity 97.4%). The IR spectrum showed peaks at 3353 (OH), and no typical tetrahydropyranyl peaks (1200–1036 cm^{-1}) were found.

(Z)-3-Dodecen-1-ol (VII). To synthesize (Z)-3-dodecen-1-ol, a solution of the alcohol (III) (5.24 g, 0.029 mol) in hexane (98.4 mole %) was hydrogenated at room temperature using a Lindlar catalyst (1.05 g, 20% by weight of the alkyne substrate) and quinoline (0.19 ml, d = 1.09, 20% by weight of the catalyst) (Ho and Liu, 1987). When no more hydrogen was absorbed, the reaction was stopped. The catalyst was removed by filtration, the hexane was evaporated at reduced pressure, and the residue was dissolved in ether. The ether solution was washed with 5% HCl, saturated aq. $NaHCO_3$, water, and brine, dried over $MgSO_4$, and concentrated in a rotary evaporator to give product VII

5.16 g (yield 97.4%; chemical purity 95.8%). IR (cm^{-1}film) 3335, 3306, 2955, 2854, 1467, 1378, 1178, 1050, 968 (*trans* HC=CH), 874, 723 (*cis* HC=CH).

(Z)-*3-dodecen-1-yl* (E)-*2-butenoate (I).* Crotonyl chloride (3.3 ml of 90% technical grade, 0.034 mol) and compound VII (5.10 g, 0.028 mol) were dissolved in methylene chloride (50 ml), and the solution was cooled in an ice bath. Pyridine (2.2 ml, 0.027 mol) was added in methylene chloride (10 ml) dropwise. The resulting mixture was stirred cold for 1 hr, and then worked up with water and ethyl acetate (methylene chloride produced serious emulsion). The organic phase was dried ($MgSO_4$) and concentrated. The crude ester (6.16 g) was purified by silica gel column chromatography (eluent: *n*-hexane: EtOAc 200:3, v/v) twice to obtain product I, 5.08 g (yield 72.7%; chemical purity: 94.2%; active isomeric purity: 82.2%) with overall yield for the synthesis of 29.8%. The EI mass spectrum showed the diagnostic peaks of fragment ions at *m/e* 167 ($M^{+\cdot}-C_4H_5O_2$, 3.4%), 166 ($M^{+\cdot}-C_4H_6O_2$, 31.7%), 87 ($C_4H_7O_2^{+\cdot}$, 5.5%), and 69 (100%). Other major fragmention ion peaks were 138 (14.2), 124 (8.8), 109 (9.5), 96 (33.8), 82 (42.1). IR (cm^{-1} film) 3012 (C=CH), 2920, 2854, 1728 (C=O), 1662 (C=C), 1459 (CH—O—C=O), 1379, 1310, 1294, 1259, 1184, 1103, 1017, 970 (*trans* HC=CH), 838 (C=CH), 723 (*cis* HC=CH), 689. The [^{1}H]NMR spectrum was identical to that of Heath et al. (1986).

The product was further purified with a 20% silver nitrate-impregnated silica gel column (eluent: 1% ether in *n*-hexane) for field tests; two fractions were collected and concentrated to give a colorless liquid of 1.12 g (85.3% isomeric purity) for the first fraction, and 1.99 g (94.6% isomeric purity) for the second fraction.

Field Tests. Field testing was carried out in Houlung, Miaoli during November 13–December 12, 1990. Polyethylene tube dispensers (5.5 cm × 1.0 mm OD, sealed at one end), and rubber septa (1.8 cm OD) were used to load the synthetic pheromones of 1.0 mg containing antioxidant BHT (10%). The dispenser was mounted 2 cm above the poly(ethylene terephthalate) (PET) bottle (Figure 2) (Hwang et al., 1989). Traps were set 10 m apart, 5 cm below the ground, and were rotated regularly every week in a randomized block design with three replications in three blocks. The number of trapped *Cylas formicarius elegantulus* (Summers) was recorded every two days. In all experiments, blank dispensers (with solvent only) were used as controls.

RESULTS AND DISCUSSION

The initial attempt to prepare the compound (*Z*)-3-dodecen-1-yl-(*E*) 2-butenoate (I) was through the alkylation of ω-tetrahydropyranyloxy-1-butyne (V) (in DMI) with 1-bromooctane (in DMI) and use of *n*-butyllithium as metalating

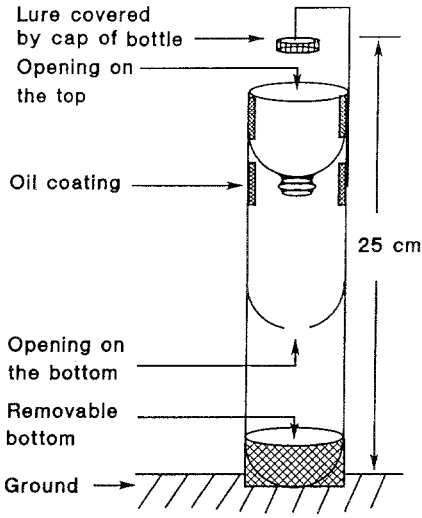


FIG. 2. Trap design of PET bottle developed by Hwang.

agent, but it was unsuccessful, no compound VI was detected. When the solvent DMI was replaced by tetrahydrofuran (THF), DMPU, or HMPA, or when 1-bromooctane was replaced by 1-chlorooctane or 1-iodooctane, the reactions were also unsuccessful.

We decided to try the alkylation in a protic basic solvent system, instead of in an aprotic one. Alkylation of compound V with 1-bromooctane was conducted in liquid ammonia, using sodamide as base (suspended in the reaction medium). We have found that this reaction produced the expected compound VI in good yield (64.0%) and finally produced the sex pheromone (compound I), 5.08 g of 82.2% active isomer. Semihydrogenation of 3-dodecyn-1-ol in 99 mole % hexane at 0°C followed by crotonation was also conducted to give the sex pheromone of 84.9% active isomer; the result indicated that a low temperature was preferred for the synthesis of the *Z* isomer.

Purification of synthetic sex pheromone on a silver nitrate-impregnated silica gel column was applied to collect purified samples for field evaluation, and two portions of isomeric purities of 85.3% and 94.6% were obtained. The purified synthetic sex pheromones were then tested for the field attraction. The influence of polyethylene tubes and rubber septa dispensers on the male attraction was also evaluated. Tests were carried out in November and December 1990. Poly(ethylene terephthalate) bottles were used to trap the male weevils.

The results of field tests indicated that the synthetic sex pheromone was effective. There was no major difference between isomeric purities of 85.3% and 94.6%, indicating that further purification with the expensive silver nitrate-

TABLE 1. INFLUENCE OF SEX PHEROMONE PURITY AND TYPE OF DISPENSER ON ATTRACTION OF MALE *C. formicarius elegantulus* (SUMMERS)

	Pheromone dispenser					
	Isometric purity					
	85.3%		94.6%		Control	
	Tube	Septum	Tube	Septum	Tube	Septum
Males attracted	4672	431	5042	454	0	0

impregnated silica gel column was not necessary. The same situation was also found when different isomeric purity of *cis*-9-*trans*-11-tetradecadien-1-yl acetate was used to trap the cotton leafworm *Spodoptera litura* (Fabricius); no significantly different attractiveness existed between isomeric purities of 86% and 95% (Lo et al., 1988). However, the total male catch was significantly influenced by the type of dispenser, and the field tests showed that the polyethylene tube was a better dispenser than the rubber septum (Table 1).

CONCLUSION

A new route to synthesize the sex pheromone of sweet-potato weevils proved to be a practical method when the carcinogenic reagent EO and carcinogenic solvent HMPA were replaced by the noncarcinogenic reagent 3-butyn-1-ol and the noncarcinogenic solvent NH_3. Polyethylene tubes with one end sealed were effective dispensers.

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EVALUATION OF FLAVONOIDS IN *Gossypium arboreum* (L.) COTTONS AS POTENTIAL SOURCE OF RESISTANCE TO TOBACCO BUDWORM[1]

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Abstract—Asiatic cottons [*Gossypium arboreum* (L.)] have been investigated as a source of resistance to the tobacco budworm [*Heliothis virescens* (Fab.)] because their diversely colored petals have been presumed to contain various allelochemicals. However, we found that larvae fed *G. arboreum* squares (buds) grew about equally compared with those fed squares from commercial *G. hirsutum* lines. The best source of resistance was found in several *G. hirsutum* double-haploid (DH) lines. In our investigation of allelochemicals, *G. arboreum* lines were found to contain much less gossypol in leaves, squares (buds), and petals than *G. hirsutum* L. lines. Flavonoids were significantly higher in *G. arboreum* lines only in petals. Of 22 *G. arboreum* lines from which squares were gathered and fed to tobacco budworm (TBW) larvae in the laboratory, larval growth was not significantly decreased on any, but larval survival was decreased on six. When the square flavonoids were isolated and incorporated in laboratory diets for the TBW, moderate toxicity was observed. However, the estimated toxicities were not greater than those of the same flavonoid isolates from *G. hirsutum* lines. The most prevalent flavonoids, all previously found in *G. arboreum* plant tissues, were gossypetin 8-0-glucoside and gossypetin 8-0-rhamnoside, neither of which were present in *G. hirsutum* tissue. Quercetin 3-0-glucoside, quercetin-3′-0-glucoside, and quercetin 7-0-glucoside were also present in significant amounts in both species. Gossypetin 8-0-rhamnoside and gossypetin 8-0-glucoside were the most toxic flavonoids tested (the ED_{50}% was estimated to be 0.007 and 0.024) and therefore may prove to be contributing factors of resistance to TBW feeding.

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Key Words—Cotton, *Gossypium arboreum*, tobacco budworm, *Heliothis virescens*, Lepidoptera, Noctuidae, resistance, allelochemicals, flavonoids, gossypetin 8-0-rhamnoside, gossypetin 8-0-glucoside.

INTRODUCTION

Cottons of *Gossypium arboreum* (L.), so-called Asiatic cottons, have been periodically investigated as a source of resistance to the tobacco budworm (TBW) *Heliothis virescens* (F) (Altman et al., 1990). There has been speculation that *G. arboreum* cottons may be resistant because of their apparently high flavonoid content, given that petals are diversely colored, generally yellows, oranges, and reds. However, these Asiatic cottons are not high in gossypol-type terpenoid aldehydes, which are the greatest source of insect resistance in other gossypium species (Altman et al., 1990).

In previous work with *G. hirsutum* cottons (Ramolho, 1983; Ramolho et al., 1984; Parrott et al., 1989; Hedin et al., 1991), we have shown that the feeding behavior of the TBW is subject to and modified by both the morphology of the plant and its chemical constituents (allelochemicals). Female moths oviposit in the terminals of the cotton plant. The hatched larvae first feed on terminals, and then migrate to small squares (buds) on which they feed, finally burrowing into the anthers, where they grow and develop. They attempt to avoid gossypol glands as they feed (Parrott et al., 1983). The flavonoids, although found to be toxic in laboratory feeding studies (Hedin et al., 1983a,b, 1988, 1991), have never been shown to have a primary role in insect resistance in field studies of *G. hirsutum*.

Parks (1965) identified 24 flavonoids in *G. arboreum* (L.), including gossypetin 7-0-glucoside, gossypetin 8-0-glucoside, quercetin 3-0-glucoside, quercetin 3-0-rhamnoglucoside, quercetin 7-0-glucoside, kaempferol 3-0-glucoside, and kaempferol 3-0-rhamnoglucoside. Waage and Hedin (1984) found two additional flavonoid glycosides in *G. arboreum* flower petals, gossypetin 8-0-rhamnoside and quercetin 3′-0-glucoside. These later compounds along with quercetin 3-0-glucoside and quercetin 7-0-glucoside showed antibacterial activity against *Pseudomonas maltophilia* and *Enterobacter cloacae* (Hedin and Waage, 1986).

Altman et al. (1990) analyzed eight typed race stocks of *G. arboreum* (L.) for terpenoid aldehydes (TAs). *G. arboreum* accession A2-2 was devoid of hemigossypolone, contained only 5.8% of total terpenoid aldehydes as gossypol, and the remainder (75.9%) was comprised of the heliocides, chiefly H_1. Four *G. arboreum* race stocks contained small amounts of hemigossypolone. H_1 was the predominant TA in each *G. arboreum* line except accession A2-198, which did not contain H_1 but had a large amount of H_2.

In this study, laboratory tests in which TBW larvae were fed squares were

conducted to determine the relative resistance of *G. arboreum* lines. Laboratory feeding tests were also carried out with the flavonoid isolates to estimate their relative contributions, if any, to insect resistance. The content of flavonoids and three other allelochemicals in squares, leaves, and petals of 22 *G. arboreum* lines and six *G. hirsutum* lines was determined to identify any correlations that might exist between content and resistance.

METHODS AND MATERIALS

Cultivars and Agronomic Practices. Seeds of cotton lines were planted in field plots in early May 1987. No insecticides were used on plants in these plots after they began to produce squares. There were 22 experimental lines of 17 *G. arboreum* accessions identified with PI numbers, three doubled haploids of *G. hirsutum*, Stoneville 213 (ST213), Stoneville 213 glandless, and Stoneville 825 (ST825) in the experiment.

Gathering and Processing of Plant Tissue. Leaves, squares, and petals were harvested on July 21 and July 24, frozen, freeze-dried, and ground through a 40-mesh screen. The powders were stored in vials at −20°C until they were evaluated.

Insect and Feeding Regimens. Neonate TBW larvae used in the various tests were obtained from a colony at the Crop Science Research Laboratory, Mississippi State, Mississippi, that was maintained as described by Jenkins et al. (1982).

Squares (8 mm diameter) were harvested throughout the summer, washed in a 0.5% sodium hypochlorite solution, and placed individually into 30-ml plastic cups with 2% agar in the bottom (6.4 mm) to prevent desiccation. For the *G. arboreum* lines, an equal number of neonate TBW larvae were placed on each square and allowed to feed. Fresh squares were introduced every two days. Larvae were weighed at seven days. The test was a completely randomized design with eight squares per line, and the test was repeated twice. Data were subjected to analysis of variance (ANOVA). Least significant differences ($P = 0.05$) were calculated from the data analyzed by ANOVA. These statistical procedures were performed with SAS software (SAS Institute, 1985).

In order to obtain estimates of ED_{50} values (percent of compound required to reduce weight gain by 50%) for various flavonoid isolates and authentic compounds, larvae were reared on the standard TBW larval diet with addition of flavonoids (Hedin et al., 1991). Severely limited availability of tissue (with no expectation of future availability) restricted the scope of the test. Generally, less than 5 g of plant tissue was available, so that total flavonoid isolates seldom exceeded 35–40 mg. Because 50 g of diet yielded only 16 growth cells (a minimum requirement) and 25 mg of isolate was generally required to decrease

growth by about 50%, typically, tests could only be conducted at two levels. The ED_{50} of the commercially available flavonoid, rutin, was obtained by conducting growth tests at six levels with the results being plotted on semilog paper to establish a straight-line growth slope ($y = 122.87 - 27.53x$). Then, on the assumption that slope lines for other flavonoids would be roughly parallel to the rutin line, ED_{50} values were determined using the available data points. The larvae were weighed after seven days.

Analysis of Allelochemicals. Analyses for gossypol and related terpenoid aldehydes (total gossypol) were performed on cyclohexane–ethyl acetate–acetic acid (500:500:1; CHEA) extracts of plant (whole bud and leaf) tissue by the phloroglucinol reaction [2% in absolute EtOH–concentrated HCl (1:1)] and were let stand for 1 hr, with subsequent spectrometric analysis at 550 nm. The concentration was determined by comparison with data obtained from authentic gossypol and is expressed as gossypol equivalents. Condensed tannin analyses were performed on 70% aqueous methanol (MW) extracts of tissue. The anthocyanidin chromophore was developed from the tannin by boiling 1 hr with 1-butanol–HCl (95:5) (Hedin et al., 1983a,b). The concentration was determined by comparison with the absorbancy at 550 nm of a purified cotton condensed tannin sample, the structure of which was elucidated by Collum et al. (1981). The anthocyanin content was determined by measuring the absorbancy at 540 nm of an extract of freeze-dried tissue extracted with methanol–water–HCl (79:19:3), using the molar extinction coefficient (E) of cyanidin 3-β-glucoside (Hedin et al., 1967). Flavonoids were determined after extraction of freeze-dehydrated tissue with 70% aqueous acetone. Diphenylboric acid–ethanolamine complex (Natural Product Reagent A, Aldrich Chemical Co., 1%) in methanol was added, and the chromophore absorptivity at 440 nm was determined and compared to that obtained from a purified sample of isoquercitrin, a representative flavonoid in *G. hirsutum*.

HPLC Analyses of Terpenoid Aldehydes. HPLC analysis of the gossypol-type terpenoid aldehydes was performed on a reverse-phase C-18 column according to the procedure of Stipanovic et al. (1988), who also kindly provided samples of hemigossypolone, and heliocides H_1 and H_2 for column calibration.

Isolation of G. arboreum *Flavonoids for Feeding Tests and Semiquantitative Analysis.* For use in feeding tests, freeze-dried leaf, square, and petal samples, 2 g of each, were extracted two times with 70% aqueous methanol, the extracts were combined, evaporated to a small volume, freeze-dried, and weighed. The samples were redissolved into 20 ml of 50% aq. methanol and chromatographed on a Polyamide 6 (J.T. Baker Chemical Co., Phillipsburg, New Jersey) column (2 cm high × 6 cm diameter), to elute amino acids, carbohydrates, salts, etc. The total flavonoids were then eluted with 70% aqueous methanol and methanol. The samples were evaporated to near dryness, resuspended in H_2O, and freeze-dried. The yields (2.5–4.0%) paralleled the analysis data (see previous section).

For semiquantitative analysis, small quantities (~5 mg) of the freeze-dried total flavonoids were subjected to two-dimensional TLC on 10 × 10-cm cellulose (EM Laboratories, Inc., Elmsford, New York) plates using *t*-butanol–acetic acid–water 3:1:1 in the first dimension and 15% aq. acetic acid in the second dimension (Mabry et al., 1970). The plate was sprayed with Natural Product Reagent (see previous section) and individual flavonoids (visualized spots) were carefully scraped into test tubes and eluted with 1 ml of 70% aqueous methanol. After addition of additional Natural Product Reagent (1 ml of 1% reagent in 70% aqueous methanol) the chromophores were analyzed at 440 nm, and the content was determined with reference to a standard curve (see previous section). Finally, the data was normalized by conversion to percent of total flavonoids.

Identification of G. arboreum *Flavonoids.* Individual flavonoids were isolated by one-dimensional TLC procedures (see previous section) and then were identified by making spectral and chromatographic comparisons with authentic samples retained from our previous work (Waage and Hedin, 1984). For verification, flavonoid isolates from both *G. arboreum* and *G. hirsutum* tissues were hydrolyzed with 1 N aq. HCl. The aglycone fraction was extracted from the reflux mixture with ethyl acetate and examined by both TLC and EI-MS methods. Gossypetin was found in *G. arboreum* samples, but not in *G. hirsutum* samples.

RESULTS AND DISCUSSION

Allelochemicals in G. arboreum *and* G. hirsutum *Plant Tissues.* The average contents of flavonoids, total gossypol, anthocyanins, and tannins in leaves, squares, and petals of multiple lines of these two species are listed in Table 1. All tissues of *G. arboreum* were low in gossypol relative to *G. hirsutum*. Anthocyanin levels were nearly identical except for the much higher level (0.05 vs. 0.45%) in petals of *G. arboreum*. Tannin levels of *G. arboreum* in squares and petals were also much higher, but tannin levels were higher in *G. hirsutum* leaves. The flavonoid content of *G. arboreum* was higher than *G. hirsutum* in squares and petals, but lower in leaves. The average contents of these allelochemicals in *G. hirsutum* were similar to those found by us in earlier work (Hedin et al., 1983a,b, 1991).

Because the distribution of terpenoid aldehydes is the most important factor of resistance in *G. hirsutum* to the TBW, it was considered important to determine their distribution in composited square and petal samples of *G. arboreum* tissue. The terpenoid aldehydes in petals were comprised of 98.1% gossypol, 1.6% hemigossypolone, and only traces of heliocides. Those in squares were comprised of 3.1% hemigossypolone, 12.9% gossypol lactone, 69.5% gossy-

TABLE 1. ALLELOCHEMICALS IN *G. hirsutum* AND *G. arboreum* PLANT TISSUES, AVERAGES FOR MULTIPLE LINES OF EACH WITH STANDARD ERROR OF MEANS[a]

Tissue	Percent content			
	Gossypol[b]	Flavonoids	Anthocyanin	Tannins
Leaves				
G. hirsutum	0.37 ± 0.02	4.38 ± 0.62	0.17 ± 0.02	11.60 ± 0.65
G. arboreum	0.10 ± 0.02	2.18 ± 0.22	0.18 ± 0.03	7.49 ± 0.57
Squares				
G. hirsutum	0.40 ± 0.02	2.12 ± 0.08	0.13 ± 0.02	6.27 ± 0.37
G. arboreum	0.26 ± 0.04	2.37 ± 0.25	0.15 ± 0.03	15.18 ± 0.66
Petals				
G. hirsutum	0.75 ± 0.06	2.09 ± 0.50	0.05 ± 0.01	4.55 ± 0.44
G. arboreum	0.27 ± 0.04	4.55 ± 0.34	0.45 ± 0.08	8.65 ± 0.44

[a] *G. hirsutum* lines: see Hedin et al. (1991); *G. arboreum* lines: see PI numbers in Table 2.
[b] Total gossypol as determined by the phloroglucinol procedure.

pol, 10.4% heliocide H_1, and 4.1% heliocide H_2. In *G. hirsutum* lines that were resistant to the tobacco budworm, hemigossypolone and heliocides H_1 and H_2 were high relative to susceptible lines (Hedin et al., 1991) and gossypol was unchanged. Therefore, resistance in *G. hirsutum* appeared to be closely associated with hemigossypolone and the heliocides. By comparison, based on gossypol content, *G. arboreum* lines would not be expected to be resistant because total gossypol was relatively low in squares and petals, and gossypol was the dominant terpenoid aldehyde in *G. arboreum* lines. Therefore it seems unlikely that terpenoid aldehydes are a decisive factor of resistance in *G. arboreum* lines.

Previously, we found that survival of TBW larvae was less on nonglanded *G. hirsutum* red petals than on the first-day (younger) white petals (Hedin et al., 1983a,b). In those lines, chrysanthemin was greatly increased as expected in the second-day red petals and gossypol was low. In glanded lines, both gossypol and anthocyanin were higher in the red petals and larval growth was lower. However, the effect of allelochemicals in the petals of intact plants on larval growth may be marginal because the larvae do not consume significant amounts of petals in their normal feeding patterns (Parrott et al., 1989). In fact, as previously discussed (Ramolho, 1983; Ramolho et al., 1984; Parrott et al., 1989; Hedin et al., 1991), feeding behavior of the TBW on cotton is subject to and modified by both the morphology of the plant and its chemical constituents in the various tissues.

Effects of Cotton Flavonoids and Other Allelochemicals on Survival and Growth of the TBW. Table 2 lists data on growth and survival of TBW larvae fed squares of several *G. arboreum* and *G. hirsutum* lines. It also lists ED_{50}

TABLE 2. TBW LARVAL SURVIVAL AND GROWTH ON *G. hirsutum* AND *G. arboreum* SQUARES AND ON ARTIFICIAL DIET WITH INCORPORATED SQUARE FLAVONOIDS AND CONTENT OF ALLELOCHEMICALS IN SQUARES

Test lines	Survival 3 days (*N*)	Survival 7 days (*N*)	Larval Weight (% of ST213)[a]	ED_{50} (%)	Flav (%)	Goss (%)	Antho (%)	Tannin (%)
G. hirsutum lines								
ST213 (control)	8.3	3.9	100.0	0.080	2.31	0.43	0.14	11.54
ST213 glandless			148.2	0.095	2.14	0.12	0.16	10.10
ST825			79.0	0.066	2.05	0.37	0.11	
DH118			67.1	0.056	2.66	0.68	0.11	
DH121			76.2	0.060	2.71	0.69	0.12	
DH126			25.2	0.040	2.75	0.70	0.16	
G. arboreum lines decreasing larval growth								
PI408770	5.6	2.6	77.6	0.056	1.43	0.31	0.12	16.48
PI408767 tan, few glands	6.4	3.1	80.8	0.048	1.79	0.11	0.05	15.41
PI408766 green	6.6	3.6	85.9	0.060	2.14	0.24	0.14	16.56
PI408769 brown	2.5[b]	1.6	87.2	0.034	1.59	0.31	0.12	16.40
PI408767 tan	4.4[b]	1.8[b]	89.5	0.060	1.72	0.37	0.13	16.46
PI408766, off type	5.4	3.6	90.2	0.060	2.02	0.30	0.14	16.79
PI408764	4.3	2.0[b]	90.2	0.090	2.43	0.29	0.17	12.58
PI408766 red	5.1	2.1	95.5	0.062	2.10	0.27	0.13	16.93
PI408761	4.8	2.8	96.7	0.080	2.19	0.31	0.19	17.10
PI408757	6.8	3.7	98.4	0.060	3.12	0.27	0.16	15.50
Mean	5.2	2.7	89.2	0.06	2.05	0.28	0.14	16.02
G. aboreum lines not decreasing larval growth								
PI408763	6.0	1.9[b]	101.9	0.076	2.58	0.28	0.15	17.95
PI408758	6.1	2.8	104.5	0.074	2.26	0.20	0.14	12.75
PI408767	6.8	2.9	106.0	0.090	1.90	0.16	0.15	15.96
PI408765	5.4	2.1[b]	108.4	0.056	2.48	0.25	0.16	13.42
PI408770, off-type red	7.4	3.6	113.5	0.066	2.12	0.31	0.13	16.24
PI408770 off-type green	6.2	3.5	113.6					
PI408762	4.3	2.2	113.9	0.070	3.51	0.24	0.23	13.53
PI408762, few glands	2.2[b]	1.5[b]	117.6	0.070	2.60	0.23	0.20	14.25
PI408754	4.9	3.0	121.4	0.052	3.77	0.39	0.16	11.92
PI408755	9.2	4.7	131.8	0.056	3.47	0.19	0.14	13.85
PI408769 white	8.2	4.4	135.5	0.076	2.17	0.31	0.14	14.24
PI408758, few glands	3.7	1.7[b]	237.2	0.085	2.46	0.11	0.12	13.16
Mean	5.9	2.9	125.4	0.07	2.67	0.24	0.16	14.30
Mean all *G. arboreum* lines	5.6	2.8	109.0	0.091	2.37	0.26	0.15	15.18
LSD 0.05[c]	3.0	1.7	37.3					

[a] ED_{50} as per cent of isolated flavonoids in larval diet required to decrease growth by 50%.
[b] Significantly less than control at 0.05 level LSD.
[c] LSD for *G. arboreum* line comparisons with control ST213.

values (percent of compound required to reduce weight gain by 50%) for larvae fed diets in which *G. hirsutum* and *G. arboreum* flavonoid isolates (a mixture of flavonoids) were incorporated. Finally, it lists the content of the four major allelochemical groups (total gossypol, flavonoids, anthocyanins, and condensed tannins) of the squares of 10 of 22 *G. arboreum* lines on which larval weight was equal to or less than on ST213, and 12 of 22 lines on which larval weight was equal to or greater than on ST213. For comparison, additional data on several other *G. hirsutum* lines, some resistant to the TBW, were included.

None of the *G. arboreum* lines, except PI 408758 (which had very few glands) was statistically significant (lower or higher) with regard to larval growth, and only six were so with regard to larval survival at seven days, while all of the double haploid *G. hirsutum* lines produced smaller larvae. Estimates of ED_{50} values of the *G. arboreum* flavonoids (when incorporated in laboratory diets) varied from 0.034 to 0.090%. The average ED_{50} for lines decreasing growth was 0.06% vs. 0.07% for lines not decreasing growth (not significant). The average total flavonoid content (2.05%) of lines producing smaller larvae was lower than that of lines on which larvae were larger (2.67%), not suggestive that flavonoids were important resistance factors. On the other hand, lines decreasing growth were higher in total gossypol and tannins.

Distribution and Toxicity (ED_{50}, %) of G. arboreum *and* G. hirsutum *Flavonoids.* Table 3 lists the major flavonoids found in *G. arboreum* and *G. hir-*

TABLE 3. DISTRIBUTION OF *G. hirsutum* AND *G. arboreum* FLAVONOIDS IN LEAVES, SQUARES, AND PETALS; PERCENT OF TOTAL FLAVONOIDS AND LARVAL TOXICITY

Flavonoid	ED_{50} (%)	*G. hirsutum* (%)			*G. arboreum* (%)		
		Leaves	Squares	Petals	Leaves	Squares	Petals
Gossypetin 8-0-glucoside	0.024	0	0	0	25.8	44.7	49.8
Gossypetin 8-0-rhamnoside	0.007	0	0	0	7.3	16.5	13.7
Quercetin 3'-0-glucoside		5.5	6.2	5.5	5.3	5.0	6.4
Quercetin 7-0-glucoside	0.045	42.6	36.8	28.9	22.1	16.0	11.3
Quercetin 7-0-rhamnoside		0	0	0	0	0	5.6
Quercetin 3-0-glucoside	0.060	51.8	53.8	55.4	28.0	7.9	7.4
Quercetin 3-0-rhamnosyl glucoside	0.031	0	3.0	3.0	3.2	4.6	4.0
Quercetin diglycoside 1		0.2	0.2	0	2.6	3.7	0.5
Quercetin diglycoside 2		0	0	0	2.8	4.6	0
Quercetin	0.042	0	0	3.9	0	0.1	0

sutum leaves, squares, and petals expressed as percent of total flavonoids and their estimated relative toxicities expressed as ED_{50}, %. These flavonoids were the most prevalent in *G. arboreum* of those reported by Parks (1965) and Waage and Hedin (1984) and in *G. hirsutum* (Hedin et al., 1968).

Gossypetin 8-0-glucoside and gossypetin 8-0-rhamnoside were the most toxic (ED_{50} = 0.024 and 0.007%). They were prominent flavonoids in *G. arboreum* plant tissues, but they were not found in *G. hirsutum*. Quercetin 3-0-glucoside and quercetin 7-0-glucoside were the most prevalent flavonoids in *G. hirsutum*, and they were also prominent in *G. arboreum*. Quercetin 3′-0-glucoside was also present in significant quantities in tissues of both species.

The ED_{50} values are of the same magnitude as those reported earlier (Hedin et al., 1983a,b). Four of the flavonoid glycosides could not be isolated in sufficient quantities for dietary tests. Of those tested, rhamnosides were somewhat more toxic than glucosides, supporting previous work by us (Hedin and Waage, 1986) in which it was shown that rhamnosides inhibited the growth of two bacterial species more than glucosides. Although *G. arboreum* square flavonoids were not more toxic in our dietary test than those isolated from *G. hirsutum* (Table 2), gossypetin 8-0-glucoside and gossypetin 8-0-rhamnoside may yet be contributing factors of resistance to TBW growth, considering that they are the most prevalent flavonoids in these lines. The development of cotton lines with higher levels of rhamnosides either by breeding or genetic strategies, may become a research objective.

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ENANTIOSPECIFIC SYNTHESIS OF (3*S*,6*R*)-3-HYDROXY-1,7-DIOXASPIRO[5.5]UNDECANE AND ITS (4*R*)-4-HYDROXY ISOMER [MINOR COMPONENTS OF OLIVE FRUIT FLY (*Dacus oleae*) SEX PHEROMONE][1]

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Abstract—The synthesis of (3*S*,6*R*)-3-hydroxy-1,7-dioxaspiro[5.5]undecane **(8)** and its (4*R*)-4-hydroxy isomer **(11)** from (3*R*,4*S*,6*R*)-3,4-dihydroxy-1,7-dioxaspiro[5.5]undecane **(1)**, obtained from D-fructose, has been accomplished by regioselective deoxygenation at C-4 and C-3, respectively, of the appropriately protected derivatives.

Key Words—Enantiospecific synthesis, enantiomers, spiroacetal, pheromone, Dacus oleae, olive fruit fly, Diptera, Tephritidae, D-fructose.

INTRODUCTION

The title compounds, minor components of the pheromone bouquet of olive fruit fly (*Dacus oleae*), were isolated and identified by Baker et al. (1982). Lately, several racemic (Kocienski and Yeates, 1983; Kay and Williams, 1983; Baker and Herbert, 1987; DeShong et al., 1988) and enantiospecific syntheses (Redlich and Francke, 1984; Mori et al., 1985; Bestmann and Schmidt, 1986) have been reported, where the D-glucose, *S*-malic acid and 2,3-*O*-isopropylidene-D-glyceraldehyde were used as chiral precursors, respectively. We report herein an enantiospecific synthesis of such compounds starting with D-fructose as the chiral pool.

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[1]Enantiospecific synthesis of spiroacetals. Part III. For Part II, see Izquierdo et al. (1991).

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METHODS AND MATERIALS

General Methods. Solutions were dried over $MgSO_4$ before concentration under reduced pressure. [^{13}C]- (75.4 MHz) and [^{1}H]NMR spectra (300 MHz, internal Me_4Si) were recorded with a Bruker AM-300 spectrometer of solutions in $CDCl_3$. The assignment of resonances for **8** and **11** were assisted by [^{13}C–^{1}H]COSY spectra. Infrared spectra were recorded with a Perkin-Elmer 782 spectrophotometer and mass spectra with a Hewlett-Packard HP-5988-A spectrometer. Optical rotations were measured on solutions in chloroform (1-dm tube) with a Perkin-Elmer 141 polarimeter. Thin-layer chromatography (TLC) was performed on silica gel G (Merck) (0.25 mm) with detection by charring with sulfuric acid. Column chromatography was done with silica gel Merck (7734). Tetrahydrofuran (THF) was distilled from sodium–benzophenone ketyl and stored over 4-Å molecular sieves prior to use. All compounds were shown to be homogeneous by chromatography and characterized by NMR and mass spectrometry.

(3R,4S,6R)-4-Benzyloxy-3-hydroxy-1,7-dioxaspiro[5.5]undecane **(3)** *via Dibutylstannylene Derivative* **(2)**. To a solution of 0.9 g (4.8 mmol) of (3*R*,4*S*,6*R*)-3,4-dihydroxy-1,7-dioxaspiro[5.5]undecane **(1)** (Izquierdo et al., 1991) in anhydrous methanol (15 ml), di-*n*-butyltin oxide (1.2 g, 4.8 mmol) was added and the suspension heated under reflux for 2 hr to give a clear solution. Then the solvent was removed to afford the 3,4-dibutylstannylenedioxy derivative **(2)** as a solid foam that was dried under vacuum over P_2O_5 overnight. Compound **2** was taken up in dry *N*,*N*-dimethylformamide (10 ml), treated with benzyl bromide (1.7 ml, 14.5 mmol) and heated at 110–120°C for 45 min. TLC (ether) revealed the presence of a faster-running product. Concentration of the solvent gave a residue that was dissolved in dichlorometane, washed with brine, water, and concentrated to give a residue. Column chromatography (ether–hexane, 1:2 to 1:1) afforded **3** as a syrup (620 mg, 47%), $[\alpha]_D$ −75° (*c* 1.2); ν^{film} 3469 (OH), 3092, 3066, and 3034 (C—H, aromatic), 2944 and 2874 (C—H), 1222, 1172, 1113, 1096, 1072, 1052, 1042, 1029, 1000, and 989 (C—O—C), 737 and 698 cm^{-1} (aromatic). [^{1}H]NMR (80 MHz): δ 7.30 (bs, 5 H, $CH_2\underline{Ph}$), 4.53 (s, 2 H, $\underline{CH_2}Ph$), 4.00–3.40 (m, 6 H, H-2,2′,3,4,8,8′), 2.43 (bs, 1 H, HO-3), 2.00–1.00 (m, 8 H, H-5,5′,9,9′,10,10′,11,11′). For the [^{13}C]NMR, see Table 1. Mass spectrum: *m/z* 279 (1, M^+; pl1), 278 (4, M^+), 217 (5), 172 (5), 157 (9), 139 (11), 127 (15), 101 (20, $C_5H_9O_2^+$), 99 (22), 91 (100, $C_7H_7^+$), 83 (11), 71 (11), 65 (13), 55 (20) and 43 (20, Ac^+).

(3R,4S,6R)-3-Benzoyloxy-4-benzyloxy-1,7-dioxaspiro[5.5]undecane **(4)**. Compound **3** (0.62 g, 2.2 mmol) was benzoylated in dry pyridine (2 ml) with benzoyl chloride (0.5 ml) in the usual manner to yield, after column chromatography (ether–hexane, 1:5), **4** as a colorless syrup (0.66 g, 77%), $[\alpha]_D$ −127° (*c* 1); ν^{film} 3067 and 3035 (C—H, aromatic), 2946 and 2873 (C—H), 1721

TABLE 1. [^{13}C] NMR DATA FOR **3–5** AND **7–11**

Compound	C-2	C-3	C-4	C-5	C-6	C-8	C-9	C-10	C-11	C=O	S-Me	$\underline{CH_2}$Ph	Ph	C=S
3	62.60	65.45	72.73	36.09	97.25	60.84	25.05	18.37	35.05			70.13	138.14 i 128.53 m 127.85 p 127.86 o	
4	61.68	67.48	71.26	37.82	97.45	61.07	25.09	18.51	35.14	166.39		70.29	138.29 i 133.03 p 130.04 i 129.24 o 128.42 m 128.38 m 127.67 p 127.59 o	
5	61.34	64.70	71.40	39.80	97.32	61.07	25.04	18.51	35.04	166.91			133.27 p 130.14 i 129.85 o 128.46 m	
7	62.13	68.06	22.84	30.70	95.04	61.09	25.30	18.58	35.34	166.23			132.97 p 130.74 i 129.78 o 128.36 m	
8	64.84	64.46	25.69[a]	30.19	95.06	60.64	25.57[a]	18.86	35.56					
9	60.99	71.04	76.00	37.96	97.28	60.69	24.95	18.37	34.88		18.69	70.37	137.00 i 128.40 m 127.61 o,p	215.76
10	58.86	32.27	71.53	42.42	97.34	60.39	25.13	18.54	35.61			69.90	138.84 i 128.40 m 127.59 p 127.50 o	
11	58.89	35.59	64.16	45.80	97.41	60.23	25.47	19.90	35.84					

[a] Assignment uncertain within the pair.

(C=O), 1453 (benzyl), 1275, 1119, 1098, 1044, and 984 (C—O—C), 737 and 712 cm^{-1} (aromatic). [^{1}H]NMR: δ 8.15–8.00 and 7.60–7.20 (2 m, 10 H, COPh and $CH_2\underline{Ph}$), 5.50 (m, 1 H, H-3), 4.69 and 4.49 (2 d, 2 H, J = 11.7 Hz, $\underline{CH_2}Ph$), 4.05 (ddd, 1 H, $J_{3,4}$ = 3, $J_{4,5a}$ = 9.4, $J_{4,5e}$ = 8 Hz, H-4), 3.94 (dd, 1 H, $J_{2a,2e}$ = 12.8, $J_{2a,3}$ = 2.1 Hz, H-2*a*), 3.82 (dd, 1H, $J_{2e,3}$ = 1.3 Hz, H-2*e*), 3.65–3.58 (m, 2 H, H-8*a*,8*e*), 2.01–1.97 1.85–1.75, and 1.65–1.50 (3 m, 8 H, H-5*a*,5*e*,9*a*,9*e*,10*a*,10*e*,11*a*,11*e*). For the [^{13}C]NMR, see Table 1. Mass spectrum: *m/z* 383 (1, M^++1), 382 (2, M^+), 260 (1, M^+-PhCOOH-C_7H_7), 154 (12), 139 (7), 105 (100, $PhCO^+$), 101 (4, $C_5H_9O_2^+$), 98 (23), 91 (43, $C_7H_7^+$), 77 (41), 55 (19), and 54 (12).

*(3*R*,4*S*,6*R*) -3-Benzoyloxy-4-hydroxy-1,7-dioxaspiro[5.5]undecane* ***(5)***. A solution of **4** (1.64 g, 4.3 mmol) in dry methanol (80 ml) was hydrogenated at 1.5 atm over palladium oxide (780 mg) for 6 hr. TLC (ether–hexane, 3:2) revealed the presence of a new compound of lower mobility. The catalyst was removed by filtration, washed with methanol, and the filtrate concentrated to a residue that was chromatographed (ether–hexane, 1:2 to 1:1) to afford **5** as a syrup (1.25 g, quantitative), $[\alpha]_D$ −121.2° (*c* 0.9); ν^{film} 3471 (OH), 3067 and 3037 (C—H, aromatic), 2948 and 2876 (C—H), 1724 (C=O), 1453, 1273, 1226, 1172, 1119, 1073, 1044, 981 (C—O—C), 734, and 714 cm^{-1} (aromatic). [^{1}H]NMR: δ 8.10–8.00 and 7.60–7.40 (2 m, 5 H, relative intensity 2:3, COPh), 5.22 (m, 1 H, H-3), 4.29 (ddd, 1 H, $J_{3,4}$ = 3.4, $J_{4,5a}$ = 12, $J_{4,5e}$ = 5.2 Hz, H-4), 3.93 (dd, 1 H, $J_{2a,2e}$ = 13, $J_{2a,3}$ = 2 Hz, H-2*a*), 3.82 (dd, 1 H, $J_{2e,3}$ = 1.4 Hz, H-2*e*), 3.60 (m, 2 H, H-8*a*,8*e*), 2.10 (bs, 1 H, HO-4), 1.95 (ddd, 1 H, $J_{5a,5e}$ = 11.8, $J_{3,5e}$ = 1 Hz, H-5*e*), 1.80 (t, 1 H, H-5*a*), 1.88–1.72 and 1.64–1.48 (2 m, 6 H, H-9*a*,9*e*,10*a*,10*e*,11*a*,11*e*). For the [^{13}C]NMR, see Table 1. Mass spectrum: *m/z* 293 (1, M^++1), 292 (5, M^+), 261 (2), 192 (7, M^+-$C_5H_8O_2$), 187 (11, M^+-PhCO), 175 (4), 170 (4, M^+-PhCOOH), 140 (7), 139 (9), 127 (6), 123 (6), 105 (100, $PhCO^+$), 101 (5, $C_5H_9O_2^+$), 98 (13), 84 (14), 77 (46), and 55 (22).

*(3*S*,6*R*)-3-Benzoyloxy-1,7-dioxaspiro[5.5]undecane* ***(7)***. To a solution of **5** (1.15 g, 4 mmol) in dry 1,2-dichloroethane (25 ml), 1,1′-thiocarbonyldiimidazole (1.1 g, 6.2 mmol) was added and the mixture heated at 90° for 45 min. TLC (ether–hexane, 2:1) revealed the presence of a new compound of slightly higher mobility. The mixture was diluted with dichloromethane (25 ml), washed with 10% aqueous hydrochloric acid, water, 10% aqueous sodium hydrogen carbonate, and water. Concentration of the solvent gave a residue that was chromatographed (ether–hexane, 1:3) to afford the corresponding 4-imidazol-1-yl-thiocarbonyloxy derivative **6** (1.55 g) as a pale yellow syrup that was slightly impure.

A solution of **6** (1.55 g) and azo-bis-isobutyronitrile (100 mg) in dry toluene (30 ml) was added dropwise to a stirred solution of refluxing tri-*n*-butyltin hydride (1.7 ml, 6 mmol) in the same solvent (20 ml) under N_2. Refluxing was

continued for 6 hr. TLC (ether–hexane, 2:1) revealed that **6** had disappeared and that a new compound of higher mobility was present. Concentration of the solvent, followed by column chromatography (ether–hexane, 1:4) of the residue afforded syrupy **7** (0.9 g, 81.5% from 5), $[\alpha]_D$ −81.5° (*c* 1); ν^{film} 3067 (C—H, aromatic), 2949 and 2872 (C—H), 1717 (C=O) 1452, 1276, 1114, 1082, 1063, 996, 986 (C—O—C), and 709 cm^{-1} (aromatic). [^{1}H]NMR: δ 8.10–8.04, 7.58–7.51, and 7.46–7.39 (3 m, 5 H, relative intensity 2:1:2, COPh), 5.05 (m, 1 H, H-3), 3.91 (dd, 1 H, $J_{2a,2e}$ = 12.7, $J_{2a,3}$ = 1.7 Hz, H-2*a*), 3.83 (dt, 1 H, $J_{2e,3} = J_{2e,4e}$ = 1.9 Hz, H-2*e*), 3.69–3.63 (m, 2 H, H-8*a*,8*e*), 2.20 (ddt, 1 H, $J_{4a,4e} = J_{4a,5a}$ = 13.3, $J_{3,4a}$ = 3, $J_{4a,5e}$ = 4.7 Hz, H-4*a*), 1.95–1.81 (m, 3 H, H-5*a*,10*a*,11*e*), 1.74 (dm, 1 H, H-4*e*), 1.67–1.51 (m, 4 H, H-5*e*,9*a*,9*e*,10*e*), 1.48 (td, 1 H, $J_{11a,11e} = J_{10a,11a}$ = 13.2, $J_{10e,11a}$ = 4.2 Hz, H-11*a*). For the [^{13}C]NMR, see Table 1. Mass spectrum: *m/z* 278 (0.1, M^++2), 277 (0.5, M^++1), 276 (3, M^+), 176 (20, M^+-$C_5H_8O_2$), 154 (5, M^+-PhCOOH), 141 (3), 124 (7), 105 (100, $PhCO^+$), 98 (60), 77 (36), and 55 (20).

(3S,6R)-3-Hydroxy-1,7-dioxaspiro[5.5]undecane ***(8)***. Conventional debenzoylation of **7** (420 mg, 1.52 mmol) in anhydrous methanol (5 ml) and 0.2 N sodium methoxide (5 ml) for 12 hr was carried out. Work-up of the reaction mixture followed by column chromatography (ether–hexane, 1:1) yielded **8** (160 mg, 61%) as a colorless syrup, $[\alpha]_D$ −134° (*c* 0.9), {lit. (Mori et al., 1985) $[\alpha]_D$ −129° (*c* 0.93, ether); lit. (Bestmann and Schmidt, 1986) $[\alpha]_D$ −135° (*c* 1, CH_2Cl_2)}; ν^{film} 3421 (OH), 2943 and 2873 (C—H), 1235, 1106, 1083, 1066, 1051, 994, 988, and 940 cm^{-1} (C—O—C). [^{1}H]NMR (C_6D_6) data were in accord with those found in the literature (Bestmann and Schmidt, 1986). For the [^{13}C]NMR, see Table 1. Mass spectrum: *m/z* 172 (2, M^+), 155 (2, M^+-OH), 149 (5), 142 (5), 141 (4), 117 (8, $C_5H_9O_3^+$), 105 (11), 101 (17, $C_5H_9O_2^+$), 98 (34), 85 (100), 84 (56), 57 (18), 55 (24, M^+-$C_5H_9O_2$), and 47 (42).

(3R,4S,6R)-4-Benzyloxy-3-[(thiomethyl)thiocarbonyloxy]-1,7-dioxaspiro[5.5]undecane ***(9)***. A solution of **3** (740 mg, 2.7 mmol) in anhydrous THF (10 ml) was added to a stirred solution of sodium hydride (330 mg, 13.7 mmol) (80% oil dispersion) in the same solvent (5 ml) and imidazole (50 mg) under N_2 at room temperature. The mixture was refluxed for 30 min, cooled, and carbon disulfide (0.4 ml, 6 mmol) was added dropwise. The reaction was refluxed again for 30 min, cooled, and methyl iodide (0.3 ml, 4.7 mmol) was added slowly and the mixture was heated under reflux for 1 hr. TLC (ether–hexane, 3:2) revealed the presence of a new compound of higher mobility. The excess hydride was destroyed by cautious addition of ether saturated with water (30 ml) and water (15 ml). The organic phase was separated, and the aqueous phase extracted with ether (2 × 10 ml). The combined extracts were washed with brine, water, concentrated, and the residue chromatographed (ether–hexane, 1:4) to yield syrupy **9** (780 mg, 80%), $[\alpha]_D$ −118° (*c* 1); ν^{film} 3092, 3065 and

3032 (C—H, aromatic), 2948 and 2873 (C—H), 1221 (C=S), 1096, 1074, 1063, 1045, and 977 (C—O—C), 737, and 698 cm^{-1} (aromatic). [^{1}H]NMR: δ 7.40–7.20 (m, 5 H, $CH_2\underline{Ph}$), 6.10 (bs, 1 H, H-3), 4.64, and 4.46 (2 d, 2 H, J = 11.8 Hz, $\underline{CH_2}Ph$), 4.04 (ddd, 1 H, $J_{3,4}$ = 3.1, $J_{4,5e}$ = 5.4, $J_{4,5a}$ = 11.6 Hz, H-4), 4.03 (dd, 1 H, $J_{2a,3}$ = 2, $J_{2a,2e}$ = 13 Hz, H-2*a*), 3.74 (dd, 1 H, $J_{2e,3}$ = 1.2 Hz, H-2*e*), 3.62–2.95 (m, 2 H, H-8*a*,8*e*), 2.56 (s, 3 H, Me-S), 2.03–1.70, and 1.70–1.45 (2 m, 8 H, relative intensity 1:1, H-5*a*,5*e*,9*a*,9*e*,10*a*,10*e*,11*a*,11*e*). For the [^{13}C]NMR, see Table 1. Mass spectrum: *m/z* 368 (0.1, M^+), 336 (2), 335 (12, M^+-SH), 321 (5, M^+-MeS), 261 (1.5, M^+-OCSSMe), 260 (5, M^+-MeSH—OCS), 169 (9), 154 (12), 98 (30), and 91 (100, $C_2H_3S_2^+$).

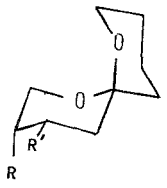
*(4*R*,6*R*)-4-Benzyloxy-1,7-dioxaspiro[5.5]undecane* ***(10)***. A solution of **9** (640 mg, 1.7 mmol) and bisazoisobutyronitrile (50 mg) in dry toluene (10 ml) was added dropwise to a stirred solution of refluxing tri-*n*-butyltin hydride (0.7 ml, 2.3 mmol) in the same solvent (5 ml) under N_2. Refluxing was continued over night. TLC (ether–hexane, 1:1) revealed the absence of **9** and the presence of a new compound of slightly lower mobility. Concentration of the solvent, followed by column chromatography (ether–hexane, 1:5) of the residue gave **10** (360 mg, 79%) as a colorless syrup, $[\alpha]_D$ −74° (*c* 1); ν^{film} 3091, 3066 and 3033 (C—H, aromatic), 2946 and 2875 (C—H), 1369, 1221, 1182, 1164, 1117, 1092, 1074, 1064, 1044, 984, and 798 (C—O—C), 736, and 697 cm^{-1} (aromatic). [^{1}H]NMR: δ 7.37–7.20 (m, 5 H, $CH_2\underline{Ph}$), 4.54 (s, 2 H, $\underline{CH_2}Ph$), 3.87 (tt, 1 H, $J_{3a,4}$ = $J_{4,5a}$ = 11.2, $J_{3e,4}$ = $J_{4,5e}$ = 4.6 Hz, H-4), 3.73 (ddd, 1 H, $J_{2a,2e}$ = 11.4, $J_{2e,3e}$ = 1.8, $J_{2e,3a}$ = 5.4 Hz, H-2*e*), 3.63 (dt, 1 H, $J_{2a,3a}$ = 11.4, $J_{2a,3e}$ = 2.3 Hz, H-2*a*), 3.63 (dt, 1 H, $J_{8a,8e}$ = $J_{8a,9a}$ = 12.7, $J_{8a,9e}$ = 2.2 Hz, H-8*a*), 3.54 (dm, 1 H, H-8*e*), 2.14 (dddd, 1 H, $J_{3e,5e}$ = 2.1, $J_{5a,5e}$ = 12.6 Hz, H-5*e*), 1.99 (ddq, 1 H, $J_{3a,3e}$ = 12.5 Hz, H-3*e*), 1.93–1.73, and 1.72–1.43 (2 m, 7 H, H-3*a*, 9*a*,9*e*,10*a*,10*e*,11*a*,11*e*) and 1.37 (dd, 1 H, H-5*a*). For the [^{13}C]NMR, see Table 1. Mass spectrum: *m/z* 263 (0.2, M^++1), 262 (1, M^+), 189 (1), 171 (7, M^+-C_7H_7), 162 (10, M^+-$C_5H_8O_2$), 161 (5, M^+-$C_5H_9O_2$), 156 (11), 155 (8, M^+-C_7H_7O), 154 (7, M^+-C_7H_8O), 141 (19), 108 (19), 107 (19), 101 (28, $C_6H_9O_2^+$), 100 (23), 99 (25), 96 (35), 91 (100, $C_7H_7^+$), 79 (32), 77 (27), 65 (18), 55 (25), and 43 (14, Ac^+).

*(4*R*,6*R*)-4-Hydroxy-1,7-dioxaspiro[5.5]undecane* ***(11)***. A solution of **10** (280 mg, 1.1 mmol) in methanol (3 ml) and triethylamine (0.1 ml) was hydrogenated over Raney-Nickel (100 mg) at 1.5 atm for five days. TLC (ether–hexane, 3:2) revealed the presence of a new compound of lower mobility. Removal of the catalyst by filtration and concentration of the filtrate gave a residue that was chromatographed (ether–hexane, 3:2) to yield **11** (110 mg, 77.5%) as a colorless mobile oil, $[\alpha]_D$ −104° (*c* 1, *n*-pentane) [lit. (Mori et al., 1985) $[\alpha]_D$ −116° (*c* 2.45, *n*-pentane)]; ν^{film} 3410 (OH), 2947 and 2876 (C—H), 1220, 1182, 1161, 1090, 1063, 1048, 985, 873, and 798 cm^{-1}

(C—O—C). [^{1}H]NMR (C_6D_6): δ 4.15 (tt, 1 H, $J_{3a,4} = J_{4,5a} = 11$, $J_{3e,4} = J_{4,5e} = 4.8$ Hz, H-4), 3.62–3.54 (m, 2 H, H-2*a*,2*e*), 3.52 (dt, 1 H, $J_{8a,8e} = J_{8a,9a} = 11.6$, $J_{8a,9e} = 2.3$ Hz, H-8*a*), 3.46–3.38 (dm, 1 H, H-8*e*), 3.16–3.04 (bs, 1 H, OH-4), 2.11 (ddd, 1 H, $J_{5a,5e} = 12.5$ Hz, H-5*e*), 1.87 (tq, 1 H, $J_{10a,11e} = J_{9e,10a} = 2$, $J_{9a,10a} = J_{10a,10e} = J_{10a,11a} = 13.6$ Hz, H-10*a*), 1.74 (ddq, 1 H, $J_{2a,3e} = J_{2e,3e} = J_{3e,5e} = 2$ Hz, H-3*e*), 1.66–1.59 (dm, 1 H, $J_{11a,11e} = 13$ Hz, H-11*e*), 1.51 (dq, 1 H, $J_{2a,3a} = J_{3a,3e} = 11.5$, $J_{2e,3a} = 6.3$ Hz, H-3*a*), 1.35 (dd, 1 H, H-5*a*), 1.46–1.28 (m, 3 H, H-9*a*,10*e*,11*a*), and 1.23–1.12 (dm, 1 H, H-9*e*). For the [^{13}C]NMR, see Table 1. Mass spectrum: *m/z* 172 (2, M^+), 155 (9, M^+-OH), 128 (9), 127 (29), 117 (100, $C_5H_9O_3^+$), 114 (45), 101 (88, $C_5H_9O_2^+$), 99 (27), 98 (62), 58 (24), 57 (34), 55 (36, M^+-$C_5H_9O_2$) and 43 (37, Ac^+).

RESULTS AND DISCUSSION

In a previous paper (Izquierdo et al., 1991), we reported on the synthesis of (3*R*,4*S*,6*R*)-3,4-dihydroxy-1,7-dioxaspiro[5.5]undecane **(1)** from D-fructose. When **1** was allowed to react with di-*n*-butyltin oxide the (not isolated) intermediate (3*R*,4*S*,6*R*)-3,4-dibutylstannylenedioxy-1,7-dioxaspiro[5.5]undecane **(2)** was obtained. Subsequent reaction of **2** with benzyl bromide occurred with



1 R = R' = OH

2 R = R' =

3 R = OH, R' = OBn

4 R = OBz, R' = OBn

5 R = OBz, R' = OH

6 R = OBz, R' =

7 R = OBz, R' = H

8 R = OH, R' = H

9 R = OCSSMe, R' = OBn

10 R = H, R' = OBn

11 R = H, R' = OH

Bn = Benzyl, Bz = Benzoyl

SCHEME 1.

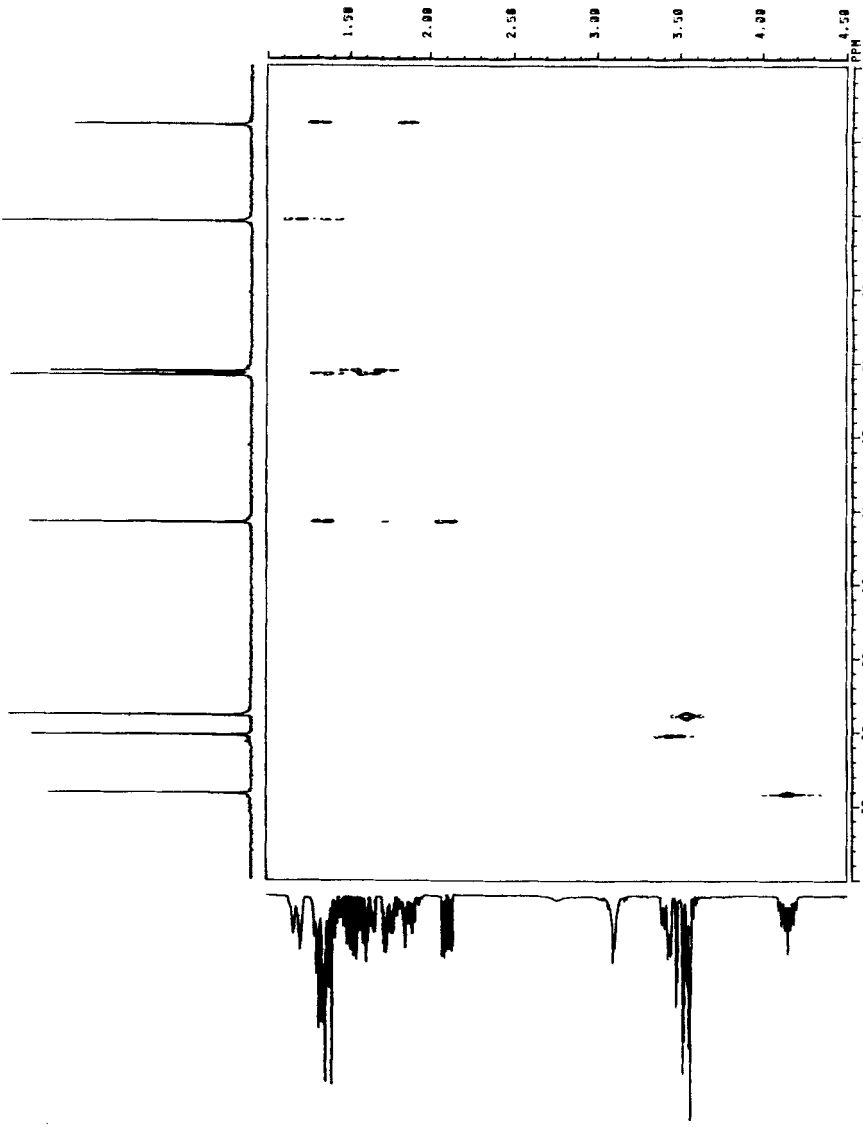


FIG. 1. Two-dimensional $^{13}C-^{1}H$ Heteronuclear shift-correlation spectrum of **8**, at a ^{1}H frequency of 300 MHz. To clarify the spectrum, [^{1}H]-and [^{13}C]NMR spectra have been included along the axes of the two-dimensional spectrum.

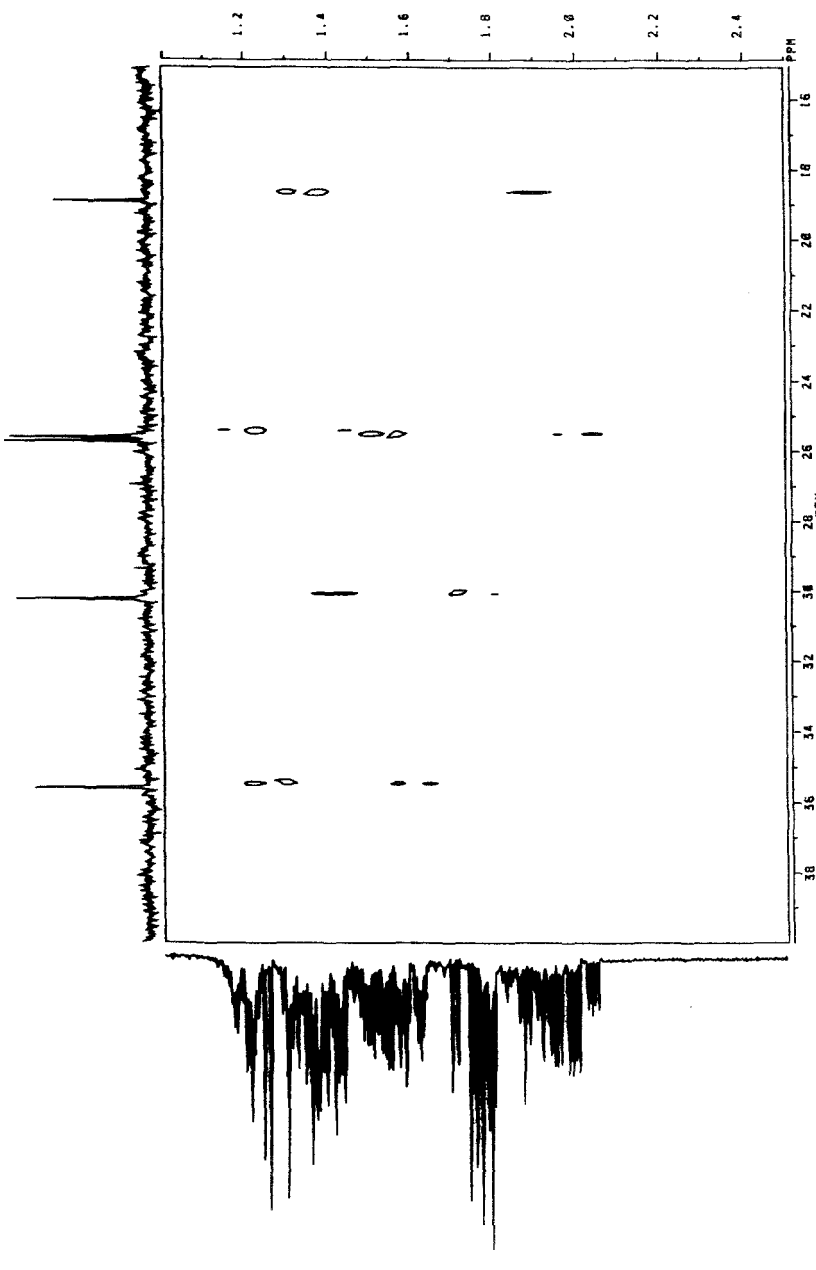


FIG. 2. Two-dimensional $^{13}C-^{1}H$ heteronuclear shift-correlation spectrum of **11**, at a ^{1}H frequency of 300 MHz. To clarify the spectrum, [^{1}H]- and [^{13}C]NMR spectra have been included along the axes of the two-dimensional spectrum.

a high regioselectivity (Nashed and Anderson, 1977; Izquierdo and Plaza, 1986, 1990), to afford the 4-*O*-benzyl derivative of **1** (**3**).

Compound **3** was transformed into the corresponding 3-benzoyloxy derivative **4**. Hydrogenolysis of the benzyl group at C-4 gave (3*R*,4*S*,6*R*)-3-benzoyloxy-4-hydroxy-1,7-dioxaspiro[5.5]undecane (**5**) that was subsequently deoxygenated by reduction (Seo, 1983) of its 4-imidazol-1-yl-thiocarbonyloxy derivative (**6**) to yield (3*S*,6*R*)-3-benzoyloxy-1,7-dioxaspiro[5.5]undecane (**7**), since an attempt to carry out the deoxygenation by a Barton's reduction (Barton and McCombie, 1975) caused the migration of the benzoyl group to the oxygen atom at C-4.

Finally, conventional debenzoylation of **7** afforded the target compound (3*S*,6*R*)-3-hydroxy-1,7-dioxaspiro[5.5]undecane (**8**), whose physical and spectroscopic data were in good agreement with those found in the literature (Mori et al., 1985; Bestmann and Schmidt, 1986) (Scheme 1).

On the other hand, compound **3** was transformed into the related (3*R*,4*S*,6*R*)-4-benzyloxy-3-[(thiomethyl)thiocarbonyloxy]-1,7-dioxaspiro[5.5]-undecane(**9**) that was reduced by the Barton's method to yield the corresponding 3-deoxy derivative (**10**). Hydrogenolysis of the benzyl group at C-4 gave the expected (4*R*,6*R*)-4-hydroxy-1,7-dioxaspiro[5.5]undecane (**11**), which showed physical and spectroscopic data in agreement with those previously reported (Mori et al., 1985). We have included the assignment of the [^{1}H]- for **11** and [^{13}C]magnetic resonance data for **8** and **11** (Figures 1 and 2).

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A NEMATICIDAL TOXIN FROM *Pleurotus ostreatus* NRRL 3526[1]

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Abstract—A nematicidal toxin was purified from *Pleurotus ostreatus* NRRL 3526 grown on moistened, autoclaved wheat straw for 30 days at room temperature (21–33°C). The active compound, at a concentration of 300 ppm, immobilized 95% of test nematodes (*Panagrellus redivivus*) within 1 hr. Immobilized nematodes did not recover, even after being rinsed with deionized water. The toxin was identified as *trans*-2-decenedioic acid.

Key Words—*trans*-2-Decenedioic acid, *Pleurotus ostreatus*, fungus, nematotoxin, *Panagrellus redivivus*, fatty acids, ostreatin, dicarboxylic acids, natural nematicides, nematoda, *Penagrellus redivivus*.

INTRODUCTION

Several species of wood-rotting basidiomycetes capture and consume nematodes as a supplementary source of nitrogen and other nutrients to the fungus (Thorn and Barron, 1984). Barron and Thorn (1987) described the mechanism used by *Pleurotus ostreatus* (Jacq. ex Fr.) Kummer (the oyster mushroom) to rapidly immobilize the nematodes, enabling fungal hyphae to penetrate, colonize, and digest the host. Droplets from specialized secretory cells produced in older portions of the fungal colony contain a potent, but unidentified, nematotoxin that was assigned the trivial name ostreatin. Once a nematode touches these

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[1]The mention of firm or trade names does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

droplets, it is immobilized in as little as 30 sec. We have confirmed their findings. In this paper, we report the isolation and structure elucidation of a nematotoxic compound from *P. ostreatus* NRRL 3526 grown on moistened, autoclaved wheat straw, a substrate on which the fungus produced massive numbers of secretory cells and toxin droplets.

METHODS AND MATERIALS

Cultures. Pleurotus ostreatus NRRL 3526 was obtained from the U.S. Department of Agriculture, Agricultural Research Service Culture Collection, Peoria, Illinois, and maintained on potato dextrose agar (PDA). We observed that this fungal strain captured nematodes when grown on water agar as described by Thorn and Barron (1984). The test nematode *Panagrellus redivivus* L. was supplied by Professor B.M. Zuckerman, Massachusetts Agricultural Experiment Station, Amherst, Massachusetts. The nematode culture was maintained on a uracil-requiring strain (OP-50) of *Escherichia coli* (Migula) Castellani & Chalmers on NG agar (Brenner, 1974) and transferred weekly.

Toxin Production. Pleurotus ostreatus was cultured on moistened, autoclaved wheat straw for the production of nematotoxin(s). We observed that *P. ostreatus* produced massive numbers of secretory cells and toxin droplets when grown on Arthur variety wheat (*Triticum aestivum* L.) straw, a lignocellulosic substrate. The wheat straw was cut into 2.5- to 7.5-cm lengths and ca. 60 g packed loosely in individual Fernbach flasks, which were then filled with tap water and the straw allowed to soak for 1 hr. The water was then decanted, and the flasks cotton-stoppered and autoclaved twice on two consecutive days at 15 psi for 60 min. After the flasks cooled, the substrate in each flask was inoculated by transferring two mycelial plugs (8 mm) excised from the growing margin of the fungal colony on a PDA plate. The cultures were incubated statically at room temperature (21–23°C) for 30 days, during which time the fungus colonized the entire mass of wheat straw.

Toxin Purification. Following incubation, 750 ml water was added to each flask, and the contents (i.e., mass of fungus-colonized straw) were agitated with a glass rod, followed by wrist-action shaking for 15 min. The aqueous extract was filtered first through a single layer of tissue paper (Kimwipes, Kimberly-Clark Corp.), and then through Whatman No. 1 filter paper. The water extract was then lyophilized in shallow trays. After the residue from 12 flasks was reconstituted in 50 ml water, 50 ml methanol was added, and the resulting precipitate was removed by centrifugation. The supernatant was evaporated to dryness in a rotary evaporator. After the residue was dissolved in 10 ml water, 90 ml methanol was added, and the resulting precipitate was removed by centrifugation. The supernatant was again evaporated to dryness, and the residue

was dissolved in 5 ml water. Forty-five milliliters of acetone was added, and insoluble materials were separated by centrifugation. The fractions from each precipitation step were redissolved or suspended in water, and then checked for nematicidal activity. The acetone-soluble fraction was purified further by high-pressure liquid chromatography (HPLC). A Beckman HPLC system equipped with a UV detector containing a preparative flow cell and a semipreparative C-18 column (10 × 250 mm) was used throughout the purification process. The system was operated isocratically with various methanol–water mobile phases at a flow rate of 2 ml/min. The elution was monitored at 210 nm. Each fraction was evaporated to dryness and redissolved in water prior to the nematotoxin bioassay. An uninoculated flask of autoclaved wheat straw was extracted and processed the same way to serve as a control.

Bioassay. The nematotoxin bioassay was performed in 400-μl flat-bottomed microtiter wells. Nematodes were removed from a 7-day-old culture with a stream of deionized water from a wash bottle. The nematodes were transferred to an 18 × 150-mm culture tube and allowed to settle to the bottom. After 15 min, most of the liquid containing the bacteria was aspirated, and fresh deionized water was added to suspend the nematodes to a population of 2000–4000/ml. No attempt was made to remove the bacteria completely. One hundred to two hundred nematodes in a volume of 50 μl were added to each well containing 50 μl aqueous solution of toxin sample. When the test material was in organic solvent, the solvent was removed in a rotary evaporator and the residue suspended in water for the assay. The nematodes were monitored for 1 hr with the aid of a dissecting microscope. Tests for toxicity were scored positive when 95% of the nematodes were immobilized within 1 hr. The relatively short test duration should limit any interference from the contaminating bacteria (*E. coli*) in the bioassay.

To quantify the nematotoxicity of pure compounds, the materials were dissolved in ethanol and serial twofold dilutions were made directly in the microtiter wells. The solvent was allowed to evaporate, and 100–200 nematodes in 100 μl water were then added. The results were recorded after 1 hr. The concentration that immobilized 95% of the nematodes within 1 hr was termed ED_{95}. Besides the *Pleurotus* toxin, other structurally related compounds and two commercial nematicides were also tested for comparison. The compounds were the simple fatty acids (C_6–C_{18}, Sigma Chemical), dicarboxylic acids (C_6–C_{12}, Sigma Chemical), chloethocarb [2-(2-chloro-1-methoxyethoxy)-phenyl methylcarbamate, BASF], and oxamyl [methyl 2-(dimethylamino)-*N*-{[(methylamino)carbonyl]oxy}-2-oxoethanimidothioate, DuPont].

Reversibility of Immobilization. After the nematodes were immobilized by *Pleurotus* toxin (300 ppm, 1 hr), they were transferred from the microtiter well to 100 ml deionized water in a 400-ml beaker. The nematodes were collected on an 8-μm Millipore membrane filter, rinsed with 10 ml deionized water, and

then transferred to a 60 × 15-mm Petri dish with 5 ml deionized water. The nematodes were observed for sign of recovery for 2 hr and were rechecked after 24 hr.

Structure Determination. Proton and carbon NMR data were obtained in CD_3OD on a Bruker WM-300 spectrometer, and chemical shifts were recorded using the signals for the residual protiated solvent and deuterated solvent as references (3.30 ppm for ^1H; 49.0 ppm for ^{13}C). Carbon multiplicities were established by a DEPT experiment. The CI mass spectrum of the toxin was obtained with the solids probe on a Finnigan TSQ 46 instrument. An aliquot of the *Pleurotus* toxin was methylated with diazomethane and analyzed by GC-MS on a Finnigan/Mat TSQ 46 instrument. The column was a DB-1 capillary column (15 m, 0.25 μm film, J & W Scientific). The temperature of the GC was 70°C for the first 2 min and was increased to 250°C at a rate of 10°/min.

RESULTS

Toxin Purification. Nematicidal activity was not detected in the initial water extract, but it was detected in the concentrated solution following lyophilization. When methanol was added to this toxin solution to a concentration of 90%, the toxic principle remained soluble, and no activity was detected in the precipitate. The methanol-soluble fraction was further purified by an acetone precipitation step. All the nematotoxic activity was found in the acetone-soluble fraction, which was further purified by HPLC. Extracts from the uninoculated flask did not have any biological activity.

With HPLC, the toxic principle was eluted in the void volume by 50% methanol. When the active fraction was rechromatographed with a mobile phase of 40% methanol, the toxic activity was eluted at 48 min. The peak was broad and nonsymmetrical. Finally, the mobile phase was changed to 0.1% acetic acid in 40% methanol. The toxic activity was eluted as a single peak at 62 min. The purified compound was a colorless solid, very soluble in methanol and acetone, and slightly soluble in water. The yield from 1800 g fermented wheat straw (36 Fernbach flasks) was 5 mg.

Structure Determination. The CI mass spectrum showed a protonated molecular ion (MH^+) peak at *m/z* 201, suggesting a molecular weight of 200 ($C_{10}H_{16}O_4$). The proton NMR data (Table 1) show a one-proton doublet at 5.79, which is coupled to a one-proton multiplet at 6.94, indicating a double bond between a carbonyl group and a CH_2 group. The observed coupling constant (15.5 Hz) demonstrates a *trans* configuration for the double bond. The carbon NMR data (Table 1) show six methylene carbons, two olefinic carbons, and two carboxyl carbons. No end methyl group was detected. After methylation with diazomethane, the methylated sample emerged from the gas chromatograph

as a single peak with a retention time of 7 min, 36 sec. The CI mass spectrum had an intense protonated molecular ion at *m/z* 229, indicating the formation of two methyl ester groups. All these data suggest a structure of *trans*-2-decenedioic acid for the toxin (Figure 1).

Nematotoxicity Test. The nematode *P. redivivus* was very active in water, wiggling from side to side. In the presence of the *Pleurotus* toxin, the movement gradually slowed, and eventually the nematodes became totally immobile. The immobilized nematodes did not recover, and after 24 hr they were considered dead because their bodies became straight and opaque. However, we do not know exactly when death occurred. At a concentration of 300 ppm, the purified *Pleurotus* toxin immobilized 95% of the nematodes within 1 hr. At higher concentrations, paralysis occurred more rapidly, often within minutes. In some tests, we rinsed the nematodes soon after immobilization and placed them in fresh deionized water but not a single nematode, out of approximately 200, recovered.

TABLE 1. [^{1}H]- AND [^{13}C]NMR SPECTRAL DATA FOR *Pleurotus* TOXIN[a]

Position	Chemical shifts, multiplicity[b] ^{13}C	^{1}H	Couplings (Hz)
1	170.1 s		
2	122.6 d	5.79 bd	2, 3 = 15.5
3	151.0 d	6.94 dt	3, 4 = 7.0
4	33.0 t	2.21 m	
5	29.9 t	1.36 m	
6	29.9 t	1.36 m	
7	29.1 t	1.48 m	
8	25.9 t	1.60 m	8, 9 = 7.3
9	34.9 t	2.28 t	
10	177.6 s		

[a] Carbon assignments are based on analogy with established chemical shift values for long chain fatty acids.
[b] Abbreviations: b, broad; d, doublet; s, singlet, t, triplet; m, multiplet.

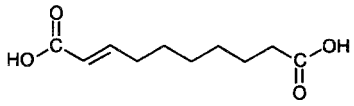

FIG. 1. The nematicidal toxin from *Pleurotus ostreatus* NRRL 3526 *trans*-2-decenedioic acid.

Several simple fatty acids and dicarboxylic acids were also tested for their toxicity to nematodes. The medium-chain fatty acids (C8–C12) were more toxic than the corresponding dicarboxylic acids and the *Pleurotus* toxin (Table 2). At high concentrations (>2500 ppm), the medium-chain fatty acids killed the nematodes instantaneously. By contrast, chloethocarb, at a concentration of 10,000 ppm, did not immobilize the nematodes within 24 hr, while oxamyl immobilized the nematodes only at concentrations of 2500 ppm or higher. However, although the nematicides did not immobilize or kill the nematodes, they effectively disabled the nematodes at low concentrations. At a concentration as low as 25 ppm, the rhythmic motion of the nematodes ceased, their bodies appeared contorted, and they moved only feebly.

DISCUSSION

Nematode-destroying fungi produce elaborate traps to capture nematodes (Barron, 1977). Once captured, the nematodes do not struggle much and become quickly paralyzed. The general belief is that fungal toxins produced in the traps paralyze or kill the captured nematodes. Several such toxins have been reported from nematode-trapping species of *Arthrobotrys* (Olthof and Estey, 1963; Balan

TABLE 2. IMMOBILIZATION OF *Panagrellus redivivus* BY FATTY ACIDS AND DICARBOXYLIC ACIDS

Compound	ED_{95} (ppm)[a]
Hexanoic acid	2,500
Heptanoic acid	625
Octanoic acid	156
Nonanoic acid	156
Decanoic acid	156
Dodecanoic acid	156
Tetradecanoic acid	10,000
Hexadecanoic acid	>10,000
Hexanedioic acid	10,000
Heptanedioic acid	5,000
Octanedioic acid	2,500
Decanedioic acid	312
trans-2-Decenedioic acid (*Pleurotus* toxin)	300
trans-2-Dodecenedioic acid	>5,000

[a]Toxin concentration that immobilizes 95% of the test nematodes within 1 hr.

and Gerber, 1972; Balan et al., 1974), *Dactylaria* (Krizkova et al., 1976), *Monacrosporium* (Balan et al., 1974), and *Nematoctonus* (Giuma and Cooke, 1971; Giuma et al., 1973; Kennedy and Tampion, 1978). Other than ammonia implicated as a nematotoxin (Balan and Gerber, 1972), none of the toxins has been purified.

Unlike the nematode-trapping fungi, *P. ostreatus* does not produce traps or adhesive cells. Instead, it produces tiny droplets of toxin from minute spatulate secretory cells. Once a nematode touches the droplets, it is immobilized in as little as 30 sec (Barron and Thorn, 1987). Because *P. ostreatus* does not produce any mechanical device to restrain a struggling nematode, it may be likely to produce a more potent toxin than those produced by the trap-forming fungi, so that the nematodes are paralyzed quickly and cannot escape.

We used a solid substrate to culture *P. ostreatus* for the production of the nematotoxin, because *P. ostreatus* does not produce toxin droplets in liquid culture (Barron and Thorn, 1987). Wheat straw provides a low-nutrient natural substrate with a large surface area for *P. ostreatus*. Massive numbers of droplet-producing secretory cells could be seen on fungal hyphae colonizing individual lengths of wheat straw. We used water to extract the nematotoxin, because the nematotoxin from *Nematoctonus* species has been reported to be water soluble (Giuma et al., 1973). In addition, our strategy was to focus on the toxin-containing droplets and to minimize our chances of removing metabolites from the fungal mycelium. While this gentle extraction procedure should cause little damage to fungal cells, we have no direct evidence that the nematotoxin we isolated was from the droplets. The toxin droplets are only 2–5 μm in diameter (Barron and Thorn, 1987). At the present time, we do not know of any practical way to harvest the droplets and to determine the toxin concentration in the droplet.

Our test nematode, *P. redivivus*, is free-living and is highly active in water, in contrast to most plant parasitic nematodes, which move feebly. *P. redivivus* is, therefore, a more suitable test organism to detect nematoxins, as sublethal toxic effects can be perceived easily by observing any reduction in motility. On the other hand, it is more difficult to count the fast-moving nematodes, and the background mortality rate was high. Therefore, accurate determination of LD_{50} is unrealistic, so we express our results as ED_{95}, which is defined as the concentration of toxin that immobilizes 95% of the nematodes. We also limited our bioassay to 1 hr and discarded any fraction that did not immobilize the nematodes within 1 hr, because we were searching for fast-acting nematotoxins that would explain the immobilization we observed on Petri plates.

The toxin was purified by HPLC and was identified as *trans*-2-decenedioic acid by NMR and mass spectrometry. This structure has been reported previously by Cram and Tishler (1948) for a microbiologically inactive compound isolated from a crude penicillin preparation from *Penicillium notatum*. Since

trans-2-decenedioic acid was fast-acting, it is probably the ostreatin described by Barron and Thorn (1987). It is probably formed by enzymic oxidation of linolenic acid via the intermediates 10-hydroperoxy-*E*-8,*Z*-12,*Z*-15-octadecatrienoic acid and 10-oxo-*E*-8-decenoic acid, as described in the fungus *Psalliota bispora* (Wurzenberger and Grosch, 1986).

trans-2-Decenedioic acid immobilized 95% of the nematodes within 1 hr at 300 ppm. Under the same test conditions, oxamyl did not immobilize or kill the nematodes within 24 hr until the concentration was increased to 2500 ppm, whereas chloethocarb was not lethal at 10,000 ppm for 24 hr. Although the commercial nematicides did not immobilize or kill the nematodes immediately, they effectively disabled 95% of the nematodes at 25 ppm. Therefore, *trans*-2-decenedioic acid acts quite differently from the acetylcholinesterase inhibitors.

After we identified the toxin as a dicarboxylic acid, we screened several structurally related compounds for nematicidal activity. Maximum biological activity was associated with a 10-carbon backbone (Table 2). *trans*-2-Dodecenedioic acid (traumatic acid), which is a plant hormone with two more carbon atoms than *trans*-2-decenedioic acid, had no nematicidal activity. The monocarboxylic acids (fatty acids) were more active than the corresponding dicarboxylic acids. Medium-chain fatty acids have been shown to be toxic to nematodes (Munkata, 1983), insects (Fogleman and Kircher, 1986; Puritch, 1978), fungi (Teh, 1974), and yeasts (Hunkova and Fencl, 1977). However, there were no prior reports of similar biological activity for dicarboxylic acids.

Because of their fast action, we believe that *trans*-2-decenedioic acid, medium-chain dicarboxylic acids, and fatty acids act by affecting membrane permeability. Many useful drugs, which are lipophilic acids, act in this way (Freese, 1978). The change in cell membrane permeability would affect the normal function of the nerve and muscle cells, resulting in a reduction and cessation of muscle contraction. After prolonged exposure, leakage of the cytoplasm into the surroundings would eventually cause death. When we rinsed the nematodes with deionized water soon after they were immobilized, they did not recover. Presumably, enough leakage of the cytoplasm had already occurred by the time they were paralyzed, and the damage became irreversible. Our result confirms the finding of Barron and Thorn (1987), who reported that when immobilized nematodes on *P. ostreatus* culture on water agar were transferred to a fresh water agar plate, only one out of 25 recovered.

trans-2-Decenedioic acid and related compounds are simple natural compounds and are, thus, biodegradable. They should be safer to use than most synthetic nematicides. Our results showed that a 10-carbon backbone is required for maximal biological activity. Better nematicides may be synthesized by adding other functional groups to the 10-carbon backbone.

So far, we have found only one nematicidal compound from *P. ostreatus*: *trans*-2-decenedioic acid. It is likely that *P. ostreatus* produces, in addition to

attractants, several toxins that may act synergistically. Even the presence of a common, nontoxic organic acid (i.e., acetic acid) in the toxin droplets should produce a synergistic effect, since the penetration of the cuticle of the nematode by *trans*-2-decenedioic acid would be enhanced at low pH. It is possible that we may have missed other more lipophilic nematotoxic compounds because we used water to extract the toxins.

To the best of our knowledge, *trans*-2-decenedioic acid is the first nematotoxin ever purified from nematode-destroying fungi. Additional investigations are needed on natural nematicides from these fungi.

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ARRESTMENT OF MALE TWOSPOTTED SPIDER MITE CAUSED BY FEMALE SEX PHEROMONE

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Abstract—A glass slide bioassay was used to evaluate male twospotted spider mite, *Tetranychus urticae* Koch, arrestment caused by quiescent deutonymph extract. Males that were guarding quiescent deutonymphs prior to being tested were arrested by a 3-mm-diameter circle of quiescent deutonymph extract; nonguarding males and adult females were not arrested. Extracts of all *T. urticae* instars tested caused male arrestment, but mean arrestment duration was longest with quiescent deutonymph extract. Arrestment by volatile perception of pheromone and upwind orientation to point sources of extract were not observed. The mono- and sesquiterpene alcohols previously identified as components of the pheromone did not arrest males. HPLC separation of extract resulted in four active fractions; a subtractive bioassay showed that three were essential to elicit maximum male response.

Key Words—*Tetranychus urticae*, twospotted spider mite, Arachnida, Tetranychidae, quiescent deutonymph, female sex pheromone, bioassay, arrestment, active components.

INTRODUCTION

Twospotted spider mite, *Tetranychus urticae* Koch, is a serious pest of more than 150 economically important hosts throughout the world. Despite numerous instances where biological control has been used for control of *T. urticae*, acaricides are the most widely used control method in commercial situations (Jeppson et al., 1975).

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Efforts to combat the resistance and registration problems that have beset many commercially available acaricides have encouraged the development of alternative control strategies. One possible *T. urticae* control method is mating disruption by use of synthetic pheromones. Numerous Acari-specific pheromones and other semiochemicals have been identified (Sonenshine, 1985 and references therein), and semiochemicals have been used to control Ixodid ticks (Ziv et al., 1981). Therefore, use of pheromones to manage *T. urticae* in commercial situations warrants investigation.

T. urticae mating behavior has been described extensively (Ewing, 1914; Lehr and Smith, 1957; Cone et al., 1971b; Potter et al., 1976; Potter and Wrensch, 1978; Cone, 1985). Female deutonymphs deposit webbing on the leaf surface and become quiescent. Males encountering quiescent deutonymphs can become arrested locally near the female; the probability of arrestment increases as ecdysis approaches (Cone et al., 1971b; Potter et al., 1976). Arrested male behavior has been described as "guarding" (Ewing, 1914; Lehr and Smith, 1957; Cone, 1979, 1985); arrested males may remain immobile, touch the idiosoma of the female with their forelegs and palps, and attack other males that encounter the quiescent female. Males assist females in emergence from the exuviae and immediately attempt mating (Laing, 1969; Potter et al., 1976; Cone, 1985).

Cone et al. (1971a) concluded that quiescent deutonymphs contained chemicals attractive to *T. urticae* males. They labeled the chemical(s) a female sex pheromone, but concluded that their bioassay was inadequate to aid in the identification of the pheromone. Subsequent efforts to identify the chemical composition of the sex pheromone are inconclusive. Regev and Cone identified the sesquiterpene alcohols farnesol (1975a) and nerolidol (1976a), and the monoterpene alcohol citronellol (1980) as possible pheromonal components. They also observed that topical applications of farnesol increased the fecundity of *T. urticae* adult females (Regev and Cone, 1976b), and suggested that the observed positive correlation between host-plant farnesol content and the plant's susceptibility to *T. urticae* was due to the gonadotropic effect of farnesol or to its pheromonal activity (Regev and Cone, 1975b; Regev, 1978). However, identification of compounds was done solely on the basis of gas chromatographic retention times; no mass spectroscopy was performed.

Our studies were done to reaffirm that a sex pheromone is involved in the guarding behavior of *T. urticae* males towards quiescent deutonymphs. Fulfillment of this objective involved extraction of female sex pheromone from quiescent deutonymphs and the development of a quick, discriminating bioassay for male *T. urticae* response. The bioassay was used to describe male response, to identify the mechanism of pheromone perception, and to bioassay the terpenoid compounds previously identified by Regev and Cone as pheromonal components. We also separated the components of the sex pheromone and determined

the combination of fractions necessary to elicit maximum male *T. urticae* response.

METHODS AND MATERIALS

Arrestment by Contact Chemoreception: Evidence of Female Sex Pheromone. T. urticae were reared on Henderson lima beans, *Phaseolus lunatus* L., at 25°C, 60% relative humidity, and on a 14:10 light–dark photoperiod. Two hundred fifty quiescent deutonymphs were immersed in 1.0 ml of HPLC grade hexane (Mallinckrodt, Inc., Paris, Kentucky) for 48 hr. All extractions used in our studies were stored under N_2 in the dark at 0–5°C to preserve potential activity.

The arrestment activity of the extract was bioassayed by placing one quiescent deutonymph equivalent (QDE) (4.0 μl) of extract on a glass microscope slide using a 5-μl syringe (Hamilton Co., Reno, Nevada). The extract was placed in a 3-mm-diameter circle. The slide was dried in air for 30 min to allow the solvent to evaporate. Controls consisted of 4.0 μl of hexane deposited in a 3-mm-diameter circle. Using this method, the response of mites observed in our studies could be attributed solely to chemicals extracted from quiescent deutonymphs (Waage, 1978).

One randomly selected *T. urticae* was placed on the edge of the circle using a camel-hair brush. Care was taken to transfer the mite as quickly and gently as possible; the mite was discarded if it stuck to the brush or was damaged during the transfer process. Arrestment was described by the amount of time the mite spent within the circle of extract. Arrestment ended if the mite left the circle of extract for >5 sec.

We tested the arrestment activity of the extract on guarding adult males, nonguarding adult males, and adult females. Arrestment of guarding and nonguarding males by the control also was measured. To ensure that mites did not respond to the hexane control, the response of 20 guarding males to the 3-mm circle of hexane was compared to their response to an untreated circle [t test (Snedecor and Cochran, 1967)].

Each treatment was replicated with 20 mites. Differences in response between sexes, guarding behaviors, and the extract and control were described by analysis of variance with a randomized complete block design and the Student-Newman-Keuls test (Snedecor and Cochran, 1967; CoHort Software, 1986). This statistical procedure was used for all subsequent bioassays unless otherwise specified.

Arrestment by Contact Chemoreception: Comparison of Solvents. Two hundred fifty quiescent deutonymphs were immersed in 1.0 ml of either hexane, diethyl ether, or methanol (HPLC grade) (Mallinckrodt, Inc.) for 48 hr. Extrac-

tions were done using solvents with differing polarities to ensure that all pheromone components were eluted from the mites.

The arrestment activity of 1 QDE of each extract–solvent mixture was bioassayed as described previously. Each solvent–extract mixture was tested with 20 adult male mites. Males that were guarding quiescent deutonymphs on lima bean leaves were selected. A 2-min preobservational period was included to allow the mites to acclimate to their new surroundings. Arrestment was described by the amount of time the male spent within the circle of extract. The male was considered unarrested if it left the circle of extract for >5 sec.

Arrestment by Contact Chemoreception: Effect of Different Extraction Times. Extracts were prepared by soaking 250 quiescent deutonymphs in 1.0 ml of methanol or hexane for one of 11 different time intervals ranging from 5 min to 96 hr. Bioassays (1 QDE) of guarding *T. urticae* adult male response to the different extraction times were done as described in the solvent comparison study. Fifteen male mites were tested for each extraction. *T. urticae* male response times were regressed on extraction times using SYSTAT MGLH (Wilkinson, 1990).

Arrestment by Contact Chemoreception: Effect of Concentration and Extractions from Other Instars. Previous bioassays demonstrated that guarding adult males were arrested by quiescent deutonymph extract. Because males occasionally are observed guarding other instars, female sex pheromone may be present in instars other than quiescent deutonymphs. We tested for the presence of *T. urticae* female sex pheromone in protonymphs, quiescent protonymphs, female deutonymphs, and adult males and females. Extracts were prepared by soaking 1000 mites of each instar in separate 250-μl aliquots of hexane for 72 hr. Nine concentrations of each extraction, ranging from 0.01 to 25 mite equivalents (ME), and a 4.0-μl hexane control were tested by the response of 22 guarding male *T. urticae*. A 2-min preobservational period was included to increase the discriminatory power of the bioassay. Data were analyzed by regressing log(duration of arrestment) on log(mite equivalents + 1) to determine the concentration ranges of extracts necessary to elicit maximum male response. CoStat Statistical Software (CoHort Software, 1986) was used for the regression analysis.

Arrestment by Volatile Chemoreception. Behavior of adult male mites in an extract-laden airstream was used to test for *T. urticae* volatile chemoreception. The airstream apparatus was constructed by placing a circular piece of Fisherbrand P5 filter paper (Fisher Scientific, Pittsburgh, Pennsylvania) in a 65-mm Buchner funnel. The funnel was attached to a 500-ml Erlenmeyer flask, and the flask connected to a cold-water aspirator to create a vacuum. A glass tube (7 mm diameter × 100 mm long) was suspended vertically 4 mm above the filter paper. The mite extract was spread on the inside surface of the tube 50 mm from the filter paper and a 5-cm/sec airstream blown downwards through

the tube. The extract-laden airstream emerging from the tube was sucked through the filter paper by the vacuum apparatus so that a 7-mm-diameter extract–air column was maintained directly beneath the mouth of the glass tube. The air was filtered with activated charcoal before entering the glass tube to remove impurities.

Each assay consisted of one adult male *T. urticae* placed on the edge of the air column. Males that were guarding quiescent deutonymphs on lima bean leaves were selected. Arrestment was described by the amount of time the male spent within the circular air column. A 1-min preobservational period was included to allow the mite to become acclimated.

Two concentrations (1 and 10 QDE) were tested for each solvent; 4.0 μl hexane was used for the control. Each assay was replicated with 19 male *T. urticae*.

Orientation by Volatile Chemoreception to an Extract Source. Previous researchers have concluded that the attraction of males to quiescent deutonymphs occurred from an approximate maximum distance of 2–3 mm (Cone et al., 1971a; Penman and Cone, 1974); therefore, our upwind orientation studies were done in moving air on glass microscope slides. A 24-mm × 35-mm rectangular arena was outlined on each slide with lanolin to prevent the mites from escaping. Two point sources (3 mm diameter) of quiescent deutonymph extract were placed on the slide approximately 8 mm apart and 7 mm from the upwind edge of the arena. The male mite was placed 1 cm downwind from the extract points, and a 5-cm/sec airstream was blown over the extract towards the mite. Two concentrations (0.5 and 5 QDE/point) were assayed for each solvent extraction (hexane, diethyl ether, and methanol). The control consisted of two 2.0-μl points of hexane. In addition, 1 QDE of the hexane extract was tested in still air for comparison.

Total time for each test was 3 min. The time the mite took to reach one of the extract deposits was subtracted from 3 min, hence a male's failure to locate the extract during the 3-min period was scored as a zero response. Twelve replicates of each treatment were tested.

Bioassay of Terpenoid Compounds. Four quantities (ranging from 4 to 4000 ng in 4.0 μl methanol) of farnesol, nerolidol, geraniol, and citronellol (Sigma Chemical Co., St. Louis, Missouri) were tested. Response of male *T. urticae* to each terpenoid, to the four terpenoids combined, and to 1 QDE of methanol extract were tested as described in the previous contact chemoreception bioassays. It was anticipated that the terpenoid compounds would not cause arrestment for the entire 2-min preobservational period. Therefore the acclimation period was not included to provide more accurate comparisons with the methanol control.

Each synthetic terpenoid was tested by the response of 14 male *T. urticae*. Mean male response times to the terpenoids and extract were compared to the

mean response to the methanol control by analysis of variance and Dunnett's procedure (1955).

Initial Isolation of Active Components. Concentrated extract (2500 QDE/250 μl hexane) was prepared as described in previous bioassays. A 100-μl (1000 QDE) sample of extract was fractionated using a Hewlett Packard model 1090 liquid chromatograph with an HP Hypersil silica column (5-μm particle size; 200 mm × 2.1 mm internal diameter). The mobile-phase program (flow rate = 1 ml/min) consisted of 2 ml hexane, an 8-ml linear conversion from hexane to diethyl ether, a 6-ml linear conversion to CH_2Cl_2, and 4 ml CH_2Cl_2 column flush. Eighteen 1-ml fractions were collected.

Response of guarding adult male *T. urticae* to the 18 fractions and the original extract was evaluated as described in the previous contact chemoreception bioassays. The concentration tested was reduced to 0.25 QDE to increase bioassay discrimination (Baker and Cardé, 1984). Each fraction was tested by the response of 16 males. Controls consisted of 1.0 μl hexane.

Subtractive Bioassay of Active Fractions. Eight 1-ml HPLC fractions from the initial separation bioassay were tested further using a subtractive bioassay technique. The purpose of the subtractive bioassay was to determine the fractions that contained components necessary to elicit maximum arrestment of male *T. urticae*. First, all eight fractions were combined (0.5 QDE of each) and bioassayed, and the observed arrestment compared to that caused by the extract. Next, individual fractions were subtracted from the combination and the remaining fractions tested. If male arrestment caused by a new combination was significantly less than that caused by all the fractions, the subtracted fraction was considered to contain an essential component of the pheromone.

Each treatment was tested by the response of 16 male *T. urticae*. We suspected that fraction 3 probably contained small amounts of the active components found in fractions 2 and/or 4; therefore, this fraction was subtracted in combination with both 2 and 4 when testing for reduced activity in the two latter fractions. Similarly, fractions 15–17 were tested as one combined fraction.

RESULTS AND DISCUSSION

Arrestment by Contact Chemoreception: Evidence of Female Sex Pheromone. Data showing the arrestment response of guarding males, nonguarding males, and adult females to the circle of extract and to the hexane control are presented in Figure 1. Males that were guarding a quiescent deutonymph immediately prior to the bioassay were arrested by the extract for a significantly longer period relative to the control [$F = 52.42$; $df = 4, 76$; $P = 0.0001$; W_p (critical value for the Student-Newman Keuls test) = 48.02 sec]. These data suggest that chemicals are present in the quiescent deutonymph extract that arrest the

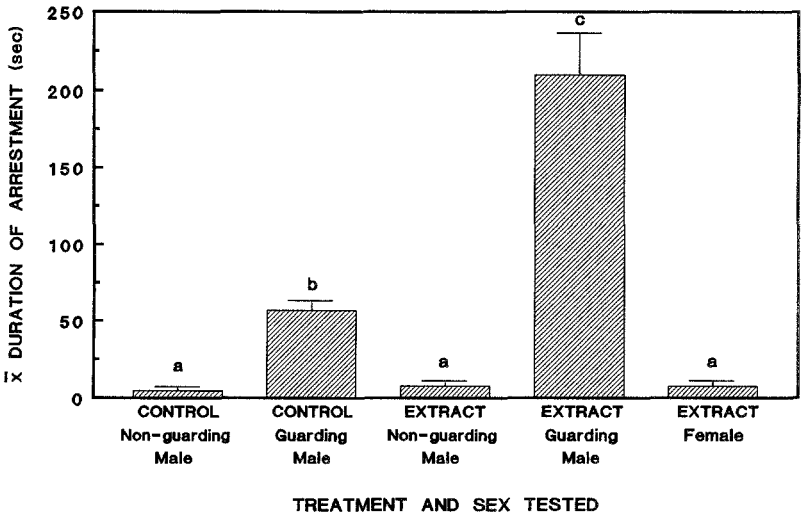


FIG. 1. Mean duration of adult male and female *T. urticae* arrestment caused by 1 QDE of extract. Error bars are standard errors of means. Means with the same letter were not significantly different [$P = 0.05$; Student-Newman-Keuls test (Snedecor and Cochran 1967)].

movement of guarding adult males. Typically, arrested males walked in tight circles within the 3-mm circle of extract. Upon moving out of the circle, the males immediately reversed direction until they reencountered the extract. Arrested males occasionally became immobile, or pivoted back and forth on their hind legs, dragging the tarsi and tibiae of the forelegs across the glass slide. Unarrested mite movement was more linear; these mites often walked off the glass slide 5–10 sec after placement. Although these responses were not quantified, the circular ambulatory movement may be a localized searching behavior; upon pheromone perception the male attempts to locate a source in the vicinity through tactile or visual stimuli. Males also exhibit circling behavior while covering quiescent deutonymphs with webbing (Penman and Cone, 1974).

The time spent in the hexane circle by guarding males also was significantly longer than the time spent by nonguarding males. Guarding males remained within the 3-mm circle for nearly a minute despite the absence of extract. Positive response to the control was not a result of the arresting effects of hexane, as guarding males also remained within an untreated 3-mm circle longer than nonguarding males ($P = 0.05$, $N = 20$; t test). Continuation of localized ambulatory behavior after stimulus removal suggests that response to the presence or absence of sex pheromone is not instantaneous. Therefore, a 2-min preobservational period was included in subsequent studies to allow the guarding males to acclimate to their surroundings.

The time spent in the extract circle by adult females did not differ significantly from the response of nonguarding males to the hexane controls. These data suggest that the chemical is sexually specific to male *T. urticae*. Response of nonguarding adult males to extract also did not differ significantly from their response to the controls. The lack of response by nonguarding males along with the positive response of guarding males to the hexane control suggest that the pheromone is context specific; initiation or cessation of the male's guarding behavioral mode is not controlled solely by the presence of female pheromone. Observations of male behavior under natural conditions support this conclusion; males often walk next to and over quiescent deutonymphs without becoming arrested (Ewing, 1914; Lehr and Smith, 1957; Cone et al., 1971b; Penman and Cone, 1974; Potter et al., 1976). Guarding behavior is initiated by perception of pheromone, tactile, and/or visual stimuli, but male sensitivity to these stimuli probably depends on other factors as well. Possible factors regulating initiation or cessation of adult male *T. urticae* guarding behavior are abiotic environmental conditions. Cone et al. (1971b) observed more frequent male response to quiescent deutonymphs at higher temperatures and lower humidities. Host plant quality also could affect the onset of mating behavior. Males frequently wander the surface of the leaves and intermale conflicts over quiescent deutonymphs are rare when *T. urticae* are colonizing nutrient-rich host plants, while during periods of host-plant decline males guard quiescent deutonymphs almost exclusively and conflicts are observed frequently (Potter et al., 1976; Potter, 1978; Potter and Wrensch, 1978). Conflict with other males often results in cessation of arrestment (Potter et al., 1976), and internal factors such as the male's sperm supply, past mating history, or physiological age (Cone and Pruszynski, 1972; Potter and Wrensch, 1978) also may affect male guarding behavior.

The fact that only guarding males were arrested may explain the low response rates observed in earlier studies (Cone et al., 1971a,b; Penman and Cone, 1974). Since nonguarding males did not respond to quiescent deutonymph extract, we used only guarding males in all subsequent bioassays.

Arrestment by Contact Chemoreception: Comparison of Solvents. No significant differences were observed in the guarding adult male *T. urticae* response to any of the quiescent deutonymph extract–solvent combinations, and all combinations arrested *T. urticae* longer than the control [$F = 6.86$; $df = 3, 57$; $P = 0.001$; $W_p = 107.03$ sec (Figure 2)]. These results show that the three solvents extracted all the pheromonal components from quiescent deutonymphs, as reported previously by Cone and Pruszynski (1972). Therefore, all subsequent bioassays were done using the hexane extract.

Arrestment by Contact Chemoreception: Effect of Different Extraction Times. Relationships between extraction time ($\hat{Y}$) and duration of arrestment (x) for the hexane and methanol extractions were best described by the Michaelis-Menton equation $\hat{Y} = ax/(b + x)$, where a_{hexane} ($\pm$SEM) = 192.82 $\pm$ 8.37,

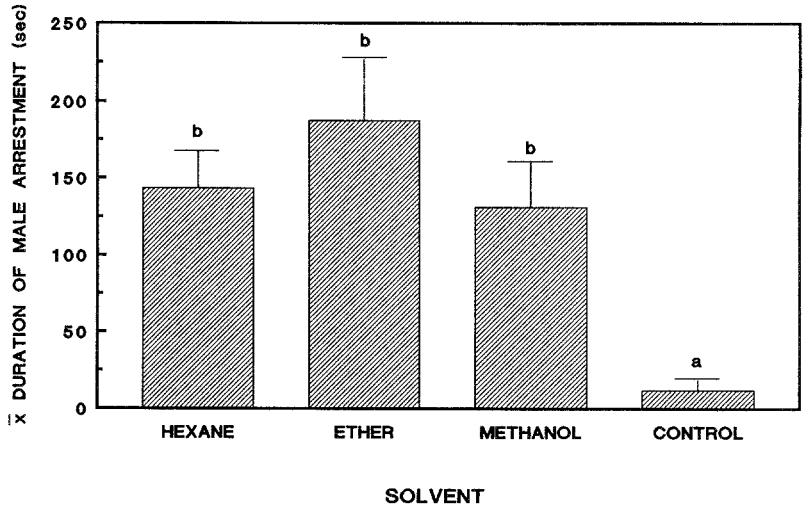
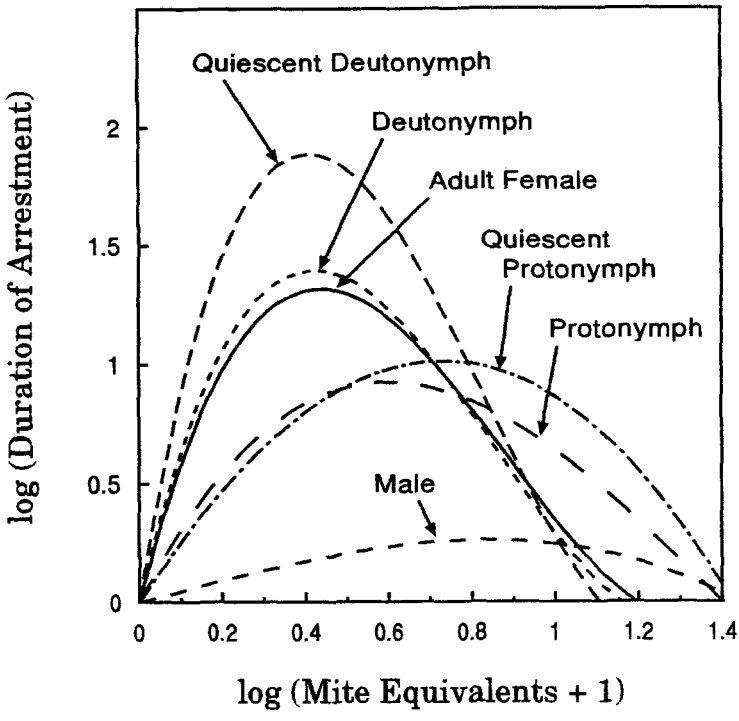


FIG. 2. Mean duration of male *T. urticae* arrestment caused by 1 QDE of extracts in different solvents. Error bars are standard errors of means. Means with the same letter were not significantly different [$P = 0.05$; Student-Newman-Keuls test (Snedecor and Cochran 1967)].

$b_{\text{hexane}} = 0.22 \pm 0.09$; $F = 355.52$, $df = 2, 163$; $P = 0.00001$; $r^2 = 0.74$, $a_{\text{methanol}} = 206.06 \pm 8.38$, $b_{\text{methanol}} = 0.16 \pm 0.06$; $F = 389.53$; $df = 2, 163$; $P = 0.00001$; $r^2 = 0.74$). No differences were observed in the extraction rates of the two solvents (b_{hexane} vs. b_{methanol}) ($t = 0.52$; $df = 2, 326$; $P = 0.05$) or in the maximum amount of pheromone extracted (a_{hexane} vs. a_{methanol}) ($t = 1.12$; $df = 2, 328$; $P = 0.05$). These results show that male *T. urticae* response to the extracts, and therefore extraction of pheromone from quiescent deutonymphs, was substantial even at short extraction times (≤ 1 hr), and did not increase substantially at times >2 hr.

Arrestment by Contact Chemoreception: Effect of Concentration and Extractions from Other Instars. Regressions of log(duration of arrestment) ($\hat{Y}$) against log(mite equivalents + 1) (x) show that extracts from all *T. urticae* instars elicited a response by guarding males (Figure 3). Relationships were best described by third-order polynomials, suggesting that maximum male *T. urticae* response to extracts occurred at a range of 1–10 ME for all instars. No male response occurred at concentrations <0.1 ME, and males usually avoided concentrations >10 ME (although a few individuals were arrested for extended time periods).

Maximum male *T. urticae* response to quiescent deutonymph, deutonymph, and adult female extracts occurred at approximately 1 ME, but mean durations of arrestment caused by deutonymph and adult female extracts were substantially



Regression Equations[a]

Adult Female	$\hat{Y} = 6.19x - 8.43x^2 + 2.65x^3$; $r^2 = 0.58$; $F = 88.19$
Quiescent Deutonymph	$\hat{Y} = 10.43x - 16.91x^2 + 6.90x^3$; $r^2 = 0.66$; $F = 127.24$
Deutonymph	$\hat{Y} = 7.65x - 12.44x^2 + 5.07x^3$; $r^2 = 0.58$; $F = 89.40$
Quiescent Protonymph	$\hat{Y} = 2.42x - 0.32x^2 - 1.44x^3$; $r^2 = 0.46$; $F = 50.88$
Protonymph	$\hat{Y} = 1.58x + 2.12x^2 + 2.86x^3$; $r^2 = 0.47$; $F = 52.52$
Male	$\hat{Y} = 0.46x + 0.02x^2 - 0.24x^3$; $r^2 = 0.11$; $F = 8.85$

[a] df = 3, 195 for all regressions.

FIG. 3. Regression of log(mean duration of male *T. urticae* arrestment) on log(mite equivalents) of extracts from different instars.

shorter than were caused by quiescent deutonymph extract. One explanation for these data is that deutonymphs and adult females lack pheromonal components essential to elicit maximum male response. It also is possible that quiescent deutonymphs contain greater quantities of female sex pheromone than adult females and deutonymphs. Higher concentrations (>2 ME) of deutonymph and adult female extracts may have contained sufficient sex pheromone to elicit arrestment comparable to that observed with 1 QDE of quiescent deutonymph extract, but the consequent increased concentrations of pigments and other chemicals in the extracts may have interfered with the guarding males' ability to perceive or respond to the sex pheromone.

Cone et al. (1971a) reported arrestment over a narrow concentration range, but observed maximum activity at 0.01–0.1 QDE. The disparity in results probably is due to the difference in experimental methods. Cone et al. (1971a) applied extract to plastic clumps approximately 1 mm in diameter, and placed the clumps on hop, *Humulus lupulus* L., leaf disks. These clumps concentrated the extract to an area smaller than the 3-mm circle used in our study, in addition to supplying the males with visual and tactile stimuli. Another difference between the two studies was the host plants used for mite cultures (hops vs. lima beans); Cone and Pruszynski (1972) reported that males reared on hops were arrested more frequently by extract-treated clumps than were males reared on beans.

The regression equations predict that maximum responses to the protonymph, quiescent protonymph, and male extracts were observed at the 5- to 8-ME range. The protonymph and quiescent protonymph extracts probably were either missing one or more of the components or contained lower concentrations of pheromone than the more mature instars. The relationship between concentration of male extract and duration of response was minimal. However, arrestment of a few males was observed at the 1-ME concentration, suggesting that male *T. urticae* probably contain small quantities of at least some of the components.

Orientation and Arrestment by Volatile Chemoreception to an Extract Source. No significant differences in *T. urticae* male response to the column of extract-laden air were observed among solvents, and none of the treatments differed significantly from the control ($F = 0.75$; $df = 6, 108$; $P = 0.05$; $W_p = 40.99$ sec). Similar data were collected for the upwind orientation study; no significant differences in the latency until arrestment [3 min − (time until male arrestment)] were observed among solvents at either 1 or 10 QDE, and no treatment differed from the control ($F = 0.70$; $df = 6, 66$; $P = 0.05$; $W_p = 22.97$ sec). Failure of male *T. urticae* to become arrested by the extract-laden airstream or to orient towards the upwind extract source suggests an inability to perceive sex pheromone in the air. Further evidence demonstrating *T. urticae*'s inability to detect extract via volatile chemoreception is the number of males that reached the point sources and became arrested during the upwind orientation

study. Initial placement of male *T. urticae* was 1 cm from the extract point sources, yet only 25% of males tested in this study became arrested at the point sources during the 3-min observation period.

The negative results of the arrestment and upwind orientation by volatile chemoreception studies disagree with Penman and Cone (1972). They observed a positive correlation between male *T. urticae* linear movement and duration of male exposure to quiescent deutonymphs, and therefore concluded that male perception of female sex pheromone volatiles initiated a searching behavior characterized by linear motion. They also observed that the mat of webbing deposited by the deutonymph prior to the final molt amplified this linear relationship. Our studies show that arrested males become immobile or move in tight circles and that unarrested male movement is more linear. A reinterpretation of Penman and Cone's results may explain this difference. Penman and Cone stopped observing males that became arrested by deutonymphs, thus failing to include the circling behavior of guarding males in their calculations. By the end of their study, they were measuring the movement only of males that had not become arrested. Consequently, what they described as linear searching behavior initiated by chemoreception of pheromone may have been a combination of orientation to quiescent deutonymphs through visual cues, orientation by tactile or chemoperception of the webbing spun by the deutonymph prior to becoming a chrysalis, and linear motion typical of unarrested males.

Bioassay of Terpenoid Compounds. Mean response time of male *T. urticae* to any of the four terpenoids did not differ from response to the control at any concentration [$F_{4ng} = 17.38$, $d'_{4ng} = 66.50$; $F_{40ng} = 17.87$, $d'_{40ng} = 63.80$; $F_{400ng} = 16.21$, $d'_{400ng} = 66.45$; $F_{4000ng} = 28.99$, $d'_{4000ng} = 56.88$; $df = 6, 78$; $P = 0.0001$; $t_{Dunnett} = 2.48$ (Table 1)]. In addition, all synthetics combined did not elicit a response that differed from the control. These results show that farnesol, nerolidol, geraniol, and citronellol probably are not components of *T. urticae* female sex pheromone.

Our results differ substantially from those obtained by Regev and Cone (1975a, 1976a, 1980). One possible explanation for the differing results are the bioassays used in the two studies. We collected data continuously for a maximum time period of 10 min, while their data were collected intermittently for several hours; no continuous observations were made. Another difference was their use of polyvinylpyrrolidone clumps as the pheromone source. Substantial numbers of males were observed guarding the control clumps, indicating male visual or mechanoperception of the clumps. Therefore, although significantly greater numbers of male guarding responses were observed with farnesol-, nerolidol-, and citronellol-treated clumps than with ethanol-treated controls, the frequency of male arrestment by control clumps suggests that the observed number of positive responses to treated clumps may have been exaggerated by the mites' tactile or visual perceptions of the source.

Initial Isolation of Active Components. Bioassays of HPLC-fractionated extract (Figure 4) showed that four of the 18 fractions (2, 4, 9, 12) caused significantly greater male *T. urticae* arrestment than the hexane control (F = 10.82; df = 19, 361; P = 0.0001; W_p = 53.01 sec). The presence of inactive

TABLE 1. DURATION OF ARRESTMENT OF MALE *T. urticae* CAUSED BY SYNTHETIC TERPENOIDS FARNESOL, NEROLIDOL, GERANIOL, AND CITRONELLOL

	$\bar{X}$ response time (sec) ± SEM[a]			
Terpenoid	4 ng	40 ng	400 ng	4000 ng
Farnesol	85.7 ± 18.9a	77.7 ± 14.3a	73.0 ± 14.3a	47.1 ± 10.9a
Nerolidol	60.4 ± 10.2a	47.3 ± 10.2a	52.1 ± 9.5a	38.4 ± 7.1a
Geraniol	49.4 ± 11.0a	64.1 ± 16.2a	81.4 ± 21.3a	32.6 ± 7.5a
Citronellol	66.4 ± 10.1a	91.6 ± 8.8a	61.6 ± 8.8a	27.3 ± 4.5a
Combined	63.3 ± 10.2a	54.4 ± 9.3a	63.2 ± 12.6a	26.9 ± 7.4a
Extract (1 QDE)	268.0 ± 38.1b			
Control	76.3 ± 13.3a			

[a]Means followed by the same letter are not significantly different from the control [P = 0.05; Dunnett's procedure (1955)].

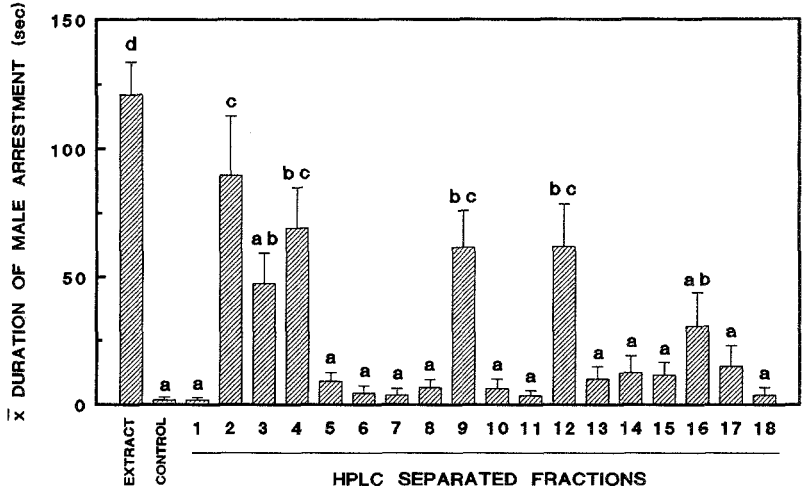


FIG. 4. Mean duration of male *T. urticae* arrestment caused by 0.5 QDE of HPLC-separated extract fractions. Error bars are standard errors of means. Means with the same letter were not significantly different [P = 0.05; Student-Newman-Keuls test (Snedecor and Cochran 1967)].

fractions between the four peaks of activity and the fact that the active fractions were eluted from the column by solvents of differing polarities suggest that each active fraction contained a different pheromonal component.

Fractions 3 and 16 also caused noticeable male *T. urticae* arrestment, although activity of these fractions did not differ significantly from the control. The apparent activity of fraction 3 may have been due to the presence of small quantities of the active components found in 2 and/or 4. Fraction 16, however, was collected in a solvent of greater polarity than the other active fractions. Therefore, the possible activity observed during the bioassay of this fraction probably was caused by a different pheromonal component.

The arrestment durations caused by the four active fractions were significantly shorter than the duration caused by the unseparated extract. The greater activity caused by the extract suggests that more than one pheromonal component is necessary to elicit maximum male response.

Subtractive Bioassay of Active Fractions. No significant differences were observed between the arrestment time caused by the extract and all of the fractions combined [$F = 6.38$; $df = 8, 152$; $P = 0.0001$; $W_p = 80.08$ sec (Figure 5)]. Therefore, all the active pheromonal components found in the extract probably were contained in the eight combined fractions.

No significant differences were observed among the durations of arrestment caused by all fractions combined and combinations lacking fractions 3, 12, or

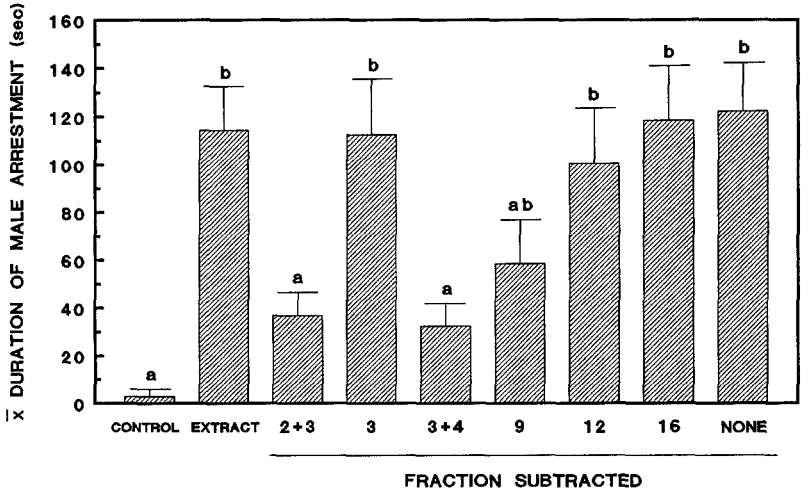


FIG. 5. Mean duration of male *T. urticae* arrestment from 0.5 QDE subtractive bioassay of active extract fractions. Error bars are standard errors of means. Means with the same letter were not significantly different [$P = 0.05$; Student-Newman-Keuls test (Snedecor and Cochran 1967)].

15–17. These results show that these three fractions were not essential to elicit maximum male *T. urticae* response. In addition, the insignificant arrestment caused by fractions 3 and 15–17 in the bioassay of individual fractions suggests that they probably do not contain pheromonal components.

Subtraction of fractions 2, 4, or 9 did cause a significant reduction in the duration of *T. urticae* male arrestment, as the duration of arrestment caused by their omissions did not differ significantly from the control. Therefore, the components of these three fractions are necessary to elicit maximum male response.

Our studies confirm that sex pheromone is present in *T. urticae* that arrests adult males. Extracts of adult females, males, and all immature instars elicited male response, but maximum duration of male response was observed with pheromone extracted from quiescent deutonymphs. These data agree with previous observations that while males frequently guard all *T. urticae* instars (including other males), they guard quiescent deutonymphs most frequently. Guarding quiescent deutonymphs is the optimum reproductive strategy for males, as mating with newly molted virgin females ensures that the male's sperm will be used for fertilization (Helle, 1967; Potter et al., 1976; Potter and Wrensch, 1978). Therefore it is logical to expect that the instar most likely to arrest males would be the quiescent deutonymph.

Only males that were guarding quiescent deutonymphs immediately prior to testing were arrested; nonguarding males did not respond. These data show that initiation of arrestment and guarding behavior probably is dependent on perception of other stimuli as well as the physiological state of the male; the primary function of sex pheromone is to prolong arrestment and guarding behavior.

Upwind orientation to point sources of pheromone and arrestment within a moving column of pheromone-laden air were not observed. These results suggest that volatile perception of pheromone by male *T. urticae* does not occur, and therefore males do not use volatile pheromone to locate quiescent deutonymphs.

HPLC separations showed that *T. urticae* female sex pheromone consists of at least four distinct active components. The three least polar fractions were necessary to elicit maximum male response, but full activity was observed when the most polar fraction was subtracted from the combination. Unlike Regev and Cone, we were unable to show that farnesol, nerolidol, and citronellol were components of *T. urticae* sex pheromone; if these alcohols are produced by *T. urticae*, they probably are not the essential components isolated in our studies.

Penman and Cone (1972, 1974) concluded that males locate quiescent deutonymph females through volatile chemoreception of sex pheromone. However, the range of male arrestment described in their studies was limited [1–2 mm (Penman and Cone, 1974)]. In addition, male response to extract sources was significantly greater on 10.3-mm leaf disks than on 21.0-mm disks (Cone and Pruszynski, 1972); the effect of disk area on source location by males

probably would have been negligible if males could perceive and/or orient to pheromone sources by olfaction. Our volatile chemoreception and upwind orientation studies do not support the hypothesis that male *T. urticae* perceive sex pheromone by olfaction. We suggest that males wandering on the leaf surface probably locate quiescent deutonymphs by short-range orientation to cues such as mechano- or chemoperception of the webbing spun by the deutonymph prior to becoming quiescent (Penman and Cone, 1972), visual perception of the quiescent deutonymph, or by chance. Males perceive sex pheromone by contact chemoreception; perception initiates localized searching behavior characterized by frequent turning and/or pivoting. Males recognize the source of pheromone as a quiescent deutonymph by assimilating tactile, visual, and contact chemoreceptive information, and guarding behavior subsequently begins. Future studies will attempt to determine the chemical composition of *T. urticae* female sex pheromone and to define more clearly the role of the various cues in the intersexual interaction of *T. urticae*.

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POSSIBLE ECOLOGICAL SIGNIFICANCE OF WITHIN-FRUIT AND SEED FUROCOUMARIN DISTRIBUTION IN TWO *Psoralea* SPECIES

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Abstract—Furocoumarin distribution patterns in the fruits and seeds of *Psoralea macrostachya* and *P. onobrychis* were investigated. Both species contain the linear furocoumarin psoralen and its angular isomer, angelicin. In the monospermous indehiscent fruit of *P. macrostachya*, furocoumarins occur in the pericarp and all seed parts. In *P. onobrychis*, the pericarp of which is easily detached at ripeness, no furocoumarins were found in the pericarp tissues and only traces occur in the embryo axis; cotyledons are the preferential accumulation site. The within-fruit and -seed furocoumarin variations associated with the developmental stages of fruit were followed in *P. onobrychis*, in view of changes in the defensive value of the pericarp before and after ripening. Rapid furocoumarin biosynthesis after fertilization was observed in both pericarp and seed; ripening is associated with furocoumarin decrease in the seed and complete disappearance in the pericarp tissues. Such findings are consistent with the chemical defense role of these substances. The cooccurrence of linear and angular isomers seems to be a chemical marker of the genus *Psoralea:* the biosynthetic pathway leading to the angular isomer as an evolutionary response to selective pressure from herbivore insects is suggested.

Key Words—*Psoralea macrostachya* DC., *P. onobrychis* Nutt., furocoumarins, psoralen, angelicin, within-fruit and -seed allocation, chemical defense, germination inhibitors, allelopathy.

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INTRODUCTION

Natural products such as furocoumarins have been regarded as protective agents in plants against predation by insects (Berenbaum and Feeny, 1981; Muckensturm et al., 1981; Berenbaum et al., 1984; Berenbaum, 1985) and by vertebrates (Ivie, 1978). A number of coumarin derivatives show antimicrobial (Fowlks et al., 1958; Fischer et al., 1976) and antifungal (Chakraborty et al., 1957) activities and may therefore prevent seed decay before germination (Rice, 1984).

Furocoumarins also act as seed germination inhibitors (Rodighiero, 1954; Fukushi, 1960) and plant growth regulators (Bennett and Bonner, 1953; Shimomura et al., 1982), and their role as allelopathic agents has been suggested (Junttila, 1976; Friedman et al., 1982). Whether furocoumarins function as autoinhibitors of germination is still an open question: they are believed to be responsible for the inhibition of seed germination in *Psoralea subacaulis* (Baskin et al., 1967) and *Angelica archangelica* (Zobel and Brown, 1991), whereas other umbelliferous species exhibit resistance or avoidance of autointoxication (Friedman et al., 1982, and references therein).

Furocoumarin distribution patterns within reproductive structures are of interest in connection with their suggested roles as germination regulators for the plant itself, as allelopathic agents, and as chemical defense against herbivores and pathogens. The importance of recording phenological changes of furocoumarin content within reproductive structures in order to evaluate the physiological and/or ecological significance of these compounds has been stressed (Berenbaum and Zangerl, 1986; Nitao and Zangerl, 1987).

As part of research on species belonging to the genus *Psoralea* as natural sources of biologically active furocoumarins of pharmaceutical interest (Cappelletti et al., 1984; Innocenti et al., 1977, 1984, 1991), we examined furocoumarin distribution patterns within the reproductive structures of *P. onobrychis* and *P. macrostachya* (for furocoumarin contents in the vegetative organs of these species, see Innocenti et al., 1991).

The fruits of *P. onobrychis* have never previously been investigated with respect to their coumarin content, whereas *P. macrostachya* fruits are known to contain psoralen and angelicin (Innocenti et al., 1977); their distribution within fruits and seeds, however, has not been studied.

In *P. macrostachya* the pericarp is free from the ripe seed and the monospermous fruit is indehiscent, which is typical of the genus according to Hutchinson (1964). In contrast, in *P. onobrychis* the pericarp is detached at ripeness, releasing the seed.

In order to evaluate the ecological significance of furocoumarins in chemical defense, we compared the distribution patterns of these compounds within

the reproductive structures of these two *Psoralea* species, in which very different ecological values and roles must be attributed to the pericarp tissues.

METHODS AND MATERIALS

Plant Material. Plants of two American *Psoralea* species [*Psoralea onobrychis* Nutt. of moist areas from Ohio to Missouri and southward to South Carolina and Tennessee (Rickett, 1967) and *P. macrostachya* DC. of moist areas and salt marshes of California (Mason, 1957)] were cultivated under identical environmental conditions in the Botanical Garden of the University of Padova. For both *Psoralea* species, ripe fruits collected in September were used.

To record phenological changes in furocoumarin contents of the reproductive structures of *P. onobrychis*, samples were collected at the following stages: stage 1: flowers; stage 2: fertilized ovaries (just after petal fall); stage 3: ovaries 5 mm long; stage 4: ovaries 8 mm long; stage 5: ovaries 11 mm long; stage 6: ripe fruits.

Fruit and Seed Morphology. Fruit and seed samples were prepared for SEM examination following the previously described procedure, which consists of sample fixation in 6% glutaraldehyde in 0.1 M cacodylate buffer (pH 6.9) in the dark at 0°C for 24–48 hr, gradual dehydration in acetone of increasing concentrations, critical-point drying in a Balzers Union critical point dryer, coating with gold in an Edwards S 150 A sputter coater, and observation under a Cambridge Stereoscan 250 at the accelerating voltage of 20 kV (Cappelletti and Trevisan, 1983).

Sample Preparation. Three different plants of the two *Psoralea* species were investigated. Each sample consisted of tissues taken from at least 10–20 fruits from a single plant. Ripe fruits (and also fruits of stage 5), were separated by microdissection under a binocular microscope, and the following subsamples were obtained: (1) pericarp, (2) seed coats, (3) cotyledons, and (4) shoot–hypocotyl–root axis of embryo. Sample and subsample weights ranged from 0.6 to 2.5 g.

Chemical Analysis. The extraction procedure takes into account the cooccurrence of both free and bound furocoumarins in the fruits of leguminous plants (Stöll et al., 1950; Innocenti et al., 1984). Samples dried *in vacuo* (20°C) were extracted with MeOH in a Soxhlet extraction apparatus for 48 hr, and the methanolic extract was concentrated (fraction I). Fraction I contained the furocoumarins occurring in the plant in the free form. The extracted marc was refluxed for 2 hr in 30 ml of 5% HCl. After cooling and filtration, an acid residue and an aqueous–acid phase were obtained. Following a previously described procedure for exhaustive psoralen extraction (Innocenti et al., 1984), the acid res-

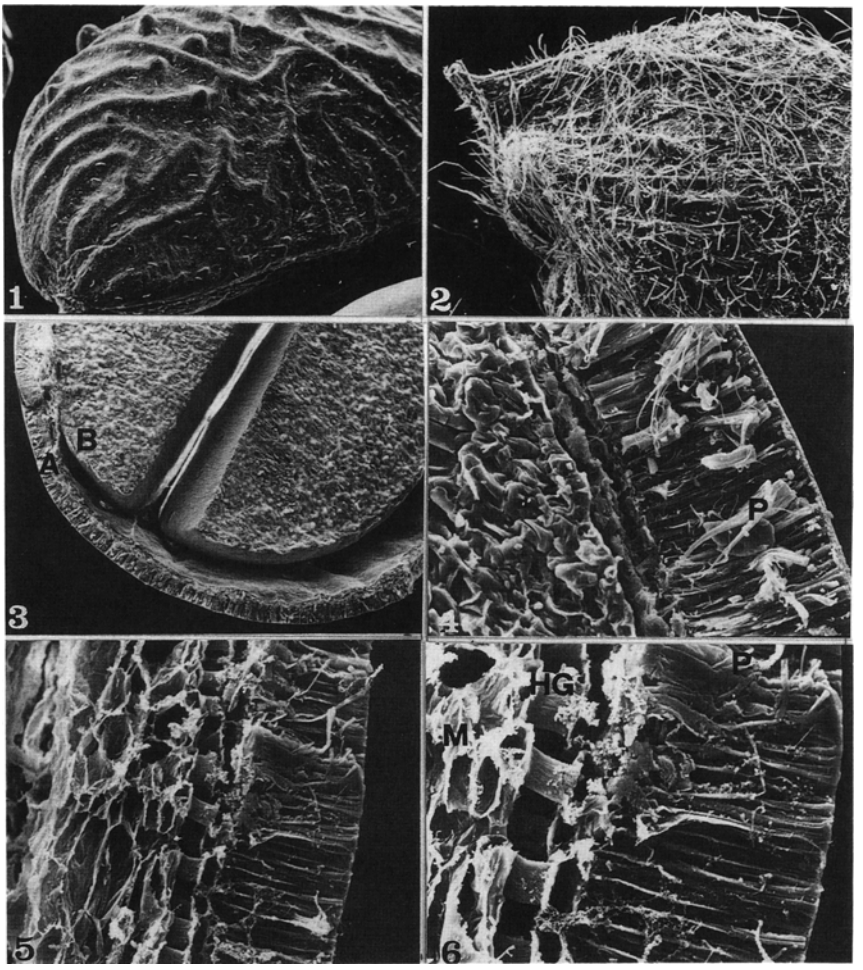
idue was extracted with MeOH for 48 hr and the methanolic extract was then concentrated (A). The aqueous-acid phase was extracted with Et_2O (48 hr); after distillation of the solvent, the residue was dissolved in MeOH (B). Methanolic solutions A and B were combined (fraction II). This fraction contained the furocoumarins occurring in the plant in bound form. Fractions containing both free and bound coumarin forms were combined and analyzed.

Psoralen and angelicin were isolated by TLC (silica gel plates with $CHCl_3$ or EtOAc–cyclohexane in different ratios as eluents) (Innocenti et al., 1989, 1991) and identified by their UV and mass spectra and by comparison with authentic samples. The furocoumarin contents in the different plant parts were determined by HPLC under the following conditions: Si gel column VIOSIL SI 10 μm (Violet) 250 × 4.6 mm ID with a small precolumn (25 × 4.6 mm ID) packed with Si gel Spherisorb 10 μm, mobile phase *n*-hexane–$CHCl_3$ (71:29; v/v); flow-rate 2 ml/min; sample size injected 10 μl, detection at 300 nm. Retention times: angelicin 2.34 min, psoralene 2.93 min. For each solution, quantification was carried out using 5,8-dimethoxypsoralen (5,8-DMOP) as the internal standard. Calibration curves were determined using a series of standard solutions containing a constant amount of internal standard and varying amounts of stock psoralen and angelicin (Innocenti et al., 1991). The quoted data are the average values of quantitative determinations performed on the three samples each taken from a single plant. Standard deviation was shown to be less than 5%. Authentic samples of psoralen, angelicin, and 5,8-DMOP were supplied by the Department of Pharmaceutical Sciences of the University of Padova.

RESULTS AND DISCUSSION

The main morphoanatomical features of the fruits and seeds of the studied *Psoralea* species are shown in Figures 1–6. In both species, the embryo (two large cotyledons and a shoot–hypocotyl–root axis) is easily detached from an outer seed part consisting of the seed coats together with a residual film of endosperm (Figure 3), as previously described for *P. corylifolia* (Cappelletti et al., 1984). The outer seed coat epidermis consists of prismatic, radially elongated, and compactly arranged cells with thickened but not lignified walls (palisade or Malpighian cells). A layer of columnar, slightly waisted cells with wide airspaces in between (so-called hourglass cells) can also be found; the mesophyll and a residual layer of endosperm follows inward (Figures 4–6).

The furocoumarin contents of the whole fruits and their distribution patterns within the reproductive organs are reported in Table 1. The total furocoumarin amounts found in the whole fruits of *P. macrostachya* and *P. onobrychis* are fairly high with respect to those occurring in other species of the same genus, although far from the very high contents present in *P. corylifolia* (both psoralen



FIGS. 1–6. (1) *Psoralea onobrychis* fruit surface (×12); (2) *P. machrostachya* fruit surface (×20); (3) *P. onobrychis* seed, seed coats, and endosperm (A) easily detached from *cotyledone* (B) (×30); (4) *P. onobrychis* seed coat with palisade or malpighian cells (P), hour-glass cells, and mesophyll (×290); (5) *P. machrostachya* seed coat (×215); (6) *P. machrostachya* seed coat with palisade (P), hour-glass (HG), and mesophyll (M) cells (×390). [Figure reduced 77% for placement.]

and angelicin about 2500 ppm) and *P. plumosa* (psoralen 2500 ppm; angelicin 3000 ppm) fruits (Innocenti et al., 1984).

P. macrostachya fruits seem to be a much more interesting natural source of furocoumarins than previously supposed. In fact, fruits of *P. macrostachya*

TABLE 1. FUROCOUMARIN DISTRIBUTION PATTERNS WITHIN REPRODUCTIVE ORGANS OF INVESTIGATED *Psoralea* SPECIES (ppm OF DRY WEIGHT)

Species	Plant parts	Psoralen (Ps)	Angelicin (Ang)	Total	Ps/Ang ratio
P. macrostachya	whole fruit	278 ± 14	928 ± 45	1206 ± 47	0.30
	pericarp	121 ± 6	334 ± 15	455 ± 16	0.36
	seed coat	53 ± 3	126 ± 6	179 ± 7	0.42
	cotyledon	98 ± 4	240 ± 11	338 ± 12	0.40
	shoot-hypocotyl-root axis	149 ± 7	367 ± 18	516 ± 19	0.40
P. onobrychis	whole fruit	267 ± 11	589 ± 24	856 ± 26	0.45
	pericarp				
	seed coat	39 ± 2	86 ± 4	125 ± 4	0.45
	cotyledon	979 ± 47	2174 ± 98	3153 ± 109	0.45
	shoot-hypocotyl-root axis	traces	traces	traces	

cultivated in the Botanical Garden of Padova were found to contain only 50 ppm of psoralen and 300 ppm of angelicin (Innocenti et al., 1977). This discrepancy is attributable to the different extractive procedure used in the present research: milder extraction conditions and hydrolysis of the bound forms, along with a more sensitive quantitative method (Innocenti et al., 1984), allowed more complete recovery of furocoumarin compounds.

In *P. macrostachya* whole fruit, furocoumarin contents comparable with those found in the vegetative organs of the same plant were observed; in contrast, in *P. onobrychis*, fruits seem to be the preferential accumulation site (Innocenti et al., 1991).

In the whole fruits of *P. onobrychis* a high proportion of the total furocoumarin is present in bound form (80% and 65% for psoralen and angelicin, respectively). In contrast, *P. macrostachya* fruits contain mainly unbound furocoumarins (bound psoralen and angelicin 23% and 20%, respectively). This proportion varies greatly among *Psoralea* species. In fact, species in which the unbound forms are most prevalent (*P. corylifolia*, *P. lacnostachys*) and species in which more than 90% of the total furocoumarins are bound (*P. plumosa*), have been described (Innocenti et al., 1984).

Very different distribution patterns of furocoumarins within reproductive structures were found in the two species (Table 1). In *P. macrostachya* a situation similar to that recorded in *P. corylifolia* (Cappelletti et al., 1984) was observed, furocoumarins occurring in the pericarp and in all seed parts. Whereas the pericarp is the preferential accumulation site in *P. corylifolia*, similar contents are found in the pericarp and embryo axis in *P. macrostachya*. No furo-

coumarins were found in the pericarp and only traces occur in the embryo axis of *P. onobrychis*; furocoumarins are present in the seed coats but accumulate mainly in the cotyledons.

In view of changes in the defensive value of the pericarp as the fruit progresses in its development, and in agreement with optimal defense theory (Nitao and Zangerl, 1987), the within-fruit furocoumarin variation associated with the developmental stages of *P. onobrychis* fruit was followed (Table 2). No furocoumarins occur in the flower (stage 1), in contrast with a previous report for *Coronilla vaginalis* (Innocenti et al., 1989). After fertilization (stage 2), rapid biosynthesis of furocoumarins takes place, as previously described for umbelliferous fruits (Nitao and Zangerl, 1987; Bicchi et al., 1990). When the fruits reach a convenient size, allowing separate sampling and analysis (stage 5), furocoumarins are found in both pericarp and seed, with significantly higher concentrations in the seed. Ripening (stage 6) is associated with a decrease of furocoumarins in the seed and their complete disappearance in pericarp tissues.

The differences in furocoumarin allocation within the fruits of these species is consistent with the suggested role of these compounds as protective agents. In fact, in *P. onobrychis*, pericarp tissues may have a defensive significance as long as they act as an envelope for the seed, i.e., until fruit ripening. In *P. macrostachya*, the persisting defensive role of the pericarp, which does not become detached from the seed, may account for the different furocoumarin distribution. In the present research, cultivated plants have been analyzed; however, in order to evaluate the actual ecological significance of furocoumarins as

TABLE 2. *P. onobrychis:* CHANGES IN FUROCOUMARIN CONTENTS (ppm OF DRY WEIGHT) ASSOCIATED WITH FRUIT DEVELOPMENTAL STAGES

Sample	Psoralen (Ps)	Angelicin (Ang)	Total	Ps/Ang ratio
Stage 1				
Stage 2	2794 ± 112	4861 ± 146	7655 ± 184	0.57
Stage 3	2347 ± 94	3711 ± 148	6058 ± 175	0.63
Stage 4	1087 ± 54	1729 ± 69	2816 ± 87	0.63
Stage 5				
Whole fruit	2085 ± 83	4104 ± 123	6189 ± 148	0.51
Pericarp	437 ± 20	719 ± 28	1156 ± 34	0.60
Seed	2328 ± 93	4740 ± 140	7068 ± 168	0.49
Stage 6				
Whole fruit	268 ± 10	609 ± 24	877 ± 26	0.44
Pericarp				
Seed	387 ± 16	903 ± 35	1290 ± 38	0.43

protective agents for these two *Psoralea* species, plants in their natural habitats should be investigated.

The linear (psoralen) and angular (angelicin) isomers were found in both species. These findings are in agreement with the analytical data from all *Psoralea* species hitherto investigated (Jois and Manjunath, 1936; Kohlmuenzer, 1965; Akubakirov and Khalmurzaev, 1967; Ahmad and Basha, 1972; Samylina and Ladygina, 1975; Cappelletti et al., 1984; Innocenti et al., 1977, 1984, 1991) and give further support to the hypothesis (Innocenti et al., 1984, 1991) that the cooccurrence of linear and angular furocoumarins is a chemical marker of the genus *Psoralea*. In contrast, in another genus of the same family, *Coronilla*, only the linear isomer, psoralen, is present (Stöll et al., 1950; (Innocenti et al., 1986, 1989).

The angular isomer, angelicin, is more abundant than psoralen in both the investigated *Psoralea* species. However, interspecific differences in ratios were described. The psoralen–angelicin ratios in the whole fruits range from 2.0–2.5, as in *P. lacnostachys* and *P. pustulata*, to about 0.3–0.4, as in the present species and in *P. cinerea* (Innocenti et al., 1984). Very few data on ontogenetic changes of the isomer ratio within the different plant organs are available; therefore, no hypothesis on the possible significance of these changes can be put forward.

Angular compounds seem to be more advanced biosynthetically than linear furocoumarins (Berenbaum and Feeny, 1981). The biosynthetic pathway leading to angular attachment of the furan ring in advanced tribes of the Umbelliferae has been regarded as an evolutionary response to selective pressure by insects adapted to feeding on plants containing linear furocoumarins (Berenbaum and Feeny, 1981). Chemical mixtures of furocoumarins are in fact more effective as toxicants than single component compounds at equivalent concentrations (Berenbaum and Feeny, 1981; Berenbaum, 1985). Similarly, the biosynthetic pathway leading to angular furocoumarins may have arisen in *Psoralea* in response to selective pressure by insect herbivores.

Although our data on furocoumarin allocation within the fruits of the investigated *Psoralea* species are consistent with the role of chemical defense, the occurrence of considerable amounts of furocoumarins in embryos raises the question of their role as germination inhibitors. In *P. subacaulis*, furocoumarins have been shown to be both inhibitory to other unrelated species and autoinhibitory (Baskin et al., 1967).

Therefore, in *Psoralea*, furocoumarins may have many ecological functions: as chemical defense against predators and as germination inhibitors to reduce interspecific competition and regulate seedling densities within the population (intraspecific inhibition).

More research is needed in order to evaluate the role of *Psoralea* furocoumarins as germination inhibitors. We believe that recording within-seed furo-

coumarin distributions and variations during imbibition and germination may provide valuable information on the ecological role of these compounds. Research on this topic is in progress.

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SEX PHEROMONE COMPONENTS OF THE GIANT LOOPER, *Boarmia selenaria* SCHIFF. (LEPIDOPTERA: GEOMETRIDAE): Identification, Synthesis, Electrophysiological Evaluation, and Behavioral Activity

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Abstract—(*Z*,*Z*)-6,9-*cis*-(3*S*,4*R*)-Epoxynonadecadiene **9a** and (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene have been identified as sex pheromone components in female ovipositor extracts and effluvia of the geometrid *Boarmia selenaria*. The identification was based on relative retention times on gas chromatographic columns, GC-MS, chemical enantioselective syntheses, EAG, wind-tunnel behavioral experiments, and field tests. A single female ovipositor extract contained an average of 42 ng of (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene and 2 ng of (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene. EAG, wind-tunnel behavioral studies, and field tests demonstrated that male *B. selenaria* exhibit preferential attraction for the (3*S*,4*R*)-enantiomer of (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene. Only in effluvia and ovipositor extracts of calling females was (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene detected, whereas relatively high amounts of (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene were found in noncalling females. Wind-tunnel behavioral studies showed that (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene did not elicit any response in the males, and when tested in combination with a mixture of monoepoxynonadecadienes, (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene decreased the observed behavioral responses. A possible precursor role for (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene in the pheromonal system is discussed.

Key Words—*Boarmia selenaria*, giant looper, sex pheromones, Lepidoptera, Geometridae, (*Z*,*Z*)-6,9-*cis*-(3*S*,4*R*)-epoxynonadecadiene, (*Z*,*Z*)-6,9-*cis*-

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(3*R*,4*S*)-epoxynonadecadiene, (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene, enantiomers, enantioselective synthesis, EAG, wind tunnel, field tests, precursor.

INTRODUCTION

Boarmia selenaria Schiffermüller, the giant looper, is a serious pest of fruit and foliage in avocado orchards in various regions of Israel (Wysoki et al., 1975). The identification of the sex pheromone of *B. selenaria* was undertaken as part of a study to develop an integrated management program for this insect species based on the use of *Bacillus thuringiensis* (Berliner) and pheromone monitoring traps. In 1982, efforts were made to replace the virgin female-based monitoring system by a monitoring system based on the synthetic pheromone of *B. selenaria*. Using material extracted from the abdomen of virgin females, Becker et al. (1983) isolated two fractions by gas chromatography and liquid chromatography, which were found to be active by electroantennograph measurements. The less polar fraction was subsequently identified as (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene whose presence was demonstrated in the female extract by GC-MS. Recently, the second fraction was identified as (*Z*,*Z*)-6,9-*cis*-(3*S*,4*R*)-epoxynonadecadiene **9a** (Becker et al., 1990). We report here, in detail, the isolation and identification of those two sex pheromone components of *B. selenaria*, together with the enantioselective synthesis of two enantiomers of (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene, and the results of electroantennogram, wind-tunnel, and field-trapping experiments using the synthetic materials.

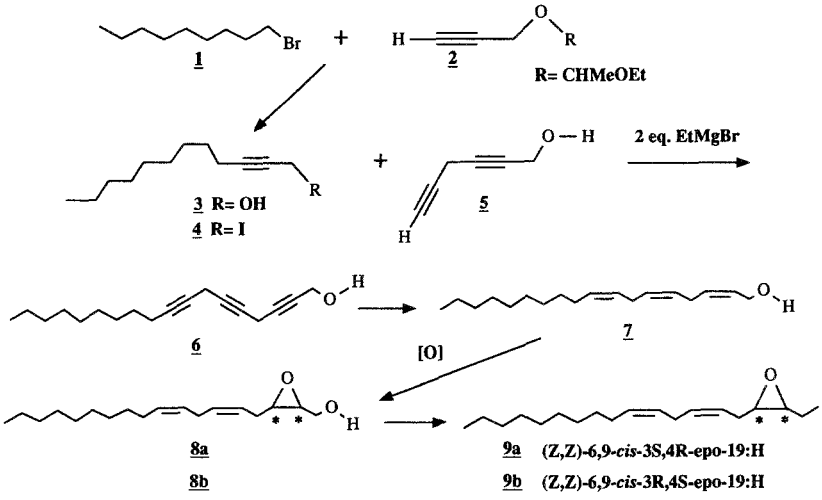
METHODS AND MATERIALS

Synthetic Chemicals. Compounds were analyzed by GC and were shown to be ≥98% pure. The two enantiomers of (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene were synthesized following the methodologies of Mori and Ebata (1986), and Millar et al. (1990c), modifications to these methods are described below (see Scheme 1).

Preparation of (Z,Z,Z)-*3,6,9-Nonadecatriene.* (*Z*,*Z*,*Z*)-3,6,9-Nonadecatriene (*Z*3,*Z*6,*Z*9–19:H) was prepared from linolenic acid and a 20-fold excess of propanoic acid by a Kolbe electrolysis according to the procedure of Conner et al. (1980). Pure *Z*3,*Z*6,*Z*9–19:H was obtained after column chromatography (silica gel, elution with hexane).

Preparation of cis-*Monoepoxynonadecadienes.* The *cis*-monoepoxynonadecadienes (MED-19:H) were prepared from *Z*3,*Z*6,*Z*9–19:H as previously described for the homologous C_{17}–C_{22} compounds (Wong et al., 1985), using *m*-chloroperbenzoic acid in dry methylene chloride.

Preparation of (Z,Z)-*6,9*-cis-*(3*S,*4*R*)-*Epoxynonadecadiene* ***9a*** *and its*



SCHEME 1.

(3R,4S)-enantiomer ***9b***. 1-Nonanol (41 g, 0.28 mol) was dissolved in HBr (100 ml, 48%) to which H_2SO_4 (25 ml) was added dropwise. After reflux overnight, methylene chloride (100 ml) and H_2O (100 ml) were slowly added. The organic layer was separated and the aqueous layer was extracted with methylene chloride. The combined organic solutions were washed with satd. $NaHCO_3$, dried ($MgSO_4$) and concentrated under reduced pressure to yield an oily compound that was distilled (115°C, 20 mm Hg) to give 56 g (96%) of 1-bromononane **1**.

Protected propargyl alcohol **2** was prepared by acid-catalyzed addition of propargyl alcohol to ethyl vinyl ether as described by Brandsma (1971).

To a solution of protected propargyl alcohol **2** (25,6 g, 200 mmol) in dry THF (500 ml) at −5°C was added dropwise a solution of Bu^nLi (2.3 M in hexane, 100 ml, 230 mmol), followed by the addition of HMPA (100 ml). After adding dropwise 1-bromononane **1** (36.5 g, 177 mmol), the reaction was heated up to room temperature. After 3 hr at room temperature, the protecting ethyl vinyl ether was cleaved by adding 2 ml of conc. HCl in 20 ml of methanol at room temperature. The mixture was stirred for 1 hr and then poured into H_2O (100 ml), followed by the removal of the solvents under reduced pressure. The residue was extracted with hexane, washed with H_2O, and dried ($MgSO_4$). The solvent was removed under reduced pressure to yield an oily compound that was distilled (90°C/0.01 mm Hg) to give 28.3 g (88%) of 2-dodecyn-1-ol **3**.

2-Dodecyn-1-ol **3** (13.4 g, 74 mmol) was converted to the corresponding 1-iodo-2-dodecyne **4** by published procedure (Corey et al., 1983), using PhP_3 (21 g, 80 mmol), imidazole (5.4 g, 80 mmol), and I_2 (20.3 g, 80 mmol) in

ether–CH_3CN 3:1 (160 ml) for 2 hr at room temperature. After work-up, the crude mixture was chromatographed over silica gel column, eluted by hexane to give 18.4 g (85%) 1-iodo-2-dodecyne **4**.

Protected propargyl alcohol **2** (10 g, 78 mmol) was converted to 2,5-hexadiyn-1-ol **5** (4.7 g) in 64% yield as described by Brandsma (1971).

To a solution of EtMgBr (36 mmol) in THF (40 ml) was added dropwise a solution of 2,5-hexadiyn-1-ol **5** (1.7 g, 18 mmol) dissolved in THF (20 ml) at room temperature. After stirring for 2 hr, CuI (0.6 mg, 3.1 mmol) was added, followed 25 min later by the addition of a solution of 1-iodo-2-dodecyne **4** (4 g, 13.7 mmol) dissolved in THF (20 ml). The mixture was stirred overnight and then poured into a solution of NH_4Cl (30 g) and NaCN (1 g) dissolved in H_2O (100 ml). The organic layer was separated and the aqueous layer was extracted with ether. The combined organic solutions were washed with satd. NH_4Cl and dried ($MgSO_4$). The solvent was removed under reduced pressure to yield a dark brown oil. The product was crystallized from hexane (0°C) to yield 2 g (58%) of 2,5,8-octadecatriyn-1-ol **6** (mp ≈ 20°C). 2,5,8-Octadecatriyn-1-ol was rather unstable and was therefore immediately reduced.

2,5,8-Octadecatriyn-1-ol **6** (1 g, 3.9 mmol) was reduced (H_2, 1 atm., hexane) over Lindlar catalyst (5% w/w) to give (*Z,Z,Z*)-2,5,8-octadecatrien-1-ol **7** in quantitative yield.

HRMS calcd. for $C_{18}H_{32}O$: 264.2453, found: 264.2462. [^{1}H]NMR (400 MHz, $CDCl_3$) δ 5.60 (1H, m, =C—H 2), 5.52 (1H, m, =C—H 3), 5.33 (4H, m, =C—H 5, 6, 8, 9), 4.20 (2H, d, J = 7 Hz, C—H 1), 2.82 (2H, t, J = 7 Hz, C—H 4), 2.78 (2H, t, J = 7 Hz, C—H 7), 2.02 (2H, q, C—H 10), 1.24 (12H, s, C—H 11–17), 0.85 (3H, t, C—H 18). IR ($CHCl_3$): 3600, 3420, 2920, 2860, 1460 cm^{-1}.

(*Z,Z,Z*)-2,5,8-Octadecatrien-1-ol **7** (0.6 g, 2.27 mmol) was epoxidized applying a Sharpless epoxidation (Katsuki and Sharpless, 1980), using $Ti(OPr^i)_4$ (0.81 ml, 2.73 mmol), L-(+)-dimethyl tartrate (566 mg, 3.18 mmol), and Bu^tOOH (7.5 M in isooctane, 0.76 ml, 5.67 mmol) in dry methylene chloride (40 ml), 6 hr at −23°C followed by 63 hr at −10°C. After work-up, the product was chromatographed over silica gel, eluted with hexane–methylene chloride 1:2 to give 0.49 mg (77%) (*Z,Z*)-5,8-*cis*-(2*S*,3*R*-epoxyoctadecadien-1-ol **8a**, ee = 68% by GC.

HRMS calcd. for $C_{18}H_{32}O_2$: 280.2403, found: 280.2393. [^{1}H]NMR (400 MHz, $CDCl_3$) δ 5.48 (1H, m, =C—H 5), 5.39 (2H, m, =C—H 6,8), 5.27 (1H, m, =C—H 9), 3.81 (1H, bd, J = 15 Hz, C—H 1), 3.68 (1H, dd, J = 15, J = 7 Hz, C—H 1), 3.15 (1H, m, C—H 2), 3.04 (1H, m, C—H 3), 2.75 (2H, t, C—H 7), 2.44 (1H, m, C—H 4), 2.25 (1H, m, C—H 4), 2.0 (2H, q, C—H 10), 1.22 (14H, s, C—H 11–17), 0.84 (3H, t, C—H 18). IR ($CHCl_3$): 3520, 2960, 1650, 1460 cm^{-1}.

(*Z,Z,Z*)-2,5,8-Octadecatrien-1-ol **7** (0.4 g, 1.52 mmol) was epoxidized as

described for (*Z*,*Z*)-5,8-*cis*-(2*S*3*R*)-epoxyoctadecadien-1-ol **8a,** using D-(−)-diethyl tartrate (437 mg, 2.12 mmol), to give 0.3 g (70%) (*Z*,*Z*)-5,8-*cis*-(2*R*,3*S*)-epoxyoctadecadien-1-ol **8b**. The IR and NMR spectra were identical to those of the (2*S*,3*R*)-enantiomer, ee = 65% by GC.

To a solution of (*Z*,*Z*)-5,8-*cis*-(2*S*,3*R*)-epoxyoctadecadien-1-ol **8a** (200 mg, 0.71 mmol) in dry pyridine (10 ml) was added *p*-toluenesulfonyl chloride (340 mg, 1.78 mmol) with stirring at 0°C. The reaction was left overnight at 4°C. The mixture was poured into ice–H_2O and extracted with ether. The ether solution was washed with 10% aqueous cupric sulfate, H_2O and brine, dried ($MgSO_4$), and the solvent was removed under reduced pressure to give 264 mg (85%) (*Z*,*Z*)-5,8-*cis*-(2*S*,3*R*)-epoxyoctadecadienyl tosylate.

(*Z*,*Z*)-5,8-*cis*-(2*R*,3*S*)-Epoxyoctadecadien-1-ol **8b** (200 mg, 0.71 mmol) was tosylated as described for (*Z*,*Z*)-5,8-*cis*-(2*S*,,3*R*)-epoxyoctadecadienyl tosylate, to give 254 mg (82%) (*Z*,*Z*)-5,8-*cis*-(2*R*,3*S*)-epoxyocadecadienyl tosylate.

To a solution of CuI (290 mg, 1.52 mmol) in dry ether (5 ml) was added dropwise a solution of CH_3Li (1.6 M, 2.38 ml, 3.80 mmol) at −20°C. The mixture was stirred for 20 min, followed by the addition of (*Z*,*Z*)-5,8-*cis*-(2*S*,3*R*)-epoxyoctadecadienyl tosylate (264 mg, 0.61 mmol). The mixture was stirred for an additional 2 hr at −20°C and then poured into aqueous NH_4Cl. After stirring for 30 min, the organic layer was separated and the aqueous layer was extracted with ether. The combined organic solutions were washed with H_2O and brine, dried ($MgSO_4$), and the solvent was removed under reduced pressure. The residue was chromatographed over silica gel, eluted with hexane–methylene chloride 6:1 to give 130 mg (77%) (*Z*,*Z*)-6,9-*cis*-(3*S*,4*R*)-epoxynonadecadiene **9a** [*Z*6,*Z*9-*cis*-(3*S*,4*R*)-epo-19:H].

HRMS calcd. for $C_{19}H_{34}O$: 278.2609, found: 278.2594. [^{1}H]NMR (400 MHz, $CDCl_3$) δ 5.5–5.42 (2H, m, =C—H 6,7), 5.42–5.3 (2H, m, =C—H 9,10), 2.93 (1H, m, C—H 4), 2.87 (1H, m, C—H 3), 2.79 (2H, t, C—H 8), 2.39 (1H, m, C—H 5), 2.2 (1H, m, C—H 5), 2.02 (2H, q, C—H 11), 1.57 (2H, m, C—H 2), 1.35 (2H, m, C—H 12), 1.25 (12H, s, C—H 13–18), 1.04 (3H, t, C—H 1), 0.86 (3H, t, C—H 19). IR ($CHCl_3$): 2960, 2840, 1740, 1460 cm^{-1}.

(*Z*,*Z*)-5,8-*cis*-(2*R*,3*S*)-Epoxyoctadecadienyl tosylate (254 mg, 0.58 mmol) was alkylated as described for (*Z*,*Z*)-6,9-*cis*-(3*S*,4*R*)-epoxynonadecadiene **9a** to give 122 mg (75%) (*Z*,*Z*)-6,9-*cis*-(3*R*,4*S*)-epoxynonadecadiene **9b** [*Z*6,*Z*9-*cis*-(3*R*,4*S*)-epo-19:H]. The IR and NMR spectra were identical to those of the (3*S*,4*R*)-enantiomer.

Insects. The *B. selenaria* breeding used in this study was started with larvae obtained from avocado orchards in Regba, Western Galilee, Israel. Newly collected larvae were added to the breeding monthly. The insects were kept under a reversed 16-hr light–8-hr dark cycle at 27 ± 1°C, relative humidity 70%, and were fed on avocado leaves or on a semiartificial diet (Shorey and Hale, 1965).

Immediately after the caterpillars pupated (approx. 25–30 days after egg hatching), pupae were gathered, sexed, and kept in separate rooms.

Extraction of Female Glands and Collection of Airborne Volatiles from Females. One- to two-day-old virgin females were placed in a wire net cage under red fluorescent light (11 lux) at the beginning of the scotophase. After 5 hr of active calling, females were placed at −20°C for 20 min prior to excising their ovipositor. Ovipositors were excised from the middle of the ninth segment. In freeze-killed females the eighth, ninth, and tenth abdominal segments remain telescoped within the seventh segment. These last parts of the abdomen were pulled out with forceps and cut from the middle of the ninth segment. The cutting edges were blotted on filter paper before transferring the tissue to solvent. The sex pheromone gland is epidermal tissue located between the ninth and tenth abdominal segment (Wysoki et al., 1985). The ovipositor tips were extracted with hexane (redistilled and passed over Al_2O_3) for about 1 hr at room temperature. For quantitative analysis, excised ovipositors were extracted individually in cone-shaped microvials containing 200 μl of hexane. The samples were concentrated to 1 μl under a slow stream of N_2. The samples were directly analyzed by GC. Recoveries of synthetic materials were reproducible and were proven to be 100%.

The collection apparatus was a modified version of the one used by Charlton and Cardé (1982). The collection device consisted of two modified glass joints (B-55) forming the insect holding chamber, which incorporated a 2 × 3-cm metal screen, hung in such a way that it did not touch the sides of the chamber. The lower half of the holding chamber was connected to a pheromone trap. The pheromone traps consisted of glass liner tubes for the GC injector (78.5 ± 0.1 mm × 6.52 mm OD) and were each filled with 100 mg Chromosorb Q (80–100 mesh, Chromopack) between two glass wool plugs. The absorbent was pretreated by repeated washes with redistilled hexane followed by baking at 200°C for 24 hr under vacuum. The glass liners were filled with the pretreated absorbent and placed in the GC injector (250°C) for 16 hr under a constant stream of helium. The injector was cooled down and the liner removed and stored in individual glass vials under nitrogen. The airflow for the collection apparatus was supplied by a water-vacuum pump and set at 0.3 liters/min. The air, before entering the holding chamber, was passed through an air filter (70 mm × 20 mm ID) containing 6 g of activated charcoal (1.5 mm). A 1- to 2-day-old virgin female was placed in the collection apparatus just before the onset of the scotophase. Once the female became acquiescent and before calling started, the airflow was initiated. The aeration period lasted a maximum of 5 hr, during which the female was observed to be calling. Visibility was provided by a red fluorescent light (11 lux). An empty collection apparatus, operated under the same conditions as mentioned above, served as a control for collected volatiles that were unrelated to the female emitted volatiles. Breakthrough of

pheromone components did not occur as evidenced by the lack of detectable pheromone in the second pheromone trap placed in series with the first. At the end of the aeration period, the pheromone trap was removed and the collected volatiles were directly analyzed by GC by placing the trap into the GC injector.

Gas Chromatography (GC) and GC-Mass Spectrometry (GC-MS). GC analyses were performed on a Hewlett Packard 5890 equipped with a HP-3393A integrator, using the following columns: (1) SE-54, 25 m × 0.22 mm ID fused silica capillary column (S.G.E. Ltd.); (2) OV-101, 50 m × 0.25 mm ID fused silica WCOT capillary column (Packard, Inc.); (3) Supelcowax-10, 60 m × 0.25 mm ID fused silica capillary column (Supelco, Inc.); and (4) BD210, 30 m × 0.25 mm ID fused silica capillary column (J&W Scientific). Columns 1, 2, and 3 were operated in splitless mode with the following program: 3 min delay on inlet purge; 3 min at 50°C; 35°/min to various isothermal temperatures of 220–300°C for various runs. Samples were injected as hexane solutions. The three isomers of MED-19:H eluted as single fully separated peaks from the Supelcowax-10 column in the order 6,7-,3,4-, and 9,10-monoepoxydiene. The enantiomeric excess of **8a** and **8b** was determined by esterification with (2*S*)-2-acetoxypropionyl chloride as described by Millar et al. (1990c) on column 4, isothermal 215°C.

Components were identified by comparison of retention times on capillary columns 1, 2, and 3, of extracted, collected, and synthetic components and by their GC-MS spectra.

Capillary GC traces of extracted components of individual virgin female ovipositors were quantified by comparison of peak areas with peak area response curves of external synthetic materials.

For structural determination a Finnigan Mat ITS40 mass spectrometer (70 eV) linked to a Varian 3400 gas chromatograph was used. Separation was achieved on a DB5, 30 m × 0.22 mm ID fused silica capillary column (J&W Scientific), maintained for 1 min at an initial temperature of 80°C and then heated to 140°C at a rate of 35°C/min, followed by 5°C/min to 250°C with an isothermal hold of 15 min.

Electroantennogram (EAG) Measurements. The EAG responses of adult male *B. selenaria* to synthetic materials were determined and standardized according to the procedures of Moore (Moore, 1981; Moore et al., 1986).

Wind-Tunnel Behavioral Tests. The wind tunnel (350 × 90 × 90 cm), a modification of earlier designs (Miller and Roelofs, 1978; Cardé and Hagaman, 1979; Sanders et al., 1981), was of clear Plexiglas and had a flight compartment of 240 cm. Its upwind and downwind ends were closed with cheesecloth screens; one side of the tunnel was covered with black plastic. Tests showed that the airflow was almost laminar and started out pencil-thin at the release point and widened out to about 20 cm in cross section at the downwind end of the tunnel. The floor consisted of a stationary orange-and-black chessboard pattern (each

square 15 × 15 cm). Air was sucked through the tunnel by means of a ventilator (air speed: 25 cm/sec), its outlet leading outside the building. Temperature was controlled by means of a heating unit in the entrance duct of the ventilator. The wind tunnel was diffusely illuminated from above by means of four red fluorescent lamps with a light intensity of about 11 lux (measured at the floor of the flight compartment).

The tested materials were applied on filter paper disks (Whatman No. 1, 2 cm diam.) or on rubber septa (Abic, Natanya, Israel) in 100 μl hexane and placed in individual glass tubes after evaporation of the solvent. A thin glass rod, 18 cm downwind of the upwind cheesecloth screen and at a height aligned with the male release platform, served as a release support for the stimuli. Calling virgin females were placed individually in wire net cages and lowered from the tunnel ceiling. Female ovipositor extracts (FE) were obtained from individually extracted ovipositors in 200 μl hexane.

A χ^2 comparison of the daily values at similar temperatures showed no significant day-to-day variabilities in any of the examined responses. Since not all treatments were carried out on one single day of testing, the data from all testing days for each treatment were combined. Percentage responses were compared by χ^2. The 0.05 level was set for the rejection of all null hypotheses.

Field Attractancy Tests. Preliminary field test were carried out in avocado orchards in Regba, Western Galilee, Israel, using sticky (Rimipot, Rimi, Tel-Aviv, Israel), open-ended, cone-shaped, white polyethylene traps (20 cm long, 13–17 cm ID). Traps, baited with synthetic compounds impregnated in rubber septa, were hung from branches, 1.5 m above the ground and approximately 40 m apart. Traps were set out in a randomized block design and trap captures were recorded weekly.

RESULTS

GC analyses on three capillary columns of hexane extracts of giant looper female ovipositors showed five well-resolved and reproducible peaks. A typical chromatogram from an analysis of a single female ovipositor extract is shown in Figure 1A. Figure 1B shows the corresponding chromatogram from an analysis of collected volatiles of a single calling female. GC-MS analysis of the five peaks showed that the mass spectrum of component **1** ($M^+ = 262$) exactly matched that of the previously reported *Z*3,*Z*6,*Z*9–19:H (Becker et al., 1983). The mass spectrum of component **2** ($M^+ = 278$) coincided exactly with that of the synthetic *Z*6,*Z*9-*cis*-(3*S*,4*R*)-epo-19:H (Figure 2) and is in full agreement with published data (Hansson et al., 1990). The mass spectra of components **3**–**5** matched those of tricosane (23:H), pentacosane (25:H), and heptacosane (27:H), respectively. The GC analyses of extractions of different abdominal

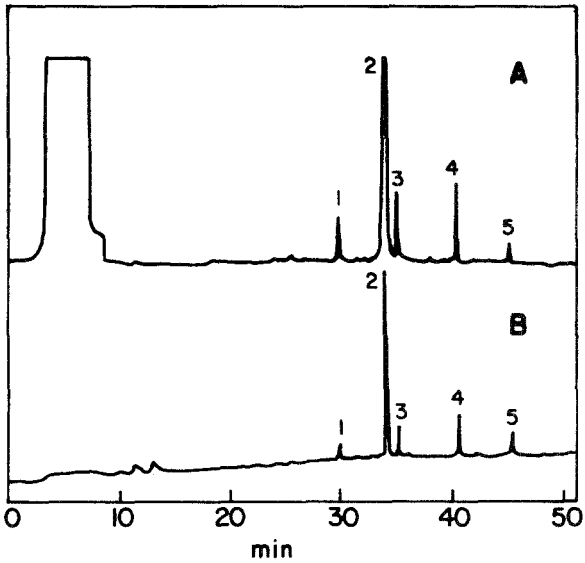


FIG. 1. Gas chromatogram of *B. selenaria* female ovipositor extract (A) and female collected effluvia (B). A 60-m Supelcowax-10 column was used.

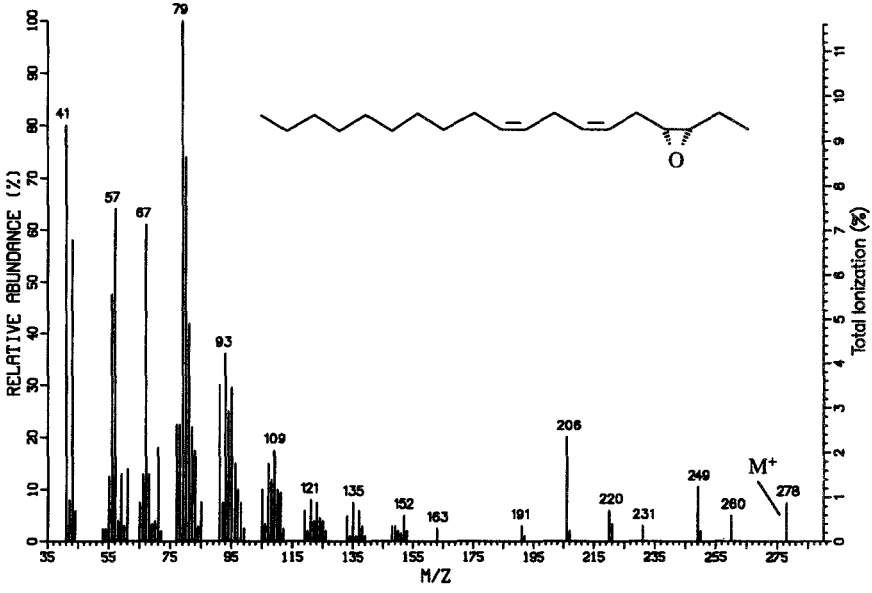


FIG. 2. Electron impact mass spectrum (70 eV) of (*Z*,*Z*)-6,9-*cis*-(3*S*,4*R*)-epoxynonadecadiene.

segments (eighth to tenth) showed that the identified alkanes were not only localized in the segment containing the pheromone gland but could be extracted from every segment. These alkanes were therefore not considered to be part of the pheromonal secretions. Comparison of peak areas with peak area response curves of Z3,Z6,Z9–19:H and Z6,Z9-*cis*-(3*S*,4*R*)-epo-19:H showed that from one female ovipositor extract an average of 42.3 ng ± 15.5 of Z6,Z9-*cis*-3,4-epo-19:H and 1.9 ng ± 0.8 of Z3,Z6,Z9–19:H ($N = 10$) could be extracted. Z6,Z9-*cis*-3,4-epo-19:H was only detected in effluvia and ovipositor extracts of calling females, whereas relatively high amounts of Z3,Z6,Z9–19:H were found in noncalling females.

EAG responses were recorded using Z3,Z6,Z9–19:H and its product after random epoxidation to a mixture of *cis*-monoepoxynonadecadienes (MED-19:H). The application of a range of doses (0.1 ng–1.0 μg) of MED-19:H resulted in responses ranging from 3.3 mV (0.1 ng) to 13.2 mV (1.0 μg) ($N = 10$). The highest dose in this range of Z3,Z6,Z9–19:H gave a stimulation of 4.4 mV, which was 3.0 times lower than that found for MED-19:H. Similarly, in EAG tests with 0.05-ng to 10-ng doses ($N = 7$) of MED-19:H and Z6,Z9-*cis*-(3*S*,4*R*)-epo-19:H, the chiral monoepoxydiene was significantly more active than MED-19:H with EAG recordings ranging from 1.4 to 2.4 mV for MED-19:H and from 4.3 to 6.8 mV for Z6,Z9-*cis*-(3*S*,4*R*)-epo-19:H. The EAG responses given by male *B. selenaria* antennae to Z6,Z9-*cis*-(3*S*,4*R*)-epo-19:H and its (3*R*,4*S*)-enantiomer showed significantly higher responses with 0.01-μg and 1.0-μg doses of Z6,Z9-*cis*-(3*S*,4*R*)-epo-19:H compared to those of the (3*R*,4*S*)-enantiomer.

Table 1 presents the results obtained when male *B. selenaria* moths were exposed in a wind tunnel to Z3,Z6,Z9-19:H, Z6,Z9-*cis*-(3*S*,4*R*)-epo-19:H, MED-19:H, and a calling female. The giant looper males exhibited wing fan-

TABLE 1. RESPONSES OF MALE *B. selenaria* IN A WIND TUNNEL TO Z3,Z6,Z9–19:H, MED-19:H, Z6,Z9-*cis*-(3*S*,4*R*)-epo-19:H, AND A CALLING FEMALE

Bait (μg)[a]	*N*	Percent		
		Wing fanning[b]	Upwind flight[b]	Landing on source[b]
Z3,Z6,Z9–19:H (250)	25	0c	0d	0b
MED-19:H (250)	26	85b	19c	12b
Z6,Z9-*cis*-(3*S*,4*R*)-epo-19:H (250)	20	85ab	55b	15b
Calling female	43	100a	100a	77a

[a] Applied on rubber septum dispensers.
[b] Percentages that have no letter in common are significantly different according to χ^2 ($P < 0.05$).

ning, plume-oriented upwind flight, and landing on source in response to MED-19:H, *Z*6,*Z*9-*cis*-(3*S*,4*R*)-epo-19:H, and a calling female. No behavioral responses could be observed with *Z*3,*Z*6,*Z*9–19:H. The onset of wing fanning was rapid for all treatments and occurred within 1–5 sec after the plume had reached test males. Of the total tested males, 19% responded to MED-19:H with plume-oriented upwind flight and 12% landed on the source, whereas 55% and 15% of the males responded to *Z*6,*Z*9-*cis*-(3*S*,4*R*)-epo-19:H with plume-oriented upwind flight and landing on source, respectively. The males that terminated their upwind progress in response to *Z*6,*Z*9-*cis*-(3*S*,4*R*)-epo-19:H did this within 15 cm in front of the source. Males that flew upwards out of the *Z*6,*Z*9-*cis*-(3*S*,4*R*)-epo-19:H plume were repeatedly observed to realign themselves with the plume and to continue with plume-oriented upwind flight. Those males that landed on source extended their claspers with vigorous wing fanning and attempted to mate with either the dispenser or with the holding cage containing the calling virgin female. Males that initiated plume-oriented upwind flight in response to a calling female generally landed on source within 25 sec after takeoff. Although the response percentages towards the synthetic materials were significantly lower than those towards a calling female, no marked differences in behavior were noted. The presence of *Z*3,*Z*6,*Z*9–19:H in the natural pheromone has been clearly demonstrated (Figure 1), but its behavioral role is at this point still unclear. Combining a calling female with a filter paper disk containing 40 μg of *Z*3,*Z*6,*Z*9–19:H caused a significant decrease in the percentage wing fanning and the elimination of plume-oriented upwind flight and landing on source (Table 2). The behavioral responses towards MED-19:H were not significantly altered with the additions of increasing amounts of *Z*3,*Z*6,*Z*9–

TABLE 2. RESPONSES OF MALE *B. selenaria* IN A WIND TUNNEL TO MED-19:H, *Z*3,*Z*6,*Z*9–19:H, CALLING FEMALE, AND COMBINATIONS THEREOF

Bait (μg)[a]	*N*	Percent		
		Wing fanning[b]	Upwind flight[b]	Landing on source[b]
MED-19:H (5)	20	65b	20bc	15b
MED-19:H (5) + *Z*3,*Z*6,*Z*9–19:H (0.05)	20	75b	25b	15b
MED-19:H (5) + *Z*3,*Z*6,*Z*9–19:H (0.5)	20	60b	10bc	0b
MED-19:H (5) + *Z*3,*Z*6,*Z*9–19:H (5)	20	5d	0c	0b
*Z*3,*Z*6,*Z*9–19:H (5)	10	0d	0c	0b
Calling female + *Z*3,*Z*6,*Z*9–19:H (40)	10	50b	0c	0b
Calling female	10	100a	100a	80a

[a] Applied on filter paper disk dispensers.
[b] Percentages that have no letter in common are significantly different according to χ^2 ($P < 0.05$).

19:H, with the exception of the 1:1 blend, where a significantly lower wing fanning percentage of 5% was noted. These data indicate no synergistic role for Z3,Z6,Z9–19:H; the results show more a trend towards a behavioral antagonist for relatively high amounts of Z3,Z6,Z9–19:H. Results of the males' behavioral responses towards various concentrations of Z6,Z9-*cis*-(3*S*,4*R*)-epo-19:H and Z6,Z9-*cis*-(3*R*,4*S*)-epo-19:H are presented in Table 3. As indicated, the behavioral responses towards the (3*S*,4*R*)-enantiomer showed a stronger decline in percentages of wing fanning and upwind flight with decreasing bait concentrations than towards the (3*R*,4*S*)-enantiomer. Furthermore, there were significant differences in behavioral activity between the 100-μg doses (wing fanning) and the 250-μg doses (upwind flight), indicating that the (3*S*,4*R*)-enantiomer is the more active compound of the two enantiomers. Landing-on-source percentages showed no difference for the three tested concentrations of enantiomers. A female ovipositor extract, containing approx. 40 ng of the natural monoepoxide and 2 ng of the triene, was highly attractive to males in the wind tunnel (Table 4). Of the total males tested, 75% completed the full behavioral sequence. Wind-tunnel experiments showed similar behavioral percentages when up to five times the female equivalent was used. Comparison between a female ovipositor extract and combinations of female ovipositor extracts with the two enantiomers of Z6,Z9-*cis*-3,4-epo-19:H showed that the addition of both enantiomers decreased the males' behavioral responses. No differences in wing fanning percentages were noted for either added concentration of enantiomers; however, there were significant differences between the 2.5 ng additions (landing on source) and the 10 ng additions (upwind flight). In both instances, addition of the (3*R*,4*S*)-enantiomer resulted in a greater reduction of the behavioral responses than with

TABLE 3. RESPONSES OF MALE *B. selenaria* IN A WIND TUNNEL TO Z6,Z9-*cis*-(3*S*,4*R*)-epo-19:H AND Z6,Z9-*cis*-(3*R*,4*S*)-epo-19:H

Bait (μg)[a]	*N*	Percent		
		Wing fanning[b]	Upwind flight[b]	Landing on source[b]
Z6,Z9-*cis*-(3*S*,4*R*)-epo-19:H (1000)	37	100a	86a	49a
Z6,Z9-*cis*-(3*S*,4*R*)-epo-19:H (250)	15	87ab	53b	13bc
Z6,Z6-*cis*-(3*S*,4*R*)-epo-19:H (100)	11	45c	0c	0c
Z6,Z9-*cis*-(3*R*,4*S*)-epo-19:H (1000)	15	100a	80ab	40ab
Z6,Z9-*cis*-(3*R*,4*S*)-epo-19:H (250)	15	67bc	7c	0c
Z6,Z9-*cis*-(3*R*,4*S*)-epo-19:H (100)	10	0d	0c	0c

[a] Applied on rubber septum dispensers.
[b] Percentages that have no letter in common are significantly different according to χ^2 ($P < 0.05$).

TABLE 4. RESPONSES OF MALE *B. selenaria* IN A WIND TUNNEL TO FEMALE OVIPOSITOR EXTRACT (FE) AND COMBINATIONS OF FE, *Z6,Z9-cis*-(3*S*,4*R*)-epo-19:H, AND *Z6,Z9-cis*-(3*R*,4*S*)-epo-19:H

Bait (ng)[a]	*N*	Percent		
		Wing fanning[b]	Upwind flight[b]	Landing on source[b]
FE	20	100a	90a	75a
FE + *Z6,Z9-cis*-(3*S*,4*R*)-epo-19:H (2.5)	15	80a	73a	60ab
FE + *Z6,Z9-cis*-(3*R*,4*S*)-epo-19:H (2.5)	16	88a	44a	25c
FE + *Z6,Z9-cis*-(3*S*,4*R*)-epo-19:H (10)	17	100a	82a	35bc
FE + *Z6,Z9-cis*-(3*R*,4*S*)-epo-19:H (10)	16	88a	38b	25c

[a] Applied on filter paper disk dispensers.
[b] Percentages that have no letter in common are significantly different according to χ^2 ($P < 0.05$).

the addition of the (3*S*,4*R*)-enantiomer. From these data, it seems likely that the natural epoxide has the 3*S*,4*R* configuration or that it is a mixture of both enantiomers with an enrichment in the (3*S*,4*R*)-enantiomer.

Overall capture of male *B. selenaria* with virgin female-baited traps was relatively low. During a three-year period (1973–1975), from March to November, the weekly average catches varied between 0 and 2 males. During the years 1986–1989, average weekly catches of males varied between 0 and 19. Preliminary field-trapping experiments, performed during two-week periods (September 1988 and May 1989), showed that on average a total of six male *B. selenaria* were attracted to baits impregnated with 500 μg of *Z6,Z9-cis*-(3*S*,4*R*)-epo-19:H, while no males were captured with baits that contained 500 μg of the (3*R*,4*S*)-enantiomer of 500 μg of *Z3,Z6,Z9*-19:H. Combinations of the (3*S*,4*R*)-enantiomer with *Z3,Z6,Z9*-19:H showed mixed results, but all tested combinations (9:1 to 1:9) attracted lower numbers of males than the (3*S*,4*R*)-enantiomer alone. However, overall capture of males was low, and field tests will have to be repeated in order to obtain statistically relevant data.

DISCUSSION

Only recently, in addition to multiunsaturated hydrocarbons, have monoepoxydienes been identified as sex pheromone components of the lepidopteran family Geometridae. (*Z,Z*)-6,9-*cis*-3,4-Epoxynonadecadiene, as part of a blend, has so far tentatively been identified in extracts of female sex pheromone glands

of the geometrid *Probole amicaria* (Millar et al., 1990b) and for several other geometrids as sex attractant (Millar et al., 1990b,c).

This study shows that female *Boarmia selenaria* moths are emitting a sex pheromone blend that is comprised of a binary mixture of (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene and (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene.

(*Z*,*Z*)-6,9-*cis*-3,4-Epoxynonadecadiene, either in the form of an enantiomerically enriched compound or as part of a mixture of monoepoxynonadecadienes, was shown to be attractive to male *B. selenaria* in the wind tunnel. We could demonstrate that male *B. selenaria* moths exhibit preferential attraction to the (3*S*,4*R*)-enantiomer of (*Z*,*Z*)-6,9-*cis*-3,4-epoxynonadecadiene in EAG tests, wind-tunnel behavioral studies, and preliminary field trials.

The wind-tunnel experiments showed that the males' behavioral responses towards the synthetic materials were significantly lower than towards a calling female or female ovipositor extract. One of the possible reasons for the low behavioral activity of the synthetic materials is that the synthetic monoepoxydiene may have a different enantiomeric composition than the natural monoepoxydiene. Our synthetic procedure used the Sharpless epoxidation in order to obtain the chiral monoepoxides. Previous studies, using the Sharpless epoxidation on similar compounds, showed enantiomeric purities to be within the range of 57–90% ee (Mori and Ebata, 1986; Mori and Takeuchi, 1989; Millar and Underhill, 1986; Millar et al., 1990c). Our analyses of the synthesized enantiomers, using known methods, such as NMR measurements in the presence of chiral shift agents, NMR measurements after derivatizations of the samples, HPLC analyses employing chiral phases, and GC and HPLC analyses after derivatizations of the samples, failed to determine the enantiomeric purities. The problem was solved by following Millar's procedure (Millar et al., 1990c), which enabled us to determine the enantiomeric purity of long-chain epoxyalcohol intermediates (see Scheme 1, **8**). Indications as to the importance of the enantiomeric purity of the synthesized enantiomers could be obtained from the experiment presented in Table 4: combinations of each of the enantiomers with a female ovipositor extract reduced the male behavioral responses in the wind tunnel. The decrease of males' behavior response can be explained by the fact that relatively low enantioselectivity has been achieved in the synthesis of *Z*6,*Z*9,-*cis*-3,4-epo-19:H (ee $< 70\%$). However, additions of the (3*R*,4*S*)-enantiomer decreased to a greater degree the behavioral responses when compared to the (3*S*,4*R*)-enantiomer.

(*Z*,*Z*,*Z*)-3,6,9-Nonadecatriene, in combination with homologs or with monoepoxide derivatives, has been shown to be attractive to male geometrids in wind-tunnel and in field tests (Wong et al., 1984, 1985; Szocs et al., 1984; Buser et al., 1985; Hansson et al., 1990; Millar et al., 1990a–d). Our results confirm the presence of the previously reported (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene (Becker et al., 1983) in female ovipositor extracts and, in the present study, it

was also identified in collected volatiles emitted by female *B. selenaria*. However, the wind-tunnel behavioral studies showed that (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene failed to elicit any response in the tested males. Furthermore, additions of (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene to a biologically active mixture of monoepoxynonadecadienes clearly decreased the behavioral responses of the males. Moreover, a combination of (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene with a calling virgin female eliminated the female's attractancy to the moths. Preliminary field trials showed that (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene alone was not attractive nor was an increase noted in number of catches when applied in combination with (*Z*,*Z*)-6,9-*cis*-(3*S*,4*R*)-epoxynonadecadiene. (*Z*,*Z*)-6,9-*cis*-3,4-Epoxynonadecadiene was only detected in effluvia and ovipositor extracts of calling females, whereas relatively high amounts of (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene were found in noncalling females. Rather than being a pheromone component, the above results corroborate earlier reports that triunsaturated hydrocarbons may function as precursors for monoepoxidienes (Kasang et al., 1974; Rule and Roelofs, 1989).

The recently reported single sensillum measurements on male antennae of the geometrid *Erannis defoliaria* showed two physiological types of sensilla: one type responded to (*Z*,*Z*)-3,9-*cis*-6,7-epoxynonadecadiene and to a lesser extent to (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene; a second type responded only to (*Z*,*Z*)-3,9-*cis*-6,7-epoxynonadecadiene, and this cell was inhibited by (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene (Hansson et al., 1990). With regard to the results discussed above, it is possible that similar types of sensilla are present in antennae of male *B. selenaria* and that (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene performs a dual role of precursor and pheromone component in the pheromonal system, but this remains to be investigated.

The synthesis of chiral methylene-interrupted epoxydienes has been based on the acetylene methodology. This approach has been used by Mori (Mori and Ebata, 1986; Mori and Takeuchi, 1989) in the syntheses of similar structures and last year by Millar et al. (1990c) in the synthesis of (*Z*,*Z*)-6,9-*cis*-(3*S*,4*R*)-epoxynonadecadiene. The main drawback in this route is the catalytic reduction of the 3,6,9-triene-1-ol over Lindlar catalyst, which often has to be repeated since the catalyst becomes poisoned. It has become clear to us that the removal of protecting groups by acid catalysis is one of the main problems. Probably at that stage some double bond rearrangement does occur and the formed conjugated systems poison the catalyst. Conjugated systems can be formed at the condensation steps that are performed in basic media. We have worked out a procedure that gives a general synthetic route for the synthesis of chiral methylene-interrupted epoxydienes and provides a solution to the problems mentioned. We have synthesized triynol from two units: 1-iodo-2-dodecyne, which has been prepared in three steps from commercially available starting materials in good yields, and a six-carbon unit, 2,5-hexadiyne-1-ol, which has been prepared in a straightforward two-step synthesis according to Brandsma (Brandsma,

1971) and remained stable at 5°C for weeks. The next step was crucial, since it led in one step to methylene interrupted triynol by condensation of 1-iodo-2-dodecyne with unprotected 2,5-hexadiyne-1-ol. The fact that no acid hydrolysis was needed after the condensation enabled us to obtain, after crystallization, a white solid compound that was reduced over Lindlar catalyst to give the stable 3,6,9-triene-1-ol in quantitative yield. It seems to us that for the time being this route is the procedure of choice for the synthesis of methylene interrupted 3,6,9-triene-1-ol.

Although the optimal synthetic pheromone formulation is not yet complete, current efforts to obtain enantiomerically pure monoepoxydienes, together with more detailed field tests and wind-tunnel experiments, should provide further information on the exact formulation of the natural pheromone.

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LEAF-CUTTING ANT *Atta laevigata* (FORMICIDAE: ATTINI) MARKS ITS TERRITORY WITH COLONY-SPECIFIC DUFOUR GLAND SECRETION

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Abstract—The workers of the leaf-cutting ant *Atta laevigata* were found to mark a territory around their nest and along trunk trails. Elsewhere, we reported that the territorial mark had orientation and agonistic functions. The presence of this mark induced an alarm behavior in intruder workers and stimulated the residents' aggressiveness. Of the parts of the body tested, only the extracts from gaster or Dufour gland were able to induce the same agonistic behavior. The compounds *n*-heptadecane, (Z)-9-nonadecene, 8,11-nonadecadiene, and (Z)-9-tricosene, secreted by Dufour gland were identified on foraging trails in the field as components of the territorial odor.

Key Words—*Atta laevigata*, Hymenoptera, Formicidae, territorial pheromone, agonistic behavior, Dufour gland.

INTRODUCTION

The existence of a territorial pheromone in Attini is still a controversial subject. A compound secreted by the valves gland of the sting (Bazire-Benazet and Zylberberg, 1979) and identified as *n*-nonadecadiene had been first proposed for *Atta cephalotes* (Jaffe et al., 1979). The same authors suggest that the mandib-

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ular gland secretions are laid down around the nest exits and propose the alarm pheromone as a colony-specific compound of the territorial mark. Hölldobler and Wilson (1986) were unable to confirm the existence of territorial pheromones in the nest vicinity of *Atta cephalotes*. They suggest that colony-specific marks of the nest entrances are used as orientation signals and find that the valves gland produces an alarm pheromone.

Vilela (1983) confirms the existence of a territorial pheromone in leaf-cutting ants, regulating intraspecific encounters. Excluding the role of the venom gland in the territorial secretion, he reports that the (Z)-9-nonadecene produced by the Dufour gland (Evershed and Morgan, 1981) is an important compound acting in the territorial behavior of *Atta sexdens rubropilosa*.

Salzemann and Jaffe (1990) made the first experimental demonstration of the existence of a colony-specific territory in field colonies of an Attini. *Atta laevigata* actively mark the area around their nests with a pheromone that is produced in the tip of the gaster (Salzemann and Jaffe, 1991), in a way similar to *Atta cephalotes* and *Atta sexdens rubropilosa* as demonstrated by Jaffe et al. (1979) and Vilela (1983). Ethological studies on *Atta laevigata* showed that the territorial mark serves two distinct but complementary functions. One is clearly agonistic, in that intruders become submissive whereas resident ants on marked territory show increased watchfulness and aggressiveness (Salzemann and Jaffe, 1991). The second function is orientation: workers from the same colony use the mark to differentiate the various nest mounds; this difference allows the ants to orient to a specific mound (Salzemann and Jaffe, 1990).

Here we report the effect of various extracts of glands on the territorial behavior of *Atta laevigata*. The ethological study was completed by a chemical analysis of the extracts of the exocrine glands tested and of the secretions collected from the foraging trails in the field.

METHODS AND MATERIALS

Extracts

Insect material was collected from nests in a 15-year-old commercial pine (*Pinus caribea*) plantation at the Estado Monagas, Venezuela. *Atta laevigata* were present at densities of more than 30 adult nests per hectare. Media workers were dissected to prepare the extracts tested. Exocrine glands and parts of body were macerated in methylene chloride (CH_2Cl_2), each extract containing six ant equivalents per milliliter of solvent. The following extracts were prepared: whole gaster, sting + valves gland, Dufour gland, poison gland, hindgut, tibia + tarsus (Figure 1).

To detect the territorial pheromone on ant trails, glass sheets (7.6 × 2.6 × 0.1 cm) were washed with acid and rinsed successively with water, acetone,

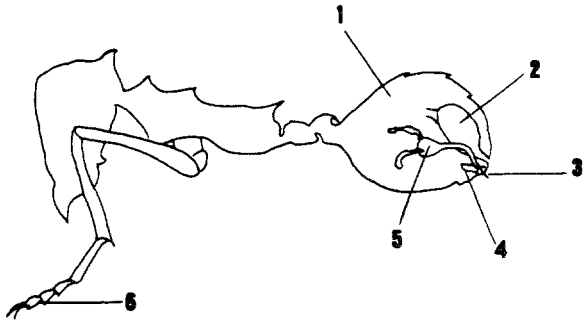

FIG. 1. Diagram of a worker of *Atta*. Parts of the body and glands dissected for the preparation of the extracts are: 1, gaster; 2, rectal sac (hindgut); 3, sting + valves gland; 4, Dufour gland; 5, poison gland; 6, tarsal gland (tibia + tarsus).

and CH_2Cl_2. The glass sheets were laid down on two trails of two close societies, near the nest entrance (first meter of the trail), in the middle, and at the end of the trail (foraging area), for 20 min each. Then they were collected once a day during the five following days and dipped in 200 ml of CH_2Cl_2 before evaporation to 4 ml with a Rotovapor. Two control samples were included: one in which the glass sheets were placed far from the nests where foraging activity was never observed (trail control); the second was a solvent control of pure CH_2Cl_2. All samples were concentrated under nitrogen to 20 μl frozen at $-30°C$ before the chemical analyses.

Ethological Study

To study the agonistic behavior of *A. laevigata* on different substrata, a confrontation test between media workers from neighboring nests was developed. The experiments were conducted in the pine tree plantations.

For each test, two ants were introduced into a Petri dish (9 cm diameter) containing clean sand. The experiment was performed in the field, far away from the nests to avoid any visual recognition of the site. Each worker was transferred to the experimental arena by making it climb a small pine branch and then placing it with the branch into the Petri dish without disturbing it. Encounters were studied on virgin territory (with only clean sand in the dishes) or on marked territories (sand impregnated with different gland extracts).

The first worker was always introduced 5 min after the impregnation of the sand by 1 ml of the extract. Its alarm behavior, characterized by rapid and uncoordinated movements and opening of mandibles, was quantified. Then, the agonistic behavior of ants was observed during the 5-min period following the introduction of the second worker into the Petri dish. Resident workers were

nestmates of the dissected ants used to prepare the extracts; intruder workers were taken from a different conspecific colony.

Results were compared to a control prepared by spreading the sand with pure solvent and to a series of tests performed on a natural marked territory (marked by workers in the field). In this case, the experimental arena was a bottomless plastic cylinder (9 cm diameter, 8 cm high) placed at the beginning of an active foraging trail (0–1 m from the resident nest mound).

Reference Compounds

n-Heptadecane and (*Z*)-9-tricosene were commercial standards provided by Sigma Co. Their purity (99%) was checked by capillary gas chromatography.

(*Z*)-9-Nonadecene was synthesized by palladium-catalyzed cross-coupling reactions between nonylzinc bromide and (*Z*)-1-iodo-1-decene, according to the following procedure.

(*Z*)-*1-Iodo-1-Decene.* One tenth mole of an etheral solution of octyllithium (prepared from 1-bromooctane and Li) was added to a stirred suspension of cuprous iodide (0.052 mol) in 100 ml Et_2O at −50°C. Stirring was maintained for 30 min at −35°C. Acetylene (0.11 mol) was bubbled into this blue solution at −35°C and after 30 min at −25°C, a greenish solution of (*Z*)-1-decenyl-cuprate was obtained. To this reagent was added at −60°C, 0.1 mol of iodine, and the stirred mixture was warmed to −15°C until discoloration. The mixture was hydrolyzed with NH_4OH–NH_4Cl solution (50:50), 100 ml, admixed with pentane (100 ml), filtered, and extracted with Et_2O. The organic phase was washed successively with $Na_2S_2O_3$, $NaHCO_3$, and NH_4Cl saturated aqueous solutions. It then was dried over $MgSO_4$ and concentrated on a Rotovapor. The residue was distilled over Cu powder to produce 1-iodo-(*Z*)-1-decene (80% yield) with isomeric purity >99.5%, Bp = 61°C/0.01 mm Hg.

(*Z*)-*9-Nonadecene.* To an etheral solution of nonyllithium (0.03 mol) in 50 ml of THF were added at 0°C an anhydrous $ZnBr_2$ solution (0.035 mol/50 ml THF). The stirred mixture was allowed to warm up to 20°C and at 15°C and 0°C was added successively a solution of tetrakis triphenyl phosphine palladium (0.4 mmol/20 ml THF) (2%) and 0.02 mol of (*Z*)-1-iodo-1-nonadecene. Stirring was maintained overnight at room temperature. The reaction mixture was hydrolyzed with H_2SO_4 solution (1 N). The crude residue then was filtered through a small column packed with silica gel in order to remove the palladium catalyst (eluting with pentane). The solvent was evaporated, and the crude residue was distilled to produce (*Z*)-9-nonadecene (87% yield), bp: 110–120°C/0.01 mm Hg; (Z) purity (>99.5%); *m/z:* 266 ($M^{+\cdot}$), 55 (100%); IR (neat) ν cm-1: 1450, 710 (HC=CH) *cis*; [^{1}H]NMR (δ ppm): 0.9 (t, 6H) CH_3, 1.3 (m, 26H) CH_2, 2.0 (m, 4H) CH_2−CH=, 5.3 (t, 2H) CH= (J = 4.5 Hz); [^{13}C]NMR (δ ppm): 129.9 (HC=CH).

Identification

All gland, trail, and control extracts were analyzed under the same conditions by gas chromatography (GC) on a nonpolar capillary column. A Fractovap 2900 (Carlo Erba) was equipped with a split/splitless injector (275°C) and a FID detector (275°C). The column was a fused silica capillary Ultra 2 (Hewlett Packard) measuring 25 m × 0.2 mm ID. The oven was programmed from 40 to 60°C at 20°C/min, 60°C for 5 min, then reprogrammed from 60 to 250°C at 6°C/min and held isothermally at 250°C for 20 min. The pressure of the carrier gas (helium) in the column was 1.2 bars, and there was a splitless period of 20 sec during each injection. Gas chromatography–mass spectrometry (GC-MS) studies of the Dufour gland components were conducted on a Girdel 32 GC (CP Sil5 CB, Chrompack, 25 m × 0.32 mm ID, 60–250°C at 8°C/min) coupled with a Nermag 10-10C quadrupole mass spectrometer incorporating a Sidar data system and using the NIH spectra library. Mass spectra were recorded at 70 eV. The interface was maintained at 150°C and the carrier gas (Helium) at a 0.5 bar pressure.

Identification of the components of the Dufour gland was made by comparison with reference spectra, interpretation of the fragmentation pattern, and by coinjection with appropriate standards on a nonpolar column (previously described) and a polar column (FFAP CB, 25 m × 0.32 mm ID, Chrompack, from 80 to 220°C at 6°C/min; other conditions identical to the previous analyses).

Positions of the double bonds in the mono- and diethylenic compounds were determined by ozonolysis of fractions obtained by micropreparative GC using a Wide Bore nonpolar capillary column (Malosse, 1990). The purified fractions checked by GC-MS and cooled in an ice bath were submitted for 5 min to a slow stream of ozone generated by a microozonolyzer. The production of derivatives was controlled by GC-MS.

RESULTS

Ethological Study

At the beginning of an active foraging trail, a clear dominance of the resident worker over an intruder could be observed, regardless of the order in which they entered the arena (Table 1: natural marked territory). The presence of the territorial mark also induced a significant difference between the alarm behavior and its duration (alarm prolonged over 1 min) in the intruder and the resident ant.

On virgin territory (clean sand without extract), the first ant to enter the arena seemed to have an advantage in aggressive encounters (frequency of men-

TABLE 1. ALARM BEHAVIOR SHOWN BY WORKER 1 INTRODUCED TO ARENA AND FREQUENCIES OF AGGRESSIVE BEHAVIORS OF BOTH ANTS (% OF TESTS PERFORMED) AFTER INTRODUCTION OF WORKER 2[a]

	Natural marked territory (N = 40)			Virgin territory (N = 30)			Pure CH_2Cl_2 (N = 20)		
	R-I		I-R	R-I		I-R	R-I		I-R
Menaces 1-2	57.5		47.5	53		63	70		65
Menaces 2-1	22.5	**[b]	60	30		30	40		50
Bites 1-2	57.5	***	10	17		13	30		10
Bites 1-2	0	***	30	0		10	15		0
Alarm 1	25	*	50	50		30	40		35
Prolonged alarm 1	0	***	25	20		13	20		20
	Gaster (N = 20)			Sting + valves (N = 20)			Dufour (N = 20)		
	R-I		I-R	R-I		I-R	R-I		I-R
Menaces 1-2	80		70	60		60	80		70
Menaces 2-1	30	*	70	20		35	10	**	60
Bites 1-2	55	**	15	5		10	35	*	10
Bites 2-1	0	*	30	0		0	0		15
Alarm 1	50		75	45		70	50		65
Prolonged alarm 1	20	*	50	5	**	45	10	*	40
	Venom (N = 20)			Hindgut (N = 20)			Tibia + Tarsus (N = 20)		
	R-I		I-R	R-I		I-R	R-I		I-R
Menaces 1-2	55		45	60		65	70		65
Menaces 2-1	25		10	30		25	25		15
Bites 1-2	10		0	15		5	5		20
Bites 2-1	5		0	0		0	5		0
Alarm 1	30		40	40		25	35		25
Prolonged alarm 1	0		20	15		10	10		15

[a]Intruder was introduced into the arena with a 5-min delay after the resident (R-I) or vice versa (I-R).

[b]*, **, *** indicates $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, if R-I is compared to I-R with a χ^2 or Fisher test when theorical samples were less than five.

acing shown by the first worker introduced in the arena). The intruder, instead of submission, menaced the resident worker. In this case, no significant difference could be detected between the alarm behavior of the two ants. The same results could be observed when encounters were studied on sand spread with pure solvent (Table 1: virgin territory and CH_2Cl_2).

Only two series of extracts (gaster and Dufour gland) indicated the presence of a territorial mark when spread on the bottom of the dishes. The resident ant dominated the encounters, whatever the order of entry into the arena, and the alarm behaviors were statistically significantly different ($P < 0.05$ in the repetition of the behavior during the test; Table 1: gaster and Dufour between resident and intruder). When the sand of the dishes was impregnated with the other extracts, the same agonistic behavior as that reported on virgin territory was observed. The extract of sting + valves gland was only able to induce a statistically significant difference ($P < 0.01$) when comparing the repetition of alarm behavior between both ants (Table 1: sting + valves, venom, hindgut, and tibia + tarsus).

Identification of Pheromone

The ethological study suggested that the Dufour gland is the site of production for the territorial pheromone. Gas chromatography of this extract (Figure 2) revealed the presence of 14 compounds, mainly long-chain hydrocarbons (Table 2). Only four products (peaks 2, 4, 5, 10) were found to be present on the trail extracts (Table 3, Figure 3). Compound 1 was present in very low amounts (0–18% of mixture 1, 2, 4, 5, 10 at the beginning of the trail). The proportion of the four products found on naturally marked trails suggested a possible colony-specific function for products 2, 5, and 10 (Table 4). Stable ratios of products 2 and 10 and 5 and 10 from different trails of the same nest (Table 5) suggested a colony specificity of products 5 and 10.

Compound 2 had a retention time on the two columns corresponding to the *n*-heptadecane. Retention times of compounds 5 and 10 matched those of nonadecene and tricosene on both columns. Their identity was confirmed by GC-MS, which gave a molecular weight for nonadecene of $M^{+\cdot} = 266$ ($C_{19}H_{38}$) and for tricosene of $M^{+\cdot} = 322$ ($C_{23}H_{46}$). The position of the double bond was given by the ozonolysis of the corresponding fractions: nonanal and decanal, nonanal and tetradecanal, respectively, were produced from (*Z*)-9-nonadecene and (*Z*)-9-tricosene.

The retention time of compound 4 compared to that of nonadecene and nonadecane, combined with the GC-MS data ($M^{+\cdot} = 264$, $C_{19}H_{36}$), suggested it to be nonadecadiene. The ozonolysis of the corresponding fraction only gave the octanal resulting from the cleavage of the symmetrical 8,11-nonadecadiene, as also found by Evershed and Morgan (1981) in *Atta sexdens* Dufour gland.

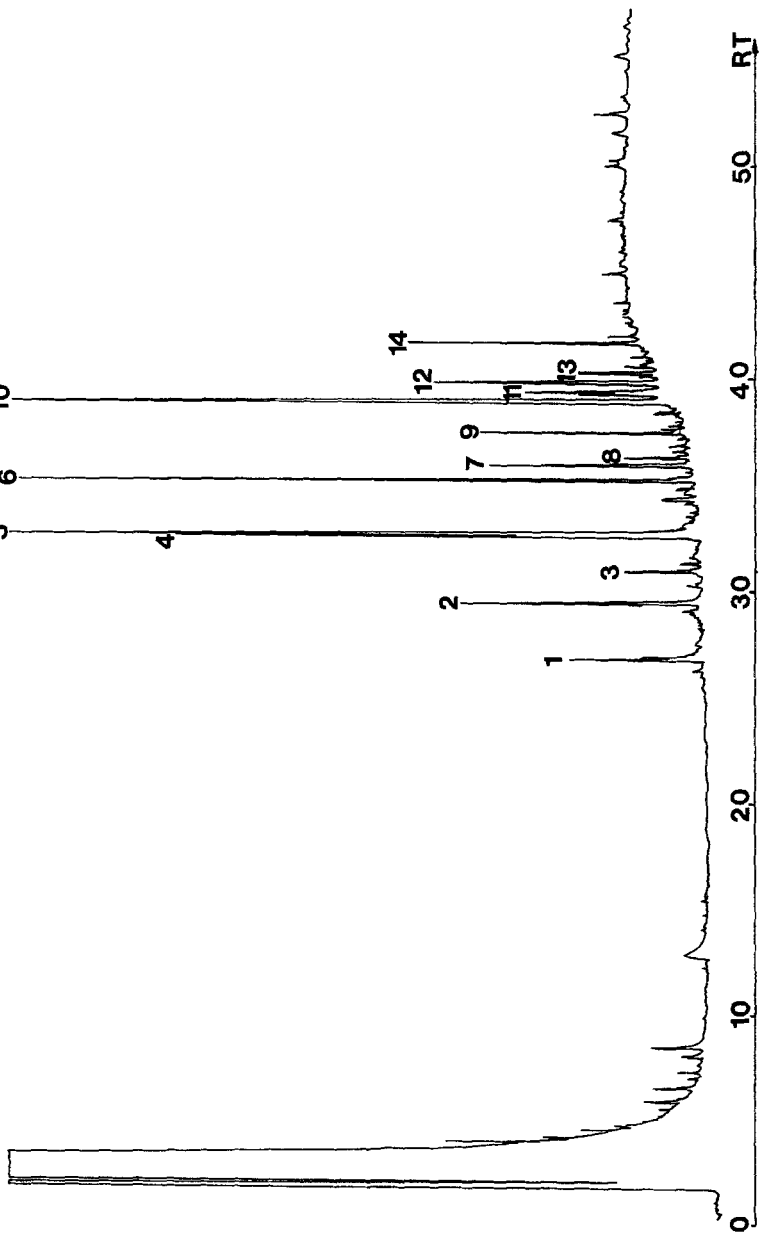

FIG. 2. Gas chromatographic trace of *Atta laevigata* Dufour gland extract (nonpolar column Ultra 2, Hewlett Packard, 25 m × 0.2 mm ID, all GC conditions in text). For the assignment of numbers, see Table 2).

TABLE 2. DUFOUR GLAND COMPOSITION AND MEANING OF GC PEAK ASSIGNMENT[a]

Peak	Compound	Evidence
1	Homofarnesene	MS
2	Heptadecane	GC, MS
3	Octadecene	MS
4	8,11-Nonadecadiene	GC, MS, OZ
5	(Z)-9-Nonadecene	GC, MS, OZ
6	Diterpene C_{20}	MS
7	Eicosene	MS
8	Eicosane	GC, MS
9	Docosene	MS
10	(Z)-9-Tricosene	GC, MS, OZ
11	Terpene	MS
12	Unknown	
13	Tetracosene	MS
14	Pentacosene	MS

[a]GC: retention times of the compound matched with the standard on polar and nonpolar columns. MS: identity was determined or confirmed by GC-MS coupling. OZ: the position of the double bonds was determined by ozonolysis after micropreparative GC.

TABLE 3. PRESENCE (+) OR ABSENCE (−) OF DUFOUR GLAND COMPOUNDS IN EXTRACTS

	Gaster	Sting + Valves	Cuticle	Tibia + Tarsus	Hindgut	Venom	Trail
1	+	+	−	+	−	−	+
2	+	+	+	−	+	−	+
3	+	+	−	−	−	−	−
4	+	+	+	−	+	−	+
5	+	+	+	−	+	−	+
6	+	+	+	?[a]	+	−	−
7	+	+	+	?	+	−	−
8	+	+	+	?	+	+	−
9	+	+	+	?	−	−	−
10	+	+	+	?	+	−	+
11	+	+	+	?	+	?	−
12	+	+	+	?	+	−	?
13	+	−	−	+	−	−	?
14	+	−	+	+	−	−	−

[a]? indicates doubtful presence of the compound.

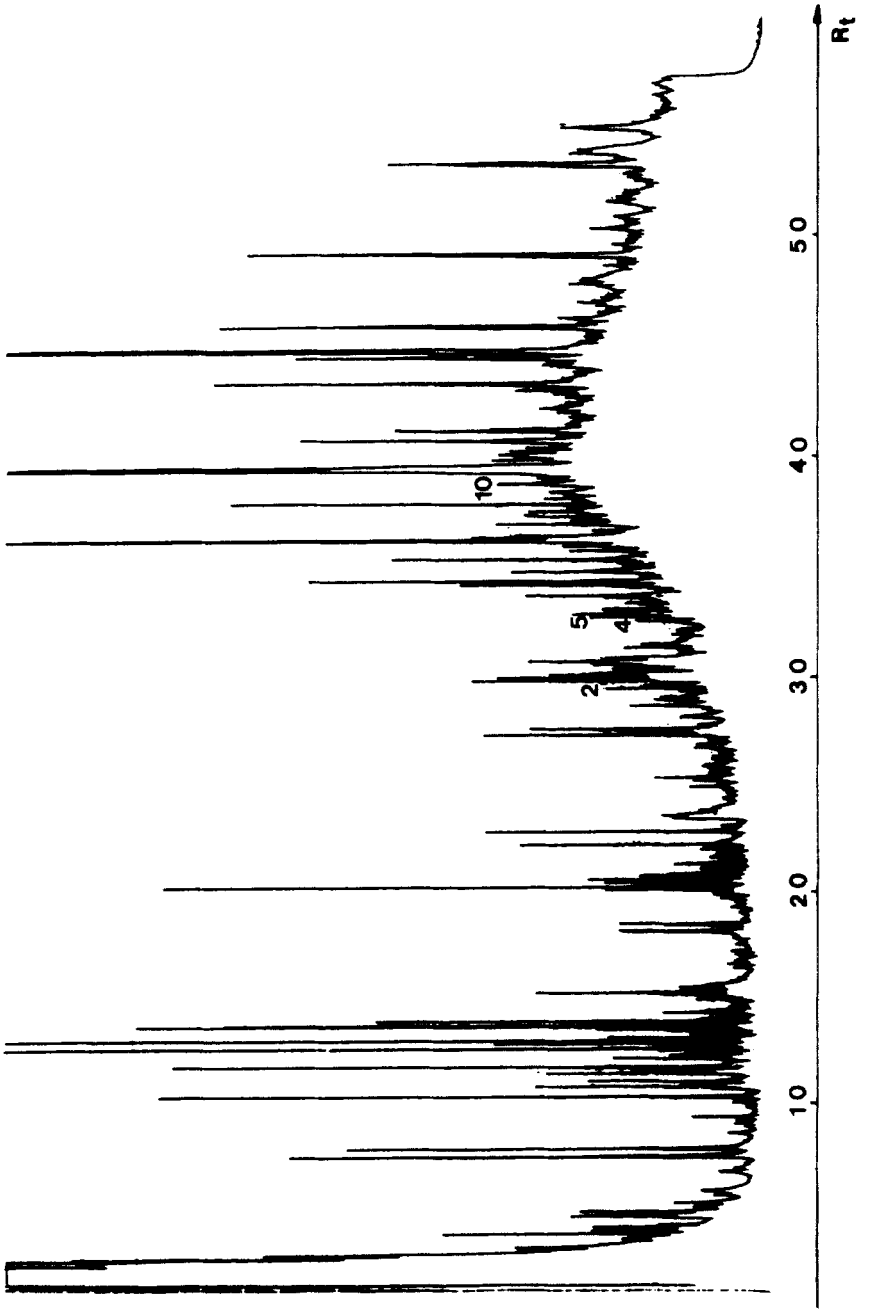

FIG. 3. Gas chromatographic trace of the washing extract of the glass sheets laid down at the beginning of *Atta laevigata* foraging trail: the components 2, 4, 5, and 10 from the Dufour gland also appear on the trail (nonpolar column Ultra 2, Hewlett Packard, 25 m × 0.2 mm ID, all other GC conditions in the text).

TABLE 4. RATE OF FOUR COMPOUNDS SECRETED BY DUFOUR GLAND (% OF AREA DETECTED BY GC) IN MIXTURE 2-4-5-10 AT BEGINNING OF TRAIL OF COLONIES A AND B

	Nest A			Nest B	
	Trail 1	Trail 2		Trail 1	Trail 2
Component 2	30	29	**[a]	8	9
Component 4	5	8		11	9
Component 5	39	37	***	13	13
Component 10	26	26	***	68	69

[a] ** and *** indicate $P < 0.01$ and $P < 0.001$, respectively, if the compound frequencies from trails of nest A are compared to those of nest B with a χ^2 test.

TABLE 5. RATES OF DUFOUR GLAND COMPOUNDS 2, 4, 5, AND 10 (IN AREA DETECTED BY GC AND NORMALIZED TO 10.000) IN DIFFERENT FORAGING TRAIL EXTRACTS FOR TWO COLONIES[a]

	Nest A			Nest B			
Compound	BT_1	BT_2	ET_1	BT_1	BT_2	MT_1	ET_1
2/4	633	374	4830	70	97	168	(2)
2/5	78	79	1088	62	67	3	(2)
2/10	118	111	(1)	12	13	29	(1) (2)
4/5	12	21	0.6	88	69	2	112
4/10	19	30	(1)	17	13	17	(1)
5/10	150	140	(1)	19	19	11	(1)

[a] BT_1, BT_2: beginning of two trails 1 and 2; MT_1: middle of the trail 1; ET_1: end of the trail 1; (1): absences of compound 10; (2): absence of compound 2.

DISCUSSION

Many compounds detected on the foraging trails of *Atta laevigata* may be involved in territorial recognition. Ethological tests showed that the Dufour gland of the ant is the best candidate to interfere in the territorial marking. The principal compounds of the Dufour gland are alkenes in *Atta laevigata*; alkanes were reported by Evershed and Morgan (1980) in *Atta cephalotes*. The four compounds also found on natural marked territories [from major to minor: (*Z*)-9-tricosene, (*Z*)-9-nonadecene, 8,11-nonadecadiene, and *n*-heptadecane] were reported in various Attini by Evershed and Morgan (1980, 1981), but only nonadecadiene (Jaffe et al., 1979) and (*Z*)-9-nonadecene (Vilela, 1983) were

suggested as territorial compounds in *Atta*. The latter alkene was described in *Atta cephalotes* by Bradshaw et al., (1983) as a product regulating chain transport of leaves. (Z)-9-Tricosene is known as a sex pheromone of the house fly (Carlson et al., 1971). The absence of this compound in the sites where the territorial behavior of workers was never observed (end of the trails in the pine tree plantations) suggests its importance in the constitution of the territorial pheromone of *Atta laevigata*.

None of these compounds alone could define a clear species, colony, or nestmound specificity. Other compounds of the Dufour gland secretion could be laid down on the trails and chemically modified (e.g., oxidation of terpenes, Funes et al., 1973). Such compounds could interfere in the territorial behavior of the ants but their transformation makes difficult their detection in the gas chromatographic trace of the trails. Only the quality of the mixture could lend significance to the mark laid down around the nest. In *Atta laevigata*, proportions of (Z)-9-nonadecene and (Z)-9-tricosene showed a colony-specific property and 8,11-nonadecadiene could define, according to the other compounds, a nestmound specificity of the territorial pheromone.

Elsewhere, Salzemann and Jaffe (1990) reported that the territorial pheromone in *Atta laevigata* presented such high specificity that it was able to help the workers, without visual cues, differentiate the mounds of their nest. Therefore, this pheromone has a double function: to repel conspecific intruders and to orient resident ants on their return to the nest. All *Atta* species seem to show a nestmound fidelity of foraging workers (Porter and Bowers, 1982). We suggest that the Dufour gland secretion has this function in Attini where the territorial pheromone is also an orientation pheromone. Cammaerts et al. (1977) observed a similar phenomenon in *Myrmica rubra* and showed that workers used their Dufour gland secretions to conquer new territory and to recruit nestmates to it.

Our results excluded the role of the venom gland in territorial marking and confirmed observations reported by Vilela (1983) on *Atta sexdens rubropilosa*. Observing an attractive effect of the venom gland extract, Hölldobler and Wilson (1986) suggested that in *Atta cephalotes* a role for this secretion is in orientation. The same authors reported that other colony-specific substances are laid down around the nest exits but ignored their origin or relation to the orientation pheromones. They observed that the perception of these substances by workers of a foreign colony increased their marking behavior of the territory. This behavior, allowing covering the foreign mark and maintenance of the territorial property of the resident colony as reported by Vilela (1983) in *Atta sexdens rubropilosa* and Jaffe and Sanchez (1984) in *Camponotus rufipes*, indicates the value of this substance.

As was true for Hölldobler and Wilson (1986), we could find no evidence for a function in territorial behavior of the valves gland or sting sheet gland secretion. The only statistically significant that could be detected was difference

in the duration of the alarm behavior, showing that this extract had a colony-specific property, probably from contamination of the sting duct by the content of the Dufour gland. The valves gland was not the source of a territorial pheromone as suggested by Jaffe et al. (1979) in *Atta cephalotes*. The results reported by these authors are probably due to contamination of the gland extracts used in their experiments. In fact, we have observed that *n*-nonadecadiene, produced by the valves gland according to Jaffe (1979), was secreted by the Dufour gland, as reported by Evershed and Morgan (1981). We found that the venom gland, the Dufour gland, and the hindgut were easy to dissect but, as Hölldobler and Wilson (1986) reported, we found the valves gland difficult to isolate without portions of the sting or fragments of the adjacent tissues. Thus, contamination of the sting sheet gland extract by other gland secretions is inevitable. Due to this problem of obtaining clean preparations, we did not consider the tergal and the sternal glands.

Tarsal glands, recently observed in the attines (Billen, unpublished results) are not the source of a territorial pheromone in *Atta laevigata*. Few data are available about this glandular structure: first reported in *Apis mellifera* (Arnhart, 1923), their ultrastructure was studied by Lensky et al. (1985). In ants, tibial glands and tarsal glands were observed in *Crematogaster* (Pasteels et al., 1970; Billen, 1984) and *Solenopsis* (Billen, 1990), respectively. A possible function of this structure could be the production of compounds allowing the adherence of workers on smooth substrata as suggested by Lensky and Slabezki (1981) in *Apis* and by Billen (1986) in *Polistes*. Adhesive substances were also described in Diptera (Stork, 1983; Walker et al., 1985). In *Atta*, the structure and function of these glands will be the subject of future study (Salzemann and Billen, in preparation).

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SORGOLEONE FROM ROOT EXUDATE INHIBITS MITOCHONDRIAL FUNCTIONS[1]

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Abstract—The aim of this investigation was to determine if sorgoleone (SGL), a hydrophobic compound in *Sorghum bicolor* (L.) Moench root exudate, interferes with mitochondrial functions. Tests were conducted on mitochondria isolated from etiolated soybean [*Glycine max* (L.) Merr.] and corn (*Zea mays* L.) seedlings. The data show SGL is a potent inhibitor of state 3 and state 4 respiration rates in both soybean and corn. Using either NADH, succinate, or malate as substrate, the I_{50} was about 0.5 μM SGL for state 3 and 5.0 μM for state 4 based on 0.3–0.5 mg mitochondrial protein. Absorption spectra indicate SGL blocks electron transport at the b-c_1 complex. These data show that disruption of mitochondrial function may be a mechanism of SGL-mediated growth inhibition previously reported and demonstrate a probable role of SGL in *Sorghum* allelopathy.

Key Words—Sorgoleone, mitochondria, inhibitor, allelochemical, allelopathy, root exudate, *Sorghum bicolor*, electron transport.

INTRODUCTION

Cultivated *Sorghum* species have a reputation for suppressing weed growth (Overland, 1966; Putnam et al., 1983; Putnam and DeFrank, 1983; Forney et

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al., 1985; Einhellig and Rasmussen, 1989). Roots of *Sorghum* are known to exude several biological products, both hydrophilic and hydrophobic, that exhibit allelochemical activity (Panasiuk et al., 1986; Netzly and Butler, 1986; Einhellig and Souza, 1992). Some of the hydrophilic exudates include phenols, protein, and 3-deoxanthocyaninidin derivatives (Netzly and Butler, 1986). A major component of the hydrophobic exudate of grain sorghum [*Sorghum bicolor* (L.) Moench] roots is a compound identified as 2-hydroxy-5-methoxy-3-[(8′Z,11′Z)-8′,11′,14′-pentadecatriene]-*p*-hydroquinone, which oxidizes readily into the quinone form (Figure 1), and three minor *p*-benzoquinones that are structurally similar (Netzly and Butler, 1986; Chang et al., 1986; Netzly et al., 1988). The unstable dihydroquinone form of this compound was shown to be a potent germination stimulant for witchweed [*Striga asiatica* (L.) Kuntz. #STRLU]. The stable quinone form, which we will refer to as sorgoleone (SGL), causes inhibition of root elongation in lettuce and some weed species (Netzly et al., 1988). Recent studies in our laboratory indicate that SGL inhibits the growth of several weed species at micromolar concentrations (Einhellig and Souza, 1992).

The mechanisms through which allelopathic chemicals cause their effects remain obscure. Most of the literature reflects quantitative effects of individual allelochemicals on plant growth parameters, with only a limited number of investigations directed toward determining the actual physiological role of an inhibitor that leads to the growth reduction response. Specific allelochemicals have been shown to interfere with phytohormones, plant–water relations, mineral nutrition, stomatal function, carbon fixation and distribution, and respiration (Einhellig, 1986). It is unlikely that the mode of action of the large diversity of individual inhibitors is the same. However, understanding the specific physiological action of a compound is valuable information for potential applications

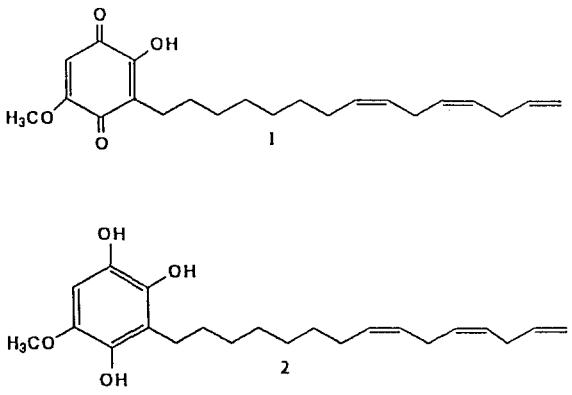

FIG. 1. Structures of **(1)** sorgoleone and **(2)** dihydroquinone of sorgoleone (Chang et al., 1986).

in agroecosystems. This information is also important because allelopathy is generally not the isolated effect of a single compound, and it is affected by the abiotic environment (Rice, 1984; Einhellig, 1986, 1989).

Several allelopathic compounds, as well as some synthetic herbicides, show an effect on respiration and oxidative phosphorylation (Stenlid, 1968, 1970; Koeppe, 1972; Demos et al., 1975; Moreland and Huber, 1979; Jacobson and Jacobson, 1980; Moreland and Novitzky, 1987). Koeppe (1972) found that juglone, a naphthoquinone, is a potent inhibitor of corn mitochondrial respiration. The lipophilicity of SGL suggested it would readily dissolve in plant membranes, and we suspected that SGL might interfere with mitochondrial respiration. The structural similarity between SGL and coenzyme Q, an intermediate in the mitochondrial electron transport chain, was another rationale to investigate possible effects of SGL on respiration.

The initial intent of this investigation was to determine if SGL impacts on oxygen uptake in isolated plant mitochondria. If this occurred, the aim was to determine if the site of inhibition was located in the electron transport pathway. The third objective was to see if response differences exist between mitochondria isolated from monocots and dicots.

METHODS AND MATERIALS

Extraction and Preparation of Sorgoleone. Sorghum root exudate was collected from the roots of grain sorghum seedlings [*Sorghum bicolor* (L.) Moench., Dekalb Hybrid DK 28] in a procedure modified from Netzly et al. (1988). The seeds were rinsed with distilled water and surface sterilized by soaking for 10 min in a 20% bleach solution. They were then grown on moistened filter paper in 9-cm petri dishes (10 seeds per dish) at approximately 27°C for five to seven days. The roots were individually dipped for 1 sec in an extraction solution consisting of 20 ml of methylene chloride acidified with 50 μl of glacial acetic acid, and the extract was filtered through Whatman No. 1 filter paper in a syringe–filtration system. The extract was then evaporated to dryness, resulting in 12–15 mg of residue per 1000 roots. Fate et al. (1990) indicated that the exudate collected in this way is remarkably clean and contains only two major components: sorgoleone and its dihydroquinone. The dihyroquinone readily oxidizes into the quinone. Analysis of this exudate by high-pressure liquid chromatography (HPLC) (Spectra Physics, model SP8800) using a Spherisorb 5 μM ODS2 column (4.6 $\times$ 250 mm) showed over 90% correlated to the SGL peak published by Netzly and Butler (1986) and Netzly et al. (1988). Since no commercial SGL is available, Dr. Larry Butler (Purdue University) provided the initial sample for comparison. This exudate, which we will call SGL, was stored at -4°C until used.

Isolation of Mitochondria. Mitochondria were isolated from 4- to 5-day-old dark-grown, etiolated corn (*Zea mays* L., Pioneer #3615) coleoptiles or soybean [*Glycine max* (L.) Merr., Pioneer #9202] hypocotyls grown in 10^{-4} M $CaCl_2$-saturated vermiculite at 25°C according to procedures modified from Miller et al. (1970). The isolation procedures consisted of grinding tissue in a defined aqueous medium followed by a series of refrigerated centrifugations (1–4°C). Approximately 80 g of tissue were placed in 100 ml of chilled grind mix (0–4°C) containing 0.3 M mannitol, 0.1% bovine serum albumin (BSA), 50 mM HEPES-KOH (pH 7.5), 4 mM L-cysteine, and 5 mM EDTA. Coleoptiles or hypocotyls were homogenized with a chilled mortar and pestle. The homogenate was strained through four layers of cheesecloth. The liquid suspension was placed in each of four, 50-ml tubes and centrifuged (Sorvall RC-5, SS-34 rotor) at 1500*g* for 12 min. The supernatant was reserved and centrifuged at 28,000*g* for 6 min to isolate the mitochondria in the pellet. Pellets were resuspended in chilled, 0.4 M sucrose using a soft camel-hair brush to gently loosen the pellet, and this suspension was centrifuged at 1500*g* for 12 min. The supernatant was reserved, underlain with 0.6 M sucrose, and centrifuged at 17,500*g* for 18 min. The supernatant was carefully aspirated to avoid disruption of the pellet containing the mitochondria. The pellets were resuspended for a final time with enough of a 0.4 M sucrose solution to yield a suspension that contained optimal concentrations for study.

The final mitochondrial suspension used for oxygen uptake studies contained 7–10 mg protein/ml and that used for cytochrome studies was 10–15 mg protein/ml. Mitochondrial suspensions were kept on ice until used. A small amount of suspension was frozen and saved for protein analysis using the Bio-Rad Protein Analysis Method (Bio-Rad Chemical Division) with BSA used as the protein standard.

Experimentation. Oxygen utilization was measured polarographically with a Hansatech instrument (King's Lynn, Norfolk, England), which has a Clark-type oxygen electrode. The 2-ml, water-thermostated reaction chamber was maintained at 25°C. The mitochondrial reaction medium consisted of: 2 mM KH_2PO_4, 10 mM TES (pH 7.6), 5 mM $MgCl_2$, 0.3 M mannitol, and either 10 mM succinate, 33 mM malate, or 1 mM NADH as substrate. Preliminary experiments included 0.1% BSA in this reaction medium. BSA was subsequently found unnecessary, and it was deleted from the reaction medium. A mitochondrial concentration of 0.3–0.5 mg was maintained in 2 ml of reaction medium. For certain tests, 1 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) was employed as an uncoupling agent.

SGL was dissolved in EtOH for use in mitochondrial studies. The concentration of SGL was adjusted so no more than 20 μl of EtOH were injected into the 2-ml reaction chamber. Controls run with 20 μl of EtOH had no impact on mitochondrial respiration rates.

The ADP-stimulated rate of respiration will be referred to as state 3, and the ADP-limited rate will be referred to as state 4 (Chance and Williams, 1955). A respiratory control ratio (RCR) and ADP/O ratio were determined at the onset of each set of experimental trials. The RCR was computed as state 3/state 4. The ADP/O ratio was computed from the moles of ADP utilized per moles of oxygen used in state 3 respiration. Percentage of inhibition was determined as: (control rate minus rate at inhibition)/control rate. An I_{50} is defined as the concentration required to inhibit respiratory rates by 50%.

Spectrographic methodology was used to determine if restricted electron flow occurred at specific cytochrome sites in the electron transport chain. A dilution of approximately two parts mitochondrial suspension (10–15 mg protein/ml) and one part aerated reaction medium was prepared in a 1-ml cuvette. The sample was scanned (Beckman DU70) from 500 to 650 nm using sodium dithionite to reduce all cytochromes, allowing definition of the region of the *b* (560–557) and *c* (554–550) peaks (Douce, 1985; Keightley, 1991). A similar mitochondrial preparation without sodium dithionite was scanned following amendments of SGL. The scans were compared for evidence of location of inhibition of electron transport.

RESULTS AND DISCUSSION

Oxidation of Substrates. Representative polarographic traces of NADH, succinate, and malate oxidation by mitochondria containing approximately 0.3–0.5 mg protein are depicted in Figure 2A–C. Corn mitochondria had average RCR values of 2.5, 2.3, and 2.4 for NADH, succinate, and malate, respectively. The ADP/O ratios for these substrates were 1.2, 1.3, and 1.9. The RCR and ADP/O values for soybean mitochondria were similar. Addition of 1 μM FCCP during state 4 elicited an increased rate of oxygen utilization that persisted until anaerobiosis, indicating the electron transport system could be uncoupled from its requirement for ADP (Douce, 1985).

Sorgoleone Inhibition. Numerous tests were conducted with SGL additions to the reaction medium during state 3 and state 4 mitochondrial respiration after a linear slope was established (Figure 2D and E). As illustrated, SGL is a strong inhibitor of oxygen uptake, and this inhibition is not overcome by FCCP. Although soybean was generally more affected, there were no trend differences between the responses of isolated corn and soybean mitochondria to the addition of SGL. In an average of six or more trials, the I_{50} for NADH substrate state 3 respiration for corn and soybean mitochondria was between 0.5 and 0.7 μM (Figure 3). This lack of differential action between effects on monocot and dicot mitochondria corresponds with our previous study, which showed that SGL strongly inhibited growth in both broadleaf and grass weeds (Einhellig and

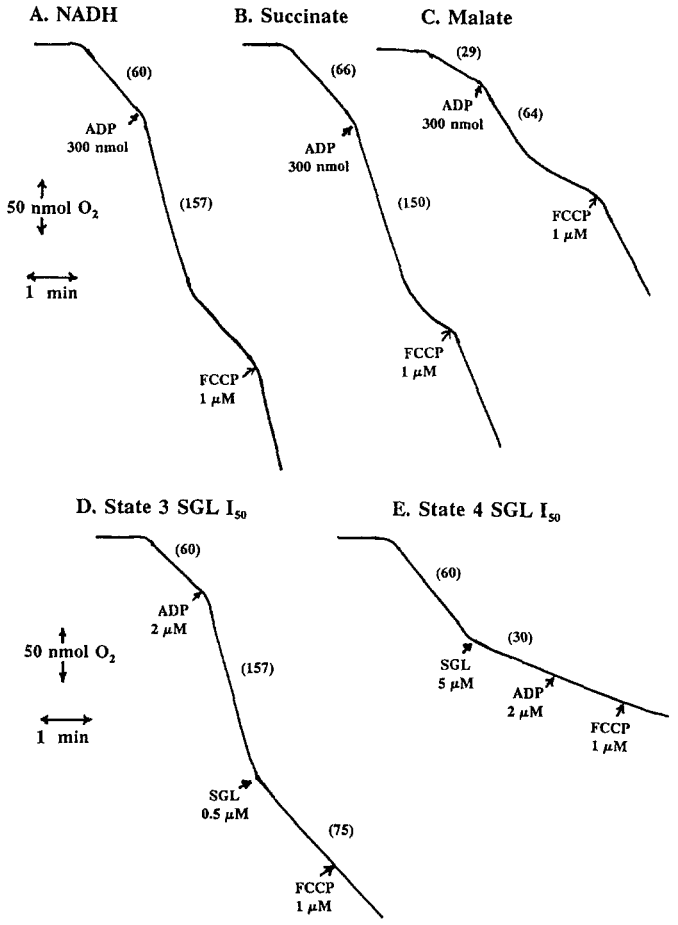

FIG. 2. Representative polarographic traces of oxygen utilization by soybean mitochondria. (A) oxidation of NADH; (B) oxidation of succinate; and (C) oxidation of malate. Traces D and E show sorgoleone inhibition of state 3 and state 4 respiration, respectively.

Souza, 1992). Hence, it appears that the action of SGL on mitochondria can not explain the differences that we found in broadleaf and grass weed abundance following a crop of *Sorghum bicolor* (Einhellig and Rasmussen, 1989).

SGL is a strong inhibitor of mitochondrial oxidation for all three substrates tested in these experiments (Figure 4). NADH, succinate, and malate oxidation were similarly inhibited by SGL concentrations between 0.1 and 0.5 μM, with the latter being the I_{50}. However, SGL approaches maximum inhibition of state-3 succinate oxidation at lower concentrations (0.8–1.0 μM) than when NADH and malate are the substrates. Perhaps this is because succinate provides elec-

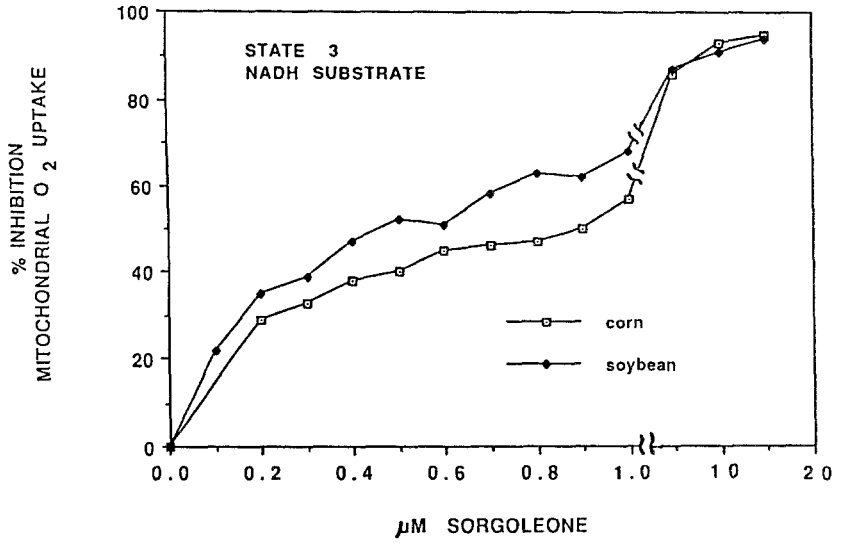

FIG. 3. Effects of sorgoleone on NADH state 3 oxygen uptake of isolated soybean and corn mitochondria, approximately 0.4 mg protein.

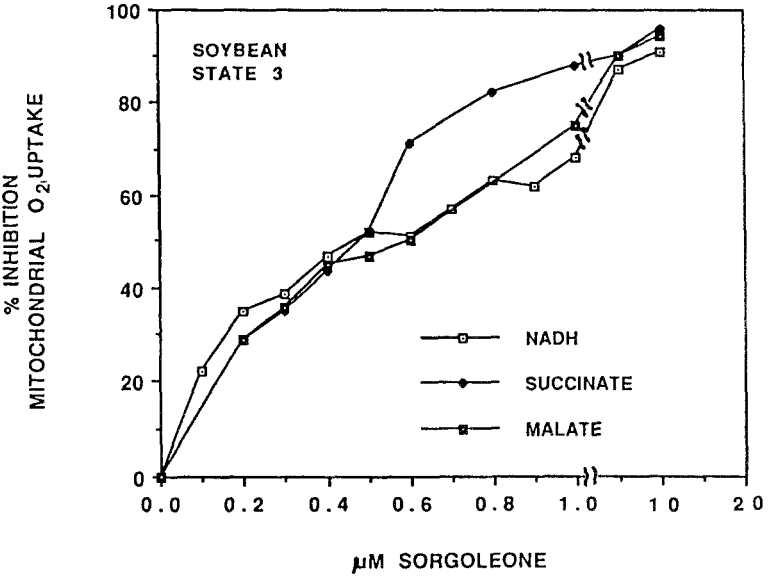

FIG. 4. Effects of sorgoleone on NADH, succinate, and malate state 3 oxygen uptake of isolated soybean mitochondria, approximately 0.4 mg protein.

trons to the electron transport system via complex II, whereas NADH and malate feed electrons through complex I (Douce, 1985). The maximum SGL inhibition of oxidation for each of the three substrates is approximately 90% even when the SGL concentration is raised 10-fold to 10 μM (Figure 4).

The extreme toxicity of SGL to state 3 mitochondrial respiration is highlighted by comparisons to other compounds reported in the literature (Table 1). It is in the same inhibitory range as rotenone, a commonly used mitochondrial respiratory inhibitor. These comparisons also show SGL is effective at several orders of magnitude below other reported allelochemicals.

State 4 respiration is also inhibited by SGL, but it is much less sensitive to the effects of this compound (Figure 5). The state 4 I_{50} was approximately 5.0 μM, which is 10 times the I_{50} of state 3 (Figures 4 and 5). It should be noted that SGL inhibition of state 4 soybean respiration is essentially identical with all three substrates. After the injection of SGL during state 4, state 3 respiration could not be elicited by the addition of ADP (Figure 2E). This is logical given the high concentrations required to inhibit state 4. As in all previous work, there was no material difference between effects of SGL on corn and soybean mitochondrial functions.

In both state 3 and state 4 respiration studies, the introduction of SGL elicited an immediate inhibitory response that persisted during the course of the experiment. The strongly hydrophobic nature of SGL with its long nonpolar, hydrocarbon chain (Figure 1) makes it very lipid soluble. Hence, it should readily solubilize in the mitochondrial membrane, providing a mechanism for immediate and persistent interaction with the site of electron transport.

TABLE 1. COMPARISON OF SORGOLEONE AND OTHER INHIBITORY COMPOUNDS ON STATE 3 PLANT MITOCHONDRIAL RESPIRATION[a]

Compound	Malate oxidation (μM) I_{50}	Reference[b]
Sorgoleone	0.5	
Rotenone	0.5	1
2,6-Dinitroaniline Herbicides	12 to 43	1
Selected Allelochemicals:		
Quercetin	20	2
Naringenin	110	2
Umbelliferone	1,180	2
Vanillin	4,030	2
Ferulic Acid	4,530	2
Vanillic Acid	17,670	2

[a] Mitochondria containing approximately 0.4 mg protein.
[b] 1 = Moreland and Huber (1979); 2 = Moreland and Novitzky (1987).

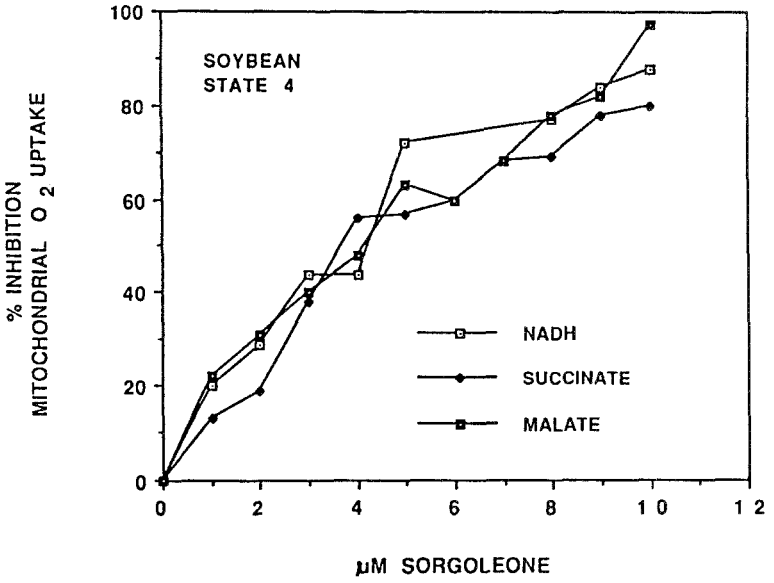

FIG. 5. Effects of sorgoleone on NADH, succinate, and malate state 4 oxygen uptake of isolated soybean mitochondria, approximately 0.4 mg protein.

The long hydrocarbon chain of SGL probably had another impact in the preliminary stages of the experiments when BSA was used in the reaction medium. In our early experiments BSA was utilized because Douce (1985) had reported its ability to bind free fatty acids. However, we found the presence of BSA in the reaction medium caused a profound effect on the I_{50} of SGL. With BSA in the reaction medium, the I_{50} for state 3 respiration of NADH in soybeans was 75 μM SGL compared to 0.5 μM SGL without BSA. It seems very probable that BSA in the medium binds to SGL, limiting the activity of SGL.

Site of Inhibition. The failure of FCCP to circumvent inhibition of state 3 and state 4 respiration by SGL (Figure 2D and E) suggests that SGL acts primarily as an electron-transport inhibitor (Moreland and Novitzky, 1987). When sodium dithionite was used to reduce the cytochromes of isolated mitochondria, characteristic peaks of the cytochrome *b* and *c* regions were revealed (Figure 6). The *b*-type cytochromes are reduced with the addition of SGL, but there is no evidence of a cytochrome $c_{1\text{-}554}$ peak. Since details of the cytochrome *b* complex are elusive, we can conclude that SGL blocks electron flow between the cytochrome *b* and c_1 complex. Several inhibitors of animal mitochondrial electron transport, including hydroquinone analogs, are known to block in this region (Von Jagow and Link, 1986).

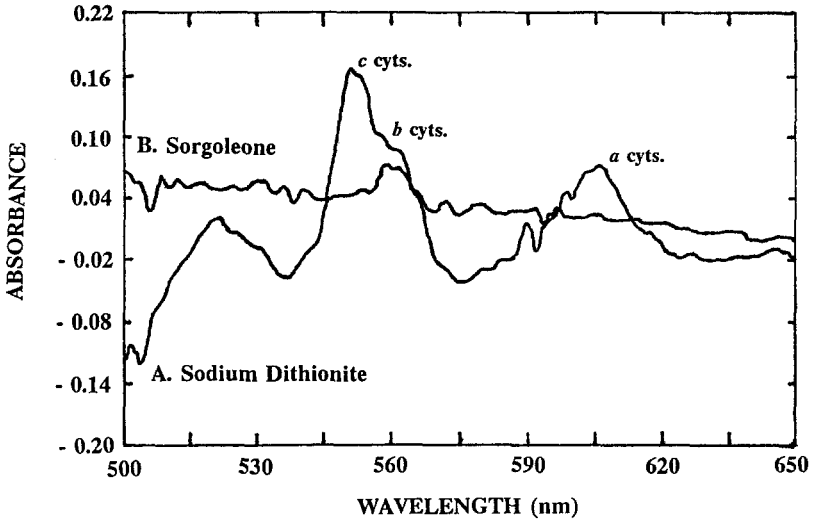

FIG. 6. Spectrophotometric traces of mitochondrial cytochromes (cyts.) after amendments of sodium dithionite (A) and sorgoleone (B).

CONCLUSION

The data demonstrate that SGL, a recently reported *Sorghum bicolor* root exudate, is a powerful inhibitor of respiration in isolated soybean and corn mitochondria. Spectrophotometric studies suggested one mechanism of SGL inhibition is a blockage of electron flow between the cytochrome *b* and c_1 complex.

The effects of SGL on mitochondrial respiration may be an important mechanism responsible for causing the inhibition of growth of several weed species that we have reported (Einhellig and Souza, 1992). This mitochondrial work, plus the evidence that SGL is abundant in *S. bicolor* root exudate, has been isolated from soil, and can persist for a substantial period in soil (Netzly et al., 1988), demonstrates SGL has a role in *Sorghum* allelopathy.

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COMPARATIVE CHEMISTRY OF ELAIOSOMES OF THREE SPECIES OF *Trillium*

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Abstract—This study was designed to compare the chemistry of elaiosomes of three sympatric species of *Trillium* to discover if there are correlations between chemistry and dispersal rates by ants. We quantified the amount of proteins and neutral lipids and qualitatively analyzed the fatty acids occurring in triglyceride, diglyceride, monoglyceride, and free fatty acid fractions. *T. grandiflorum* had the highest lipid concentration per milligram of protein and the highest total lipid per elaiosome, followed by *T. erectum* and then by *T. undulatum*. Oleic acid (18:1) was abundant in all lipid fractions of each species. This fatty acid, which is responsible for corpse-carrying behavior in some ants, probably causes ants to pick up diaspores. Linoleic acid (18:2) is relatively abundant in *T. erectum* and *T. grandiflorum* but not in *T. undulatum* and may cause ants that have picked up diaspores to move (carry) those diaspores back to the nest. Total elaiosome mass and fatty acid composition appear to explain why *T. erectum* is dispersed most rapidly and why ants that have picked up elaiosomes move more *T. grandiflorum* than *T. undulatum* diaspores.

Key Words—*Trillium*, ant dispersal, Hymenoptera, Formicidae, elaiosomes, lipids, diglycerides, triglycerides, oleic acid, linoleic acid.

INTRODUCTION

The dispersal units (diaspores) of ant-dispersed plants consist of a seed and an attached elaiosome that is usually rich in lipids (Beattie, 1985). Ants grasp the

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elaiosome and carry or drag the diaspore to their nest. The ants feed the elaiosome to larvae and discard the remaining seed in their refuse piles (Beattie, 1985). Such dispersal may provide the plant with escape from predation (O'Dowd and Hay, 1980; Culver and Beattie, 1978; Kjellsson, 1985a), escape from competition (Handel, 1978; Kjellsson, 1985b), enhanced germination (Culver and Beattie, 1980; Horvitz, 1981), or nutrient-rich germination sites (Beattie and Culver, 1983; Hanzawa et al., 1988; but see Horvitz and Schemske, 1986; Bond and Stock, 1989).

The elaiosome is the ant-attractive portion of the diaspore, as shown by numerous experiments where intact diaspores and elaiosomes were removed by ants but seeds without elaiosomes were not (Mesler and Lu, 1983; Kjellsson, 1985a; Ohara and Higashi, 1987; Skidmore and Heithaus, 1988; Brew et al., 1989). Elaiosomes vary in size (O'Dowd and Gill, 1986; Gunther and Lanza, 1989), as well as in chemical content, with sympatric species differing in amino acid content (Brew et al., 1989) and lipid composition (Bresinsky, 1963; Soukup and Holman, 1987; Brew et al., 1989; Kusmenoglu et al., 1989).

Most studies have shown that the lipid fraction, particularly the diglyceride fraction, is the major attractant to ants. Bresinsky (1963) concluded that the fatty acid ricinoleic acid (18:1) was the most important ant attractant of *Viola odorata* elaiosomes but later experiments have failed to support this conclusion. Instead, experiments by Marshall et al. (1979), Skidmore and Heithaus (1988), and Brew et al. (1989) have focused attention on the free fatty acid oleic acid (also 18:1) and the diglyceride containing two oleic acid chains (1,2-diolein). In addition, amino acids may contribute to ant attraction in *Tetratheca stenocarpa* (Brew et al., 1989).

Ants disperse up to 30% of the spring-flowering herbaceous species in the deciduous forests of eastern North America, including *Trillium* spp., *Hepatica americanum*, *Asarum canadense*, *Sanguinaria canadensis*, and *Viola* spp. (Berg, 1958; Beattie and Culver, 1981; Heithaus, 1981; Gunther and Lanza, 1989; Smith et al., 1989a,b). Ants respond differently to diaspores of different species; for example, *Aphaenogaster rudis* is more likely to disperse *Hepatica acutiloba* than *Viola* sp. (Beattie et al., 1979) and *Myrmica punctiventris* is more likely to disperse *Trillium erectum* than *T. grandiflorum* (Gunther and Lanza, 1989). The cause of these differences may be differences in elaiosome mass, morphology, chemistry, or, according to Smith et al. (1989a,b), spatial distributions and densities. Elaiosome size seems to explain the preference of *Lasius niger* for three species of plants in The Netherlands (Oostermeijer, 1989). Gunther and Lanza (1989) attempted to answer the question with regard to elaiosome mass by measuring elaiosome mass and elaiosome/diaspore mass ratios in three sympatric species of *Trillium* (*grandiflorum*, *erectum*, and *undulatum*) and correlating these comparisons with ant response to the diaspores of the three species. They discovered that elaiosome mass characteristics could account for only some

of the observed differences in dispersal rates and concluded that ants respond to a complex of characters, including elaiosome mass, morphology, and chemistry.

Might differences in lipid content explain the observed differences in dispersal of sympatric *Trillium* species? This study was designed to compare the chemistry of elaiosomes of three sympatric species of *Trillium* (*grandiflorum*, *erectum*, and *undulatum*) from the same populations studied by Gunther and Lanza (1989) to discover if there are correlations between chemistry and dispersal rates by ants.

METHODS AND MATERIALS

Diaspores of three species of *Trillium* were collected and stored at −18°C prior to chemical analysis. Fruits of *Trillium erectum* and *T. grandiflorum* were collected from the SUNY College at Fredonia campus woodlot, and fruits of painted trillium (*T. undulatum*) were collected from the Herbert C. Mackie Camp in Brocton, New York, approximately 16 km away. These populations were the ones Gunther and Lanza (1989) used for collecting diaspores for their study.

Prior to chemical analysis, elaiosomes were scraped from the diaspores, weighed to 16.0 ± 0.4 mg, transferred to microcentrifuge tubes, and stored at 0°C. Six samples were collected for each of the three species. Samples usually consisted of elaiosomes from a single fruit; in a few cases, when fruits had few seeds, elaiosomes from two fruits were pooled to make a single sample. Each elaiosome sample was then homogenized in 2.0 ml physiological saline (8.5 g NaCl/liter distilled, deionized water) for 10 sec using a Tissumizer (model TR-10, Tekmar Co., Cincinnati, Ohio) at 70% maximum speed. The sample was placed on ice for 1 min, homogenized for another 10 sec at 70% maximum speed, and rinsed with 1.0 ml physiological saline.

For total protein determinations, aliquots of the elaiosome homogenates were assayed in duplicate according to Lees and Paxman (1972). Lipids were extracted from the homogenate by the method of Folch et al. (1957). For total neutral lipids (triglycerides, diglycerides and monoglycerides) determination, 0.25-ml aliquots of the lipid extracts were assayed with a Sigma Diagnostics Kit (Procedure No. 335-UV). Total phospholipids concentrations were measured by the method of Raheja et al. (1973) but were too low to measure reliably and no further quantitative analysis was conducted; qualitative analyses were conducted but are not reported here because of the low concentrations.

Thin-layer chromatography (TLC) was used to separate the different groups of neutral lipids (Garland and Randle, 1963) in the lipid extracts of four randomly selected replicates of each of the three species. Silica gel containing triglycerides (TG), diglycerides (DG), monoglycerides (MG), and free fatty

acids (FFA) was scraped from the plates, and silica was deactivated with one drop of water; the lipids were eluted with 2.0 ml methanol:chloroform (2:1 v/v). The resultant lipid fractions were stored in Teflon-capped tubes at 4°C under nitrogen.

For fatty acid analysis, lipid fractions (TG, DG, MG, and FFA) were methylated (Lepage and Roy, 1984), and their fatty acid composition was examined as described by Awad et al. (1989). Briefly, fatty acid methyl esters were separated on a GC-9A Shimadzu (Columbia, Maryland) using a 6-ft × 1/8-in. glass column packed with 10% SP-2330 on 100–120 Chromosorb WAW (Supelco, Bellefonte, Pennsylvania). The temperatures of the injection port and oven were maintained at 260°C and 190°C, respectively. Nitrogen was used as the carrier gas. Peaks were identified using standards obtained from Supelco and Nu-Chek Prep (Elysin, Minnesota). Areas under the peaks were measured using an integrator and expressed as percent concentrations. Percentages were arc-sine transformed (Zar, 1984) and analyzed with two-factor ANOVAs on the Statview 512+ package for the Macintosh. Tukey's test for multiple comparisons (Zar, 1984) was used to determine significant differences among the three *Trillium* species in the amounts of individual fatty acids.

Statistical comparisons could only be made on the amounts of fatty acids, but two data summaries were useful for examining trends in lipid chemistry of the three species. We first calculated the percent of fatty acids that were saturated, monounsaturated, and polyunsaturated; some insects require polyunsaturated fatty acids in their diet to complete normal development (Chippendale, 1972). We also calculated an unsaturation index {[% monounsaturated + 2(% fatty acids with two double bonds) + 3(% fatty acids with three double bonds)]/(% saturated + % monounsaturated + % polyunsaturated)} to provide a single estimate of the average number of double bonds per fatty acid.

RESULTS

The three *Trillium* species differed in their protein content (Table 1; ANOVA, $F = 13.56$, $df = 2,15$, $P < 0.05$). *T. undulatum* had the lowest protein concentration per gram of elaiosome tissue and was significantly lower than both *T. erectum* (Scheffé $F = 5.33$; $P < 0.05$) and *T. grandiflorum* (Scheffé $F = 13.24$; $P < 0.05$). The protein content of *T. erectum* and *T. grandiflorum* did not differ significantly (Scheffé $F = 1.77$; $P > 0.05$).

The three *Trillium* species also differed in their total neutral lipids in relation to the protein concentration (Table 1; ANOVA, $F = 42.44$, $df = 2,15$, $P > 0.001$). *T. undulatum* had the lowest lipid concentration per milligram of protein and was significantly lower than both *T. erectum* (Scheffé $F = 16.82$; $P < 0.05$) and *T. grandiflorum* (Scheffé $F = 41.40$; $P < 0.05$). The lipid concen-

TABLE 1. ELAIOSOME CHARACTERISTICS OF *Trillium erectum*, *T. grandiflorum*, AND *T. undulatum*[a]

Species	Elaiosome mass (mg)[b]	Protein concentration (mg/g elai)	Neutral lipids concentration (mg/mg protein)	Total lipids/ elaiosome (mg)
T. grandiflorum	3.50 (0.93)c	117.55 (24.2)b	2.7 (0.04)c	1.10
T. erectum	3.90 (1.66)b	96.40 (23.2)b	1.9 (0.02)b	0.71
T. undulatum	1.05 (0.35)a	59.67 (3.8)a	0.7 (0.05)a	0.04

[a] Means (±SD) are shown for elaiosome mass, protein concentration per gram of elaiosome tissue, and neutral lipids concentration per milligram of protein; for protein and lipid measurements, $n = 6$. Total lipids per elaiosome = (elaiosome mass, g/elai) × (protein concentration, mg/g elai tissue) × (lipid concentration, mg/mg protein). Means in a column with different letters were significantly different.

[b] From Gunther and Lanza (1989).

tration of *T. erectum* was also lower than that of *T. grandiflorum* (Scheffé $F = 5.45$; $P < 0.05$).

The elaiosomes of *T. undulatum* contained much lower total amounts of lipids than *T. grandiflorum* and *T. erectum*, approximately 5% of the lipid content of *T. grandiflorum* elaiosomes (Table 1). The total lipids per *T. erectum* elaiosome was approximately 70% of the amount occurring in *T. grandiflorum*.

The fatty acid composition of the three *Trillium* species differed significantly in all four classes of lipids tested (Table 2). In all classes of lipids, concentrations of the 11 fatty acid types differed significantly. Furthermore, there was always a significant interaction between fatty acid type and species, meaning that the concentration of different fatty acids differed among species. Because the interaction effect was significant, we conducted Tukey multiple comparison tests to discover which fatty acids differed in concentration among the three species (Zar, 1984).

The triglyceride fraction (Tables 3 and 4) of the elaiosomes of *T. undulatum* had a lower unsaturation index (i.e., fewer fatty acids containing double bonds) than that of either *T. erectum* or *T. grandiflorum*. This difference was due to a lower concentration of linoleic acid (18:2) in *T. undulatum* than in the other two species and a concomitant increase in palmitic (16:0) and stearic (18:0) acids. *T. undulatum* also contained higher concentrations of myristic (14:0), heptadecanoic (17:0), and erucic (22:1) fatty acids than its congeners. The concentrations of only two fatty acids differed between *T. erectum* and *T. grandiflorum*, with *T. erectum* being richer in palmitoleic (16:1) and poorer in stearic than *T. grandiflorum*. In *T. erectum* and *T. grandiflorum*, the most abundant fatty acids, in decreasing order of concentration, were oleic (18:1), linoleic,

TABLE 2. RESULTS OF TWO-FACTOR ANOVAS ON PERCENTAGE OF DIFFERENT FATTY ACIDS IN VARIOUS LIPIDS IN ELAIOSOMES OF THREE SPECIES OF *Trillium*[a]

Source	*df*	MS	*F*	*P*
Triglycerides (TG)				
Species (A)	2	20.93	3.19	0.04
Fatty acid type (B)	11	1634.77	249.42	0.0001
AB	22	71.99	10.98	0.0001
Error	108	6.55		
Diglycerides (DG)				
Species (A)	2	9.72	0.76	0.47
Fatty acid type (B)	11	1137.30	88.59	0.0001
AB	22	98.37	7.66	0.0001
Error	108	12.84		
Monoglycerides (MG)				
Species (A)	2	2.12	0.15	0.86
Fatty acid type (B)	11	1109.30	80.69	0.0001
AB	22	43.96	3.20	0.0001
Error	108	13.75		
Free fatty acids (FFA)				
Species (A)	2	36.49	1.89	0.16
Fatty acid type (B)	11	1475.53	76.38	0.0001
AB	22	73.31	3.80	0.0001
Error	108	19.32		

[a] ANOVAs were conducted on percentages that had been subjected to an arcsine transformation (Zar 1984).

TABLE 3. UNSATURATION INDEX VALUES FOR FOUR LIPID CLASSES OF THREE SPECIES OF *Trillium*[a]

	T. erectum	*T. grandiflorum*	*T. undulatum*
TG	1.09	1.04	0.60
DG	0.98	0.84	0.40
MG	0.62	0.54	0.42
FFA	0.85	0.55	0.80

[a] The unsaturation index represents the mean number of double bonds per fatty acid in a lipid class for a given species.

and palmitic; the most abundant fatty acids in *T. undulatum* were oleic, palmitic and stearic.

The diglyceride fraction (Tables 3 and 4) of *T. undulatum* was also considerably more saturated than that of either *T. erectum* or *T. grandiflorum*.

TABLE 4. FATTY ACID (CHAIN LENGTH: NUMBER OF DOUBLE BONDS) COMPOSITION OF TRIGLYCERIDES, DIGLYCERIDES, MONOGLYCERIDES, AND FREE FATTY ACIDS OF ELAIOSOMES FROM *T. erectum*, *T. grandiflorum* AND *T. undulatum*[a]

Fatty acid	Lipid class	*T. erectum*	*T. grandiflorum*	*T. undulatum*
12:0 laurate	TG	tr a	tr a	0.94 ± 0.52a
	DG	tr a	0.74 ± 0.41a	0.62 ± 0.49a
	MG	tr a	1.17 ± 0.40a	1.44 ± 1.55a
	FFA	tr a	tr a	tr a
14:0 myristic	TG*	3.18 ± 2.73a	3.47 ± 2.26a	6.71 ± 3.27b
	DG	13.44 ± 5.59a	16.78 ± 5.65a	12.56 ± 5.17a
	MG	28.53 ± 3.62a	21.35 ± 10.26a	20.28 ± 7.03a
	FFA	2.29 ± 0.68a	3.96 ± 2.04a	0.90 ± 0.71a
14:1 myristoleic	TG	tr a	tr a	0.55 ± 0.72a
	DG	2.61 ± 4.28a	0.92 ± 0.66a	0.75 ± 0.55a
	MG*	0.00 ± 0.00a	2.21 ± 1.89b	3.72 ± 4.49b
	FFA	tr a	tr a	tr a
16:0 palmitic	TG*	19.23 ± 1.35a	16.92 ± 1.75a	26.34 ± 2.46b
	DG*	10.82 ± 6.69a	10.54 ± 2.09a	31.02 ± 2.21b
	MG*	11.49 ± 1.32a	12.74 ± 0.81a	20.71 ± 2.84b
	FFA	15.70 ± 1.01a	17.11 ± 2.78a	17.18 ± 2.35a
16:1 palmitoleic	TG*	5.76 ± 1.86b	2.98 ± 1.77a	2.22 ± 1.15a
	DG	1.22 ± 0.87a	1.62 ± 1.32a	1.65 ± 1.12a
	MG	1.07 ± 0.34a	0.83 ± 0.59a	1.12 ± 2.25a
	FFA	2.92 ± 1.59a	0.56 ± 0.16a	5.67 ± 3.70a
17:0	TG	0.00 ± 0.00a	0.00 ± 0.00a	1.07 ± 0.80b
heptadecanoic	DG	0.00 ± 0.00a	tr a	0.94 ± 1.09a
	MG	0.00 ± 0.00a	0.00 ± 0.00a	1.13 ± 2.26a
	FFA	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
18:0 stearic	TG*	2.75 ± 1.31a	7.92 ± 3.21b	12.57 ± 5.45c
	DG*	5.21 ± 3.99a	5.26 ± 0.38a	14.82 ± 2.71b
	MG*	4.22 ± 0.85a	11.24 ± 8.06b	10.54 ± 3.57b
	FFA*	15.38 ± 4.75ab	27.49 ± 6.00b	7.90 ± 1.74a
18:1 oleic	TG	32.84 ± 1.20a	34.40 ± 5.38a	35.87 ± 2.81a
	DG	24.16 ± 4.54a	34.10 ± 7.23b	23.93 ± 3.28a
	MG*	17.85 ± 2.61ab	24.45 ± 7.17b	13.62 ± 2.83a
	FFA*	38.35 ± 6.20a	34.13 ± 6.76a	58.63 ± 8.55b
18:2 linoleic	TG*	33.22 ± 5.63b	30.82 ± 1.27b	8.08 ± 5.81a
	DG*	29.60 ± 12.52c	18.48 ± 2.40b	3.18 ± 0.92a
	MG*	12.90 ± 0.54b	5.76 ± 2.44a	4.34 ± 2.78a
	FFA*	18.81 ± 11.31b	6.02 ± 6.20a	5.99 ± 4.63a
18:3 linolenic	TG	0.96 ± 0.17a	1.35 ± 0.92a	0.59 ± 0.22a
	DG	0.98 ± 0.25a	1.15 ± 1.12a	tr a
	MG	tr a	tr a	0.82 ± 1.59a
	FFA	0.93 ± 0.14a	tr a	0.78 ± 0.15a
22:0 behanate	TG	tr a	tr a	1.08 ± 0.92a
	DG	3.11 ± 1.33a	2.87 ± 0.57a	2.01 ± 0.93a
	MG	5.86 ± 0.96a	4.24 ± 1.90a	3.66 ± 1.70a
	FFA	1.49 ± 0.67a	1.72 ± 0.31	0.66 ± 0.55a

TABLE 4. Continued

Fatty acid	Lipid class	*T. erectum*	*T. grandiflorum*	*T. undulatum*
22:1 erucic	TG*	0.72 ± 0.38a	0.79 ± 0.21a	3.02 ± 1.43b
	DG	4.98 ± 3.37a	5.83 ± 1.88a	5.75 ± 1.63a
	MG	16.52 ± 2.13a	13.49 ± 6.02a	11.69 ± 3.82a
	FFA	3.60 ± 1.11a	6.98 ± 3.90a	0.66 ± 0.58a
Trace and unidentified	TG	1.34	1.35	0.96
	DG	3.87	1.35	2.77
	MG	1.56	2.52	7.75
	FFA	0.53	2.03	1.63

[a] Values are mean percentages ± SD ($N = 4$) for each fatty acid as a fraction of each lipid type (TG, DG, MG or FFA). * after a lipid type indicates the presence of significant differences in a given row. Means within a lipid class with different letters were significantly different (Tukey's multiple comparison test on transformed data, $P < 0.05$).

Again, this difference was primarily due to a lower concentration of linoleic (18:2) fatty acid and a higher concentration of palmitic (16:0) and stearic (18:0) acids in *T. undulatum* as compared to both *T. erectum* and *T. grandiflorum*. *T. grandiflorum* had higher levels of oleic (18:1) than did either of the other two species. The most abundant fatty acids in *T. erectum* and *T. grandiflorum* were oleic, linoleic, and myristic (14:0), whereas *T. undulatum* contained the largest amounts of palmitic, oleic, stearic, and myristic acids.

The degree of saturation of the fatty acids from monoglycerides (Tables 3 and 4) was more similar among the three *Trillium* species than in the tri- and diglyceride fractions but was still highest in *T. undulatum*. *T. undulatum* was lower in oleic (18:1) than *T. grandiflorum* and lower in linoleic (18:2) than *T. erectum*; it was higher than both in palmitic (16:0) and higher than *T. erectum* in stearic (18:0). *T. erectum* and *T. grandiflorum* also differed, with *T. erectum* having lower concentrations of myristoleic (14:1) and stearic acids but a higher concentration of linoleic. The three most abundant fatty acids varied among the species: myristic, oleic, and erucic (22:1) in *T. erectum*; oleic, myristic, and erucic in *T. grandiflorum*; and palmitic (16:0), myristic, and oleic in *T. undulatum*.

The free fatty acids (Tables 3 and 4) of *T. grandiflorum* were more saturated than those of *T. erectum* and *T. undulatum*. This difference was caused by a higher concentration of stearic acid (18:0) in *T. grandiflorum*, more oleic acid (18:1) in *T. undulatum*, and more linoleic acid (18:2) in *T. erectum*. *T. undulatum* was also lower in linoleic acid concentration than *T. erectum*. The most abundant fatty acid in all three species was oleic acid. The next most abundant fatty acids varied among the three species: linoleic, palmitic (16:0), and stearic

in *T. erectum*; stearic and palmitic in *T. grandiflorum*; and stearic in *T. undulatum*.

In comparing the three species, the largest difference in fatty acid chemistry occurred between *T. undulatum* and *T. erectum*. The unsaturation index for *T. undulatum* was lower than that of *T. erectum* in all four classes of lipids.

The most consistent differences between species occurred in the concentrations of fatty acids with 18 carbons. *T. undulatum* tended to have a higher concentration of stearic (18:0—three of the four classes of lipids) but lower concentrations of oleic (18:1—two of the four lipid classes) and especially linoleic (18:2—all four of the lipid classes).

DISCUSSION

The elaiosomes of all three *Trillium* species contained protein, triglycerides, diglycerides, monoglycerides, and free fatty acids. Elaiosomes of *T. undulatum*, the smallest of the three species (Gunther and Lanza, 1989), were lowest in protein and lipid content, both in absolute measurements (per elaiosome) and in concentration (per gram of elaiosome). *T. erectum* was lower in lipid content than *T. grandiflorum*. Our analyses appear comparable to two other less-detailed studies of *Trillium* elaiosomes. When the fatty acids of all neutral lipids are analyzed together, the three most common fatty acids of *T. grandiflorum*, *T. erectum*, and *T. undulatum* are oleic (18:1), linoleic (18:2), and palmitic (16:0) (Soukup and Holman, 1987). These fatty acids were common in our analyses as well, but we cannot make direct comparisons of amounts because Soukup and Holman did not separate the different neutral lipid fractions. Oleic, linoleic, and palmitic acids are also the most common in the other *Trillium* species that Soukup and Holman analyzed, although two species have substantial amounts of palmitoleic (16:1). In the triglyceride fraction of *T. sessile*, the three most abundant fatty acids in triglycerides are again oleic, linoleic, and palmitic (Kusmenoglu et al., 1989); these authors did not analyze the fatty acids of other neutral lipids in *T. sessile*.

Our analyses, in which we first separated the classes of neutral lipids and then assayed the fatty acids, allow more detailed comparisons of the elaiosomes of three species of *Trillium*. In all classes of neutral lipids, *T. erectum* produced the most unsaturated elaiosomes, although *T. grandiflorum* had similar unsaturation levels in the triglyceride fraction and *T. undulatum* had similar unsaturation levels in the free fatty acid fraction. In all lipid classes, polyunsaturated lipids were more abundant in *T. erectum* than in *T. undulatum*; similarly, in three of the lipid classes, polyunsaturated lipids were more abundant in *T. erectum* than in *T. grandiflorum*. In all lipid classes, *T. undulatum* contained less linoleic acid (18:2), a dietary requirement for some insects (Chippendale, 1972), than did *T. erectum*. Oleic acid (18:1) was a major fatty acid component of all three *Trillium* species in all four classes of lipids.

Differences in *Trillium* elaiosome mass and elaiosome/diaspore mass ratio account for some, but not all, of the differences in ant behavior observed by Gunther and Lanza (1989). The high dispersal rate of *T. erectum* is correlated with large elaiosome mass and high elaiosome/diaspore mass ratio; however, large differences in elaiosome mass and elaiosome/diaspore mass ratio between *T. grandiflorum* and *T. undulatum* are not correlated with overall differences in removal rates by ants. The diaspores of *T. undulatum* are initially very attractive to *Myrmica punctiventris*, for these ants repeatedly pick up the diaspores, but the diaspores do not maintain ant attention, for the ants remove them relatively infrequently from experimental arenas (Gunther and Lanza, 1989).

The total lipid content of elaiosomes may influence ant behavior but does not appear to be a major force controlling ant response to *Trillium* diaspores. Elaiosomes of *T. grandiflorum* represent the greatest lipid reward to potential seed-dispersing ants; even though *T. grandiflorum* elaiosomes are smaller than those of *T. erectum*, ants can collect a greater nutrient reward by gathering *T. grandiflorum* elaiosomes. However, *T. grandiflorum* is removed less quickly by *M. punctiventris* than is *T. erectum* (Gunther and Lanza, 1989). *T. undulatum* elaiosomes contain less total lipid than either *T. erectum* or *T. grandiflorum* but are intermediate in and not significantly different from either species in overall removal rate (Gunther and Lanza, 1989).

The fatty acid profiles of the three species were more closely correlated with ant behavior than was total lipid content. The abundance of oleic acid in our analyses and those of others (Soukup and Holman, 1987; Skidmore and Heithaus, 1988; Brew et al., 1989; Kusmenoglu et al., 1989) supports the idea that it is a major attractant of ants. Behavioral experiments of Marshall et al. (1979), Skidmore and Heithaus (1988), and Brew et al. (1989) showed that oleic acid, in diglyceride and free fatty acid fractions, is an important ant attractant. This is the fatty acid that, as a free fatty acid, elicits corpse-carrying behavior in several ants (Wilson et al., 1958; Haskins and Haskins, 1974) and foraging behavior in some social contexts (Gordon, 1983). The high concentration of oleic acid (59%) in the free fatty acid fraction of *T. undulatum* elaiosomes may account for their initial attractiveness to *M. punctiventris*. However, the fact that picked-up *T. undulatum* diaspores are not removed as frequently as those of *T. grandiflorum* (Gunther and Lanza 1989) suggests that some additional chemical components are important in seed dispersal behavior. Also, the concentrations of oleic acid do not, in any of the lipid fractions, parallel the ant preferences measured by Gunther and Lanza (1989), again indicating that oleic acid is not the only cue to which ants respond.

Linoleic acid, in several lipid fractions, may be a second fatty acid cue in *Trillium* diaspores to which ants may respond. Vinson et al. (1967) showed that linoleic acid (18:2), both as a triglyceride and as a free fatty acid, stimulates feeding by fire ants (*Solenopsis saevissima*). Skidmore and Heithaus (1988)

showed that two lipid fractions extracted from *Hepatica americanum* elaiosomes were attractive to ants and contained various fatty acids, including linoleic acid, either as a diglyceride or as a free fatty acid; however, Marshall et al. (1979) measured little response by ants to linoleic acid as a free fatty acid.

Linoleic acid concentrations, in the various lipid fractions of the three *Trillium* species, appeared correlated with *Myrmica punctiventris* behavior observed by Gunther and Lanza (1989). The linoleic acid concentrations were significantly lower in *T. undulatum* than in all four lipid fractions of *T. erectum* elaiosomes and in two of the four lipid fractions of *T. grandiflorum* elaiosomes, including the important diglyceride fraction. The low concentration of linoleic acid in *T. undulatum* may explain why ants were more likely to remove picked-up *T. grandiflorum* elaiosomes than picked-up *T. undulatum* elaiosomes from the experimental arenas of Gunther and Lanza (1989). This decrease in linoleic acid is concomitant with an increase in the proportion of saturated fatty acids, a group that in various lipid forms, does not contribute substantially to ant attraction (Skidmore and Heithaus, 1988; Brew et al., 1989).

The elaiosomes of three *Trillium* species vary chemically, but the ecological significance of this variation is uncertain. We do not know if this variation has been caused by the preferences of ants for elaiosomes of particular chemical composition, but our results, and those of Gunther and Lanza (1989), suggest that movement of diaspores involves at least two chemical cues: (1) diglyceride and other forms of oleic acid (18:1) and (2) diglyceride and other forms of linoleic acid (18:2). Although this idea must, at this point, remain an hypothesis, it is experimentally testable. Full evaluation of this idea will require: (1) tests of response by other ant species to *Trillium* diaspores, (2) detailed chemical analyses of elaiosomes from other ant-dispersed plants, (3) tests of responses by ants to the diaspores of these other ant-dispersed plants, and (4) observations of ant response to experimentally manipulated mixtures of diglycerides of oleic, linoleic, and other fatty acids.

We suggest that future workers investigate the effect on ant behavior of several fatty acids, especially in diglyceride form. Simply focusing on concentrations of oleic acid is unlikely to further our understanding of the factors involved in ant dispersal.

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COMPARISON OF VOLATILES EMITTED BY MALE CARIBBEAN AND MEXICAN FRUIT FLIES

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Abstract—Volatiles emitted by *Anastrepha suspensa* and *Anastrepha ludens* males were trapped and compared by GC-MS. Both species emitted previously reported nine-carbon alcohols, (*Z*)-3-nonenol and (*Z,Z*)-3,6-nonadienol, and epimeric lactones, anastrephin and epianastrephin. A third isomeric lactone, the macrolide suspensolide, which was previously known only from *A. suspensa* is reported here for the first time in the volatiles of *A. ludens*. We also report that both species released different proportions of the same three sesquiterpene hydrocarbons: α-farnesene, β-bisabolene, and α-*trans*-bergamotene. The β-bisabolene was isolated in sufficient quantity from *A. suspensa* volatiles to establish its absolute configuration as the (*R*)-(+)-enantiomer. Some plausible biosynthetic relationships of farnesol to the isoprenoid lactones and sesquiterpenes identified in this study are discussed. Finally, we report that *A. suspensa* produces the monoterpene (*Z*)-β-ocimene while *A. ludens* volatiles contained limonene.

Key Words—Diptera, Tephritidae, Caribbean fruit fly, *Anastrepha suspensa*, Mexican fruit fly, *Anastrepha ludens*, male volatiles, GC-MS, suspensolide, farnesene, bisabolene, bergamotene, ocimene, limonene.

INTRODUCTION

The Caribbean fruit fly, *Anastrepha suspensa* Loew, and the Mexican fruit fly, *Anastrepha ludens* Loew, display very similar male courtship and calling behav-

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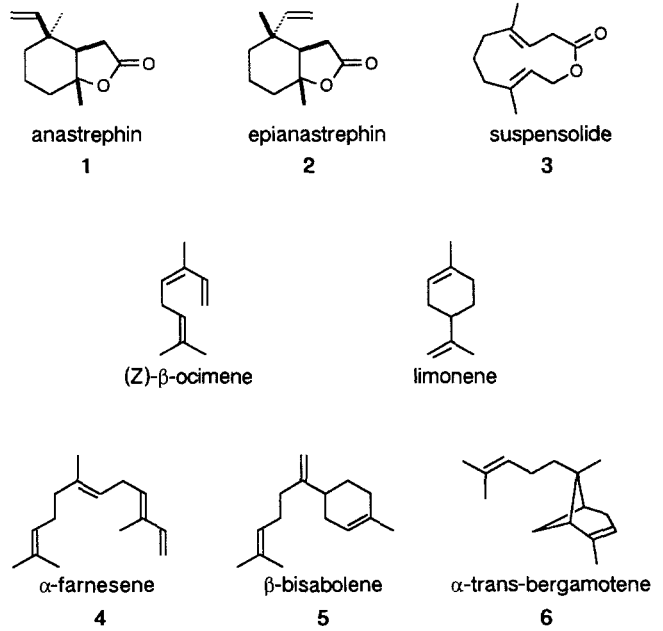
iors (Nation, 1972, 1990; Robacker and Hart, 1985a). Two nine-carbon alcohols, (Z)-non-3-en-1-ol and (3Z,6Z)-nona-3,6-dien-1-ol, have previously been identified from the multicomponent male sex pheromones of both *A. suspensa* (Nation, 1975, 1983) and *A. ludens* (Esponda-Gaxiola, 1977). The two isoprenoid 12-carbon γ-lactones shown in Scheme 1, anastrephin **(1)** and epianastrephin **(2)**, have also been characterized as components of the male pheromonal volatiles of *A. suspensa* (Battiste et al., 1983) and of *A. ludens* (Stokes et al., 1983). While behavioral roles for these four components have been demonstrated both in *A. suspensa* (Nation, 1989 and references therein) and in *A. ludens* (Robacker and Hart, 1985b, 1986; Robacker, 1988), the existence of unidentified or possible additional unknown compounds in the volatiles has also been mentioned for each species.

More recently, the 11-membered macrolide **(3)** shown in Scheme 1, isomeric with anastrephin and epianastrephin, was isolated from the volatiles of *A. suspensa* males and identified and named suspensolide by Chuman et al. (1988). Working closely with those who first identified this macrolide, Battiste



SCHEME. 1. Some of the volatile substances from *Anastrepha suspensa* and *Anastrepha ludens* males. The isoprenoid lactones, **1–3** ($C_{12}H_{18}O_2$, M = 194), and the sesquiterpenes, **4–6** ($C_{15}H_{24}$, M = 204), are present in the volatiles of both species. Of the two monoterpenes ($C_{10}H_{16}$, M = 136), the ocimene is from *A. suspensa* and limonene is from *A. ludens*.

et al. (1988) confirmed its structure and stereochemistry with the first synthesis of (3*E*,8*E*)-suspensolide. Our experience with suspensolide indicated that it was difficult to collect from whole-body extracts of *A. suspensa* because of its lability on contact with polar (acidic) solvents or materials. Thus, we wondered if it might also be present in *A. ludens* males, but not previously identified because the earlier work had been done with whole-body extracts; one of our objectives was to look specifically for this compound using gas chromatography–mass spectrometry (GC-MS). Another objective was to identify and compare additional components in the volatiles from *A. suspensa* and *A. ludens*. These new compounds include the monoterpenes and sesquiterpenes (**4, 5,** and **6**) shown in Scheme 1.

METHODS AND MATERIALS

Collection of Fruit Fly Volatiles

Volatiles from *A. suspensa* males were collected by a published procedure (Nation, 1989, 1990). Volatiles from 50 mature *A. ludens* males were entrained in an airstream and trapped on Porapak Q for 3 hr in Weslaco, Texas. The holding and aeration chamber for the flies was a 500-ml side-arm flask. The trapped volatiles were eluted from the Porapak Q with hexane, and the flask holding the flies was washed with hexane at the end of the collection period to determine if any material was left on the glass. These samples were sealed in vials and mailed to Gainesville, Florida. Before chemical analyses, the samples were diluted to a known volume (1–5 ml), depending upon the sample size and based on previous experience with similar samples from *A. suspensa*. The solutions were analyzed by GC without delay, using a procedure described by Nation (1990), to determine the suitability of the dilution and to ensure that the addition of internal standards would not mask any components in the samples. When satisfactory results were obtained in these preliminary procedures, the following quantitative standards were added: decanol, 4.6 μg; tetradecane, 6.4 μg; and methyl tridecanoate, 9.0 μg. These solutions were then analyzed by GC or GC-MS on the columns and spectrometers described below.

Instrumentation

Gas Chromatography. Samples of volatiles were examined by GC on the following two column–instrument combinations. GC-1: A 10-m × 0.5-mm, large-bore, RSL-160 (Alltech) capillary was fitted to a Varian 2400 chromatograph equipped with an FID. Data were recorded on a Hewlett-Packard 3390-A integrator. GC-2: A 25-m × 0.25-mm, narrow-bore, bonded-phase RSL-150 (Alltech) capillary was fitted to a Shimadzu G-14A chromatograph

equipped with an FID. Samples were introduced splitless into a capillary injector port, followed after 30 sec by a split-purge flow of helium. A typical temperature program started at 60°C, then immediately rose at 10°/min to 120°, followed by a 6 °/min rise to 150°C and hold. Data were recorded on a Spectra Physics 2400 integrator.

GC-MS System-1. This bench-top system (GC-MS-1) was used to produce the total ion current (TIC) chromatograms and mass spectra presented in Figures 1–5 below. Samples of volatiles were analyzed as dilute solutions (ca. 10–100 ng/μl) on a Hewlett-Packard 5995-B gas chromatograph–direct inlet quadrupole mass spectrometer system. Approximately 0.5–1 μl of solution was injected using the splitless-split mode into a 30-m × 0.25-mm fused-silica capillary with a 0.25-μm bonded methyl silicone stationary phase (HP-1), and eluted with helium at a rate of 0.5–1 ml/min. A typical temperature program for the GC was: 50° for 3 min, then 4°/min to 180°C and hold. The electron ionization (EI) mass spectra were recorded at 70 eV over a mass range of 40–350 amu with nominal unit resolution in mass–charge (m/z) ratio. The GC-MS software included a library search-and-match routine for comparing acquired spectra with various databases. The algorithm used for the library search compared the 10 most significant lines in the mass spectrum (significance = mass × abundance) with a library entry's 10 most significant lines. An estimate of the quality of the match between the reference data and the actual spectrum was given and is presented here as percent match, with a 100% match representing identity.

GC-MS System-2. Additional mass spectra were obtained with this bench-top system (GC-MS-2) consisting of a Perkin Elmer gas chromatograph interfaced directly to a Finnigan ion trap mass detector (ITD). The column used was a 30-m × 0.25-mm capillary containing a bonded DB-1 (J & W) stationary phase, eluted with helium at a linear flow velocity of 25 cm/min. The samples were introduced splitless into a capillary injector port at 180°C, followed after 30 sec by a split-purge flow of helium. The column was initially at 60°C, and the temperature was programmed to rise immediately at 10°/min to 120°, followed by a 6°/min rise to 150°C and hold. Data were stored on the hard disk of a Compuadd 286 computer. This system also compared acquired spectra with the data in several libraries, including approximately 40,000 spectra in the National Bureau of Standards Mass Spectral Library.

NMR Spectroscopy. Samples for ^{1}H nuclear magnetic resonance spectra were examined in deuteriochloroform ($CDCl_3$), at 300 MHz with digital resolutions of 0.25–0.5 Hz, using standard single-pulse techniques and routine homonuclear decoupling procedures on a Nicolet (General Electric) NT-300 spectrometer. As a reference, the residual $CHCl_3$ resonance was assigned a chemical shift of 7.24 δ.

Polarimetry. Samples of reference compounds and β-bisabolene from *Anastrepha suspensa* were analyzed in hexanes on a Rudolph Research Corp. (40

Pier Lane, P.O. Box 1446, Fairfield, New Jersey 07006) AUTOPOL III polarimeter with the following specifications: path length = 10 cm, λ = 589 nm, resolution = $\pm 0.001°$, reproducibility and accuracy up to 1° = $\pm 0.002°$.

RESULTS

Partial gas chromatograms of the volatiles trapped from males of these two *Anastrepha* species are presented in Figure 1A (*A. suspensa*) and Figure 1B (*A. ludens*). The figures are labeled with the same numbers used for the structures in Scheme 1. Although these chromatograms were obtained on a methyl silicone capillary column, we have found the MS compilation by Adams (1989) a useful reference for GC comparison to chemicals chromatographed on a similar, relatively nonpolar, phenylmethyl methyl silicone capillary column.

Suspensolide in Mexican Fruit Fly Volatiles

Peaks labeled 1, 2, and 3 in Figure 1A represent the isomeric lactones ($C_{12}H_{18}O_2$,M = 194) anastrephin, epianastrephin, and suspensolide, respectively. Synthetic samples of **1, 2,** and **3** were previously prepared in these laboratories and shown to correspond to volatiles from *A. suspensa* males (Battiste et al., 1983, 1988). These three lactones in the *A. suspensa* volatiles were compared with the corresponding peaks in Figure 1B, present in roughly similar proportions from *A. ludens*; they were found to have essentially identical mass spectra for peaks with the same retention times. This confirms the presence of the γ-lactones **1** and **2** (Stokes et al., 1983), previously identified as anastrephin and epianastrephin (Battiste et al., 1983), while demonstrating for the first time that the macrolide **3,** suspensolide, is also present in the volatiles from *A. ludens*. Figure 2 shows a mass spectrum for suspensolide (peak **3**) emitted by *A. ludens* males. The suspensolide peak is relatively large in the volatiles of *A. ludens*, as it is in the *A. suspensa* volatiles, and its retention time on all of the GC columns used in this comparison was always identical to that for (*E*,*E*)-suspensolide from *A. suspensa*. Hence, we conclude that (3*E*,8*E*)-suspensolide is a major component in the volatiles emitted by these *A. ludens* males.

Sesquiterpenes in Volatiles of Both Species

Volatiles from *A. suspensa* males were eluted from Porapak Q traps with pentane. Liquid chromatography of that solution on a small column of silica (monitored by GC) showed the presence of unidentified hydrocarbons eluting with the void volume of the column. GC-MS indicated that the major hydrocarbon ($C_{15}H_{24}$,M = 204) from *A. suspensa* was a sesquiterpene, and β-bisa-

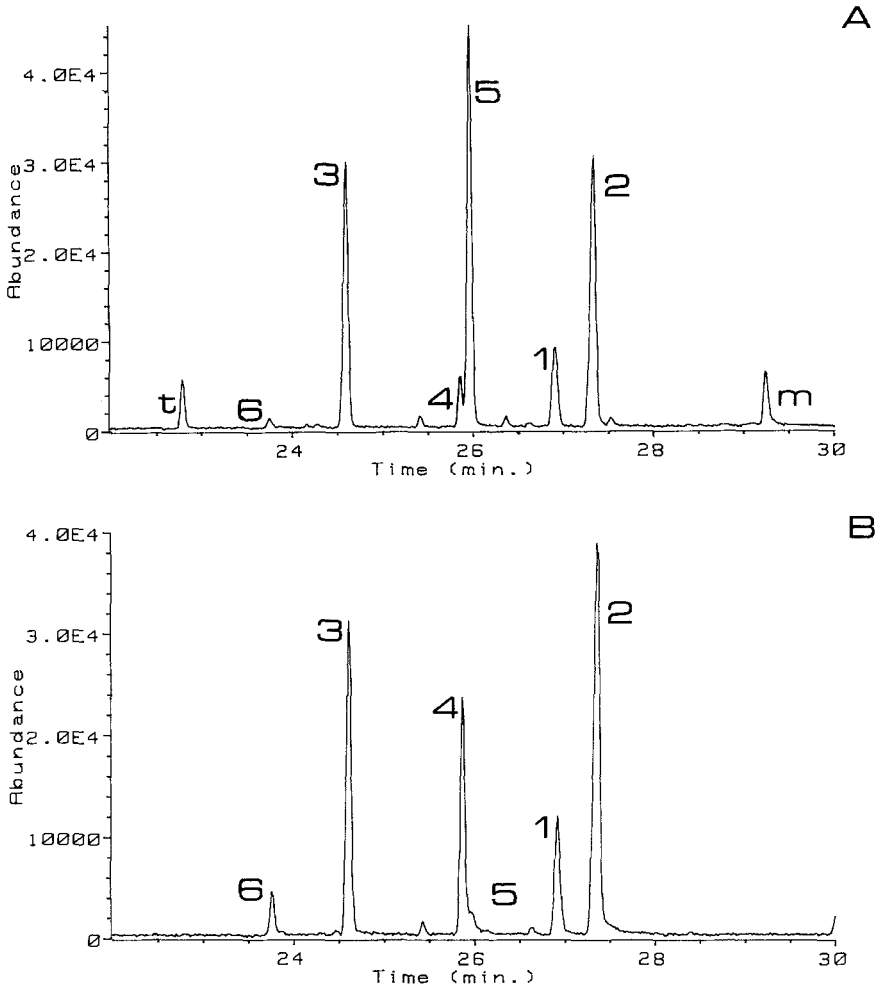


FIG. 1. Capillary gas chromatograms (GC-EI-MS) of the volatile compounds (M = 194–204) collected from *Anastrepha suspensa* and *Anastrepha ludens*. Peaks are numbered according to the structures in Scheme 1. (A) Volatiles from *A. suspensa*; t and m are synthetic internal standards, tetradecane and methyl tridecanoate, added to the volatiles solution. (B) Volatiles from *A. ludens*.

bolene has already been mentioned as a likely candidate (cf. Nation, 1989, p. 196 and Nation, 1990, p. 554).

β-Bisabolene. A 1.29-mg sample containing the major sesquiterpene present in the volatiles of *A. suspensa* males was isolated by short-column chromatography on silica. In addition to allowing polarimetry, this quantity was

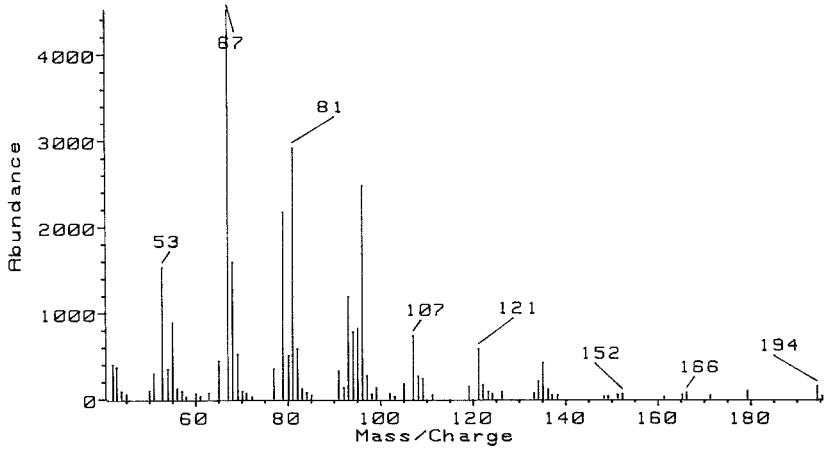


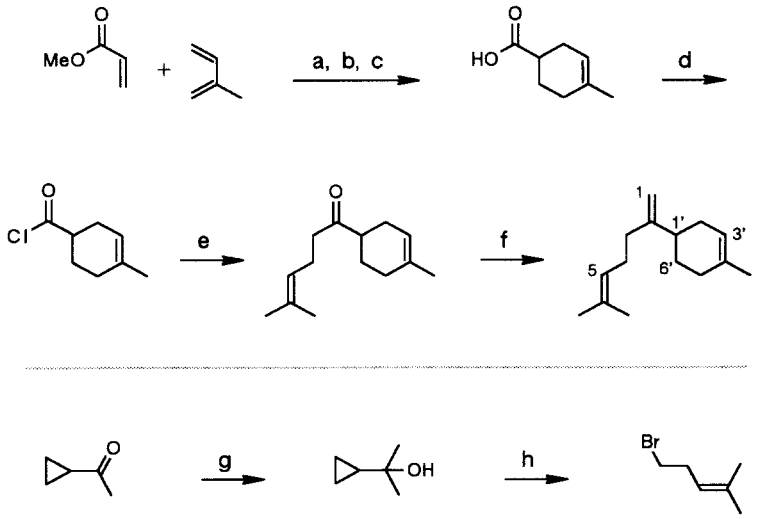
FIG. 2. Mass spectrum (EI) for the (*E*,*E*)-suspensolide peak (3) in Figure 1B from *Anastrepha ludens* males. The ion at $m/z = 41$ (not shown), with an abundance of 2600, is slightly smaller than the ion at $m/z = 81$ (abundance = 2928).

sufficient to permit comparisons of its NMR and mass spectra with those of authentic β-bisalolene as described in the following paragraphs.

A synthetic sample of (±)-β-bisabolene **(5)** was prepared with slight modification of the procedure described by Manjarrez and Guzmán (1966) as shown in Scheme 2. The Diels-Alder reaction between methyl acrylate and isoprene gave the expected mixture of methylcyclohexenyl esters, which upon hydrolysis and work-up gave the desired carboxylic acid in good yield after recrystallization. Conversion to the acid chloride, followed by coupling with the homoprenyl Grignard reagent in the presence of cuprous bromide (ether soluble, dimethyl sulfide complex), provided the expected ketone. That ketone was treated with a Wittig reagent, methylene triphenylphosphorane, to give racemic β-bisabolene. The requisite homoprenyl bromide was available from cyclopropyl methyl ketone in two steps: treatment with methyl Grignard reagent, and Julia rearrangement of the resultant α,α-dimethylcyclopropanemethanol in the presence of aqueous 48% hydrobromic acid.[4]

The [^{1}H]NMR spectrum of 1.2 mg of synthetic β-bisabolene in 0.5 ml of $CDCl_3$ contained the following resonances at 300 MHz [chemical shift, (integral, multiplicity, assignment/C-n according to Scheme 2)]: $\delta = 5.40$ (1H, broad line, C-3′); 5.12 (1H, triple multiplet, C-5); 4.74/4.76 (2H, broad AB pattern, C-1); 2.27–1.73 (9H, several multiplets, C-3, C-4, C-1′, C-2′, C-5′);

[4] Subsequent to the actual work described in this paper, one of us (J.R.R.) has also prepared this homoallylic bromide in excellent yield from the same alcohol using anhydrous $MgBr_2$ (commercial or from $BrCH_2CH_2Br$ and Mg) in diethyl ether.



SCHEME. 2. Synthesis (±)-β-bisabolene **(5)** after the method of Manjarrez and Guzmán (1966); 5-bromo-2-methyl-2-pentene required for the Grignard reagent in step (e) was prepared as shown in steps (g) and (h). Reagents and conditions: (a) 125–135°C for 2–5 hr; (b) KOH, CH_3OH, H_2O, 60°C for 1.5 hr followed by 12 N HCl; (c) warm hexane, slowly cool to −30°C, recrystallize 4×; (d) $(COCl)_2$, benzene; (e) $(CH_3)_2C{=}CH(CH_2)_2MgBr$, CuBr · $S(CH_3)_2$, Et_2O, 0°C for 2 hr then 23°C overnight followed by 5 NH_2SO_4; (f) $(Ph)_3PCH_3Br$, BuLi, Et_2O, reflux for 12 hr; (g) CH_3MgBr, Et_2O, 0°C for 1 hr then 23°C overnight followed by 10% NH_4Cl at 0°C; (h) 48% HBr at 0°C for 10 min.

1.69 (3H, singlet, vinyl methyl); 1.65 (3H, singlet, vinyl methyl); 1.61 (3H, singlet, vinyl methyl); and 1.47 (2H, multiplets, C-6′). Assignments of the vinyl protons are based on decoupling experiments as well as empirical calculations using the relevant table in the text of Silverstein et al. (1981). These data agree fairly well with the 100-MHz data accompanying the syntheses of Crawford et al. (1972), with the exceptions of the integral for the region of 2.3 to 1.7 δ and chemical shift for the doubly homoallylic protons at C-6′. Our spectrum for synthetic β-bisabolene accounted for all of the significant lines present in the [^{1}H]NMR spectrum of the major hydrocarbon from *A. suspensa* volatiles, excluding resonances due to solvent and other small impurities in the spectrum of the material from the flies.

The synthetic sample of β-bisabolene eluted at the same time as the natural material on three different capillary columns: GC-1, GC-2 and GC-MS-1. A mass spectrum of this synthetic sample is shown in Figure 3A. It compares favorably with the data given by Crawford et al. (1972) for β-bisabolene, as well as with the reference spectra in the compilations by Stenhagen et al. (1974,

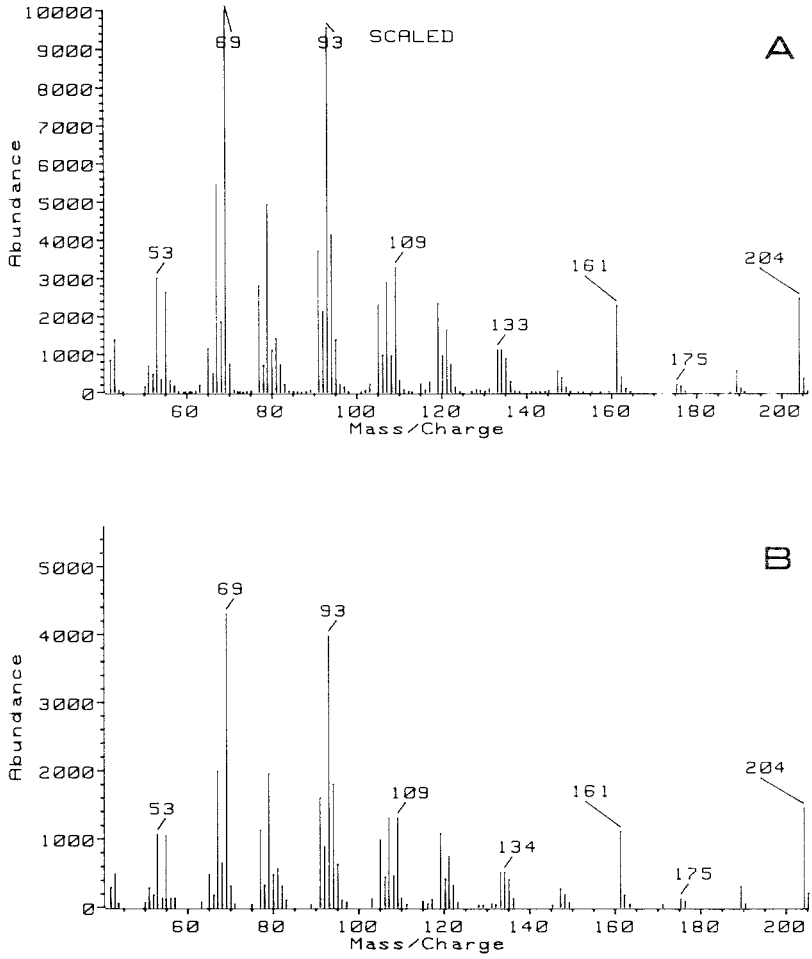


FIG. 3. Mass spectra (GC-EI-MS) of β-bisabolene. The ion at $m/z = 41$ (not shown) is the base peak in each spectrum with an approximate abundance of 1.4 times that of the $m/z = 93$ ion. (A) Top is from a synthetic sample of (±)-β-bisabolene prepared according to Scheme 2. (B) Bottom is for the major hydrocarbon peak (5) in Figure 1A, identified as (*R*)-(+)-β-bisabolene, from *Anastrepha suspensa* males.

entry A1-503-1) and by Adams (1989, entry DB5-1667). Figure 3B shows the mass spectrum for the major hydrocarbon (peak 5 in Figure 1A) in the volatiles of *A. suspensa*, which is essentially identical with that of the synthetic β-bisabolene. The small peak labeled 5, seen as a shoulder on peak 4 in Figure 1B of the *A. ludens* volatiles, was tentatively identified as β-bisabolene on the basis of its retention time and its relatively low abundance mass spectrum. Thus,

we conclude that β-bisabolene **(5)** is the major hydrocarbon component from the male *A. suspensa* volatiles, while it is probably present as a very minor component in the volatiles from *A. ludens* males.

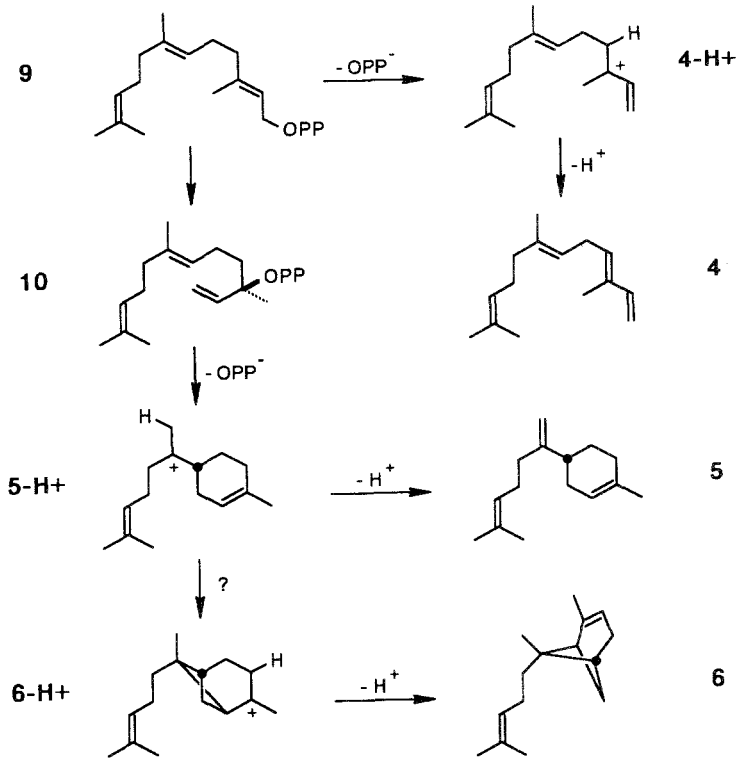
The natural material from *A. suspensa* was optically active and shown to be dextrorotatory by polarimetry at 23°C in hexanes (concentration = 1.29 mg/6 ml = 0.215 mg/ml, path length = 1 dm). The average measured rotation angle from four trials (± SD) with this solution was $\alpha_D = +0.0218° \pm 0.0023°$, and the specific rotation was then calculated as follows:

$$[\alpha]_D^{23} = \frac{(+0.0218° \pm 0.0023°) \times (1000 \text{ mg/g})}{(0.215 \text{ mg/ml}) \times (1 \text{ dm})} = +101° \pm 11°$$

The calculation above only accounts for variations in the actual measurement of α; allowing for a reasonable error in concentration of ±10%, a correct range for the true value of our specific rotation might easily be as much as ±20°. The (*R*)-(+)- and (*S*)-(−)-limonenes were synthetically correlated with the (*R*)-(+)- and (*S*)-(−)-β-bisabolenes by Crawford et al. (1972). The specific rotation they found for (*R*)-β-bisabolene, $[\alpha]_D^{30} = +74°$, is one of the highest that has been reported for this enantiomer at the sodium D line. Those same authors measured a specific rotation of slightly lower magnitude, $[\alpha]_D^{30} = -68°$, for the opposite enantiomer, although they did cite the value of Soucek et al. (1961), $[\alpha]_D^{20} = -84.4°$. That value, the highest-magnitude specific rotation recorded for either antipode, has also been cited by Buckingham (1982, entry B-01388) for the apparently more common (−)-enantiomer, while showing its absolute configuration as (*S*)-β-bisabolene. Given the small amount of material available from the fruit fly volatiles, the magnitude of our value agrees qualitatively with those previously reported rotations, and its sign indicates that the major sesquiterpene from the *A. suspensa* males is shown in Scheme 3 with the correct absolute configuration as (*R*)-(+)-β-bisabolene.

α-Farnesene. On the basis of approximate GC retention times, we also suspected that β-bisabolene might be emitted by *A. ludens*. Surprisingly, we found that these species differ with respect to the presence of two major sesquiterpenes in their volatiles. The mass spectrum for the major hydrocarbon ($C_{15}H_{24}$, M = 204) in the male *A. ludens* volatiles (peak 4 in Figure 1B) is shown in Figure 4. It is very similar to the spectrum for α-farnesene[5] given by Stenhagen et al. (1974) in the *Registry of Mass Spectral Data*. Furthermore, the mass spectrum and relative retention time of the natural substance from *A. ludens* are also quite similar to those given for a compound, DB5-1657, identified as (*E*,*E*)-α-farnesene (CAS #502-61-4) in the GC-MS compilation by Adams (1989). The stereochemistry for this common isomer of α-farnesene is

[5]Note that more than one farnesene isomer is given the same entry number, B22-199-2, in this edition of the *Registry of Mass Spectral Data.*



SCHEME. 3. A possible biosynthetic route from farnesol pyrophosphate **(9)** or nerolidyl pyrophosphate **(10)**, shown formally via the cations **4+**, **5+** and **6+**, to (*E*,*E*)-α-farnesene **(4)**, β-bisabolene **(5)**, and α-*trans*-bergamotene **(6)** in *Anastrepha suspensa* and *Anastrepha ludens* males.

shown in Scheme 1 as (3*E*,6*E*)-**4**; however, it would be difficult to distinguish this structure from its isomers on the basis of its mass spectrum alone. Baker et al. (1985) had demonstrated that (*E*,*E*)-α-farnesene is a major pheromonal component in the volatiles of male Mediterranean fruit flies, *Ceratitis capitata*, and we fortunately had access to an extract, collected by one of us (J.L.N.) in Europe, of *C. capitata* males. Thus, a comparison of the peak labeled 4 from the *A. ludens* volatiles within the (*E*,*E*)-α-farnesene in the *C. capitata* extract showed them to have identical retention times and mass spectra on GC-MS-2; coinjection of the two samples firmly established this assignment. The mass spectrum of a minor hydrocarbon (peak 4 in Figure 1A) in the male *A. suspensa* volatiles was virtually identical to that of the peak with the corresponding retention time from *A. ludens*. We therefore conclude that (3*E*,6*E*)-α-farnesene **(4)**

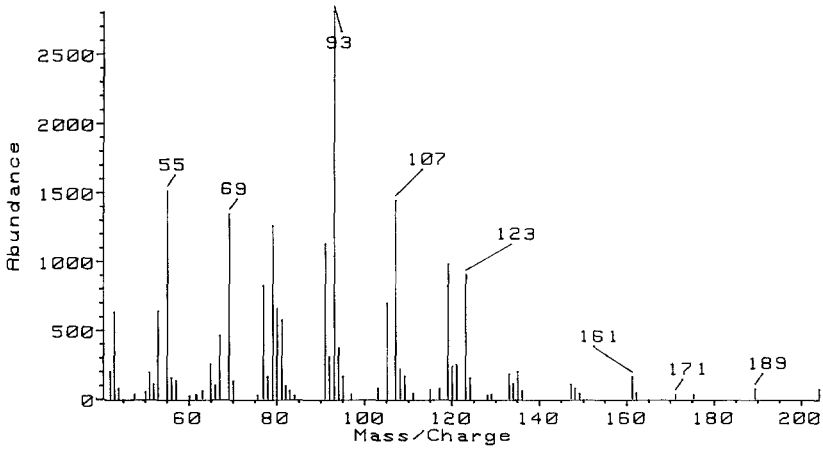


FIG. 4. Mass spectrum (EI) for the major hydrocarbon peak (4) in Figure 1B, identified as (*E*,*E*)-α-farnesene, from *Anastrepha ludens* males. The ion at *m*/*z* = 41 (not shown), with an abundance of 2796, has about the same intensity as the ion at *m*/*z* = 93 (abundance = 2811).

is a major component in the volatiles of *A. ludens* males and is probably a minor component in the *A. suspensa* volatiles.

α-trans-Bergamotene. The chromatograms of the volatiles from both *Anastrepha* species showed several small peaks representing previously unidentified compounds. The mass spectrum of a hydrocarbon ($C_{15}H_{24}$, M = 204) of moderate abundance in the male *A. ludens* volatiles (peak 6 in Figure 1B) is shown in Figure 5. This spectrum gave a 96% match with the that of the α-bergamotene in the National Bureau of Standards Mass Spectral Database (Library Index #16057, CAS #17699-05-7). Visual comparison of this spectrum with the one given by Stenhagen et al. (1974) for α-bergamotene, A1-501-1 in the *Registry of Mass Spectral Data*, provides additional support for the assignment of this basic structure (cf. Snider and Beal, 1988) to the peak labeled 6. This does not unambiguously define the substitution pattern at C-6 to be either *trans*, as in structure **6** (Buckingham, 1982, entry B-00924), or *cis* to the three-carbon bridge (Newman, 1972). However, based on matching the mass spectrum and relative retention time of peak 6 from GC-MS-1 with those of the corresponding isomer reported by Adams (1989; entry DB5-1485, CAS #13474-59-4), it can be assigned as α-*trans*-bergamotene. A comparison of these GC data is presented in Table 1. While our data were acquired with a 30-m HP-1 (Hewlett-Packard) column from 50° to 180°C at 4°/min, and Adams used a 30-m DB-5 column (J & W) from 60° to 240°C at 3°/min, the relative retention times of the hydrocarbons agree well enough to discount the *cis* isomer. Nevertheless, this

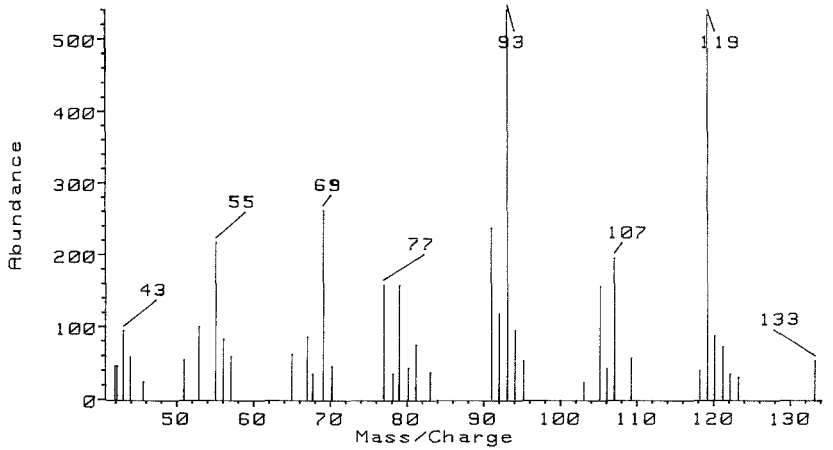


FIG. 5. Mass spectrum (EI) for the minor hydrocarbon peak (6) in Figure 1B, identified as α-*trans*-bergamotene, from *Anastrepha ludens* males. The ion at m/z = 41 (not shown), with an abundance of 512, is slightly smaller than the ion at m/z = 93 (abundance = 540).

TABLE 1. GC COMPARISON OF RELATIVE RETENTION TIMES (RT) OF SESQUITERPENE HYDROCARBONS (M = 204) FROM VOLATILES OF MALE CARIBBEAN FRUIT FLIES (*Anastrepha suspensa*) WITH STANDARDS FROM TEXT BY ADAMS (1989)

		Adams data		*A. suspensa*	
Peak label in Figure 1	Substance name	RT (sec)	RF[a]	RT (sec)	RF[a]
t[b]	*n*-tetradecane	1398	0.00	1367	0.00
	α-*cis*-bergamotene	1434	0.13		
6	α-*trans*-bergamotene	1485	0.32	1424	0.30
4	(*E*,*E*)-α-farnesene	1657	0.96	1552	0.97
5	β-bisabolene	1667	1.00	1558	1.00

[a] Retention factor = (RT of x − RT of t)/(RT of 5 − RT of t).
[b] Synthetic internal standard.

led us to obtain an authentic sample of α-*trans*-bergamotene in order to produce further evidence for this structure. That sample did indeed elute at the same time as the natural material from *A. ludens* on GC-MS-2, and our assignment was secured. A small analogous hydrocarbon peak, labeled 6 in Figure 1A, also occurred in the male *A. suspensa* volatiles, but the quality of its mass spectrum was poor because of low abundances. However, we believe that this peak can

also be assigned to *α-trans*-bergamotene, based on the match in retention time with the corresponding peak in the *A. ludens* volatiles. Thus, we identify *α-trans*-bergamotene as a minor component in the volatiles of *A. ludens* males and suggest that it may be present as an even smaller component in the volatiles from *A. suspensa*. Its presence with the other sesquiterpenes would be consistent, as described later, with the biosynthesis presented in Scheme 3.

Monoterpenes in Volatiles of Each Species

β-Ocimene. A minor hydrocarbon ($C_{10}H_{16}$, M = 136) in the volatiles from *A. suspensa* males has already been described as a monoterpene and a β-ocimene (cf. Nation, 1989, p. 196 and Nation, 1990, p. 554). Depending upon the source, the name ocimene has been used to describe various acyclic monoterpene trienes.[6] We presently refer to stereoisomers of the acyclic monoterpene shown in Scheme 1 as β-ocimenes, following the nomenclature of Dev (1986). We noticed that the mass spectrum of the monoterpene from *A. suspensa* was rather similar to the one accompanying a structure, which we would name as (*Z*)-β-ocimene, in Swigar and Silverstein's (1981) spectral compilation from Aldrich Chemical Co. A commercial sample described as β-ocimene, and characterized as a mixture of approximately 30% *E* and 70% *Z* isomers, was compared to the natural material from the flies. The (*E*)- and (*Z*)-β-ocimenes could be resolved on all the chromatograph columns used in this study, with the *E* isomer always eluting before the *Z* isomer. Using the conditions described in Methods and Materials, the authentic (*Z*)-β-ocimene eluted from GC-MS-2 at 484 sec, and the corresponding peak in the *A. suspensa* volatiles eluted at 487 sec (GC and MS data not shown, but available upon request). A coinjection of the fruit fly volatiles with the authentic sample confirmed that the natural substance eluted with the *Z* isomer. The mass spectrum of the fruit fly substance was significantly different from that of the (*E*)-β-ocimene, which eluted at 474 sec, but it matched the spectrum of the *Z* isomer fairly well. Thus, we conclude that *A. suspensa* males release (*Z*)-β-ocimene.

Limonene. The solution we received of volatiles from *A. ludens* males also contained a hydrocarbon ($C_{10}H_{16}$, M = 136) peak that eluted from the GC columns used in this study somewhat earlier than the monoterpene of the other species. This substance was identified as limonene, the cyclic monoterpene shown in Scheme 1, by comparison on GC-MS-2 with a genuine sample (GC and MS data not shown). The retention time, 473 sec using the conditions for

[6] For example, in the *Handbook of Chemistry and Physics, 55th Edition* (Weast, 1974) the name "ocimene" was given to a molecule with a terminal isopropenyl group, 3,7-dimethyl-1,3,7-octatriene. Swigar and Silverstein (1981), however, showed the structure (*Z*)-3,7-dimethyl-1,3,6-octatriene, which bears a terminal isopropylidene group, as "*cis*-ocimene." According to Dev (1986) the triene with an isopropenyl terminus is an α-ocimene, and the triene bearing the isopropylidene terminus is a β-ocimene.

the ocimenes described above, and mass spectrum of that hydrocarbon in the volatiles from *A. ludens* were essentially identical to those of authentic limonene.

Blend of Components in Mexican Fruit Fly Volatiles

The quantities of some of the components in the sample of *A. ludens* volatiles were determined by methods published for analyses of the *A. suspensa* components (Nation, 1990). The data, which were acquired using GC-1 and are presented in Table 2, show that two of the substances identified in this paper, (*E*,*E*)-suspensolide and (*E*,*E*)-α-farnesene, represent a significant fraction of the material trapped from *A. ludens* males. Since a single collection of volatiles from 50 individuals is involved here, such data should obviously be taken as an initial indication of the identities and quantities of the various compounds released by *A. ludens* males and used primarily for comparisons to similarly acquired data already published on the blend on components from *A. suspensa*.

DISCUSSION

The results from our comparison of these fruit flies, in conjunction with the information that has previously been published concerning their sex pheromones, indicate that males of both *Anastrepha* species synthesize and release the same volatile compounds with the exception of the two monoterpenes. The common substances, in order of elution from the HP-1 column, are: (*Z*)-non-3-enol with (*Z*,*Z*)-nona-3,6-dienol, α-*trans*-bergamotene, (*E*,*E*)-suspensolide, (*E*,*E*)-α-farnesene, β-bisabolene, anastrephin, and epianastrephin. We cannot make comprehensive statements about proportions among the principal components at present, because *A. ludens* has not yet been extensively sampled. Furthermore, because the data in Table 2 represent a single sampling of the

TABLE 2. RECOVERED VOLATILES FROM MALE MEXICAN FRUIT FLIES (*Anastrepha ludens*), INCLUDING A WASH OF CONTAINER THAT HELD FLIES DURING COLLECTION

Substance name	Quantity (ng/male-hour)
Nonenol/nonadienol[a]	186.1
(*E*,*E*)-Suspensolide	213.5
α-Farnesene	154.5
β-Bisabolene	trace
Anastrephin	99.9
Epianastrephin	463.1

[a] (*Z*)-3-Nonenol + (*Z*,*Z*)-3,6-nonadienol. The two nine-carbon alcohols do not separate from each other on the nonpolar RSL-160 column used here.

volatile compounds from *A. ludens* males, we should note that age, circadian rhythm, and density of flies have strong influences upon the ratio of components in *A. suspensa* volatiles (Nation, 1990). Such factors apparently influence *A. ludens* as well (Robert R. Heath, personal communication, USDA, Gainesville, Florida), and more exhaustive analyses of its volatiles will clearly be needed. Nevertheless, assuming that substances released in these experiments might serve semiochemical functions, we should identify the overt similarities and differences that emerged in this first direct comparison of the volatiles from *A. ludens* and *A. suspensa* males. Some of the GC-MS results are thus qualitatively summarized in Table 3, which emphasizes the compounds with 14 or 15 major atoms identified as common to the two fruit fly species.

Among these six common substances from the males, the relative proportions of the three isoprenoid lactones seemed roughly similar in this comparison, as each species presented larger quantities of both suspensolide and epianastrephin, along with a smaller amount of anastrephin. However, the two species differed quite markedly with regard to the hydrocarbon blend released, most notably in the relative proportions of the bisabolene and the farnesene. The volatiles of *A. suspensa* contained β-bisabolene, a cyclic sesquiterpene, as the major hydrocarbon component, a minor amount of (*E*,*E*)-α-farnesene, and a trace of α-*trans*-bergamotene. These proportions were redistributed in the *A. ludens* volatiles, which contained the acyclic (*E*,*E*)-α-farnesene as the major sesquiterpene, a minor amount of α-*trans*-bergamotene, and an even smaller quantity of β-bisabolene. The males of the two *Anastrepha* species also differed

TABLE 3. QUALITATIVE SUMMARY FOR THIS GC-MS COMPARISON OF VOLATILES (M ≈ 200) FROM MALE CARIBBEAN (*Anastrepha suspensa*) AND MEXICAN (*Anastrepha ludens*) FRUIT FLIES

Molecular formula and substance name	*Anastrepha suspensa*	*Anastrepha ludens*	Method of comparison
$C_{12}H_{18}O_2$ (M = 194)			
Anastrephin	++[a]	++	synthetic MS[b]
Epianastrephin	+++	+++	synthetic MS[b]
(*E*,*E*)-Suspensolide	+++	+++	synthetic MS[c]
$C_{15}H_{24}$ (M = 204)			
(*E*,*E*)-α-Farnesene	××	×××	reference MS[d]
β-Bisabolene	×××	×	syn./ref. MS[d]
α-*trans*-Bergamotene	×	××	reference MS[d]

[a] +, × = trace; ++, ×× = minor; +++, ××× = major.
[b] Battiste et al. (1983), Stokes et al. (1983).
[c] Chuman et al. (1988), Battiste et al. (1988).
[d] Adams (1989), Stenhagen et al. (1974).

with respect to the monoterpenes present in their volatiles; *A. suspensa* evidently released (*Z*)-β-ocimene, an acyclic compound, while *A. ludens* apparently emitted a cyclic isomer, limonene.

Possible Evolutionary Significance of These Volatiles

The similarity of the volatiles produced by these two flies, their similar behavioral biology during pheromone release, and the similarity in female and male response to the pheromones (Robacker and Hart, 1985a,b, 1986; Nation, 1972, 1989) suggest an interesting and close evolutionary relationship. The two species do not overlap in their distributions anywhere today, and how they might interact with each other in such a situation can only be conjectured. The *A. ludens* female is physically larger and also has a longer ovipositor sheath than the *A. suspensa* female. Thus, it is questionable whether the two species could actually intermate or whether they would even attempt to do so in the wild, but the differences in their terpenes may be related to the reproductive isolation of these flies. It seems probable that the two species shared a common ancestor that produced the C_9 alcohols with the C_{12} lactones and possible that it also produced at least one each of the monoterpenes and sesquiterpenes. We can only guess that the hydrocarbons may have functioned as semiochemicals for the ancestor, because it remains to be shown how the terpenes revealed in this comparison, or even the macrolide, serve these modern *Anastrepha* species. However, if the two monoterpenes and the different sesquiterpene proportions from *A. suspensa* and *A. ludens* males do prove behaviorally significant, this would allow the possibility that elaboration of the hydrocarbon blends occurred while the present species diverged.

We shall consider the relevance here of two mechanisms of speciation with distinct origins: (1) environmental disruption of a widely distributed ancestral species into isolated, divergent populations; or (2) reproductive isolation within an ancestral species by virtue of divergent host preferences in different population segments. It is unlikely that one of these processes occurs exclusively, because one can imagine scenarios that incorporate elements of each mechanism.

As mentioned before, the ranges of these species do not now overlap; *A. suspensa* is found principally in Florida as well as in islands of the northeastern Caribbean Sea, and *A. ludens* is distributed principally along the western end of the Gulf of Mexico. The first mechanism might therefore seem more obvious in this case, and it is also one that appears to be accepted more generally as a process of speciation.[7] Hence, because their ranges are now roughly divided by the Gulf of Mexico, it is possible that a once-continuous distribution of these species' ancestor became fragmented due to environmental change, and the

[7]This means of species divergence seems more likely to an anonymous reviewer of this paper. We acknowledge the reviewer's version of the description given here.

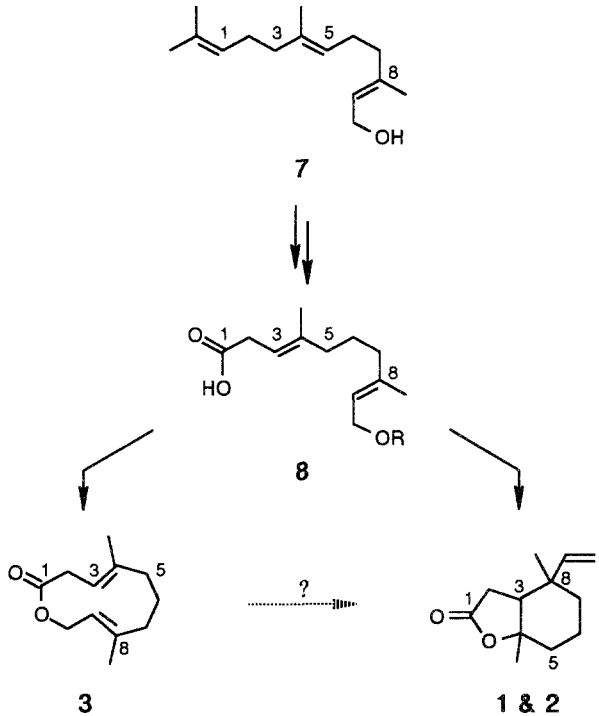
resulting isolated populations diverged. Differences in emitted hydrocarbons may have resulted from various external factors, including later contact with other divergent populations or even other *Anastrepha* species.

On the other hand, the two present species evidently also differ with respect to preferred ovipositional hosts, and these fruit preferences are the basis for invoking the second, albeit probably less well accepted, mechanism of species divergence. The immature fruit of chapote, *Sargentia greggii*, and the mature fruit of mango, *Mangifera indica*, have been described (e.g., Robacker and Garcia, 1990) as larval hosts for *A. ludens*, while guava, *Psidium guajava*, is a favored oviposition fruit for *A. suspensa*. Thus, a host race mechanism, such as discussed by Bush (1974), may have led to the initial reproductive isolation of the two (sub)species and consequently allowed time for pheromonal changes, physical size, and subtle differences in acoustical and visual displays to evolve further as reproductively isolating influences.

The authors recognize that our observations alone do not discriminate between these two mechanisms of speciation, but given the interest in evolutionary origins of natural products within the chemical ecology community, we wished to raise them as arguments in the context of this discussion. Scenarios that combine elements of both processes raise the question of which came first: was the environmentally driven segregation followed by the development of different host preferences, or was the earlier reproductive segregation of host races subsequently amplified by environmental changes? Evidence to support or reject the different mechanisms may ultimately lie in diverse aspects of the biochemistry, cytogenetics, and physiology of the insects or their host fruit plants. Perhaps even geological or paleontological data from the perimeters of the Caribbean Basin and the Gulf of Mexico could be pertinent. Additional information or experiments might reconcile our observations with one of these evolutionary hypotheses or might provide another explanation that accommodates these results.

Biosynthetic Implications for These Volatiles

A reasonable biosynthetic route from farnesol to the terpenoid lactones **1**, **2**, and **3** is presented in Scheme 4, with the numbering of all structures keyed to the central structure **8**. Saito and coworkers (1984) have shown that this intermediate (3*E*,8*E*)-10-hydroxy-4,8-dimethyldeca-3,8-dienoic acid (**8**, R = H) undergoes electrophilic cyclization to the anastrephins, **1** and **2**, while Battiste and others (1988) have carried out the macrolactonization of **8** to suspensolide, **3**. Farnesol could be converted enzymatically to this hydroxy acid in two steps: (1) oxidative loss of the terminal isopropylidene fragment, providing the carboxy carbon 1; and (2) isomerization of the double bond between carbons 4 and 5 into a new double bond between carbons 3 and 4. Derivatization of the terminal



SCHEME. 4. A possible biosynthetic route from farnesol (**7**) to suspensolide (**3**) and the anastrephins (**1** and **2**) in *Anastrepha suspensa* and *Anastrepha ludens* males via (3*E*,8*E*)-10-hydroxy-4,8-dimethyldeca-3,8-dienoic acid (**8**, R = H). All structures are numbered according to structure **8** for continuity.

hydroxy residue into a suitable leaving group (i.e., OH → OR) would allow cyclization, presumably via at least two enzymes and possibly in different glandular tissues (Nation, 1989), to either the γ-lactones or the macrolide. Feeding experiments with appropriate ^{2}H or ^{13}C precursors, such as mevalonolactones, might provide enough of these materials, isolated from trapped volatiles, to determine by NMR if this biosynthetic scheme is consistent with the fate of the labels. Finally, we also point out the possibility of some in vitro conversion of suspensolide to the anastrephins, indicated by the light arrow. In principle, this could occur via an open-chain structure like the hydroxy acid, but it raises the interesting question of additional isomeric lactone structures, which remain undetected as highly labile or trace materials.

Scheme 3 shows a likely biosynthetic route (cf. Newman, 1972) from farnesyl pyrophosphate to the sesquiterpene hydrocarbons **4, 5,** and **6**. The loss of pyrophosphate from **9** could give the 3°, allylic, farnesyl cation (**4+**), which,

after elimination of a proton from C-4, would give (3*E*,6*E*)-α-farnesene **(4)**. Although the α-farnesene from *A. ludens* males was identified as the *E*,*E* isomer on the basis of GC-MS, the possible presence of minor amounts of the isomeric (3*Z*,6*E*)-α-farnesene, formed via proton elimination from a different conformation of **4+**, should also be considered. Cane et al. (1989) have recently implicated the stereoselective enzymatic rearrangement of farnesyl pyrophosphate to nerolidyl pyrophosphate in the synthetic course to β-*trans*-bergamotene and structurally related antibiotics from fungi. We suppose a similar rearrangement occurs in these fruit flies of **9** to nerolidyl pyrophosphate, **10**, based upon the isolation of optically active β-bisabolene from *A. suspensa*; we can only presume that the minor substance from *A. ludens* has the same configuration. A facile cyclization of **10** following loss of pyrophosphate (cf. Cane et al., 1989, 1990) results in the bisabolyl cation **(5+)**, and elimination of the proton at C-1 from this cation gives β-bisabolene **(5)**. Note that obtaining (*R*)-(+)-β-bisabolene from the fruit fly by this cyclization requires (3*R*)-nerolidyl pyrophosphate as shown, and this would be opposite to the absolute configuration required by the experimental results of Cane et al. using the fungal enzyme. Further cyclization from a favorable conformation of the bisabolyl cation gives the *trans*-bergamotyl cation **(6+)**, which could eliminate a ring proton from C-3 giving α-*trans*-bergamotene **(6)**. If the bergamotene from these flies actually has its origin in the same cation as the bisabolene, i.e. **5+** with the chiral carbon's hydrogen shown beta as required for the *A. suspensa* substance, it should be possible in principle to correlate the absolute stereochemistry of the two hydrocarbons (cf. Cane et al., 1990).

The information in Table 3 suggests that these molecules may be significant taxonomically. From this point of view it would be interesting to compare the volatiles from these two flies with those from other *Anastrepha* species and other genera of tephritid fruit flies using GC-MS. It will require many more experiments to give a detailed understanding of the functions and glandular sources of these chemicals, especially with regard to learning how the hydrocarbons serve the flies. Still, one point clearly emerges from the results of this study and the structures in Schemes 3 and 4—farnesol must be a very important chemical precursor for the Caribbean and Mexican fruit flies. The males of these two species appear to exploit the basic farnesol structure for the production of at least six volatile substances.

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FIELD TRAPPING OF *Migdolus fryanus* WESTWOOD (COLEOPTERA: CERAMBYCIDAE) USING NATURAL SEX PHEROMONE

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Abstract—*Migdolus fryanus* is a sugarcane pest restricted to South America that is becoming more important due to the inefficacy of control methods against it. The larvae bore into the plant root system and the available insecticides can not give adequate protection. So, the search for alternate control strategies is mandatory. However, the literature on bioethological studies of the species is scarce. This work investigates the attraction of adult males in the field by females that is mediated by a sex pheromone that remains to be identified. Dissected female thoraces were more attractive than other body parts. A trap design for monitoring field populations of this species also is presented.

Key Words—Natural attractant, pheromone, *Migdolus fryanus*, Coleoptera, Cerambycidade, male response, sugarcane pest.

INTRODUCTION

Every year *Migdolus fryanus* is becoming more important as a pest of sugarcane, coffee, and eucalyptus in Brazil. The lack of more detailed knowledge on the biology and behavior of this pest as well as of adequate methodology to evaluate its damage in infested areas has contributed to the poor efficacy of control methods.

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These insects were first reported in sugarcane in 1927 (Lane, 1937) in Ibaté County, São Paulo State, Brazil. Taxonomic studies on this genus were carried out by Lane (1972) and Dias (1984). These insects have been associated with "cerrado" areas used for crops such as coffee, eucalyptus, and lately sugarcane (Fonseca, 1958; Roccia, 1977). They also have been reported from mullberry, grape, beans, and pasture (Arrigoni et al., 1986).

In sugarcane the larvae feed on the root system and, even though the attack may be localized, destruction of the infested sugarcane field may occur (Terán et al., 1983, 1984).

Little has been done on the biological aspects of *Migdolus fryanus*, which is one of the most common pest species in the genus (Nakano and Joko, 1969; Roccia, 1977; Terán et al., 1983, 1984).

Adult males are alate and live, on average, two to three days; adult females are wingless and live for 30 days or more (Roccia, 1977). From November to March in São Paulo, the newly emerged individuals mate after a characteristic male nuptial flight.

The orientation of the males towards the females is thought to be mediated by a female sex pheromone (Roccia, 1977; Terán, 1984; Pizano, 1991), since traps containing virgin females have caught up to 192 males (Roccia, 1977); however conclusive proof has been lacking.

The objectives of the present paper were to determine if the female produces a sex pheromone as well as the body part responsible for it. It was also our aim to develop a more efficient trap to capture the males and to determine the ideal number of females per trap.

METHODS AND MATERIALS

The experiment was set up in Olímpia County, São Paulo State (latitude 20° 46′ S; longitude 49° 40′ W) during February and March 1991 in sugarcane plantations.

The total area planted was 21,000 ha. Field bioassays were divided in two steps. The first had eight treatments and 12 replicates: (A) virgin female (trap design—OAL); (B) virgin female (trap design—UFV); (C) virgin females and virgin male (trap design—UFV); (D) virgin male (trap design—UFV); (E) virgin female head (trap design—UFV); (F) virgin female thorax (trap design—UFV); (G) virgin female abdomen (trap design—UFV); and (H) control (unbaited).

The experiments were run for three consecutive days (four replicates per day). The traps used were plastic water containers, 20-liter volume, with sections of the large sides cut out, leaving two rectangular windows (Figure 1). The OAL design had an acrylic box (60 mm diameter × 20 mm thick) with small holes centered in the top and the bottom and was hung by wire in the

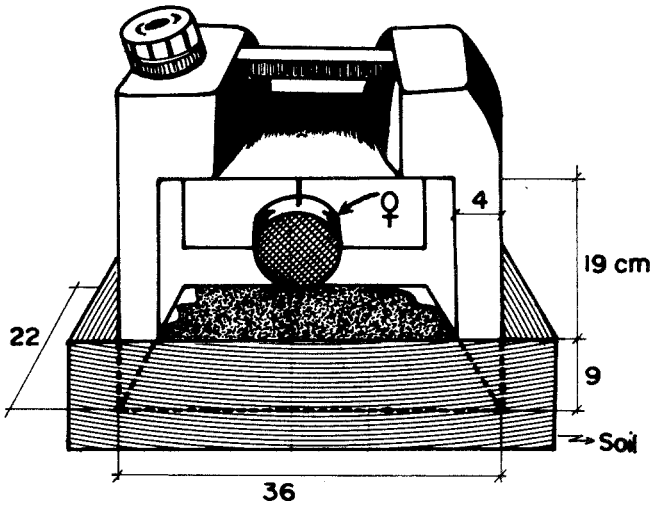

FIG. 1. Trap design used to capture males of *Migdolus fryanus* (Coleoptera: Cerambycidae) in sugarcane fields.

center of the container. One virgin female was placed inside the box together with a fine layer of soil. The UFV trap design was modified from the OAL by replacing the acrylic box with a PVC tube (85 mm diameter × 40 mm thick), which had the ends covered with a nylon screen. All traps were placed beside the sugarcane rows and 0.50 m inside these rows at the soil level with a distance of 22 m between.

The male buzzes the ground until he senses the females' presence. Then he lands and locates her with antennal movements. Finally he mounts her (Roccia, 1977; Arrigoni, 1986).

All traps without the attractants were placed the day before the data were to be collected. The attractant was then placed in the traps slightly before 7 AM, the time at which the nuptial flights normally started. The males were captured in water containing 5% detergent, which was placed in the base of the trap. The females had been collected and placed in individual containers the day before while the males were collected on the day of the experiment. Pinned specimens of both male and female *M. fryanus* are deposited in the Cerambycidae Collection of the Zoology Museum of the University of São Paulo, Brazil.

The experimental design was completely randomized and the average temperature and relative humidity on the three days were: 26.8°C, 25.7°C, 24.4°C, and 20.4%, 18.9%, 18.7%, respectively.

The second step of the field bioassay consisted of aerating 30 virgin females through 1 g Porapak Q for 24 hr at a flow rate of 9 liters/min, followed by

washing of the material in 1 ml dichloromethane. This solution was used to prepare six rubber septa by placing 40 μl of the washed material, which corresponded to 1.2 female equivalents, on each septum. These were immediately stored in a freezer for three days until taken to the field.

Six treatments and six replicates were carried out in the same sugarcane plantation following the methodology already described. The treatments were: (A) one virgin female; (B) two virgin females; (C) four virgin females; (D) rubber septum impregnated with material extracted from virgin female + dichloromethane; (E) rubber septum + dichloromethane; and (F) control (unbaited).

The trap design was UFV in all treatments. The captured males were counted after 24, 48, and 72 hr, and there was no substitution of virgin females or septa during this period. Average temperature and relative humidity on the three days were: 24.8°C, 25.2°C, 26.3°C, and 20.6%, 20.3%, 21.3%, respectively.

RESULTS AND DISCUSSION

Live virgin females of *Migdolus fryanus* strongly attracted males (Table 1). Far fewer males were attracted to the other treatments. The UFV trap design caught more male *M. fryanus* than the OAL. The soil layer in the acrylic box with the female, as in the OAL trap, seemed to interfere with female calling, as shown by the reduced number of males captured by that trap.

The presence of a male with the virgin female, even though they mated,

TABLE 1. MALES OF *Migdolus fryanus* (COLEOPTERA: CERAMBYCIDAE) CAPTURED BY TRAPS BAITED WITH EIGHT ATTRACTANTS DURING A 24-HR PERIOD (12 REPLICATES)

Attractants	Males Captured	
	Total	Average
A. Virgin female[a]	851	70.9 c[b]
B. Virgin female	2047	170.5 a
C. Virgin female + male	1418	118.2 b
D. Virgin male	84	7.0 f
E. Virgin female head	263	21.9 e
F. Virgin female thorax	636	53.0 d
G. Virgin female abdomen	343	28.5 e
H. Control	110	9.1 f

[a]This trap design was OAL. The other treatments used the UFV trap type.
[b]Means followed by the same letter are not different $P \leq 0.05$, by Wilcoxon's rank-sum test.

TABLE 2. MALES OF *Migdolus fryanus* (COLEOPTERA: CERAMBYCIDAE) CAPTURED AT 24, 48, AND 72 HR AFTER SETTING DIFFERENT TRAPS (6 REPLICATES)

Treatments	After 24 hr		After 48 hr		After 72 hr	
	Total	Average	Total	Average	Total	Average
A. 1 Virgin female	1193	198.8 c[a]	590	98.3 b	258	43.0 c
B. 2 Virgin females	1495	249.2 b	520	86.7 b	314	52.3 b
C. 4 Virgin females	1876	312.7 a	588	98.0 b	283	47.2 bc
D. Septum + extr + dichlor	727	121.2 d	660	110.0 a	479	79.8 a
E. Septum + dichlor	76	12.7 e	94	15.7 c	81	13.5 d
F. Control	55	9.2 e	79	13.2 c	66	11.0 d

[a]Means followed by the same letter are not different $P < 0.05$, by Wilcoxon's rank-sum test.

slightly reduced the capture of males, although this bait still proved to be superior to others. One female mates with more than one male before she returns to the soil to oviposit (Roccia, 1977; Terán et al., 1984), thus she remains attractive to the males after her first mating.

Treatments using dissected parts of a female were significantly different among themselves. The female thorax was more efficient than the other body parts in attracting males (Table 1). It has been shown that in the field, the receptive female remains at soil level with only the head and thorax exposed (Roccia, 1977); therefore it appears that the mating signal, although general over the body, is more pronounced on the thorax.

The results obtained with the rubber septa impregnated with female odor in dichloromethane (Table 2) strongly indicate the presence of a sex pheromone. Although this had been suggested previously (Roccia, 1977; Terán et al., 1984; Pizano, 1991), this is the first proof of the chemical origin of the attractiveness of female *M. fryanus*. The impregnated rubber septum continued to attract males after 24 hr, at levels greater than one, two, or four virgin females. Males also were observed trying to copulate with the rubber septa in the field.

Twenty-four hours after the virgin females were exposed in the field they died, probably due to the hot and sunny weather. However, they were still attractive for at least 72 hr after the beginning of the tests, although the number of males falling in the traps was much reduced. This attraction observed in the dead females suggests various possibilities. The pheromone could simply be retained on the insect bodies or substrate, or the sex pheromone could be released from a gland in one region and bathe the body with material. Finally, it could also suggest an association with cuticular hydrocarbons.

Based on the results, the use of two females per trap is recommended when the distance between traps is 22 m because it is more practical than four and safer than one female. Daily female replacement in the traps is also suggested.

The attraction potential of the female sex pheromone is so great that we believe monitoring could be a valuable tool, especially to determine population levels in the infested areas. However, pheromone identification and synthesis still have to be carried out.

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FATE OF [^{14}C]XANTHOTOXIN (8-METHOXYPSORALEN) IN LAYING HENS AND A LACTATING GOAT

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Abstract—The metabolism of xanthotoxin, a naturally occurring furanocoumarin photosensitizer, was studied in laying hens and a lactating goat treated with single oral doses equivalent to 10 mg xanthotoxin/kg of body weight. Within 48 h, essentially all of the administered radiocarbon was eliminated in the excreta of the laying hens, while in the goat 92% and 3% were excreted in the urine and feces, respectively. Radiocarbon residues in the milk, egg white, and egg yolk were low. Xanthotoxin, 8-hydroxypsoralen (xanthotoxol), 6-(7-hydroxy-8-methoxycoumaryl)-acetic acid (HCA) and 6-(7-hydroxy-8-methoxycoumaryl)-hydroxyacetic acid (HCHA) were identified in the excreta of laying hens. In the goat, xanthotoxin was metabolized to HCA, HCHA, xanthotoxol, 5,8-dihydroxypsoralen, psoralenquinone, 5-hydroxy-8-methoxypsoralen and 3[5-(6-hydroxy-7-methoxybenzofuryl)]-propanoic acid. Thus, identified metabolites in one or both of these species arose through *O*-demethylation, oxidative cleavage of the furan ring, hydroxylation, reduction, oxidation, and hydrolysis of the lactone ring.

Key Words—Xanthotoxin, 8-methoxypsoralen, furanocoumarin, metabolism, detoxification, chicken, goat, egg, milk ^{14}C radiolabel.

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INTRODUCTION

Linear furanocoumarins (psoralens) are photoactive compounds that occur naturally in many species of plants, particularly in the families *Umbelliferae* and *Rutaceae* (Pathak et al., 1962; Scott et al., 1976; Towers, 1984). Some of these plants are consumed occasionally by livestock or pasture- or field-raised poultry, which can lead to outbreaks of photosensitization (Egyed et al., 1977; Dollahite et al., 1978; Ivie, 1982; Perelman et al., 1987).

Direct exposure of humans to furanocoumarins occurs by handling or ingesting furanocoumarin-containing food plants such as parsnip, parsley, celery, figs, limes, and some spices (Scott et al., 1976). These compounds are not destroyed by normal cooking procedures (Ivie et al., 1981). In addition, furanocoumarins are phytoalexins in some food plants, and certain plant stresses, particularly diseases, induce biosynthesis of these toxic products (Beier et al., 1983; Ashwood-Smith et al., 1985; Chaudhary et al., 1985; Surico et al., 1987). Human exposure to appreciable levels of furanocoumarins may thus result from the consumption of "spoiled" or otherwise diseased plant foodstuffs. Furanocoumarins and long-wavelength ultraviolet radiation (320–400 nm) are used in human medicine for the treatment of vitiligo, psoriasis, mycosis fungoides, and cutaneous T-cell lymphoma (Bleehen et al., 1978; Edelson et al., 1987; Parrish et al., 1974), but with some apparent increase in risk of skin cancer (Stern and Lange, 1988; Stern, 1990).

Studies on the disposition and metabolism of linear furanocoumarins have been reported in mammalian species (including man), in insects, and in fungi. Most of these studies have focused on one of the linear furanocoumarins (xanthotoxin) because it is well known for its photoactivity, it occurs commonly and often predominantly in furanocoumarin-containing plants, and it is the furanocoumarin of choice in many medical applications. Studies on the metabolism of xanthotoxin have shown that the furan ring is an important site for metabolic transformation in man (Schmid et al., 1980), in the dog (Kolis et al., 1979), in rats (Mays et al., 1986), and in insects (Ivie et al., 1983).

In view of the biological effects associated with furanocoumarins, there is a need to obtain definitive data on the interaction of these compounds with avian and mammalian species, both to evaluate their ecological significance to wildlife and to determine the possibility of potentially toxic residues entering the human food chain. Although episodes of photosensitization in domesticated avian species are rare, furanocoumarin-caused photosensitization of pasture-raised birds has been observed (Eilat et al., 1974; Egyed et al., 1977; Perelman et al., 1987). Acute photosensitization in whitetail deer has been reported (Dollahite et al., 1978), although a link with furanocoumarins has not been established (Rowe et al., 1987).

A limited study of xanthotoxin metabolism in the domestic goat has been

reported (Ivie et al., 1986) but, because the radiolabel was on the *O*-methyl moiety, which was easily lost upon metabolism of the parent compound, >50% of the radioactivity was not recovered or accounted for. In the present studies, the radiolabel was on the metabolically stable 4 position of the coumarin ring (Figure 1), and thus a more complete picture of the fate of xanthotoxin in the goat, as well as the chicken, was determined.

METHODS AND MATERIALS

Chemicals. Specifics of the procedures used in these studies are detailed in a thesis by the senior author (Pangilinan, 1990). Unlabeled authentic samples of furanocoumarins included xanthotoxin (8-methoxypsoralen), xanthotoxol (8-hydroxypsoralen), psoralenquinone (PQ), 5,8-dihydroxypsoralen (DHP), and 5,8-dimethoxypsoralen (DMP, isopimpinellin). [^{14}C]Xanthotoxin labeled at the 4 position of the coumarin ring (26.47 mCi/mM, New England Nuclear, Boston, Massachusetts) was determined by thin-layer chromatography (TLC) to have a radiochemical purity >98% (Pangilinan, 1990).

Chromatography. [^{14}C]Xanthotoxin and its metabolites were analyzed by TLC on silica gel F-254 chromatoplates (0.25 mm or 0.5 mm gel thickness, 20 × 20 cm, with fluorescent indicator, E. Merck, Darmstadt, Germany). The plates were developed in one or more of 19 solvent systems, depending upon the sample and specific compound of interest (Pangilinan, 1990). In addition to TLC techniques, high-performance liquid chromatography (HPLC) was used in the isolation of [^{14}C]xanthotoxin metabolites. Mobile phases consisted of four buffered water, methanol–acetonitrile, and methanol–water systems, with gradients used in some cases. Detection was by absorbance at 248 nm or, in many instances, by the collection of eluent fractions (0.5–1.0 ml), which were subsequently assayed by liquid scintillation counting (LSC) (Pangilinan, 1990).

In Vivo Metabolism of [^{14}C]Xanthotoxin in Laying Hens. White leghorn laying hens were purchased from a local grower and held in individual cages housed in an environmentally controlled room. Throughout the study, the birds were maintained on a commercial pelleted feed, with water available ad libitum. The birds were allowed to acclimate one week prior to treatment.

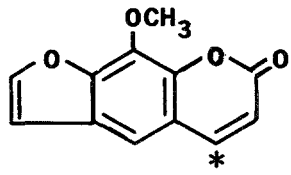


FIG. 1. Structure of xanthotoxin (8-methoxypsoralen), with the site of radiocarbon label incorporation indicated by the asterisk.

Two groups of birds were studied. The first group of four birds was kept for seven days after treatment and was used primarily to characterize residues in egg white, egg yolk, and tissue samples. The second group of eight birds was maintained for 24 hr posttreatment and was used primarily for the characterization of metabolites in the excreta. All birds were treated with single [^{14}C]xanthotoxin doses equivalent to 10 mg xanthotoxin/kg of body weight and a specific activity of 6000 dpm/μg for the first group of birds and 1500 dpm/μg for the second. The dose was dissolved in a small amount of acetone and added to pulverized feed in order to facilitate dispersion. The individual doses were sealed inside gelatin capsules (0.25 oz) and delivered orally to the birds. All the birds were in apparent good health and high (>90%) egg production.

Excreta from the first group of birds were collected at intervals until the birds were killed seven days posttreatment. Eggs were collected daily and egg yolk and white were separated. Selected tissue samples were quickly collected when the birds were killed for analysis. Excreta from the second group of birds were collected at intervals but analyzed as pooled 24-hr samples. Aliquots of excreta, egg yolk, egg white, and tissue were taken in triplicate, air dried, and pelleted. The remainder of each sample was stored in a glass jar and frozen at −60°C for future assay. The pelleted samples were analyzed for radiocarbon content by oxygen combustion; all values obtained were corrected for instrument efficiency and quench (Pangilinan, 1990).

Isolation and Identification of Chicken Excreta Metabolites. Because >90% of the administered dose was eliminated during the first 24 hr posttreatment, analytical efforts were limited to a composite of 24-hr excreta. Four-gram composites of chicken excreta were diluted with water, acidified, and extracted four times with ethyl acetate. The extracts were subsequently concentrated and analyzed by TLC and/or HPLC. In TLC studies, the ^{14}C-labeled components were visualized by exposing the developed plates to X-ray film, and specific radioactive components were quantitated by scraping the appropriate gel regions and subjecting them to LSC. For HPLC studies, the ethyl acetate extracts of chicken excreta were cleaned up prior to analysis using C_{18}Sep-Pak cartridges (recovery from the Sep-Pak was always 90–99%). Quantitative measurements were made by LSC of eluent fractions.

Sufficient quantities of xanthotoxin metabolites for characterization studies were obtained by ethyl acetate extraction of larger excreta samples. The ethyl acetate extracts were applied as bands to TLC plates, and the plates were developed in an appropriate solvent system. Major metabolite bands were scraped and eluted with methanol, ethyl acetate–glacial acetic acid (99:1), or ethyl acetate, depending upon metabolite polarity. Individual metabolites were purified further by HPLC. Metabolite identification was accomplished by TLC and HPLC cochromatographic techniques using authentic standards and in some

cases by mass spectrometry (MS) and nuclear magnetic resonance spectrometry (NMR) techniques (Pangilinan, 1990).

Isolation and Identification of Metabolites in Egg White and Yolk. Composite samples of egg white were lyophilized and then extracted with methanol. The extracts were then concentrated under vacuum and analyzed by TLC. Egg yolk composites were extracted with hexane–tetrahydrofuran (1 : 1), cleaned up by partitioning between hexane and acetonitrile, and the acetonitrile extracts were subsequently analyzed by TLC (Pangilinan, 1990).

In Vivo Metabolism of [^{14}C]Xanthotoxin in Lactating Goat. A lactating Nubian goat was purchased from a local source and allowed to acclimate for one week before treatment. The goat was provided an appropriate ration and water ad libitum and was hand milked twice daily, producing 0.7–1.2 liters of milk daily.

The administered dose consisted of [^{14}C]xanthotoxin diluted with unlabeled xanthotoxin to a specific activity of 500 dpm/μg. The dose was dissolved in acetone and adsorbed on a small amount of pulverized feed, which was administered orally to the animal via a gelatin capsule; the dosage was equivalent to 10 mg xanthotoxin/kg body weight. [Note: A second goat was identically treated but this animal performed poorly under confinement conditions and voided urine very sporadically. Data obtained from this animal are reported elsewhere (Pangilinan, 1990).]

Following treatment, total urine, milk, and excreta were collected at intervals until the animal was killed seven days posttreatment; selected tissue samples were collected at this time. Samples of urine, milk, feces, and tissue were analyzed for total radiocarbon content by LSC or oxygen combustion as appropriate. Other sample portions were held frozen for future analysis.

Isolation and Identification of Goat Urinary Metabolites. Aliquots of 24-hr goat urine were acidified and extracted three times with ethyl acetate. The extracts were cleaned up using C_{18} Sep-Pak cartridges and the purified samples were then analyzed by HPLC. Certain samples were, prior to extraction and analysis, subjected to acid hydrolysis or enzyme hydrolysis procedures to determine the presence and nature of metabolite conjugates. Certain goat urine metabolites were obtained in sufficient quantity through HPLC isolation techniques to allow their study by MS and NMR techniques (Pangilinan, 1990).

RESULTS

Excretion Patterns and Tissue Residues in Laying Hens. The elimination of radiocarbon in the excreta of hens treated orally with [^{14}C]xanthotoxin was rapid. Within 24 hr posttreatment, $>90\%$ of the dose had been eliminated in the excreta (Figure 2). Low levels of radiocarbon continued to be eliminated in

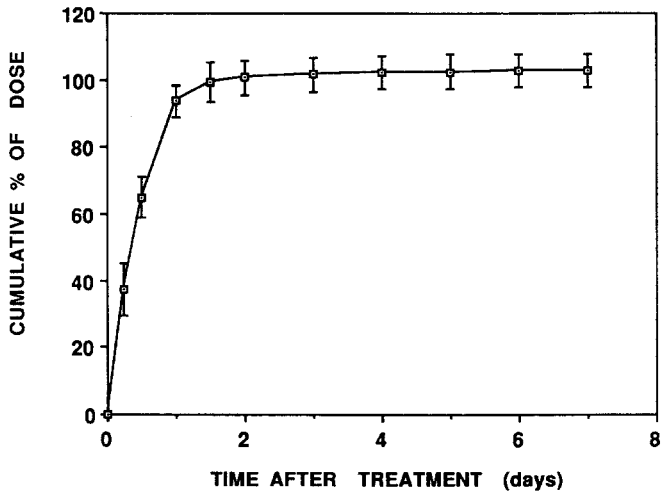


FIG. 2. Elimination of radiocarbon in excreta of laying hens treated with [^{14}C]xanthotoxin as a single oral dose equivalent to 10.0 mg xanthotoxin/kg body weight. Data points are mean ± standard deviation of four hens.

the excreta during the succeeding days until termination of the studies; however, radiocarbon elimination was almost total within just two days after dosing.

Very low levels of radiocarbon were eliminated in the egg yolk and white. Highest levels were observed two days after treatment for both egg fractions (0.8 ppm and 0.5 ppm xanthotoxin equivalent in yolk and white, respectively), and residues in the egg yolk were consistently higher than those detected in the egg white. The cumulative secretion of radiocarbon into the egg during the seven days posttreatment amounted to only about 0.5% of the total [^{14}C]xanthotoxin administered to the laying hens.

Of the different tissues collected from birds killed seven days posttreatment, only the blood, kidney, liver, and ovarian egg contained as much as 0.01% of the administered [^{14}C]xanthotoxin dose. Radiocarbon residues, expressed on a ppm basis, were highest in the liver, followed by kidney and lungs (Table 1).

Characterization of Metabolites in Excreta of Laying Hens. Ethyl acetate extraction of acidified excreta samples resulted in an average of 78% recovery of the radiocarbon present in the samples. Two-dimensional TLC analysis of the concentrated ethyl acetate extracts showed at least 13 distinct radioactive components, only six of which comprised 1% or more of the total radiocarbon in the sample. HPLC analysis of the ethyl acetate extracts similarly revealed six radioactive components that each comprised 1% or more of the radioactivity in the sample (Table 2).

Mass spectral analysis of the dimethyl ester derivative of metabolite C-1

TABLE 1. RADIOCARBON RESIDUES IN TISSUES 7 DAYS AFTER TREATMENT OF LAYING HENS WITH [^{14}C]XANTHOTOXIN AS A SINGLE ORAL DOSE EQUIVALENT TO 10.0 mg XANTHOTOXIN/kg OF BODY WEIGHT

Tissue	Xanthotoxin equivalent (ppm)[a]	Percent of dose[a]
Blood[b]	0.04 ± 0.02	0.02 ± 0.01
Fat[b]	<0.01	<0.01
Gizzard	<0.01	<0.01
Heart	<0.01	<0.01
Kidney	0.14 ± 0.01	0.01 ± 0.0
Liver	0.51 ± 0.05	0.14 ± 0.01
Lungs	0.06 ± 0.01	<0.01
Muscle[b]	<0.01	<0.01
Ovarian follicle	0.02 ± 0.01	<0.01
Ovarian egg	0.03 ± 0.02	0.01 ± 0.0
Skin[b]	0.01 ± 0.01	<0.01

[a]Results are expressed as mean ± standard deviation of four laying hens.
[b]Based on relative body composition of these tissues (Card and Nesheim, 1973).

TABLE 2. XANTHOTOXIN AND METABOLITES IN EXCRETA OF LAYING HENS 24 HOURS AFTER TREATMENT WITH A SINGLE ORAL DOSE OF [^{14}C]XANTHOTOXIN EQUIVALENT TO 10.0 mg XANTHOTOXIN/kg OF BODY WEIGHT

Radiolabeled component	HPLC retention time (min)[a]	Percent of sample radiocarbon
C−1 (HCHA)	13	50
C−2	14	3
C−3	15	3
C−4 (HCA)	27	13
C−5 (xanthotoxol)	33	1
C−6 (xanthotoxin)	41	1
Others[b]		7
Unextractable[c]		22

[a]For specifics of HPLC analysis procedures, see Pangilinan (1990).
[b]Refers to a group of HPLC resolved metabolites, none of which individually contained as much as 1% of the radiocarbon in the sample.
[c]Radioactivity remaining in the water/residue slurry after ethyl acetate extraction of acidified samples.

(prepared by reaction with diazomethane) revealed a molecular ion at *m/z* 294 (Table 3) and a fragmentation pattern that was essentially identical to that of the dimethyl derivative of [6-(7-hydroxy-8-methoxycoumaryl)]-hydroxyacetic acid (HCHA) isolated from the insect *Papilio polyxenes* (Ivie et al., 1983). The NMR spectrum of the nonderivatized metabolite C-1 (Figure 3) was very comparable to that of the methyl ester derivative of HCHA isolated from the urine of the dog (Kolis et al., 1979). It was therefore concluded that chicken metabolite C-1 was in fact HCHA.

Metabolite C-4 was identified as 6-(7-hydroxy-8-methoxycoumaryl)-acetic acid (HCA). Mass spectral analysis of HPLC-purified C-4 revealed a molecular ion of *m/z* 250 (Table 3) and a fragmentation pattern that matched that of HCA previously isolated from the dog (Kolis et al., 1979), man (Schmid et al., 1980), insect (Ivie et al., 1983), goat (Ivie et al., 1986), and rat (Mays et al., 1986).

The mass spectra (Table 3) and HPLC retention times of C-5 and C-6 were identical to those of authentic xanthotoxol and xanthotoxin, respectively. Metabolite C-5 was identical to authentic xanthotoxol in TLC behavior using eight different solvent systems. Similarly, the TLC behavior in nine different solvent systems supported the assignment of C-6 as unmetabolized xanthotoxin (Pangilinan, 1990).

Characterization of Metabolites in Egg Fractions. Cochromatographic analysis of the acetonitrile extract of egg yolk with authentic xanthotoxin using eight TLC solvent systems revealed that >85% of the radiocarbon in the sample was unmetabolized xanthotoxin. Similarly, in the egg white, one radioactive component accounted for >92% of the radioactivity. Cochromatographic analysis of the egg white product also revealed it to be identical in TLC behavior (seven different solvent systems) to authentic xanthotoxin. Isolation of sufficient quantities of this metabolite for confirmation by GC-MS was not successful due to the very low levels present in the egg yolk and egg white and to the relatively large amount of interfering materials.

Excretion Patterns and Tissue Residues in Goat. Radiocarbon elimination in the urine of the goat was rapid (Figure 4). Within 24 hr posttreatment, >80% of the dose was excreted through the urine, although very low levels of radiocarbon continued to be eliminated in urine during the following six days.

Radiocarbon elimination in the feces of the goat was much lower than that in the urine (Figure 4). Maximum radiocarbon elimination in the feces occurred between 24 and 48 hr after treatment; however, low levels of radiocarbon continued to be eliminated in the succeeding days until the study was terminated.

Radiocarbon residues in milk of the goat were very low. Highest levels (<2 ppm xanthotoxin equivalent) were observed at 12 hr posttreatment; within two days after treatment, no radiocarbon residues were detected in the milk. Less than 0.4% of the administered radiocarbon was secreted into the milk.

Analysis of tissues from the goat killed seven days after treatment showed

TABLE 3. MASS SPECTRAL DATA OF METABOLITES OF XANTHOTOXIN IN LAYING HENS AND LACTATING GOAT

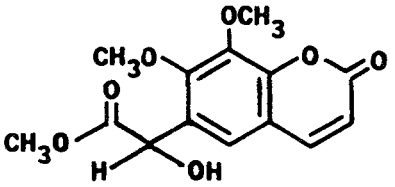
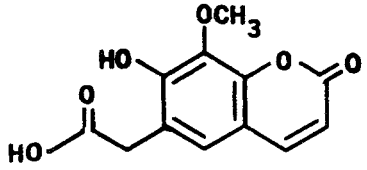
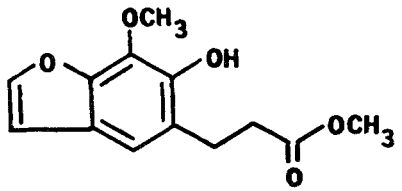
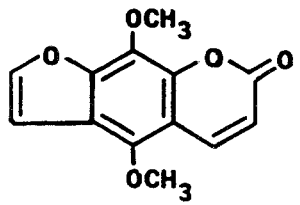
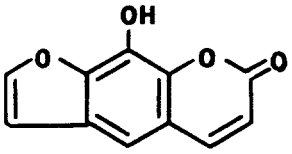
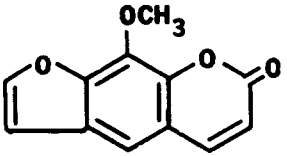
Metabolite and structure	m/z	Relative intensity	Fragment
Metabolite C−1, G−2 HCHA [6-(7-hydroxy-8-methoxycoumaryl)-hydroxyacetic acid] (identified after methylation to dimethyl derivative shown)			
	294	15	$[M]^+$
	235	100	$[M-CO_2CH_3]^+$
	220	10	$[M-CO_2CH_3,CH_3]^+$
	207	5	$[M-CO_2CH_3,CO]^+$
	192	12	$[M-CO_2CH_3,CO,CH_3]^+$
Metabolite C−4, G−8 HCA [6-(7-hydroxy-8-methoxycoumaryl)-acetic acid]			
	250	78	$[M]^+$
	232	85	$[M-H_2O]^+$
	204	100	$[M-H_2O,CO]^+$
	176	42	$[M-H_2O,2CO]^+$
	148	57	$[M-H_2O,3CO]^+$
Metabolite G−11 BFPA {3-[5-(6-hydroxy-7-methoxybenzofuryl)]-propanoic acid} (identified after methylation to derivative shown			
	250	100	$[M]^+$
	218	92	$[M-CH_3OH]^+$
	190	20	$[M-CH_3OH,CO]^+$
	176	50	$[M-CH_3OH,C_2H_2O]^+$
Metabolite G−15 5-HMP (5-hydroxy-8-methoxypsoralen) (identified after methylation to derivative shown)			
	246	95	$[M]^+$
	231	100	$[M-CH_3]^+$

TABLE 3. Continued

Metabolite and structure	m/z	Relative intensity	Fragment
Metabolite C−5, G−12 xanthotoxol (8-hydroxypsoralen)			
	202 174	100 70	$[M]^+$ $[M-CO]^+$
Metabolite C−6, G−17 xanthotoxin (8-methoxypsoralen)			
	216 201 173	100 23 52	$[M]^+$ $[M-CH_3]^+$ $[M-CH_3,CO]^+$

that only the blood, liver, and muscle contained levels of radiocarbon equivalent to 0.01% of the administered dose or higher (Table 4). Radiocarbon residues, expressed on a parts per million basis, were highest in the liver (0.25 ppm), followed by the kidney (0.07 ppm).

Characterization of Metabolites in Goat Urine. HPLC analysis of the ethyl acetate extracts of goat urine indicated the presence of 18 radioactive components that each contained at least 1% of the sample radiocarbon. Five HPLC-resolved components each contained at least 5% each of the total sample radiocarbon in the urine samples analyzed (Table 5).

The HPLC retention time of metabolite G-12 matched that of authentic xanthotoxol. The mass spectrum and HPLC retention time of the methylated derivative of G-12 were identical to those of authentic xanthotoxin, confirming that G-12 was indeed xanthotoxol. The HPLC retention times of G-2, G-6, G-8, G-9, and G-17 matched those of authentic HCHA, psoralenquinone (PQ), HCA, dihydroxypsoralen (DHP), and xanthotoxin, respectively. Studies with the methylated derivative of G-8 indicated the product to have the same HPLC retention time as authentic methylated HCA.

Two metabolites from urine treated with deactivated enzyme prior to extrac-

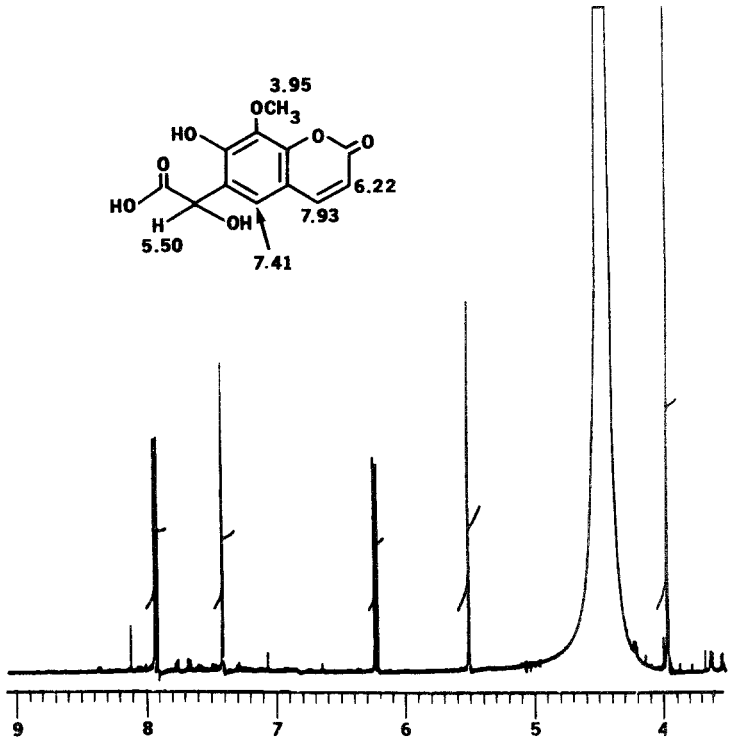


FIG. 3. NMR spectrum of chicken metabolite C-1 (deuteroacetone).

tion were poorly resolved by HPLC as two overlapping peaks. One of the peaks, however, was absent in extracts of active enzyme-hydrolyzed urine samples, suggesting that the product was a glucuronide or sulfate conjugate. The remaining component of this metabolite pair (G-11) was identified as 3[5-(6-hydroxy-7-methoxy-benzofuryl)]-propanoic acid (BFPA). The mass spectrum of methylated G-11 yielded a molecular ion of *m/z* 250 (Table 3) and a fragmentation pattern consistent with that of the major goat metabolite isolated in the previous work by Ivie et al., (1986).

The mass spectrum of metabolite G-15 (after methylation) matched that of authentic 5,8-dimethoxypsoralen (5,8-DMP) (Table 3). The unmethylated G-15 was clearly not dihydroxypsoralen (DHP) on the basis of chromatographic behavior; thus G-15 was identified as 5-HMP.

When the aqueous phase of extracted urine was subjected to acid hydrolysis and then reextracted with ethyl acetate, about 12% and 60% of the radiocarbon present in the extracts exhibited HPLC retention times identical to those of authentic xanthotoxin and xanthotoxol, respectively. These data are most con-

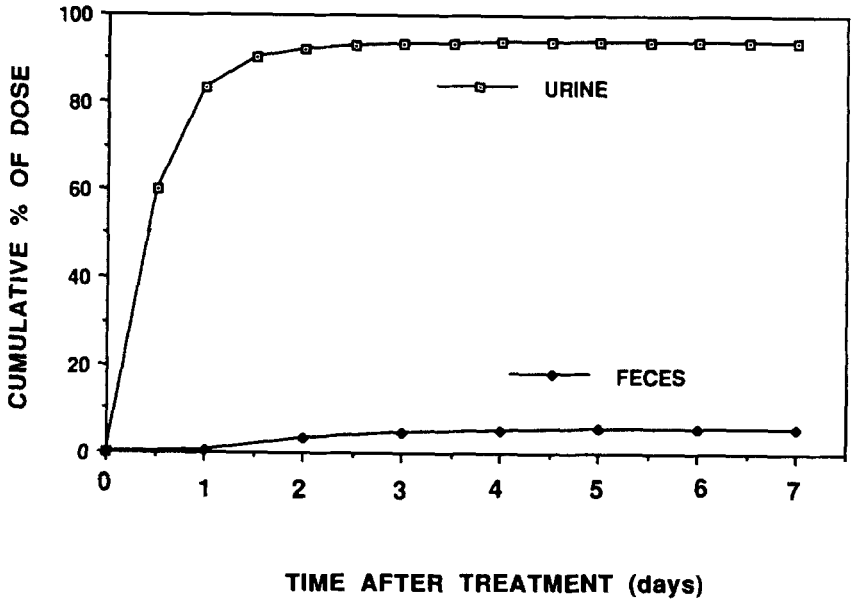


FIG. 4. Elimination of radiocarbon residues in urine and feces of a lactating goat treated with [^{14}C]xanthotoxin as a single oral dose equivalent to 10.0 mg xanthotoxin/kg body weight.

TABLE 4. RADIOCARBON RESIDUES IN TISSUES 7 DAYS AFTER TREATMENT OF LACTATING GOAT WITH [^{14}C]XANTHOTOXIN AS A SINGLE ORAL DOSE EQUIVALENT TO 10.0 mg XANTHOTOXIN/kg OF BODY WEIGHT

Tissue	Percent of Dose	Xanthotoxin equivalent (ppm)
Blood	0.01[a]	0.02
Brain	<0.01	0.03
Fat	<0.01[a]	<0.01
Heart	<0.01	0.04
Kidney	<0.01	0.07
Liver	0.05	0.25
Muscle	0.04[a]	0.01
Tongue	<0.01	0.01

[a] Based on relative body composition of these tissues (Davis et al., 1975).

TABLE 5. XANTHOTOXIN AND METABOLITES IN URINE OF LACTATING GOAT 24 HOURS AFTER TREATMENT WITH A SINGLE ORAL DOSE OF [^{14}C]XANTHOTOXIN EQUIVALENT TO 10.0 mg XANTHOTOXIN/kg OF BODY WEIGHT

Compound	HPLC retention time[a] (min)	Urinary metabolites (%)	
		Control[b]	Treated[c]
G−1	4	1	<1
G−2 (HCHA)	13	2	2
G−3	15	1	2
G−4	17	1	1
G−5	23	2	1
G−6 (PQ)	24	1	2
G−7	26	2	2
G−8 (HCA)	27	5	4
G−9 (DHP)	28	2	<1
G−10	29	25	24
G−11 (BFPA)	31	9	7
G−12 (xanthotoxol)	33	11	12
G−13	34	5	6
G−14	36	2	3
G−15 (5-HMP)	38	4	9
G−16	39	2	1
G−17 (Xanthotoxin)	41	<1	1
G−18	53	4	5
Others[d]		3	4
Unextractable[e]		18	14

[a] For specifics of HPLC analysis procedure, see Pangilinan (1990).
[b] Metabolites in ethyl acetate extracts of urine treated with deactivated glucuronidase–sulfatase enzyme.
[c] Metabolites in ethyl acetate extracts of urine treated with active glucuronidase–sulfatase enzyme.
[d] HPLC resolvable metabolites, none of which individually contained as much as 1% of the radiocarbon in the sample analyzed.
[e] Radioactivity remaining in the aqueous phase after ethyl acetate extraction of the sample.

sistent with the hypothesis that these two compounds occurred in urine partly as lactone-ring-opened derivatives (probably conjugates), which were cleaved and then recyclized under strong acid treatment (Kolis et al., 1979).

DISCUSSION

Xanthotoxin was rapidly absorbed, metabolized, and excreted after oral administration of single 10 mg/kg doses to laying hens and to a lactating goat. Radiocarbon content in the feces of the goat was very low, indicating that absorption was nearly complete and that elimination of metabolites through the

bile or other fluids secreted into the gastrointestinal tract is a minor excretory route. In man given an oral dose of 40 mg of [*methoxy*-^{14}C]xanthotoxin, 71% of the dose was excreted in the urine and 6% in the feces within 24 hr post-treatment (Schmid et al., 1980). Almost all of a [*methoxy*-^{14}C]xanthotoxin dose administered orally to the insect, *Papilio polyxenes*, was found in the excreta 24 hr after treatment (Ivie et al., 1983).

Radiocarbon elimination in the egg yolk and white was very low. In both fractions, at least 85% of the radioactivity present was as unmetabolized xanthotoxin.

In the hen and goat, there was very little retention of unmetabolized xanthotoxin or its metabolites in tissues. At seven days after treatment, only the livers of both laying hens and lactating goat contained 0.05% or more of the administered dose. Similar studies on the tissue distribution of [^{14}C]xanthotoxin in beagle dogs and miniature pigs given single oral doses (10 mg/kg body weight) showed rapid clearance of the compound from the bodies of the animals (Ikeda et al., 1990). In the dog, small amounts of radioactive components were found in the liver (0.47% of dose) and blood (0.15% of dose) after four days, while in the pig seven days after treatment, highest levels of radioactivity were observed in the liver (0.5% of dose) and fat (0.27% of dose).

Ivie et al. (1986) reported the appearance of substantial radiocarbon residues (10.6% of administered dose) in the milk of a goat treated with [*methoxy*-^{14}C]xanthotoxin. The authors, however, hypothesized that this radiocarbon occurred as the result of biosynthetic incorporation of [^{14}C]CO_2 into normal milk constituents. Results from the present study showed that $<0.4\%$ of the administered radiocarbon was eliminated in the milk, results that seemingly confirm the earlier hypothesis.

In the chicken, xanthotoxin is rapidly metabolized via dealkylation, oxidation, hydrolysis, and conjugation mechanisms (Figure 5). Structural characterization of the major xanthotoxin metabolites in the chicken, HCHA and HCA, indicated that the major mechanism of metabolic degradation of xanthotoxin in the laying hen is through oxidative opening of the furan ring. These furan oxidation products also have been isolated as products of xanthotoxin metabolism in dogs (Kolis et al., 1979), humans (Schmid et al., 1980), and in the insects *P. polyxenes* and *Spodoptera frugiperda* (Ivie et al., 1983). Rats also metabolize xanthotoxin by oxidative cleavage of the furan ring; HCA but not HCHA is generated as the final product (Mays et al., 1986). In the chicken, HCHA and HCA account for $>60\%$ of the radiocarbon eliminated in the excreta of the laying hen. In *P. polyxenes* treated with [^{14}C]xanthotoxin, $>80\%$ of the dose is eliminated in the excreta as these two furan oxidation products (Ivie et al., 1983), and in humans treated with [^{14}C]xanthotoxin more than half of the urine radioactivity was products of oxidative cleavage of the furan ring (Schmid et al., 1980). The intermediate pathways involved in the formation of the furan

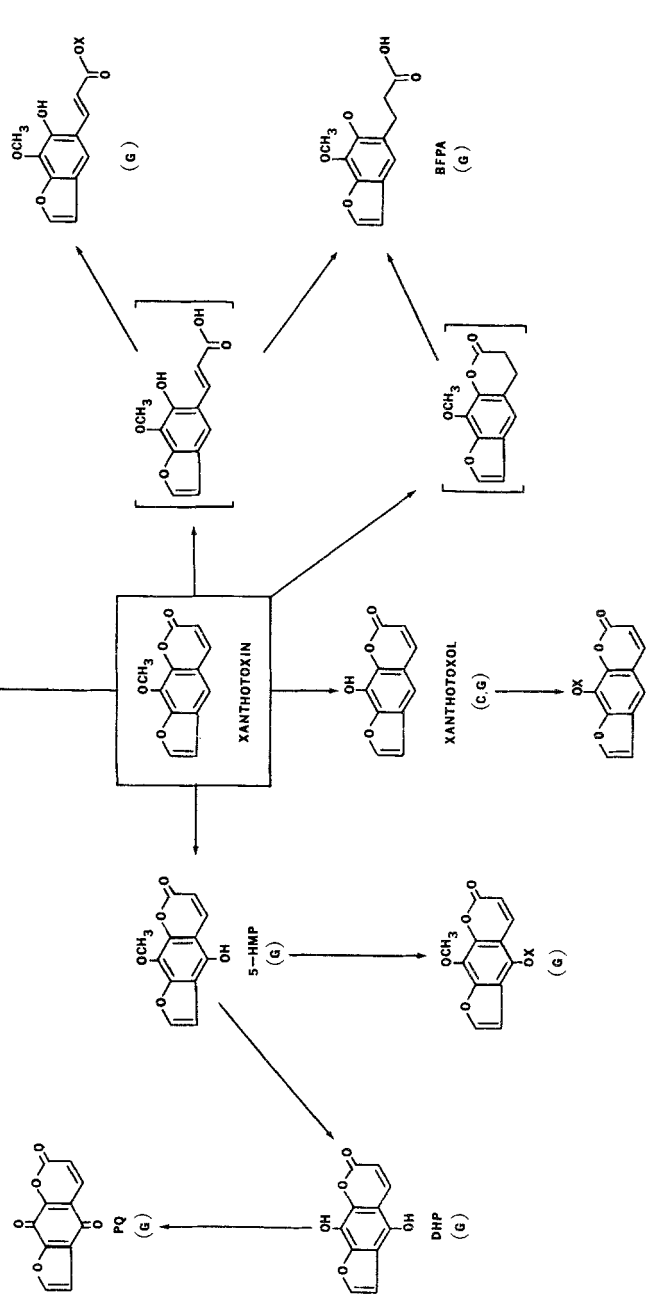


FIG. 5. Proposed pathways of xanthotoxin metabolism in laying hens and a lactating goat. Structures in brackets indicate logical intermediates leading to certain metabolites identified. Conjugating moieties are indicated by X. Letters in parentheses indicate that specific compounds were identified as xanthotoxin metabolites in the chicken (C) and/or goat (G).

oxidation products have not been definitely established; however, Kolis et al. (1979) postulated the generation of a short-lived reactive epoxide intermediate (shown in brackets, Figure 5) in the formation of these two metabolites.

In the goat, pathways for xanthotoxin metabolism include: (1) *O*-demethylation of the 8-methoxy group, (2) hydroxylation at position 5 to form 5-HMP, (3) hydrolysis of the lactone ring, (4) saturation of the lactone olefinic moiety, (5) oxidative opening of the furan ring, and (6) conjugation. At least one metabolite, 5-HMP, occurred partially as a conjugate on the evidence that its concentration was increased after hydrolysis of the urine prior to extraction. The apparent conjugation of xanthotoxin observed in these studies likely occurs after hydrolysis of the lactone ring (Kolis et al., 1979).

Low levels of unmetabolized xanthotoxin were found in the urine of the goat. Polarity considerations make it unlikely that xanthotoxin itself would be excreted in the urine; more likely these residues resulted either from low-level fecal contamination or from lactone hydrolysis followed by recyclization (Kolis et al., 1979).

The furan oxidation (HCA and HCHA) and lactone hydrolysis (BFPA) products identified in this study were also isolated in the previous goat metabolism study (Ivie et al., 1986). Although not established experimentally, formation of BFPA in the goat may be catalyzed in the rumen, a highly reductive environment (Van Soest, 1982; Ennis et al., 1989). The goat, along with the fungus *Gibberella pulicars* (Spencer et al., 1990), are apparently the only organisms thus far known to metabolize xanthotoxin by a reductive mechanism leading to BFPA.

The hydroxylation products, 5-HMP and DHP, as well as PQ, have also been identified as metabolites of xanthotoxin in rats (Mays et al., 1986) but apparently not in other species studied. We consider the identities of PQ and DHP in the goat as tentative because these metabolites were identified only by cochromatography with authentic standards using HPLC techniques and the structures were not confirmed by spectral analyses.

The present studies add to the body of knowledge on xanthotoxin metabolism in vertebrates and represent the first reported data on furanocoumarin metabolism by an avian species. We conclude that xanthotoxin is not likely to be secreted into milk at appreciable levels; thus, animals ingesting furanocoumarin-containing plants probably will not produce milk with sufficient photosensitizing residues to present significant toxicological or ecological risks. However, in eggs of xanthotoxin-treated chickens, the furanocoumarin residues were shown to consist largely of unmetabolized xanthotoxin, raising the issue that under certain circumstances, ingestion of furanocoumarin-containing plants by avian wildlife might result in toxicological consequences to developing embryos.

Because only low levels of radiocarbon residues occurred in the tissues,

eggs, and milk of the xanthotoxin-treated chickens and goat, it seems unlikely that human exposure to furanocoumarins through animal-derived foodstuffs will be appreciable or toxicologically significant.

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WEAK RESPONSES OF PINE SAWFLY LARVAE TO HIGH NEEDLE FLAVONOID CONCENTRATIONS IN SCOTS PINE

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Abstract—Responses of sawfly larvae (Hymenoptera, Diprionidae) to the flavonoid taxifolin glucoside in their host plant were studied in a laboratory experiment. Larvae of *Neodiprion sertifer* and *Diprion pini* were raised from egg hatch to cocoon spinning on two Scots pine (*Pinus sylvestris*) chemotypes, one without needle taxifolin glucoside (−) and the other containing 2–4% taxifolin glucoside (+). The (+) chemotype had somewhat lower concentrations of needle terpenoids (resin acids) than the (−) chemotype. Current-year needles had higher taxifolin glucoside concentrations than mature needles. There were no differences in survival or body size between *N. sertifer* larvae that fed on the (+) chemotype and those that fed on the (−) chemotype. Female *D. pini* larvae raised on (+) needles developed 6% more slowly than larvae fed (−) needles. The results from this study are contrary to earlier findings showing that flavonoid glucosides have strong negative effects on insect performance. Possible explanations for the different outcomes are discussed.

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Key Words—Plant-insect interaction, *Pinus sylvestris*, *Neodiprion sertifer*, *Diprion pini*, Hymenoptera, Diprionidae, insect performance, flavonoids, taxifolin glucoside, resin acids.

INTRODUCTION

Flavonoids are polyphenolic compounds found in many species of plants, where they usually occur as glycosides (Harborne, 1979). Little is known about the function of flavonoid compounds. Some (e.g., quercitrin, rutin, and maysin) are known to have deleterious effects on agricultural insects (Shaver and Lukefahr, 1969; Waiss et al., 1979; Elliger et al., 1980, 1981; Isman and Duffey, 1982; Hedin et al., 1983; Duffey et al., 1986). However, rutin has also been shown to enhance insect growth (McFarlane and Distler, 1982), and other flavonoid compounds (e.g., morin and kaempherol) act as feeding stimulants for certain insects (Hamamura et al., 1962; Nielsen et al., 1979).

Coniferous trees have a great variety of flavonoid compounds in their needles (Nieman, 1988), yet their function in the plant is poorly known. There are some indications that they confer resistance against microorganisms (Nomura and Kishida, 1978; Szweykowski and Urbaniak, 1982). Their effects on the feeding of folivorous insects have not been studied.

Two Scots pine (*Pinus sylvestris* L.) chemotypes exist with respect to one particular flavonoid, the dihydroflavonol glucoside taxifolin 3′-*O*-β-D-glucopyranoside (taxifolin glucoside) (Lundgren and Theander, 1988; Laracine-Pittet and Lebreton, 1988). One of the chemotypes lacks taxifolin glucoside, while the other can have a needle taxifolin glucoside concentration of 3–4% dry weight (Lundgren, unpublished data). The two chemotypes cooccur over large parts of Europe, and the relative frequencies in a given tree population seem to vary with climatic conditions (Laracine-Pittet and Lebreton, 1988).

These chemotypes offer an interesting opportunity to study flavonoid–insect interactions in natural plant tissue. In some cases, variation in plant phenolics may have an important influence on insect population dynamics. For example, it has been suggested that stress-induced changes in needle chemistry may trigger outbreaks of needle-eating insects (Larsson and Tenow, 1984). Environmental stress can alter phenolic glycoside concentrations in leaves of some woody plants (Larsson et al., 1986b; Bryant et al., 1987). It is not known, however, whether this also applies to flavonoids in conifers. If it can be shown that insect performance is affected by flavonoids, then it would be rewarding to investigate how needle flavonoid concentrations are influenced by environmental factors.

In this paper we report the results from an experiment in which two species of pine sawflies [*Diprion pini* L. and *Neodiprion sertifer* (Fourcroy)] were fed the two Scots pine chemotypes. Insects were raised on the two types of needles,

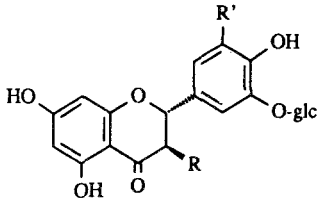
and responses were evaluated in terms of larval survival, developmental time, and cocoon weight.

METHODS AND MATERIALS

Study Organisms. Plant material was obtained from a 15-year-old Scots pine plantation at Hägerdal, 30 km north of Uppsala, central Sweden. Trees in this plantation had been investigated earlier with respect to variation in needle flavonoid chemistry (Lundgren unpublished data). Based on these results, trees with [(+) chemotype] and without [(−) chemotype] taxifolin glucoside and chemically related compounds (Figure 1) were selected for use. The experiment was performed in 1986 and 1987, using a different insect species each year (see below). From each study tree (N = 12 trees in 1986, N = 5 trees in 1987) one branch (three branches in 1987) was sampled from the lower half of the crown, which was immediately placed in a bucket of water and taken to the laboratory.

Larvae of *Neodiprion sertifer*, collected from an outbreak area south of Sandviken, central Sweden, were used in the 1986 experiment, and larvae of *Diprion pini* from a small outbreak at Närsholmen, Gotland, southeast Sweden, were used in the 1987 experiment. In both years, insects for the experiments were obtained by collecting pine shoots containing egg batches just prior to hatching. The shoots were taken to the laboratory and checked daily for hatched larvae. The transfer of larvae to test shoots began as soon as the first emerging larvae were observed.

Larval Performance Experiments. Sawfly larvae were raised from egg hatch to cocoon spinning on experimental shoots in an environmental chamber (18:6 light–dark photoperiod, 18°C, 70% relative humidity). Larvae were presented their preferred needle type, i.e., 1-year-old needles for *N. sertifer* and both



R=R'=H	Eriodictyol glucoside
R=OH; R'=H	Taxifolin glucoside
R=R'=OH	Ampelopsin glucoside

FIG. 1. Flavonoid glucosides present in the needles of the (+) chemotype (cf. Lundgren and Theander, 1988).

1-year-old needles (early instars) and current-year needles (late instars) for *D. pini*. Each shoot was placed in a plastic container filled with tap water. To prevent mixing of the larvae, each shoot was enclosed in a transparent plastic cylinder with a muslin cover. Larvae were given fresh shoots at weekly intervals.

At the start of the experiment newly emerged larvae or eggs just about to hatch were transferred to shoots representing each of the two types of trees. About 30 larvae were transferred to each shoot. In 1986, 12 replicates were studied (one shoot from each of 12 trees). In 1987, three shoots from each of five trees were studied (always one terminal and two lateral shoots). Larvae that had started to feed on a terminal shoot were always given terminal shoots, and larvae that had started feeding on lateral shoots were always given lateral shoots.

In order to avoid pseudoreplication, means were calculated for male and female larvae fed needles from the same tree. Thus, there were 12 independent observations in 1986 and five in 1987. Statistical analyses were performed on these mean values.

Chemical Analyses. Needles intended for chemical analyses were sampled from branches offered to the larvae in the performance experiments. In 1986, 1-year-old needles were sampled fresh from trees on June 17. In 1987, 1-year-old needles were sampled fresh from trees on July 16. After one week in the laboratory, samples were again taken from the same shoots to check whether the laboratory treatment had any effects on needle phenolic chemistry. Current-year foliage was sampled fresh from trees on July 27, 1987, and again from shoots in the laboratory on July 30. Since no effects of the laboratory treatment on phenolic chemistry were found, data were pooled in all subsequent analyses.

Phenolic compounds were analyzed by reverse phase high-performance chromatography (HPLC). In the 1986 experiment, needle extracts were treated with pectinase and the resulting aglycone mixtures were extracted with ethyl acetate and examined by HPLC. The taxifolin content was determined, and the concentration of taxifolin glucoside in the needles calculated, assuming that taxifolin glucoside is quantitatively hydrolyzed and extracted to the organic layer. Acetovanillon was used as internal standard. In 1987, the intact extractives were analyzed, and picein was used as internal standard. The internal standard (0.5 mg) was added to the needle sample (1000 mg fresh weight), and the sample was homogenized with an UltraTurrax with 2 × 5 ml of 95% ethanol (5 and 0.5 min, respectively) and with 2 × 5 ml of 80% ethanol in the same way. The combined aqueous ethanol extract was evaporated to dryness, dissolved in water (15 ml) and extracted with light petroleum (bp 40–60°C) (2 × 20 ml). In 1987 the aqueous layer was evaporated to dryness and the residue dissolved in methanol (1 ml). In 1986 the aqueous layer was treated with pectinase (0.5 ml, No. P-5146, Sigma) overnight at room temperature, acidified with three drops of 25% phosphoric acid, and extracted with ethyl acetate (3 × 10 ml). The combined ethyl acetate extract was dried with sodium sulfate and

evaporated to dryness, after which the residue was dissolved in methanol (1 ml).

The HPLC separation was performed with a Spectra Physics liquid chromatogram system with a Nova-Pak Rad-Pak C-18 column (Waters Associates). The separated components were detected with a UV detector at 280 nm. For the 1986 experiment, the mobile phase (mixture by volume of 0.01 M, pH 2.8, aqueous sodium phosphate buffer and methanol) consisted of a linear gradient from 20% to 30% methanol for 15 min, then maintained isocratically for 10 min, followed by a linear gradient to 55% methanol for 25 min and to 80% methanol for 10 min. The flow rate was 1.5 ml/min. For the 1987 experiment, the mobile phase (mixture by volume of 0.01 M, pH 2.8, aqueous sodium phosphate buffer and acetonitrile) consisted for the initial 7 min of 5% acetonitrile. The concentration of acetonitrile was increased linearly to 25% in 43 min and subsequently to 70% during 10 min. The flow rate was 1.5 ml/min.

Diterpenoids were extracted and treated according to the methods reported by Gref and Ericsson (1985). Samples were analyzed on a Varian 3700 gas chromatograph equipped with a fused silica capillary column (15 m × 0.25 mm ID) with 0.25-μm film of DB-1 (J & W Scientific). The chromatograph was equipped with a split injector and a flame ionization detector. Hydrogen was used as a carrier gas at a flow rate of 1.60 ml/min. The chromatograph was operated isothermally at 210°C or temperature programmed to increase from 160°C to 240°C at 4°/min. Peak areas, relative to the internal standard, and retention times were measured with an electronic integrator.

Total nitrogen (on a dry weight basis) was measured with a CHN-elemental analyzer (Perkin-Elmer model 2400 CHN).

RESULTS

Needle Chemistry. As expected, the two types of trees differed in their needle concentrations of taxifolin glucoside. No taxifolin glucoside was found in the (−) chemotype, whereas taxifolin glucoside made up 1.7–3.8% of the needle dry weight in the (+) chemotype (Figure 2; Table 1). In 1987, taxifolin glucoside concentrations were twice as high in current-year needles as in 1-year-old needles. Other hydrophilic UV-absorbing compounds (presumably phenolics) were not specifically quantified, but their total chromatogram peak areas were determined. Concentrations of these compounds in 1-year-old needles were higher in the (+) chemotype than in the (−) chemotype in both 1986 and 1987, i.e., 26% and 40%, respectively (Table 1). No differences were found with respect to these compounds in current-year needles.

In 1986, concentrations of total resin acids were, on average, 69% higher in the (−) chemotype compared with the (+) chemotype (Table 1). Pinifolic

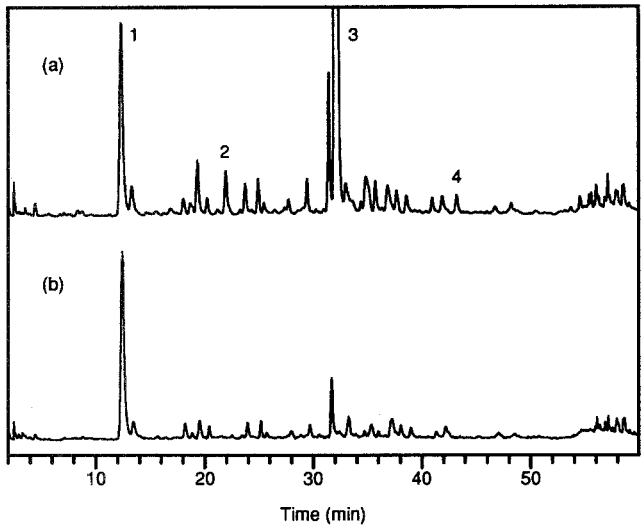


FIG. 2. HPLC separation of phenolic extractives from needles of (a) a pine tree containing taxifolin glucoside [= (+) chemotype] and (b) a pine tree lacking taxifolin glucoside [= (−) chemotype]. Peak numbers refer to (1) picein (internal standard), (2) ampelopsin glucoside, (3) taxifolin glucoside, and (4) eriodictyol glucoside (cf. Figure 1).

acid contributed most to the total resin acid content (66.5%, averaged over the two types of trees). In 1987, a subset of five trees of each type was compared. These trees were selected in a way that minimized variation in resin acid concentrations. As a result, there was much less variation in resin acid concentrations in the 1987 experiment. No differences were found in total resin acid or pinifolic acid concentrations, but there were significantly higher concentrations of 4-epiimbricatolic acid and neoabietic acid in the (+) chemotype.

There were no differences in concentrations of total nitrogen between the two types of trees (Table 1).

Larval Performance. No differences were found in survival or cocoon weight between *N. sertifer* larvae raised on the (+) chemotype and those raised on the (−) chemotype (Table 2). No detailed measurements of larval development were made. However, according to a survey made on June 27 (late cocoon spinning stage), the proportion of larvae that had spun cocoons was 79% on the (+) chemotype and 71% on the (−) chemotype.

D. pini larvae developed more slowly on the (+) chemotype (Table 2). The difference was small (6%) and significant for female larvae ($P < 0.05$), but not significant for male larvae. No effects on body size were found.

TABLE 1. CONCENTRATIONS (% dw) OF PHENOLIC COMPOUNDS, TERPENOIDS, AND TOTAL NITROGEN IN NEEDLES OF TWO SCOTS PINE CHEMOTYPES WITH (+) AND WITHOUT (−) TAXIFOLIN GLUCOSIDE[a]

	1986			1987					
	Old needles			Old needles			New needles		
Compounds	(−)	(+)	$p<$	(−)	(+)	$p<$	(−)	(+)	$p<$
Taxifolin glucoside	0.00(0)	3.33[b](0.81)	0.001	0.00(0)	1.67(0.174)	0.001	0.00(0)	3.822(0.680)	0.001
Other phenolics[c]	1.10(0.30)	1.39(0.29)	0.05	2.55(0.20)	3.57(0.43)	0.01	1.58(0.35)	1.81(0.21)	ns
Abietadienoic acid[d]	0.186(0.054)	0.104(0.053)	0.001	0.068(0.086)	0.191(0.086)	0.05	0.121(0.052)	0.227(0.086)	0.05
Dehydroabietic acid	0.095(0.061)	0.109(0.075)	ns	nd	nd	—	nd	nd	—
4-epiimbricatolic acid	0.105(0.046)	0.113(0.053)	ns	0.090(0.024)	0.205(0.079)	0.05	0.051(0.018)	0.070(0.021)	ns
Pinifolic acid	1.060(0.320)	0.586(0.113)	0.001	0.734(0.154)	0.712(0.234)	ns	0.442(0.105)	0.350(0.074)	ns
Total resin acids	1.540(0.356)	0.913(0.200)	0.001	1.125(0.476)	0.940(0.301)	ns	0.794(0.390)	0.647(0.169)	ns
Isoabienol	0.176(0.332)	0.040(0.141)	ns	0.574(0.533)	0.00(0)	ns	0.346(0.325)	0.00(0)	ns
Total nitrogen	no data	no data	—	1.31(0.13)	1.35(0.15)	ns	1.66(0.08)	1.59(0.11)	ns

[a] n = 12 trees (1986) and n = 5 trees (1987), nd = no detectable amounts; standard deviation within parentheses.
[b] Calculated from concentration of taxifolin.
[c] Total hydrophilic UV-absorbing compounds; relative amounts, data not comparable between years owing to differences in extraction methods.
[d] Sum of levopimaric, abietic, and neoabietic acids.

TABLE 2. PERFORMANCE OF LARVAL SAWFLIES RAISED ON SCOTS PINE CHEMOTYPES WITH (+) OR WITHOUT (−) TAXIFOLIN GLUCOSIDE[a]

	Neodiprion sertifer			*Diprion pini*		
Insect trait	(−)	(+)	$p<$	(−)	(+)	$p<$
Survival (%)	52(23)	54(20)	ns	no data		—
Male cocoon weight (mg dw)	29.4(4.2)	29.9(3.7)	ns	88.7(4.6)	84.0(5.0)	ns
Female cocoon weight (mg dw)	73.8(7.9)	70.7(7.4)	ns	179.1(14.0)	183.8(5.2)	ns
Male developmental time (*d*)	no data		—	33.7(1.8)	34.2(1.7)	ns
Female developmental time (*d*)	no data		—	35.7(1.4)	37.7(0.4)	0.05

[a] $n = 12$ (*N. sertifer*) and $n = 5$ (*D. pini*); standard deviations within parentheses.

DISCUSSION

Three findings with ecological implications came out of this study: First, trees of the (+) chemotype tended to have lower concentrations of needle resin acids than those of the (−) chemotype. Second, concentrations of taxifolin glucoside in current-year needles were about twice as high as those in 1-year-old needles. Third, despite the great differences in flavonoid concentrations between the two chemotypes, differences in performance between larvae fed the (+) chemotype and those fed the (−) chemotype were surprisingly small.

In 1987, we selected trees in a way that minimized differences in resin acid content between chemotype groups in order to isolate the taxifolin glucoside effect on sawfly performance. This explains why a chemotype-related difference in resin acid concentrations was found in 1986 but not in 1987. The negative relationship between resin acid and taxifolin glucoside concentrations in 1986 is difficult to explain because these two types of substances are synthesized along completely different metabolic pathways (Goodwin and Mercher, 1983). Since both types of compounds are examples of carbon-based substances, their synthesis might have been constrained owing to limited substrate availability (e.g., Steward and Keeler, 1988).

In 1987, concentrations of taxifolin glucoside were found to be higher in young needles than in 1-year-old needles. Although this type of comparison could not be made in 1986, we were able to compare concentrations in 1-year-old needles between 1986 and 1987 and found them to be somewhat higher in 1986. This difference suggests that concentrations may vary between years, but it could also reflect within-season variation since needles were sampled at an

earlier date in 1986. Young plant tissue often has higher concentrations of secondary metabolites than mature tissue, although the reverse also occurs (Ikeda et al., 1977; Niemelä et al., 1982; Raupp and Denno, 1983). It may be adaptive for the plant to invest strongly in the defense of young tissue, which will contribute most to future growth and reproduction (e.g., McKey, 1979). Thus, it appears likely that the high concentrations of taxifolin glucoside in young needle tissue is an example of such an adaptation, since it is known that this compound has a protective function in some plant species (Nomura and Kishida, 1978).

The results of the present experiments, however, provide little support for the hypothesis that taxifolin glucoside helps to protect *P. sylvestris* against pine sawflies. It is possible, however, that the performance of *N. sertifer* larvae fed the (+) chemotype was reduced, but that this reduction was masked by the higher resin acid concentrations in the (−) chemotype, which should have also adversely affected performance (Larsson et al., 1986a).

In the *D. pini* experiment, where the two chemotype groups had similar resin acid concentrations, there was a significant difference in the larval performance of females (developmental time), but the difference was small. Developmental time was the insect trait most affected in earlier sawfly–needle quality experiments in which *N. sertifer* larval performance was related to resin acid content (Larsson et al., 1986a) and nitrogen content (Björkman et al., 1991). Thus, in response to suboptimal food quality, it appears as if, in general, sawfly larvae extend the length of their feeding period rather than accepting a reduction in body weight.

It is somewhat surprising that the great difference in taxifolin glucoside concentrations did not result in stronger effects on sawfly performance. Effects of flavonoids on other insects have been reported at much lower concentrations. For example, flavonoid concentrations of 0.1–0.4% reduced growth in *Heliothis zea*, *H. virescens*, and *Pectinophora gossypiella* (Shaver and Lukefahr, 1969). Similarly, *Heliothis zea* larval weight was reduced by about half on control medium when 0.3% (fresh weight) of rutin was added (Isman and Duffy, 1982). Pine sawflies, which are oligophagous, might be less sensitive than polyphagous insect species to variation in the composition of their preferred host plants (cf. Barbosa, 1988). Most insect species for which flavonoids have been shown to reduce performance have a broader host range than the sawflies studied here, i.e., *Heliothis zea*, *H. virescens*, *Pectinophora gossypiella*, and *Schizaphis graminae* (Shaver and Lukefahr, 1969; Waiss et al., 1979; Elliger et al., 1980, 1981; Isman and Duffey, 1982; Hedin et al., 1983; Duffey et al., 1986).

In addition, there may be differences in bioactivity between flavonoid compounds depending on their molecular structures. Elliger et al. (1980) reported that *H. zea* larvae fed an artificial diet containing 3.5 mmol/kg (=0.11% fresh weight) of dihydroquercetin (=taxifolin), the aglycone of taxifolin glucoside, grew 50% more slowly than controls. However, there are probably important

differences in the mode of action between taxifolin and the taxifolin glucoside. Flavonoids possessing a catecholic B-ring seem to be active against insects, whereas those with other substitution patterns generally are not (Elliger et al., 1980). Taxifolin has a catecholic B-ring while the 3′-*O*-glucoside is glucosylated on the B-ring, and therefore does not possess the structure that presumably is active.

Thus, as in certain other plants (e.g., Yu, 1989) the aglycone appears to be more toxic to insects than the glucoside. Free taxifolin occurs in very small concentrations in Scots pine needles (L. Lundgren, unpublished data). Many herbivorous insects are known to enzymatically cleave glycosidic bonds, releasing sugar and the aglycone (Lindroth, 1991). Further studies will be needed to determine whether such a process takes place in sawfly larvae, which would indicate that they have evolved a mechanism for tolerating taxifolin (cf. Lindroth et al., 1987). Presently, all that we can conclude is that high needle concentrations of taxifolin glucoside have a minor effect on sawfly larval performance.

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BIOLOGICAL ACTIVITY AND IN VIVO DEGRADATION OF TRITIATED FEMALE SEX PHEROMONE IN THE MALE EUROPEAN CORN BORER

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Abstract—Isomers of [11,12-$^3H_2$]-11-tetradecenyl acetate (57 Ci/mM) were synthesized. Behavioral assay of the two compounds using Z- and E-type European corn borer (ECB) males showed that introduction of tritons into the double bond of the pheromone caused a significant isotope effect in the E-type ECB but not in the Z-type ECB. Measurements of tritium associated with the male antennae after a 3-min exposure showed that radioactivity equivalent to 10^{-17} mol pheromone was adsorbed onto male antennae. Time-course in vivo metabolic studies with picogram amounts of compound applied topically to antennae of E- and Z-type males and Z-type females showed that they metabolized pheromone similarly but females degraded pheromone more slowly than males. Pheromone was hydrolyzed, and the only other major radiolabeled metabolite observed by combined high-pressure liquid chromatography-radiodetection was tritiated water. Capillary gas chromatography and radiomonitoring permitted detection of a trace amount of 11-tetradecenoic acid, which indicated alcohol oxidase activity is associated with the antennae. Evidence shows that clearing of pheromone from the ECB male antennae involves hydrolysis and oxidation of the alcohol to fatty acid, which in turn is degraded, probably via β-oxidation, to carbon dioxide and water.

Key Words—Lepidoptera, Pyralidae, *Ostrinia nubilalis*, European corn borer, tritiated pheromone, 11-tetradecenyl acetate, sensory biochemistry, catabolism.

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INTRODUCTION

Ostensibly, the role of the catabolic enzymes in moth olfaction is to maintain chemoreceptive sensitivity to pheromone through rapid clearing of resident pheromone from antennal structures and to thereby maintain a high level of sensitivity to fresh incoming chemical signals. Radiolabeled sex pheromones have proven to be useful in the study of pheromonal catabolism in a number species of moths. The radioactive probes have expedited discovery and elucidation of esterase, epoxide hydrase, alcohol oxidase, and aldehyde dehydrogenase activities in the moths (Prestwich et al., 1989a; Tasayco and Prestwich, 1990; Ding and Prestwich, 1986). However, with the exception of studies with the polyphemus moth (*Antheraea polyphemus*) and the silk moth (*Bombyx mori*) by Kasang et al. (1989a,b), most pheromone catabolism studies have involved identification of only the first intermediary catabolic product formed after pheromone contacts the antenna. The intent of our present work was to elucidate degradative pathways and metabolites beyond the first product.

Much has been accomplished in studies of first-product metabolites of pheromones. As examples, Prestwich et al. (1989b) discovered that antennae of gypsy moth (*Lymantria dispar*) males contain epoxide hydrolase activity, which transforms the epoxide moiety of the gypsy moth pheromone to a diol, but the metabolic fate of the diol was not investigated. Tasayco and Prestwich (1990) demonstrated conversion of pheromonal aldehydes of the tobacco budworm to carboxylic acids but did not determine the fate of the acids. Ferkovich (1982) monitored hydrolysis of the cabbage looper pheromone component, (*Z*)-7-dodecenyl acetate, but the fate of the alcohol was not studied. In these examples, the research was focused upon the first step of pheromone catabolism because the first product was either known or assumed to be behaviorally inert and a simple one-step conversion presumably fulfilled the pheromone-clearance requirement for maintenance of optimal olfactory sensitivity. However, the assumption that a first product is behaviorally inert can be faulty. It is known, for instance, that such an assumption is not valid for the cabbage looper; males possess antennal chemoreceptor cells that are specifically responsive to (*Z*)-7-dodecenol (Grant and O'Connell, 1986), and the compound is a potent inhibitor of male behavior (Tumlinson et al., 1972; McLaughlin et al., 1974). Inasmuch as cabbage looper males show considerable electrophysiological and behavioral sensitivity to (*Z*)-7-dodecenol, then, provided the hypothetical role of catabolic enzymes in moth olfaction is correct, mechanisms for clearance of that compound from the chemosensory sensilla of the antennae must also exist and it is imperative to know what they may be. An analogous situation exists in case of the ECB (*Ostrinia nubilalis*). Gas chromatographic analyses of extracts of Z- and E-type ECB (Klun and Huettel, 1988) male antennae treated with 11-tetradecenyl acetate have shown that antennal esterase effectively hydrolyzes

the pheromone and that the alcohol subsequently also disappears from the antennae (Klun et al., 1991). Flight-tunnel assays with Z-type ECB males have shown that when (*Z*)-11-tetradecenol was coevaporated with its pheromone, male upwind flight response was inhibited (Schwarz et al., 1990). Thus, the alcohol formed by esterase operating on ECB pheromone is a behaviorally relevant substance, and it must necessarily be cleared from the antennal chemosensory sensilla; otherwise it could interfere with response to pheromone. We present evidence that provides insight into the metabolic fate of the alcohol in the ECB.

It is well known that compounds labeled with tritium, like all compounds containing isotopes, possess physical properties that are different from compounds composed of natural stable isotopes and these can cause what is referred to as an isotope effect (Evans, 1974). To our knowledge no one has ever attempted to ascertain what effect tritium might have upon the biological activity of an insect pheromone, despite the fact that a number of high specific activity pheromones have been prepared. Therefore, it became of interest to see if tritiated ECB pheromone might express an isotope effect at a behavioral level in the insect.

METHODS AND MATERIALS

[11,12-$^3H_2$]-11-Tetradecenyl Acetate. Preparation and purification of tritiated pheromone was conducted at the National Tritium Labeling Facility (NTLF) in Berkeley, California, where appropriate facilities exist for handling carrier-free tritium. All synthetic and purification procedures used at NTLF were developed and tested in advance at Beltsville using deuterium. The semitritiation method was patterned after that reported by Ding and Prestwich (1986). 11-Tetradecynyl acetate (0.03 mM) in 2 ml hexane was transferred to a reaction vessel fitted with a magnetic stirring bar and attached to a gas manifold system. Five milligrams of Lindlar catalyst (5% palladium on $CaCO_3$-Pb poisoned; Strem Chemicals Inc., Newburyport, Massachusetts) were placed in a glass spoon above the hexane solution. The reaction vessel was closed, the hexane solution degassed by three freeze–pump–thaw cycles with nitrogen and liquid nitrogen cooling. Tritium, desorbed from uranium, was then delivered to the evacuated reaction vessel at room temperature until its internal pressure was at atmospheric pressure. The Lindlar catalyst was added to the hexane solution, and the reaction mixture was stirred for 5 min. In model reduction reactions with deuterium, the 5-min reaction time caused reduction of one half of the alkyne; partial reduction minimized the formation of secondary products and facilitated isolation of pure product. Tritium and hexane were removed from the reaction vessel under vacuum. The residue was washed with three separate 1-ml aliquots of methanol that were successively added to the reaction mixture and

removed under reduced pressure to rid the preparation of exchangeable tritium. Toluene (2 ml) was added to the residue, and the mixture was filtered through a 0.5-μm Millex-SR filter (Millipore Corp., Bedford, Massachusetts) into a Luer-lock syringe. The toluene solution was concentrated to 0.4 ml under a nitrogen gas flow and injected onto two 25-cm × 10-mm-ID 10-μm Spherisorb silica (Phase Sep, Norwalk, Connecticut) chromatographic columns plumbed in series and eluted with toluene pumped at 4 ml/min. The chromatography was designed to resolve reduction products from unreacted alkyne. Column effluent was monitored using a solid scintillant detector cell and a refractive index detector. The reduction products were collected in ca. 8 ml toluene from 10 min to 12 min after injection. The toluene solution containing products was concentrated to 0.3 ml and injected onto a 31-cm × 10-mm-ID 20% $AgNO_3$–5-μm Spherisorb silica column eluted with toluene at 3 ml/min to obtain ca. 0.56 Ci pure *Z* isomer (T_r = 14.5 min).

Isomerization and Tritiated trans *Isomer Isolation.* One half of the purified [11,12-$^3H_2$]-(*Z*)-11-tetradecenyl acetate in 0.25 ml toluene was transferred to a 2-ml screw-cap vial and 60 μg thiophenol in 2 μl toluene and 34 μg 2,2′-azobis-(2-methylpropanenitrile)–2 μl benzene were added to the sample. The vial was closed, placed into a high-pressure containment flask and warmed at 103°C in a sand bath for 1.5 hr to cause formation of a 6:4 *E*:*Z* equilibrium mixture (Schwarz et al., 1986). Approximately 0.17 Ci pure *E* isomer (T_r = 9.9) was isolated by using argentation chromatography described previously.

The specific activities of the *Z* and *E* isomers were determined by NMR. Specific activity measurements are usually made by liquid scintillation (LS) counting of a known mass of compound, by mass spectrometry, or by determination of the [^{3}H]- and [^{1}H]NMR spectrum of a compound. The NMR technique has been used to analyze compounds such as [^{3}H]$NaBH_4$, where the other methods are not facile, and NMR is most readily applied when the level of replacement of ^{1}H by ^{3}H at specific positions in the molecule is greater than 10% (Bloxsidge et al., 1971; Altman and Thomas, 1980). In our case, the method involved integration of residual protons at the tritiated site, carbons 11 and 12 (δ = 5.5 ppm) relative to integration of protons at carbon 1 (δ = 4.0 ppm). Requisites and assumptions for use of NMR in specific activity determinations are: the compound must be chemically and radiochemically pure, the residual protons are assumed to have the same relaxation time as the internal reference protons, the proton spectrum must be sufficiently resolved to allow integration of the sites of interest, and the specific radioactivity of the compound containing one tritium per molecule is assumed to be 28.72 Ci/mM. Specific activity determinations by NMR spectroscopy are subject to an error of ±10%. (Dr. Philip Williams, NTLF personal communication). [^{1}H]- and [^{3}H]NMR spectra were recorded in deuterated benzene, on an IBM AF-300 NMR spectrometer (^{3}H at 320 MHz, ^{1}H at 300 MHz), using a ^{3}H/^{1}H 5-mm dual probe.

Samples were made to a volume of 200 μl in Teflon tubes (Wilmad, #6005), which were then placed into 5-mm glass NMR tubes having screw caps (Wilmad, 507-TR-8″). A high-quality ^{3}H band stop–^{1}H band pass filter (Cir Q-Tel Inc., FBT/20-300/3-6/50-3A/3A) was placed in the proton decoupling line of the instrument, and the observed channel had an in-line ^{1}H band stop–^{3}H band pass filter. Tritium and proton spectra were acquired over ca. 12 ppm, using a 5-sec total recycle time and excitation pulse of 3.6 μsec (^{3}H, 65°) and 2 μsec (^{1}H, 25°). All spectra were acquired at 297K with the sample spinning. Referencing of tritium chemical shifts was achieved by generation of a ghost ^{3}H tetramethylsilane (TMS) signal from internal TMS in the [^{1}H]NMR spectrum.

Glass ampoules containing approximately 100 mCi *Z* isomer or *E* isomer in 5 ml toluene were transported to Beltsville and stored at −80°C to await use. During the course of study, all tritiated compounds were repurified every four to eight weeks.

Derivatives of Tritiated Pheromone. The radiolabeled acetates were used to prepare corresponding 11,12-^{3}H alcohols and aldehydes using synthetic methods similar to those described by Ding and Prestwich (1986). [11,12-^{3}H]-(*Z*)-11-tetradecenoic acid was prepared by oxidation of aldehyde using Ag_2O as oxidizing agent (Campaigne and LeSuer, 1963). Tritiated derivatives were used in metabolic studies and as chromatographic references.

Liquid Chromatography and Radioisotope Detection. All high-pressure liquid chromatographic (HPLC) analyses or preparative purifications of compounds involving tritium were conducted using a Beckman System Gold (Beckman Instruments, Fullerton, California 92634) chromatographic system equipped with a Beckman model 171 radiochromatography detector that was fitted with either a solid scintillator (Ultra-Scint) or a liquid cell. In liquid cell applications, Beckman Ready Flow liquid scintillant was pumped at 2 ml/min and mixed with the chromatographic column effluent (1 ml/min). The solid scintillator cell was used when compounds were purified by using a 43.5-cm × 3-mm 5-μm Spherisorb silica column eluted with an appropriate mixture of hexane–ethyl acetate. The hexane–ethyl acetate was removed in a nitrogen gas flow, and the sample was taken up into heptane solution to form a stock solution whose tritium composition was quantified by liquid scintillation counting. The stock solution was then used in assays and in preparation of appropriate dilute heptane solutions.

Most analyses of the extracts of insect tissues for tritiated pheromone and its metabolites were performed using a 25-cm × 3-mm-ID column packed with 5-μm Spherisorb ODS (octadecylsilane coated silica), acetonitrile–water (85 : 15) pumped as eluant, and the liquid cell detector. Tritium detection efficiency of the liquid cell was 20% for organics and 17% for water. The minimum detection limit for tritiated pheromone was 400 dpm. The retention times of pheromone and other reference compounds in HPLC were established by using tritiated

reference compounds that we synthesized or purchased from NEN, DuPont Company, Boston, Massachusetts 02118. In instances where reference compounds were not available with a radiolabel, the HPLC effluent was monitored by using a Waters Associates model 401 differential refractometer to determine their retention characteristics relative to radiolabeled components in insect extracts.

Normal liquid scintillation (LS) counting was done by using a Beckman model LS 6000 counter and 20 ml Ecoscint LS cocktail (National Diagnostics, Manville, New Jersey 08835). Detection efficiency was 23% for tritiated water and 39% for tritiated organic compounds. Counting efficiency was verified by using external-standard tritiated NEN toluene or water calibration compounds.

Gas Chromatography and Tritium Detection. The chromatography was conducted using a Hewlett-Packard model 5880A gas chromatograph that was vented to a chemical fume hood. The instrument was fitted with a splitless injector, flame ionization detector (FID), and either a polar 60-m × 0.25-mm DB-Wax column (J & W Scientific, Folsom, California 95630) or a nonpolar 50-m × 0.32-mm Ultra 1 (cross-linked methyl silicone gum, Hewlett-Packard, Palo Alto, California 94304). The oven temperature program for both columns was 90°C for 0.4 min after injection, heating at 2°C/min to 120°C followed by heating to 175°C at 30°C/min. Hydrogen was used as carrier gas at 50 cm/sec flow. Gas flows through the FID detector were 300 ml air, 60 ml hydrogen, and 60 ml nitrogen. A detector-insert tube normally used for measurement of FID gas flows was positioned in the chimney of the detector, and the occurrence of tritiated water, formed by hydrogen-flame combustion of compounds eluting from the column, was monitored throughout each chromatographic run. FID water was collected at 1-min intervals into disposable 23-cm glass Pasteur pipets that were inserted through a 17-cm × 8-mm-ID glass sleeve cooled with Dry Ice and over the detector effluent flow tube (3.2 mm OD). The collections were made from the time of injection to 2500 (Kovats retention index) on DB-Wax and from injection to 2200 on Ultra 1. Water collected in each pipet was rinsed with Ecoscint into a LS vial and assayed in the LS counter. Tests in which picogram amounts of tritiated pheromone were injected onto the columns showed that ca. 30% of the radioactivity injected was recovered in the FID water coincident with the retention time of the compound on the column. This methodology permitted us to analyze antennal extracts for tritiated pheromone and its metabolites by capillary gas chromatography (GC). In advance of such analyses, retention time reference points were established by injecting unlabeled standard mixtures of C_7–C_{14} saturated fatty acid methyl esters, 11-tetradecenyl acetate, 11-tetradecenol, 11-tetradecenal, and methyl-11-tetradecenoate isomers onto the capillary columns.

Analyses of Pheromonal Metabolites via GC. Previous pheromone catabolism studies in several species of moths showed that pheromones were often

oxidized to carboxylic acids (Kasang et al., 1989a,b; Lonergan, 1986; Prestwich et al., 1989a; Rybczynski et al., 1989). Therefore, we conducted a specific search for fatty acid metabolites of pheromone in the ECB by conducting an experiment with a large amount of radiolabeled pheromone. A pooled chloroform extract of the antennae of Z-type male ECB was prepared by treating the antennae of each of 30 males with 123.5×10^3 dpm *Z* pheromone. After a 6-min in vivo incubation, antennae were excised into 300 μl chloroform. The combined extract was filtered, 10 μl was analyzed on the ODS HPLC to determine tritium recovery and composition of the sample, and the remainder was reacted with an excess of diazomethane in diethyl ether to convert putative fatty acids in the extracts to methyl esters. The methylated sample was reanalyzed (10 μl) on ODS, concentrated in a nitrogen gas flow to 10 μl, and chromatographed on the DB-Wax and Ultra capillary columns as described earlier to determine the retention times of radiolabeled metabolites in the extracts. This experiment was replicated twice.

Bioassay of Tritiated versus Nonradioactive Pheromone. A glass closed-system device (Figure 1) for bioassay of the pheromones was fabricated using a 65/40 standard glass joint. The tube size on the socket end was 6 cm × 40 mm ID, and it ended with 3 cm × 4 mm ID tube to which a Luer connector was cemented. The tube length on the ball end of the joint was 8.5 cm, and it was constricted and ended with a 18/9 socket joint. A sintered glass 40-mm-diam. disk drilled with 21 1-mm-diam. uniformly distributed holes was fitted

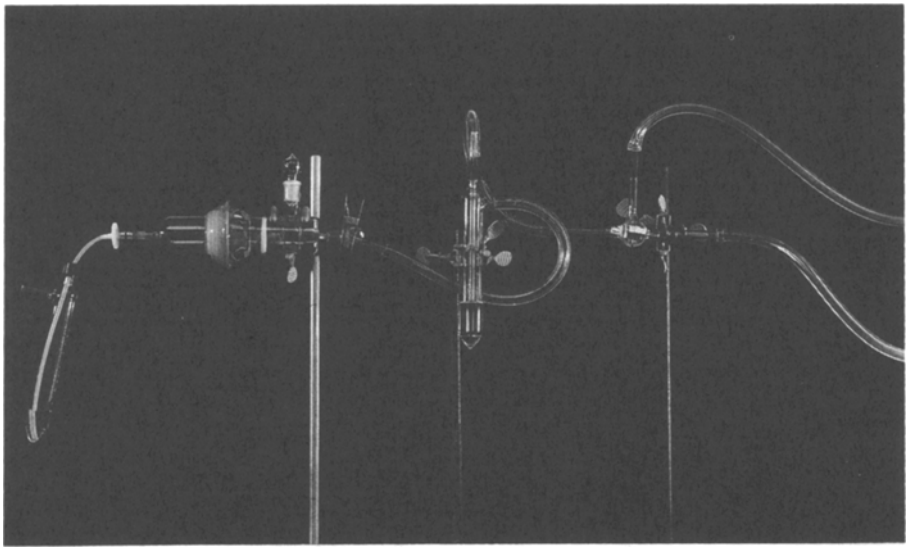

FIG. 1. Apparatus for bioassay of high specific activity tritiated sex pheromone.

across the diameter of the tube 5.4 cm from the end of the ball joint. The eventual function of the disk was to serve as a barrier between a pheromone source and moths used in the assays. A 14/20 standard taper joint and stopper was located perpendicular to the tube of the ball joint and 8.5 cm from the ball joint end. The 14/20 stopper was fitted with a nichrome wire to which an alligator clip was attached. The ball-joint half of the device was clamped to a ring stand in a fume hood. Tygon tubing was used to connect a tank of compressed breathing-quality air and a tank of CO_2 to a water-filled bubbler via a three way T valve, and the bubbler effluent was connected to a 18/9 ball joint, which was attached to the bioassay device with a pinch-type clamp. A Millex filter was loaded with 2 mg KB grade Darco activated carbon and attached to the Luer connector of the socket portion of the 65/40 joint to contain tritiated pheromone. Tritium containment was verified in advance of conducting any bioassays by passing tritiated pheromone laden air through the system for 20 min while effluent from the filter was bubbled through ethylene glycol; LS counting showed no tritium in the ethylene glycol.

In the bioassays, a 48- to 72-hr-old male moth was transferred to the socket portion of the device, and it was connected to the ball joint with a pinch-type clamp. All assays were conducted at 20–21 °C under low-intensity red light (ca. 2.5 lux) when males were in the second to fourth hour of their 8-hr scotophase. Radioactive pheromones and nonradioactive pheromones were assayed in separate but identical apparatus. Geometrically pure *Z*- (14 ng) or *E*- (12.5 ng) tritiated or an equal amount of the respective nonradioactive isomer in 1 μl heptane was applied to a 6-mm × 6-mm filter paper that was attached to the alligator clip and inserted upwind of the male and just beyond the sintered disk. Each pheromone-treated filter paper was used successively to test four to six males before it was discarded. Humidified air was delivered through the bioassay unit at 33 ml/sec, and each male was observed and exposed to stimulus for 3 min. A positive response was recorded if the male displayed precopulatory behavior (extension of genitalia, wing vibration, and clasper response) in the exposure period and a negative response was recorded if he did not display the behavior. The unit was then purged with CO_2, the anesthetized moth was removed, and the male's antennae were excised into Ecoscint for LS counting. Antennae of males exposed to nonradioactive pheromone were processed in the same way and LS counts obtained with them served as a measure of background radiation. Testing was replicated over time, each male represented a replicate, and equal numbers of males within ECB pheromonal types were tested against equal amounts of radioactive and nonradioactive pheromone. In all, the tests with Z-type and E-type males were replicated 52 and 84 times, respectively. The behavioral responses to radioactive and nonradioactive pheromones were compared, for both types of males, with a logistic analysis using the SAS CATMOD procedure (SAS, 1988). The LS counts per minute (cpm) for the two treatments within male types were compared by analysis of variance using

the SAS GLM procedure (SAS, 1988). The cpm difference observed between antennae exposed to tritiated pheromone and antennae exposed to nonradioactive pheromone represented above-background radiation, and it was converted to disintegrations per minute (dpm) by assuming that the tritium was in the form of water, a 23% LS efficiency. With a specific activity of 57 Ci/mM, 1 dpm represented 2.04 fg pheromone, and thereby the amount of radiolabeled pheromone adsorbed onto male's antennae in the 3-min exposure was calculated.

[11,12-$^3H_2$]-11-Tetradecenyl Acetate Metabolism in ECB Types. In the time-course studies of *Z* and *E* pheromone metabolism in Z- and E-type ECB males, respectively, and in the study of *Z* pheromone metabolism in Z-type females, one antenna of a CO_2-anesthetized moth (24–96 hr old) was treated with 106.5 $\times$ 10^3 dpm tritiated pheromone (ca. 220 pg) in 0.5 μl heptane using a micrometer-driven 10 μl syringe and a dissection microscope. Heptane was used as solvent because previous study showed that treatment of the antennae of moths with this solvent did not impair male behavioral sensitivity to sex pheromone (Klun et al., 1991). Following the topical treatment, the antenna was then either immediately excised (T_0) at its base into 50 μl chloroform–methanol (2:1, v/v) or the moth was enclosed in a plastic jelly cup and permitted to incubate for 3 min (T_3), 6 min (T_6), or 12 min (T_{12}). The antenna was then excised into chloroform–methanol (CM), soaked 30 min, and removed from the solvent. The volume of the extract remaining was measured by drawing all of the solvent into a 50-μl Hamilton syringe and then half the sample was analyzed by ODS HPLC. Using this technique, we monitored degradation of pheromone, formation of radiolabeled metabolites, estimated tritium recoveries, and followed changes in the proportional distribution of tritium between pheromone and its metabolites as a function of time. Mean proportions of tritium were plotted as a function of incubation time. Arbitrarily, best-fit simple linear regression equations for the data points were computed and drawn by using a personal computer with Cricket Graph software for all metabolic data. Tests were replicated 12 times in the case of E-type males and six times with the other male type and females.

[11,12-$^3H_2$]-11-Tetradecen-1-ol Metabolism. Alcohols were applied to the male ECB types using the same techniques as were used with the acetates. E-type males were treated with 128.4 $\times$ 10^3 dpm *E* isomer (287 pg) and Z-types were treated with 126.5 $\times$ 10^3 dpm *Z* isomer (258 pg). The tests were replicated six times with each type of insect.

RESULTS AND DISCUSSION

Reduction of alkyne with tritium proceeded more rapidly than it had with deuterium; no residual alkyne remained after 5 min of tritiation and significantly more saturated product was formed than had been observed in reductions using

deuterium. The yield was 13.4% tetradecyl acetate, 23.5% *E* isomer, and 63.1% *Z* isomer according to high-pressure liquid chromatography. However, the purification procedures yielded the desired pure high specific activity isomers of the ECB pheromone.

NMR spectroscopy of *E* pheromone showed a ^{1}H integrated intensity of 0.0985 at 5.5 ppm and 0.9215 at 4 ppm (protons of carbon 1). This indicated a specific activity (SA) of 51.29 Ci/mM at carbons 11 and 12. Comparison of the ^{3}H integrated intensity at 5.5 ppm (25.082) and 2 ppm (2.59) indicated that 5.29 Ci/mM was associated with carbons 10 and 13. Therefore the total estimated SA for the *E* pheromone was 56.6 Ci/mM. In the proton spectrum of the *Z* pheromone, the signal-to-noise ratio was such that an integral for residual protons at carbons 11 and 12 could not be obtained. We therefore assumed that the SA associated with those carbons was the same as observed in the *E* pheromone since it was derived from the *Z* compound. For the *Z* pheromone, the ^{3}H integrated intensity at 5.5 ppm was 20.690 and the integral at 2 ppm was 2.391, indicating 5.93 Ci/mM was associated with carbons 10 and 13. Thus, the SA estimated for the *Z* pheromone was 57.2 Ci/mM. Inasmuch as SA values for both isomers are subject to a 10% error, we concluded that the nominal SA for each isomer was 57 Ci/mM.

Results of behavioral assays with tritiated and nonradioactive pheromones showed that tritiated *Z* isomer elicited behavioral responses from Z-type males as effectively as natural pheromone. On the other hand, tests with tritiated *E* isomer showed that it was significantly less effective in evoking behavioral responses from the E-type ECB than the protonated counterpart (Table 1). Thus, incorporation of tritium into the *E* pheromone caused a significant isotope effect; the Z-type borer was deceived by our tritiated analog, but the E-type was not. Previous pheromone-structure × activity relationship studies (Schwarz et al., 1990) have proven that the olfactory systems of the E- and Z-type ECB are quite dissimilar in their organization, inasmuch as the types often responded differently to identical analogs of their pheromones. Therefore, we were not

TABLE 1. COMPARATIVE BIOLOGICAL ACTIVITY OF TRITIATED AND NATURAL EUROPEAN CORN BORER FEMALE SEX PHEROMONES IN BIOASSAYS WITH *E*- AND *Z*-TYPE MALES[a]

	Mean percentage positive response	
Male type	Tritiated Pheromone	Natural Pheromone
Z (*N* = 52)	53.8 a	65.4 a
E (*N* = 84)	36.3 b	66.1 a

[a]Means followed by the same letter in each row are not significantly different according to the logistic analyses of variance. *N* = number of replicates.

surprised that the two types also reacted dissimilarly to the tritiated analogs. It is not known whether the mechanistic basis for the differential responses lies at the receptor level, at the central nervous system processing level, or in a combination of the levels.

Liquid scintillation counting data from antennae exposed to airborne pheromone in the 3 min bioassays showed that the above-background count associated with antennae exposed to tritiated pheromone was 60.4 ± 12.5 dpm ($SE_{diff.}$; $P = 0.05$) and 43.4 ± 8.7 dpm ($SE_{diff.}$; $P = 0.05$) for Z- and E-type males, respectively. These data indicate that a minimum of 123 ± 25 fg pheromone was adsorbed by Z-type male antennae and 88 ± 18 fg was adsorbed by E-type males. This represents 10^{-17} mol pheromone/pair of antennae/3 min exposure for both types. The amount provides insight as to how little compound impinges on the moth's antennae when it is exposed to a physiologically relevant dose of pheromone. The amount estimated to be associated with antennae is probably an underestimation because some tritium may have been internalized and not extracted by the LS cocktail. Owing to the low energy of the tritium β emission, such radiolabel would have gone undetected.

In an earlier study with tritiated pheromone, Kasang et al. (1989) estimated that a pair of male silk moth (*Bombyx mori*) antennae adsorbed 40–400 pg pheromone (bombykol)/10 sec from a 50 ml/sec pheromone-laden airstream. This value is a 1000-fold greater than we observed to be adsorbed on ECB antennae. Although it is difficult to compare results directly, the greater amount adsorbed by the silk moth may be due, in part, to the comparatively larger size of its antennae, but it is unlikely that this can account for the 1000-fold difference in observed values.

Results of time-course metabolism studies with tritiated pheromone applied to antennae of the two types of males and Z-type females showed formation of two radiolabeled metabolites according to ODS HPLC analyses. The retention time of one metabolite was coincident with the tritiated water standard; it eluted at void volume. The other metabolite was coincident with the 11-tetradecenol and 11-tetradecenal standards on ODS. We rechromatographed an aliquot sample of the extracts on the Spherisorb silica HPLC column and found that the metabolite eluted coincident with alcohol. Gas chromatographic analyses, described subsequently, also confirmed identity of the alcohol.

Data in Figure 2A and 2B show that at T_0 (ca. 5–10 sec incubation) almost all acetate applied to the E- and Z-type ECB males was converted to alcohol. With increasing incubation time, the proportion of tritiated alcohol and acetate declined as the proportion of tritiated water increased. The rate of increase in the tritiated water proportion (slope = 4.8) was similar in the two types of ECB males, showing that catabolism proceeded at a similar rate in each type. Measurements of tritium recoveries from the male antennae in each incubation period (Figure 3) showed that they were similar in each type of male; only ca. 40%

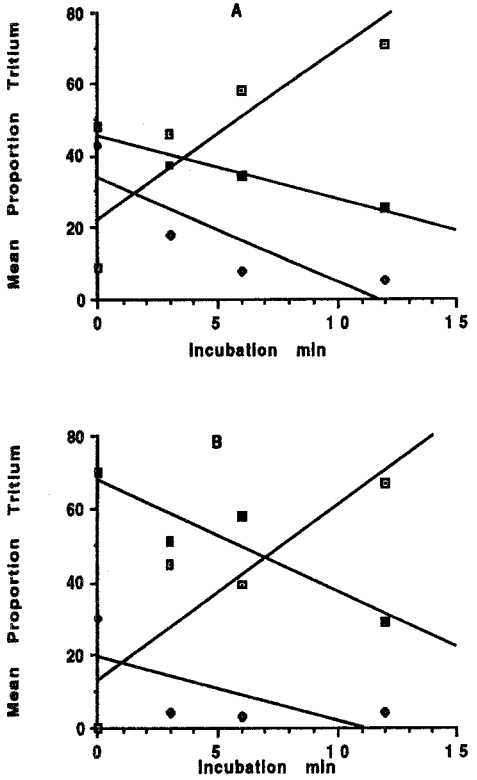

FIG. 2. Mean proportion tritium in antennal extracts of ECB as a function of incubation time. (A) E-type males; (B) Z-type males. Diamond = 11-tetradecenyl acetate Open square = water, Closed square = 11-tetradecenol.

was recovered at T_0, and by T_{12} recovery fell to ca. 15%. The radiolabel loss is attributed to evaporation and diffusion of the tritium from the antennae to the body of the insect. Data obtained from females (Figure 4) showed that females catabolized pheromone in the same manner as the males. However, the relatively slower proportional rate of increase of tritiated water in the females (slope = 2.8) indicated that the catabolism proceeded more slowly in the females than in males.

When isomers of tritiated 11-tetradecenol were applied to the male antennae and incubated 0–12 min, results showed (Figure 5) that changes in the proportion of tritium in alcohol and the proportion in water were inversely related over time. This is evidence that the alcohol serves as an immediate substrate for an aggressive oxidative process.

In our search for intermediary fatty acid metabolites of pheromone, an

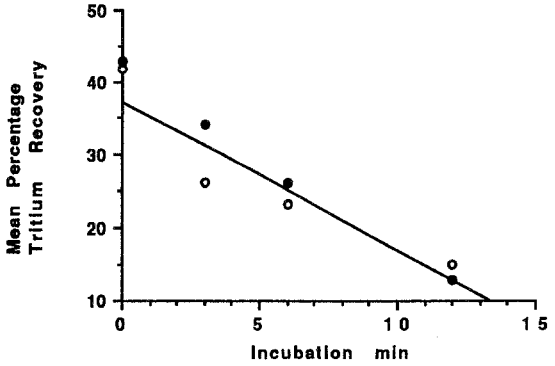

FIG. 3. Percentage recovery of tritium applied to antennae of E-type (solid dot) and Z-type ECB (open dot) males in 0- to 12-min incubations.

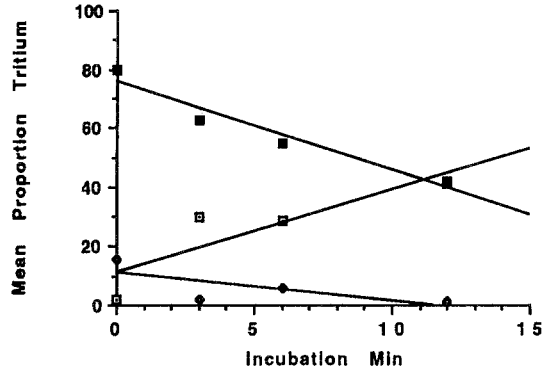

FIG. 4. Time-course conversion of tritiated (Z)-11-tetradecenyl acetate on the antennae of Z-type ECB females. Symbols: see Figure 2.

average of 9×10^5 dpm were recovered (24% of total applied tritium) in the combined extracts of 30 male antennae. ODS HPLC showed that the extracts contained 81.5% tritium in the form of 11-tetradecenol and 18.5% in water. Neither acetate nor any other labeled product was detected before or after the extract was treated with diazomethane. Capillary GC analyses of the derivatized extracts on the polar and nonpolar columns consistently showed elution of tritium at retention times coincident with (Z)-11-tetradecenol, (Z)-11-tetradecenyl acetate, and methyl-(Z)-11-tetradecenoate. More than 90% of the FID-recovered tritium resided with the alcohol, 6% was in tetradecenoate, and the remainder was in acetate. Small amounts of radioactivity, barely discernible above background, were seen in early FID-effluent collections (2–4 min) where water elutes. Most of the tritiated water detected in HPLC analyses of the extracts was prob-

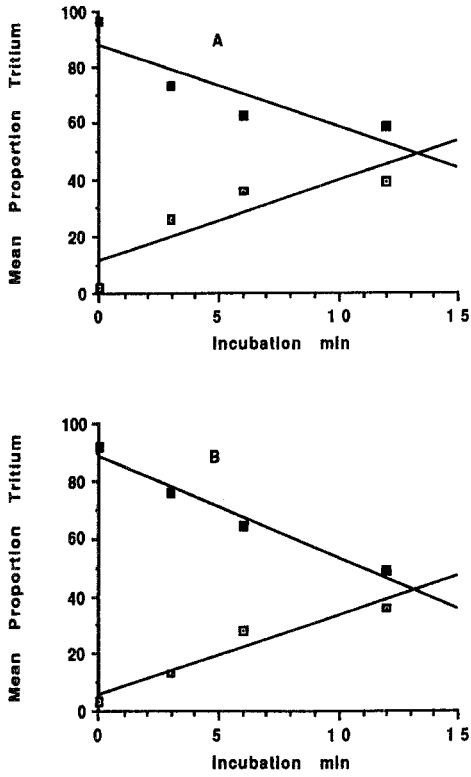

FIG. 5. Mean proportion tritiated alcohol and water in extracts of antennae of E-type (A) and Z-type (B) males treated with tritiated 11-tetradecenol and incubated in vivo for 0–12 min. Symbols: see Figure 2.

ably lost through evaporation when the extracts were concentrated for the GC analyses. Moreover, the capillary GC results proved that pheromone is converted to the corresponding fatty acid, but the size of this metabolic pool was well below detection limits in HPLC analyses of extracts from individual moths.

The data suggest two possible catabolic pathways for pheromone: the degradation may have proceeded via hydrolysis followed by direct attack upon the double bond of the alcohol to rapidly yield radiolabeled water, or by hydrolysis, conversion of the alcohol to acid and β-oxidation of the fatty acid chain. If oxidation of the double bond or ω-oxidation was a primary catabolic pathway, we should have detected a metabolite indicative of an attack upon the terminal end of the molecule. This was not the case, but having detected tetradecenoic acid, there is good evidence for oxidative attack of the functional end of the molecule. Therefore, β-oxidation is the more plausible catabolic pathway. The

free fatty acid probably represents a metabolic pool that is intermediate to transfer of the fatty acyl to coenzyme A, and the tritiated water is likely a result of rapid β-oxidation of the carbon chain in the mitochondrial matrix (Stumpf, 1969). Studies of the ultrastructural characteristics of chemosensilla (Zacharuk, 1980) have shown that mitochondria are among the most abundant organelles in cells that comprise the antennal chemosensory sensilla. A pair of male ECB antennae are known to be clothed with more than 14×10^3 chemosensory sensilla (Cornford et al., 1973), and their abundance and heavy provisions of mitochondria make them the probable site for the β-oxidation.

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FILAMENTOUS NATURE OF PHEROMONE PLUMES PROTECTS INTEGRITY OF SIGNAL FROM BACKGROUND CHEMICAL NOISE IN CABBAGE LOOPER MOTH, *Trichoplusia ni*

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Abstract—(Z)-7-Dodecenol failed to interrupt pheromone-mediated anemotactic responses by male cabbage looper moths, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) in a wind tunnel when released 5 cm crosswind on both sides of the pheromone source or 10 cm upwind of the source to create an overlapping plume downwind. Significant inhibitory effects of (Z)-7-dodecenol were observed when released with the six-component pheromone blend from the same septum or abutting septa. These results indicate that (Z)-7-dodecenol needs to be received simultaneously with the pheromone blend to inhibit the anemotactic responses of males to the sex pheromone. We suggest that this feature and the filamentous nature of pheromone plumes render pheromone signals relatively protected from background chemical noise that may originate from pheromone plumes of other insect species. Unless filaments from a pheromone signal and an inhibitor arrive simultaneously, the integrity of the signal is maintained.

Key Words—*Trichoplusia ni*, Lepidoptera, Noctuidae, sex pheromone, inhibitor, (Z)-7-dodecenol, wind tunnel, orientation.

INTRODUCTION

A male moth orienting upwind in a pheromone plume does not encounter a uniform pheromone signal, as some time-averaged models of plume structure might suggest (Wright, 1958; Bossert and Wilson, 1963; Sower et al., 1973;

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Nakamura, 1976). Rather, pheromone plumes are discontinuous in nature and are composed of many filaments (Wright, 1958; Murlis and Jones, 1981; Baker et al., 1985). In fact, the filamentous nature of pheromone plumes appears to be important in sustaining upwind flight (Baker et al., 1985). The filamentous nature of pheromone plumes may also render pheromone signals relatively protected from background chemical noise. Although the pheromone plumes of different species may overlap spatially, it is less likely that individual pheromone filaments within the overlapping plumes will mix to a great extent. The integrity of the signal may be maintained within some filaments. Thus the pheromone-mediated communication system within a species may be relatively protected if insects can sense and respond to those intact pheromone filaments without being affected by subsequent detection of filaments that contain chemical noise or contaminated pheromone signals.

This concept was tested using the cabbage looper moth, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae). The pheromone blend of female *T. ni* consists of (Z)-7-dodecenyl acetate (Z7-12:Ac) (Berger, 1966), dodecyl acetate (12:AC), (Z)-5-dodecenyl acetate (Z5-12:Ac), 11-dodecenyl acetate (11-12:Ac), (Z)-7-tetradecenyl acetate (Z7-14:Ac), and (Z)-9-tetradecenyl acetate (Z9-14:Ac) (Bjostad et al., 1984). (Z)-7-Dodecenol (Z7-12:OH) inhibits the anemotactic responses of male *T. ni* to pheromone sources (Tumlinson et al., 1972; McLaughlin et al., 1974; Bjostad et al., 1984; Linn et al., 1984). This compound has been found in gland extracts and volatile collections from forcibly extruded pheromone glands of female *T. ni* (Bjostad et al., 1984; Haynes and Hunt, 1990), but apparently it does not play a role in the normal pheromone signal since it is not released by calling females (Haynes and Hunt, 1990). Male *T. ni* have specialized sensory receptors for Z7-12:OH (O'Connell et al., 1983; O'Connell, 1985). It is possible that responding to Z7-12:OH may play a role in preventing responses to other species. In this wind-tunnel study, we demonstrate that the inhibitor Z7-12:OH must be received simultaneously with the pheromone blend to interrupt the normal anemotactic response of the moth to the pheromone source. This finding supports the concept that pheromone signals are relatively protected from background chemical noise.

METHODS AND MATERIALS

Insects. Cabbage loopers were reared on a semisynthetic diet (Shorey and Hale, 1965). Males were separated from females as pupae. Emerged adults were removed daily and provided with 8% sugar water. Pupae and adults were kept in 3.8-liter paper cartons under 16:8 light-dark photoperiod and 27 ± 1°C.

Chemicals. All pheromone components and Z7-12:OH were of >99% purity (Pesticide Research Institute, Wageningen, The Netherlands). The pro-

portions of blend components in the six-component blend for rubber septa were as follows: 12:Ac (5%), Z5-12:Ac (10%), Z7-12:Ac (100%), 11-12:Ac (3%), Z7-14:Ac (3%), and Z9-14:Ac (2%). This blend ratio resulted in a released blend from a loaded rubber septum similar to that emitted by female *T. ni* (Hunt et al., 1990). All solutions were prepared in hexane (HPLC grade) and checked by gas chromatography (30-m Carbowax 20 M capillary column in a Hewlett-Packard 5890A gas chromatograph linked to a Hewlett-Packard 5970B mass selective detector) to ensure purity and blend ratios of pheromone components. Dilutions were made for all solutions to yield 0.2 μg/μl Z7-12:Ac or Z7-12:OH. A 50-μl aliquot of each solution (containing 10 μg Z7-12:Ac or Z7-12:OH) was added to the wide end of a rubber septum (Thomas Scientific, Swedesboro, New Jersey; 5 × 9 mm, preextracted with hexane). The loaded rubber septa were kept in a laboratory exhaust hood for 24 hr before being used in wind-tunnel flight experiments.

Test Procedures. Male *T. ni* (3 to 4 days old) were confined individually in 5-cm-diam. × 10-cm-high hardware cloth cages during the last hour of the photophase and placed in an environmental chamber under the same conditions as above. They were tested in a wind tunnel during the fifth through seventh hours of the scotophase under conditions of about 0.3 lux light intensity, 50 cm/sec wind velocity, and 27–30°C temperature.

A moth was released from the cage on a flat metal mesh surface 20 cm above the tunnel floor and 1.5 m downwind from the pheromone source, which was positioned 20 cm above the tunnel floor. Each moth was allowed about 1 min to respond after it left the cage. Behaviors of moths were scored as: wing fanning, taking flight, locking on (initiation of counter-turning), and source contact. In addition, the distance of closest approach to the pheromone source was recorded.

Experiment 1. Effects of Z 7-12:OH Released 5 cm Crosswind from Pheromone Source on Male Responses. The four treatments of this experiment included: (1) the full six-component blend, (2) the full blend and Z7-12:OH in the same septum, (3) the full blend and Z7-12:OH in different but abutting septa, and (4) the full blend with septa containing Z7-12:OH 5 cm from it across the wind line on both sides. A randomized complete-block design was used in both experiments. The rubber septa containing the pheromone blend or Z7-12:OH were fixed at the downwind edge of a 15 × 15-cm metal plate positioned 20 cm above the tunnel floor. A total of 75 males was tested for each treatment. Ryan's (1960) test was used to compare proportions of behavioral responses among treatments in both experiments.

Experiment 2. Effects of Z 7-12:OH Released 10 cm Upwind of Pheromone Source on Male Responses. The four treatments included: (1) the full blend, (2) the full blend and Z7-12:OH in the same septum, (3) the full blend and Z7-12:OH in different but abutting septa, and (4) the full blend with a Z7-12:OH

septum placed 10 cm upwind. Rubber septa corresponding to different treatments were supported with 2.5-mm-diam. copper wire 8 cm above the downwind edge of a 15 × 15-cm metal plate placed on a metal mesh surface 12 cm above the tunnel floor. In the last treatment, a rubber septum loaded with Z7–12:OH solution was supported with the copper wire 10 cm upwind of the pheromone source. For all treatments, a 15 × 15-cm metal plate was positioned further upwind with the downwind edge angled up 15° using a metal stand. The downwind edge was 1 cm above the septa level and 17 cm from pheromone sources. This created turbulence, which improved the behavioral responses to the six-component blend.

Using cigarette smoke, we demonstrated that this arrangement resulted in converging of the two smoke plumes about 10–15 cm downwind of the source. The two smoke plumes were generated 10 cm apart along the wind line at the positions of the pheromone source and Z7–12:OH as in the last treatment. The plumes appeared to completely overlap further downwind. A total of 65 male *T. ni* was tested for each treatment.

RESULTS AND DISCUSSION

Z7–12:OH reduced locking-on rates to pheromone plumes when it was released from the same septum as the pheromone or when the two septa, one containing Z7–12:OH and another with the full blend, were placed together. This resulted in significantly lower source contact rates than for the full blend (Figures 1 and 2). When released 5 cm crosswind on both sides of the pheromone source, Z7–12:OH failed to interrupt pheromone-mediated anemotactic flight. There were no significant differences in locking-on rate or source contact rate between the full blend with Z7–12:OH 5 cm crosswind and the full blend alone. The locking-on rate and upwind flight for the treatment with Z7–12:OH and the full blend from different but abutting septa were significantly higher than those for the treatment with Z7–12:OH and the full blend from the same septum, although the difference in source contact rate was not significant. For all four treatments, most moths that locked on to plumes succeeded in contacting the pheromone sources (Figure 1). Similar results were achieved in experiment 2 in which Z7–12:OH, when released 10 cm upwind of the pheromone source, failed to inhibit upwind flight of male *T. ni* (Figure 2).

The ineffectiveness of Z7–12:OH in interrupting anemotactic responses to the pheromone source by male *T. ni* when it was released only 5 cm crosswind on both sides of or 10 cm upwind of the source indicated that Z7–12:OH, as an inhibitor, must be detected simultaneously with the pheromone to influence the behavioral responses of the moth. Witzgall and Priesner (1991) drew the same conclusion based on a wind-tunnel study with *Coleophora laricella*. The

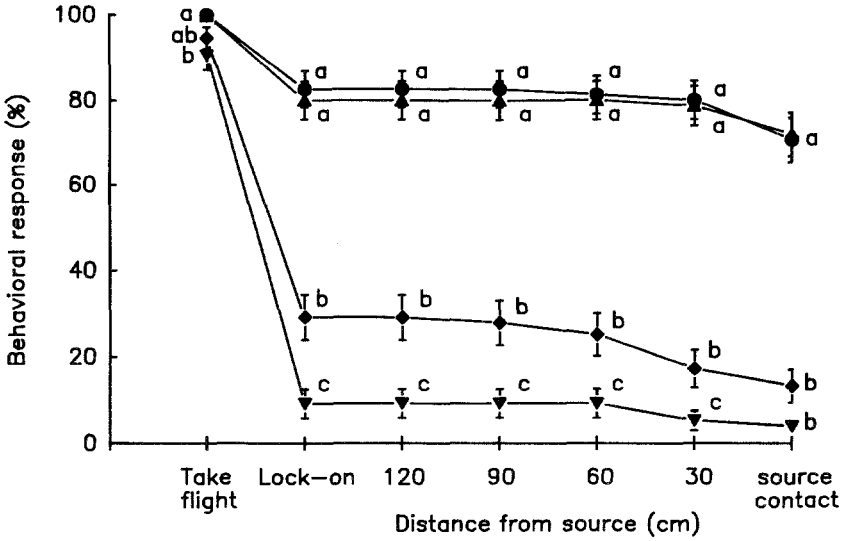

FIG. 1. Effects of Z7-12:OH released 5 cm crosswind on both sides of the pheromone source on responses of male *T. ni*. The four treatments were: (1) the full six-component blend (●), (2) the full blend and Z7-12:OH in the same septum (▼), (3) the full blend and Z7-12:OH released from different but abutting septa (♦), and (4) the full blend with Z7-12:OH septa 5 cm apart crosswind on both sides (▲). Vertical lines represent standard error of means. For each behavioral category, values with the same letter were not significantly different based on Ryan's multiple comparison test on proportions ($P > 0.05$) (Ryan, 1960).

inhibitor (Z)-5-decenyl acetate failed to interrupt responses of male moths to the pheromone (Z)-5-decenol when released 5 cm crosswind on one side of the pheromone source.

Several field studies support the conclusion that an inhibitor and a pheromone must be detected simultaneously to interrupt behavioral responses of moths. Z7-12:OH failed to inhibit the attractant responses by male *T. ni* when released in the proximity of a pheromone source (McLaughlin et al., 1974). Z9-12:Ac, Z9-14:Ac, or (Z,E)-9,12-tetradecadienyl acetate inhibits attractant responses of male *T. ni* to Z7-12:Ac when they were released separately within the same trap at a comparable release rate. When evaporated into the atmosphere surrounding pheromone traps, none of the three chemicals had a significant influence on the capture of male *T. ni* (Mitchell, 1976). In *Rhyacionia buoliana*, the geometrical isomer of its pheromone, (E)-9-dodecenyl acetate, inhibited attractant responses of males to the pheromone when they were released together. When dispersed around pheromone- or virgin female-baited traps, the inhibitory isomer did not suppress male orientation to the pheromone source (Daterman et

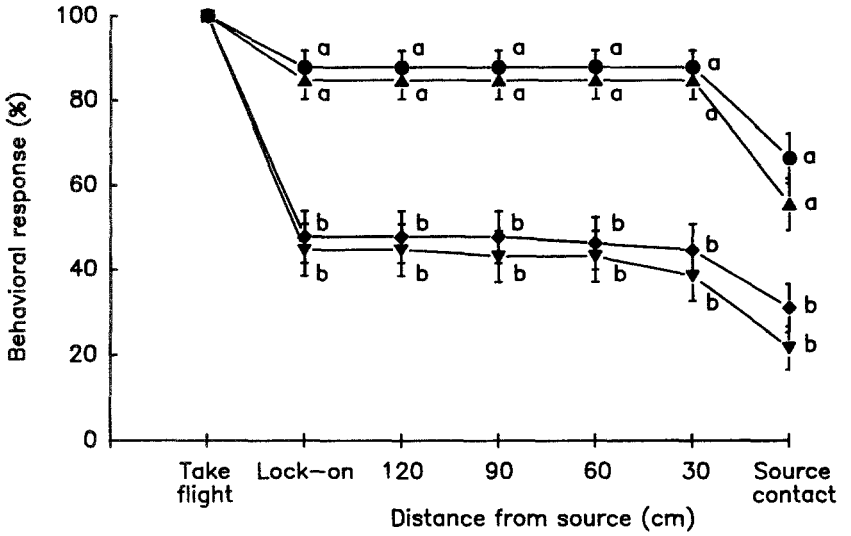

FIG. 2. Effects of Z7-12:OH released 10 cm upwind of the pheromone source on responses of male *T. ni*. The four treatments were: (1) the full blend (●), (2) the full blend and Z7-12:OH in the same septum (▼), (3) the full blend and Z7-12:OH released from different but abutting septa (♦), and (4) the full blend with Z7-12:OH septum placed 10 cm upwind (▲). Vertical lines represent standard error of means. For each behavioral category, values with the same letter were not significantly different based on Ryan's multiple comparison test on proportions ($P > 0.05$) (Ryan, 1960).

al., 1975). In *Coleophora laricella*, anemotactic flight of males towards the pheromone (Z)-5-decenol was not fully suppressed even with the same amount of the inhibitor (Z)-5-decenyl acetate placed only 2–5 cm apart. However, male responses to (Z)-5-decenol were totally suppressed when it contained only 3% (Z)-5-decenyl acetate (Priesner and Witzgall, 1984).

In the wind tunnel, plumes of Z7-12:OH and the pheromone overlapped each other as demonstrated with smoke. However, a plume is composed of many entangled filaments (Murlis and Jones, 1981; Baker et al., 1985). Although some mixing may occur between filaments containing pheromone and Z7-12:OH, respectively, especially far downwind from sources, large portions of these individual pheromone filaments may maintain their integrity. Thus, male moths may still detect intact pheromone filaments and detect Z7-12:OH filaments or Z7-12:OH-contaminated pheromone filaments sequentially as they fly upwind towards the pheromone source. Studies have revealed that moths are able to respond rapidly to individual pheromone filaments at the sensory level (Kaissling, 1986; Baker and Haynes, 1989). This suggests that a moth is able to detect closely spaced filaments of its pheromone and chemical noise as dis-

crete signals contained within individual filaments rather than as a signal which is averaged.

There is probably a threshold on the time interval within which two sequentially arriving signals would be perceived as one signal. The threshold is likely dependent in part on the speed of biochemical processes related to receiving and disposing of pheromone molecules in the insect antenna. When pheromone components are released from separated points to form an overlapping plume downwind, it is likely that some filaments may mix to such an extent that insects perceive them as a signal of the pheromone blend rather than discrete signals of individual pheromone components. This may explain the increased responses by male *T. ni* when 12:Ac was released close to *Z*7–12:Ac resulting in an overlapping plume downwind (Linn and Gaston, 1981). Our own study (unpublished) also revealed that responses to *Z*7–12:Ac by male *T. ni* were enhanced when minor pheromone components were released near *Z*7–12:Ac. As long as a complete signal is perceived within the plume, the moth will exhibit appropriate behavioral responses.

The fact that an inhibitor such as *Z*7–12:OH interrupts the response by male *T. ni* to the pheromone only if detected simultaneously protects the integrity of the pheromone signal. This mechanism may help to ensure species specificity in *T. ni* as well as other lepidopteran species. In natural habitats, it is common that many lepidopteran species have spatially overlapping distributions and temporally overlapping diurnal calling rhythms. A moth that orients upwind within a pheromone plume of its own species may encounter pheromones produced by other species. On a time-averaged scale, a pheromone plume may be contaminated by these chemical noise signals. However, a moth is likely to detect an intact and uncontaminated pheromone blend contained in individual filaments. Because individual filaments within a plume are less likely to overlap and sequential reception of chemical noise such as *Z*7–12:OH in different filaments does not interrupt normal anemotactic responses of a moth to its pheromone, pheromone signals should be relatively protected from background chemical noise.

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CHEMISTRY AND CHEMICAL ECOLOGY OF THE BAHAMIAN SPONGE *Aplysilla glacialis*[1]

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Abstract—Chemical investigation of the secondary metabolites of the marine sponge *Aplysilla glacialis* collected at French Wells, Crooked Island, Bahamas, resulted in the isolation and characterization of four diterpenes, two sterol endoperoxides, and two methylated primary metabolites. Some of these compounds, along with crude extracts of the sponge, were investigated for their ability to deter fish predation, affect the fouling of surfaces, and inhibit the growth of marine microorganisms. The diterpene manoöl **(3)**, cholesterol endoperoxide **(4)**, and the crude nonpolar extract of *A. glacialis* were shown to deter feeding by a natural assemblage of fish predators in an in situ assay conducted at French Wells. Pure secondary metabolites and crude extracts of *A. glacialis* also were tested in a laboratory fish feeding assay employing the wrasse *Thalassoma lunare*. A mixture of sterol endoperoxides was isolated from the mucus that coats the surface of *A. glacialis* and is exuded in large quantities when the sponge is disturbed. These compounds are thereby distributed in a manner in which they can best serve a defensive role for the sponge. An in situ assay was designed to determine the effect that pure secondary metabolites and crude extracts have on the fouling of surfaces. Manoöl **(3)** and cholesterol endoperoxide **(4)** were determined to increase the rate of fouling when compared to control surfaces. 1-Methyladenine **(5)** was identified as an antimicrobial constituent of *A. glacialis* that inhibited the growth of four marine bacteria isolated from seawater samples collected at French Wells.

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Key Words—*Aplysilla glacialis*, Dendroceratida, marine sponge, cholesterol endoperoxide, sterol endoperoxides, manoöl, 1-methyladenine, marine chemical ecology, fish feeding deterrence, fouling deterrence, antifouling.

INTRODUCTION

Marine sponges have proven to be an extremely rich source of natural products (see reviews by Minale et al., 1976; Faulkner, 1984, 1986, 1987, 1988, 1990, 1991). Sponge secondary metabolites provide examples of many unique carbon skeletons and include compounds derived from terpenoid, amino acid, alkaloid, acetogenin, and mixed biosynthetic origins. Most of the interest in natural products from sponges has come from the desire to isolate new compounds with potential pharmaceutical applications. Sponge metabolites have been shown to possess hypotensive (Baslow, 1977), antiinflammatory (Kernan, 1988; Glaser et al., 1989), antiviral (Rinehart et al., 1981), cytotoxic (Rinehart et al., 1981; Jacobs et al., 1981), antimicrobial (Rinehart et al., 1981), antifungal (Baker and Wells, 1981), and many other potentially useful pharmacological properties (Baker and Wells, 1981; Kaul, 1982). To a much lesser extent, studies have been undertaken to determine the roles that these chemicals may play in the natural environment. Many of the reports of secondary metabolites from sponges include speculation regarding the defensive purposes of the compounds isolated, but there has been relatively little effort to experimentally determine the natural functions of sponge metabolites.

There are many ecological roles that may be played by secondary metabolites in the marine environment. Conspecific and interspecific interactions are often mediated by the presence of secondary metabolites in ways that enhance the survival of the producing organism. Most of these aspects have been discussed in recent reviews on marine chemical ecology (Bakus et al., 1986; Van Alstyne and Paul, 1989; Coll and Sammarco, 1989). Secondary metabolites may provide a competitive advantage for a sponge by deterring predation by fish or invertebrates, deterring settlement or overgrowth by other benthic organisms competing for substrate space, preventing invasion by pathogenic microorganisms, and attracting conspecific gametes.

Our continuing interest in the chemistry of sponges of the order Dendroceratida (Bobzin and Faulkner, 1989a, b, 1991a, b; Bobzin, 1990) led us to collect the pink encrusting sponge *Aplysilla glacialis* from the mangrove lagoon at French Wells, Crooked Island, Bahamas, in June 1988 and July 1989. *Aplysilla glacialis* is a cosmopolitan species that has been reported from the Caribbean, North and South America, Australia, and the Arctic Ocean (Zea, 1987). Our investigation of sponges of the order Dendroceratida was motivated by the hypothesis that the absence of siliceous spicules, which is characteristic of this

order, may be compensated by an increase in the variety and quantity of defensive secondary metabolites produced by these animals. This hypothesis originates from the observation that the loss of a physical defense mechanism, such as spicules, may be the result of an evolutionary development of an alternate, chemical defense mechanism (Faulkner and Ghiselin, 1983).

Aplysilla glacialis was found only on the prop roots of the mangrove trees that line the lagoon at French Wells in depths of 0–2 m. *Aplysilla glacialis* is rarely overgrown by the invertebrates and algae that compete for substrate space in this diverse environment, and it was never observed to be eaten by fish despite the fleshy, aspiculous nature of its tissue. In situ and laboratory assays were designed to determine whether crude extracts of *A. glacialis*, or pure secondary metabolites isolated from these extracts, were responsible for these observations. The assays employed in this work were designed to determine whether the extracts or metabolites deterred feeding by fish, affected the rate of fouling of surfaces, and inhibited the growth of marine microorganisms.

A sample of *Aplysilla glacialis* collected in British Columbia has been reported to possess a number of spongian diterpene metabolites (Tischler and Anderson, 1989; Tischler et al., 1991). Two of these diterpenes, glaciolide **(1)** and cadlinolide A **(2)**, were also isolated from the dorid nudibranch *Cadlina luteomarginata*, which was collected in the same locality. It was suggested that the glaciolide and cadlinolide A found in *C. luteomarginata* were obtained by the nudibranch from its diet, which includes *A. glacialis*. Dorid nudibranchs often sequester secondary metabolites from their sponge diets, and these compounds are widely believed to serve a defensive role for both animals. Unfortunately, no effort was made to determine whether glaciolide **(1)** and cadlinolide A **(2)** served any ecological role for the sponge and the nudibranch.

Chemical investigation of our samples of *Aplysilla glacialis* collected at French Wells, Crooked Island, Bahamas, did not result in the isolation of any of the spongian diterpenes previously reported from this species. Instead, four diterpenes, two sterol endoperoxides, and two methylated primary metabolites were isolated from the Bahamian collections made during 1988 and 1989. Crude extracts and the pure metabolites manoöl **(3)** and 5α,8α-epidioxycholest-6-en-3β-ol **(4)** ("cholesterol endoperoxide") obtained from the June 1988 collection of *A. glacialis* were assayed in San Diego Bay, California, in January 1990 to determine their effect on the fouling of surfaces in situ. Manoöl **(3)**, cholesterol endoperoxide **(4)**, and the dichloromethane-soluble portion of the fresh methanol extract of *A. glacialis* were tested for their effect on fish feeding in an in situ assay carried out at French Wells in July 1989. Crude extracts and pure secondary metabolites isolated from a sample of *A. glacialis* collected in July 1989 were tested in a laboratory fish feeding deterrence assay employing the wrasse *Thalassoma lunare*. These crude extracts and pure compounds were also screened for antimicrobial activity against nine bacterial isolates obtained from seawater

samples collected in the mangrove lagoon at French Wells in July 1989. 1-Methyladenine (**5**) was isolated as an antimicrobial metabolite from *A. glacialis* that inhibited the growth of four of the nine marine bacterial isolates screened. Several other secondary metabolites were also isolated from the collections of *A. glacialis*, but these compounds were not obtained in large enough quantity to allow testing in these assays.

METHODS AND MATERIALS

Collection of Aplysilla glacialis. The pink encrusting sponge *A. glacialis* was collected by hand at a depth of 0–2 m from the mangrove prop roots in the lagoon at French Wells, Crooked Island, Bahamas (Lat. 22°42'N, Long. 74°23'W) in June 1988 (sample 88-275) and July 1989 (samples 89-150B and 89-150C). Numerous individuals were combined in each of these collections. The samples were either stored frozen (−10°C) or directly extracted in methanol.

Extraction and Chromatography of Aplysilla glacialis. The sponge (sample 88-275) was stored frozen for approximately six months and then freeze-dried. The lyophilized sponge tissue (54.4 g) was successively extracted with hexane (1 × 600 ml), hexane–dichloromethane (1:1, 2 × 800 ml), dichloromethane (4 × 600 ml), ethyl acetate (1 × 600 ml), and methanol (4 × 600 ml). The hexane and dichloromethane extracts were combined to yield a crude nonpolar extract (1.9 g, 3.5% dry weight) that was separated by flash chromatography on silica[2] (column size 39 × 4 cm) using a solvent gradient from hexane–ethyl acetate (9:1) to ethyl acetate to elute the mixture. A fraction (42 mg) that eluted with hexane–ethyl acetate (4:1) was purified by HPLC on μ-Partisil in hexane–diethyl ether (4:1) to yield manoöl (**3**, 21.0 mg, 0.039% dry weight). Manoöl (**3**) was identified by comparison of the [^{13}C]NMR data with published values (Buckwalter et al., 1975). A second fraction (157 mg) that eluted with hexane–ethyl acetate (2:3) was separated by HPLC on μ-Partisil in hexane–ethyl acetate (1:1) to yield two nearly pure sterol endoperoxides[3] along with a mixture of several other sterol endoperoxides. Both of the nearly pure fractions (34 and 35 mg) were purified by HPLC on μ-Partisil in hexane–ethyl acetate (1:1) to yield 5α,8α-epidioxy-24ξ-ethylcholest-6-en-3β-ol (**6**, 33.8 mg, 0.062% dry weight) ("24-ethylcholesterol endoperoxide") and 5α,8α-epidioxycholest-6-en-3β-ol (**4**, 33.2 mg, 0.061% dry weight) (cholesterol endoperoxide), respectively. These two compounds were identified by their characteristic [^{1}H]NMR resonances and

[2]Kieselgel 60, 230–400 mesh (Gallade Chemical, Escondido, California) was used for all silica flash chromatography separations (Still et al., 1978).

[3]We define the term "sterol endoperoxide" as any compound possessing a tetracyclic sterol skeleton (cf. cholesterol) and an endoperoxide bridge between carbons 5 and 8.

fragments in the low-resolution mass spectrum (Gunatilaka et al., 1981). The ethyl acetate and methanol extracts were combined to form a polar extract (10.3 g, 19.0% dry weight). This polar extract was triturated with dichloromethane (3 × 100 ml) to yield a polar triturate (1.2 g, 2.2% dry weight).

The second collection of *Aplysilla glacialis* (sample 89-150B, 6.5 g dry weight) was directly extracted in methanol (3 × 250 ml). The methanol extract was partitioned between dichloromethane (2 × 200 ml) and water (200 ml), the organic soluble portion dried over sodium sulfate, and the solvent removed to yield a green oil (202 mg, 4.1% dry weight) that exhibited antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus*. This mixture was separated on Sephadex LH-20 (column size 95 × 3 cm) in dichloromethane–methanol (1:1). The active fractions were combined and separated by flash chromatography on silica (column size 17.5 × 1.7 cm) using a solvent gradient from hexane–ethyl acetate (9:1) to ethyl acetate to elute the mixture. This procedure yielded an impure sample of manoöl (**3,** 12.7 mg, 0.26% dry weight), a mixture of sterol endoperoxides (11.7 mg, 0.24% dry weight), and two fractions that appeared by [^{1}H]NMR to consist of diterpenes that had not been isolated from the previous sample (88-275) of *A. glacialis*. The least polar of these two fractions (12 mg) was separated by HPLC on μ-Partisil in hexane–diethyl ether (7:3) to yield spongia-16-one (**7,** 1.9 mg, 0.039% dry weight). Spongia-16-one (**7**) was identified by comparison on the [^{1}H]- and [^{13}C]NMR data with published values (Kernan et al., 1990). The second fraction (16 mg) was purified by HPLC on μ-Partisil in hexane–ethyl acetate (1:1) to yield atisane-3β,16α-diol (**8,** 4.1 mg, 0.083% dry weight). Atisane-3β,16α-diol (**8**) crystallized from diethyl ether–methanol and its structure was determined by single-crystal X-ray diffraction analysis.

The third collection of *Aplysilla glacialis* (sample 89-150C) was stored frozen for approximately three months and then freeze-dried. The lyophilized sponge tissue (8.8 g) was extracted successively with hexane (3 × 100 ml), dichloromethane (3 × 100 ml), ethyl acetate (3 × 100 ml), and methanol (3 × 100 ml). The hexane extract (176 mg, 2.0% dry weight) was separated by flash chromatography on silica (column size 17 × 2 cm) using a solvent gradient from hexane to hexane–ethyl acetate (3:7) to elute the mixture. This produced an impure sample of manoöl (**3,** 8.6 mg, 0.098% dry weight), a mixture of sterol endoperoxides (8.2 mg, 0.093% dry weight), and pure spongia-15α,16α-diacetate (**9,** 43.5 mg, 0.49% dry weight). The diacetate **9** was identified by comparison of the [^{1}H]- and [^{13}C]NMR data with published values (Cimino et al., 1982). The dichloromethane extract (141 mg, 1.6% dry weight) was determined by [^{1}H]NMR to contain a mixture of sterol endoperoxides and fatty acids. The ethyl acetate extract (17.1 mg, 0.19% dry weight) appeared by [^{1}H]NMR to contain mostly fatty acids.

Separation of the methanol extract of sample 89-150C was bioassay-directed

by using the antimicrobial activity against *Flavobacterium* sp. (strain H-73) in the standard disk assay (Acar, 1980). The methanol extract (1.66 g, 18.8% dry weight) was separated on Sephadex LH-20 (column size 95 × 3 cm) in methanol, the bioactive fractions combined, and the solvent removed. The resulting white solid (76 mg) was triturated with dry ethyl acetate (7 ml) and dry methanol (7 ml) to yield *N,N'*-dimethylglycine bisulfite (**10,** 9.7 mg, 0.11% dry weight) as an inactive, insoluble component of the mixture. *N,N'*-Dimethylglycine bisulfite (**10**) was identified from [^{1}H]NMR, [^{13}C]NMR, and mass spectral data. The soluble, active portion was partitioned between hexane (25 ml) and methanol (25 ml). The methanol-soluble portion retained the antimicrobial activity and was further partitioned between ethyl acetate (25 ml) and water (25 ml). The water-soluble portion was triturated with dry methanol (10 ml) to yield methanol-soluble and -insoluble fractions that both retained strong activity against *Flavobacterium* sp. The methanol-insoluble fraction was identified as pure 1-methyladenine (**5,** 3.6 mg, 0.04% dry weight), while the methanol-soluble fraction was identified as impure 1-methyladenine (45.5 mg, 0.52% dry weight). 1-Methyladenine (**5**) was identified from [^{1}H]NMR, [^{13}C]NMR, UV, and mass spectral data (Brookes and Lawley, 1960).

Extraction and Chromatography of Aplysilla glacialis *Mucus.* A solution of seawater and mucus (ca. 250 ml) that was recovered from the plastic bags in which *A. glacialis* (sample 89-150C) had been collected was separated from the sponge tissue and stored frozen (−10°C) for one week. The solution was freeze-dried, and the lyophilized residue was extracted with dry dichloromethane–methanol (1:1, 3 × 100 ml). This extract was partitioned between dichloromethane (75 ml) and water (75 ml), the organic soluble portion dried over sodium sulfate, and the solvent removed to yield a yellow oil (6.7 mg). This oil was separated on a silica Sep-pak (Waters Associates, Milford, Massachusetts) using hexane–ethyl acetate (1:1) to elute the mixture. The fractions that appeared by TLC to possess sterol endoperoxides were combined and separated by HPLC on μ-Partisil in hexane–ethyl acetate (3:2) to yield a clean mixture of sterol endoperoxides (0.69 mg, 10.3% of CH_2Cl_2 solubles). TLC and [^{1}H]NMR analyses were used to identify the mixture, but the GC-MS data were too complex to identify specific sterol endoperoxides from this mixture.

Detection of Secondary Metabolites Exuded by Aplysilla glacialis. A sample of *A. glacialis* was collected intact by cutting off a 6-in. length of mangrove prop root upon which a relatively large specimen of the sponge was growing. The sponge-encrusted root was placed in a 32-oz jar underwater to avoid air exposure and left undisturbed for approximately 24 hr. After this length of time, the root and sponge were removed and the seawater (ca. 650 ml) filtered through three (ca. 220 ml each) disposable C_{18} mini-extraction columns (6 ml HC Bakerbond spe*, J.T. Baker Inc., Phillipsburg, New Jersey). Each column then was washed successively with methanol (25 ml) and dichloromethane (25 ml), the

respective washes combined, the solvents removed, and the residues stored at 0°C. After 10 days the methanol wash was triturated with dry methanol (4 ml) and filtered through a cotton plug to yield a white solid (46.2 mg). The dichloromethane wash was dissolved in dry dichloromethane (4 ml) and filtered through a cotton plug to yield a yellow oil (5.6 mg). TLC and [^{1}H]NMR analysis of the two washes indicated that they contained mostly fatty acids and salt. None of the secondary metabolites reported from samples of *A. glacialis* were detected. This procedure was repeated and again failed to detect any of the secondary metabolites from *A. glacialis*.

Fish Feeding Deterrence Assays. In situ fish feeding deterrence assays were carried out in July 1989 in the mangrove lagoon at French Wells, Crooked Island, Bahamas, in the same locations where *Aplysilla glacialis* was found. The assays were carried out in a manner similar to that previously described (Harvell et al., 1988; Pawlik and Fenical, 1989). Artificial fish food strips (5 × 1 × 0.5 cm) were prepared from a mixture of carrageenan agar (Gelcarin FF 961L, FMC Corp., Philadelphia, Pennsylvania), water, and a homogenate of tuna to make the food palatable to fish. The tuna homogenate was prepared from canned chunk light tuna packed in oil (6.5 oz) and water (250 ml). Batches of 10 food strips were prepared by stirring the carrageenan agar (4.0 ml) and tuna homogenate (10 ml) into water (40 ml) and heating the mixture to boiling. Dichloromethane solutions (1.5 ml) of manoöl **(3)**, cholesterol endoperoxide **(4)**, or the dichloromethane-soluble portion of the crude methanol extract of *A. glacialis* were then stirred into this mixture to prepare experimental samples with a concentration of 1.6% dry weight (0.37% wet weight) of the pure metabolites or a "natural concentration" (see below) of the dichloromethane extract. Control strips were prepared by adding an equal volume of dichloromethane to the food preparation. The hot, fluid agar mixture was then poured into a plastic mold (10 × 5 × 0.5 cm) crossed by five lengths (40 cm) of cotton string. After the mixture cooled and solidified, ten strips were sliced to size (5 × 1 × 0.5 cm), leaving lengths of string (15 cm) free from one end to be used to secure the strips to a substrate.

The manoöl **(3)** that was used in the in situ feeding assay was obtained from a commercial source (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin), while cholesterol endoperoxide **(4)** was prepared by singlet oxygen addition to 7-dehydrocholesterol obtained from Aldrich. 7-Dehydrocholesterol (5.1 g) was dissolved in absolute ethanol (600 ml) and oxygen gas bubbled through the solution. A solution of 10% eosin in absolute ethanol (20 ml) was added as a photosensitizer, and the reaction mixture was irradiated with a 500-W tungsten lamp. The reaction mixture was stirred and refluxed for 5.0 hr, whereupon more 10% eosin in ethanol solution (20 ml) was added and the reaction was continued for an additional 7.5 hr. The solvent was removed and the reaction mixture was separated by flash chromatography on silica (column size 34 × 4.5 cm) using

a solvent gradient from hexane–ethyl acetate (4:1) to ethyl acetate to elute the material. Cholesterol endoperoxide (**4**, 1.77 g, 35% yield) was crystallized (MeOH, mp 149–152°C) from the fractions that eluted in hexane–ethyl acetate (3:7 and 1:4).

The dichloromethane-soluble portion of the crude methanol extract was prepared by extracting a volume of *Aplysilla glacialis* tissue that was equal to the volume of the fish food preparation used in the assay, therefore providing the experimental samples with a volumetric concentration of extract equal to that found in the sponge. A portion (ca. 80 ml) of *A. glacialis* tissue (sample 89-150B) was extracted with methanol (150 ml) for 24 hr. The methanol extract was partitioned between dichloromethane (125 ml) and water (125 ml), the organic-soluble portion dried over sodium sulfate, and the solvent removed to obtain a yellow oil. Half of this material was dissolved in dichloromethane (1.5 ml) and added to the fish food preparation as described above.

Control and experimental food strips were deployed in pairs using the string to tie them directly to the mangrove prop roots (0 to −2 m) at various locations in the lagoon at French Wells where *Aplysilla glacialis* was found. The strips were retrieved after 2–8 hr depending on the predator pressure at each time and place of deployment. Each strip was then measured to determine the amount consumed by estimating the percentage of the area of each 5 × 1 × 0.5 cm strip that had been eaten. The two-tailed Wilcoxon paired-sample test (Zar, 1984) was used to analyze the assay results after first excluding pairs where both the experimental and control samples had been either completely consumed or not eaten at all.

Laboratory fish feeding assays were carried out in a similar manner to that described for the determination of the feeding deterrent properties of metabolites isolated from the intertidal limpet *Collisella limatula* (Pawlik et al., 1986). The assay animals, a group of nine wrasses (*Thalassoma lunare*), were kept in separate cubicles in flowing seawater aquaria. *Thalassoma lunare*, an Indo-Pacific species, was used due to the unavailability of an endemic Caribbean species. Freeze-dried krill (4–10 mg, *Euphausia pacifica*, Aqua-Stock Inc., Bayonne, New Jersey) were weighed and then injected with solutions of crude extracts or pure secondary metabolites from *Aplysilla glacialis* to produce experimental food samples of the appropriate concentration. Control food samples were prepared by only injecting the corresponding solvent into the krill. The solvents were then evaporated using a stream of nitrogen. Experimental and control food samples were fed in pairs to each fish individually, randomly alternating the order of control and experimental samples. A sample was determined to be rejected if the fish repeatedly (three times) took the food particle into its mouth and regurgitated it.

Extracts of *Aplysilla glacialis* (sample 89-150C) were used in the laboratory fish feeding assay. The hexane, dichloromethane, and ethyl acetate extracts of

A. glacialis were assayed at a concentration of 100 μg extract/mg krill, while the methanol extract was assayed at a concentration of 200 μg extract/mg krill. The pure secondary metabolites manoöl **(3)**, cholesterol endoperoxide **(4)**, and spongia-15α,16α-diacetate **(9)** were assayed at a concentration of 50 μg metabolite/mg krill. The results from these experiments were analyzed using the G test for independence (Sokal and Rohlf, 1969).

In Situ Fouling Assay. The fouling assay design employed unglazed ceramic plates (2 × 2 in. surface area 55.6 cm^2) that were coated with crude extracts or pure metabolites from *Aplysilla glacialis* (sample 88-275) by immersing the plates in a solution of a known concentration of the extract or compound for approximately 1 min. The semiporous plates were found to absorb approximately 0.3 ml of solvent in this length of time, thereby providing an estimate of the surface concentration achieved by this method. Sample plates were back-extracted to verify the exact surface concentration of each extract or pure metabolite assayed. Control plates were prepared by immersing the plate in solvent only. The plates were then suspended in the water column by hanging them from wooden dowels (36 in. long × 1/2 in. diam.) with Dacron fishing line (50 lb test, 24 in.) tied through holes (1/16 in.) that had been drilled in the plates. The dowels were suspended at a depth of about 3 ft, leaving the plates at a depth of approximately 5 ft. Each experiment consisted of a group of five experimental plates and one control plate hung in random order 6 in. apart along the wooden dowel.

Plates were immersed in solutions of manoöl (**3,** 50 mg/ml dichloromethane), cholesterol endoperoxide (**4,** 40 mg/ml dichloromethane), the nonpolar extract (22 mg/ml dichloromethane), polar extract (133 mg/ml methanol), and the polar triturate (55 mg/ml dichloromethane) of *Aplysilla glacialis* (sample 88-275) to produce surface concentrations of 241, 203, 199, 585, and 300 $\mu g/cm^2$, respectively. Surface concentrations were checked by back-extracting (3 × 20 ml) sample plates with their respective solvent to determine the amount of material that had been adsorbed to the plate. Sample plates were also back-extracted (3 × 20 ml) with their respective solvent after being left in beakers of sterile, filtered seawater (120 ml) for approximately 24 hr in order to determine the extent to which the compounds were desorbed off of the plate's surface when immersed in water. Manoöl (**3,** 89.5% recovery) and cholesterol endoperoxide (**4,** 94.1% recovery) were only slightly affected by immersion in seawater, while the nonpolar extract (13% recovery), polar extract (3% recovery), and polar triturate (21% recovery) were substantially desorbed off of the plate's surface over this period of time. The manoöl **(3)** used in this assay was obtained from a commercial source (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin), while cholesterol endoperoxide **(4)** was prepared by singlet oxygen addition to 7-dehydrocholesterol (see above).

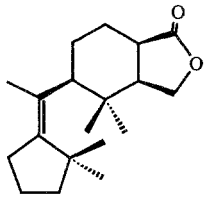
Nineteen experiments were deployed under a boat dock at Coronado Cays

in southwest San Diego Bay in January 1990. Ten experiments were retrieved after a period of two weeks, while the remaining nine experiments were retrieved after five weeks. The plates were preserved by rinsing them in sterile, filtered seawater and then stored individually in plastic Petri dishes (9 cm diam.) at −10°C until analyzed.

The plates were analyzed for total adsorbed protein using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, Illinois) (Smith et al., 1985) to provide an indication of the total biomass that was present on each plate. Each plate was ultrasonically agitated (0.5 hr) in a solution of 1.0 M sodium hydroxide (10 ml) to desorb the particulate matter from the plate surface. The protein from each plate was then extracted by heating the plate in this solution for half an hour at 80°C in a water bath. This technique was similar to that used to extract protein from plankton and seawater samples for quantitative analysis (Mayzaud and Martin, 1975; Barlow and Swart, 1981). After adjusting the volume back to 10 ml with distilled water, the solutions were centrifuged (15 min), and an aliquot (1.00 ml) of each of the sodium hydroxide extracts was neutralized with 1.0 M hydrochloric acid (1.00 ml). An aliquot (0.20 ml) of each neutralized assay solution was mixed with the BCA working reagent (4.00 ml) and the solutions incubated for 30 min in a water bath at 60°C. Each sample was prepared in duplicate. After the samples were cooled to room temperature, the absorbance at 562 nm of each solution was recorded against a reagent blank prepared in 1.0 M sodium chloride. A standard curve was constructed from standards (5, 10, 25, 50, and 100 μg/ml) of bovine serum albumin (BSA) prepared in 1.0 M sodium chloride in order to obtain the concentration of protein in each of the experimental samples. The two-tailed Wilcoxon paired-sample test (Zar, 1984) was used to analyze the assay results.

RESULTS

Nonpolar Secondary Metabolites from Aplysilla glacialis. Chemical investigation of *A. glacialis* collected from the mangrove lagoon at French Wells, Crooked Island, Bahamas, in 1988–1989 has resulted in the isolation and characterization of a variety of nonpolar secondary metabolites (Scheme 1 and 2). Different samples of *A. glacialis* were found to possess different mixtures of secondary metabolites (Table 1). *Aplysilla glacialis* (sample 88-275) collected in June 1988 was found to possess the labdane diterpene manoöl **(3),** 5α,8α-epidioxycholest-6-en-3β-ol **(4,** "cholesterol endoperoxide"), and 5α,8α-epidioxy-24ξ-ethylcholest-6-en-3β-ol **(6,** "24-ethylcholesterol endoperoxide"), along with a mixture of several other sterol endoperoxides that were not separated. Manoöl **(3)** has been isolated from numerous terrestrial sources, but this is the first report of manoöl from a marine source. Cholesterol endoperoxide **(4)**



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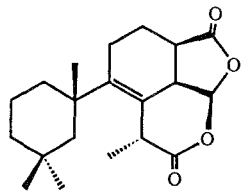

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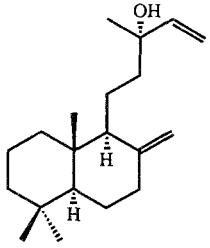

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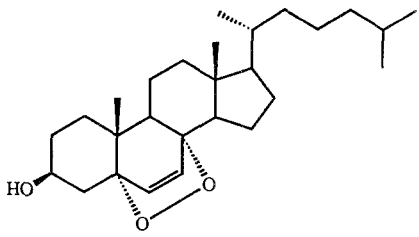

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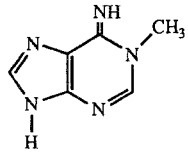
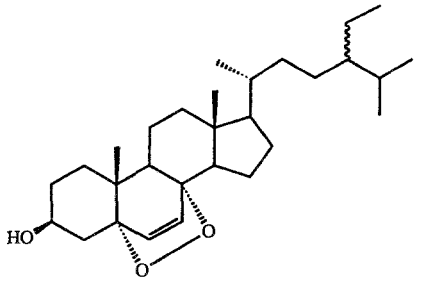


5

SCHEME 1.

and 24-ethylcholesterol endoperoxide **(6)** have been reported from a variety of marine sources and appear to be especially common in sponges (Gunatilaka et al., 1981).

A second collection of *Aplysilla glacialis* (sample 89-150B) was made in July 1989 from the same location in the Bahamas as the collection of the previous year. Separation of the dichloromethane-soluble portion of the crude methanol extract of the sponge yielded a sample of impure manoöl **(3)**, a mixture of sterol endoperoxides, and two diterpenes, spongia-16-one **(7)** and atisane-3β,16α-diol **(8)**, not found in the previous collection of *A. glacialis*. Spongia-16-one **(7)** has been reported recently from the dendroceratid sponge *Dictyodendrilla cavernosa* (Kernan et al., 1990), while atisane-3β,16α-diol **(8)** had been isolated previously



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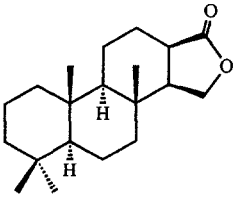

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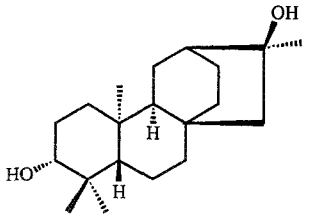

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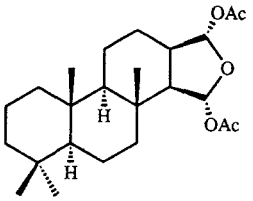

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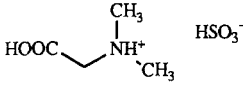

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SCHEME 2.

from the sponge *Tedania ignis* collected in the Caribbean Sea (Schmitz et al., 1983).

A third sample of *Aplysilla glacialis* (sample 89-150C) was collected from the same location in July 1989 and stored frozen for three months. The hexane extract of the lyophilized sponge tissue was separated to yield impure manoöl **(3)**, a mixture of sterol endoperoxides, and pure spongia-15α,16α-diacetate **(9)**. Spongia-15α,16α-diacetate **(9)** had been reported previously from *Spongia officinalis* collected in the Mediterranean Sea (Cimino et al., 1982).

A colloidal mixture of seawater and clear mucus from *Aplysilla glacialis*

TABLE 1. CONCENTRATIONS OF NONPOLAR SECONDARY METABOLITES ISOLATED FROM DIFFERENT COLLECTIONS OF *Aplysilla glacialis* MADE AT FRENCH WELLS, CROOKED ISLAND, BAHAMAS

Secondary metabolite	Collection[a] June 1988 (88-275)	July 1989 (89-150B)	July 1989 (89-150C)
Manoöl (**3**)	0.039	0.26	0.098
Cholesterol endoperoxide (**4**)	0.061	[c]	[c]
24-Ethylcholesterol endoperoxide (**5**)	0.062	[c]	[c]
Sterol endoperoxides		0.24	0.093
Spongia-16-one (**7**)	[b]	0.039	[b]
Atisane-3β,16α-diol (**8**)	[b]	0.083	[b]
Spongia-15α,16α-diacetate (**9**)	[b]	[b]	0.49

[a]Values are in percent dry weight.
[b]Metabolite not detected during isolation procedure.
[c]Metabolite not isolated in pure form.

was recovered from the plastic bags in which *A. glacialis* (sample 89-150C) was collected. Chemical investigation of this mucus yielded a mixture of sterol endoperoxides. TLC and [^{1}H]NMR analyses were used to identify the mixture, but the GC-MS data were too complex to identify specific sterol endoperoxides from this mixture. Experiments were conducted in order to determine whether any of the nonpolar secondary metabolites isolated from the tissue of *Aplysilla glacialis* might be actively exuded directly into the water column. These experiments failed to detect the secondary metabolites isolated from *A. glacialis* in the water around the sponge.

Fish Feeding Deterrents from Aplysilla glacialis. In situ fish feeding assays were carried out in the lagoon at French Wells, Crooked Island, Bahamas, in July 1989. The results (Figure 1) show that the pure secondary metabolites manoöl (**3**) and cholesterol endoperoxide (**4**) deter fish feeding by a natural assemblage of fish predators at a concentration of 1.6% dry weight (0.37% wet weight). Although these concentrations are approximately 10 times higher than was found in the sponge tissue, the dichloromethane-soluble portion of the crude methanol extract of *A. glacialis* (sample 89-150B) also was found to deter fish feeding when assayed at a natural volumetric concentration (Figure 1). Chemical investigation of the dichloromethane-soluble portion of the crude methanol extract of *A. glacialis* (sample 89-150B) revealed that this collection contained a mixture of sterol endoperoxides, manoöl (**3**), spongia-16-one (**7**), and atisane-3β,16α-diol (**8**). Spongia-16-one (**7**) and atisane-3β,16α-diol (**8**) were unavailable for testing in their pure form.

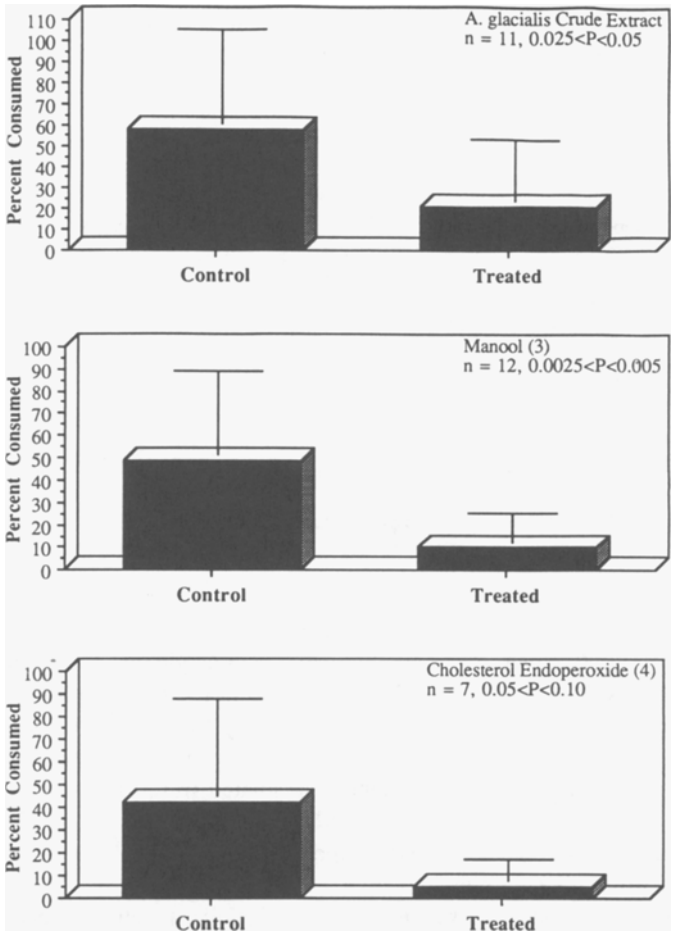

FIG. 1. In situ fish feeding deterrence assay results for manoöl (**3,** 1.6% dry weight), cholesterol endoperoxide (**4,** 1.6% dry weight), and the dichloromethane soluble portion of the crude methanol extract of *Aplysilla glacialis* ("natural concentration"—see text). Error bars indicate one standard deviation from the mean, n = number of experiments, P = significance value calculated using the two-tailed Wilcoxon paired-sample test.

Additional fish feeding assays were carried out in aquaria using a group of nine wrasses as the assay animals. *Thalassoma lunare*, an Indo-Pacific species, was used due to the unavailability of an endemic Caribbean species. The hexane, dichloromethane, and ethyl acetate extracts of *Aplysilla glacialis* (sample 89-150C) were assayed at a concentration of 100 μg extract/mg krill, while the methanol extract was assayed at a concentration of 200 μg extract/mg krill. Each

of the crude extracts appeared to exhibit a deterrent effect on the feeding of *Thalassoma lunare* at these concentrations, although the methanol extract was the only extract assayed in enough replicates to achieve favorable significance values (Table 2). Manoöl **(3)**, spongia-15α,16α-diacetate **(9)**, and a mixture of sterol endoperoxides were isolated from the hexane extract of this collection of *A. glacialis*, while *N,N'*-dimethylglycine bisulfite **(10)** and 1-methyladenine **(5)** were isolated from the methanol extract. The dichloromethane extract was determined by [^{1}H]NMR to contain a mixture of sterol endoperoxides and fatty acids, while the ethyl acetate extract appeared by [^{1}H]NMR to contain mostly fatty acids. The pure secondary metabolites manoöl **(3)**, cholesterol endoperoxide **(4)**, and spongia-15α,16α-diacetate **(9)** were assayed at a concentration of 50 μg metabolite/mg krill. None of these pure compounds had an appreciable effect on the feeding of *Thalassoma lunare* (Table 2). *N,N'*-Dimethylglycine bisulfite **(10)** and 1-methyladenine **(5)** were not available for testing in this assay.

Effect of Secondary Metabolites of Aplysilla glacialis *on Surface Fouling.* An in situ assay was carried out to determine the effect of manoöl **(3)** and cholesterol endoperoxide **(4)** on the fouling of surfaces at a concentration of 241 and 203 μg/cm^2, respectively. Ten experiments were deployed for a period of two weeks in southwest San Diego Bay in January 1990. After this period of

TABLE 2. LABORATORY FISH FEEDING ASSAY RESULTS FOR MANOÖL, CHOLESTEROL ENDOPEROXIDE, SPONGIA-15α,16α-DIACETATE, AND CRUDE EXTRACTS OF *Aplysilla glacialis*[a]

Sample	Assay concentration (μg/ml)	Number of replicates	Treated samples consumed	Control samples consumed	Significance value
Manoöl (**3**)	50	7	6	7	$0.975 < P < 0.99$
Cholesterol endoperoxide (**4**)	50	12	7	12	$0.10 < P < 0.25$
Spongia-15α,16α-diacetate (**9**)	50	11	8	9	$0.975 < P < 0.99$
A. glacialis hexane extract	100	8	3	8	$0.10 < P < 0.25$
A. glacialis CH_2Cl_2 extract	100	7	3	7	$0.10 < P < 0.25$
A. glacialis EtOAc extract	100	4	0	4	$0.10 < P < 0.25$
A. glacialis methanol extract	200	10	1	8	$0.025 < P < 0.05$

[a]Assay organism: *Thalassoma lunare*. Significance values were calculated using the G test of independence.

time, visual inspection of the plates indicated that the surfaces were very sparsely settled by invertebrate larvae but were quite heavily colonized by bacteria, fungi, diatoms, and other unicellular algae. Therefore, the fouling of the plates was not assessed by visual inspection, but by a technique that would provide an index of the total biomass that had settled on the plates. The plates were analyzed for total adsorbed protein using the bicinchoninic acid (BCA) protein assay (Smith et al., 1985) to provide an indication of the total biomass that was present on each plate. The results indicate that manoöl **(3)** and, to a less significant extent, cholesterol endoperoxide **(4)** enhanced the rate of fouling when compared to the control plates (Figure 2).

The results obtained for the experiments that used the crude extracts of *Aplysilla glacialis* were discarded due to the loss of material from the plates' surface upon immersion in seawater. The results for the experiments that were allowed to remain submerged for five weeks were also discarded due to uncertainties in the stability and solubility of the materials over this period of time.

Antimicrobial Activity of Secondary Metabolites and Crude Extracts of Aplysilla glacialis. Nine pure bacteria cultures were isolated from seawater samples collected in the lagoon at French Wells, Crooked Island, Bahamas, in July 1989. These seawater isolates were used for antimicrobial screening of the crude extracts and pure compounds from *A. glacialis*. The hexane, dichloromethane, ethyl acetate, and methanol extracts of *Aplysilla glacialis* (sample 89-150C) were assayed against the nine bacterial isolates in the standard disk assay at a concentration of 250 μg/disk. The hexane and dichloromethane extracts were inactive at this concentration against all nine of the bacterial isolates

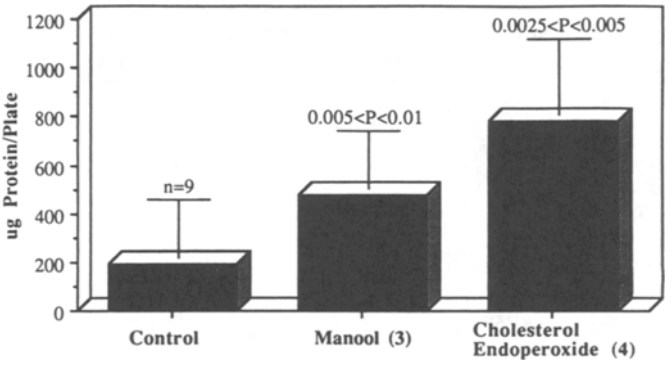

FIG. 2. In situ fouling assay results for manoöl (**3**, 241 μg/cm^2) and cholesterol endoperoxide (**4**, 203 μg/cm^2). Assay conducted for two weeks in southwest San Diego Bay, California, in January 1990. Error bars indicate one standard deviation from the mean, n = number of experiments, P = significance value calculated using the two-tailed Wilcoxon paired-sample test.

screened. The ethyl acetate extract showed slight activity (8- to 10-mm zone of growth inhibition) against *Vibrio* sp. (strain H-46), *Acinetobacter* sp. (strain H-60), and *Flavobacterium* sp. (strain H-73), while the methanol extract showed strong activity (17- to 21-mm zone of growth inhibition) against these three isolates in addition to slight activity against another *Vibrio* sp. (strain H-12). The active, methanol extract of *Aplysilla glacialis* was separated using the activity against *Flavobacterium* sp. (strain H-73) to guide the isolation process. This yielded *N,N'*-dimethylglycine bisulfite **(10)** as an inactive component of the mixture and 1-methyladenine **(5)** as an active component. 1-Methyladenine inhibited the growth (11-mm zone) of *Flavobacterium* sp. (strain H-73) at a concentration of 50 μg/disk.

DISCUSSION

Chemistry of Aplysilla glacialis. Chemical investigation of *Aplysilla glacialis* from French Wells, Crooked Island, Bahamas, revealed that different collections of the sponge contained different mixtures of nonpolar secondary metabolites (Table 1). Manoöl **(3)** and sterol endoperoxides were isolated from each of the three collections of *A. glacialis* made in June 1988 and July 1989, while the diterpenes, spongia-16-one **(7),** atisane-3β,16α-diol **(8),** and spongia-15α,16α-diacetate **(9),** were each isolated from only one of these collections. These differences may be due to the different procedures that were used in the storage, extraction, and isolation of each of the samples, although the high concentration of spongia-15α,16α-diacetate in sample 89-150C (0.49% dry weight) makes it appear unlikely that this compound could have gone undetected in the other collections. Alternatively, there may be a high degree of variability in the secondary metabolism of sponges in the French Wells population of *A. glacialis* or the species may be affected by unknown spatial, temporal, and environmental factors. The reports of several different spongian diterpene metabolites that were isolated from *A. glacialis* collected in British Columbia (Tischler and Anderson, 1989; Tischler et al., 1991) indicate that the secondary metabolism of *A. glacialis* does exhibit a high degree of variability. These compounds, exemplified by glaciolide **(1)** and cadlinolide A **(2),** would have been detected by the isolation procedure used in this work. Despite this, we believe that it would be premature to speculate as to which one of these possibilities has led to the observed differences in the chemistry of *A. glacialis*.

We observed that *Aplysilla glacialis* exudes a mucus that is distributed as a thin film over the exterior of the sponge surface and that large quantities of this mucus are exuded when the sponge is handled or disturbed. The presence of sterol endoperoxides in the mucus of *A. glacialis* provides circumstantial evidence for the hypothesis that these compounds serve a defensive role. By

distributing these compounds in the mucus, the sponge deploys them in a manner in which they could most effectively deter predation, overgrowth, or invasion by pathogens. The use of mucus exudates containing secondary metabolites is quite common in the defensive strategy of marine animals, especially among the opisthobranchs (Faulkner and Ghiselin, 1983). The secondary metabolites isolated from the mucus of the sponge *Siphonodictyon coralliphagum* were proposed to be responsible for the zones of dead coral polyps adjacent to this burrowing species (Sullivan, 1985).

The isolation of sterol endoperoxides from the mucus of *Aplysilla glacialis* suggested that these compounds may also be actively exuded by the sponge into the surrounding water. The exudation of secondary metabolites by a sponge has been demonstrated in one case (Walker et al., 1985). This type of allelochemical defense strategy has also been observed in soft corals (Coll et al., 1982). The negative results from our field experiments do not eliminate the possibility that *A. glacialis* actively exudes its secondary metabolites into the surrounding environment, but it is apparent that when left undisturbed *A. glacialis* does not exude large quantities of compounds. Experiments with the sponge *Aplysina fistularis* indicated that this species exuded secondary metabolites at a rate of 10^{-3}–10^{-4} μg/min/g dry weight of sponge (Walker et al., 1985). If *A. glacialis* exuded its metabolites at a similar rate, our experiments would have retrieved a quantity of metabolites well below the detection limits of TLC and [^{1}H]NMR.

Chemical Ecology of Aplysilla glacialis. Our field observations of *Aplysilla glacialis* indicated that this sponge was rarely eaten by fish, despite the fleshy, aspiculous nature of its tissue. This observation suggested that *A. glacialis* may be protected by a chemical defense provided by its secondary metabolites. There have been no reports in the literature of fish predation on dendroceratid sponges, but the absence of spicules in sponges of this order may be responsible for this observation because most of the studies of sponge predation by fishes have analyzed the gut contents of fishes for spicules in order to determine the species of sponges consumed (Randall and Hartman, 1968). Alternatively, the abundance of secondary metabolites that is common in dendroceratid sponges may provide this group with a very effective chemical defense against fish predation.

The lagoon at French Wells, Crooked Island, Bahamas, is populated by yellowtail snappers (*Ocyurus chrysurus*), angelfishes (*Pomacanthus* spp. and *Holacanthus* spp.), grunts (unidentified species), bluehead wrasses (*Thalassoma bifasciatum*), foureye butterflyfishes (*Chaetodon capistratus*), juvenile barracudas (*Sphyraena barracuda*), and numerous schools of unidentified juvenile fishes. Of these fish species, only angelfishes are known to prefer sponges as a major component of their diet (Randall and Hartman, 1968), although butterflyfishes have also been identified as occasional spongivores (Dawson et al., 1955). Bluehead wrasses are omnivores that may include sponges in their diverse

diet, while grunts, snappers, and barracudas are carnivores that probably do not normally include sponges in their diet.

The results from the in situ fish feeding assay conducted at French Wells show that the pure secondary metabolites manoöl **(3)** and cholesterol endoperoxide **(4)** deter fish feeding by a natural assemblage of fish predators at a concentration of 1.6% dry weight (0.37% wet weight) (Figure 1). This dry weight concentration is approximately an order of magnitude higher than that found in samples of *Aplysilla glacialis*, but the identification of sterol endoperoxides in the mucus of *A. glacialis* indicates that these compounds may be present at much higher concentrations at the sponge surface where they would be encountered by potential fish predators. This argument may not be applied to manoöl **(3)**, but the high degree of significance of these data (Figure 1) indicates that this compound may be deterrent at lower concentrations. In support of this hypothesis, the dichloromethane-soluble portion of the crude methanol extract of *A. glacialis* (sample 89-150B), from which manoöl **(3)**, spongia-16-one **(7)**, atisane-3β,16α-diol **(8)**, and sterol endoperoxides were isolated, was shown to deter fish feeding in situ when assayed at a natural volumetric concentration (Figure 1). Pure spongia-16-one **(7)** and atisane-3β,16α-diol **(8)** were not tested due to lack of material. These results provide the first data on the effect of sponge extracts and pure secondary metabolites on fish feeding in situ at a location where the sponge species naturally occurs.

The results from the laboratory fish feeding assay show that the crude methanol extract of *Aplysilla glacialis* (sample 89-150C) deterred feeding by *Thalassoma lunare* (Table 2). The other crude extracts also appear to deter feeding by *T. lunare*, but the limited number of replicates for these experiments resulted in poor significance values (Table 2). Manoöl **(3)**, spongia-15α,16α-diacetate **(9)**, and a mixture of sterol endoperoxides were isolated from the hexane extract of this collection of *A. glacialis*, while *N,N'*-dimethylglycine bisulfite **(10)** and 1-methyladenine **(5)** were isolated from the methanol extract. Pure samples of manoöl **(3)**, cholesterol endoperoxide **(4)**, and spongia-15α,16α-diacetate **(9)** did not have an appreciable effect on the feeding of *Thalassoma lunare* at a concentration of 50 μg/mg (Table 2). *N,N'*-Dimethylglycine bisulfite **(10)** and 1-methyladenine **(5)** were not available for testing in this assay due to lack of material. The lack of a deterrent effect of the pure secondary metabolites may be an indication that these compounds must be present together, or present with some unknown component(s) of the crude extract, to elicit a strong negative response. Unfortunately, mixtures of the pure metabolites were unavailable for testing the hypothesis that the metabolites act synergistically to deter fish predation.

We observed that *Aplysilla glacialis* was rarely overgrown by other invertebrates or algae and hypothesized that the secondary metabolites of *A. glacialis*

may prevent fouling of the sponge's surface. The benthic environment in the lagoon at French Wells, Crooked Island, Bahamas, is a diverse and abundant habitat. The sea grass *Thallassia testudinum* carpets the sand–sediment bottom wherever the depth is less than 15 ft. Several species of tunicates, hydroids, green algae, and brown algae are found at French Wells, while 23 species of sponges have been collected from this lagoon (Bobzin and Faulkner, unpublished data). Although many of these organisms co-occurred with *A. glacialis* on the mangrove prop roots, none of them were observed to overgrow this sponge. In fact, it was often found that *A. glacialis* would overgrow adjacent benthic species.

An in situ assay was designed to determine the effect of manoöl **(3)**, cholesterol endoperoxide **(4)**, and crude extracts of *Aplysilla glacialis* on the fouling of surfaces. The results from the two week experiment carried out in San Diego Bay, California, indicate that manoöl **(3)** and, to a less significant extent, cholesterol endoperoxide **(4)** enhance the rate of fouling when compared to control plates (Figure 2). The results for the crude extracts of *A. glacialis* were discarded due to the interference of components in these chemical mixtures with the BCA assay used to quantify the results and due to the inherent water solubility of these extracts, which allowed most of the plate coatings to be desorbed in a short period of time. The results for the five week experiment were also discarded due to the loss of compounds and extracts off of the plates after this period of time.

These results indicate that manoöl **(3)** and cholesterol endoperoxide **(4)** most likely serve no role in the maintenance of the unfouled surface of *Aplysilla glacialis*. The observation that these metabolites actually enhance the rate of fouling may be simply due to the organic nature of the coatings that are applied to the experimental plates. The organic material may provide a nutritional source for bacteria and thereby increase the rate of colonization by bacteria, which is believed to be the first step in the succession of fouling (O'Neill and Wilcox, 1971). This point illuminates one of the shortcomings in the experimental design of the control plates. These plates should have possessed an equivalent organic coating similar to that of the experimental plates, thus providing the control plates with similar organic content and surface wettability characteristics. Despite these shortcomings, we believe that a truly deterrent metabolite would have overcome these factors.

There have been few investigations into the antifouling role of marine secondary metabolites. Relevant in situ assays to determine the effect of secondary metabolites on fouling have been difficult to design (Bakus et al., 1983). Therefore, most investigations have employed pure cultures of a fouling species to determine the effect of secondary metabolites in laboratory experiments (Thompson et al., 1985; Rittschof et al., 1985; Targett et al., 1983). Our experimental design provides a quick and easy, albeit crude, method for deter-

mining the effect that pure nonpolar secondary metabolites have on surface fouling in situ. Although we acknowledge that the experimental design requires some refinements, we found that the BCA assay technique was a very quick and accurate method for quantifying the results from such an experiment.

The secondary metabolites produced by *Aplysilla glacialis* may also serve to protect the sponge from invasion by pathogenic marine microorganisms that the sponge filters out of the water while feeding. 1-Methyladenine **(5)** was identified as the antimicrobial constituent of *A. glacialis* (sample 89-150C) responsible for the growth inhibition of two strains of *Vibrio* sp., a *Flavobacterium* sp., and an *Acinetobacter* sp. These potentially pathogenic bacteria were isolated from seawater samples collected from within the lagoon at French Wells and would therefore presumably come into contact with *A. glacialis* in the natural environment. The antimicrobial activity of 1-methyladenine **(5)** may also be useful to *A. glacialis* as an aid to feeding by causing the agglutination of bacteria in the incurrent canals of the sponge (Bergquist and Bedford, 1978), in addition to a defense against invasion by pathogenic microorganisms.

The field of sponge chemical ecology has received relatively little attention when one considers the amount of effort that has been invested in the isolation and characterization of the secondary metabolites produced by sponges. We have investigated several aspects of the chemical ecology of *Aplysilla glacialis* in an effort to determine whether the secondary metabolites produced by this sponge may serve ecologically relevant functions for the animal. Our results show that *A. glacialis* produces compounds, most notably manoöl **(3)** and cholesterol endoperoxide **(4)**, that deter predation by fish in situ. A mixture of sterol endoperoxides, which are also believed to deter fish predation, were also isolated from the mucus which is exuded by *A. glacialis*. The distribution of these compounds in this mucus layer that coats the exterior of the sponge allows them to be deployed in a manner in which they could most effectively deter an attack by a fish predator. Although the results from the surface fouling experiments indicated that the compounds tested had no role in the maintenance of the unfouled surface of *A. glacialis*, the exudation of mucus may provide a physical means of continuously renewing the sponge surface, thereby sloughing off any growth that may start on the sponge. Another compound, 1-methyladenine **(5)**, was found to inhibit the growth of several strains of marine bacteria, although the ecological purpose for this antimicrobial activity was not determined. It is quite apparent that *A. glacialis* employs a variety of strategies, both physical and chemical, to enhance its survival, and that the chemical aspect consists of more than one compound.

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ELECTROANTENNOGRAM RESPONSE OF ALFALFA SEED CHALCID, *Bruchophagus roddi* (HYMENOPTERA: EURYTOMIDAE) TO HOST- AND NONHOST-PLANT VOLATILES

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Abstract—The alfalfa seed chalcid (ASC), *Bruchophagus roddi*, is a monophagous pest of alfalfa that parasitizes developing seeds. To further understand the olfactory basis of host-plant recognition by ASCs, we recorded electroantennograms (EAGs) from females and males to 39 volatiles from both alfalfa and red clover. The chemoreceptive sensitivity of ASCs was selective for certain general classes of compounds, defined by their carbon-chain length (C_6 and C_8), structure (aliphatics and phenolics), isomerism, and/or functional group (acetates, ketones, and alcohols). The compounds that elicited the largest EAGs were ranked as follows: (Z)-3-hexenyl acetate > hexyl acetate ≥ acetophenone ≥ octan-3-one ≥ methyl salicylate > octan-3-ol > oct-1-en-3-one > oct-1-en-3-ol ≥ (*E*)-β-ocimene ≥ (*Z*)- and (*E*)-3-hexen-1-ols. Over half the test compounds elicited significantly different responses between the sexes, and female antennal responses exceeded those of males for twice the number of these volatiles. Relationships of the tested volatiles to host-plant composition, EAG responses, and ASC behaviors showed no consistent correlations. However, nearly all of the host-plant volatiles known to stimulate behavioral activity also elicited moderate to potent EAG responses.

Key Words—Alfalfa seed chalcid, *Bruchophagus roddi*, Hymenoptera, Eur-

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ytomidae, alfalfa volatiles, plant odors, electroantennogram, chemoreception, olfaction, kairomones, host plant selection, orientation, oviposition.

INTRODUCTION

The alfalfa seed chalcid, *Bruchophagus roddi* (Gussakovsky) is a monophagous pest of alfalfa (*Medicago sativa* L.). Alfalfa seed chalcids (ASC) will oviposit only in alfalfa seed pods and not in pods of red clover (*Trifolium pratense* L.) or other sympatric forage legumes (Strong, 1962). When ASC females were exposed to the odors of alfalfa and red clover (either whole plant parts or extracts) in a flight-tunnel bioassay arena, they flew to, landed on, and explored targets emanating alfalfa but not red clover odors (Kamm and Buttery, 1986a; Kamm, 1989). Moreover, when female ASCs were exposed to individual volatile components of alfalfa, they oriented to, alighted on, and explored targets impregnated with certain specific components, some of which also stimulated oviposition (Kamm and Buttery, 1983, 1986b).

More than half the volatile components in the odors of alfalfa and red clover are common to both species (Kami, 1978, 1983; Buttery and Kamm, 1980; Buttery et al., 1982, 1984; Srinivas, 1986) (Table 1). Hence, host-plant recognition based on olfaction by ASCs is a complex task of both sensory reception and integration. An array of relationships between plant volatile production by these two legumes and associated chalcid behaviors have been identified for ASC females (Kamm and Buttery, 1983, 1986b) (Table 1). Certain plant-specific volatiles have chalcid species-specific semiochemical activity (Kamm and Buttery, 1986a,b).

To further understand the olfactory basis of host-plant detection, we determined the chemoreceptive responsiveness and sensitivity of antennae of female and male ASCs, by recording the electroantennogram (EAG) responses to both alfalfa and red clover volatiles. The EAG responses of ASCs were then correlated to the composition of the plant emissions and the behaviors evoked by these volatiles, as determined in previous laboratory bioassays.

METHODS AND MATERIALS

Insects. Test insects emerged in the laboratory from infested alfalfa seeds from Ontario, Oregon, as previously described (Kamm and Buttery, 1983). Chalcids were collected on the day of emergence and put into vials (95 × 24 mm) segregated by gender, provisioned with 10% sucrose water solution, and exposed to a photoperiod regime of 16 hr light–8 hr dark, at 24°C for 48–72 hr before a test.

Olfactory Stimuli. Table 1 lists the 40 test compounds (36 legume volatiles

TABLE 1. COMPOSITIONAL PERCENTAGE OF VOLATILES IDENTIFIED IN HEADSPACE ODORS OF PLANT PARTS OF ALFALFA AND A NONHOST PLANT, RED CLOVER,[a] BEHAVIORAL RESPONSE THRESHOLDS (IN μg AND $\mu g/\mu l$) AND POTENCY RANKS OF THESE VOLATILES, WHEN PRESENTED SINGLY TO ELICIT FLIGHT AND PEDESTRIAN ORIENTATIONS, ARRESTMENT, AND OVIPOSITION RESPONSES FROM FEMALE ALFALFA SEED CHALCIDS IN PREVIOUS LABORATORY OLFACTOMETER STUDIES,[b] AND EXPERIMENTAL RANKS OF THESE COMPOUNDS AS TO THEIR POTENCY IN ELICITING ELECTROANTENNOGRAM RESPONSES FROM ALFALFA SEED CHALCIDS

	Relative percent of headspace volatiles						Olfactometer behavioral thresholds of female ASCs					Volatility indexes:		Electroantennogram data analyses			
	Alfalfa			Red clover			Orientation			Oviposition				Male ASCs ranking		Female ASCs ranking	
Compounds in headspace trappings	Leaves	Flowers	Pods	Leaves	Flowers	Pods	Flight (μg: rank)	Walking (μg: rank)	Arrestment (μg: rank)	Stimulation ($\mu g/\mu l$: rank)	Inhibition ($\mu g/\mu l$: rank)	Mol. wt. (g/mole)	R. index /subset[c]	10 μg	100 μg	10 μg	100 μg
Aliphatic compounds																	
Acetic acid[d]						3.0	[g]					60.1	580 A	27	27	34	24
Pent-1-en-3-one						4.0						84.1	655 A	24	18	35	10
2-Methylbutanol						4.0	NR[h]	NR	NR		NR	88.2	718 AB	32	17	38	22
3-Methylbutanol[e]	0.5	7.0	3.5[i]				NR	NR	NR		NR	88.2	714 AB	28	16	33	9
2-Hydroxybutan-3-one					3.0	3.0						88.2	AB	38		27	
2,3-Dihydroxybutane					2.0		NR	NR	NR			90.2	AB	30		31	
Hexan-2-one					0.2							100.2	761 ABC	35	26	30	20
Hexanal			2.0				NR	NR	NR		NR	100.2	722 ABC	26	11	36	8
(*E*)-2-Hexenal[f]	0.2	0.5	1.3		6.0	3.0	25.0: 10	NR	NR		NR	98.2	822 BC	20	1	26	28
Hexan-1-ol			0.5	0.5	0.7							102.2	848 BC	10	9	17	14
(*Z*)-3-Hexen-1-ol	7.0	4.5	25.0	24.0	3.0		250.0: 12	250.0: 12	2.5: 4		NR	100.2	834 BC	8	13	9	15
(*E*)-2-Hexen-1-ol			4.0	4.0	0.4		0.25: 7	2.5: 9	NR		NR	100.2	845 BC	9	10	13	7
Hexyl acetate	0.2						25.0: 6	25.0: 7	25.0: 5	0.1: 1		144.2	995 C	2	4	7	2
(*Z*)-3-Hexenyl acetate	78.5	8.1	14.5	33.0	3.0	2.0	250.0: 11	2.5: 6	2.5: 6	0.1: 3		142.2	986 C	1	8	1	1
Heptan-2-one					0.7		NR	NR	NR			114.2	865 BC	23	3	19	13
Octan-3-one	<0.1	1.0	1.5				0.025: 1	0.025: 2	0.025: 1	0.01: 4		128.2	964 C	7	6	2	17
Oct-1-en-3-ol	0.4	0.5	6.0	0.8	3.0	4.0	2.5: 8	NR	NR		NR	128.2	962 C	12	7	8	3
Decyl acetate		7.0				3.0						200.3	1393 D	14	20	25	25
Dodecyl acetate		4.0				3.0						228.1	D	19	21	29	26
Analogs																	
Oct-1-en-3-one											NR	126.2	953 C	13		5	
Octan-3-ol											0.1: 2	130.2	980 C	5		6	
Monoterpenoids																	
Myrcene	1.1	3.0					NR	25.0: 11	NR	0.1: 2		136.2	981 C	33	28	32	29
Limonene		0.5										136.2	1020 C	21	12	16	6
(*Z*)-β-Ocimene				5.0	3.0	6.0	NR	NR	NR			136.2	1026 C	17	15	14	19
(*E*)-β-Ocimene	1.9	25.0	4.0	22.0	8.0	35.0	NR	NR	NR		NR	136.2	1037 C	6	14	10	4

TABLE 1. Continued

Compounds in headspace trappings	Relative percent of headspace volatiles: Alfalfa Leaves	Alfalfa Flowers	Alfalfa Pods	Red clover Leaves	Red clover Flowers	Red clover Pods	Olfactometer behavioral thresholds of female ASCs: Orientation Flight (μg: rank)	Orientation Walking (μg: rank)	Orientation Arrestment (μg: rank)	Oviposition Stimulation (μg/μl: rank)	Oviposition Inhibition (μg/μl: rank)	Volatility indexes: Mol. wt. (g/mole)	Volatility indexes: R. index /subset[c]	Electroantennogram data analyses: Male ASCs ranking 10 μg	Male ASCs ranking 100 μg	Female ASCs ranking 10 μg	Female ASCs ranking 100 μg
Monoterpenoids																	
Neryl 2-methylbutrate		1.0					NR	NR	NR			238.2	D			24	
Analog: Linalool							NR	NR	NR			154.3	1083 C	11	19	12	11
Aromatic compounds																	
Benzyl alcohol	0.4	0.6	5.0				NR	NR	NR	NR		108.2	1004 C	27		22	
Acetophenone					24.0	4.0	NR	NR	NR			120.2	1031 C	4	5	4	5
1-Phenylethanol					8.0		NR	NR	NR			122.2	1031 C	22	23	18	18
2-Phenylethanol	0.3	1.0	5.5		1.0		NR	25.0: 10	25.0: 7		1.0: 3	122.2	1081 C	15	25	15	23
Methyl salicylate	0.8	2.5	1.5	0.4			NR	NR	NR	NR		152.1	1166 C	3	2	3	12
Methyl cinnamate					11.0							162.2	1348 D	25		11	
Sesquiterpenes																	
α-Copaene[j]	0.1	1.4	0.3				2.5: 5	2.5: 8	NR	NR		204.4	1373 D	37		23	
Longifolene						5.0	NR	NR	NR			204.4	1402 D	31	29	37	27
Caryophyllene	<0.1	0.2	0.5	0.1	0.4	3.0	25.0: 9	2.5: 4	2.5: 3	10.0: 6		204.4	1414 D	16	22	20	16
(*E*)-β-Farnesene	0.1	0.1	4.0	2.0		2.0	0.025: 2	0.025: 1	NR	1.0: 5		204.4	1447 D	18	24	21	21
γ-Muurolene	0.3	0.7	5.5				2.5: 4	2.5: 5	NR		0.1: 1	204.4	1469 D	29		39	
Analog: Farnesol							0.025: 3	0.025: 3	0.025: 2	NR		222.4	1675 E	34		28	
Total percentage:	92.0	68.6	87.3	91.8	77.4	84.0											
Number of compounds	16	20	18	10	17	15	28: 12	28: 12	28: 7	22: 6	22: 3	39		38	29	39	29

[a]The headspace volatiles in the atmosphere associated with the plant's intact leaves, flowers, and pods were adsorbed on Tenax substrates, extracted in back-washes of ether, concentrated, and then identified by comparisons of the complete mass spectrum and Kovats' GLC retention index of authentic samples, followed by GLC/FID integrative analysis of the percent relative occurrence in the trapped plant-odor; alfalfa analysis (Buttery et al., 1982) and Red clover analysis (Buttery et al., 1984).

[b]Kamm and Buttery (1983, 1986a, b).

[c]Retention index on a nonpolar DB-1 column (a bonded, cross-linked polymethylsiloxane coated fused-silica capillary column); this retention index is an approximate reflection of the volatility of a component relative to a scale of normal saturated, aliphatic hydrocarbons, where the assigned values are: e.g., butane = 400, pentane = 500, hexane = 600, etc.

[d]Compounds fully indented were found only in red clover analyses.

[e]Compounds not indented were found only in alfalfa analyses.

[f]Compounds partially indented were found in analyses of both plant species.

[g]Blank spaces represent the compound having not been found or tested.

[h]NR: no response elicited behaviorally by the compound.

[i]For the alfalfa composition analysis the 2- and 3-isomers of methylbutanol were not distinguished.

[j]The EAG responses reported for α-copaene are an average of those recorded to separate samples of the (+) and (−) enantiomers.

and four analog compounds), their presence in different plant parts of the two legume species, and the type and threshold of behavioral responses of females when exposed to individual compounds in laboratory bioassays. Test chemicals were either obtained from commercial sources, synthesized, or isolated from plant extracts. All compounds were repurified by preparative gas chromotography separation and their identities verified by mass and infrared spectrometries. Purity was >95% for all compounds except the sesquiterpenes. The sesquiterpenes are not commercially available and therefore were separated from hop oils (Buttery et al., 1982) with the following purities: α-copaene (>81%), caryophyllene (>85%), (*E*)-β-farnesene (>93%), and γ-muurolene (>80%).

Test compounds were dissolved in spectrometric grade hexane to form volume-to-volume dilutions resulting in 10% and 1% solutions of compounds. For nine of the compounds the dilution series were taken to the 0.0001% or 0.00001% levels. For each test, 1 μl of a compound solution was pipetted onto a 1 × 2-cm piece of folded glass-fiber, Whatman No. 1 filter paper. For the 10% and 1% solutions approximately 100 μg and 10 μg, respectively, of test compound were placed on separate filter-paper odor sources. For the dose–response tests the dilution series spanned from 100 μg to 1 ng of test compound on the filter papers. Due to insufficient amounts of octan-3-ol and oct-1-en-3-one, their dilution series had to span from 10 μg to 0.1 ng. After the solvent was allowed to evaporate for 10 sec, the filter paper was inserted into a glass "test cartridge" (a disposable Pasteur pipet), which was then immediately attached to the test odorant delivery system. Each test cartridge (including the control and standard, see following) was prepared just prior to its testing, tested, and then discarded. Compounds were tested in random order for each chalcid replication. All solutions were stored at −50°C.

Electrophysiological Recording Technique. The electroantennogram procedure utilized glass capillary Ag–AgCl electrodes filled with insect saline, as described by Light et al. (1988). Test insects were immobilized by imbedding their dorsal side into low-melting-point wax affixed to one end of brass rod (3 × 100 mm). Antennae were secured with a strand of aramid fiber (Kevlar, DuPont DeNemours Co., Wilmington, Delaware). Under 128× microscope magnification, the brass rod mount was then positioned for ease of insertion of the recording electrode into the distal antennal tip. The indifferent electrode was inserted into the frons near the base of the scape. The signal was amplified 1000× by a microelectrode amplifier (P-16, Grass Instruments, Inc., Quincy, Massachusetts) and viewed on a digital storage oscilloscope (310, Nicolet Instruments, Inc., Madison, Wisconsin). EAG deflections were measured directly from the screen image and stored on floppy diskettes for later retrieval.

Odor Delivery. The odor delivery system was similar to that previously described (Light et al., 1988). Briefly, charcoal-filtered and humidified compressed air, delivered via Teflon tubing, was continuously passed over the

antenna at the rate of 1 liter/min through a disposable (pipet tip) nozzle positioned 1 cm from the antenna. Activation of a three-way solenoid valve diverted the purified air through a test cartridge where the evaporated volatiles were purged by the airflow and carried onto the antenna. The duration of an odor stimulation was 1.0 sec, as controlled by a precision time-delay relay circuit.

Experimental Procedure. The following experiments were conducted on ASC antennae: (1) 39–40 different volatiles at 10-μg dose, (2) 29 of those volatiles at 100 μg dose, and (3) nine of the volatiles (determined to be the most potent in the 10-μg experiment and/or prior behavioral bioassays) over a six-decade step series of doses. All three experiments were conducted on females, while only the two set-dose experiments were conducted on males. At least 3 min elapsed between stimulations to allow for adequate receptor recovery of ASC antennae. In the dose–response experiments compounds were randomly chosen for testing and then test doses for each compound were presented sequentially from lowest to highest. Different cohorts of individual chalcids were used for the three separate experiments. Each experiment was replicated on five different individuals per sex.

Interspersed between every fifth test odor stimulation were "control" stimulations (odor cartridges with filter papers impregnated with 1 μl of hexane solvent) and "standard" stimulations [odor cartridges with filter papers impregnated with 1 μl of a 1% solution of hexyl acetate (a 10-μg dose)]. For each individual chalcid, EAG responses were evaluated by measuring the maximum amplitude of the negative polarity deflection ($-$mV) elicited by each test volatile stimulation, minus the amplitude of the response to the accompanying solvent control. Then these "control-corrected" $-$mV responses to the test volatiles were converted for each individual to percentage values of the response to the accompanying 10-μg hexyl acetate standard. This normalization of each response to a percentage of a standard response permits comparison of responses within and between preparations (Payne, 1975).

Data Analysis. For the two set-dose experiments, statistical differences between the evoked EAG responses to the ensemble of legume volatiles were evaluated by ANOVA followed by Duncan's multiple-range test. Certain large differences between means were evaluated by paired t test statistics for the dose–response data. Statistical differences between the sexes in their EAG response magnitudes for a set volatile at a set test dose were evaluated by t test statistics.

Analysis of antennal sensitivity for the nine dose–response test compounds was based on three dose–EAG response curve parameters: threshold dose, maximum response, and the slope of the curve over its dynamic response phase (Light, 1983). The "threshold dose" is defined as the interpolated dose of a test compound that elicited an EAG response that just exceeded the average response to control stimulations (delineated by the horizontal zero lines in Figure 3 below). The "maximum EAG response" was elicited usually to the highest

(100 μg) dose of compound tested. The curve slope was calculated for the exponential "dynamic response phase" of each curve, when the rate of change in response is the greatest (i.e., steepest slope). Thus, higher antennal sensitivity to a compound is represented by lower threshold, greater slope, and greater maximum percent response values.

RESULTS

Antennal Responses to Control and Standard

The mean $-$mV responses of both female and male antennae to the solvent control stimulations were < -0.20 mV and uniform. The mean $-$mV responses to the standard stimulations (10 μg hexyl acetate) for both sexes were of a moderate magnitude. No significant difference in EAGs to standard was found between males ($X = -0.84$ mV $\pm$ 0.02 SEM, $N = 10$) and females ($X = -0.80$ mV $\pm$ 0.02 SEM, $N = 15$). However, a great degree of sexual dimorphism in response to many of the 38 legume volatiles was evident, with significant differences between the sexes found in response to 58% of the volatiles tested at the two set-doses.

Antennal Responsiveness

Limitations in Comparative Analyses. Most test volatiles in Table 1 and Figures 1 and 2 are arranged by increasing molecular weight and GC retention index (nonpolar capillary column). The relative volatility of these compounds is generally correlated to these two physical property indexes. Thus, the relative number of molecules of a test volatile delivered to the antennae in these EAG tests varied tremendously over the full range of volatiles tested, from the three- and four-carbon aliphatic compounds to the 15-carbon sesquiterpenes.

Therefore, we have limited direct comparisons in EAG responsiveness to subsets of compounds, each subset comprised of compounds possessing a common narrow range in volatility, as delineated in Table 2 by a common capital letter following the retention time index values. Thus, EAGs to volatiles in the subsets at the extremes of volatility (i.e., the $\leq C_5$ volatiles and the sesquiterpenes) can be compared validly only with members of their subset. The EAGs elicited by the C_6 acetates (e.g., the experimental standard) can be compared to C_7–C_8 volatiles, monoterpenes, and most aromatic volatiles. However, valid relative comparisons in potency of EAGs are possible between compounds in different volatility subsets, if the compound with a lower volatility (i.e., far fewer numbers of molecules per stimulation puff) elicited EAG responses of equal or greater magnitude than those elicited by the compound with a higher volatility (i.e., far greater numbers of molecules per stimulation puff). One may

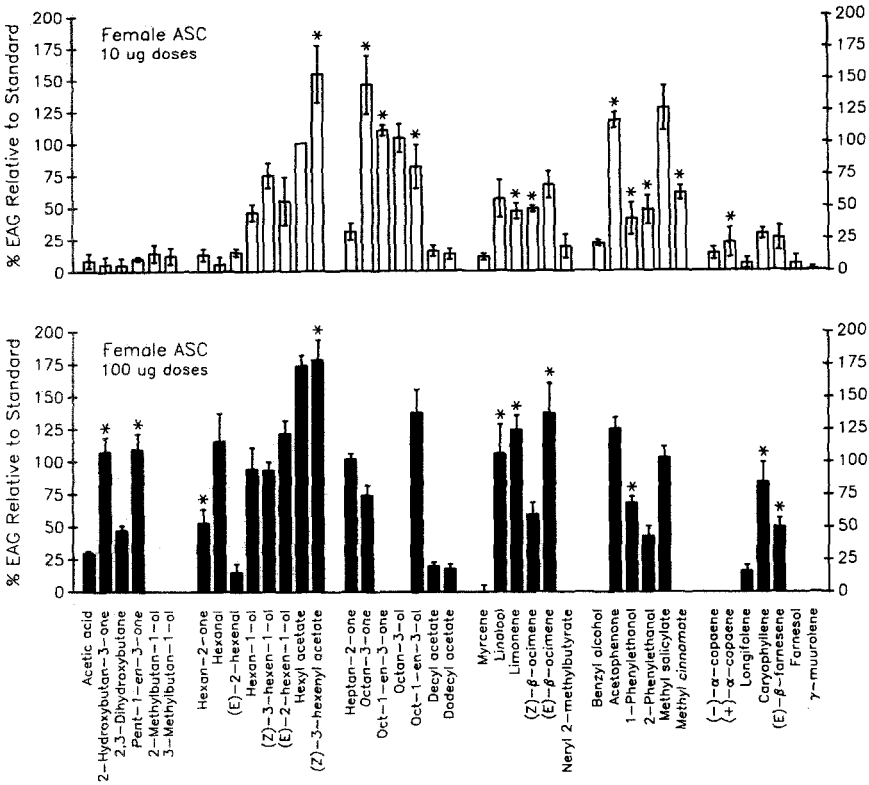

FIG. 1. Mean EAGs (±SEM, $N = 5$) of female alfalfa seed chalcids to alfalfa and red clover volatiles at 10 μg and 100 μg doses. A 100% response is approx. −0.80 mV. Asterisks identify compounds that elicited significantly larger EAGs ($P < 0.05$) from females than males.

then conclude that the less volatile compound is a superior EAG stimulant (e.g., higher transductory affinity of acceptor sites, greater degree and/or numbers of receptors and/or acceptors depolarized or "excited", etc.) than the more volatile compound, because the EAG magnitude per molecule delivered was proportionately far greater.

Legume Volatiles. With these analysis limitations considered, EAG recordings for both sexes show C_6 acetates, phenolic ketone compounds, and C_8 secondary ketones and alcohols (Table 1, Figures 1 and 2) elicited the largest EAGs. The compounds found to elicit the largest EAGs (i.e., >100% of standard) may be ranked as follows: (Z)-3-hexenyl acetate > hexyl acetate ≥ acetophenone ≥ octan-3-one ≥ methyl salicylate > octan-3-ol > oct-1-en-3-one. The compounds that elicited EAGs of moderate magnitudes

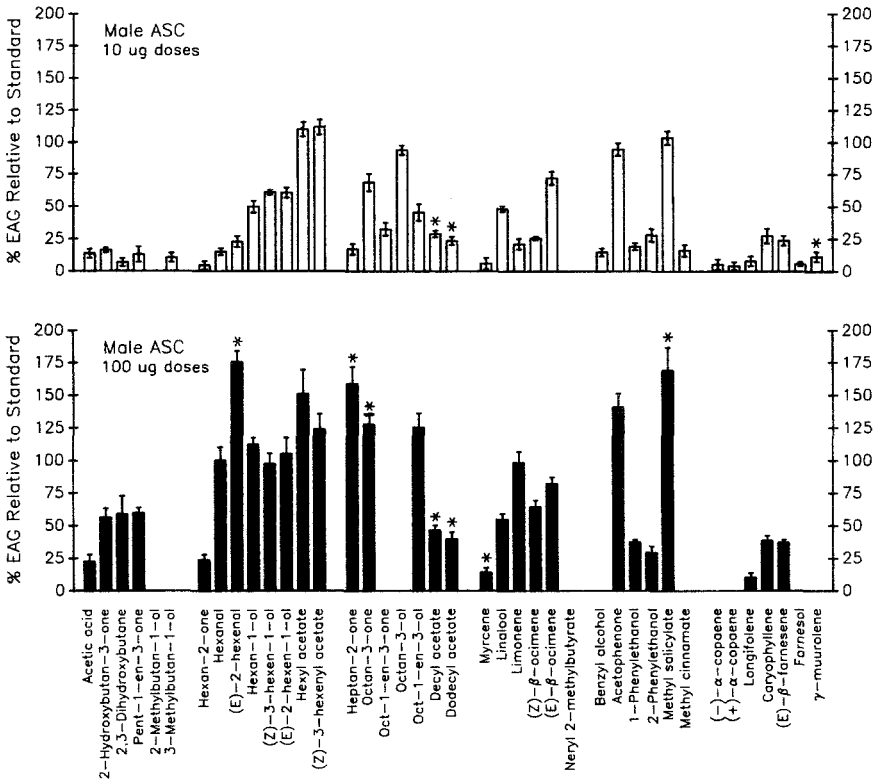

FIG. 2. Mean EAGs (±SEM, $N = 5$) of male alfalfa seed chalcids to alfalfa and red clover volatiles at 10-μg and 100-μg doses. A 100% response is approx. −0.84 mV. Asterisks identify compounds that elicited significantly larger EAGs ($P < 0.05$) from males than females.

(i.e., 50–100% of standard) were: oct-1-en-3-ol ≥ (*E*)-β-ocimene ≥ (Z)-3- and (*E*)-2-hexen-1-ols. These rankings reflect both set-dose experiments (10 and 100 μg) for both females and males (Table 1). However, males varied from females in the order of compound ranking, in that male antennae appear to be relatively less responsive to the C_8 secondary ketones and alcohols than to the C_6 alcohols (Table 1).

Sexual Dimorphism. Analysis of EAG responsiveness to the two set-dose presentations reveals that 58% of the test compounds elicited responses that varied significantly between the sexes (Figures 1 and 2). Further, female antennal responsiveness exceeded that of males for twice the number of volatiles found to elicit sexual dimorphic responses. For the 38 compounds tested in the 10-μg dose experiments, EAG responses by females significantly exceed those

TABLE 2. SENSITIVITY PARAMETERS[a] FOR ANTENNAE OF FEMALE ALFALFA SEED CHALIDS DERIVED FROM DOSE VS. MEAN EAG RESPONSE CURVES (FIGURE 3)

Compound	Threshold $\log_{10}$ dose (μg)	Maximum response (% of response to standard) (100-μg doses)	Dynamic response phase slope [% EAG response/ $\log_{10}$ dose (μg) step][b]
(Z)-3-Hexen-1-ol	−0.5	101.5	57.7
Hexyl Acetate	−1.4	152.5	48.8
(Z)-3-Hexenyl Acetate	−2.2	162.1	86.1
Octan-3-one	< −3.0	140.2	77.9
Oct-1-en-3-one	≤ −4.0	(> 108.4)[c]	76.6
Octan-3-ol	−3.2	(> 104.9)	82.3
Oct-1-en-3-ol	−1.6	134.1	78.2:55.8[d]
Acetophenone	−2.8	138.4	65.9
Methyl Salicylate	−2.6	117.5	59.4

[a] See Materials and Methods for definitions of parameters.
[b] The dose-step interval over which slope was determined was the segment between 1-μg and 10-μg doses for all but one compound (see [d]).
[c] Percent response values in parentheses were to 10-μg dose stimulations.
[d] For this compound the slope values are for the dose-step intervals of 0.1 to 1.0 μg: an average over two $\log_{10}$ steps, 0.1 μg to 10 μg.

of males for 15 compounds (four compounds $P < 0.01$ and 11 compounds $P < 0.05$), while male responses exceed those of females for six compounds ($P < 0.05$) (Figures 1 and 2). Similarly, for the 29 compounds tested in the 100-μg dose experiments, female EAGs exceed those of males for 11 compounds (for seven, $P < 0.01$; remainder, $P < 0.05$) and the opposite was observed for seven compounds (for four, $P < 0.01$; remainder, $P < 0.05$). The compounds that selectively evoked larger EAGs from females than males can be ranked (from highest to lowest degree of sex difference) as: oct-1-en-3-one, octan-3-one, methyl cinnamate, (Z)-3-hexenyl acetate, oct-1-en-3-ol, (Z)- and (E)-β-ocimenes, limonene, acetophenone, caryophyllene, 3-methylbutan-1-ol, pent-1-en-3-one, 1-phenylethanol, hexan-2-one, linalool, 2-phenylethanol, (+)-α-copaene, and (E)-β-farnesene. The rank of compounds that evoked larger EAGs from males than females (but primarily at the higher 100-μg dose; Table 1) was: (E)-2-hexenal, methyl salicylate, heptan-2-one, octan-3-one, decyl acetate, dodecyl acetate, γ-muurolene, and myrcene.

Relative Responsiveness of Structural Analogues

Positional Isomers. Antennal responsiveness of female ASCs was greater for the three-than the two-position methylbutanols (100-μg doses) (Figure 1, Table 1), but for the hexen-1-ols, the (E)-2- geometric isomer elicited larger

EAGs from females than the (*Z*)-3- isomer (100-μg doses). Both sexes were significantly more responsive to the (*E*)- than the (*Z*)-β-ocimene geometric isomer or its positional isomer, myrcene. Antennal responsiveness of ASCs was greater for 1-phenylethanol than 2-phenylethanol (100-μg doses).

Carbon-Chain-Length Analogs. Both sexes of ASCs were more responsive to the C_8 and C_7 saturated ketones than to the C_6 analog (Figures 1 and 2, Table 1). Similarly, the C_8 monounsaturated ketone elicited significantly greater EAGs than the C_5 analog. Hexyl acetate was a more potent EAG stimulant than either decyl or dodecyl acetates. 2-Phenylethanol, with its two-carbon side chain, elicited significantly larger EAGs than benzyl alcohol, with its single-carbon moiety.

Saturation vs. Unsaturation Analogs. Antennal responsiveness of females was greater for hexanal than (*E*)-2-hexenal at the 100-μg dose level, while the opposite was observed for males (Figures 1 and 2, Table 1). Antennal responsiveness was only slightly greater for both the (*E*)-2- and the (*Z*)-3-hexen-1-ols than hexan-1-ol. Only female antennae were significantly more responsive to (*Z*)-3-hexenyl acetate than hexyl acetate. Both sexes were more responsive to the C_8 saturated ketone, octan-3-one, than its unsaturated analog, oct-1-en-3-one. Similarly, octan-3-ol elicited significantly larger EAGs than oct-1-en-3-ol but just for males.

Functional-Group Analogs. Antennal responsiveness to the four functional-group moieties associated with the saturated and unsaturated six-carbon compounds tested increased in the order of their listing (by generally decreasing volatility) in Table 1. Thus, the least volatile compounds, the C_6 acetates, elicited significantly greater EAGs than the increasingly more volatile C_6 alcohols, aldehydes, and ketone tested (Figures 1 and 2, Table 1). Antennal responsiveness was greatest for (*Z*)-3-hexenyl acetate followed by (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, and (*E*)-2-hexenal, and similarly was greatest for hexyl acetate followed by hexan-1-ol and hexanal. However, EAGs of females were significantly greater for C_8 ketones than for C_8 alcohols, while the opposite was observed for males at the 10-μg dose. Both sexes were significantly more responsive to the ketone, acetophenone, than to its alcohol analog, 1-phenylethanol. Methyl salicylate (with its additional alcohol moiety) elicited significantly larger EAGs than methyl cinnamate (with its two-carbon longer side chain). The hydrocarbon, myrcene, was a far less potent EAG stimulant than its alcohol analog, linalool; conversely, (*E*)-β-farnesene was a more potent EAG stimulant than its alcohol analog, farnesol.

Antennal Sensitivity

Relative Sensitivity to Legume Volatiles. Pertinent to the analysis of antennal sensitivity to specific compounds are three parameters (threshold dose, maximum response, and the slope of the dynamic response phase; Table 2) that are

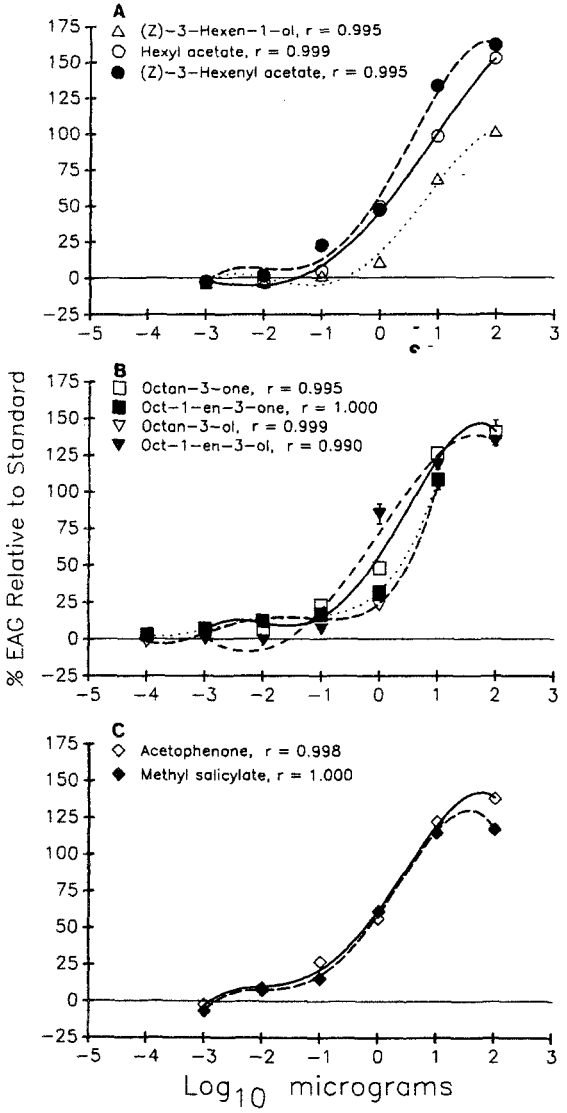

FIG. 3. Dose-EAG curves of female alfalfa seed chalcids to nine compounds found to be potent EAG elicitors: (A) six-carbon alcohol and acetates, (B) eight-carbon ketones and alcohols, and (C) phenolic ketones. Points are means of recordings from five females and vertical lines are ±SEM.

derived from the dose–EAG response curve data (Figure 3). The dose–response curves for the nine compounds tested on female ASC antennae were all sigmoidal (Figure 3), but varied in their threshold, slope, and maximum height values (Table 2). The lowest threshold doses observed were in response to oct-1-en-3-one, octan-3-ol, and octan-3-one. The largest EAG response maxima were to 100 μg stimulations by (*Z*)-3-hexenyl acetate, hexyl acetate, octan-3-one, and acetophenone. The greatest or steepest slopes of the EAG response curves were attained by doses of (*Z*)-3-hexenyl acetate, octan-3-ol, oct-1-en-3-ol, octan-3-one, oct-1-en-3-one, and acetophenone. Combining the analyses of these three sensitivity parameters, the tested compounds may then be ranked as to female ASC antennal sensitivity in the following order: oct-1-en-3-one $\geq$ octan-3-one $\geq$ (*Z*)-3-hexenyl acetate $\geq$ octan-3-ol $\geq$ oct-1-en-3-ol $\geq$ acetophenone $>$ hexyl acetate $\geq$ methyl salicylate $>$ (*Z*)-3-hexen-1-ol.

Sensitivity to Six-Carbon Compounds. Female antennae were more sensitive to (*Z*)-3-hexenyl acetate than the saturated C_6 acetate followed by the (*Z*)-3-alcohol, as evidenced by significantly higher mean response values (except at the 1-μg dose), greater response rates (steeper curve slope), and lower threshold values.

DISCUSSION

Antennal Chemoreceptive Sensitivity

The EAG is thought to be the summational expression of generator potentials of many simultaneously stimulated receptor cells with potentially different acceptor specificities and/or affinities (Boeckh et al., 1965). The amplitude of the EAG deflection has been interpreted to be a measure of the relative number of acceptors responding to an odor stimulus (Payne, 1975). Because both the position of the electrodes and the presentation of the odor stimuli spanned the entire length of the antennae, the EAGs recorded should reflect the overall population size of chemoreceptors and/or acceptors present. Thus, the EAG results reported here for alfalfa seed chalcids suggest significant differences in size of antennal receptor and/or acceptor populations for the various alfalfa hostplant and nonhost-plant (red clover) volatiles examined. In general, both female and male ASCs possess antennal chemoreceptive sensitivities that were keen, dynamic, and selective to certain specific compounds and to certain classes of compounds, defined by their carbon-chain length, structure, isomerism, and/or functional group (Table 1). The antennae of ASCs are stimulated to a greater extent and thus possess larger populations of responsive receptors and/or acceptors for constituents of three (of the seven) structural classes of compounds investigated: (1) the six-carbon acetates, primary alcohols, and aldehydes; (2)

the eight-carbon, three-position ketones and secondary alcohols; and (3) the aromatic methyl ketones and methyl esters.

ASC Sexual Dimorphism. There appear to be significant differences between the ASC sexes in the size of their receptor and/or acceptor populations responsive to a majority (58% overall) of the alfalfa and legume volatiles tested. Overall, the receptivity of female ASCs significantly exceeded that of males for 18 of the 38 tested compounds, while the reverse was observed for just eight of the 38 compounds (Figures 1 and 2, Table 1). Furthermore, the dominance of female receptivity to legume volatiles over that of males is evident for: (1) six of the top 10 EAG stimulants (for males > females, 1/10) (Figure 3), (2) six of the 14 demonstrated behavioral attractants (for males > females, 3/14) (Table 1), and (3) four of the six demonstrated ovipositional stimulants (for males > females, 1/6) (Table 1).

The observed sexual dimorphism in antennal receptivity to volatiles of host- and nonhost-plant odors might be a consequence of either (1) chromosomal genetics or (2) natural selection. First, because male–female sex determination in Hymenoptera is due to a haploid–diploid chromosomal endowment, the apparent sexual dimorphism in antennal receptivity to volatiles of host- and nonhost-plant odors might be an expression of the different genotypes of the sexes, potentially creating differences in numbers of receptors. We suggest the second possibility, that this dimorphism in reception reflects differences between the sexes in perception of these compounds that has evolved through the selective advantage of the use of these olfactory cues in the survival requisites of host finding, food foraging, and oviposition.

Both sexes share some of these survival requisites and roles that are reflected in some common searching and foraging behaviors. Because ASCs are monophagous, host finding is selective and critical; however, they forage for nectar on host and nonhost plants. The primarily floral volatiles from alfalfa are: (*E*)-β-ocimene, 3-methylbutanol, decyl and dodecyl acetates, and methyl salicylate; those from red clover are: acetophenone, methyl cinnamate, (*E*)-2-hexenal, 2-hydroxybutan-3-one, and 2,3-dihydroxybutane (Table 1). None of these alfalfa floral volatiles were attractive or stimulatory to females in laboratory behavioral bioassays (Kamm and Buttery, 1983, 1986a,b). Because methyl salicylate was a potent elicitor of EAGs, we suspect it could have an impact on behavior but was overlooked in previous bioassays (Kamm and Buttery, 1983, 1986a,b).

The obvious specialization and diversification between the sexes is in their reproductive physiology and behavior. Since mating and oviposition of ASCs are restricted to their alfalfa host plant, one might assume that alfalfa odors that are indicative of the phenological quality or suitability of pods and/or seeds for exploitation might influence the selection by female ASCs of not just oviposition sites (Kamm and Buttery, 1986b; Kamm, 1989) but also calling and/or mating sites and/or release of pheromone (Riddiford and Williams, 1967; Riddiford,

1967; Raina, 1988). Similarly, because females call and release pheromone in the immediate context of host kairomones, the perception of these two distinct semiochemical odors may have coevolved in chemical communication to the degree that alfalfa odors might influence, enhance, or synergize the attractiveness of the female-released pheromone to orienting males. Such enhancement or synergism of the attractiveness of pheromones by plant volatiles has been observed for a number of insect species (e.g., Dickens et al., 1990; Light, unpublished).

Green Leaf Volatiles. The saturated and unsaturated [(*E*)-2- and (*Z*)-3-] C_6 acetates, alcohols, and aldehydes are the most common class of botanical volatiles, having been termed the general "green leaf volatiles" (GLVs). The GLVs are ubiquitous in the plant kingdom and are produced from the general oxidative breakdown of simple fatty acids (Buttery, 1981; Tressel et al., 1981; Schreier, 1984; Visser et al., 1979). Plant species and their plant parts differ in composition (occurrence and ratio) of these GLV compounds (Visser et al., 1979; Buttery, 1981; Van Straten and Maarse, 1983). (*Z*)-3-Hexenyl acetate and (*Z*)-3-hexen-1-ol are the dominant contributors to the odors of many forage legumes, especially alfalfa (Kami, 1978, 1983; Buttery and Kamm, 1980; Buttery et al., 1982, 1984; Srinivas, 1986) (Table 1). These two particular GLVs were potent EAG stimulants and were moderate to low in potency in eliciting behavioral responses (Figure 4), and they should be included in future behavioral tests with various chalcid species.

Because the GLVs, in particular the C_6 acetates, were found to be very potent EAG stimulants, we conclude that ASC antennae possess large populations of receptor and/or acceptors responsive to these compounds. Furthermore, this substantial olfactory receptor and/or acceptor endowment suggests a selective detection and perception of the GLVs by ASCs. Similarly, the GLVs have been found to be potent EAG and receptor cell stimulants for a great number of diverse insects (see Light et al., 1988; Visser, 1983, 1986), e.g. *Ceratitis capitata* (Light et al., 1988), *Dacus dorsalis* (Light and Jang, 1987), *Anthonomus grandis* (Dickens, 1984), *Leptinotarsa decemlineata* (Visser, 1979), and *Trirhabda bacharides* (Dickens and Boldt, 1985).

Antennal Responsiveness of Other Hymenoptera. Half the legume compounds tested here have also been the subject of electrophysiological investigations on other species of Hymenoptera from diverse families and resource ecologies—from ichneumonid parasitoids, nyssonine wasps, horntail wood wasps, to eusocial hymenopteran ants and bees. Many of these compounds have been tested on the eusocial Hymenoptera because of their importance to chemical communication, having been identified as pheromones or constituents and/or products of various glands (anal, cephalic, Dufour's, mandibular, poison, and/or sting) (Wheeler and Duffield, 1988). Single-cell electrophysiological recordings on both an ant species, *Lasius fuliginosus* (Dumpert, 1972), and *Apis*

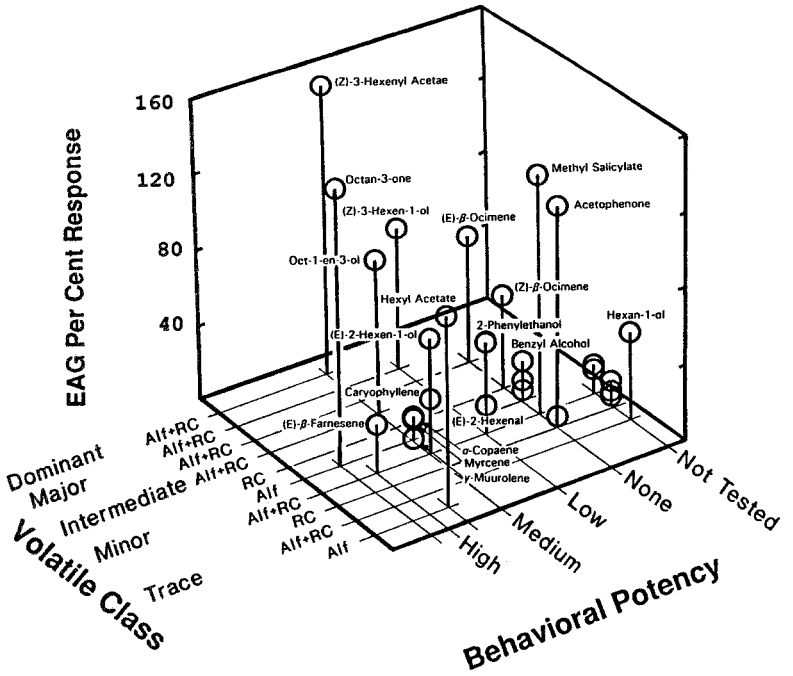

FIG. 4. Relationship of the tested volatiles to their: evoked EAGs by female ASCs, behavioral potency classification for female ASCs, and contribution classification to the odor composition of alfalfa, red clover, or both legumes. The EAG data are the mean percent response relative to standard for 10-μg doses of volatiles. Behavioral potency classification is based on threshold doses (TD): "high" potency (TD $\geq$ 0.025 μg), "medium" potency (TD $\geq$ 2.5 μg), and "low" potency (TD $>$ 25.0 μg). Contribution classification or "volatile class" for the odor constituents of leaves and/or pods is delineated as: "dominant" ($>$30% of odor), "major" ($>$10%), "intermediate" ($>$5%), "minor" ($>$1%), and "trace" ($<$1%). " Alf," "RC," and "RC+Alf" designate the presence of those volatiles in alfalfa, red clover, or both odors.

mellifera (Lacher, 1964; Vareschi, 1971) have established that the sensitivity to many of these compounds is based on the antennae being endowed with an array of different "generalist" cell types, each possessing a rather narrow specificity for certain structural, chain-length, and functional-group classes of compounds. The presence of an array of generalist cell types might also occur for ASCs.

Plant Odor Composition, Chemoreceptivity, and Behavior

No correlations were found between either the specific occurrence and concentration of a plant volatile and ASC behavior (Table 1 and Figure 4). ASCs were highly receptive to the dominant odorants of alfalfa, (Z)-3-hexenyl

acetate and (*Z*)-3-hexen-1-ol, but these compounds individually elicited only medium and low potency (respectively) overall behavioral responses (low attractancy, but moderate arrestment and ovipositional stimulation) (Table 1 and Figure 4). However, the three compounds that were most potent behaviorally (volatiles that elicited significant levels of behavior at the lowest threshold doses) were merely minor or trace odor constituents of either alfalfa or red clover. For example, octan-3-one and hexyl acetate are minor and trace components of alfalfa odor, but they are potent elicitors of both EAG responses and behavioral attraction, arrestment, and oviposition responses. In contrast, (*E*)-β-farnesene is a minor to trace component of both alfalfa and red clover odors and low in EAG potency, but it is second overall to octan-3-one as a very potent elicitor of ASC behavioral attraction and a potent elicitor of oviposition.

Nearly all of the plant components that were behaviorally active elicited moderate [e.g., (*E*)-2- and (*Z*)-3-hexen-1-ols and oct-1-en-3-ol] to potent [e.g., (*Z*)-3-hexenyl acetate, octan-3-one and hexyl acetate] EAGs. Three exceptions are minor constituents of alfalfa odors that are relatively impotent as EAG stimulants. Two of these volatiles, α-copaene and γ-muurolene, are minor components of alfalfa pod odor and are moderately potent in eliciting close-range flight and pedestrian orientation, while γ-muurolene inhibits oviposition (Table 1) (Kamm and Buttery, 1983, 1986b). Myrcene (a minor component of both alfalfa flower and leaf odors) also elicited a very low EAG response but was a very potent stimulant for oviposition but not flight. These behaviors were observed only when females were in contact with the host (or an evaporative substrate, i.e., filter paper) (Kamm and Buttery, 1983, 1986b). Considering both the low antennal olfactory responses and the low volatility of these compounds, female ASCs may detect these semiochemicals with contact chemoreceptors on the tarsi, ovipositor, or tip of antennae (Kamm and Buttery, 1986b). Chalcids constantly tap plant surfaces with the tips of their antennae in a manner suggesting both a chemo- and mechanoreceptive evaluation of substrates.

The detection and recognition of host from nonhost plant is crucial for seed chalcids, and thus both the presence of host species-specific volatiles and their selected activity as kairomones and allomones is expected. The seed chalcids might follow sequential perceptual assessments and behavioral responses to olfactory information in their immediate environment. The activation of flight orientation by ASCs might be elicited by general volatiles of clover-related legume species, but the maintenance of the anemotaxis (i.e., the flight to or arrival on the target plant) might require the discrimination of alfalfa-specific volatiles. Moreover, ASCs might be initially attracted to legume kairomones, but this attraction might be repelled (inhibited or interrupted) by allomonal nonhost volatiles. However, we doubt that nonhost components can confound host finding because chalcids readily identify their hosts from nonhosts in mixed stands in nature. Finally, close-range orientation and alightment might be elicited by alfalfa volatiles followed by contact host-assessment and either stimu-

lation or inhibition and/or interruption of oviposition. The odorants in common between alfalfa and red clover can be segregated into four classes based on behavioral response of female ASCs: (1) volatiles that elicited no responses [(*E*)-β-ocimene and methyl salicylate], (2) volatiles that elicited only flight orientations (attraction) [oct-1-en-3-ol and (*E*)-2-hexenal], (3) volatiles that elicited flight and pedestrian orientations [(*E*)-2-hexen-1-ol and (*Z*)-3-hexen-1-ol], and (4) volatiles that elicited both of these orientations and oviposition [(*Z*)-3-hexenyl acetate, (*E*)-β-farnesene and caryophyllene] (Table 1, Figure 4). Some of these compounds common to both legumes, for which ASCs have a keen chemoreceptivity and which elicit orientation behavior, are believed to be involved in initial host (or legume) finding; then the alfalfa-specific volatiles with demonstrated behavioral impact [hexyl acetate, γ-muurolene, α-copaene, and myrcene (Table 1)] are perhaps close-range cues that females use for host identification and/or discrimination. Conversely, ASC antennae were keenly sensitive to acetophenone (a red clover-specific volatile) and thus it might be a distinguishing cue for identification of nonhost legumes. Without sensitivity to such nonhost compounds, ASC females could expend considerable time and energy maladaptively responding to compounds present in both legumes. Appropriate flight bioassays are now required to identify the specific behaviors (initiation of flight, orientation, landing, and assessment of the source) elicited by specific host and nonhost volatiles when presented individually and in various binary and multicomponent combinations, as was accomplished using plant parts (Kamm, 1989). Information herein will provide insight into how chalcids perceive and use olfactory cues to exploit their host plant.

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BIOLOGICAL EVIDENCE OF AN OVIPOSITION-DETERRING PHEROMONE IN *Lobesia botrana* DEN. ET SCHIFF. (LEPIDOPTERA, TORTRICIDAE)

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Abstract—Females of the European grapevine moth (*Lobesia botrana* Den. et Schiff.) usually deposit isolated eggs on flowers and berries of the grapevine (*Vitis vinifera* L.). We have investigated whether an epideictic pheromone could be present on the egg surface to explain this spacing behavior. About 21,000 eggs of *L. botrana* were washed in cold methanol, and the biological activity was tested in a two-choice bioassay offering treated and nontreated areas. Different dilutions of the extract were tested in methanol, which was inactive alone. At the dose of four egg equivalents per microliter of extract, the number of eggs laid by the *L. botrana* females was reduced by as much as 57% on the treated areas compared to nontreated areas. A longer-term suppression of oviposition (at least 24 hr) following an exposure to the extract occurred for two doses (0.6 and 4.0 eggs/μl) of egg extract. Our results strongly suggest the occurrence of an oviposition-deterring pheromone (ODP) on the eggs of *L. botrana*. The ecological value of these results is discussed.

Key Words—Pheromone, oviposition-deterring pheromone, oviposition, *Lobesia botrana*, Lepidoptera, Tortricidae.

INTRODUCTION

The study of insect oviposition behavior in response to plant semiochemicals has been a focus of numerous studies (Renwick and Radke, 1987; Schöni et al., 1987; Thompson and Pellmyr, 1990). However, little is known about the role

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and the nature of conspecific host-marking chemicals that mediate the oviposition behavior of females. Host-marking has been reported in insect parasitoids (van Lenteren, 1981) and in more than 30 phytophagous species (Anderson, 1986; Roitberg and Prokopy, 1987). The biological activity of epideictic pheromones extracted from Lepidoptera eggs has been reported in only a few species (Rothschild and Schoonhoven, 1977; Klijnstra, 1986; Schoonhoven et al., 1990; Thiéry and Le Quéré, 1991).

Lobesia botrana Den. et Schiff. (European grapevine moth, EGM) is a polyphagous insect *stricto sensu* that develops on a range of host plants that belong to different families (Stoeva, 1982). The oviposition behavior depends on the adult generation in the year and on the available plants (Bovey, 1966; Stoeva, 1982). Females usually lay isolated eggs on flower buds (first flight), immature (second flight), and matured grapes (third flight). This suggests that egg marking might exist and that it might influence the oviposition site selection and egg dispersion in this insect.

This paper reports on the mediation of the egg laying by *L. botrana* in response to conspecific semiochemicals extracted from the egg surface.

METHODS AND MATERIALS

Biological Material and Extraction. The insects originated from a stock culture annually infused with wild insects (INRA, Bordeaux, France) reared on a semisynthetic diet under controlled conditions (16:8 light–dark; 22 ± 1°C; 65 ± 5% relative humidity). Females were allowed to oviposit inside precleaned glass tubing (1 cm diam.) 48 hr after mating. Two females were placed in each tube for 24 hr, then the tubes were replaced for an additional 24 hr period. A total of 21,363 eggs laid by 386 females were collected. Each tube was rinsed with 2 ml of purified methanol (purity >99.99%). Scales were removed from the extract by filtration on a Millipore filter (type GW, 0.22 μm). The total extract was then reconcentrated under a flow of nitrogen (U quality), which was previously purified by a Tenax trap. Three dilutions expressed in egg equivalent (ee) per microliter were obtained: 4 ee, 0.6 ee, and 0 ee (pure solvent).

Bioassay. The bioassay consisted of a two-way choice. The oviposition arena was a Plexiglas tube (ID 6.5 cm, length 13.6 cm) in which females could oviposit on a sheet of cardboard (10.7 × 13.6 cm) (Figure 1A). The cardboard covered the upper half of the inside oviposition arena, and the other half was covered with white tissue to avoid oviposition (females are able to lay the eggs only on smooth surface). To test the different dilutions, 32 drops of 5 μl each were deposited on the oviposition substrate. In order to make equidistant circles approximating the size of small berries, we applied on the oviposition substrate a Plexiglas mask to limit marking to the circles on the substrate (Figure 1B).

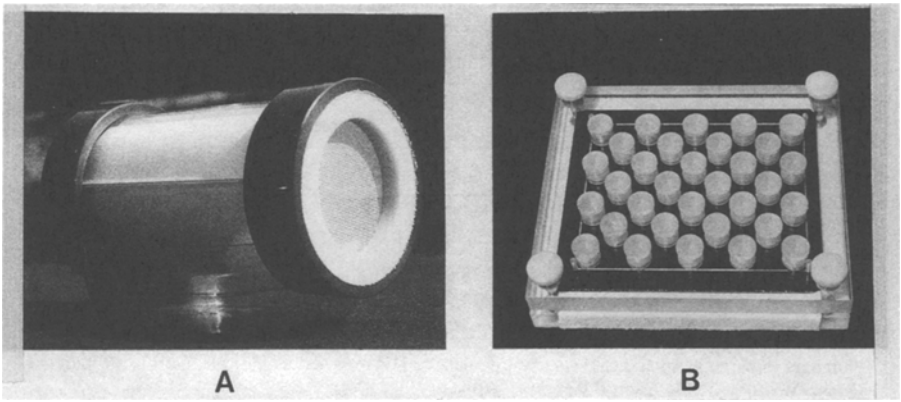

FIG. 1. (A) Oviposition arena used with *Lobesia botrana*; (B) Plexiglas mask with 32 holes (1.1 cm diam.) used to deposit the extract. Each hole is covered with a cap pierced in its center for insertion of the syringe needle.

Circles were 1.1 cm diameter (centers 2.1 cm apart). Ten females per replicate were placed for one night inside the arena. The number and the position of the eggs were then recorded. The ovipositing status of the females was determined by placing them separately in glass tubing 24 hr after the exposure to the various treatments and observing whether or not they oviposited. The number of eggs laid by each female was then counted.

At least 10 groups of females were used for each treatment. A deterrence index (DI) was calculated according to the area ratio between the untreated and treated area (B area $= 3 \times A$ area), DI $= [(3A - B)/(3A + B)] \times 100$, where A is the total number of eggs laid on the treated areas and B on the untreated area for all the replicates. The number of eggs laid by females 24 hr after exposure to the egg extract was also noted.

RESULTS AND DISCUSSION

The mean of the ratio of the number of eggs per ovipositing female during the exposure to the egg extract was not significantly affected by the various treatments. This mean varied from 38.91 ± 7.98 (methanol) to 41.2 ± 4.84 (4 eggs/μl dose). These differences were not statistically significant (Mann-Whitney U test) (Table 1).

The oviposition preferences are reported in Table 2. In the blank experiment (cardboard without solvent), females deposited about 1/4 of their eggs on the area corresponding to the position of the deposits of solvent or extract. The value of DI (0.06%) indicates no preference between these two areas, and the

TABLE 1. NUMBER OF EGGS PER OVIPOSITING FEMALE IN *Lobesia botrana* DURING AND 24 HR AFTER EXPOSURE TO DIFFERENT DILUTIONS OF EGG EXTRACT[a]

	Treatments			
	Blank	Methanol	0.6 egg/μl	4 eggs/μl
Mean number of ovip. females	8.43 (1.27)	8.33 (1.05)	8.80 (1.32)	9.44 (0.88)
During exposure	39.05 (5.07) a	38.91 (7.98) a	39.87 (5.69) a	41.20 (4.84) a
24 hr after exposure	38.5 (2.62) a	38.27 (6.37) a	32.33 (2.87) b	32.90 (2.33) b

[a]Numbers in parentheses indicate standard deviation. Different letters indicate statistical differences (Mann-Whitney U test, $P < 0.02$).

TABLE 2. EFFECT OF EXPOSURE OF *Lobesia botrana* FEMALES TO DIFFERENT CONCENTRATIONS OF CONSPECIFIC EGG EXTRACTS[a]

	Treatments			
	Blank	Methanol	0.6 egg/μl	4 eggs/μl
Replicates	10	16	11	10
Treated area (36.17 cm^2)	80,30 (11,61)	78,38 (21,85)	69,73 (26,59)	36,50 (10,71)
Untreated area (109.35 cm^2)	240,60 (34,16)	232,06 (65,67)	286 (102,44)	331,00 (87,37)
DI (%)	0.06	0.65	−15.51	−50.28
Statistical analysis	(a)	(a)	(b)	(c)

[a]Numbers in parentheses represent standard deviations. Differences letters indicate statistical differences on egg numbers (χ^2 analysis, $P < 0.01$). The deterrence index (DI) varies from +100% in case of maximal stimulation to −100% in case of maximal deterrency, the 0% value indicates no preference.

deterrency index obtained in the methanol treatment (DI = 0.65%) was very similar to that for the blank (χ^2). The observation that methanol does not affect oviposition is consistent with that on other Lepidoptera (Dittrick et al., 1983; Klijnstra, 1986; Thiéry and Le Quére, 1990). At the dose of 20 egg equivalents (ee) per circle (4 ee/μl), females strongly preferred to oviposit on the untreated areas (χ^2, $P < 0.001$) (DI = −50.28%). The observed deterrency was apparently dose dependent, as the deterrence index obtained with the 0.6 ee/μl dilution (DI = −15.51%) was significantly higher to that obtained with solvent (χ^2, $P < 0.01$), but lower than that obtained with the dose of 4 ee/μl (χ^2, $P < 0.001$).

We never obtained total suppression of oviposition in any replicate. The maximum reduction obtained with the highest dose was 57% (DI) (i.e., 309 eggs deposited on the untreated area vs. 28 eggs on the treated area; control methanol: 169 vs. 61 eggs). This observation is consistent with experiments reported on other lepidopteran species in which total deterrency was never observed (Schoonhoven, 1990; Thiéry and Le Quéré, 1990). In *L. botrana*, we can observe a slight but insignificant increase in the mean number of eggs laid by females with the higher dose of egg extract (Mann Whitney U test) (Table 1). Klijnstra and Schoonhoven (1987) observed that, after contacting ODP, *Pieris brassicae* increased its dispersal activity. This might influence the number of eggs laid by females. The presence of egg extract on the substrate might also lure the females, providing them with chemical information that corresponds to a "better quality substrate" because it releases egglike signals. The slight increase we have observed at the high dose needs confirmation by further observation. In such a two-choice bioassay, the effect of *L. botrana* ODP is interpreted as discrimination enhancing more than oviposition suppressing.

The mean number of eggs laid by *L. botrana* females was depressed 24 hr after exposure to the dose of 0.6 ee/μl (Mann-Whitney U test, $P < 0.02$) and to the dose of 4 ee/μl (Mann-Whitney U test, $P < 0.002$), although no statistical differences were observed between these two doses (Table 1). This effect was not observed in either the blank or the methanol treatment, where no statistical differences were observed between the two consecutive days of oviposition (Mann-Whitney U test) (Table 1). These results suggest a persistent effect of the deterrency in *L. botrana*. The mechanism underlying this reduction in the number of eggs laid after exposure to the ODP is not yet understood. We lack data to conclude whether peripheral saturation of the neuroreceptors, integrative processing, or motor control of egg deposition are involved in this suppression. The ecological significance of a lasting effect of the ODP exposure is still unknown. The effect we observed needs to be checked over longer periods and has to be confirmed with single female observations instead of grouped females.

In Europe, *L. botrana* is considered one of the major pests of vineyards (Bovey, 1966). ODP could be useful in future integrated pest management tactics against this insect. However, insect control programs utilizing ODP will need additional study of chemical identification, learning capacities of females, polymorphism of ODP production, and perception by females.

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MAJOR ANTITERMITIC COMPONENTS OF THE HEARTWOOD OF SOUTHERN CATALPA

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Abstract—The heartwood of southern catalpa, *Catalpa bignonioides* Walt., is resistant to attack by the eastern subterranean termite *Reticulitermes flavipes* (Kollar), but extraction with a ternary solvent mixture of acetone-hexane-water (54:44:2) by volume removed the antitermitic characteristics from the heartwood. Four compounds comprised approximately 98% of the antitermitic fraction of the extract: the sesquiterpene alcohol, catalponol (67%); its epimer, epicatalponol (5%); a structurally related ketone, catalponone (1%); and the phthalide, catalpalactone (25%). Pure compounds were isolated by semipreparative scale reversed-phase HPLC and identified by GC-MS and UV spectroscopy. The structure of catalponol was further confirmed by the formation of derivatives. Bioassays indicated that catalponol had the greatest toxicity in cellulose pad tests, but in tests using vacuum impregnation of these compounds into termite-susceptible wood blocks at levels approximating those found in catalpa heartwood, catalpalactone exhibited the highest antitermitic activity.

Key Words—*Catalpa bignonioides*, Isoptera, *Reticulitermes flavipes*, termite, sesquiterpenoids, wood extractives, catalponol, epicatalponol, catalponone, catalpalactone, antitermitic activity.

INTRODUCTION

Southern catalpa, *Catalpa bignonioides* Walt., is well known for its durability and has long been used for fence posts. Our studies on conifers (McDaniel, 1989; McDaniel et al., 1989), a recent study on bald cypress by Scheffrahn (1989), and previous studies by Carter and coworkers (Carter, 1976, 1979; Carter and Dell, 1981; Carter and Smythe, 1972, 1974; Carter et al., 1978) have all indicated that antitermitic properties of heartwoods are related to the

amount and chemical characteristics of the extractable material contained in the heartwood. Except for the identification of catalpalactone (Inouye et al., 1967), little work has been done on the chemical nature of *C. bignonioides* extractives, but Carter and Beal (1982) did show that an acetone extract of *C. bignonioides* heartwood protected susceptible southern pine sapwood from subterranean termite attack in a one-year field test.

Extensive studies on the biosynthetic pathways of naphthoquinone congeners have led to the identification of the extractives of a similar species, *C. ovata* G. Don. (Inoue et al., 1980; Inouye et al., 1965, 1967, 1971, 1975a,b, 1981; Shingu et al., 1971; Ueda et al., 1980), which is native to Japan and is known as "kisasage."

In this study, the extractive components of *C. bignonioides* were found to be nearly identical to those of *C. ovata*. The major components were isolated and the antitermitic properties of these components were investigated.

METHODS AND MATERIALS

Preparation of Test Material. Heartwood boards were cut from two *C. bignonioides* trees: one felled in the Harrison Experimental Forest in southern Mississippi, the other in Copiah County in central Mississippi. The boards were oven-dried overnight at 50°C, after which shavings were made with a jointer and then ground in a Wiley mill. A 500-g portion of freshly milled wood was extracted six times with 1000-ml volumes of a ternary solvent mixture of acetone–hexane–water (AHW) that was 54:44:2 by volume. The extract was vacuum filtered and the solvent removed from the extracted components in vacuo at 40°C. Residual water was removed by azeotroping with ethanol in vacuo at 50°C.

Separation of Extractive Components. The extracted material was stirred for 2 hr with 200 ml of toluene and then vacuum filtered again. The beige-colored insoluble portion was washed four times with 10-ml portions of toluene and the filtrates were combined.

The toluene solvent was removed in vacuo at 50°C, and 100 ml of acetonitrile were added. A white gelatinous precipitate formed and was removed by vacuum filtration. The dark yellow–brown filtrate was placed in a freezer at −10°C for one week. Crude yellow crystals that formed during this time were then removed by vacuum filtration, and subsequent recrystallization from methanol yielded white crystals.

The components of the acetonitrile filtrate were further separated by semi-preparative, reversed-phase high-performance liquid chromatography (HPLC)

with a Perkin-Elmer 3B Liquid Chromatograph[1] and a Whatmann M-9 ODS-3 column. The solvent employed was a 1:1 (v/v) mixture of acetonitrile–water at a flow rate of 2.5 ml/min. The eluant was monitored with a Hewlett-Packard 1037A refractive index detector and a Hewlett-Packard 3392A recording integrator. HPLC fractions were collected, and the volumes were increased twofold by the addition of distilled water and then partitioned with methylene chloride. The methylene chloride extract was dried over anhydrous sodium sulfate, and the solvent removed in vacuo.

Analysis of Components. The extractive components were identified with a Hewlett-Packard 5890A gas chromatograph (GC) coupled to a 5970 Mass Selective Detector, which is a quadrupole mass spectrometer (MS) that uses 70-eV electron-impact ionization. The system is interfaced to a HP 9000-216 computer and a 9133 disk drive. The column employed was a 30-m × 0.25-mm-ID DB-5 (J&W Scientific, Folsom, California) fused silica capillary column. The carrier gas was ultrapure helium at a head pressure of 105 kPa. The oven temperature was programmed from 50°C to 150°C at 25°C/min, and then to 285°C at 5°C/min. The injection port temperature was 250°C and the transfer line was 280°C.

The components were quantified with a Shimadzu 6AM gas chromatograph and a flame ionization detector (FID) interfaced to a Hewlett-Packard 3392A integrator. The column employed was a 30-m × 0.53-mm Megabore DB-1 (J&W Scientific), with ultrapure nitrogen as the carrier gas at a flow rate of 7 ml/min. The oven temperature was programmed from 150°C to 325°C at 5°C/min. The injection port and detector temperatures were 285°C.

Ultraviolet (UV) spectra were obtained using a Perkin-Elmer Hitachi 200 spectrophotometer with ethanol as the solvent.

Bioassays. Bioassays were performed using workers beyond the third instar from field-collected colonies of the subterranean termite *Reticulitermes flavipes* (Kollar). The colonies were collected in the Desoto National Forest in southern Mississippi.

Preliminary tests were performed by dissolving the material in acetone and applying 1.0-ml aliquots of the solutions to cellulose pads (ca. 0.5 g, 47 mm diameter, Gelmann Instrument Company) at concentrations of 2500 μg/ml, 1,250 μg/ml, 500 μg/ml, and 250 μg/ml. The solvent was allowed to evaporate, the pads were moistened with 1.0-ml portions of distilled water and placed in cylindrical plastic test chambers that measured 4.0-cm high by 5.3-cm in diameter, and 25 *R. flavipes* workers were added to each container. Pads treated with 1.0-ml volumes of acetone served as controls, and all tests were run in triplicate.

[1] Mention of a company, trade name, or commercial products is for identification purposes only and does not imply endorsement by the USDA.

The condition of the termites was observed at 24 hr, 48 hr, 72 hr, one week, and then twice a week for the duration of the four-week test.

Further bioassays were performed to assess the wood-protectant qualities of the extractive components. The test material was dissolved in acetone (or, in one case, tetrahydrofuran) and vacuum-impregnated into sapwood blocks of sweetgum, *Liquidambar styraciflua* L., according to ASTM methodology (Anon., 1976), at concentrations approximating that of the material in catalpa heartwood.

The control blocks were prepared by treating them with acetone only. They were weighed, vacuum-impregnated with acetone, and then weighed again. The mean uptake of acetone in the controls was used to estimate the concentrations of the extract materials necessary to prepare wood at given concentrations.

Two protocols were used in the laboratory tests: no-choice tests and choice tests. In no-choice tests, a mixture of sand–vermiculite–water (1:1:0.4) by volume (Haverty, 1979) was placed into a cylindrical plastic test chamber as described above. The treated blocks were oven-dried for 3 hr at 50°C, cooled for 30 min, and weighed to the nearest 0.01 g. They were then placed into the test chambers with the sand–vermiculite–water mixture completely covering them. Then 100 *R. flavipes* workers were added to each test chamber and tops were placed on the chambers. The choice test was similar to the no-choice test except that two chambers connected by a 10-cm × 0.5-cm-ID polypropylene foraging tube were employed in the former. An untreated block was buried in one chamber and a treated block in the other. The 100 *R. flavipes* workers were then placed into the chamber with the treated block, and tops were placed on both chambers.

In both the choice and no-choice protocols all treatments were run in triplicate, and blocks treated with solvent alone served as controls.

After four weeks the test chambers were dismantled and the surviving termites were counted. The blocks were cleaned with a small brush, dried as before, and the weight loss determined. The physical condition of each block was examined and assigned a rating based on both weight loss and physical condition (Table 1).

RESULTS AND DISCUSSION

The initial separations yielded a toluene-insoluble brown solid, an acetonitrile-insoluble material, and an acetonitrile-soluble fraction. A total ion chromatogram of the acetonitrile-soluble material is shown in Figure 1. Identifications of the major components of this mixture, quantified by FID detector response, and their Kovats' indices are shown in Table 2. Relevant UV and MS data of these major components are presented in Table 3. In general, they are in excel-

TABLE 1. BLOCK DAMAGE RATING FOR EXTRACTIVES OF *Catalpa bignonioides*

Damage rating	Description	Weight loss (%)
0	No feeding	0–2
1	Slight feeding	3–5
2	Moderate feeding	6–20
3	Extensive feeding	21–50
4	Very extensive feeding	>50

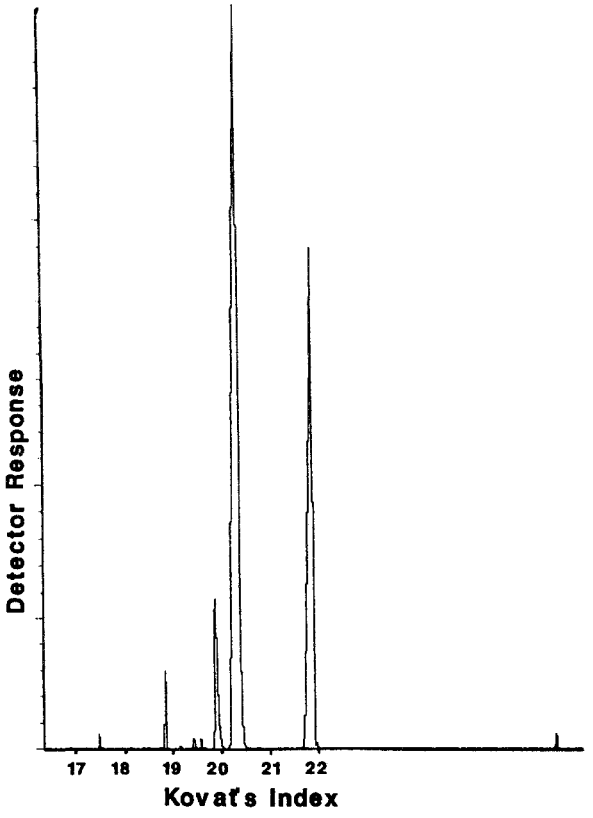

FIG. 1. Total ion chromatograph of *Catalpa bignonioides* acetone extract.

TABLE 2. CHROMATOGRAPHIC DATA OF MAJOR COMPONENTS OF SOUTHERN CATALPA HEARTWOOD

Component	Kovats' index (DB-5)	Composition (%)[a]
Catalponone	1892	1
Deoxylapachol	1921	tr[b]
$C_{15}H_{16}O_2$	1948	tr[b]
Epicatalponol	1988	5
Catalponol	2036	67
Catalpalactone	2196	25

[a]Determined by integration of FID GC detector response.
[b]Trace (<0.5%).

TABLE 3. SPECTROSCOPIC DATA OF MAJOR COMPONENTS OF SOUTHERN CATALPA HEARTWOOD

Component	Molecular formula	UV absorbances[a] [λ_{max} nm (log ϵ)]	Mass spectra m/z (%)
Catalponone	$C_{15}H_{16}O_2$	224 (4.59) 253 (4.13)	M+ 228 (23), 173 (32), 160 (100)
Epicatalponol	$C_{15}H_{18}O_2$	205 (4.48) 248 (4.05) 286 (3.24)	M+ 230 (33), 197 (22), 161 (81), 105 (61)
Catalponol	$C_{15}H_{18}O_2$	211 (4.07) 247 (3.97) 286 (3.26)	M+ 230 (3), 197 (35), 105 (63)
Catalpalactone	$C_{15}H_{14}O_4$	207 (4.20) 224 (4.13) 275 (3.13) 281 (3.11)	M+ 258 (1), 240 (100), 197 (55), 133 (74), 105 (37)

[a]All UV spectra were run in ethanol solvent.

lent agreement with data on these compounds obtained from *C. ovata* (Inouye et al., 1971, 1975b). The identifications of catalponol, epicatalponol, catalponone, and deoxylapachone were further substantiated by the isomerization of catalponol to epicatalponol with NaOH, the oxidation of catalponol to catalponone with Jones reagent, and the oxidation of catalponol to deoxylapachone with manganese dioxide (Inouye et al., 1975a). Additional amounts of epica-

talponol and catalponone were generated by these methods as needed for bioassays.

The HPLC chromatogram of the acetonitrile-soluble extract is shown in Figure 2. The major compounds from Table 2 were isolated in >95% purity for bioassays.

The results of the bioassays on cellulose pads are shown in Table 4. The most active termiticidal components were catalponone and catalponol; epicatalponol was somewhat less active. Catalpalactone was less termiticidal than the above compounds, but it did have moderate activity at higher concentrations. The toluene-insoluble fraction showed no toxicity toward *R. flavipes*, even at the highest concentration. The acetonitrile-insoluble material turned brown during vacuum filtration and became only slightly soluble in acetone. It was not tested on the cellulose pads because the evidence suggested that changes were taking place in the material and it was no longer a heartwood component, but was an artifact instead. This material was, however, tested later in the vacuum impregnation tests.

The results of the choice and no-choice vacuum-impregnation bioassays are presented in Tables 5 and 6. These tests indicated that the primary antiter-

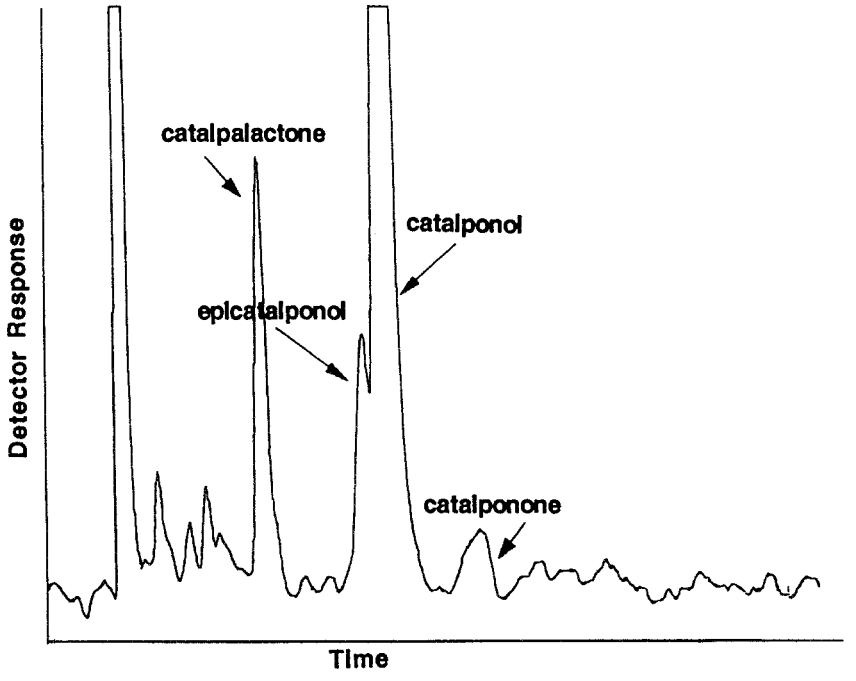

FIG. 2. HPLC chromatogram of *Catalpa bignonioides* acetone extract.

TABLE 4. CELLULOSE PAD BIOASSAY OF EXTRACTS AND COMPONENTS OF SOUTHERN CATALPA HEARTWOOD AGAINST *Reticulitermes flavipes* WORKERS

Test material	Concentration (ppm)	Termite survival (%) after treatment									
		1 day	2 days	3 days	7 days	11 days	14 days	18 days	21 days	25 days	28 days
Control	0	100	100	100	100	100	100	100	99	99	99
Catalponone	5000	100	52	15[a]	0						
	2500	100	65	27[a]	0						
	1000	100	81	35[a]	12	0					
	500	100	69	64	32	0					
Epicatalponol	5000	100	96	93	89	44	23[a]	16	0		
	2500	100	100	100	100	100	100	67	19[b]	19	9
	1000	100	100	100	99	99	99	99	99	80	37
	500	100	100	100	100	100	100	99	99	99	83
Catalponol	5000	100	100	72	0						
	2500	100	100	100	13[a]	11	0				
	1000	100	100	100	40	5[b]	0				
	500	100	100	100	72	47	0				
Catalpalactone	5000	100	100	100	89	83	49	27	8[a]	3	0
	2500	100	99	99	97	88	81	71	61	40	16
	1000	100	100	100	99	97	92	88	85	83	64
	500	100	100	99	99	99	99	97	97	96	85
AHW[c] extract	5000	89	[d]	45[a]	29	0					
	2500	100	[d]	100	99	5[b]	0				
	1000	100	[d]	100	99	73	36[a]	28[b]	19	4	0
	500	100	[d]	100	100	96	64	19[a]	15	9[b]	0
Toluene-insoluble	5000	100	100	100	100	100	99	97	97	97	95
	2500	100	100	100	100	100	100	99	99	99	99
	1000	100	100	100	100	100	100	100	100	100	100
	500	100	100	100	100	99	99	99	99	99	95

[a] All termites in one replicate died.
[b] All termites in two replicates died.
[c] Acetone–hexane–water mixture (54:44:2) by volume.
[d] Not determined because of laboratory oversight.

mitic compounds in *C. bignonioides* are the most abundant compounds in the acetonitrile-soluble extract fraction, catalponol and catalpalactone. When they were placed into susceptible wood at levels approximating those found in the heartwood of *C. bignonioides*, both of these compounds imparted antitermitic qualities that were comparable to those of the heartwood itself.

TABLE 5. NO-CHOICE TEST OF *Catalpa bignonioides* HEARTWOOD AND HEARTWOOD EXTRACTIVES[a]

Test material	Concentration (%)	Termite survival (%)	Damage rating
Catalponone	0.02	79	2.0
Epicatalponol	0.20	83	1.0
Catalponol	2.67	0	0.0
Catalpalactone	1.01	1	0.0
Toluene-insoluble material	0.80	82	1.0
Acetonitrile-insoluble material[b]	1.07	80	1.3
Catalpa wood		0	0.0
Control		90	2.0

[a]Extractives were vacuum impregnated into sapwood blocks of sweetgum *Liquidambar styraciflua* L.
[b]Material was acetone insoluble and was dissolved in tetrahydrofuran.

TABLE 6. CHOICE TEST OF *Catalpa bignonioides* HEARTWOOD AND HEARTWOOD EXTRACTIVES[a]

Test material	Concentration (%)	Termite survival (%)	Damage rating	
			Treated	Untreated
Catalponone	0.02	88	0.7	1.3
Epicatalponol	0.20	82	0.7	1.0
Catalponol	2.80	88	0.0	2.0
Catalpalactone	1.98	86	0.0	2.0
Toluene-insoluble material	0.86	86	0.0	1.7
Acetonitrile-insoluble material	1.09	89	0.3	1.3
Catalpa wood		76	0.0	1.0
Control		85	1.0[b]	1.3

[a]Extractives were vacuum impregnated into sapwood blocks of sweetgum *Liquidambar styraciflua* L.
[b]Not treated, but corresponds to the chamber into which the termites were introduced.

Insufficient amounts of some of the minor compounds, including deoxylapachone, were isolated for these tests and, therefore, they were not tested further. The toluene-insoluble material and the acetonitrile-insoluble material both seemed to impart little or no antitermitic character to the test wood at the concentrations examined.

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ELECTROANTENNOGRAM RESPONSES OF SYNTHETIC PERIPLANONE-A AND PERIPLANONE-B IN THE AMERICAN COCKROACH

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Abstract—The electroantennogram responses of synthetic (−)-periplanone-B (PB) and (−)-periplanone-A (PA) were evaluated by using the antennae of the unmated male American cockroach *Periplaneta americana* L. The threshold concentrations of the pheromone activities for the two chemicals were similar. Neither a synergistic nor an inhibitory response was noted, and only an additive effect was detected when PA and PB were applied as a mixture.

Key Words—American cockroach, *Periplaneta americana*, sex pheromone, EAG response, periplanone-A, periplanone-B, Orthoptera, Blattidae.

INTRODUCTION

Nishino and coworkers have extensively studied EAG responses of sex pheromones (SP) and their mimics (SPM) in the American cockroach and other *Periplaneta* spp. In 1979, Nishino and Takayanagi showed that the middle region of male antenna was the most strongly stimulated by SPM. The male–female index of EAG responses was a good indicator for the response of sexually active components (Nishino and Kimura, 1981; Nishino and Takayanagi, 1981). Nishino et al. (1983) isolated the SP of the American roach, periplanone-A (PA) and periplanone-B (PB), from female feces and soiled filter paper, and the quantities of PA and PB were estimated to be 6.8 and 1.5 μg, respectively. Nishino and Kuwabara (1983) evaluated the threshold dose of PA and PB. In

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the EAG response, both pheromones induced significant responses from the male antennae at 1×10^{-4} μg. By applying a differential saturation EAG technique (DS-EAG), Nishino and Manabe (1983, 1984) showed that there were two types of SP receptors specifically responsible for PA and PB in their experiment. Additionally, Manabe and Nishino (1985) studied the affinity and intrinsic activity of the stimulants to the PB receptor and categorized those chemicals into four types. Nishino and Usui (1985) dealt with the comparative olfactory sensitivity of *Periplaneta* cockroaches to general odors. Nishino et al. (1988) further found that the ratio of PA and PB in the feces and soiled filter paper was 8:5. Recently, Okada et al. (1991) evaluated the biological activities of synthetic periplanone analogs, including four candidates of PA by behavioral and EAG assays and suggested that Hauptmann's PA is a natural PA produced from female cockroaches.

In 1990, Okada et al. (1990a) showed that the activity of synthetic PB was two orders of magnitude lower than that of PA, and the mixtures of PA and PB (PA + PB) elicited only a simple additive effect in the sexual response of the American cockroach when a special behavioral bioassay was used. In this study, the authors used standard EAG techniques to investigate the olfactory responses of *P. americana* to synthetic PB, Hauptmann's PA, and PA + PB to see if there is a synergism or inhibition when PA and PB are used together.

METHODS AND MATERIALS

Insects. Unmated male cockroaches (3–4 months old) from the Institute of Zoology colonies were selected and kept in a room that was isolated from females and female odor. Water and commercial dry dog food were provided ad libitum. Room temperature was 26 ± 2°C and relative humidity was 55 ± 5%. The light regime was a 16:8 light–dark photocycle, with the dark phase beginning at 8:00 A.M. The insects were chosen on the basis of behavioral sex responses when stimulated by the pipet method with 10^{-2} μg PB (Chow and Wang, 1981).

Pheromone Sources. Synthetic pure crystalline compounds of PB and Hauptmann's PA were kindly provided by Dr. Kenji Mori. Gas chromatography–mass spectrometry analysis of the compounds showed that both compounds were chromatographically pure and had the same mass spectra as reported for PA (Kuwahara and Mori, 1990) and for PB (Talman et al., 1978). The GC-MS system consists of a Varian 3400 GC coupled with a Finnigan MAT Incos 50. A 30-m DB-5 fused silica capillary column was used for the analysis and elution was programmed from 50°C (hold for 5 min), then 50°C to 280°C with a heating rate of 2°C/min (carrier gas: helium). The injector temperature was set at 200°C, and the ion source temperature at 180°C. PA eluted at 23:46 and

with mass fragmentation of *m/z* 41(65), 55(32), 65(24), 77(55), 79(45), 91(100), 105(53), 117(40), 131(28), 147(20), 159(28), 171(10), 189(10), 202(8). Compound PB eluted at 25:32 and with mass fragmentation of *m/ z* 41(70), 55(40), 69(30), 77(70), 91(100), 105(43), 117(25), 131(22), 145(20), 159(20), 175(10), 187(20), 204(8), 248(1).

EAG Recording. PA and PB were tested on excised male antennae that were snipped off at the pedical; 1 mm of the distal tip was cut off. The EAG recording system used was based on the early methods used by Chow et al. (1980) and Nishino and Takayanagi (1979). PA and PB were diluted hexane and applied to a 1-cm^2 filter paper at doses of 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 10^0, 10^1, 10^2 μg; the solvent was allowed to evaporate for 5 minutes. Twenty-five combinations of PA and PB were made from 10^{-3}, 10^{-2}, 10^{-1}, 10^0, 10^1 μg (see Figure 2 below). Preparation of 8:5 mixtures of PA and PB (Nishino et al., 1988) at doses of 10^{-5}, 10^{-4}, and 10^{-3} μg were completed. Stimulation with air alone and hexane alone (10 μl) were applied as check samples. The needles and stimulation syringes were only used once. Routinely, tests were repeated at 3-min intervals. An antenna was tested by PA, then PB at the same dose, or was tested by PB, then PA. In tests of single PA and PB samples, each antenna was used for eight pairs of tests from the lowest to the highest dose. In tests of PA + PB, each antenna was used for eight tests. During the testing period, the temperature of the EAG room was controlled at 21 $\pm$ 1°C. The EAG response was preamplified by an amplifier (100×/1000×) and analyzed with a V1000 video, 4035 digital storage oscilloscope and ES1000 recorder (Gould Co. Ltd.).

Analytical Method. Calculation of the expected value of PA + PB was adapted from the method described in Okada et al. (1990a), except that EAG measurements were used in place of the total activity counts per minute for their behavioral assays. The observed data of PA and PA + PB were plotted according to the patterns described by Ariens et al. (1964a,b; cited in Manabe and Nishino, 1985) in order to get the relationship between PA, PB, and their antennal receptors.

RESULTS

Neat samples of both of PA and PB elicit a negative EAG response on excised male antennae of the American cockroach. The effect of the increasing amount of PA and PB on EAG amplitudes is summarized and illustrated in Figure 1. There was no significant difference in EAG amplitude to be detected between 10^{-5} μg PA (or PB) and the control. The threshold quantity is approximately 10^{-4} μg. The doses of PA and PB that elicited maximal EAG response were about 10^2 μg. Generally, the EAG amplitude elicited by lower doses of

PB was somewhat greater than that of PA, whereas the reverse situation was observed when higher doses of PA were used. A linear increase of the range of PA activity, from 10^{-3} to 10^{1} μg, was noted to be similar to that of PB. The EAG amplitude for each dosage of PA and PB could be related to the log of the pheromone amount to give two regression equations (Figure 1): $Y_{PA} = 1.005 + 0.249 \log_{10} (X_{PA})$ (for PA, $R^2 = 0.929$, interval 10^{-3} to 10^{1} μg) and $Y_{PB} = 0.962 + 0.224 \log_{10} (X_{PB})$ (for PB, $R^2 = 0.889$, interval 10^{-3} to 10^{1} μg). The regression slope of PA was larger than that of PB.

To estimate the effects of addition of PA to PB on the male cockroach antennae, the EAG response was detected using samples with 25 combinations

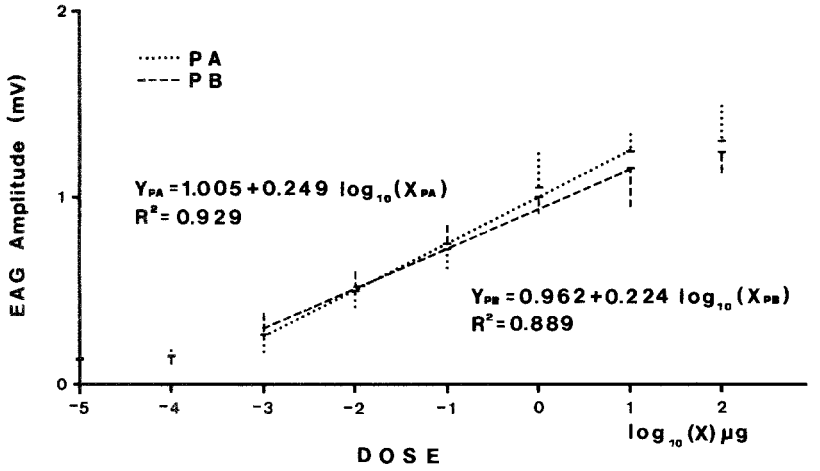

FIG. 1. Regression lines of dose–response for PA and PB. Data used in the calculation are the EAG amplitude of each dose. The vertical bar indicates the standard error of the mean.

TABLE 1. EAG AMPLITUDES (mV) OF UNMATED MALE AMERICAN COCKROACH TREATED WITH PA, PA + PB, AND PB

Dose (μg)	EAG amplitude (mV)		
	PA	PA + PB (8:5)	PB
10^{-5}	0.13 ± 0.03	0.13 ± 0.03	0.13 ± 0.04
10^{-4}	0.15 ± 0.04	0.14 ± 0.04	0.14 ± 0.04
10^{-3}	0.27 ± 0.08	0.27 ± 0.07	0.27 ± 0.11

as well as the 8:5 blend of PA and PB. Table 1 shows the comparative results among PA, PA + PB, and PB at doses of 10^{-5}, 10^{-4}, and 10^{-3} μg. Figure 2 shows the observed EAG amplitudes for the mixed samples of PA and PB. The samples were compared with those expected, which were calculated by the above regression equations for PA and PB.

The study indicated that no significant difference existed in the comparison of PA, PA + PB, and PB, between the observed and the expected EAG ampli-

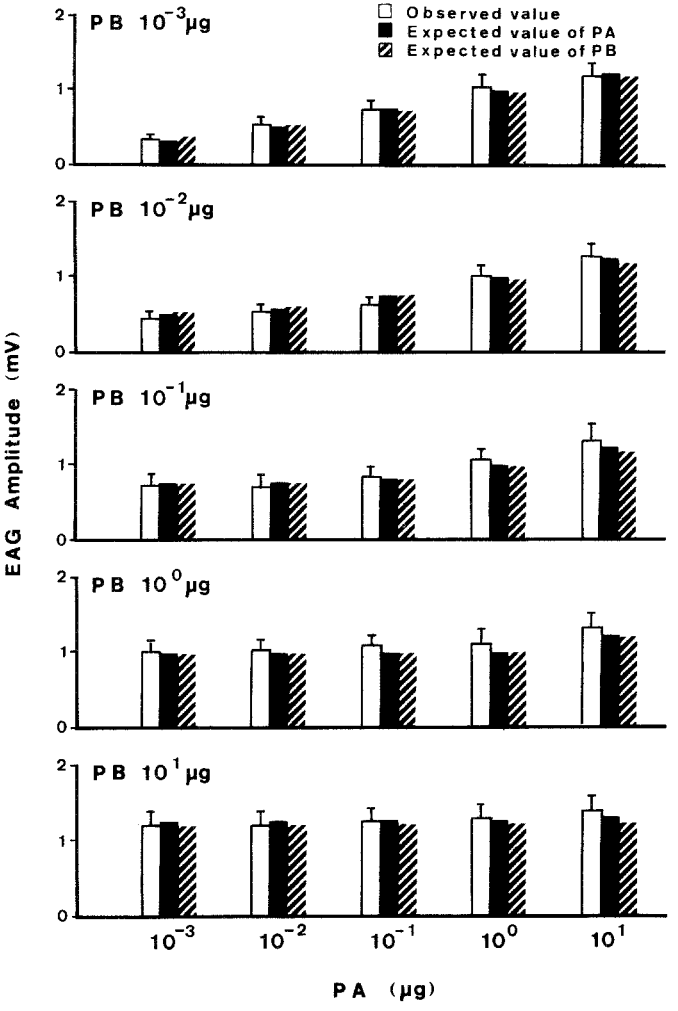

FIG. 2. EAG response of male *P. americana* antennae to the mixture of PA and PB. The vertical bar indicates the standard error of the mean.

tudes of the mixtures by Student's t test (significant level 5%) (Steel and Torrie, 1980). There was no particular ratio of the mixture of PA and PB that suppressed or reinforced the EAG amplitude. The addition of PA and PB elicited only an integrated total response.

In light of Ariens' theory that different receptors for different chemical drugs was considered, our curve of the dose of PA + PB vs EAG response is shown in Figure 3. The curves revealed that when different quantities (10^{-1}, 10^{0}, 10^{1} μg) of PB were added to PA, there is an increase on EAG response, while at higher concentrations the response approaches the maximal values.

DISCUSSION

In these studies with synthetic PB and Hauptmann's PA, the results were the same as those obtained by Nishino and Kuwabara (1983), but different from those obtained by Okada et al. (1991). This difference might be explained as resulting from different pheromone application systems, since in Okada's experiment the test solution was applied directly to the inner side of a disposable glass pipet, while in the experiments of Nishino and Kuwabara (1983) and our experiments, the test solution was impregnated on a filter paper. The filter paper can absorb the test chemicals in large quantities, so a higher dosage of the test chemicals is needed to obtain similar EAG response.

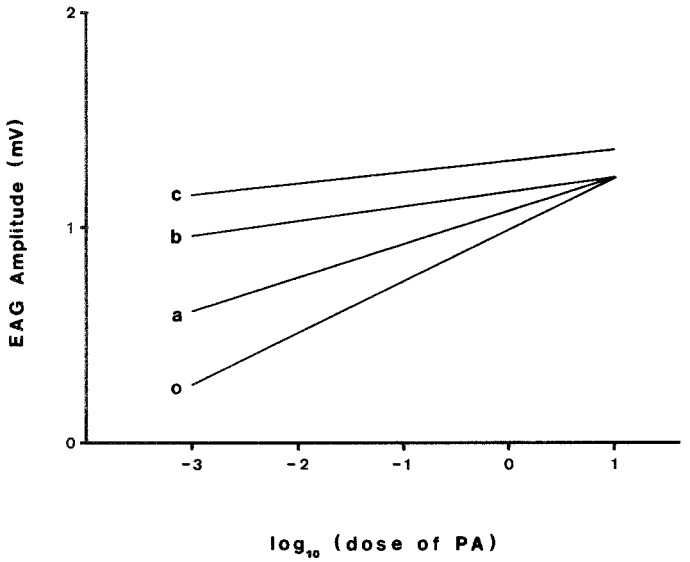

FIG. 3. Dose–EAG response curves of PA alone (o) and mixtures of PA with 10^{-1} (a), 10^{0} (b) and 10^{1} (c) μg PB.

The EAG response in this study was determined by lower doses of PB as being larger than that of PA, whereas higher doses of PA gave greater responses than those of PB. This phenomenon was also shown by Nishino and Kuwabara (1983); this supports the data of Seelinger (1985) that PB was responsible for long-distance attraction and PA was responsible for short-distance attraction.

According to the result from Sass (1983), PA and PB were detected by different individual olfactory cells. Nishino and Manabe (1983, 1984) and Nishino et al. (1988) also showed that there were two types of receptors specifically responsible for PA and PB by applying the DS-EAG technique. Moreover, in molecular pharmacology, Ariens et al. (1964a,b) determined that when drugs A and B occupy their own receptors, the curves of the addition of different quantities of drug B to drug A were categorized as pattern 3 (cited in Manabe and Nishino, 1985). In the EAG studies, the curves (Figure 3) produced by PA + PB coincided with the pattern 3. Thus this study confirms that the specificities of the receptors for PA and PB are different using the filter paper method for analysis.

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MALE-SPECIFIC TETRAENE AND TRIENE HYDROCARBONS OF *Carpophilus hemipterus:* STRUCTURE AND PHEROMONAL ACTIVITY

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Abstract—Males of *Carpophilus hemipterus* (L.), the dried-fruit beetle, (Coleoptera: Nitidulidae) were found to emit nine all-*E* tetraene and one all-*E* triene hydrocarbons in addition to two pheromonally active tetraenes that had been reported previously. The previously known compounds are (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene **(1)** and (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene **(2)**. The new tetraenes were all related to structure **1** by having one additional carbon at either one or two of the following four locations: at carbon 1 of the chain, at carbon 10 of the chain, at the 5-alkyl branch, or at the 7-alkyl branch. (Structure **2** also fits within this pattern.) The triene in *C. hemipterus* is (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene. Also identified from volatile collections from the beetles were the 2*Z* and 4*Z* isomers of **1.** All structures were proven by synthesis, with NMR and mass spectral data for the compounds provided. Two of the newly discovered compounds, (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-decatetraene and (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-undecatetraene, were quite active in the wind-tunnel bioassay, but others, such as (2*E*,4*E*,6*E*,8*E*)-5-ethyl-3,7-dimethyl-2,4,6,8-decatetraene and (2*E*,4*E*,6*E*,8*E*)-4,6,8-trimethyl-2,4,6,8-undecatetraene were not. Structure–activity relationships are explored among the natural compounds and additional, synthetic analogs, which were never detected from the beetles. Some of these analogs, such as (2*E*,4*E*,6*E*,8*E*)-3,5-dimethyl-7-propyl-2,4,6,8-undecatetraene, were quite active in the bioassay. The biosynthesis of the beetle-derived compounds is discussed. A single biosynthetic scheme that lacks complete enzyme specificity at four specific steps could account for the

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entire series of compounds found in the beetles and their relative proportions. The definition of "pheromone" is discussed in relation to these hydrocarbons.

Key Words—Pheromone, aggregation, hydrocarbon, tetraene, triene, NMR, mass spectra, dried-fruit beetle, *Carpophilus hemipterus*, Coleoptera, Nitidulidae.

INTRODUCTION

The dried-fruit beetle *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae) has a male-produced aggregation pheromone that was found to include two unusual tetraene hydrocarbons (Figure 1 below, structures **1** and **2**) (Bartelt et al., 1990a). In that earlier paper, the existence of at least eight additional male-specific hydrocarbons was reported, but no chemical structures or information about pheromonal activity was presented. The male-specific hydrocarbons of *C. hemipterus* have been studied further, and additional structures are reported here.

These compounds were identified by mass spectrometry (MS), gas chromatography (GC), and high-performance liquid chromatography (HPLC). The minute amounts of the natural chemicals from the beetles precluded the use of additional spectral methods. However, structure assignment was possible using only these techniques as long as appropriate model compounds were available for comparison. A key problem then, became one of choosing the correct model structures for synthesis. Such choices were difficult at first because interpretation of the limited analytical data for the natural compounds was ambiguous. However, the first identifications did suggest a biosynthetic model for the *C. hemipterus* hydrocarbons, similar to those proposed for *C. lugubris* and *C. freemani* (Bartelt et al., 1991, 1990b). The model was found to have great predictive value and became very useful in guiding the later hydrocarbon syntheses. NMR spectroscopy was used to verify that synthesis of the intended structures had been achieved, and the NMR and mass spectral characteristics of the synthetic tetraenes are summarized in the paper.

The large series of synthetic compounds gave us the opportunity to explore relationships between structure and pheromonal activity, and these bioassay results are also presented.

METHODS AND MATERIALS

Beetles and Volatile Collections. The beetle culture, originally from the Fresno area of California, was maintained on an artificial, pinto-bean diet (Dowd, 1987) or on a modified diet in which additional brewer's yeast was substituted for the pinto beans. Volatiles were collected from feeding beetles as previously described (Bartelt et al., 1990a). Approximately 50,000 beetle-days of volatiles

were available for analysis for this study. Silica gel and $AgNO_3$ HPLC were used to obtain the unsaturated hydrocarbons from these samples. Three separate preparations were analyzed for the additional male-specific compounds; two of these were separated into fractions by $AgNO_3$ HPLC before detailed analysis, and the other was examined without prior HPLC fractionation.

Identification of Male-Derived Hydrocarbons. Initial study was directed at the male-specific compounds for which GC retention indices and mass spectra had been obtained previously but for which structures were still undetermined (Bartelt et al., 1990a). It was known that many of these compounds could be separated from each other by applying both $AgNO_3$ and size-exclusion HPLC (Bartelt et al., 1990a). The initial effort was to isolate as many of these as possible for hydrogenation followed by GC-MS analysis. The resulting additional information, along with knowledge of pheromones in related species, allowed structures to be suggested, but the beetle-derived compounds were considered "identified" only when a synthetic standard was found that matched the natural one by all available criteria (GC retention, HPLC retention, mass spectrum, and when possible, GC retentions and mass spectra of hydrogenation products). Later on, the natural samples were examined further by GC-MS for still other tetraenes that had not been detected earlier (Bartelt et al., 1990a) but that were predicted to be present by a biosynthetic model. As before, structure identification of the additional compounds entailed matching the properties with authentic standards, although for these, too little material was available for the hydrogenation experiment.

Synthetic Compounds. The compounds used in this research are shown in Figure 1. The following chemicals were prepared in the course of this work: (2*E*,4*E*,6*E*,8*E*)-5-ethyl-3,7-dimethyl-2,4,6,8-decatetraene **(4)**, (2*E*,4*E*,6*E*,8*E*)-4,6,8-trimethyl-2,4,6,8-undecatetraene **(5)**, (3*E*,5*E*,7*E*,9*E*)-4,6,8-trimethyl-3,5,7,9-dodecatetraene **(8)**, (2*E*,4*E*,6*E*,8*E*)-5,7-diethyl-3-methyl-2,4,6,8-decatetraene **(9)**, (2*E*,4*E*,6*E*,8*E*)-4-ethyl-6,8-dimethyl-2,4,6,8-undecatetraene **(10)**, (2*E*,4*E*,6*E*,8*E*)-6-ethyl-4,8-dimethyl-2,4,6,8-undecatetraene **(11)**, (2*Z*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene **(13)**, (2*E*,4*Z*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene **(14)**, (2*E*,4*E*,6*E*,8*Z*)-3,5,7-trimethyl-2,4,6,8-decatetraene **(16)**, (2*E*,4*E*,6*E*,8*E*)-3-ethyl-5,7-dimethyl-2,4,6,8-decatetraene **(18)**, (2*E*,4*E*,6*E*,8*E*)-3,5-dimethyl-2,4,6,8-decatetraene **(19)**, (2*E*,4*E*,6*E*,8*E*)-3,5-dimethyl-2,4,6,8-undecatetraene **(20)**, (2*E*,4*E*,6*E*,8*E*)-3,5-dimethyl-7-propyl-2,4,6,8-decatetraene **(21)**, (2*E*,4*E*,6*E*,8*E*)-3,5-dimethyl-7-propyl-2,4,6,8-undecatetraene **(22)**, and (4*E*,6*E*,8*E*)-2,3,5,7-tetramethyl-2,4,6,8-decatetraene **(26)**. Other compounds were available from earlier research: (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene **(1)**, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene **(2)**, (2*E*,4*E*,6*Z*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene **(15)**, and (2*E*,4*E*,6*Z*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene **(17)** (Bartelt et al., 1990a); (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-undecatet-

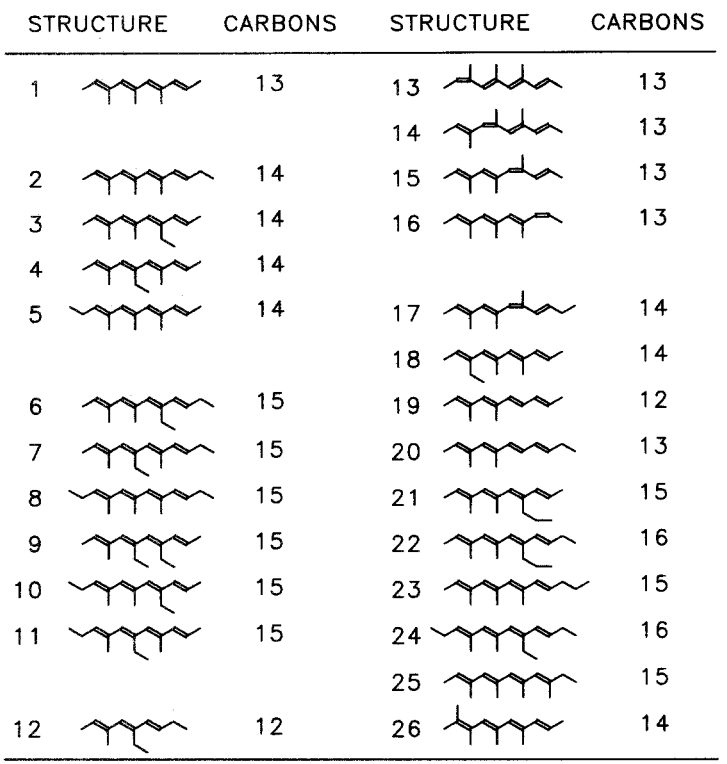


FIG. 1. Structures, structure numbers, and numbers of carbons for the compounds used in this research.

raene **(6)**, (2*E*,4*E*,6*E*,8*E*)-5-ethyl-3,7-dimethyl-2,4,6,8-undecatetraene **(7)**, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-dodecatetraene **(23)**, and (2*E*,4*E*,6*E*,8*E*)-3,5,7,9-tetramethyl-2,4,6,8-undecatetraene **(25)** (Bartelt et al., 1991); (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-decatetraene **(3)** (Bartelt et al., 1990c); and (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene **(12)** and (3*E*,5*E*,7*E*,9*E*)-8-ethyl-4,6-dimethyl-3,5,7,9-dodecatetraene **(24)** (Bartelt et al., 1990b).

Hydrocarbon syntheses generally followed methods described previously (Bartelt et al., 1990a,c). The conjugated double bond systems were built up using Wittig-Horner and Wittig reactions. The propyl side chains of **21** and **22** were introduced during a Wittig-Horner olefination step by alkylating the phosphonate anion from triethyl 2-phosphonoacetate with propyl iodide (Boutagy and Thomas, 1974). The resulting reagent was then treated with a second equivalent of base and allowed to react with the aldehyde substrate in the usual way.

These syntheses also afforded structures **19** and **20** because the alkylation with propyl iodide was incomplete.

Two mono-*Z* isomers of **1**, structures **13** and **14**, were obtained in usable amounts from a synthesis of **1** in which NaOEt in EtOH was employed as the base–solvent system for the Wittig Horner reactions. [Less of the *Z* isomers were produced when BuLi and THF were the base and solvent (Bartelt et al., 1990c)].

The synthetic compounds were purified by passage through open columns of silica gel with hexane, followed by HPLC on a $AgNO_3$-coated silica column. Purities of compounds were 90–99% by capillary GC, except for **3, 9, 17,** and **23,** which were between 83% and 88%. Impurities were generally small amounts of *Z* isomers. An aliquot of each purified synthetic compound was diluted to 1.3 ng/10 μl with hexane for bioassays. NMR spectra were obtained to verify structures of the purified compounds; the solvent was deuterobenzene. Proton shifts were assigned by analogy to previously assigned compounds in the series and by double irradiation and COSY experiments. Nuclear Overhauser experiments were conducted with the *Z* isomers, **13** and **14,** to determine configurations at the trisubstituted double bonds. Mass spectra were also obtained for all synthetic compounds. GC retention indices (I) relative to *n*-alkanes were determined on a 15-m × 0.25-mm-ID DB-1 capillary column with 1.0-μm film thickness (J & W Scientific, Folsom, California); the temperature program was 100–200°C at 10°/min, and linear interpolation was used (Poole and Schuette, 1984, p. 25). Instrumentation was as described previously (Bartelt et al., 1990a).

Bioassays with Synthetic Hydrocarbons. The wind-tunnel bioassays were conducted as described by Bartelt et al. (1990a). The wind tunnel contained ca. 500–1000 beetles, and ca. 50–100 were in flight at any time during bioassays. Two treatment preparations to be compared were applied to pieces of filter paper, and these were hung side by side in the upwind end of the wind tunnel. Each test lasted 3 min, and the numbers of beetles alighting on the pieces of filter paper were recorded. The synthetic hydrocarbons were tested for activity both with and without adding a "host-type" coattractant to the bioassay treatments. The series of experiments are summarized in Table 1. In experiment 1, compounds **1–26** were evaluated versus controls. Every fourth test, the synthetic pheromone blend (SPB) was tested versus a control to ensure that the beetles in the wind tunnel remained responsive. (SPB included the following structures and amounts per dose: **1,** 1000 pg; **2,** 135 pg; **3,** 76 pg; **4,** 39 pg; **5,** 64 pg; and **6,** 29 pg. SPB was the best approximation to the actual male-specific hydrocarbon blend midway through the project, and it was used as a standard for the bioassay comparisons.) Additional experiments (2–8) were conducted to evaluate some compounds more extensively. These were all run as balanced incomplete block experiments with two treatments per block (two treatments in the wind tunnel during each test). Experiment 2 explored the effect of (conceptually)

TABLE 1. SUMMARY OF BIOASSAY EXPERIMENTS CONDUCTED WITH SYNTHETIC HYDROCARBONS

Experiment	Description[a]	Treatments[b]	N^c
1	Evaluation vs. control	**1–26,** SPB, control	≥4
2	Effect of adding "extra carbon" to **1**	**1, 2–5, 18, 26,** SPB, control	8
3	Activity of *Z* isomers of **1**	**1, 13–16,** SPB, control	12
4	Size of alkyl group at 7 position on 10-carbon chain	**1, 19, 3, 21,** SPB, control	10
5	Size of alkyl group at 7 position on 11-carbon chain	**2, 20, 6, 22,** SPB, control	20
6	SPB vs. natural pheromone	Male-derived HCs, SPB, control	16
7	Effect of adding **14** to SPB	SPB + **14,** SPB, control	16
8	SPB vs. mix of **1** and **2**	**1** + **2,** SPB, control	24
9	Evaluation with coattractant, constant dose (1.3 ng)	**1–26** + PA, SPB + PA, PA	≥8
10	Evaluation with coattractant, "natural" dose (ca. 1 male equivalent)	**1–7** + PA, SPB + PA, PA	8

[a] All tests in experiment 1 were versus controls (see text). In experiments 2–8 a balanced incomplete block design was used (treatments tested in pairs in all possible ways). In experiments 9 and 10, a balanced incomplete block experiment was run for *each* hydrocarbon; the treatments were hydrocarbon + PA, SPB + PA, and PA alone.

[b] SPB = synthetic pheromone blend, 1.3 ng dose (ca. 1 beetle equivalent); see text for composition; PA = propyl acetate, 10% solution in mineral oil, 20 μl/test. In most cases, the dose of each compound was 1.3 ng. In experiment 6, the total dose of male-derived tetraenes was 1.3 ng. In experiment 7, the dose of 14 was 400 pg; in experiment 8, the doses of **1** and **2** were 1000 and 130 pg, respectively.

[c] Number of replications for each hydrocarbon treatment in the experiment. In experiments 1, 9, and 10, the treatments, SPB, SPB + PA, PA, and control were replicated many more times; see text.

placing an additional carbon on various positions of structure **1.** Experiment 3 compared the mono-*Z* isomers of **1.** Experiments 4 and 5 examined the effect of alkyl substituent size at position 7 in 10- and 11-carbon chains. SPB was also compared to the natural pheromone (experiment 6), to a mixture of SPB + **14** (experiment 7), and to a mixture of just **1** and **2** (experiment 8). For experiments 9 and 10, each hydrocarbon [alongside propyl acetate (PA) on the same filter paper] was tested in a balanced incomplete block experiment with PA alone and with SPB plus PA. For experiments 1–6 and 9, the hydrocarbon dose was always 1.3 ng; in experiments 7 and 8, component mixtures had slightly different total doses; and in experiment 10, the "natural" dose was used (ca. 1 beetle equivalent) (see Table 1).

Wind-tunnel responses to combinations of pheromone and coattractants

such as propyl acetate are greater than to either attractant alone (Bartelt et al., 1990a; Dowd and Bartelt, 1991). Nevertheless, meaningful counts (e.g., >10 per 3-min test) can be obtained to the pheromone alone if the beetles are allowed to "warm up" for a sufficient period of time before testing and if they are not exposed to treatments containing the host volatiles during the experiments. Tests without the coattractant (experiments 1–8) are the most sensitive in that the blank filter paper controls receive consistently low numbers of visits (almost always ≤ 1 per test). However, if the compounds were active only in the presence of a coattractant, then their activity could be overlooked. The additional experiments (9 and 10) explored this possibility. Because the control for these latter experiments, PA by itself, is an attractant in its own right, responses to it were more variable, and the effect of the test compound over and above PA was more difficult to detect. Taken together, experiments with both types of controls were expected to provide a fairly comprehensive view of activity in the compounds.

Statistical Analysis. The bioassay data were analyzed by categorical methods (Fienberg, 1977). Initially, the data set for each of experiments 1–8 was analyzed separately. A parameter was fitted for each treatment and for the total number of responders for each 3-min test. Because only two of the experimental treatments were compared in any given test, the overall contingency table was of the incomplete type, and the model of quasiindependence was applied (Fienberg, 1977). For all eight experiments the model fitted well ($P > 0.05$, chi-square test using the likelihood ratio statistic, G^2), indicating that the data were well-behaved for this sort of analysis. Because there was no evidence for interactions involving replications in these experiments, the data sets were collapsed (summed) over the replications of each two-treatment comparison to give simpler data sets for subsequent calculations. All eight experiments were then combined into one overall quasiindependence model. By combining all of the data into one model, all information about each treatment was used, and the most powerful statistical tests for differences among treatments could be obtained. Conditional chi-square tests (analogous to t tests in ANOVA) were used to detect these differences. Comparisons to the control were usually of greatest interest.

The quasiindependence model for experiments 1–8 did not quite fit ($G^2 = 129$ with 87 df). The 0.95 percentage point of the chi square distribution with 87 df is 110. Nevertheless, it did account for almost all of the variability among treatments; when the parameters for differences among treatments were removed from the model, G^2 became very large (3096, with 119 df). This very simple model was believed adequate to give reasonable conditional tests for differences among treatments and reasonable descriptions of treatment effects relative to the controls and SPB. The lack of fit of the model was due almost entirely to slightly different relationships between controls and SPB in experiment 1 versus experiments 2–8. In experiment 1, the control counts were 1.1% of SPB, whereas in

experiments 2–8, they were 3.3% of SPB. When an extra parameter was included in the model for the controls of experiment 1, G^2 was decreased to 110 (86 *df*).

For experiments 9 and 10, a separate categorical analysis was run for each treatment. In these experiments, each treatment was linked only to SPB + PA and to PA, and there was nothing to be gained by combining the data into one large model.

With categorical analysis, relationships among the treatments are often expressed as ratios of fitted values. For this study two were of particular interest: the ratio between a test compound and the control (*t/c*) and the ratio between SPB and the control (*spb/c*). The effect of each test compound was summarized for presentation by combining these values into a measure of relative activity. Relative activity = $100 \times [(t/c - 1)/(spb/c - 1)]$, and it expresses the effect of a compound as a percentage of the activity of SPB, correcting for controls. (Recall that for experiments 9 and 10, the "control" was PA.)

RESULTS AND DISCUSSION

Identification of Novel 14-Carbon Tetraenes from Males. Hydrogenation following isolation was successful for three previously unidentified 14-carbon compounds (I = 14.22, 14.28, and 14.63) (Bartelt et al., 1990a). The GC and mass spectral data for the 14-carbon compounds are given in Table 2. Multiple GC peaks after hydrogenation were due to the creation of asymmetric centers; the mass spectra for these peaks were always nearly identical. These data indicated that the carbon skeletons were distinctly different from each other and from the previously identified 14-carbon tetraene **2.** The molecular weights of all three new compounds increased by 8 after hydrogenation (from 190 to 198). Therefore, each had four double bonds (if no triple bonds) but no rings.

The compound with I = 14.63 produced a readily interpreted MS fragmentation pattern after hydrogenation. Enhanced fragments at *m/z* 71, 113, and 155 suggested a 4,6,8-trimethylundecane carbon skeleton. Structure **5** was chosen as a synthetic target for the natural tetraene by analogy to the previously identified **1** and **2** with regard to the positions and configurations of double bonds. Synthetic **5** matched the natural compound in every way, both before and after hydrogenation. (The carbon skeleton of **5** has symmetry about carbon 6 after hydrogenation; thus only three distinct diastereoisomers can be produced, in a 2:1:1 ratio. Formation of three asymmetric centers during hydrogenation normally results in four possible diastereoisomers.)

Mass spectrometry of the hydrogenated tetraenes with I = 14.22 and 14.28 was less informative. The dominant fragment was the *m/z* 169 (M-29) peak in these spectra. While loss of an ethyl group was evident, the positions of the alkyl group(s) were not obvious. A similar situation was encountered previously

TABLE 2. ANALYSIS OF FOUR MALE-DERIVED 14-CARBON TETRAENES BY HYDROGENATION, GC, AND MASS SPECTROMETRY; COMPARISON TO TETRADECANE

Description of data	Structure				
	2	**3**	**4**	**5**	Tetradecane
GC retention index					
Before H_2	14.76	14.28	14.22	14.63	14.00
After H_2	12.52	12.44	12.40	12.42	14.00
	12.55	12.47	12.44	12.50[a]	
	12.61	12.50	12.47[b]	12.57	
	12.63	12.52			
Mass spectral data after H_2					
m/z	Intensity (% of base peak)				
43	53	54	58	75	92
57	100[c]	100[c]	100[c]	99	100
71	54	43	97[c]	100[c]	57
85	41[c]	28[c]	43	30	37
99	16[c]	11[c]	12	6	7
113	5	6	12[c]	15[c]	4
127	9[c]	4[c]	9[c]	4	4
141	7[c]	<1	<1	<1	3
155	ND	3[c]	1[c]	8[c]	2
169	3[c]	10[c]	16[c]	ND	1
183	<1	ND	ND	<1	ND
198	1	1	1	1	7

[a]Sharp peak, double intensity.
[b]Broad peak, double intensity.
[c]Fragments expected to be intense, based upon rules for methyl-branched alkanes.

with the 15-carbon pheromone of *C. lugubris* (Bartelt et al., 1991). The usual rules for interpreting the mass spectra of methyl branched hydrocarbons (Nelson, 1978) do not apply clearly to ethyl-branched hydrocarbons because the loss of the ethyl group so dominates the spectrum. The compound at I = 14.28 before hydrogenation produced a relatively intense (12%) key spectral peak at *m/z* 83 (Table 3). By analogy to the *C. lugubris* pheromone, the compound was believed to be the 7-ethyl tetraene **3.** The compound at I = 14.22 produced only a small *m/z* 83 peak but a large (57%) *m/z* 69 peak, suggesting it to be either the 5-ethyl tetraene **4** or the 3-ethyl tetraene **18.** However, the 3-ethyl isomer **(18)** would have only two asymmetric centers after hydrogenation and therefore could not

produce more than two GC peaks. (The natural compound produced four.) Thus the initial synthetic targets were **3** and **4,** and they matched the natural tetraenes in every way. The fragment ions expected to be enhanced, if the "rules" for methyl-branched hydrocarbons are followed, are noted in Table 2. Many of these for structures **3** and **4** were not especially large. Even though the spectra were not readily rationalized, the fragmentation patterns and GC retentions after hydrogenation did provide diagnostic "fingerprints" for the carbon skeletons, as long as authentic standards were available for comparison.

15-Carbon Tetraenes. A 15-carbon compound with molecular weight 204 and I = 15.15, probably another tetraene, was reported previously from male *C. hemipterus* (Bartelt et al., 1990a). Fortuitously, this compound matched perfectly the GC retention and mass spectrum of the pheromone of *C. lugubris* (Bartelt et al., 1991), and structure **6** is concluded to be a component of the emission from male *C. hemipterus* as well. A search for additional 15-carbon tetraenes was motivated by the biosynthetic argument detailed below. The additional 15-carbon tetraenes **7–11** were synthesized as standards. With the GC retentions, HPLC retentions, and mass spectra of the synthetic standards as guides, the volatile collections from male beetles were examined carefully by GC-MS, and compounds **7, 8, 10,** and **11** were clearly detected. A compound was also present from the beetles with all the correct chromatographic properties to be **9,** but too little material was available for a definitive mass spectrum. The major peaks (*m/z* 105, 119, 133, 147, 175, and 204) were in the proper ratios, but smaller peaks were lost in background noise.

In the volatile collections from both male and female beetles, there were a number of additional compounds with molecular weight 204. Many of these were probably sesquiterpenes originating in the artificial diet. The compounds appearing with both sexes had large mass spectral fragments at one or more of *m/z* 69, 91, 93, 133, 147, and 161, but the 175 peaks were always very small. Tetraenes **6–11** all have intense *m/z* 175 fragments in the mass spectra, and this series is not evident in volatiles from females. The amount of the "sesquiterpenes" in the samples varied considerably, and the GC peak at I = 14.91 reported by Bartelt et al. (1990a) now appears to have been one of these. By GC, it was not resolved from the smaller amount of tetraene **11** at I = 14.95, but the selected-ion chromatogram for *m/z* 175 clearly indicated that the tetraene was hidden in the tail of the GC peak. The male-specific peaks with molecular weights of 204 and large *m/z* 175 fragments were always present and in very similar ratios in the samples examined.

Z *Isomers.* The two male-derived 13-carbon compounds at I = 12.44 and 13.08 (Bartelt et al., 1990a) were concluded to have the same carbon skeleton as **1,** based on analysis after hydrogenation. Thus the compounds differed from **1** only in double bond position or configuration. The compound with I = 12.44 has not yet been identified, but the more abundant one (I = 13.08) clearly

matched structure **14,** by GC retention, HPLC retention, mass spectrum, and by the peculiar GC peak shape (see Figure 5 below). Furthermore, the beetle-derived peak at I = 13.39 matched structure **13** by GC retention, HPLC retention, and mass spectrum. Thus both the 2*Z* and 4*Z* isomers of **1** were concluded to be present in the volatile collections from the beetles; however, the 6*Z* and 8*Z* isomers were not detected.

Just as *Z* isomers of major component **1** were present in small amounts in the volatile collections, *Z* isomers of the less abundant 14- and 15-carbon tetraenes also appeared to be present at correspondingly lower levels. Careful GC-MS revealed a large number of these in minute amounts, but the structures remain undetermined. In general, the *Z* isomers have identical mass spectra to those of the all-*E* isomers, elute well before the corresponding all-*E* isomers by GC and, as long as the *Z* double bond is trisubstituted, by $AgNO_3$ HPLC as well.

Apparently, isomerization of double-bond configuration can occur fairly readily. For example, when all-*E* **1** is applied to a rubber septum and the released volatiles analyzed, mono-*Z* **14** is invariably present (typically 10–50% the amount of **1**). Other examples of isomerization are given below, where gas chromatography of standards is discussed. It is unknown whether the beetles actually release *Z* isomers or whether their presence is due solely to isomerization of the all-*E* tetraenes during volatile collections and subsequent handling. Because the amount of *Z* isomers detected in samples varied considerably and because the freshest samples tended to have less, the latter explanation is favored.

Other Structures. One additional compound, triene **12,** was identified from male *C. hemipterus*, by HPLC, GC, and MS. This compound is of special interest because it is the major pheromone component of *C. freemani* (Bartelt et al., 1990b). This compound was overlooked originally (Bartelt et al., 1990a) because it elutes from the $AgNO_3$ column before the pheromonally active compounds. None of the other structures **(15–26)** in Figure 1 were detected from the beetles. The abundances of compounds **1–14** in volatile collections from beetles are summarized in Figure 3 (below).

Biosynthesis. Proposed biosynthetic schemes were published previously for the pheromones of *C. lugubris* (Bartelt et al., 1991) and *C. freemani* (Bartelt et al., 1990b). A similar scheme can account well for the hydrocarbons of *C. hemipterus* (Figure 2). Under this scheme, the carbon skeletons are built up in cyclic fashion, through a series of elongation, reduction, and dehydration steps. At some point after the final acylation, a decarboxylation occurs, and the free hydrocarbon is released. A methyl branch is introduced if the second, third, or fourth acyl unit is propionate. Correspondingly, butyrate addition leads to ethyl branches.

Although 12 different carbon skeletons are produced by male *C. hemipterus*, it is suggested that there are not 12 different biosynthetic pathways. Instead,

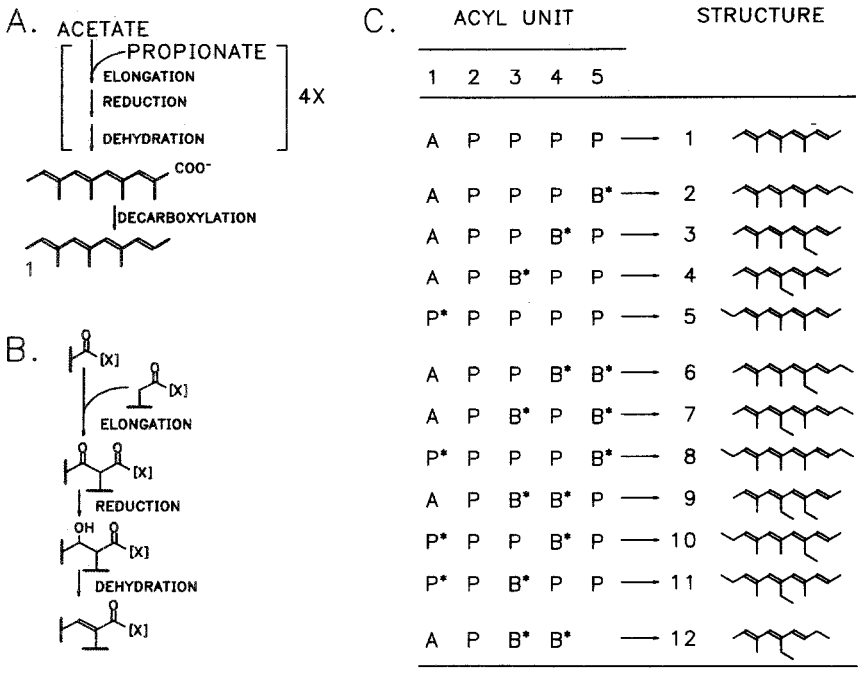


FIG. 2. Proposed biosynthetic scheme for the male-specific hydrocarbons of *C. hemipterus*. The biosynthesis of **1** is summarized in (A). The details of the elongation, reduction, and dehydration steps are given in (B); [X] is an acyl carrier such as coenzyme A. The acyl units needed to form **1–12** are shown in (C). A = acetate, P = propionate, and B = butyrate; points of deviation from the "usual" biosynthetic route (that of **1**) indicated by (*).

all of these could be produced by just one pathway. If **1** is the usual biosynthetic product (suggested by its abundance), then **2–5** would each result from one biosynthetic "mistake" (e.g., lack of rigorous enzyme specificity during the elongation step). These four points of deviation from the usual route are indicated in Figure 2. The six 15-carbon tetraenes **6–11** would result if two of these mistakes occurred in one compound. There are six ways of combining the four possibilities two at a time, and there was evidence for all six of the structures in the beetles. If the mistakes occurred independently, then one would expect compounds with two of them to be far less abundant than those with only one, and this is just the pattern seen in the beetles. Triene **12** would result from the biosynthetic sequence leading to **9** if chain termination occurred after the fourth acyl addition rather than after the fifth. Apparently, two successive butyrate units tend to lead to chain termination, regardless of the chain length when the second one is added. This could explain the relatively small amount of **9.**

Tetraenes with 16 carbons, such as **24,** would result if three mistakes occurred during biosynthesis. These were never detected, but in line with the model, the occurrence of three of these independent events in one molecule would be even less likely, and the apparent absence of these compounds is not surprising. If a greater amount of beetle-derived material were available for careful analysis, they might well be detected. Of the other compounds in Figure 1, **18–20** could easily be rationalized from acetate, propionate, and butyrate units, but they were not detected from the beetles. Compounds **21–23** could be rationalized if valerate units were used to account for the propyl branches and the long chain terminus in **23,** but again, there was no evidence for these. Compounds **25** and **26** cannot be constructed from acetate, propionate, and butyrate units as outlined in Figure 2.

Bioassays. The results of the bioassay experiments are summarized in Figure 3. Significant differences between the treatments and the control at the 0.05, 0.01, and 0.001 levels are indicated by one, two, and three asterisks, respec-

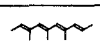
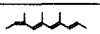
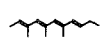
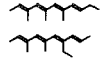
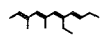
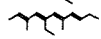
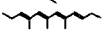
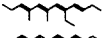
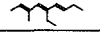
	STRUCTURE	ABUNDANCE (PG)	RELATIVE ACTIVITY EXP.1-5	EXP.9	EXP.10		STRUCTURE	ABUNDANCE (PG)	RELATIVE ACTIVITY EXP.1-5	EXP.9
1		1000	24%***	18%***	13%***	13		60	5%*	3%
						14		130	11%***	3%*
2		130	29%***	60%***	38%***	15		ND	11%***	8%*
3		80	21%***	41%***	9%*	16		ND	13%***	17%***
4		30	0%	2%	-1%					
5		60	2%	3%	-2%	17		ND	7%*	4%
						18		ND	6%**	6%*
6		20	16%***	49%***	9%**	19		ND	0%	-1%
7		5	2%	8%	2%	20		ND	0%	1%
8		10	-2%	17%***		21		ND	8%***	1%
9		2	-2%	1%		22		ND	35%***	41%***
10		10	-2%	-3%		23		ND	14%*	12%**
11		12	4%	5%		24		ND	0%	11%
						25		ND	0%	8%
12		25	5%	5%		26		ND	3%*	4%

FIG. 3. Bioassay activity for 26 individual hydrocarbons. In experiments 1–5, the compounds were tested at 1.3 ng per dose, without a coattractant; in experiment 9, at 1.3 ng per dose, with propyl acetate (PA) as a coattractant; and in experiment 10, at doses as in the abundance column (in pg), with PA added. Activity is expressed relative to the standard synthetic pheromone blend, corrected for controls (see text). Significant differences from the controls at the 0.05, 0.01, and 0.001 levels denoted by *, **, and ***, respectively (conditional G^2 tests). Abundances of the compounds in the beetles are summarized, as the approximate number of picograms per male; ND, not detected.

tively (conditional G^2 tests). The beetles were ready to respond during all of these experiments because 1.3 ng of SPB was always clearly different from controls. Overall means for SPB and control were 24.5 and 0.4, respectively, in experiments 1–8. The overall means for SPB + PA and PA were 17.4 and 1.0 for experiment 9 and 18.0 and 0.6 for experiment 10.

Most striking was that of the 11 all-*E* tetraenes found in *C. hemipterus*, only five (**1–3, 6,** and **8**) ever showed significant activity relative to controls. Compounds **2, 3,** and **6** were active in experiment 10 even in doses of well under 1 ng. In general, the results when the hydrocarbons were tested in conjunction with PA (experiments 9 and 10) paralleled those when no PA was present (experiments 1–8), but the results for **8** were unusual in that significant activity was seen only in the presence of PA. Compounds **4, 5, 7,** and **9–11** were never significantly active, nor was triene **12.**

All four of the mono-*Z* isomers of **1 (13–16)** showed significant activity, with **16** being the most active and **13,** the least. Only **13** and **14** were ever detected from the beetles. Given the instability of **14** especially, it is unclear whether the responses were to **14** itself or if partial isomerization to **1** occurred during the bioassay. The results for **14** are as one might expect from a lower dose of **1.** By GC at least, compound **16** is more stable, and it is believed to be active in its own right.

Compounds **1** and **2,** the most active tetraenes at natural doses, were not significantly different from SPB when combined in natural proportions (experiment 8, **1** + **2** was 87% of SPB). The male-derived hydrocarbons were also equivalent to SPB (95% as active as SPB, experiment 6). Adding **14,** a relatively abundant constituent of the volatile collections from beetles, to SPB did not significantly enhance the activity of SPB (combination was 112% of SPB, experiment 7). Compounds **1** and **2** are clearly major pheromone components, as concluded earlier (Bartelt et al., 1990a). Compounds **3** and **6** are also potentially important under natural conditions. The other natural compounds **4** and **5** and **7–14** would appear to have, at best, very subtle roles under natural conditions.

Of the other model compounds not found in the volatile collections, **22** was strikingly active, and five others, **17, 18, 21, 23,** and **26,** had significant activity under some conditions.

Structure–Activity Relationships. Several generalizations can be made about structure–activity relationships: (1) An ethyl group as the middle alkyl branch (e.g., the 5-ethyl group in **4**) renders the compound inactive. (2) The right-hand terminal alkyl group (as drawn in Figure 3) can be methyl, ethyl, or propyl and the compound still have activity; the ethyl group seems most consistent with high activity, however. (3) The right-hand alkyl branch (e.g., the 7 position in **1**) can be methyl, ethyl, or propyl and the compound still be active, but having only a hydrogen in that position (**19** and **20**) renders the compound inactive. (4) The left-hand terminal alkyl group (as drawn in Figure 3) should be methyl for

maximal activity, although there was some evidence of activity with ethyl (i.e., **8**). Because structure **8** is analogous to **2** and because **2** is, perhaps, the most active of the tetraenes, the high activity with PA of **8** relative to **5, 10,** and **11** is not surprising. (5) The left-hand alkyl branch should be methyl, but the one example with an ethyl group at that location **(18)** did have slight activity. (6) Alkyl groups in other locations (e.g., **25** and **26**) greatly reduce activity.

The Pheromone of C. hemipterus. It is debatable whether the entire male-specific series of compounds should be called a pheromone. We suggest the situation is as modeled in Figure 4. There is a set of compounds produced only by male beetles, and there is another set of compounds to which the pheromone receptors can respond. It is only the intersection of these two sets that should be considered as the pheromone. Inaccurate biosynthesis could nicely account for the variety of structures encountered in *C. hemipterus*, and it may be incorrect to assume that each compound has biological function merely because it is there. The pheromone receptors do detect the major tetraene **1** and minor tetraenes **2, 3,** and **6** very well, but other structures in the series seem to be unused. These structures could be retained in the emission from *C. hemipterus* as long as they pose no selective disadvantage. On a weight basis, the inactive compounds account for a very small proportion of the male-specific emission, thus their production would not be especially wasteful energetically.

At present, we believe the pheromone of *C. hemipterus* to include **1, 2, 3,** and **6.** Whether other compounds for which activity has not yet been demonstrated should be included must await careful field testing of mixtures of components.

The inactive compounds from the beetles could serve as a pool of new pheromones during speciation. If pheromone receptors evolved to detect these

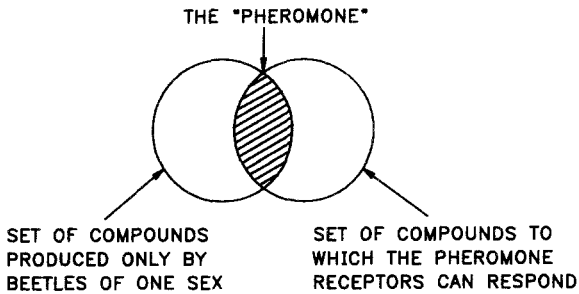


FIG. 4. Relationship of the male-specific hydrocarbons of *C. hemipterus* to the functional aggregation pheromone. As presently understood, not all of the male-specific hydrocarbons are pheromonally active, and the beetles do respond to some pheromone analogs that they do not produce. The intersection of these sets represents the pheromone blend that serves for communication under natural conditions.

compounds, then they could become biosynthetically enhanced through natural selection, and the new species could then develop a very different pheromone profile from the parent species. It is interesting that **12,** which is apparently inactive in *C. hemipterus*, is the major pheromone component in *C. freemani* and that **6,** which is active in *C. hemipterus* but is only a minor portion of the pheromone blend, is nevertheless the major pheromone component of *C. lugubris* and is an important minor component in *C. freemani*.

Gas Chromatography of Standards. Retention indices (I) relative to *n*-alkanes are given in Tables 2 and 3 and Figure 5 for the tetraenes. Triene **12** has an index of I = 12.24. While the all-*E* tetraenes always produced sharp GC peaks, some of the *Z* isomers did not. Examples are shown in Figure 5. The isomers with "internal" *Z* double bonds (**14** and **15**) produced distorted GC peaks, probably due to thermal rearrangement or other degradation, while those with a *Z* double bond at either end (**13** and **16**) generated sharp peaks. The GC peak of compound **14** rose slowly at the leading edge, then dropped off sharply. Compound **15** showed an initial sharp peak at I = 12.06, a second sharp peak at I = 13.26, and a broad hump between them. If the injector temperature was set at 250°C, only the peak at 12.06 was seen for **15;** with the injector at 100°C, no I = 12.06 peak appeared. If the sample was run on a thinner film (0.25 μm vs. 1.0 μm) column, which allowed the compound to elute at a cooler temperature (115°C vs. 160°C), only the I = 13.26 peak was evident; thus I = 13.26 most likely represented the unchanged compound. Compound **14** also chromatographed as a single, sharp peak on the thin-film column. The chromatogram for **17** (not shown) had a similar appearance to **15,** but I = 14.06 (Bartelt et al., 1990a). Of greatest importance to this study was that the diagnostic peak shape for some of these standards aided in the identification of compounds in volatile collections.

All of the *Z* isomers could be prepared in essentially pure form by $AgNO_3$ HPLC, but after storage for several months in a freezer (which was true of the samples used as examples for this figure), analysis by GC revealed additional peaks (e.g., I = 13.39, 13.83). These apparently corresponded to partial isomerization to **13** and **1,** respectively. Isomerization of **1** to **13** and **14** was also observed, although not in the sample shown in Figure 5.

Mass Spectra of Standards. The mass spectra of 20 all-*E* tetraenes are summarized in Table 3. Examples of whole spectra appeared previously (Bartelt et al., 1990a,b, 1991), but the data for these are shown here also for comparative purposes. The molecular ions were prominent (27–95% of base peak), and the masses, *m/z* 77, 91, 105, 119, 133, and 147, were very evident in all spectra (14–100% of base peak). Structural correlations were not obvious in the intensities of these peaks, but the series served as fingerprints for the structures.

Certain features of the spectra did give specific structural information, however. The most intense of the M-15, M-29, and M-43 peaks usually indi-

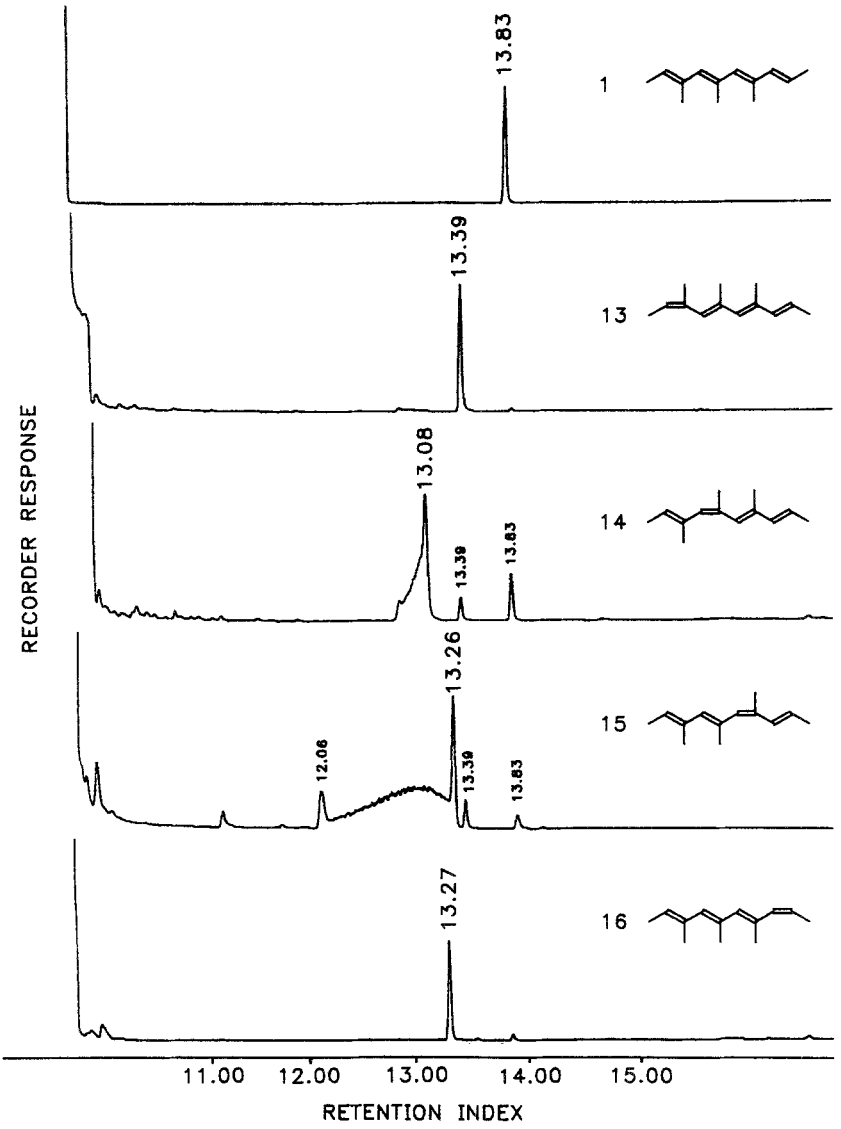


FIG. 5. Gas chromatograms of five geometrical isomers of 3,5,7-trimethyl-2,4,6,8-decatetraene. GC conditions: 15-m × 0.25-mm-ID DB-1 capillary column (1.0-μm film thickness), programmed from 100°C at 10°/min; injector temperature 170°C. Retention indices relative to *n*-alkanes; 14.00 corresponds to a retention time of 6.31 min.

TABLE 3. GC RETENTION AND MASS SPECTRAL FRAGMENTATION DATA FOR 20 ALL *E*-TETRAENES[a]

Structure	Retention index	Mass spectral peaks (*m/z*)													
		55	69	83	97	77	91	105	119	133	147	161	175	189	M^+ (mol wt)
1	13.83	25	62[b]	3	0	26	56	76	99	82	26	100[c]			95 (176)
2	14.76	100	15	26[b]	0	33	66	71	91	92	17	62[c]	27		47 (190)
3	14.28	69	16	12[b]	0	25	47	52	88	100	14	74[c]	29		49 (190)
4	14.22	42	57[b]	2	0	28	48	58	100	72	21	72[c]	33		48 (190)
5	14.63	37	72[b]	4	1	36	60	77	100	76	33	52[c]	40		53 (190)
6	15.15	53	10	2	6[b]	14	29	31	46	100	24	8	67[c]	12	42 (204)
7	15.13	100	18	25[b]	1	27	42	51	91	83	48	9	82[c]	16	45 (204)
8	15.56	82	31	34[b]	1	28	51	58	100	99	52	20	81[c]	29	71 (204)
9	14.66	82	19	18[b]	1	31	52	53	95	68	50	10	100[c]	17	44 (204)
10	15.03	49	23	17[b]	1	26	46	47	100	69	44	17	84[c]	24	55 (204)
11	14.95	29	51[b]	5	2	25	48	50	95	84	42	21	100[c]	26	65 (204)
18[d]	14.45	36	84[b]	4	1	31	47	54	71	53	25	31[c]	32		42 (190)
19	13.52	36	9	2	0	34	87	83	100	20	71[c]				66 (162)
20	14.54	30	14	0	0	26	57	58	100	20	47[c]	19			41 (176)
21	14.92	100	22	6	12[b]	33	58	57	83	80	24	71[c]	21	16	34 (204)
22[e,f]	15.75	66	51	5	1	27	47	62	61	100	39	11	47[c]	27	27 (218)
23	15.77	100	20	3	20[b]	23	46	44	66	70	20	40[c]	7	14	29 (204)
24[f]	15.89	86	36	7	15[b]	31	52	54	97	90	94	25	21	100[c]	61 (218)
25	14.98	100	34	5	48[b]	25	37	32	48	71	26	4	44[c]	19	24 (204)
26	14.32	37	54[b]	14	2	26	45	53	58	47	27	7	49[c]		37 (190)

[a] GC retention index relative to *n*-alkanes; abundances of MS ions expressed as percentage of base peak.
[b] Expected to be enhanced because of fragmentation through third double bond; see text.
[c] Expected to be enhanced; represents loss of largest alkyl group attached to the tetraene system. See text.
[d] Base peak was *m/z* 41.
[e] Peak at *m/z* 111 had intensity of 4%; no other compounds had detectable 111 peak.
[f] Peak at *m/z* 203 had intensity of 10% in **22** and 15% in **24**; no other compounds had 203 peak.

cated the largest alkyl group attached anywhere on the conjugated tetraene system, either at an end or as a side chain. Thus in **1** and **19,** a methyl was the largest alkyl group, but in **2** and **7** it was an ethyl, and in **21** and **23,** a propyl, and the corresponding fragments were relatively intense (Table 3). This generalization did not work well for **18** or **19,** however. For **19,** the large M-43 peak corresponded to *m/z* 119; because *m/z* 119 was intense in all of the compounds, it was not diagnostic for a propyl group. For the higher molecular weight compounds, this complication did not arise.

A second spectral feature involved the peaks in the C_nH_{2n-1} series, *m/z* 69, 83, 97, and 111. As noted previously (Bartelt et al., 1991), cleavage through the double bond at the 6 position in structure **6,** accompanied by hydrogen migration, corresponded to an enhanced *m/z* 97 peak, and cleavage through the analogous double bond in other structures led to enhancement of other fragments in this series. The fragments expected to be enhanced are marked in Table 3. Within each column, the marked fragments are relatively intense except with the dialkyl tetraenes **19** and **20,** for which the pattern fails. Apparently, unless there is an alkyl group at this double bond, the characteristic cleavage is not observed.

The mass spectra for the mono-*Z* tetraenes **13–17** appeared identical to the respective all-*E* isomers, **1** and **2.** The mass spectrum for triene **12** was presented elsewhere (Bartelt et al., 1990b).

NMR Spectra for Synthetic Standards. The NMR data for the standards are presented in Figures 6–9. The proton shift assignments for the all-*E* tetraenes are presented in Figure 6. The shifts for the mono-*Z* 13-carbon tetraenes related to **1** are shown in Figure 7. The data for **1, 2, 3, 6, 15,** and **24** (Bartelt et al., 1990a–c; 1991) are reproduced in these figures to facilitate comparison. The NMR data for triene **12** and tetraene **17** appear elsewhere (Bartelt et al., 1990a,b).

The proton splitting patterns were usually simple, and most could be interpreted by first order rules, although this was complicated by overlapping signals in some compounds. The coupling constants are summarized in Figure 8 for the trialkyl, all-*E* tetraenes. The large coupling (15.4–15.7 Hz) between H_D and H_E was consistent with the *E* configuration for the double bond (Silverstein et al., 1981, p. 235). In the one compound with a disubstituted double bond in the *Z* configuration **(16),** the coupling constant was 11.6 Hz. Slight long-range coupling (J = 0.5 Hz) was sometimes observed between pairs of signals not noted in Figure 8, such as between H_C and H_D. Such interactions led to line broadening but not usually to distinct splitting, and these were not included in the figure. The splittings between R_4 and H_C and between R_3 and H_B were larger (ca. 1.2–1.4 Hz) when R was methyl but were <1 Hz when R was ethyl or propyl. Configuration at the trisubstituted double bonds did not affect these coupling constants.

The spectra of four compounds **(19–22)** had second-order features. Figure

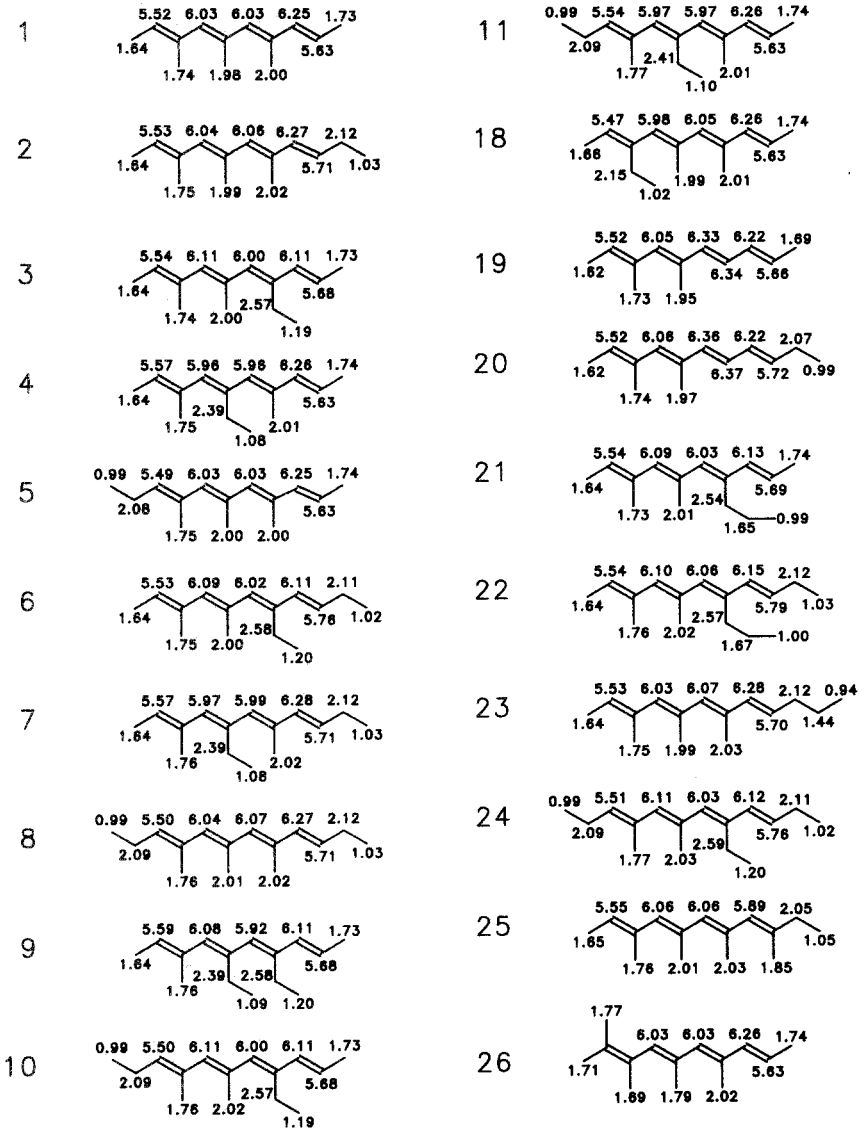


FIG. 6. Assignments of proton NMR shifts for 20 all-*E* tetraenes.

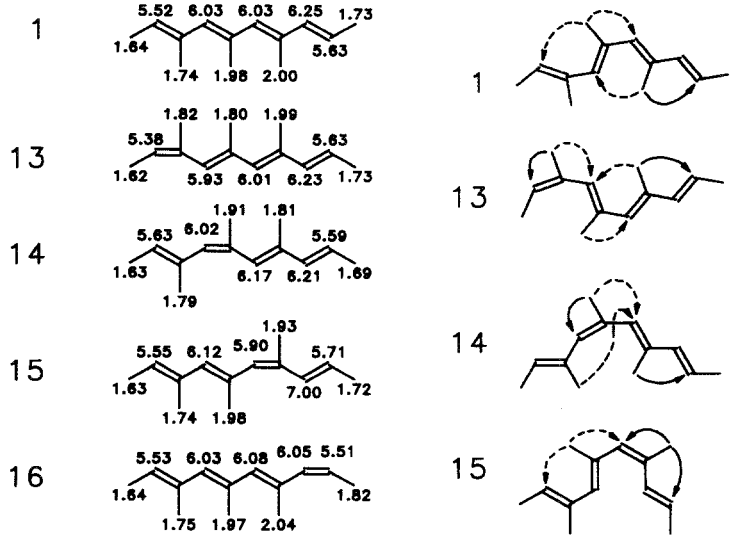
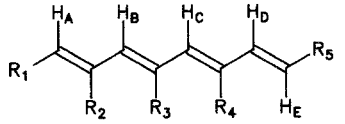


FIG. 7. Assignments of proton NMR shifts for the four mono-*Z* isomers of structure **1**, in comparison with those for **1**, and results of nuclear Overhauser experiments with **13** and **14** (see text). Results for **1** and **15** were published previously but are included here for comparison. Solid arrows indicate relatively greater enhancements than the dashed arrows.



$R_{1\text{-}3}$ = -C<u>H</u>$_3$ or -C<u>H</u>$_2$-Me

$R_{4\text{-}5}$ = -C<u>H</u>$_3$ or -C<u>H</u>$_2$-Me or -C<u>H</u>$_2$-Et

J (Hz)	
H_D,H_E: 15.4-15.7	R_4,H_C: <1.4
R_1,H_A: 6.8-7.3	R_1,R_2: ~1
R_5,H_E: 6.6-7.0	R_2,H_A: ~1
R_5,H_D: 1.2-1.6	H_A,H_B: ~1
R_3,H_B: <1.4	
WITHIN R GROUPS: 7.3-7.6	

FIG. 8. NMR coupling constants observed in the trialkyl all-*E* tetraenes.

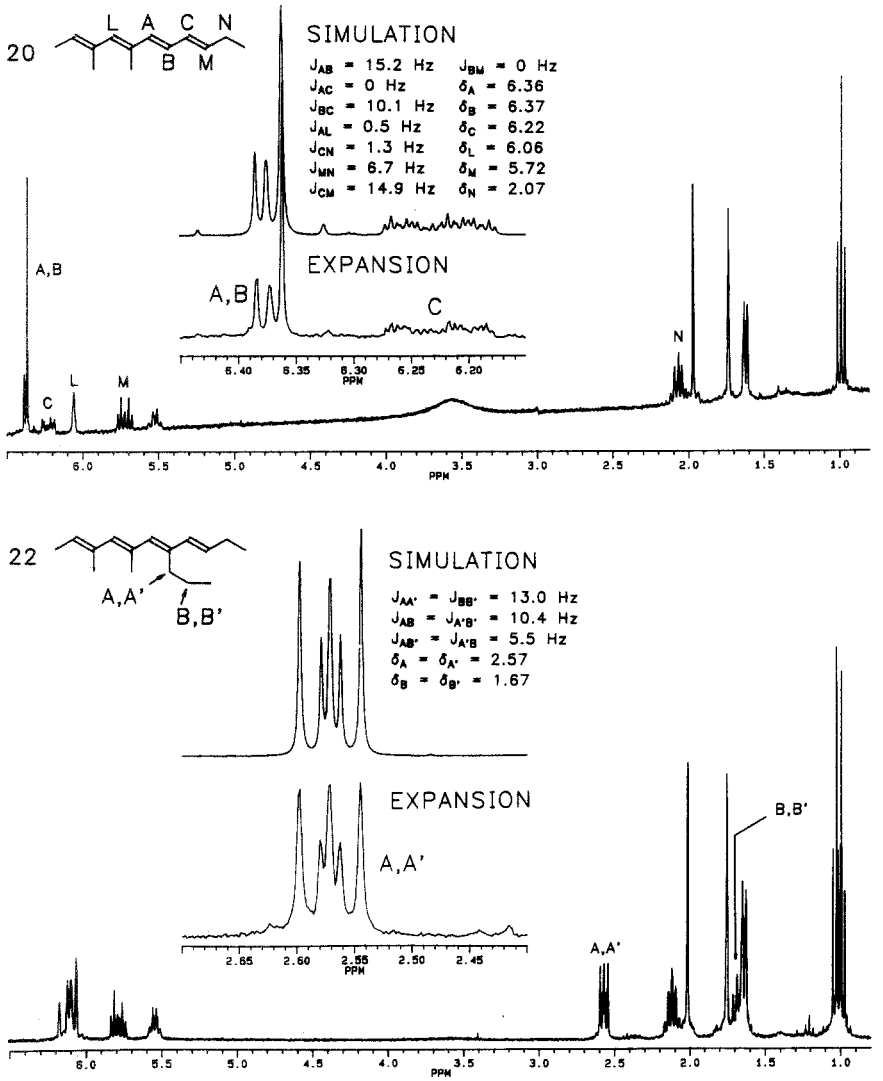


FIG. 9. NMR spectra of two tetraenes not interpretable by first-order rules. Insets show expansion of the regions with second-order character and computer simulations of these regions, based on the listed coupling constants and shifts.

9 shows examples of these. In the dialkyl tetraene **20** (Figure 9, upper), the signals at δ 6.36, 6.37, and 6.22 had ABC character, with additional splitting and line broadening by the protons at δ 5.72, 2.07, and 6.06. Through simulation, it became apparent that the coupling constant between the protons at δ 6.36 and 6.37 was $J = 15.2$ Hz, which supported the *E* configuration for this double bond. Other estimated coupling constants are given in the figure. In **22** (Figure 9, lower), the methylene protons of the propyl side chain produced an unusual multiplet. This was apparently due to hindered rotation about the bond between methylene groups in the side chain. Computer simulation of these methylenes as an AA′BB′ system accounted for the appearance of the signal. The BB′ portion is partially obscured by a methyl signal, making its features difficult to see. Although these spectra were fairly complex, they did support the postulated structures.

For the 13-carbon tetraenes, **13** and **14,** NOE experiments were used to determine the configurations at the trisubstituted double bonds (Figure 7). Each olefinic proton on a trisubstituted double bond was observed for enhancement when the methyl group sharing the same double bond was irradiated. In each compound only one such enhancement was observed; thus each had exactly one trisubstituted *Z* double bond. The locations of the *Z* double bonds were known because all proton shifts had been assigned in the structures. Enhancements of protons *not* on the same double bond as the irradiated methyl group were also observed (Figure 7) and gave some information about the conformations of these compounds in solution. The four double bonds are probably not coplanar as drawn in the figure. Molecular models and molecular mechanics calculations suggest that certain nonplanar conformations would be less strained. For example, in **13** and **14,** the double bond in the 4 position probably lies in a plane nearly perpendicular to those of the 2 and 6 double bonds. NOE data for **1** and **15** (Bartelt et al., 1990a) are also included in Figure 7 for comparison.

Acknowledgments—The assistance of D.G. Carlson, K.L. Smith, C.M. Weber, and B.W. Zilkowski is gratefully acknowledged. We also thank J.W. Hagemann of this laboratory for doing the molecular mechanics calculations for structures **13** and **14;** Chem X molecular modeling software, developed and distributed by Chemical Design, Ltd., Oxford, England, was used for this purpose.

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FEMALE SEX PHEROMONE OF ORIENTAL TOBACCO BUDWORM, *Helicoverpa assulta* (GUENEE) (LEPIDOPTERA: NOCTUIDAE): IDENTIFICATION AND FIELD TESTING

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Abstract—Analysis of ovipositor washings from virgin female *Helicoverpa assulta* (Guenée) (Lepidoptera: Noctuidae) from Korea by gas chromatography (GC) linked to electroantennography and GC linked to mass spectrometry resulted in the identification of nine compounds, hexadecanal, (Z)-9-hexadecenal, (Z)-11-hexadecenal, hexadecyl acetate, (Z)-9-hexadecenyl acetate, (Z)-11-hexadecenyl acetate, hexadecan-1-ol, (Z)-9-hexadecen-1-ol, and (Z)-11-hexadecen-1-ol. However, ovipositor washings from females from Thailand contained mainly the 16-carbon aldehydes with very small amounts of (Z)-9-hexadecenyl acetate. Field tests conducted in Korea, China, and Thai-

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land indicated that a binary blend of (Z)-9-hexadecenal and (Z)-11-hexadecenal was sufficient for attraction, although the most attractive ratio of compounds varied with location. In Korea a 20:1 blend of compounds was the most attractive, while in Thailand a 7.5:1 blend was most attractive. In China both blends of hexadecenal isomers were equally attractive. Addition of the hexadecenyl acetates to the 20:1 blend of hexadecenals in the ratio of 1:3.3 increased the trap catch of male *H. assulta* compared to lures containing the aldehydes alone in Korea but reduced trap catch in China. Addition of the hexadecenyl acetates to the 7.5:1 blend of hexadecenals had no significant effect on trap catch in Thailand or China compared to the aldehydes alone. The addition of the 16-carbon alcohols to the aldehydes had a significantly inhibitory effect in all three countries, suggesting they are not pheromone components. Taken together these results indicate that *H. assulta* is polymorphic with at least two populations responding to different sex pheromones.

Key Words—*Helicoverpa assulta*, *Heliothis*, Lepidoptera, Noctuidae, hexadecanal, (Z)-9-hexadecenal, (Z)-11-hexadecenal, hexadecyl acetate, (Z)-9-hexadecenyl acetate, (Z)-11-hexadecenyl acetate, hexadecan-1-ol, (Z)-9-hexadecen-1-ol, (Z)-11-hexadecen-1-ol, sex pheromone.

INTRODUCTION

The Oriental tobacco budworm, *Helicoverpa assulta* (Guenée) is found throughout the Far East, Africa, and Australasia, where it is a pest of such crops as peppers (Cho and Boo, 1988) and tobacco and onions (Hill, 1983). Control of this pest in the larval stages with insecticides is particularly difficult because of its habit of feeding within the fruits of host plants, and these difficulties have been further aggravated by the increasing resistance of larvae to the insecticides currently used for its control (Lee and Boo, 1985). In order to achieve effective control strategies, there is a need for a sensitive, species-specific monitoring system. An attractive bait based on the sex pheromone of the species would fit this requirement and have the added advantage that traps based on such a lure are highly mobile and cheap to operate, requirements that are particularly important to pest monitoring systems when utilized in developing countries (Beevor et al., 1983).

The present study describes our efforts to identify components of the female sex pheromone and develop a species-specific lure for monitoring *H. assulta* by conducting field trials of the compounds identified in Korea, China, and Thailand.

METHODS AND MATERIALS

Insect Material. Larvae were collected in the Suwon area of Korea and reared on either tobacco leaves or an artificial diet (Kim et al., 1984). Pupae were sexed and dispatched by air to England. On arrival they were separated

into single sex groups of 10–15 individuals and placed in Perspex containers (30 × 15 × 15 cm) with access to 10% sucrose solution and tissue paper in vertical strips to allow them to expand their wings on emergence (Cho and Boo, 1988). The containers were placed in an environmental cabinet maintained at 25°C and 60% humidity during the reversed 12-hr light period and 22°C and 60% humidity in the 12-hr dark period. In Thailand, insects were reared on artificial diet (Kim et al., 1984) and maintained on a 12-hr: 12-hr light–dark cycle.

Pheromone Collection. Pheromone gland washings were prepared in England from Korean insects using hexane and 0- to 2-day-old females 4–6 hr and 10 hr into the scotophase as described by Sower et al. (1973). In Thailand gland extracts were prepared 10 hr into the scotophase (10 glands/sample) and dispatched to Israel for chemical analysis.

Gas Chromatography (GC). GC analyses of pheromone gland extracts made from Korean insects were conducted on a Carlo Erba Mega series 5300 instrument fitted with two Grob split/splitless injectors (200°C) and flame ionization detector (FID) (220°C). Fused silica capillary columns (25 m × 0.32 mm ID) were used throughout the study, coated with either nonpolar CP Sil 5CB (chemically bonded methylsilicone; Chrompack) or polar CP Wax 52CB (chemically bonded Carbowax 20 M equivalent; Chrompack). The carrier gas was helium with an inlet pressure of 0.4 kg/cm^2. All injections were made with the split valve closed for 40 sec onto a column held at 70°C for 2 min then temperature programmed at 20°C/min to 120°C, and then at 4°C/min to 210°C.

GC analyses of Thai pheromone gland extracts were conducted on a Varian 3700 instrument equipped with a FID detector, splitless injector system, and polar fused silica capillary column (30 m × 0.25 mm ID) coated with DB-225 (chemically bonded cyanosilicone; J & W). Samples were injected onto the analytical column, held at 60°C for 2 min, and then temperature programmed to 150°C at 10°C/min. The purge valve was opened 1 min after injection, and the inlet pressure of the helium carrier gas was 1.0 kg/cm^2.

Retention times of compounds identified in the ovipositor washings are summarized in Table 1 and quoted as equivalent chain lengths (ECLs) relative to the retention times of straight-chain acetates; thus, for example, tetradecyl acetate = 14.00 (Christie, 1988; Harris and Habgood, 1966).

Electroantennography (EAG). Linked GC-EAG analyses were carried out essentially as described by Cork et al. (1990) by splitting half of the analytical column effluent to the FID detector and the other half to a silanized glass reservoir in the GC oven. Column effluent was expelled from the reservoir at 17-sec intervals for 3-sec with a stream of nitrogen (250 cm^3/min) into a constant stream of nitrogen (250 cm^3/min) over the EAG preparation. The series of EAG responses generated were then compared to identify those that were significantly above background responses in order to determine the presence of electrophysiologically active compounds.

TABLE 1. GC RETENTION TIMES AND RELATIVE COMPOSITION OF EAG-ACTIVE COMPOUNDS IDENTIFIED IN PHEROMONE GLAND WASHINGS OF *H. assulta* FROM KOREA

	Retention times (ECL)[a]				Composition[b] normalised to Z9-16:Ald	
	Nonpolar column[c]		Polar column[d]			
Compound	Synthetic	Natural	Synthetic	Natural	4–6 hr dark	10 hr dark
Z9-16:Ald	13.77	13.78	14.65	14.65	100	100
Z11-16:Ald	13.86	13.86	14.76	14.76	7.5	4.4
16:Ald	14.02	14.02	14.39	14.39	26.5	17.8
Z9-16:OH	14.49	14.50	17.00	16.97	1.7	55
Z11-16:OH	14.59	ND	17.09	17.09	0.1	6.5
16:OH	14.73	ND	16.71	16.70	0.2	2.5
Z9-16:Ac	15.75	15.75	16.23	16.23	31.6	254
Z11-16:Ac	15.86	15.85	16.36	16.36	1.4	2.3
16:Ac	16.00	16.00	16.00	16.00	4.0	25

[a]Retention times in equivalent chain length units relative to the retention times of straight chain acetates.
[b]ND = not detected.
[c]Nonpolar column, chemically bonded methylsilicone liquid phase, CP Sil 5CB.
[d]Polar column, chemically bonded Carbowax 20 M, CP Wax 52CB.

Mass Spectrometry (MS). Electron impact (EI) and chemical ionization (CI) mass spectra with isobutane as reagent gas were obtained on a Finnigan MAT Ion Trap Detector, model 700, under continuous ion monitoring between *m/z* 30–250 at 220°C for EI-MS and *m/z* 60–300 at 200°C for CI-MS as described by Cork et al. (1991). EI mass spectra of dimethyl disulfide (DMDS) adducts were obtained in the multiple ion detection (MID) mode on a Finnigan 5100 EI machine at 70 eV, coupled to a Super Incos data system and interfaced with a Finnigan MAT 9610 GC. Samples were injected onto a fused silica capillary column (30 m × 0.25 mm ID) with low-polarity SE 54 (chemically bonded phenylmethylsilicone; J & W) held at 60°C for 4 min and then temperature programmed at 20°C/min to 230°C. The injector was operated in the splitless mode with the purge valve opened 0.5 min after injection. The inlet pressure of the helium carrier gas was maintained at 0.7 kg/cm^2.

Synthetic Chemicals. Monounsaturated acetates, alcohols,and aldehydes were obtained by standard Wittig and acetylenic coupling reactions (Henrick, 1977) or purchased from Shin Etsu Chemical Co. (Tokyo, Japan). Compounds used for field testing were further purified by argentation chromatography (Houx

et al., 1974) to give material at least 99.9% isomerically and chemically pure by GC analysis.

Microreactions. Dimethyl disulfide derivatives were prepared essentially as described by Buser et al. (1983) and Dunkelblum et al. (1985).

Field Trials. Field trials in China were conducted in Hunan province in tobacco and hot-pepper fields using water traps (Campion et al., 1974). Related trials in Korea were conducted in hot-pepper fields using cone traps (Hartstack et al., 1979), while those in Thailand were conducted in tobacco fields with sleeve traps (Dunkelblum and Kehat, 1989; Neumark and Teich, 1980). In Korea and China treatments were placed out in randomized complete blocks with at least 20 m between treatments in a block and 100 m between blocks. The position of treatments within a block was rotated in a clockwise direction daily when trap catches were recorded and insects discarded. Female moths tested in Korea were provided with sugar solution and changed daily. In Thailand traps were rotated and trap catches counted once or twice a week.

Pheromone dispensers were white rubber septa (Aldrich, catalog No. Z10,072-2) impregnated with 0.1 ml of hexane solution containing the synthetic pheromone blend and an equivalent weight of 2,6-di-*tert*-butyl-4-methylphenol as antioxidant.

The results were statistically analyzed by analysis of variance (ANOVA) after combining catches for each treatment and transforming them to log $(x + 1)$. If differences in treatment means were significant at the 5% level or lower, then they were ranked by Duncan's multiple-range test (DMRT) (Duncan, 1955).

RESULTS

Structure Determination. GC-EAG analyses of extracts prepared from insects collected in Korea using typically 0.1 pheromone gland equivalents on a nonpolar CP Sil 5CB column under temperature programmed conditions indicated three regions of EAG activity significantly above background. GC retention times of compounds eluting in these regions were consistent with those of saturated and monounsaturated 16-carbon, straight-chain aldehydes, alcohols, and acetates, respectively (Table 1).

Analysis of the 16-carbon aldehyde region by EI-MS indicated the presence of three compounds (Figure 1). The compounds had MS consistent with synthetic hexadecanal and two hexadecenal isomers. CI-MS of the compounds confirmed these results, with significant M + 1 ions at *m/z* 241 and 239 for hexadecanal and the two hexadecenal isomers, respectively, and significant M − 1 and M + 1 − 18 ions associated with the hexadecenal isomers at *m/z* 237 and *m/z* 221, respectively. Comparison of GC retention times of the 16-carbon aldehydes with synthetic analogues on polar and nonpolar phases under iso-

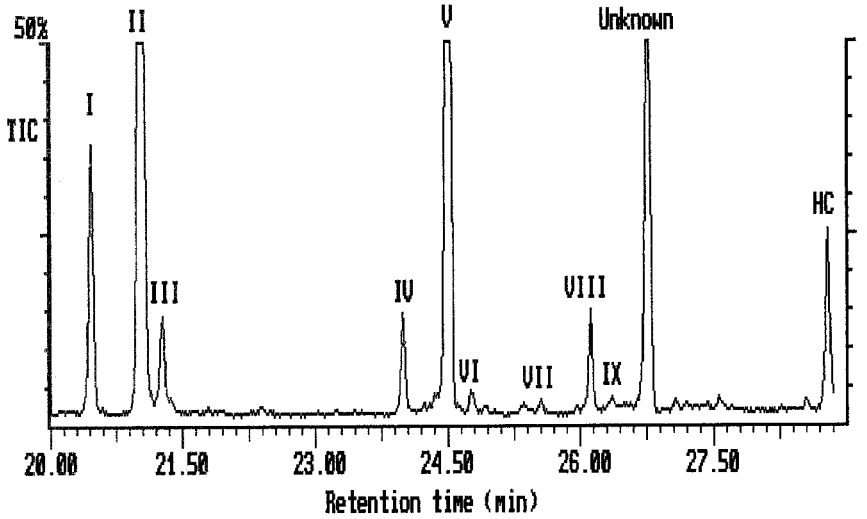

FIG. 1. Total ion chromatogram in EI mode, *m/z* 30–250, of 0.2 equivalents of *H. assulta* ovipositor extract prepared from insects reared in Korea and chromatographed on a polar GC column. The mass spectra and GC retention times of the labeled compounds corresponded with I, 16:Ald; II, Z9–16:Ald; III, Z11–16:Ald; IV, 16:Ac; V, Z9–16:Ac; VI, Z11–16:Ac; VII, 16:OH; VIII, Z9–16:OH; IX, Z11–16:OH; Unknown, unidentified compound; and HC, saturated straight-chain hydrocarbon.

thermal conditions (Cork et al., 1988) confirmed the presence of hexadecanal (16:Ald), (Z)-9-hexadecenal (Z9–16:Ald) and (Z)-11-hexadecenal (Z11–16:Ald) in the approximate ratio of 3.5:13.3:1 for pooled extracts prepared between 4 and 6 hr into the scotophase. In a related study of 10 individual female ovipositor extracts prepared 10 hr into the scotophase, the average ratio of 16:Ald, Z9–16:Ald, and Z11–16:Ald was 4:23:1. Extracts prepared 4–6 hr into the scotophase contained on average 150 ng/female equivalent of Z9–16:Ald, whereas ovipositor extracts prepared 10 hr into the scotophase contained on average 60 ng of Z9–16:Ald, but this varied from 4 to 180 ng.

Similarly three 16-carbon alcohols were observed by EI-MS (Figure 1) that had the same GC retention times on a polar column (Table 1) and mass spectra as hexadecan-1-ol (16:OH), (Z)-9-hexadecen-1-ol (Z9–16:OH), and (Z)-11-hexadecen-1-ol (Z11–16:OH). The alcohols were present in amounts approximately 2% of the corresponding aldehydes in extracts prepared 4–6 hr into the scotophase, but this increased to an average of 55% in extracts prepared 10 hr into the scotophase. Only the most abundant alcohol, Z9–16:OH, could be confirmed on both GC phases and by CI-MS with M + 1 ions at *m/z* 241.

EAG activity elicited by compounds eluting in the 16-carbon acetate region was shown by EI-MS to be associated with three compounds that had characteristic ions at *m/z* 61 $(CH_3COOH_2)^+$ and M−60 ions at *m/z* 224 and 222, indicative of hexadecyl acetate (16:Ac) and two hexadecenyl acetate isomers, respectively. CI-MS was only able to confirm the major hexadecenyl acetate with M+1 ions at 283 and M−60 ions at *m/z* 222. The GC retention times of these compounds on polar and nonpolar GC columns were consistent with those of 16:Ac, (Z)-9-hexadecenyl acetate (Z9-16:Ac), and (Z)-11-hexadecenyl acetate (Z11-16:Ac) (Table 1). The amount of Z9-16:Ac relative to Z9-16:Ald was found to be approximately 26% in extracts prepared 4–6 hr into the scotophase, but this increased dramatically to an average of 232% in extracts prepared 10 hr into the scotophase, although there was considerable variation between ovipositor extracts prepared from individual female moths of from 20% to 600%.

There was no evidence from GC-EAG or GC-MS analyses for the presence of any tetradecenal isomers that are observed in the sex pheromones of some Heliothidinae (Dunkelblum and Kehat, 1989; Klun et al., 1980a).

Ovipositor washings from insects collected in Thailand were analyzed by capillary GC and by GC-MS after conversion to the dimethyl disulfide adducts (Buser et al., 1983; Dunkelblum et al., 1985). The latter analyses confirmed the presence of Z9-16:Ald and Z11-16:Ald with characteristic retention times and ions at *m/z* 145, 187, and 332 (M^+) for Z9-16:Ald, and 117, 215, and 332 (M^+) for Z11-16:Ald. The average ratio of 16:Ald, Z9-16:Ald, and Z11-16:Ald was 1.5:5:1, respectively, and pheromone yields were 1–5 ng per female. Small amounts of Z9-16:Ac were detected in some extracts, but the alcohols were never observed.

Field Tests. Preliminary field trials were conducted in China, Korea, and Thailand with different ratios of Z9-16:Ald and Z11-16:Ald. Different results were obtained at each location. In Korea the most attractive blend tested was the 20:1 ratio of components, in Thailand the 7.5:1 ratio of compounds was the most attractive, and in China there was no significant difference in catches with lures containing either the 50:1 or 5:1 ratio of hexadecenal isomers, although the 1:1 ratio of hexadecenal isomers was significantly less attractive (Table 2). In Korea, the 20:1 blend of hexadecenal isomers was shown to be significantly more attractive to male moths than two virgin females in field trials (Table 2).

The 20:1 blend of Z9-16:Ald and Z11-16:Ald was then tested in China and Korea at different loadings and in both instances the highest catch was obtained with the highest loading tested, 5000 μg, although differences between catches for each treatment were not significant in the trial conducted in China (Table 3). A 7.5:1 blend of compounds was tested in China and Thailand with

TABLE 2. CATCHES OF MALE *H. assulta* IN CHINA, THAILAND, AND KOREA WITH DIFFERENT BLENDS OF Z9–16: ALD AND Z11–16: ALD

Ratio of compounds (1000 μg loading)		Total catch/treatment[a]			
				Thailand[d]	
Z9–16: Ald	Z11–16: Ald	China[b]	Korea[c]	Test I	Test II
50	1	279a	219b	0c	0c
25	1	367a	393a	9bc	11bc
20	1	NT	450a	NT	NT
10	1	205a	213b	16b	21b
7.5	1	317a	NT	54a	46a
5	1	239a	249b	22b	20b
1	1	11b	NT	1bc	6bc
2 females		NT	180b	NT	NT

[a]Totals followed by the same letter are not significantly different $P > 0.05$ by DMRT using log $(x + 1)$-transformed data. NT = not tested.
[b]Four replicates, 30 nights.
[c]Three Replicates, eight nights.
[d]Test I, two replicates, 14 nights; test II, two replicates, 49 nights.

TABLE 3. CATCHES OF MALE *H. assulta* IN CHINA AND KOREA WITH DIFFERENT LOADINGS OF 20: 1 BLEND OF Z9–16: ALD AND Z11–16: ALD

Loading of dispenser (μg)	Total catch/treatment[a]	
	China[b]	Korea[c]
5000	284a	107a
1000	169a	51b
500	181a	29bc
100	103a	1d
2 females	NT	13cd
Blank	0b	NT

[a]Totals followed by the same letter are not significantly different $P > 0.05$ by DMRT using log $(x + 1)$-transformed data. NT = not tested.
[b]Four replicates, 57 nights.
[c]Three replicates, seven nights.

the same result (Table 4). Lures with 1000 μg or more of the 20:1 ratio of hexadecenal isomers were found to be significantly more attractive than two female moths in Korea (Table 3).

The effect on attractiveness of adding Z9-16:OH, Z9-16:Ac, and Z11-16:Ac to a 20:1 blend of Z9-16:Ald and Z11-16:Ald was tested in China and Korea (Table 5). The compounds were added at levels comparable to those found in ovipositor extracts prepared 4–6 hr into the scotophase, when the highest levels of mating were observed in Korea (Kim and Boo, 1986). At both locations Z9-16:OH was found to reduce trap catch significantly when added at 2% of Z9-16:Ald. However, the effect of adding a 20:1 blend of Z9-16:Ac and Z11-16:Ac to an equivalent blend of Z9-16:Ald and Z11-16:Ald in the ratio of 1:3.3 was to increase trap catch significantly in Korea, but reduce trap catch significantly in China. In a related experiment in China and Thailand with a 7.5:1 ratio of Z9-16:Ald and Z11-16:Ald, the addition of the related alcohols in the same isomeric ratio reduced trap catch significantly, while the addition of the related acetates had no statistically significant effect on trap catch (Table 6).

DISCUSSION

The female sex pheromones of Heliothidinae moths characterized so far are blends of saturated and monounsaturated 14-carbon aldehydes, 16-carbon aldehydes, 16-carbon acetates, and 16-carbon alcohols (Table 7). The EAG-

TABLE 4. CATCHES OF MALE *H. assulta* IN CHINA AND THAILAND WITH DIFFERENT LOADINGS OF 7.5:1 BLEND OF Z9-16:ALD AND Z11-16:ALD

Loading of dispenser (μg)	Total catch/treatment[a]		
		Thailand[c]	
	China[b]	Test I	Test II
5000	141a	55a	133a
2000	NT	NT	NT
1000	NT	31ab	23b
500	10b	19b	11bc
100	7b	4b	0c
50	NT	13b	12bc

[a]Totals followed by the same letter are not significantly different $P > 0.05$ by DMRT using $\log (x + 1)$-transformed data. NT = not tested.
[b]Four replicates, 25 nights.
[c]Test 1, two replicates, 14 nights; test II, two replicates, 49 nights.

TABLE 5. CATCHES OF MALE *H. assulta* WITH 16-CARBON ACETATES AND ALCOHOLS ADDED TO BLEND OF Z9–16:ALD AND Z11–16:ALD IN 20:1 RATIO

Composition (μg)									
16:Ald			16:Ac			16:OH		Total catch/ treatment[a]	
Sat	Z9-	Z11-	Sat	Z9-	Z11-	Sat	Z9-	China[b]	Korea[c]
0	1000	50	0	0	0	0	0	503a	48b
0	1000	50	0	300	15	0	0	238b	129a
0	1000	50	0	300	15	0	20	20c	0c
0	1000	50	0	0	0	0	20	23c	3c
250	1000	50	85	300	15	5	20	32c	1c
Blank								0d	NT

[a]Totals followed by the same letter are not significantly different $P > 0.05$ by DMRT using log $(x + 1)$-transformed data. NT = not tested.
[b]Three replicates, 57 nights.
[c]Three replicates, four nights.

TABLE 6. CATCHES OF MALE *H. assulta* WITH DIFFERENT LOADINGS OF 7.5:1 BLEND OF Z9–16:ALD AND Z11–16:ALD AND RELATED ACETATES AND ALCOHOLS

Composition (μg)							
16:Ald		16:Ac		16:OH		Total catch/treatment[a]	
Z9-	Z11-	Z9-	Z11-	Z9-	Z11-	China[b]	Thailand[c]
880	120	9	0	0	0	116a	392a
880	120	265	35	0	0	115a	668a
880	120	265	35	17.6	2.4	2b	44b
880	120	0	0	17.6	2.4	0b	3c
Blank						0c	NT

[a]Totals followed by the same letter are not significantly different $P > 0.05$ by DMRT using log $(x + 1)$-transformed data. NT = not tested.
[b]Three replicates, 32 nights.
[c]Five replicates, 64 nights.

TABLE 7. CHEMICALS IDENTIFIED IN OVIPOSITOR WASHINGS OF *Helicoverpa* AND *Heliothis* SPECIES

Compund	Species[a]							
	a	b	c	d	e	f	g	h
14:Ald	0	0	0.8	0	0	0	2	0
Z9-14:Ald	0	0	14.6	0	0	0	3.9	0
Z9-14:OH	0	0	6.5	0	0	0	0	0
Z9-14:Ac	0	0	2	0	0	0	0	0
16:Ald	49	353	3.7	8.2	9	18	11.7	4.8
Z7-16:Ald	0	0	1.1	0	tr	0	1.6	1.2
Z9-16:Ald	7	1333	1.5	0.5	tr	66	1.2	18.4
Z11-16:Ald	100	100	100	100	100	100	100	100
Z9-16:OH	0	22.7	0	0	0	48	0	0
Z11-16:OH	21	tr	24.3	7.2	75	40.6	3.9	0
16:Ac	0	53.7	0	0	0	0	0	0
Z7-16:Ac	0	0	0	0	0	5.5	0	0
Z9-16:Ac	0	421.3	0	0	0	14.3	0	0
Z11-16:Ac	0	18.4	4.8	0	50	41	0	0

[a]	Species	Origin	Reference
a	*H. armigera*	Malawi	Nesbitt et al., 1980.
b	*H. assulta*	Korea	This study.
c	*H. peltigera*	Israel	Dunkelblum and Kehat, 1989.
d	*H. phloxiphaga*	United States	Raina et al., 1986.
e	*H. punctiger*	Australia	Rothschild et al., 1982.
f	*H. subflexa*	United States	Teal et al., 1981.
g	*H. virescens*	United States	Klun et al., 1980a.
h	*H. zea*	United States	Klun et al., 1980b.

active compounds identified in ovipositor washings of *H. assulta* moths collected in Korea and Thailand were similar 16-carbon aldehydes, acetates, and alcohols, but the relative abundances of the compounds differed both in comparison with other species and between the populations studied.

In all other *Helicoverpa* and *Heliothis* pheromones characterized, the major component is Z11-16:Ald, but Z9-16:Ald was the most abundant hexadecenal isomer found in ovipositor washings from *H. assulta* obtained from Korea and Thailand with Z11-16:Ald detected as a minor component (Table 1). Significant differences in the ratio of Z9-16:Ald to Z11-16:Ald were found in ovipositor washings prepared from insects obtained from Korea and Thailand, and this could not be attributed to differences in sampling protocols as samples were prepared by the same method (Sower et al., 1973) and in the same time period

in the scotophase. Extracts were prepared at times when calling behavior or mating had been observed, notably 4–6 hr into the scotophase for Korean insects (Kim and Boo, 1986) and 10 hr for Thai insects. Extracts prepared from Thai insects at earlier periods in the scotophase contained no detectable quantities of pheromone.

Ovipositor washings made from Korean insects also contained significant amounts of hexadecenyl acetates and hexadecen-1-ols, and again the (Z)-9 isomer predominated over the (Z)-11 isomer. The relative abundance of Z9–16:Ald, Z9–16:Ac, and Z9–16:OH changed from 1:0.3:0.02 in extracts prepared from females 4–6 hr into the scotophase to 1:2.5:0.55 in extracts prepared 10 hr into the scotophase (Table 1). This is in line with similar observations by Heath et al. (1991) working with the (Z)-11 isomers from *H. subflexa* ovipositor washings. In contrast, ovipositor washings prepared from Thai insects 10 hr into the scotophase contained only trace amounts of Z9–16:Ac and the hexadecen-1-ols were not detected.

In view of the large differences in ratios of EAG-active compounds found in the ovipositor washings of female *H. assulta*, it was not possible to define the natural blend of EAG-active compounds that would elicit the optimal mate-finding response from receptive male moths without either collecting volatiles released from calling female moths or field testing different blends of the compounds identified. In this instance the latter course of action was taken.

Field tests in Korea, China, and Thailand showed male *H. assulta* moths were attracted by blends of the two hexadecenal isomers alone in which Z9–16:Ald predominated, but the optimum ratio varied with the location and reflected differences in the ratios found in the ovipositor washings. The most attractive blend of Z9–16:Ald and Z11–16:Ald tested in Thailand was 7.5:1, while 20:1 was significantly more attractive in Korea. In China there was no difference in attractiveness with blends containing between a 50:1 and 5:1 ratio of Z9–16:Ald and Z11–16:Ald although the 1:1 blend was significantly less attractive. Taken together the laboratory and field data suggest there may be two populations of *H. assulta* and that both were present in the Hunan province of China when the field tests were conducted.

Addition of corresponding blends of the two hexadecenyl acetates to the optimum blend of aldehydes increased attractiveness in Korea (Table 5) but did not significantly affect attractiveness in Thailand (Table 6). Addition of the hexadecenyl acetates to a 20:1 blend of Z9–16:Ald and Z11–16:Ald significantly decreased attractiveness in China (Table 5), but addition of the acetates to a 7.5:1 blend had no significant effect on trap catches (Table 6). These results provide further evidence for the existence of different populations of *H. assulta*. However, addition of Z9–16:OH to blends of the hexadecenal isomers with or without the hexadecenyl acetate isomers at a level of only 2% of the Z9–16:Ald dramatically reduced trap catches in China, Korea, and Thailand (Tables 5 and

6), suggesting that the alcohol found in ovipositor washings might have been a pheromone precursor (Teal and Tumlinson, 1986).

This study has provided another example of the way in which related species achieve species specificity by varying the relative amounts of a limited range of related chemicals in their sex pheromones (Kaae et al., 1973). In India *H. assulta* coexists with both *H. armigera* and *H. peltigera*. *H. armigera* is attracted by mixtures of Z11-16:Ald with 1-10% Z9-16:Ald (Kehat et al., 1980), in agreement with relative amounts of pheromone components found in analyses of ovipositor washings (Nesbitt et al., 1979, 1980). Ten potential pheromone components were detected in female ovipositor washings from *H. peltigera* by Dunkelblum and Kehat (1989), with Z11-16:Ald, Z11-16:OH, and Z9-14:Ald the most abundant components. Laboratory and field tests showed Z9-16:Ald was not necessary for attraction of this species, and a 2:1 mixture of Z11-16:Ald and Z9-14:Ald attracted male *H. peltigera* moths and only a few male *H. armigera*. Addition of Z11-16:OH further reduced the attractiveness to *H. armigera* without affecting attraction of *H. peltigera*. Presumably male *H. assulta* moths would not be attracted to *H. armigera* female moths because of the relative amounts of Z11-16:Ald and Z9-16:Ald, nor would they be attracted to *H. peltigera* female moths because of the virtual absence of Z9-16:Ald and the presence of Z11-16:OH. The Z9-14:Ald is vital for attraction of *H. peltigera* and reduces attractiveness to *H. armigera*, but it was not detected in ovipositor washings from *H. assulta* and its effect on the behavior of this species was not tested.

In Australia, *H. assulta* is sympatric with both *H. armigera* and the native budworm, *H. punctiger*. Rothschild et al. (1982) showed that a 20:20:1 mixture of Z11-16:Ald, Z11-16:Ac, and Z9-14:Ald provided an attractive lure for male *H. punctiger* moths, again in line with analyses of ovipositor washings from virgin female moths. Cross-attraction with *H. armigera* would not be expected because of the absence of Z9-16:Ald and the presence of Z9-14:Ald in the *H. punctiger* pheromone, while male *H. assulta* moths would not be attracted because of the absence of Z9-16:Ald.

From this work, the best blends recommended for monitoring *H. assulta* are a 20:1 mixture of Z9-16:Ald and Z11-16:Ald with 30% of a corresponding mixture of Z9-16:Ac and Z11-16:Ac in Korea and a 7.5:1 mixture of the two aldehydes with 30% of the corresponding acetates in Thailand. In China the situation is complicated by the apparent mixing of these populations and, until insect material is analyzed from China, only the blend recommended for Thailand should be used. Pheromone polymorphism involving different ratios of pheromone components has been observed previously in a number of lepidopterous species (e.g., Guerin et al., 1984; Bailey et al., 1986; Löfstedt et al., 1986; Löfstedt, 1990), but whether the different populations can be described as different species will depend, in part, on the degree of homogeneity of the

pheromone blends produced by each population and the degree of cross-attractiveness of the pheromones (Foster and Roelofs, 1987). Although this study provides evidence for the possible existence of at least two separate populations of *H. assulta*, confirmation will have to await further analyses of the pheromone produced by insects collected in different regions and further field testing of the different blends. Obviously, this is particularly important if pheromone lures are to be used for reliable monitoring of *H. assulta* in different areas.

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IDENTIFICATION AND SYNTHESIS OF INSECT PHEROMONE XXX. SEX PHEROMONE OF MULBERRY CLEARWING MOTH *Paradoxecia pieli* LIEU[1]

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Abstract—(*E,Z*)-3,13-Octadecadienyl acetate **(1a)** and (*E,Z*)-3,13-octadecadien-1-ol **(2a)** were identified from the sex pheromone gland of the virgin female mulberry clearwing moth *Paradoxecia pieli* L., by GC analysis, EAG, SCR survey, and field bioassay. One female equivalent contained 250 ng of **1a** and 30 ng of **2a.** In the field tests, 100 μg of synthetic **1a** was attractive to male moths of the species.

Key Words—Insect sex pheromone, mulberry clearwing moth, Lepidoptera, Sesiidae, *Paradoxecia pieli* L. (*E,Z*)-3,13-octadecadien-yl acetate, (*E,Z*)-3,13-octadecadien-1-ol, single cell recording, EAG.

INTRODUCTION

The mulberry clearwing moth, *Paradoxecia pieli* L. (Sesiidae), is distributed in Jiang Su, Zhe Jiang, Si Chuan and Gui Zhou provinces in China. The larvae

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TABLE 1. POSSIBLE CHEMICAL STRUCTURE OF SEX PHERONOME OF SESIIDAE[a]

$$n\text{-}C_4H_9\overset{13}{C}H{=}CH(CH_2)_8\overset{3}{C}H{=}CH(CH_2)_2OR$$

R = Ac	**1a** *E,Z*	R = H	**2a** *E,Z*
	1b *Z,E*		**2b** *Z,E*
	1c *Z,Z*		**2c** *Z,Z*
	1d *E,E*		**2d** *E,E*

[a] *E,Z* describes the *E* form of configuration at Δ3 and the *Z* form at Δ13. Similar meanings apply to *Z,E*, *Z,Z* and *E,E*.

damage mulberry trees by making holes in the twigs. In the most severe situations, 30% of the twigs and leaf sprouts could be killed. The larvae and nymphs remain inside the twigs, therefore, it is difficult to kill this insect by spraying with insecticides. Because the insect has only one generation each year, it may be possible to use a mass-trapping technique with insect sex pheromone as a control.

The sex pheromone of Sesiidae has been identified as 3,13-octadecadien-1-ol, or its acetate, with few exceptions (Zhang et al., 1986). In our previous reports, the major sex pheromones of the poplar twig clearwing moth, *Paranthrene tabaniformis* Rott, (Zhang et al., 1986); the poplar pole clearwing moth, *Sphecia siningensis* Hsu (Guo et al., 1990a); and the vine tree borer, *Paranthrene regalis* Bulter, (Guo et al., 1990b) were identified as (*E,Z*)-3,13-octadecadien-1-ol **(2a)**, (*Z,Z*)-3,13-octadecadien-1-ol **(2c)**, and (*E,Z*)-3,13-octadecadien-1-ol **(2a)**,[5] respectively (Table 1). Here we report the identification of the sex pheromone of the mulberry clearwing moth.

METHODS AND MATERIALS

Pheromone Collection. Twigs in which either nymphs or the last instar larvae were located were removed from mulberry trees in Ru Guo County in late May, and they were stored in the laboratory under normal conditions. Care was taken to keep adequate humidity by occasionally watering some pieces of cotton placed adjacent to the twigs. Adults were sexed immediately upon emergence. The ovipositors of 1- to 2-day-old virgin female moths (altogether 25 virgin females were collected for use) were removed and dipped in acetyl acetate.

[5] (*E,Z*)-3,13-Octadecadien-1-ol and its Z,Z-isomer were abbreviated as (*E,Z*)-3,13–18:OH and (*Z,Z*)-3,13–18:OH, respectively. Similar abbreviations were used for other OH isomers and their acetate isomers.

The extract was then filtered over glass wool and the clean filtrate was concentrated, sealed in a glass tube (ID 1.0 mm), and stored in a freezer until needed for EAG survey, GC analysis, and field tests. The antennae of 1- to 2-day-old unmated male moths were cut off according to the published paper for EAG bioassays (Roelofs et al., 1971).

GC Analysis. GC 102 instrument (Shanghai Analytical Instrument Factory) was employed for GC analysis under two conditions: condition A—glass column 5 m × 3 mm ID, packed with 3% OV-1 on 80–100 mesh Gas Chrom Q, column temperature 210°C; condition B—glass column 5 m × 3 mm ID, packed with 5% DEGS on 80–100 mesh Gas Chrom Q column temperature 180°C.

Single Cell Recording (SCR). The electrophysiological recording technique used was essentially the same as that applied by Kaissling and Thorson (1980). Each antenna was cut off from a male moth that had emerged two days before. One capillary (about 10 μm diameter) was placed over the end of the sensillum trichodeum olfactory hair where the tip had been removed to record the potential and nerve impulses of the receptor cell. The stimulus source was a piece of filter paper treated with 0.01 μg of the test compound. In our case, this was the minimum amount used for SCR so that the effect of possible impurity isomers could be diminished.

Field Bioassays. Field tests have been conducted since 1985 in Jiang Su Province during the adult flight season. Water traps were placed on the ground; each trap was made from a large bowl (25 cm diam.) filled with water and a lure. The lure was fastened with a piece of wire and placed in the middle of the bowl 3 cm above the water surface. Each lure was charged with either 100 μg or 200 μg of the synthetic chemical using rolled filter paper or polyethylene hollow tubes as dispensers. The crude extract was also employed as the natural lure for comparison under the same conditions.

Chemicals. All geometric isomers of 3,13-octadecadien-1-ol **(2a–d)** and their acetates **(1a–d)** were synthesized at purities of over 95% as previously reported by Zhang et al. (1985). Satisfactory spectroscopic data (IR, [^{1}H]NMR, MS) were obtained with all the above compounds.

RESULTS AND DISCUSSION

EAG Survey. Among the eight compounds (**1a–d** and **2a–d**) tested using the EAG survey technique on the mulberry clearwing moth, (*E,Z*)-3,13-18:OAc **(1a)** gave the highest response, and (*E,Z*)-3,13-18:OH **(2a)** exhibited the next highest in comparison with the untreated control. The EAG responses and calculated response index are shown in Table 2. The results indicate that the pheromone system might consist of several components with (*E,Z*)-3,13-18:OAc **(1a)** as the major component.

Field Bioassays. The eight compounds (**1a–d** and **2a–d**) were bioassayed individually in the field (Table 3). Only (*E,Z*)-3,13–18:OAc (**1a**) caught significant numbers of male moths; the other seven compounds (**1b–d** and **2a–d**) were not active during five days of testing in 1985. The preliminary test also showed that the addition of either (*Z,Z*)-3,13–18:OAc (**1c**) or (*E,Z*)-3,13–18:OH (**2a**) to (*E,Z*)-3,13–18:OAc (**1a**) gave no obvious increase in the number of male moths captured. Therefore, only (**1a**) was used in the subsequent tests.

GC Analysis. The crude washing from female sex gland (0.4–1 FE) was performed on GC on either column A (nonpolar column) or column B (polar column). For comparison, four synthetic acetates (**1a–d**) and their corresponding alcohols (**2a–d**) were performed on GC under the same conditions. There were

TABLE 2. EAG RESPONSES OF MULBERRY CLEARWING MOTH, *Paradoxecia pieli* L., TO ALL GEOMETRIC ISOMERS OF 3,13-OCTADECADIEN-1-OL (**2**) AND THEIR ACETATES (**1**)

Chemicals	EAG responses (mv)				Index[a]
	I	II	III	IV	
(*E,Z*)-3,13–18:OAc (**1a**)	1.0	1.0	1.0	1.0	1.0
(*Z,E*)-3,13–18:OAc (**1b**)	0.5	0.3	0.5	0.43	0.3
(*Z,Z*)-3,13–18:OAc (**1c**)	0.2	0.1	0	0.1	0
(*E,E*)-3,13–18:OAc (**1d**)	0.2	0.1	0.2	0.17	0
(*E,Z*)-3,13–18:OH (**2a**)	0.3	0.8	0.8	0.63	0.5
(*Z,E*)-3,13–18:OH (**2b**)	0.3	0.3	0.1	0.23	0.1
(*Z,Z*)-3,13–18:OH (**2c**)	0.2	0.2	0.3	0.23	0.1
(*E,E*)-3,13–18:OH (**2d**)	0.3	0.1	0.1	0.17	0
CK	0.2	0.2	0.1	0.17	0

[a]Based on the response value (in mv) of (*E,Z*)-3,13–18:OAc (**1a**) as 1.0 and that of check CK as zero.

TABLE 3. FIELD ATTRACTION TESTS OF SYNTHETIC COMPOUNDS TO MALE MOTHS[a]

	Compounds								
	3,13–18:OAc (**1**)				3,13–18:OH (**2**)				
	E,Z	*Z,E*	*Z,Z*	*E,E*	*E,Z*	*Z,E*	*Z,Z*	*E,E*	CK
Male moths captured	33	1	1	1	1	0	1	0	0

[a]Date: June 29–July 5, 1985; place: Campus of Su Zhou College of Sericulture; dosage: each lure contained 200 μg of the test compound.

two diagnostic peaks from the crude washing that appeared on column A with retention times of 11.3 min (peak I) and 16.0 min (peak II), respectively, and two peaks on column B with retention times at of 15.2 min and 18.9 min. The retention times for **2a–d** under the same conditions were 11.3 min on column A and 18.9, 20.0, 20.9, and 18.1 min on column B. For **1a–d,** the retention time were 16.0 min on column A and 15.2, 15.0, 15.8, and 14.6 min on column B (see Table 4). Thus the above data showed that the natural sex pheromone consisted of **2a,** which appeared on GC as peak I, and **1a,** as peak II (Table 4).

Calculation of the peak areas on GC with the standard compounds revealed that 1 FE contained 30 ng (peak I) and 250 ng (peak II). Therefore, 250 ng of **1a** and 30 ng of **2a** was obtained from the crude washings of the female's sex pheromone gland.

In Figure 1, receptor potentials and nerve impulses were recorded from an olfactory hair on the male moth antenna of *Paradoxecia pieli*. Each cell is activated by only one pheromone component (distinguishable by the amplitude of the nerve impulses). The stimuli were (*E*,*Z*)-3,13–18:OAc (Figure 1A) and (*Z*,*Z*)-3,13–18:OAc (Figure 1B). Each response is AC- and DC-amplified (upper and lower trace, respectively).

The single cell recording (Figure 1) from the mulberry clearwing moth indicated that each sensilla trichodeum contained two receptor cells. The nerve impulses came from receptor cells that could be distinguished from each other by their amplitudes. One type of receptor cell producing the larger spikes was

TABLE 4. RETENTION TIME OBTAINED FROM NATURAL SEX PHEROMONE AND FROM SYNTHETIC CHEMICALS **1a–d** AND **2a–d**

Samples		Retention time (min)	
		Condition A	Condition B
(*E*,*Z*)-3,13–18:OAc **(1a)**		16.0	15.2
(*Z*,*E*)-3,13–18:OAc **(1b)**		16.0	15.0
(*Z*,*Z*)-3,13–18:OAc **(1c)**		16.0	15.8
(*E*,*E*)-3,13–18:OAc **(1d)**		16.0	14.6
(*E*,*Z*)-3,13–18:OH **(2a)**		11.3	18.9
(*Z*,*E*)-3,13–18:OH **(2b)**		11.3	20.0
(*Z*,*Z*)-3,13–18:OH **(2c)**		11.3	20.9
(*E*,*E*)-3,13–18:OH **(2d)**		11.3	18.1
n-18 OAc		17.8	11.0
Natural sex pheromone	Peak I	11.3	18.9
	Peak II	16.0	15.2

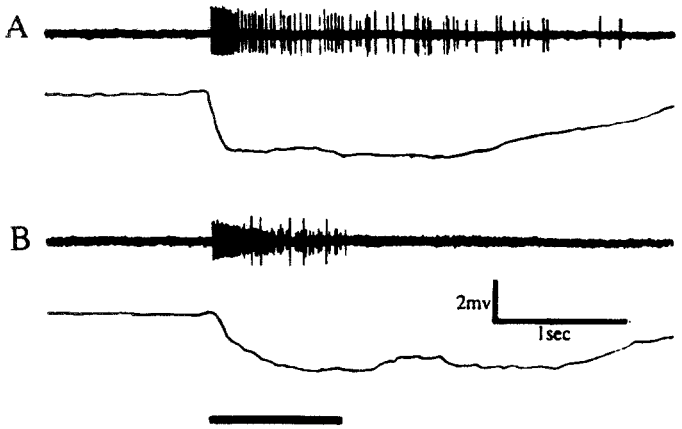

FIG. 1. Single cell recording.

sensitive to (*E*,*Z*)-3,13-18:OAc **(1a)** (Figure 1A); the other, producing smaller spikes, was reactive to (*Z*,*Z*)-3,13-18:OAc **(1c)** (Figure 1B). Each type of cell was specifically responsive and sensitive to a particular pheromone component. In general, the nerve impulses could be recorded by dosages of 0.01 μg.

The cells also responded to (*E*,*Z*)-3,13-18:OH **(2a)** and (*Z*,*Z*)-3,13-18:OH **(2c)** but exhibited lower responses than those to (*E*,*Z*)-3,13-18:OAc **(1a)** and (*Z*,*Z*)-3,13-18:OAc **(1c).** No response was observed to (*Z*,*E*)-3,13-18:OAc **(1b),** (*E*,*E*)-3,13-18:OAc **(1d),** (*Z*,*E*)-3,13-18:OH **(2b),** and (*E*,*E*)-3,13-18:OH **(2d).**

CONCLUSION

The data obtained from GC analysis field bioassays, EAG survey, and SCR strongly suggest that (*E*,*Z*)-3,13-18:OAc **(1a)** is the major sex pheromone component of the mulberry clearwing moth, *Paradoxecia pieli* L., and that (*E*,*Z*)-3,13-18:OH **(2a)** also was found in the pheromone gland. **1a** and **2a** averaged 250 ng and 30 ng, respectively, in each of the sex pheromone glands (1 FE). In the field test, only **1a** was attractive to male moths. This compound has been used for monitoring populations of this insect since 1985. The addition of **2a** to **1a** did not increase significantly captures of male mulberry clearwing moths. The behavioral function of **2a** has not yet been established. SCR investigation showed clearly that each sensilla trichodeum contained two kinds of receptor cells. One kind responded to (*E*,*Z*)-3,13-18:OAc **(1a)** and produced the larger spikes; the other was sensitive to (*Z*,*Z*)-3,13-18:OAc **(1c)** and released the smaller spikes. Further work is needed to understand the reason for the receptor

cells' response to (Z,Z)-3,13-18:OAc **(1c)**, which was not found in the sex pheromone gland.

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A POTENTIAL KAIROMONE STIMULATES PHEROMONE-RESPONSIVE RECEPTOR NEURONS IN *Utetheisa ornatrix* (Lepidoptera: Arctiidae)

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Abstract—The olfactory receptor neurons in basiconic sensilla on the antennae of *Utetheisa ornatrix*, which, in females, had earlier been shown to be responsive to stimulation with hydroxydanaidal (HD), are here shown to be responsive to volatile substances in samples of pyrrolizidine alkaloids (PAs). These latter substances are secondary plant metabolites present in the host plant of the larvae. Their sequestration during larval life serves to protect all life stages from predation. In males, the PAs also provide precursors for the production of the male pheromone, HD. In females, basiconic receptor neurons begin to respond to stimulus cartridges containing 1 ng of (*R*)-(−)-hydroxydanaidal, 100 ng of its isomer, (*S*)-(+)-hydroxydanaidal, and to volatiles emanating from 10 μg of the alkaloids monocrotaline and heliotrine. Receptor neurons in males are generally responsive to the same array of substances but with reduced sensitivity. The dietary background of the adult moths with respect to prior ingestion of PAs does not influence the response capabilities of basiconic receptor neurons to HD or to the volatile components of PAs. Earlier studies in another arctiid (*Rhodogastria*), had indicated that trace amounts of HD are present in PAs, presumably as their hydrolysis product. Thus we assume that, under natural conditions, HD may be an active component of the volatiles from PAs and may serve as both a male pheromone and a kairomone. Sensitivity to HD could thereby provide information about the location and PA content of potential mates and food plants. Mechanisms that may have resulted in the evolution of a signaling system with these properties are discussed.

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Key Words—Insect olfaction, electrophysiology, pyrrolizidine alkaloids, *Utetheisa ornatrix*, Lepidoptera, Arctiidae, hydroxydanaidal, male-produced pheromone, olfactory receptor neurons.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) play a central role in the natural history of a number of insects including *Utetheisa ornatrix* (Linnaeus). Female moths of this species oviposit exclusively on host plants of *Crotalaria* spp. (Leguminosae; cf. Eisner and Meinwald, 1987). These plants are known to be protected from general herbivory by their content of poisonous PAs (Mattocks, 1968). *Utetheisa* larvae compete for and ingest preferentially those portions of the plant that contain elevated amounts of PAs. The alkaloids thus sequestered are carried forward into the adults and the eggs of the next generation. At each of these stages, the sequestered alkaloids protect the animal from predation (cf. Eisner and Meinwald, 1987). The PAs ingested as larvae also influence the sexual life of these moths, especially males, where these chemicals provide the precursors for the production of hydroxydanaidal (HD), the male pheromone (Conner et al., 1981, 1990). In the absence of sequestered alkaloid, males fail to produce their pheromone and are less acceptable to females. The amount of HD produced by the male pheromone gland is directly proportional to the alkaloid content of the male (Conner et al., 1990). Thus, the amount of HD released from the male coremata could offer the female a direct measure of each male's sequestered alkaloid (cf. Conner et al., 1981) and an indirect measure of the prospective alkaloid gift to be obtained in the transfer of the spermatophore (Dussourd et al., 1988). Female *Utetheisa* moths possess a specialized antennal olfactory receptor neuron in basiconic sensilla responsive to the volatiles from nanogram amounts of HD (Grant et al., 1989).

In addition to its role as an actively synthesized male pheromone, HD may also modulate other communicative functions in certain insects. For example, hydrolysis of the alkaloid monocrotaline results in the production of trace amounts of HD, which can serve as a more volatile marker for the presence of PAs in plants and animals. In a series of electrophysiological and thin-layer chromatographic experiments (Bogner, 1987; Bogner and Boppré, 1989), traces of HD were reported in alkaloid sources that were stimulative in certain PA-pharmacophagous Lepidoptera such as *Rhodogastria* (Arctiidae), *Euchromia* (Ctenuchiidae), and *Danaus* (subfamily, Danainae). These species find sources of PAs attractive over long distances but do not appear to possess olfactory receptor neurons specifically responsive to stimulation with small doses (1 μg) of alkaloid (Bogner and Boppré, 1989). However, these species do possess specific olfactory receptor neurons that respond to the volatiles from sources containing large

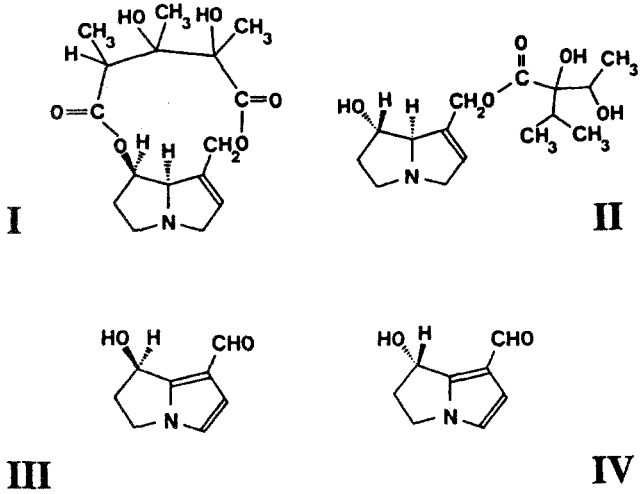
doses (10 μg) of PAs and to authentic samples of HD (10 ng) even though there is no evidence, in these species, for an HD-based male pheromone system (Boppré, 1990). Thus, it was postulated that these species are able to detect and locate sources of alkaloids by virtue of their ability to detect small amounts of the more volatile HD that may normally be present in natural sources of PAs.

Hydroxydanaidal may thus fulfill multiple functions in the life of *Utetheisa*. In addition to its direct role as a male pheromone and, thereby, an indicator of an adult male's alkaloid load, HD may also serve as a volatile marker for the presence of PAs in plants and other organisms. Consequently, we decided to expand on the concepts developed for *Rhodogastria* (Bogner and Boppré, 1989; Boppré, 1990), by using the demonstrated sensitivity of olfactory receptor neurons in female *Utetheisa* sensilla basiconica to stimulation with HD, as a bioassay for the potential presence of HD in alkaloids. Specifically, we asked four questions: (1) Do these receptor neurons respond to the volatiles emanating from mg doses of alkaloids? (2) If so, does this sensitivity depend on the moth's dietary background of ingested alkaloid? (3) How sensitive are these receptor neurons to the two stereoisomers of HD? and (4) Do male *Utetheisa* have a comparable sensitivity to the volatile components of PAs?

METHODS AND MATERIALS

Animals. The animals used in the present investigation were from a laboratory culture of *Utetheisa ornatrix*, established with stock taken from Highlands County, Florida. Voucher specimens (lot 1154) are deposited in the Cornell University insect collection. The moths were reared on two diets: (1) a pinto bean (PB) diet devoid of pyrrolizidine alkaloids (Miller et al., 1976), and (2) a modified PB diet containing 10% (by weight) dried, ground *Crotalaria spectabilis* seeds (CS diet). *C. spectabilis*, one of the principal food-plants of *Utetheisa* in the southeastern United States, contains the pyrrolizidine alkaloid monocrotaline, largely in its seeds (Johnson et al., 1985).

Stimuli. The stimuli evaluated included the volatiles swept out of odor cartridges containing: the PAs—monocrotaline (I), heliotrine (II), and monocrotaline-*N*-oxide; and the male pheromones—danaidone and the two stereoisomers of hydroxydanaidal, (*R*)-(−)-hydroxydanaidal (III), and (*S*)-(+)-hydroxydanaidal (IV) (Scheme 1). All of the stimuli were obtained from sources detailed by Bogner and Boppré, (1989). Heliotrine is not reported in either of the primary food plants of *Utetheisa* in the United States (*C. spectabilis*, *C. mucronata*) but is known to be present in other plant sources (Mattocks, 1968). All samples were diluted in spectrophotometric grade methylene chloride except for monocrotaline-*N*-oxide, which was dissolved in glass-distilled water. Aliquots of these solutions (5–50 μl) were pipetted onto filter-paper strips housed



SCHEME 1. Formula of the stimuli: monocrotaline (I); heliotrine (II); (*R*)-(−)-hydroxydanaidal (III); and (*S*)-(+)-hydroxydanaidal (IV).

in small odor cartridges (Grant et al., 1989). Control cartridges contained equivalent amounts of the appropriate solvent. The purity of individual stimuli was preserved by sealing the odor cartridges between experiments and storing them in individual glass vials held at 4°C in an opaque container purged with dry N_2 to retard oxidation and hydrolysis. Stimuli were brought to room temperature (22°C) several hours before use. The stimulus quantities stated herein indicate the total amount of material loaded into the odor cartridges and not the amounts that actually reach the olfactory receptor neurons.

Stimulation. A purge stream of purified air continuously bathed the preparation between stimulations. During stimulation, this purge stream was diverted away from the preparation and a stream of dry, O_2-free N_2 was directed through the stimulus cartridge and over the preparation. The outlets of the purge stream and the stimulus cartridge were positioned on opposite sides of the antenna, with the outlet of the stimulus cartridge approximately 6 mm from the antenna. The timing of the stimulus was controlled by computer, which simultaneously activated Teflon solenoid valves in both the purge and stimulus streams.

Recording. The procedures for electrode manufacture, as well as signal amplification, discrimination and storage, have been described previously (Grant et al., 1988, 1989). Briefly, adult animals (2–6 days old) were immobilized with wax and fixed to the stage of a microscope so that an electrolytically sharpened tungsten microelectrode (indifferent electrode) could be inserted into the lumen of a flagellar subsegment near the tip of the antenna. A second microelectrode (recording electrode) was then inserted at the base of a single

sensillum on a more proximal subsegment. Electrical signals were amplified and sent to an oscilloscope and to a microcomputer for data acquisition, analysis, and storage (O'Connell et al., 1973). Twenty seconds of neural activity were recorded for each stimulus presentation, including 10 sec of prestimulus activity, 2 sec of activity during the stimulus pulse, and 8 sec of poststimulus activity. Response values are expressed here as the rate of impulse activity (impulses per second) during the 2-sec stimulus pulse. Whenever mean response values are given, the standard error of the mean is also reported (mean $\pm$ SEM). Interstimulus periods typically ranged from 3 to 10 min, depending on the intensity of the preceding response. When multiple doses of a compound were evaluated, they were always presented in order of increasing concentration.

RESULTS

Extracellular recordings from basiconic sensilla on the antennae of both sexes usually (55%, $N = 66$) revealed the electrical activity of two receptor neurons. As demonstrated earlier, the neuron producing the smaller-amplitude action potential (the B neuron) fired infrequently, failed to respond to any of the stimuli evaluated, and therefore was not considered further in this analysis (Grant et al., 1989). This series of recordings was obtained from 23 basiconic receptor neurons sampled from each of 10 males and 13 females. Seven of the animals of each sex were raised on CS diet, the remainder on PB diet. The average spontaneous activity of the neuron producing the larger amplitude action potential (the A neuron) was 2.9 $\pm$ 0.8 impulses/sec (mean $\pm$ SEM) in females (Figure 1A) and 1.2 $\pm$ 0.4 in males. These values are comparable to those reported earlier in females (Grant et al., 1989).

The A receptor neuron is responsive to the volatiles from small amounts ($\geq$ 1 ng) of the active (*R*)-(−) isomer of HD (Figure 1D and F), larger amounts ($\geq$ 100 ng) of the (*S*)-(+) isomer (Figure 1E), and to those emanating from stimulus cartridges containing elevated doses ($\geq$ 1 μg) of the alkaloids monocrotaline and heliotrine (Figure 1B and C). The average dose–response functions for receptor neurons stimulated with the volatile components of the two alkaloids are shown in Figure 2 for females (Figure 2A) and males (Figure 2B). The average response threshold for these alkaloids was ~1 μg for females and ~10 μg for males. The volatile stimuli from cartridges containing monocrotaline were more effective than those from cartridges containing heliotrine. For example, in females, cartridges containing 1 mg monocrotaline elicited an average of 28.2 $\pm$ 2.2 impulses/sec whereas a cartridge containing 1 mg of heliotrine elicited an average of 12.8 $\pm$ 2.1 impulses/sec. These data also indicate that male receptor neurons are slightly less responsive to these stimuli (e.g., 1 mg of monocrotaline = 21.0 $\pm$ 3.2, and 1 mg of heliotrine = 6.3 $\pm$ 1.7 impulses/

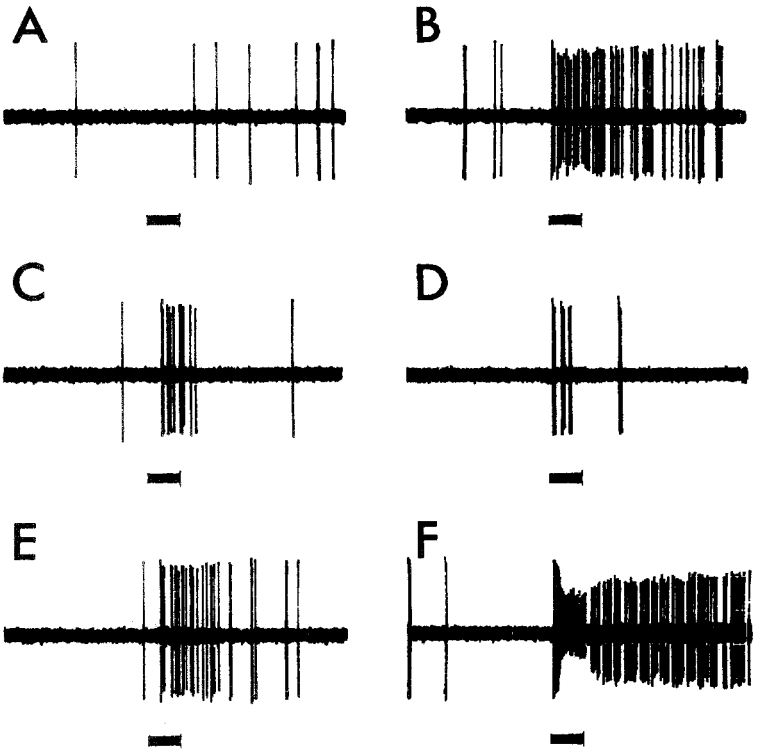


FIG. 1. Extracellular recordings from an antennal basiconic receptor neuron of a female *Utetheisa ornatrix* raised on alkaloid free diet. Each trace represents ~20 sec of electrical activity. The responses illustrated were elicited by odor cartridges containing: a solvent control (A); 1 mg monocrotaline (B); 1 mg heliotrine (C); 10 ng (*R*)-(−)-hydroxydanaidal (D); 1 μg (*S*)-(+)-hydroxydanaidal (E); and 1 μg (*R*)-(−)-hydroxydanaidal (F). The stimulus markers under each trace indicated the timing and duration of the 2-sec stimulus pulse.

sec) than are those of females. This sexual dimorphism extends to other substances including (*R*)-(−)-HD (100 ng = 15.8 ± 2.2 in males vs. 28.6 ± 3.5 impulses/sec in females) and (*S*)-(+)-HD (10 μg = 18.1 ± 2.1 in males vs. 30.2 ± 2.7 impulses/sec in females). The average responses of female receptor neurons to large amounts (1 mg) of monocrotaline-*N*-oxide, a precursor of HD, were slight (8.2 ± 3.4 impulses/sec), as was the average response (7.5 ± 2.5 impulses/sec) elicited by stimulation with a cartridge containing 1 μg of danaidone, a male pheromone of another species that is chemically similar to HD (Boppré, 1986).

The dietary background of adults did not reliably alter the responses of

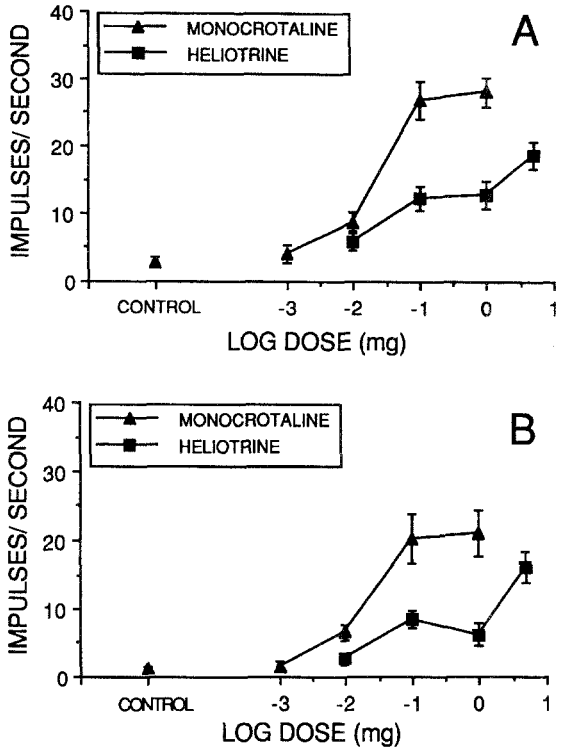


FIG. 2. Mean dose–response functions (±SEM) of antennal basiconic receptor neurons of female (A) and male (B) *Utetheisa* to stimulation with the volatiles from cartridges containing the indicated amounts of monocrotaline or heliotrine.

basiconic receptor neurons to the volatiles from PAs. Figure 3 contrasts the average dose–response function elicited by cartridges containing various doses of monocrotaline in adult females whose larvae had fed on a PA-free diet with those obtained from females whose larvae had fed on a diet containing PAs. For example, stimulation with a cartridge containing 10 μg monocrotaline elicited an average of 7.2 $\pm$ 2.4 impulses/sec in animals raised on CS diet vs. 10.8 $\pm$ 1.2 impulses/sec in those raised on PB diet. Similar data were also observed in response to stimulation with the volatiles from cartridges containing heliotrine (10 μg heliotrine = 5.1 $\pm$ 1.9 with CS diet vs. 6.9 $\pm$ 1.3 impulses/sec with PB diet). Over the range of doses and substances evaluated, systematic differences were not observed in the responses of animals, raised on diets with or without sequestered PAs, to stimulation with cartridges containing alkaloids. Similar results had been obtained earlier during an evaluation of female basiconic receptor neurons' sensitivity to stimulation with HD (Grant et al., 1989).

The differential sensitivity of basiconic receptor neurons to PAs illustrated in Figure 2 suggests that a comparable difference in sensitivity should be observed to the two isomers of HD that are the likely by-products of the hydrolysis of the alkaloids. Figure 4 details the average dose–response functions of female receptor neurons to stimulation with cartridges containing various doses of (*R*)-(−)-HD and (*S*)-(+)-HD. As expected from earlier studies, the average response threshold for (*R*)-(−)-HD was ~1 ng (3.3 ± 0.8 impulses/sec). The compa-

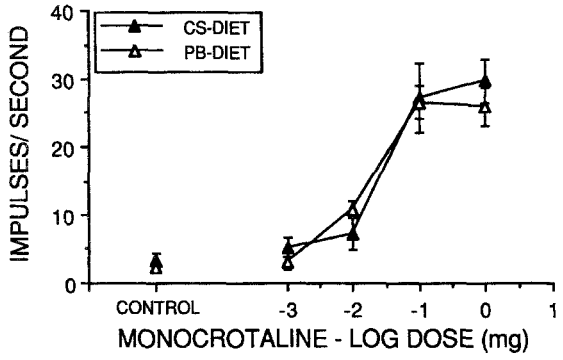


FIG. 3. Mean dose–response functions (±SEM) of antennal basiconic receptor neurons of female *Utetheisa* to stimulation with the volatiles from cartridges containing the indicated amounts of monocrotaline. The responses of adults that had been raised on larval diets containing alkaloid (CS-diet) are contrasted with those raised on alkaloid-free diet (PB-diet).

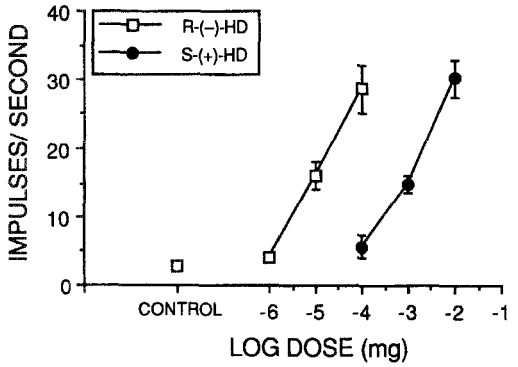


FIG. 4. Mean dose–response functions (±SEM) of antennal basiconic receptor neurons of female *Utetheisa* to stimulation with cartridges containing the indicated amounts of (*R*)-(−)-HD and (*S*)-(+)-HD.

rable threshold for (*S*)-(+)-HD was about two orders of magnitude higher (100 ng = 7.0 ± 2.4 impulses/sec).

DISCUSSION

The olfactory receptor neurons of basiconic sensilla on the antennae of female *Utetheisa ornatrix*, which had earlier been shown to be responsive to the male-produced pheromone HD (Grant et al., 1989), are also responsive to the volatiles emanating from cartridges dosed with PAs. The most parsimonious explanation for this observation involves the earlier demonstration (Bogner and Boppré, 1989) that HD is a volatile trace component in samples of PAs. Alkaloids are thought to contain, as a natural breakdown product, trace amounts of HD which, in turn, functions as the volatile signal for the presence of PAs. The (*R*)-(−)-enantiomer of HD is thought to be the active volatile component in samples of monocrotaline and the (*S*)-(+)-enantiomer is thought to result from the hydrolysis of samples of heliotrine with the appropriate configuration at the C-7 position. Thus, it was suggested that those receptor neurons, which appeared to be responsive to pyrrolizidine alkaloids in *Rhodogastria* were, in fact, responsive to the trace amounts of HD found in the PA samples, even though HD is not a natural component of the male pheromone system in this animal (Bogner and Boppré, 1989; Boppré, 1990).

This hypothesis was approached here with quantitative electrophysiological techniques using an animal (*Utetheisa ornatrix*) that has a male pheromone system involving male production and female sensitivity to HD and that engages in a vigorous competition for sources of PAs. The findings detailed in Figures 2A and 4 reveal that there are rather large differences in the absolute amounts of each material required to elicit a specified level of activity in female receptor neurons. For example, an average response of 15 impulses/sec would be expected from stimulation with cartridges containing approximately 10 ng of (*R*)-(−)-HD; 1 μg of (*S*)-(+)-HD; 20 μg of monocrotaline; and 2 mg of heliotrine. Thus, there is roughly a 2000-fold difference in the sensitivity of these receptor neurons to stimulation with each of the parent alkaloids when compared to the responses elicited by their hydrolytic by-products. There is also roughly a 100-fold difference in the sensitivity of these receptor neurons to stimulation with either the two isomers of HD (Figure 4) or with the two alkaloids (Figure 2A). These observations are all consistent with the hypothesis that these receptor neurons are differentially responsive to the two isomers of HD and that the two PAs each contain ~0.05% of the expected isomer of HD. A comparison of the semilogarithmic plots of the average dose–response functions observed in female receptor neurons shows comparable slopes to these materials and thus lends support to the notion that the PA samples contain isomers of HD. The slopes

for the two isomers of HD (Figure 4) were; (*R*)-(−)-HD = 12.2 impulses/sec/log dose (mg), and (*S*)-(+)-HD = 12.2 impulses/sec/log dose (mg). The slope of the function for monocrotaline (Figure 2A) was comparable [11.3 impulses/sec/log dose (mg)] to the slopes for the two HD isomers, consistent with the suggestion that the active material released from the alkaloid samples is HD. In contrast, the slope of the heliotrine dose–response function was considerably less [4.6 impulses/sec/log dose (mg)]. This latter fact may suggest that heliotrine naturally contains other substances that are less effective stimuli for these receptor neurons. It is also possible that the rate of release of active material from the heliotrine cartridge was reduced by one or another of the materials contained in the sample. This latter explanation may also account for the observation that the dose–response functions for the alkaloids (Figure 2A) are sigmoidal, with smaller increases in activity at elevated doses. In this case, it may be that the surface area available in the cartridge for stimulus release may be exhausted at alkaloid loads above ~1 mg.

An equally likely variant of these arguments, which will account for the observed pattern of sensitivity, involves the fact that danaidone, monocrotaline-*N*-oxide, and all four of the compounds detailed in Scheme 1 share nearly identical amino-alcohol moieties. This leaves open the possibility that all of the responses observed in basiconic receptor neurons actually reflect the differential avidity of a common odor binding site for the several variants of this common functional group (Eisner, personal communication). Resolution of these various alternatives awaits the development of biophysical and biochemical techniques with sufficient sensitivity and resolution to provide a direct evaluation of the odor binding events initiated by these stimuli at the dendritic membrane of basiconic receptor neurons.

The alkaloid content of the larval diet does not influence the sensitivity of the adult receptor neurons in females to stimulation with HD (Grant et al., 1989) or with the volatiles from cartridges containing PAs (Figure 3). A similar conclusion can be reached in evaluating the responses, observed in males raised on either diet, to stimulation with cartridges containing PAs (data not shown). This lack of a dependence on alkaloid in the diet ensures that both male and female adults have basiconic receptor neurons whose capabilities provide the ability to detect the volatile signals associated with PAs. HD thus, may fulfill a dual function in *Utetheisa*. On the one hand, it is an actively produced male pheromone and, on the other, a volatile signal that may act as a kairomone indicating the presence and location of desirable secondary plant substances. The differential sensitivity of basiconic receptor neurons to these materials (~2000-fold) also provides an amplification mechanism that allows receptor neurons responsive to the volatiles from nanogram amounts of HD to provide information on sources containing milligram amounts of relatively nonvolatile PAs. Pheromone-sensitive receptor neurons are normally housed in trichoid sensilla (Cuperus,

1985), whereas receptor neurons sensitive to other odors are normally housed in basiconic sensilla (Kaissling, 1971). Therefore, a receptor neuron responsive to a male pheromone (HD) in female basiconic sensilla is unusual at first glance (Grant et al., 1989). However, this location may be explicable in an evolutionary context, since the same stimulatory substance functions as a potential kairomone to mark the presence and location of PAs, which, in turn, are important substances in the natural history of the animal. In females, HD has a dual importance both as a volatile marker for PAs in host plants and as a sexual signal for selecting males with large amounts of PAs. Even though adult male *Utetheisa* do not appear to imbibe PAs (Eisner and Meinwald, 1987) or to engage in lek-building (c.f. Boppré, 1986), males could gain a competitive advantage with the ability to detect the volatile signals associated with locales containing PAs, simply because of the greater likelihood of encountering females.

The general utility of PAs and the importance of the volatile signals associated with them may suggest a common link in the evolution of the pheromone production and perceptual systems in this animal. For example, HD may have initially signaled the presence of PAs in plants and males could have begun to synthesize and emit this material from the alkaloid they sequestered, allowing those females with sensitivity to HD to determine both the location of food plants containing PAs and the location and relative fitness of males seeking mates. Moreover, the relative differences observed in the sensitivity of male and female receptor neurons suggests that there are differences in the selective pressures brought to bear on the sexes. In general, this evolutionary supposition is supported by the observations that: (1) male *Utetheisa* receptor neurons are responsive to their own pheromone; (2) these receptor neurons are found in basiconic sensilla instead of in trichoid sensilla where "typical" pheromone receptor neurons are normally expected; and (3) the receptor neurons may function as a potential kairomone detector with a reasonable degree of stereospecificity and an absolute sensitivity for only a selected subset of the available volatile by-products of PAs.

If HD actually functions as a kairomone for *Utetheisa ornatrix*, one may wonder how the disparate messages conveyed by this substance are encoded. Apparently HD could stem from male androconical brushes or any plant containing large amounts of PAs (Boppré, 1990). In other species these two "messages" are unambiguously signaled by two different chemicals and are received by two different receptor neuron types (e.g., *Danainae*, Bogner and Boppré, 1989). It is unlikely, in any species, that the complex behaviors triggered by mating partners and food sources are dependent on a single set or modality of sensory inputs, although baiting experiments of others (Krasnoff and Dussourd, 1989) with HD-containing coremata suggest that there are certain similarities in both courtship and host-plant selection behaviors in other species. However, we have no real evidence or explanation for a segregation of these behaviors in

nature. At this time we cannot fully explain why *Utetheisa* should have come to rely on only one sensory receptor neuron for all of these important odor-guided behaviors. We do see these observations as a promising step in the exploration of the mechanisms responsible for the evolution of the receptor neuron specificities currently seen in *Arctiidae*.

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IDENTIFICATION OF MALE CABBAGE LOOPER SEX PHEROMONE ATTRACTIVE TO FEMALES

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Abstract—A multicomponent pheromone produced by male cabbage looper moths that is attractive to female moths in a flight tunnel bioassay was isolated and identified. Based on analyses of hairpencil extracts of male cabbage loopers and volatiles emitted by males, the pheromone has been identified as a blend consisting of (*S*)-(+)-linalool, *p*-cresol, and *m*-cresol. The chirality of the major component, (*S*)-(+)-linalool, is important for behavioral response of females. These pheromonal compounds were also identified as volatiles released by males when males were exposed to the principal pheromone component of female cabbage loopers, (*Z*)-7-dodecen-1-ol acetate. The amount of male pheromone released was increased significantly when males were exposed to a combination of (*Z*)-7-dodecen-1-ol acetate and the odor from cabbage. Neither linalool nor the cresols were detected in volatiles from cabbage or from males exposed to cabbage odor.

Key Words—Male-produced pheromone, cabbage looper, *Trichoplusia ni*, Lepidoptera, Noctuidae, female attractant, linalool, *p*-cresol, *m*-cresol, enantiomers.

INTRODUCTION

Considerable research has been focused on female-produced sex pheromones of lepidopteran insects. The number of female sex attractants reported in the literature is in excess of 1700 (Mayer and McLaughlin, 1990). Male-produced sex attractants are less common in insects (Thornhill and Alcock, 1983) and unusual in Lepidoptera. In a review of male pheromones in Lepidoptera, Birch

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and Hefetz (1987) listed six species in which female attraction to male pheromones is known. There are now four reports of female flight attraction responses to males: the arctiids *Estigmene acrea* (Drury) (Willis and Birch, 1982) and possibly *Creatonotos transiens* (Walker) and *C. ganges* (L.) (Wunderer et al., 1986) and the cabbage looper *Trichoplusia ni* (Hübner) (Landolt and Heath, 1990). Female behavior often associated with the release of male-produced pheromones includes mate acceptance and occurs only at close range (Birch 1974).

Male cabbage loopers produce volatile compounds that may influence female behavior in attraction and courtship interactions. An electrophysiological response of female cabbage loopers to material extracted from male hairpencils was reported by Grant (1970). In subsequent investigations Grant (1971) and Grant and Brady (1973) speculated on the possible function of chemicals associated with hairpencils and brushes of the male cabbage looper. Jacobson et al. (1976) reported the identification of 2-phenylethanol in male cabbage looper hairpencils and a wing flutter response by females to hairpencil extracts and to 2-phenylethanol. These findings were contradicted by Hagan and Brady (1981), who were unable to find 2-phenylethanol in male cabbage loopers. Gothilf and Shorey (1976), however, were unable to demonstrate a male scent brush role in mating success of this species.

Based on reports of female catches in traps baited with female sex pheromone (Mitchell et al., 1972; Creighton et al., 1973; Birch, 1977), Birch (1977) suggested that females may have responded to chemicals released by males at the trap rather than to the female pheromone used as the trap lure. Recently, Landolt and Heath (1989, 1990) demonstrated attraction of unmated females to live males, extracts of males, and extracts of male hairpencils in flight-tunnel experiments.

We report here the isolation and identification of a multicomponent male cabbage looper pheromone attractive to females in a flight-tunnel bioassay. The release of these identified pheromone components by males exposed to female sex pheromone also was demonstrated, and the release rate and ratio of pheromone components from stimulated males were determined.

MATERIALS AND METHODS

Insects and Bioassays. All moths used were reared as larvae on artificial diet according to the procedures reported by Guy et al. (1985). Pupae were sorted by sex and placed in 22 × 22 × 22-cm aluminum and fiberglass screen cages. Cages of pupae and emerged moths were held in environmental chambers on a 14:10 light–dark cycle, at 25 ± 1°C and 60 ± 10% relative humidity. Pupae were transferred daily to new cages, providing emerged moths of discrete

age groups. Moths were held with cups of a sugar–honey solution on cotton as food.

Bioassays were conducted during the fifth to seventh hour of the 10-hr scotophase in a Plexiglas flight tunnel constructed as a 1 × 1 × 2-m box, with air drawn through by a fan at 0.22 m/sec. Lures were hung from a wire loop near the center of the upwind end of the tunnel and moths were released singly from a 20-ml polystyrene vial with a screen bottom and open top hung horizontally from a clamp on a ring stand near the center of the downwind end of the flight tunnel. Observations were aided by overhead red lights at <1 footcandles. Virgin female moths 4–6 days old were placed in the flight tunnel chamber 1–3 hr before bioassays to allow adjustment to the conditions in the chamber. All bioassays consisted of a 2-min observation period following the release of a single moth. Test material was applied to 5.5-cm-diam. filter papers and was air dried in the tunnel for 60 sec before testing. Generally, five moths were tested sequentially per test sample. Responses scored included flight, anemotactic (upwind) flight, plume tracking (zig-zagging upwind flight within the apparent odor plume), and contact. Five females were also released sequentially to solvent treated filter paper at the upwind end and observed for 2 min each, as controls.

Hairpencil Extracts and Collection of Volatiles. Extracts of male hairpencils were prepared by removing the terminal large paired abdominal hairpencils with forceps and placing them in a vial of hexane. Hairpencils from 50 males were accumulated in 2 ml of hexane. The hexane extract was pipetted to a clean vial after 20 min and concentrated when necessary under a stream of N_2.

A modified push–pull system (Landolt and Heath, 1987) was used to collect volatiles emitted by 3- to 6-day-old males. A schematic of the system is shown

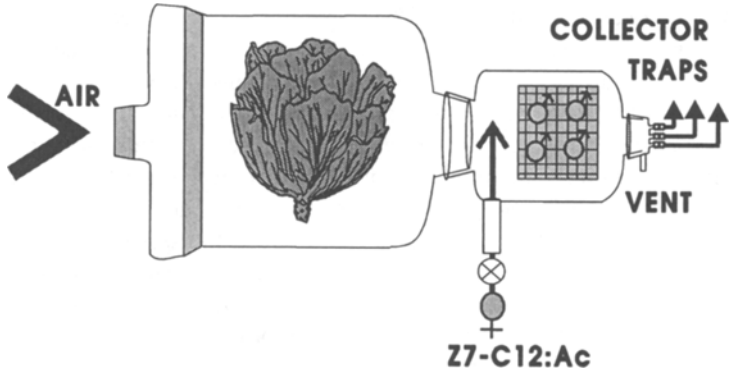

FIG. 1. Schematic of system used to collect volatiles from male cabbage loopers, suspected male stimulants, and combination of males and stimulants.

in Figure 1. Briefly, compressed air, purified by passing through two charcoal filters, was separated into two airstreams. One of these streams was saturated with water by passage through a water-filled gas dispersion cylinder. The two airstreams were recombined to provide an adjusted relative humidity of 60–65%. This purified and humidified air was introduced into a glass chamber(s) that contained a frit near the upwind end of the chamber and provided a laminar airflow. Volatiles were collected on one of three collector traps positioned at the downwind end of the chambers, any one of which could be selected for volatile collection. Vent flow was monitored with a meter from a vent in the chamber, and the vent flow was maintained at 2% of the airflow through the filter. Airflow was maintained at 1 liter/min with a slight positive pressure (+0.2 cm H_2O) established in the glass chamber(s). Pressure drop in the chamber(s) due to the collector trap was reduced to near zero by the use of vacuum downwind of the collector filter. This system was used to collect volatiles from males exposed to airflow passed over a cabbage plant, from males exposed to airflow containing (*Z*)-7 dodecen-1-ol acetate (Z7–12 : Ac), from males exposed to airflow passed over a cabbage plant and Z7–12 : Ac, from airflow over cabbage plants, and from airflow containing Z7–12 : Ac released from a rubber septum loaded with 10 μg of the acetate.

Collections were made using a 10-cm-OD × 30-cm-long glass chamber, which held the cabbage, or a 5-cm-OD × 30-cm-long chamber, which contained five moths. The smaller chamber contained a port that permitted the introduction of Z7–12 : Ac upwind of the males, via ground glass joints, and the chamber that housed the cabbage could be coupled in series with the chamber containing the insects.

Volatiles were collected on traps using Super-Q as the adsorbent. Super-Q traps were prepared by packing ca. 20 mg of the adsorbent in 4-cm-long × 4.0-mm-ID glass tubes resulting in a bed length of 5 mm. Two stainless-steel frits were used to contain the adsorbent. The Super-Q traps were cleaned by Soxhlet extraction with methylene chloride for 24 hr prior to use. Volatiles collected on the traps were eluted with 200 μl of methylene chloride and then 50 ng of tridecan-1-ol acetate was added as internal standard for subsequent analyses.

Isolation and Analysis of Pheromone. Capillary gas chromatography was done using a retention gap column prior to the capillary column. This system permitted the injection of samples without concentration in 5–100 μl of solvent. A combination of three fused silica columns connected in series using Glassseal connectors (Supelco Inc., Bellefonte, Pennsylvania) were used. The primary deactivated fused silica column, 8 cm × 0.5 mm ID, was connected between the injector and the retention gap column. This primary column permitted the use of 0.4-mm-OD stainless-steel needles with a septum injector. Selection of retention gap columns for use with the analytical column was determined by the

phase ratio of both columns (Grob, 1982; Murphy, 1989). For the purpose of this investigation the retention gap columns were 10-m × 0.25-mm-ID silane deactivated fused silica (Quadrex, New Haven, Connecticut). Analytical columns used for analyses were a 50-m × 0.25-mm-ID Supelcowax 10 (bonded Carbowax), a 50-m × 0.25-mm-ID bonded BP-1 and a 25-m × 0.25-mm Chirasil-val (Chrompak, Raritan, New Jersey). A 15-m × 0.53-mm-ID, 1.0-μm film Carbowax fused silica column (Quadrex) was used for micropreparative purification of hairpencil extracts, extracts of coriander seeds, and candidate synthetics.

Gas chromatographic analyses were conducted with a Hewlett-Packard model 5890 gas chromatograph, equipped with cool-on column capillary injector (septum injector) and flame ionization detector. Helium was used as the carrier gas at a linear flow of 18 cm/sec. The temperature program was isothermal at 60°C for 2 min, and then programmed at 20°/min to 160°C. The chromatographic data were stored and analyzed in a Nelson 4000 data system.

Initial micropreparative purification of hairpencil extracts on the 0.53-mm-ID capillary column resulted in the collection of three large fractions. Fraction 1 included the collection of compounds having an equivalent chain length unit (ECLU) (Swaboda, 1962) of heptan-1-ol acetate (ECLU = 700) to compounds eluting before nonan-1-ol acetate (ECLU = 900) (material collected from 6 to 14 min during GC run). Fraction 2 included compounds having an ECLU of 900–1400 (material collected from 18.2 to 20 min during GC run), and fraction 3 included compounds within the range of 1400–1800 (material collected from 20.2 to 30 min during GC run). Effluent from the column was split, with 2% of the effluent routed to the FID detector and 98% of the effluent collected in a cooled, 30-cm glass capillary tube. The capillary was contained in a temperature gradient collector similar to that described by Brownlee and Silverstein (1968) and liquid nitrogen was used to cool the capillary. Collected fractions were washed from the capillary using 200 μl of 1 : 1 hexane–ether. Fractions that were used in identifications were analyzed on the analytical columns described to ensure that the peak contained only one component. This same analytical method was used to further purify fractions of natural extracts, synthetics, and linalool from coriander seeds.

Identification of Male Pheromone. Mass spectra of active natural compounds and candidate compounds were obtained using the BP-1 column, operated as described above, coupled to a Nermag model R1010 mass spectrometer in either electron impact (EI-MS) or chemical ionization (CI-MS) mode. The reagent gas used for CI was either methane or isobutane. Vapor-phase infrared spectra were obtained with a Nicolet 20SXC GC-FTIR spectrometer. Samples were introduced to the FTIR spectrometer via a BP-1 column connected to the Hewlett-Packard model 5890 gas chromatograph equipped with cool-on column

capillary injector. Nuclear magnetic resonance spectra (^1H) were obtained with a Nicolet NT-300 (300 MHz) Fourier transform spectrometer using either $CDCl_3$ or C_6D_6 as the solvent and tetramethyl silane as an internal reference.

Reagents and Derivatization. The chirality of linalool (3,7-dimethyl-1,6-octadien-3-ol) was determined by formation of the diastereomeric 1-phenylethyl-urethanes with 1-phenylethyl-isocyanate (PEI) (Fluka Chemical Corp., Ronkonkoma, New York) diastereomers according to the method of Gaydou and Randriamiharisda (1987). The (*R*)-(+)- and (*S*)-(−)-PEI were used without purification. Approximately 200 ng of linalool isolated from male hairpencils, coriander, or synthetic racemic or (*R*)-(−)-linalool was dissolved in 20 μl of methylene chloride, to which approximately 1 μg of the (*R*)-(+)-PEI was added. After heating at 100°C for 1 hr, the sample was concentrated with dry N_2. The sample was redissolved in HPLC grade methanol (J.T. Baker) containing 100 ng of *n*-tetradecanol as the internal standard for GC analysis.

Racemic (Aldrich Chemical Corp., Milwaukee, Wisconsin) and (*R*)-(−)-linalool (K&K Laboratories, Cleveland, Ohio) were purified using preparative GC. (*S*)-(+)-linalool was obtained from coriander seeds (Harris Seeds, Rochester, New York). Seeds were crushed and then soaked in GC-grade methylene chloride over night. The concentrated residue was filtered and purified by preparative GC. Ortho-, meta-, and paracresol were purchased from Aldrich Chemicals and purified by preparative GC prior to use. Linalool from coriander seed and synthetic compounds used for bioassays were >98% purity based on GC analyses.

Synthetic pheromone components and blends were tested, using flight tunnel bioassays, for attractiveness to unmated female cabbage loopers and were compared to hairpencil extracts and solvent blanks. All materials tested were applied to 5.5-cm-diam. filter paper at dosages of 5 male equivalents (ca. 122.5 ng of linalool, 25.5 ng of *p*-cresol, and 3.5 ng of *m*-cresol) in hexane. A comparison (at amounts equal to 5 male equivalents) was conducted of attraction to: (1) racemic linalool, *p*-cresol, and *m*-cresol; (2) (*R*)-(−)-linalool, *p*-cresol, and *m*-cresol; (3) (*S*)-(+)-linalool, *p*-cresol, and *m*-cresol; and (4) hairpencil extracts from five males. Fifty females were tested per treatment, as 10 sets of five. Using a randomized complete block design, a complete treatment comparison of five females tested per treatment, covering all five treatments, was conducted per day for 10 days to provide the data set. In a second test, the following treatments were similarly compared: hexane (200 μl); *p*-cresol and *m*-cresol at 25.5 and 3.5 ng per aliquot; (*R*)-(−)-linalool at 122.5 ng per aliquot; the complete identified blend of (*R*)-(−)-linalool, *p*-cresol, and *m*-cresol at 122.5, 25.5, and 3.5 ng, respectively; and hairpencil extracts from five males.

Odors from cabbage plants, *Brassica oleraceae* (L), and Z7–12 : Ac loaded at 10 μg on rubber septa were tested as stimulants to elicit pheromone release from male cabbage loopers. Cabbage plants were grown in a greenhouse and

were 8–12 weeks old, 20–25 cm wide and 15–20 cm tall when used, with no head development. The Z7–12:Ac was purchased from Aldrich Chemicals and purified (>98% purity) prior to use by $AgNO_3$ high-performance liquid chromatography (Heath and Sonnet, 1980). Volatiles from males without stimuli, males exposed to cabbage odor, males exposed to Z7–12:Ac released from a rubber septum, and a combination of Z7–12:Ac and cabbage odor were collected and analyzed. Additionally, volatiles from the septum containing Z7–12:Ac and cabbage were analyzed.

Data on female bioassay response to fractions, isolates, synthetic, and linalool from coriander were subjected to ANOVA after arcsin transformation, with significant differences between treatment response means determined by Student's *t* test (Steel and Torrie, 1960) or Duncan's new multiple-range test (Duncan, 1955).

RESULTS

Response of female cabbage loopers in the flight tunnel to fraction 1 (material collected from 6 to 14 min during GC run), fraction 2 (material collected from 18.2 to 20 min during GC run), fraction 3 (material collected from 20.2 to 30 min during GC run), and combined fractions collected from the GC of male hairpencil extracts indicated that no loss in activity had occurred during chromatography (Table 1). Further testing of combinations of fractions indicated

TABLE 1. PERCENTAGES OF VIRGIN FEMALE CABBAGE LOOPERS RESPONDING IN FLIGHT TUNNEL BIOASSAYS TO GC FRACTIONS OR HAIRPENCIL EXTRACTS (A, $N = 45$) OR COMBINATIONS OF GC FRACTIONS (B, $N = 50$)[a]

	Gas chromatographic fractions[b]				
Behavior	1	2	3	Combined	Extract
A					
Attraction	0a	0a	13b	44d	31c
Contact	0a	0a	9b	31c	16b
	1 + 2	1 + 3	2 + 3	3	1 + 2 + 3
B					
Attraction	2a	26c	6a	12b	24c
Contact	0a	20c	4ab	6b	18c

[a] Amounts bioassayed were equal to that found in extracts of five males.
[b] Numbers in rows followed by the same letter are not significantly different by Duncan's (1955) new multiple-range test at $P \leq 0.05$.

that all activity was accounted for by fractions 1 and 3 (Table 1), containing three peaks eluting with equivalent chain lengths (Swaboda, 1962) of 838.50, 1434.77, and 1439.61 (designated A, B, and C in Figure 2). Chemical ionization mass spectra of peak A, in which methane was used as the reagent gas, established that the molecular weight of this compound was 154 with diagnostic ions at *m/e* 153 (M − 1) and 155 (M + 1). Diagnostic ions at *m/e* 135 (M + 1 − 18) suggested the loss of water from the parent molecule. Electron impact did not provide an ion indicative of the molecular weight of the compound. Based on this information, peak A was proposed to be a 10-carbon alcohol containing two degrees of unsaturation. Further analysis by infrared spectroscopy indicated an alcohol stretch at 3636 cm^{-1}, and a terminal methylene characterized by $C{=}CH_2$ stretch at 3090 cm^{-1}. Proton NMR spectra with decoupling experiments on ca. 4 μg of material provided the information for the identification of the compound as linalool based on the following spectral data: [^{1}H]NMR (C_6D_6) δ1.28 (s, 3 H), 1.51–1.65 (m, 3H), 1.68 (s, 3H), 1.96–2.11 (m, 2H), 5.07 (dd, 1H J = 1.9, 11.2 Hz), 5.08–5.17 (m, 1H), 5.21 (dd, 1H, J = 1.9, 16.9 Hz),

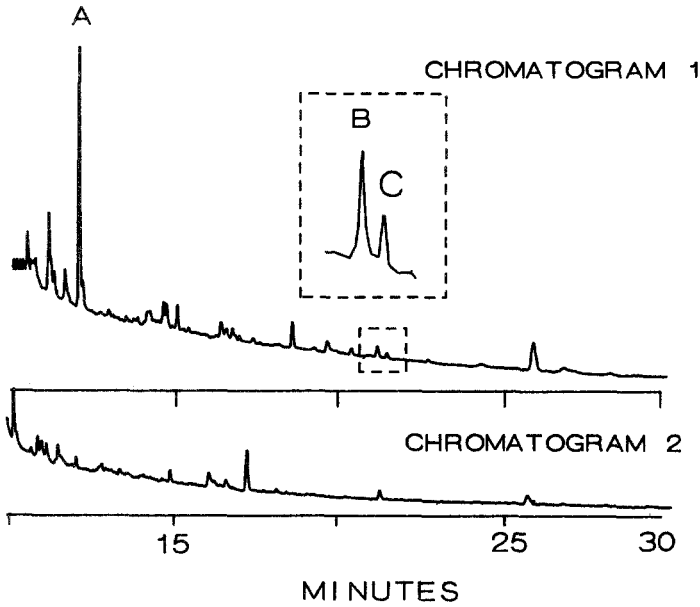

FIG. 2. Chromatograms of volatiles collected from males in the presence of Z7-12:Ac and cabbage odor (chromatogram 1) and volatiles from males in the presence of cabbage (chromatogram 2). Peak identification is A = linalool, B = *p*-cresol, and C = *m*-cresol. Gas chromatograph analysis was done on a 50-m × 0.25-mm-ID Supelcowax 10 column.

5.92 (dd, 1H, J = 11.2, 16.9 Hz). These data are in agreement with that reported for linalool by Ohwa et al. (1986).

Preliminary experiments using (*R*)-(+)- and (*S*)-(−)-1-phenylethyl isocyanate as the derivitizing agent indicated that the (*R*)-(+)-urethane derivative was enantiomerically more pure (97%) and provided greater separation factor then the (*S*)-(−) derivative. Thus, the chirality of isolated linalool was determined by formation of the (*R*)-(+)-1-phenylethyl urethane diastereomer of the natural linalool and comparison of its retention time with the linalool diastereomers of known configuration. The products were chromatographed on a Chiralsil-val column and coeluted with the diastereomer formed with the linalool obtained from coriander seeds, previously reported as (*S*)-(+)-linalool (Cornforth et al., 1962; Ohloff and Klein, 1962). The elution time of the diastereomer formed with the linalool from male hairpencils was 14.2 min compared with 14.2 min for the (*S*,*R*) diastereomer and 16.3 min for the (*R*,*R*) diastereomer.

Electron impact mass spectra of the smaller active peaks (B and C in Figure 2) found in the hairpencil extracts gave identical spectra. The molecular weight of these compounds were 108 with diagnostic ions at *m/e* 108 (M^+) and 107 ($M^+ - 1$). Diagnostic ions at *m/e* 90 ($M^+ - 18$) suggested the loss of water from the parent molecule. A computer search of the National Institute of Standards and Technology mass spectral library resulted in a significant match with the spectra of cresol. Confirmation of the identity of these two compounds was obtained by comparison of vapor-phase IR spectra of the natural product with synthetic material and GC retention time comparisons. Based on these analyses, compounds B and C were identified as para- and meta-cresol, respectively.

The average amount of (*S*)-(+)-linalool, *p*-cresol, and *m*-cresol found in male hairpencils was 24.5, 5.1, and 0.7 ng for each compound, respectively (Table 2). Wind-tunnel bioassays of the synthetic cresols and (*S*)-(+)-linalool obtained from coriander seeds resulted in comparable female response when compared to the hairpencil extracts (Table 3). Synthetic blends prepared using (*R*)-(−)-linalool and racemic linalool in combination with *p*- and *m*-cresols were significantly less attractive. In a comparison of a blend of the three components—(*S*)-(+)-linalool, *p*-cresol, and *m*-cresol—and hairpencil extracts, (*S*)-(+)-linalool alone and *p*- and *m*-cresol were significantly less attractive than the complete blend or extracts.

Having identified pheromonal components from male hairpencils that elicited positive behavioral responses of females, we investigated the emission of these compounds from males. Results of the GC analysis of the volatiles collected are shown in Table 2. The identified male-produced pheromonal components were found only in volatiles collected from males exposed to Z7-12:Ac. In a previous study (Landolt and Heath, 1990) we showed that males exposed to cabbage odor were much more attractive to females than were males alone or cabbage alone. Similar results were obtained with males exposed to

TABLE 2. AMOUNT IN OF LINALOOL, *p*-CRESOL, AND *m*-CRESOL IN 15-MIN COLLECTIONS FROM MALE CABBAGE LOOPERS OR POTENTIAL MALE STIMULANTS[a]

	Source		
	(*S*)-(+)-Linalool (ng/male)	*p*-Cresol (ng/male)	*m*-Cresol (ng/male)
Male hairpencil extracts (N = 12)	24.5 ± 5.1	5.1 ± 2.4	0.7 ± 0.2
Males (N = 5)	0.0a	0.0	0.0
Males + Z7-12:Ac (N = 5)	5.0 ± 2.3b	0.3 ± 0.2	0.1 ± 0.1
Males + cabbage (N = 5)	0.0a	0.0	0.0
Males + cabbage + Z7-12:Ac (N = 5)	9.0 ± 4.3c	0.6 + 0.6	0.2 ± 0.1
Cabbage (N = 5)	0.0a	0.0	0.0

[a]Mean amounts of linalool collected followed by the same letter are not significantly different by Duncan's (1955) new multiple-range test at $P \leq 0.05$. Values are ±SD.

TABLE 3. PERCENTAGES[a] OF VIRGIN FEMALE CABBAGE LOOPERS RESPONDING IN FLIGHT TUNNEL BIOASSAYS TO NATURALLY DERIVED OR SYNTHETIC MALE PHEROMONE CHEMICALS, BLENDS, AND HAIRPENCIL EXTRACTS (N = 50 FOR PART 1, 35 FOR PART 2)

Behavior	Racemic linalool + cresols	(*R*)-(−)-Linalool + cresols	(*S*)-(+)-Linalool + cresols	2× racemic + cresols	Hairpencil extract
Part 1					
Plume tracking	34b	20a	52c	24a	54c
Contact	16a	10a	42b	16a	44b
	p and *m* cresols	Hexane	(*S*)-(+)-Linalool	Synthetic blend	Hairpencil extract
Part 2					
Plume tracking	14b	3a	23b	60c	54c
Contact	0a	0a	9b	37c	34c

[a]Numbers in rows followed by the same letter are not significantly different by Duncan's (1955) new multiple-range test at $P \leq 0.05$.

Z7-12:Ac, which were much more attractive to females than were unexposed males. We were unable to detect the presence of linalool or cresols in volatiles from males, cabbage, or males placed in the odor of cabbage (Table 2 and Figure 2). There was, however, an increase in the amount of pheromonal components released by males exposed to both cabbage and Z7-12:Ac compared

to males exposed to Z7-12:Ac alone (Table 2). It was also noted that less pheromonal material was obtained from volatiles collected from these stimulated males than was found in analyzed hairpencil extracts. This difference in amount is due to the experimental design. It is important to note however, that the active materials isolated from male hairpencils were emitted from stimulated males.

DISCUSSION

Our results demonstrate that unmated female cabbage looper moths are attracted to a blend of three components identified in the terminal genital hairpencils of conspecific males. Linalool, the major component of the pheromone blend, is ubiquitous in plants and is often the major component of essential oils (Masada, 1976). It occurs in both the dextrorotatory [(*S*)-(+)-linalool or coriandrol] and levorotatory [(*R*)-(−)-linalool or licerol] forms. Additionally, linalool has been reported as a chemical component involved in the attraction of many other insects. For example, it is reported as a component of the attractant of the carrot fly larva, *Psila rosae* (Ryan and Guerin, 1982), a component of a female attractant of the spined soldier bug, *Podisus maculiventris* (Aldrich et al., 1984), and a component of a synthetic blend that attracts eastern yellowjackets, *Vespula maculifrons* (Aldrich et al., 1986). The effect of the chirality of linalool on the response of the carrot fly larvae, spined soldier bug, and eastern yellowjackets has not been reported. The chirality of linalool is of significance to the behavioral response of female cabbage loopers to the male pheromone.

In addition to linalool, two cresols were found as components in the pheromonal blend attractive to female cabbage loopers. *p*-Cresol has been reported as a component of female pheromone of two hard ticks, *Rhipicephalus appendicalatus* and *R. pulchellus* (Wood et al., 1975) and as a component of an oviposition attractant of the tree-hole mosquito, *Aedes triseriatus* (Bentley et al., 1981). Bioassays conducted with the cresols were based on the ratio found either in hairpencil extracts or as collected volatiles. Studies are currently being conducted to determine the importance of the ratio of these components to the response of females to blends containing altered ratios.

We have also demonstrated that, in addition to the identification of (*S*)-(+)-linalool, *p*-cresol, and *m*-cresol in male hairpencils, these compounds are released by males when males are exposed to Z7-12:Ac, and the amount of pheromone released was increased when males were exposed to Z7-12:Ac and the odor from cabbage. Neither linalool nor the cresols were evident in volatiles from cabbage or unstimulated males in the presence of cabbage odor. This suggests that either these compounds are not responsible for previously reported female attraction to cabbage and increased attraction of females to males on cabbage or that they occur in amounts not detectable with the analytical methods employed in these experiments.

Although male pheromone in the cabbage looper may function as an aphrodisiac or have some other courtship role, under the conditions of our studies the hairpencil blend of (*S*)-(+)-linalool and *p*- and *m*-cresols attracts unmated females. Field studies will be required to fully determine the potential use of these compounds as a female attractant. Captures of female cabbage loopers in traps baited with *Z*7–12 : Ac reported by Mitchell et al. (1972), Creighton et al. (1973), and Birch (1977) may have been due to female attraction to (*S*)-(+)-linalool, *p*-cresol, and *m*-cresol in male hairpencils displayed at or in the traps.

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ELEVATION IN TONGUE-FLICK RATE AFTER BITING PREY IN THE BROAD-HEADED SKINK, *Eumeces laticeps*

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Abstract—A postbite elevation in tongue-flicking (PETF) rate occurs in adult male broad-headed skinks, *Eumeces laticeps*. Males having bitten neonatal mice showed significantly higher tongue-flicking rates in the 2 min following experimental removal of the prey than did males in several control conditions. In a second experiment designed to separate the effects of tactile and chemical stimulation of the oral cavity during biting, males tongue-flicked at significantly higher rates in response to swabs bearing surface chemicals from neonatal mice than to identical swabs lacking the surface chemicals. These findings agree with previous data showing that PETF and searching movements occur in those families of lizards that can detect prey chemicals and use the tongue to do so during active foraging. The occurrence of PETF and putative searching movements supports the interpretation that PETF represents an attempt to relocate lost prey by chemosensory means. PETF was much briefer in *E. laticeps* than in many snakes and in a representative species from another lizard family and was not detectable after the first minute. This brevity is consistent with the prediction that PETF should be brief in squamates that feed on prey not likely to be located by scent-trailing.

Key Words—Prey chemicals, tongue-flicking, feeding behavior, Sauria, Scincidae, *Eumeces laticeps*.

INTRODUCTION

Reliance on chemical cues sampled by tongue-flicking has been established in the broad-headed skink, *Eumeces laticeps*, for various ecologically important

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functions. Pheromonal communication is highly developed in this species, allowing discrimination of conspecific lizards from heterospecific congeners (Cooper and Vitt, 1986a,b; 1987a,b; Cooper and Garstka, 1987), recognition of sex (Cooper and Vitt, 1984a,b, 1987c, 1989) and reproductive condition (Cooper et al., 1986), and location of reproductively active females by scent-trailing males (Cooper and Vitt, 1986c). Other important functions of tongue-flicking are detection and selective response to ophidian predators (Cooper and Vitt, 1990) and responses to prey.

Broad-headed skinks may rely heavily on the chemical senses in searching for cryptic prey (Vitt and Cooper, 1986). They are capable of detecting and responding selectively to integumentary prey chemicals (Cooper and Vitt, 1989). In comparison with responses to control chemicals, adult male *E. laticeps* that are presented with prey chemicals tongue-flick at higher rates. They are also more likely to bite cotton swabs on which the chemicals are presented than control swabs (Cooper and Vitt, 1989, 1990).

In this paper I report further investigation of chemosensory behavior related to feeding in *E. laticeps*, focusing on tongue-flicking behavior after prey has been bitten and then removed from the lizard's mouth by the experimenter. Following similar removal of bitten prey from the mouth, the tongue-flicking rate increased for variable durations in several species of nonvenomous squamate reptiles (e.g., Cooper 1989a, 1991a,b, 1992a,b; Cooper et al., 1989).

The increase is the poststrike elevation in tongue-flicking (PETF) (Cooper, 1991a, 1992b). In the species studied, PETF is accompanied by locomotion interpreted as searching movements (Cooper, 1989b, 1991a,b, 1992a,b; Cooper et al., 1989). In venomous snakes the increased tongue-flicking rate and searching movements are called strike-induced chemosensory searching (SICS), which normally occurs following voluntary release of envenomated prey (Chiszar and Scudder, 1980; O'Connell et al., 1985).

Release usually occurs during predatory attacks on large, potentially dangerous prey (Radcliffe et al., 1980). Upon releasing the prey, a venomous snake may remain motionless for a brief interval and then begin tongue-flicking at a high rate that may not return to the prestrike baseline level for an extended period, sometimes more than 2 hr if the prey has not been relocated (Chiszar and Scudder, 1980; Chiszar et al., 1982). While tongue-flicking, the snake locates and follows the chemical trail to the prey after the prey has been rendered harmless (Chiszar and Scudder, 1980).

Lacking venom to subdue their prey, nonvenomous snakes and lizards do not voluntarily release prey and thus do not exhibit the strike–release–trail strategy of many venomous snakes (Chiszar and Scudder, 1980; Chiszar et al., 1985). PETF in lizards combined with apparent searching movements appears to be SICS without voluntary release or scent-trailing, which has not been possible in the experimental paradigm used to detect PETF.

The hypothesis that SICS in lizards and nonvenomous snakes is an attempt to use chemical cues to relocate lost prey predicts that PETF should occur in a wide variety of nonvenomous squamates that use the tongue–vomeronasal system during foraging. This applies to several families of active foragers, including Scincidae, that are capable of prey chemical discrimination based on lingually sampled stimuli (e.g., Cooper, 1990a, 1992b). Thus, I predicted that PETF and searching movements would occur in *E. laticeps* following forcible removal of prey. An additional prediction was that chemical cues induce greater PETF than can be attributed to oral tactile stimulation from the prey while the lizard bites. Finally, because broad-headed skinks feed primarily on cryptic invertebrate prey that is likely to be discovered and consumed on the spot rather than scent-trailed, it was predicted that the duration of PETF would be brief.

METHODS AND MATERIALS

Subjects and Maintenance. Sixteen adult male *E. laticeps* were captured on Kiawah Island and Seabrook Island, Charleston County, South Carolina, in May 1987. They were housed at the Savannah River Ecology Laboratory (SREL) in a building having translucent walls and were exposed to the natural light cycle of the region. A retractable roof was opened on warm, sunny days to allow natural insolation. During the study, the maximum daytime temperature was maintained in the low thirties Celsius by heat lamps. Because tongue-flicking rates in lizards vary with temperature (Cooper and Vitt, 1986d; Van Damme et al., 1990), it is necessary to control temperature in experiments using numbers of tongue-flicks as a response variable. In *E. laticeps* the tongue-flicking rate in response to pheromones is maximal near 30°C. After being maintained for over a year before the experiments began, the skinks were habituated to laboratory conditions and to the close proximity of a human being. Each lizard was housed individually in a 49 × 49 × 32-cm glass terrarium containing a water bowl, a wooden shelter board, a sand substrate, and a removable wire screen top. The skinks were fed crickets to satiation three times per week and neonatal mice several times prior to the experiments. Water was continuously available.

The skinks were transported to Auburn University at Montgomery (AUM) in early September 1988, where they were housed for several more months prior to further testing. Lizards were housed and fed as at SREL, with the exception that cage dimensions were 50 × 31 × 27 cm. At AUM, indirect natural light was supplemented by fluorescent lighting on a 14:10 light–dark cycle. Air temperatures varied from 22 to 31°C. Even at low air temperatures, undercage heaters allowed the lizards to thermoregulate.

Poststrike Elevation in Tongue-Flick Rate. The effect of biting prey on tongue-flicking rate was assessed by counting tongue-flicks emitted in two con-

secutive minutes in four experimental conditions. In the strike condition a neonatal mouse held in forceps was positioned just above the substrate 10 cm anterior to a lizard's snout. When the lizard bit the mouse, the experimenter removed the mouse from the lizard's mouth as gently as possible and began to count tongue-flicks immediately. The pull condition was similar until the lizard approached, tongue-flicked, and prepared to bite the mouse. At that time the experimenter withdrew the mouse with one hand, simultaneously moved the lizard a short distance with the other hand to simulate removal of prey in the strike condition, and began counting tongue-flicks upon releasing the lizard. In the sight condition, the experimenter held a mouse in forceps at the far side of the cage in clear view of the lizard. The mouse was removed after a maximum of 10 sec if the lizard did not approach or, to prevent the lizard from tongue-flicking the mouse, as soon as the lizard approached. The forceps condition was identical to the sight condition except that the forceps were empty. In the sight and forceps conditions, counts of tongue-flicks began simultaneously with removal of the experimental stimuli.

The strike condition is necessary to observe the effects of biting on tongue-flicking because *E. laticeps* does not release prey such as mice voluntarily prior to swallowing. However, because the experimental removal of prey from a lizard's mouth requires contact between the lizard and the experimenter's hand, aspects of the lizard's behavior other than tongue-flicking may be affected. The pull condition is a control for effects of handling in the strike condition. The crucial difference between the strike and pull conditions is that chemical and possibly other cues associated with biting are limited to the strike condition. The effect of seeing the prey on tongue-flicking rate is assessed by the sight condition. The forceps condition reveals tongue-flicking rates expected in the experimental milieu. The experimental procedures and interpretation of results are discussed in greater detail in Cooper et al. (1989) and Cooper (1989a).

Each lizard was tested in all four experimental conditions in a randomized blocks (repeated measures) design. The sequence of presentation of experimental conditions was randomized. Each lizard was tested once per day. Hunger was equalized over trials by testing two days after feeding to satiation. Thirteen lizards were tested from 1200 to 1400 hr EST in late August 1988. Air temperatures 1 cm above the substrate were 31.1 $\pm$ 0.8 SD in the strike condition, 31.0 $\pm$ 0.8 in the pull condition, 31.2 $\pm$ 0.9 in the sight condition, and 31.3 $\pm$ 1.3 in the forceps condition.

For the entire 2 min, significances of differences in numbers of tongue-flicks emitted among conditions were tested by nonparametric Friedman two-way analysis of variance (Siegel, 1956). Parametric analysis of variance could not be used because variances were unequal among conditions and the distributions of tongue-flicks were nonnormal in the forceps and mouse conditions due to frequent zero values. After determination that the main condition effect

was significant, individual comparisons between pairs of conditions were made using procedures described in Hollander and Wolfe (1973). Additional sign, Wilcoxon (Siegel, 1956), and paired t tests (Winer, 1962) were conducted to examine the difference between the strike and pull conditions. Significance tests were conducted with $\alpha = 0.05$ and were two-tailed for main effects and one-tailed for directional individual comparisons unless otherwise stated.

Control for Tactile Oral Cues. In a study of garter snakes, an additional control for the effects of tactile stimuli during biting was used to show that the chemical cues received during biting produced PETF (Cooper et al., 1989). To test the possibility that PETF in broad-headed skinks is a response to tactile cues received during biting rather than to chemical stimuli, a second experiment was conducted at SREL on 1 September 1988. To begin a trial, the experimenter grasped a skink, inserted a cotton-tipped applicator in its mouth (most individuals readily bit it), removed the applicator, returned the skink to its home cage, and counted its tongue-flicks for 2 min. The two experimental conditions consisted of presenting applicators (1) dipped in deionized water and (2) dipped in deionized water and then rubbed on the skin of a neonatal mouse to impart prey chemicals.

Sixteen lizards were tested in both conditions in counterbalanced sequence with an intertrial interval of approximately 1 hr. Air temperatures were 31.1 $\pm$ 1.0°C SD in the mouse condition and 31.2 $\pm$ 1.1°C SD in the water condition. Prior to analysis of the experimental effects, the tenability of the assumption of homogeneity of variance was examined by a Hartley's F_{max} test. Significance of the difference between condition means was assessed by a t test for correlated samples.

Duration of PETF. A preliminary examination of the duration of PETF was made by recording tongue flicks performed by seven skinks for five consecutive minutes in the strike and pull conditions. This study was conducted at AUM on 22–23 March 1989 at 27.4 and 27.0°C room temperature, respectively (only one measurement was taken each day). Significance of the difference between conditions was determined by Wilcoxon signed-ranks matched pairs tests for each minute. The criterion for presence of PETF in a particular minute was a significantly greater number of tongue-flicks in the strike condition than in the pull condition. One-tailed tests with $\alpha = 0.05$ were used because the prediction was directional.

A different approach to determining the duration of effects of biting prey on tongue-flicking was conducted at AUM on 20 February and 3 March 1989 at an air temperature of 29.5 $\pm$ 0.2°C SD. Numbers of tongue-flicks were recorded each minute for six individuals for a 5-min baseline interval. At the end of the baseline period, the lizard was allowed to bite a neonatal mouse, which was removed from the lizard's mouth as in the strike condition. Following removal of the mouse, tongue-flicks were recorded for 10 min or until the lizard

failed to emit any tongue-flicks for five consecutive minutes, whichever came first. The significance of elevation in tongue-flicking rate for each minute after biting was assessed by a binomial test of the numbers of individuals emitting more tongue-flicks during the experimental interval than the mean number of tongue-flicks per minute in the baseline period.

RESULTS

PETF. Substantial numbers of tongue-flicks occurred only in the strike and pull conditions, only two individuals responding in each of the other conditions. By far the highest mean tongue-flicking rates occurred in the strike condition (Table 1). The main condition effect was highly significant ($\chi_r^2 = 28.59$, $df = 3$, $P < 0.001$). Individual comparisons revealed no difference between the sight and forceps conditions ($P > 0.10$). Significant differences were found between the forceps and pull conditions ($P < 0.001$), the sight and pull conditions ($P < 0.01$), the forceps and strike conditions ($P < 0.001$), and the sight and strike conditions ($P < 0.001$).

Using the extremely conservative procedure (Hollander and Wolfe, 1973) for individual comparisons, the large difference between the strike and pull conditions is not significant ($P < 0.09$). However, because this comparison was planned as the single critical one for establishment of PETF, because the response rates in these two conditions were over an order of magnitude greater than in the forceps and sight conditions, and because the distributions in the sight and forceps conditions differed drastically from those in the strike and pull conditions, additional statistical tests of the significance of the mean difference between the strike and pull groups were conducted. Results of sign and Wilcoxon signed-ranks matched-pairs tests both revealed significance ($P = 0.01$ and $T = 10.5$, $P < 0.01$, respectively, one-tailed). Variances in the strike and pull conditions were homogeneous ($F_{max} = 2.63$; $df = 2,12$; $P > 0.05$), allowing a t test for correlated samples, which corroborated the findings of the two previous tests ($t = 2.52$, $df = 12$, $P < 0.025$).

The highest numbers of tongue-flicks were emitted by individuals that

TABLE 1. MEANS, STANDARD ERRORS (SE) AND RANGES OF NUMBERS OF TONGUE-FLICKS BY *Eumeces laticeps* IN 2 MIN IN FOUR EXPERIMENTAL CONDITIONS

Statistic	Strike	Pull	Sight	Forceps
Mean	52.2	22.8	1.8	0.8
SE	10.2	6.3	6.1	0.4
Range	8–108	1–55	0–22	0–9

moved about the cages or attempted to burrow in the sand. The individuals that had the three highest tongue-flicking rates in the pull condition burrowed persistently and were clearly not foraging, whereas the locomotion of lizards in the strike condition was consistent with searching for food. No quantitative data are available, but skinks burrowed less in the strike condition than in the pull condition and no burrowing occurred in the sight and forceps conditions. Individuals that did not tongue-flick at all in the sight and forceps conditions tended to remain motionless.

Tactile Oral Cues. Skinks tongue-flicked at fairly high rates in both conditions and exhibited consistently higher rates in trials with applicators bearing integumentary chemicals from mice than with applicators immersed only in water. Lizards performed 52.6 $\pm$ 1.2 SE (range 19–97) tongue-flicks in the mouse condition and 34.8 $\pm$ 1.0 SE in the water condition. Variances were homogeneous ($F_{max} = 1.81$; $df = 2, 15$; $P > 0.05$) and significantly more tongue-flicks were emitted in the mouse condition than in the water condition ($t = 2.55$, $df = 15$, $P < 0.02$, one-tailed).

Duration of PETF. Tongue-flick rates were much higher in the strike condition than in the pull condition during the first minute. Thereafter, the mean tongue-flicking rate was only slightly higher in the strike condition than in the pull condition until the fifth minute, when the rates were nearly identical (Figure 1). The only significant difference occurred in the first minute (Wilcoxon $T = 0.016$, one-tailed); for the remaining minutes, $T = 11$, 8, 11, and 7, respectively ($P > 0.10$).

During each of the first 3 min after biting a mouse, the tongue-flicking rate of all six lizards was elevated above baseline (binomial $P = 0.016$, one-tailed for each minute). Thereafter, tongue-flicking was above the mean baseline rate for four of six individuals in minute 4, three in minute 5, one in minute 6 and two in minute 7 ($P > 0.10$ each). No lizard tongue-flicked in minutes 8–10.

DISCUSSION

PETF, SICS, and Their Duration. The significantly greater tongue-flicking rate in the strike condition than in the pull, sight, and forceps conditions demonstrates the existence of PETF in *E. laticeps*. The similarity of tongue-flicking rates in the sight and forceps conditions suggests that seeing the mouse briefly did not induce an increase in tongue-flicking rate above the baseline rate for the experimental setting. On the other hand, the opportunity to approach the prey (sometimes while tongue-flicking) combined with handling of the lizard by the experimenter induced a substantial increase in tongue-flicking rate, as indicated by the significantly higher tongue-flicking rate in the pull condition than in the sight and forceps conditions. As in water snakes of the genus *Nerodia*, handling

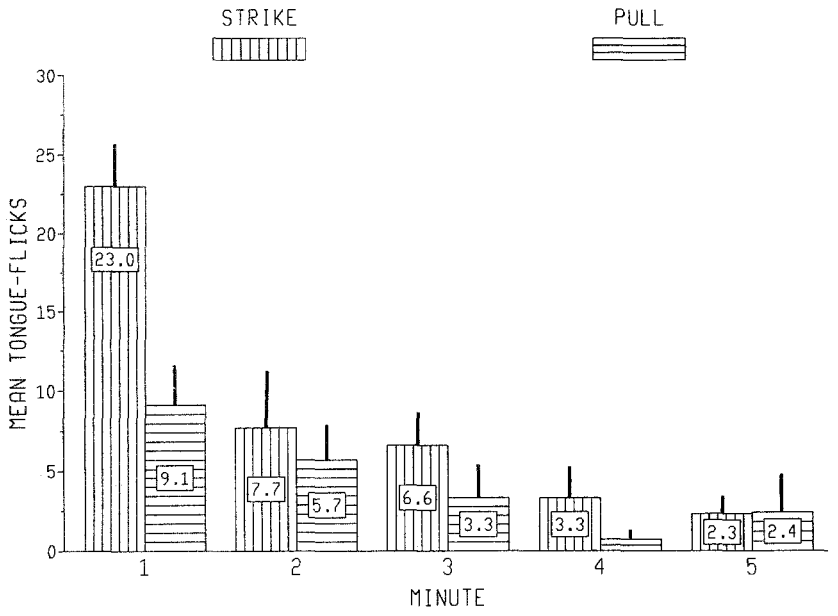

FIG. 1. Vertical bars represent the mean number of tongue-flicks emitted by adult male *Eumeces laticeps* in five successive minutes in two experimental conditions. Vertical lines above the bars represent 1 SE.

alone is followed by an increase in the tongue-flicking rate (Scudder and Burghardt, 1983).

The excess in tongue-flicking rate in the strike condition over that in the pull condition presumably represents a specific response to cues associated with biting, which could be tactile or chemical. Further evidence demonstrating the effectiveness of oral chemical stimuli is that the tongue-flick rates of lizards stimulated on the oral epithelia by moistened cotton swabs bearing prey chemicals were greater than when the swabs bore water alone. A similar effect has been reported in garter snakes (Cooper et al., 1989).

Tactile cues associated with biting prey appear to be less effective than chemical cues in eliciting elevated tongue-flicking, but they may stimulate some increase. Tongue-flicking rates were nearly identical in the strike condition and following oral stimulation by swabs bearing prey chemicals, suggesting that the combined effects of tactile and chemical stimulation to the mouth were the same in the two experiments. That the rate of tongue-flicking was somewhat higher among lizards picked up by the experimenter and stimulated by swabs bearing water only than in the pull condition suggests (1) that being held and having a swab inserted in the mouth may be more stressful than treatment in the pull

condition or (2) that biting the prey may stimulate an increase in tongue-flicking due to oral tactile stimuli independent of chemical stimuli. However, this apparent difference might or might not be significant in a planned experimental test.

The greater effectiveness of chemosensory cues than tactile cues in eliciting PETF raises the question of the degree of increase in tongue-flicking rate that can be induced by tongue-flicking prey in the absence of mechanical stimuli occurring during biting and by tongue-flicking prey chemicals on a substrate. There was some opportunity to tongue-flick prey in the pull condition, which may account at least in part for the greater tongue-flicking rate in that condition than in the sight and forceps conditions. Because movement and tongue-flicking are correlated in lizards (Burghardt et al., 1986), chemical stimulation alone might induce both PETF and searching movement, i.e., SICS. Nevertheless, the variability of relationships between locomotion and tongue-flicks directed to air and substrates in different experimental settings (Burghardt et al., 1986) emphasizes the necessity for careful experimentation. From this perspective, another possibility ignored heretofore is that approach during the pull condition increases the likelihood of subsequent movement and therefore of tongue-flicking. This could explain at least some of the differences between the pull condition and the other control conditions.

SICS appears to be present in *E. laticeps* because, in addition to their demonstrated PETF, males in the strike condition moved more frequently than they did in the other conditions. This interpretation is strengthened by the qualitative differences in movements in the strike and pull conditions, the movements in the strike condition alone resembling searching movements.

The duration of PETF as determined by comparisons of tongue-flicking rates in the strike and pull conditions is only 1 min. Such comparisons are very important for determination of the duration of PETF demonstrated by the experimental methods used here because the pull condition itself induces an increase above baseline rates. When the tongue-flicking rates in the strike condition are compared with baseline rates for undisturbed lizards, the apparent duration may be much longer. In this study, the duration of increased tongue-flicking was 3 min for the comparison between baseline and poststrike rates, with an indication that it might extend a few minutes longer if a larger sample size were tested.

Because the experimental method used to determine the duration of PETF in this and my previous related studies (Cooper, 1991a,b, 1992a,b) is based on comparison of tongue-flicking rates in the strike conditions with those in the pull condition, the durations reported are not directly comparable with those reported by Chiszar and his colleagues in a long series of papers on venomous snakes (e.g., see references above and Radcliffe et al., 1986). Indeed, although these studies have established beyond doubt the importance of chemical cues in the SICS and scent-trailing of prey by venomous snakes that release prey voluntarily after envenomation, it would be of interest to know how great a portion

of the duration of SICS relative to undisturbed baseline conditions in venomous species is attributable to tactile cues imparted while striking and releasing prey. A recent study shows that SICS occurs after striking in rattlesnakes and water moccasins having ligated and severed venom ducts; this finding eliminates stimuli arising from injection of venom as necessary antecedents of PETF (Chiszar et al., 1991). However, potentially important roles of other mechanical stimulations during the process of strike and release by venomous snakes remain to be investigated.

Comparative Data for Nonvenomous Squamates. PETF and apparent SICS have been documented in three nonvenomous species of snakes representing two families: Colubridae (Cooper et al., 1989) and Pythonidae (Cooper, 1992a); and in four species from four families of lizards: Cordylidae (Cooper, 1992c), Lacertidae (Cooper, 1991), Scincidae (this study), and Varanidae (Cooper, 1989a, 1992b). All of these squamates are also capable of detecting prey chemicals and discriminating them from control chemicals (Burghardt, 1970, 1973; papers just cited and references therein). Nonvenomous snakes use the tongue to locate prey while foraging (Burghardt, 1970). Among the lizards, all are known to be active foragers, except the cordylid, for which the foraging mode is uncertain. All available data are consistent with the hypothesis that PETF (and SICS) in lizards and nonvenomous snakes constitute use of the tongue in the attempt to relocate by chemosensory means prey that has escaped recently.

Little is known regarding the importance of foraging mode in lizards to the existence and duration of PETF and SICS, but it may be predicted that PETF and SICS are reduced or do not occur in lizards incapable of discriminating prey chemicals from control substances and in those that do not use the tongue to detect chemical prey cues during foraging. So far as is known, families of active foragers do so use the tongue and discriminate prey from nonprey chemicals (e.g., Evans, 1961; Nicoletto, 1985; Cooper, 1990a). On the contrary, insectivorous lizards that are strict ambush or sit-and-wait foragers do not discriminate chemical prey cues or tongue-flick frequently while foraging (Curio and Mobius, 1978; Cooper, 1989b, 1990b). It thus may be predicted that PETF and SICS are reduced or absent in insectivorous agamids, polychrids, and phrynosomatids.

The duration of PETF varies considerably among the few lizards studied. It is quite brief in three species of fairly small, primarily insectivorous lizards, the lacertid *Podarcis muralis* (1 min; Cooper, 1991a), the scincid *E. laticeps* (1 min; this paper), and the cordylid *Gerrhosaurus nigrolineatus* (2 min; Cooper, 1992b), despite differences in the openness of foraging habitats and methods of locating food. The scincid, for example, forages more often under forest litter for hidden prey (Vitt and Cooper, 1986) in less open habitats than the other two species. The duration of PETF is much greater in *Varanus exanthematicus*, at least 25 min (Cooper, 1992a). Although *V. exanthematicus* preys primarily on invertebrates, the occasional large vertebrate prey item may be energetically

important. Highly mobile vertebrates such as small mammals seem much more likely than insects to be located by scent-trailing. Furthermore, several other varanids, most notably *V. komodoensis*, regularly consume vertebrates and appear to locate them by scent-trailing (Auffenberg, 1981). Thus, the relatively long interval of PETF in *V. exanthematicus* might reflect a current selective regime, a historical one in which the ancestral varanids specialized in vertebrates, or both. Because snakes are believed to have evolved from varanid lizards or their predecessors (McDowell, 1972; Schwenk, 1989), the long-lasting PETF of the varanid strengthens the hypothesis that the highly developed SICS of snakes is an elaboration of the ancestral condition in lizards (Cooper et al., 1989).

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EFFECT OF DIMBOA, AN APHID RESISTANCE FACTOR IN WHEAT, ON THE APHID PREDATOR *Eriopis connexa* GERMAR (COLEOPTERA: COCCINELLIDAE)

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Abstract—DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), a secondary metabolite found in cereal extracts, confers resistance in wheat to aphids. Its effect on beneficial organisms was tested on larvae of the aphid predator *Eriopis connexa* Germar. Larvae were fed until pupation on artificial diets to which different concentrations of DIMBOA (2–200 μg/g diet) were added, as well as on aphids that had been feeding on wheat seedlings with different DIMBOA levels (140–440 μg/g fresh tissue). In diets, the effect of DIMBOA was greatest on survival of third-instar larvae and on the duration of the second and fourth instars. When aphids were provided as food, those that had fed on a wheat cultivar with an intermediate DIMBOA level led to a significantly longer larval duration in the predator than did those that fed on either low or high DIMBOA cultivars. Shortest predator development times were obtained with aphid prey that had fed on high DIMBOA seedlings. Higher DIMBOA levels in the plant appear to reduce aphid feeding rates (and rates of DIMBOA ingestion), decreasing aphid survival and minimizing the effect of the toxin on the predator.

Key Words—Wheat, hydroxamic acids, DIMBOA, plant resistance, cereal aphids, Homoptera, Aphididae, aphid predators, *Eriopis connexa*, Coleoptera, Coccinellidae, tritrophic interactions.

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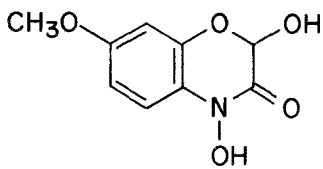
INTRODUCTION

The most prevalent cereal aphids in Chile are *Metopolophium dirhodum* (Walk.), *Sitobion avenae* (Fab.), *Schizaphis graminum* (Rond.), and *Rhopalosiphum padi* (L.) (Ramírez, 1990). Although an array of natural enemies, which includes predators, parasitoids, and pathogens limit their populations to a great extent (Zúñiga, 1987), aphids constitute a potential problem due to their ability to transmit virus diseases, such as the barley yellow dwarf virus (BYDV) (Herrera and Quiroz, 1988). *Eriopis connexa* Germar (Coccinellidae) is one of the most important aphid predators in Chile because of its high populations in spring and summer, wide range of prey, and predatory efficiency (Macfarlane, 1985).

DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), the main hydroxamic acid (Hx) present in wheat extracts (Niemeyer, 1988a), has been associated with resistance of the plant to aphids through antibiosis (Argandoña et al., 1980; Bohidar et al., 1986; Thackray et al., 1990; Wratten et al., 1991) and feeding deterrency (Argandoña et al., 1983; Niemeyer et al., 1989b; Givovich and Niemeyer, 1991).

Biological control and host-plant resistance are often compatible and even complementary (Emden and Wratten, 1991), as has been found to be the case of resistance in varieties of barley and sorghum that complemented the activity of the parasitoid *Lysiphlebus testaceipes* Cresson (Starks et al., 1972). However, variations in host plants may have dramatic effects on biological control agents. Thus, prey confined to resistant host plants may represent a low-quality food for predators and parasitoids, affecting their biology (Bergman and Tingey, 1979). Secondary metabolites in the plants may be the cause of detrimental effects on organisms at the third trophic level, as is the case of α-tomatine, an alkaloid in *Solanum* and *Lycopersicon* species, that is toxic to the endoparasitoid *Hyposoter exiguae* (Viereck), which takes up the compound through its host *Heliothis zea* (Boddie) (Campbell and Duffey, 1979). Nicotine, an alkaloid from *Nicotiana* species confers plant resistance to *Manduca sexta* L. but has negative effects on the parasitoids *Hyposoter annulipes* (Cresson) and *Cotesia congregata* (Say) developing on *M. sexta* larvae reared in nicotine-containing diets (Thorpe and Barbosa, 1986; Barbosa et al., 1986). Plant secondary metabolites might



DIMBOA

SCHEME 1.

confer resistance in the herbivore towards the predator, as is the case of *Macrosiphon albifrons*, an aphid that is protected from attack by *Carabus problematicus* by quinolizidine alkaloids ingested from its lupin host plants (Wink and Römer, 1986).

An increase in Hx levels in wheat, proposed as a mechanism to enhance resistance towards aphids (Niemeyer, 1988b; Copaja et al., 1991a,b; Wratten et al., 1991), could affect beneficial organisms. Hence, the effect of DIMBOA on the development of *E. connexa* was examined.

METHODS AND MATERIALS

A mass rearing technique and an artificial diet for *Eriopis connexa* were developed; the direct effect of DIMBOA was studied by adding the compound in different concentrations to the diet; finally, larvae were fed aphids reared on wheat lines containing different DIMBOA levels.

Mass Rearing of E. connexa. Rearing was started with 25 pairs of coccinellids collected in fields near the laboratory. Beetles were fed on the aphid *R. padi* reared on oat seedlings in a room maintained at 21°C with a 10°C range, 50% relative humidity with a 10% range and a 16:8 light–dark photoregime.

All stages of the predator were maintained separately in plastic containers of 12 cm diameter and 7.5 cm height fitted with a cover with fine holes. Adults were confined at a density of five to eight pairs per container. Aphids on oat leaves were placed as food on a sheet of paper towel inside the container. Egg groups were collected daily and maintained separately in containers. Larvae were also fed with aphids from oats; these aphids were offered as soon as egg hatch occurred. The fourth instars were supplied with a sheet of paper towel for pupation. The mean number of aphids provided daily per larva from first to fourth instar were 10, 25, 20, and 15, respectively. Pupae were collected daily and were placed on a sheet of paper towel inside a container. Adults that had emerged within the same 24-hr period were sexed and maintained in pairs in the containers.

Artificial Diet for E. connexa. Diets were based on those developed for coccinellids using liver and meat extracts (Herrera, 1960; Atallah and Newson, 1966; Ferran and Laforge, 1975) and were tested by rearing first-instar larvae in 5.5-cm-diam. Petri dishes, each larva receiving 0.5 g diet every two or four days. Table 1 shows the best formulation of those tested.

Pollen with polyvitamins and wheat germ were pulverized separately with a mortar and pestle. These constituents were mixed with sorbic acid, ascorbic acid, and vitamin E, and then bee honey was added. This mixture was blended for 30 sec with liver in 50 ml water in a domestic blender. Agar was dissolved in 50 ml water at 80°C and cooled down before being mixed into the previous

TABLE 1. COMPOSITION OF ARTIFICIAL DIET FOR *E. connexa*

Component	Quantity (g)
Cooked pig liver	68.1
Pollen	10.0
Honey	10.0
Wheat germ	5.0
Agar	4.0
Polyvitamins	1.0
Sorbic acid	1.0
Ascorbic acid	0.5
Vitamin E	0.4
Water	100.0

mixture for 1 min. The homogeneous dough of semisolid texture obtained was dispensed to sterilized containers and stored at 6°C.

The diet was tested with 10 first-instar larvae and was renewed every two days. For comparison, 14 first-instar larvae were reared individually with natural diet using *R. padi* nymphs ad libitum as food.

Effect of DIMBOA in Artificial Diets on Development of E. connexa. First-instar larvae of *E. connexa* were reared on artificial diet containing DIMBOA, isolated from ethereal extracts of *Zea mays* cv. T129s, as previously described (Queirolo et al., 1983). This compound was incorporated to the diet at the required concentration in 50 ml of water before adding the agar suspension. The latter was cooled down before use in order to minimize decomposition of DIMBOA. Analysis of the diet after four days at room temperature revealed that at least 85% of DIMBOA remained in it. Experiments were carried out in a completely randomized design with six treatments and two replicates. Treatments consisted of five DIMBOA concentrations in artificial diet plus a control without DIMBOA. Concentrations tested were 0, 2, 5, 10, 50, and 200 μg DIMBOA/g diet. Each replicate consisted of 10 recently hatched larvae of *E. connexa* maintained separately in 5.5-cm-diam. Petri dishes. The diet (0.5 g) was renewed every four days for the first and second instars and every two days for the third and fourth instars. The Petri dishes were sealed with Parafilm to keep larvae from escaping and diet from drying. Larval mortality, duration of each instar, pupal mortality, pupal duration, weight of pupae, and time to reach adulthood were evaluated.

Study of E. connexa *Larvae Fed on Aphids Reared on DIMBOA-Containing Wheat Lines.* Three wheat lines with different contents of the compound were chosen (Millaleu: 140 μg DIMBOA/g fresh tissue, Nobo: 270 μg, and Maiten: 440 μg). Oats were used as a control without DIMBOA. Aphids (*R. padi*) were

allowed to feed on the plants for 24 hr. They were placed within a clip cage located in the middle third of the leaf of a wheat seedling in the one-leaf stage, the stage at which DIMBOA was analyzed. One first-instar larva of *E. connexa* was placed inside each Petri dish with a piece of wet filter paper and allowed to develop to the adult stage. Aphids were removed from the plants and placed inside the Petri dish ad libitum for the larvae. For each wheat cultivar and the oat control there were 10 replicates. Survival of larvae, number of aphids eaten per larva, and the duration of each larval developmental stage were determined. The larvae employed were obtained from eggs laid by field-collected females that had been kept together with males for two weeks under the rearing conditions described above.

Plant Material and Hydroxamic Acid Analysis. Wheat seedlings used were in the one-leaf stage (G.S. 11) (Zadoks et al., 1974). Hydroxamic acids were analyzed by reverse-phase high-performance liquid chromatography, as previously described (Niemeyer et al., 1989a).

RESULTS

Mass Rearing of E. connexa. This technique was developed in order to obtain uniform individuals in high quantities for laboratory assays. Mating was frequent and occurred from early adulthood up to just before death. Females began oviposition on average 16.5 days after reaching adulthood, and the oviposition frequency was every three to four days. Eggs were laid in groups, usually on the surface of paper towel, each batch comprising about 25 eggs. Individuals older than three months laid a higher proportion of infertile than fertile eggs. Eggs were collected daily by cutting the paper around the batch; they hatched five days later.

Larvae were voracious, consuming on average 54, 39, 55, and 72 nymphs of *R. padi* in first, second, third and fourth instars, respectively. Development times for each stage are given in Table 2. The pupal stage lasted 5.4 days and the sex ratio was 1:1. These adults consumed 15–20 aphids per day. The life cycle was completed in 44 days.

Artificial Diet for E. connexa. The rearing of *E. connexa* under laboratory conditions is relatively easy and cheap with aphids as food. However, experiments in which the effect of a given compound is being investigated demand the use of an artificial diet for rearing. Table 2 shows comparative data on larval and pupal development of *E. connexa* on natural and artificial diets. Larvae were reared on artificial diets from first instar to adulthood. Molting was normal, with no mortality recorded. However, the larval developmental period was different from that observed with natural diet, and adults, although being as large as the controls, were whitish in color, possibly due to a lack of appropriate pigments in the diet.

Effect of DIMBOA in Artificial Diet on Development of E. connexa. Table 3 shows the effect of DIMBOA in an artificial diet on the development and survival of larvae of *E. connexa*. Survival was not significantly affected by DIMBOA, with the exception of that of the third-instar larva. In this case, progressively lower survival was obtained with diets with higher DIMBOA concentrations.

The durations of instars two and four were significantly increased by higher DIMBOA concentrations in the diet, albeit only at the highest concentration in the latter instar.

Table 4 shows the overall effect of DIMBOA on larval development and survival and also the effect on pupae and adults obtained from treated larvae. While an increase in time to reach adulthood seems apparent at the lowest

TABLE 2. COMPARATIVE DEVELOPMENT TIMES FOR LARVAE AND PUPAE OF *Eriopis connexa* IN NATURAL AND ARTIFICIAL DIETS

		Time period (days)								
		Larvae						Larva to adult	Survival (%)	
Diet	N^a	I	II	III	IV	Total	Pupae	Total	Larvae	Pupae
R. padi	14	2.9	2.2	4.3	7.6	17.0	5.4	22.4	100	100
Diet	10	5.4	4.7	5.4	9.0	24.5	5.6	30.1	100	100

[a]Number of individuals studied.

TABLE 3. EFFECT OF DIMBOA ON LARVAE OF *Eriopis connexa* REARED ON ARTIFICIAL DIET[a]

Treatment	Instar 1		Instar 2		Instar 3		Instar 4	
(μg DIMBOA/ g diet)	Duration (days)	Survival (%)	Duration (days)	Survival (%)	Duration (days)	Survival (%)	Duration (days)	Survival (%)
0	6.2 a	95.0 a	7.3 a	95.0 a	7.0 a	94.5 a	8.7 a	83.0 a
2	5.3 a	90.0 a	8.7 ab	89.0 a	9.3 a	81.3 ab	9.3 a	85.0 a
5	5.0 a	90.0 a	9.0 ab	100.0 a	8.4 a	60.0 b	8.8 a	100.0 a
10	5.2 a	80.0 a	9.1 ab	90.0 a	7.5 a	79.0 ab	8.8 a	100.0 a
50	6.3 a	100.0 a	10.4 bc	100.0 a	7.9 a	73.5 b	8.2 a	94.0 a
200	7.3 a	95.0 a	12.4 c	84.5 a	9.3 a	18.8 c	14.0 b	75.0 a

[a]The experiment was begun with 20 first-instar larvae. Means within a column followed by the same letter are not significantly different ($P = 0.05$; Duncan's multiple-range test).

TABLE 4. EFFECT OF DIMBOA ON LIFE CYCLE OF *Eriopis connexa* IN ARTIFICIAL DIETS[a]

Treatment (μg DIMBOA/g diet)	Larvae		Pupae			Adult	
	Duration (days)	Survival (%)	Duration (days)	Survival (%)	Weight (mg)	Time to reach adulthood (days)	Emergence (%)
0	29.2 a	70 a	4.4 a	100	7.0 a	33.6 a	100
2	32.4 a	55 a	4.8 a	100	6.1 a	37.3 a	100
5	31.0 a	55 a	4.5 a	100	6.5 a	35.5 a	100
10	30.6 a	55 a	4.3 a	100	6.1 a	34.8 a	100
50	32.8 a	60 a	5.3 a	100	6.7 a	38.0 a	100
200	43.0 b	10 b	4.5 a	100	6.1 a	47.5 b	100

[a]The experiment was begun with 20 first-instar larvae. Means within a column followed by the same letter are not significantly different ($P = 0.05$; Duncan's multiple-range test).

concentration of DIMBOA tested, the effect was statistically significant only for the highest concentration of DIMBOA. This concentration also decreased larval survival but had no effect on pupal survival nor on adult emergence.

Effect of Aphids Reared on DIMBOA-Containing Wheat Lines on E. connexa *Larvae.* The development time of *E. connexa* larvae differed significantly depending on the wheat cultivar on which their aphid prey had fed, the duration being longer in the case of the cultivar with an intermediate level of DIMBOA. Pupal developmental time was not affected by the level of DIMBOA in the wheat cultivar. Larval survival was lower with aphids that had fed on the wheat cultivar with the intermediate DIMBOA level (Table 5); aphid consumption was also lower in this cultivar (Table 6).

DISCUSSION

DIMBOA has been shown to be a feeding deterrent towards aphids. Thus, survival of aphids feeding on diets (Argandoña et al., 1983; Niemeyer et al., 1989b) or on wheat plants (Niemeyer et al., 1989b) differing in DIMBOA level showed a biphasic behavior: in the lower DIMBOA range survival was inversely correlated with DIMBOA concentrations while at the higher end survival was positively correlated with DIMBOA concentrations. Dual choice tests showed that aphids preferentially settled on leaves of wheat seedlings containing lower DIMBOA levels (Givovich and Niemeyer, 1991). Finally, electronic monitoring of aphid feeding behavior showed that fewer aphids reached the phloem, and those that did took longer to reach it in seedlings with higher DIMBOA levels

TABLE 5. DEVELOPMENT TIMES OF LARVAE AND PUPAE OF *E. connexa* REARED WITH APHIDS (*R. padi*) FED ON OATS AND WHEAT LINES CONTAINING DIFFERENT DIMBOA LEVELS[a]

		Development time (days)								
		Larvae						Larva to adult	Survival	
Plant	DIMBOA (μg/g fr. wt.)	I	II	III	IV	Total	Pupae	Total	Larvae	Pupae
Oat		2.1a	1.8a	2.1a	4.4a	10.4a	3.9a	14.3b	100	100
Millaleu	140	2.0a	2.0a	2.8b	3.6b	10.4a	4.0a	14.4b	98	100
Nobo	270	2.3b	1.8a	2.3a	4.3ab	10.7b	4.0a	14.7a	93	100
Maiten	440	2.5b	1.9a	2.3a	2.4c	9.1c	3.8a	13.9c	100	100

[a]The experiment was begun with 10 first-instar larvae. Means within a column followed by the same letter are not significantly different ($P = 0.05$; Duncan's multiple-range test).

TABLE 6. APHID CONSUMPTION BY *E. connexa* LARVAE OF APHIDS REARED ON OATS AND ON WHEAT LINES CONTAINING DIFFERENT LEVELS OF DIMBOA[a]

		Instar				
Plant	DIMBOA (μg/g fr. wt.)	1	2	3	4	Total
Oats		14.3ab	39.0a	27.6a	34.3a	115.2a
Millaleu	140	14.5a	38.8a	26.0b	33.7a	113.0b
Nobo	270	14.4ab	37.8b	26.3b	31.6c	110.1c
Maiten	440	14.0b	38.2b	26.2b	32.7c	111.5d

[a]The experiment was begun with 10 first-instar larvae. Means within a column followed by the same letter are not significantly different ($P = 0.05$; Duncan's multiple-range test).

than in those with lower ones (Givovich and Niemeyer, 1991). Hence, it appears that below a given DIMBOA level, the aphid ingests the compound, while above this level DIMBOA deters the aphid from feeding, leading to higher short-term survival rates.

An aphid feeding for 24 hr on a wheat seedling will accumulate DIMBOA in concentrations on the order of 1 μg/g aphid (Niemeyer et al., 1989b). Longer feeding times, as may occur under field conditions, should not radically affect this estimation since: (1) DIMBOA levels in the plant decrease as the plant develops (Argandoña et al., 1981), thus exposing the aphid to lower DIMBOA levels when feeding; (2) the instability of DIMBOA in aqueous solutions (Niemeyer et al., 1982) will hinder its accumulation; and (3) metabolization of

DIMBOA by the insect may occur, as has been shown in the case of a lepidopteran (Campos et al., 1989), which would also hinder its accumulation.

The lowest concentration of DIMBOA in diets tested on *E. connexa* is comparable with that expected in aphid bodies (Niemeyer et al., 1989b). This concentration led to a detectable, albeit not significant, increase of larval and pupal time periods of *E. connexa* with respect to control diet (Tables 3 and 4). When the predator fed on aphids, development times were also altered, being longest when the aphid prey had fed on the cultivar that had an intermediate DIMBOA level. An explanation of this finding is that these aphids were more deleterious to the predator. This is supported by the facts that: (1) two other cereal aphid species, *M. dirhodum* and *S. avenae*, feeding on wheat seedlings with intermediate DIMBOA levels accumulated the most DIMBOA in their bodies (Niemeyer et al., 1989b), and (2) *R. padi* feeding on wheat seedlings with intermediate DIMBOA levels accumulated the most DIMBOA (in glucosylated form) in their honeydew (Givovich et al., 1992). Alternatively, longer development times may have resulted from larvae ingesting fewer aphids (Table 6), which would still suggest that the compound in the plant was acting on the third trophic level, in this case through feeding deterrency. The fact that the development time of the predator significantly decreased when it fed on aphids from wheat seedlings containing high DIMBOA levels with respect to those containing low DIMBOA levels suggests that an increase in DIMBOA concentration in the plant, useful for aphid resistance, could result in the additional benefit of minimizing the effect of the toxin on the predator. This is a subtle and hitherto unreported mechanism in a tritrophic interaction between plant, herbivore, and predator (see Emden and Wratten, 1991).

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EFFECTS OF COLORADO POTATO BEETLE AND POTATO LEAFHOPPER ON AMINO ACID PROFILE OF POTATO FOLIAGE

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Abstract—Feeding by Colorado potato beetles and artificial defoliation had a minor effect on the profile of amino acids in potato foliage, whereas feeding by potato leafhoppers caused an increase in about one half of the amino acids measured.

Key Words—Amino acids, *Solanum tuberosum*, *Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae, *Empoasca fabae*, Homoptera, Cicadellidae, feeding damage.

INTRODUCTION

Two herbivores may be considered competitors if their feeding behavior reduces the relative nutritional quality of their common host plant (Kareiva, 1982). This is the probable mechanism through which the Colorado potato beetle (*Leptinotarsa decemlineata* Say) (Coleoptera: Chrysomelidae) and the potato leafhopper (*Empoasca fabae* Harris) (Homoptera: Cicadellidae) compete on potato (*Solanum tuberosum* L.) (Tomlin and Sears, in preparation). The effect of feeding damage caused by potato beetles and potato leafhoppers on the levels of certain nutrients, such as individual amino acids, in potato foliage is poorly

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documented. Changes in concentrations of amino acids in damaged plants that might lead to altered protein synthesis or production of secondary compounds was investigated.

Potato beetles have chemoreceptors specific to amino acids such as alanine, serine, proline, and γ-aminobutyric acid (GABA) (Mitchell and Schoonhoven, 1974; Hare, 1983). Pea aphids, which feed in a similar manner to leafhoppers, are also sensitive to certain amino acids (Srivastava et al., 1983). Alanine and GABA are feeding stimulants for *Acyrthosiphon pisum*, while asparagine and aspartic acid, cysteine, cystine, glutamine, glycine, proline, serine, and tyrosine are inhibitory. A negative correlation that occurs between populations of potato beetles and potato leafhoppers in the field (Tomlin, 1991) may be caused by perceived differences in the amino acid profile of potato plants or by a change in foliar nitrogen metabolism that could be indicated by a change in the profile of amino acids. Potato beetles fed on plants damaged by potato leafhoppers exhibited reduced growth and survival (Tomlin and Sears, in preparation). Jansson and Smilowitz (1985) found that the growth and development of field populations of potato beetles were negatively correlated with the concentration of total foliar nitrogen, but they did not measure the levels of free amino acids.

The objectives of this study were: (1) to determine the effect of feeding by both the Colorado potato beetle and the potato leafhopper on the profile of amino acids in potato leaves, and (2) to compare the effect of artificial defoliation with defoliation caused by potato beetles, and the effect of drought stress with damage caused by potato leafhoppers.

METHODS AND MATERIALS

Measurement of Several Factors Affecting Amino Acid Profile of Potato Foliage

Feeding by Colorado Potato Beetle and Potato Leafhopper. Pieces of potato seed (cv. Kennebec) consisting of a single eye were planted in plastic pots (10 cm diam.) in a greenhouse. Each pot, containing a 5-week-old single haulm, was covered with a cylindrical Plexiglas cage, and assigned randomly to treatment groups in a randomized complete-block design. Treatments placed on the plants consisted of 0, 1, 2, or 5 first instar beetles for 10 days or 0, 5, 10, or 20 potato leafhopper adults for 20 days replicated 10 times for beetle larvae and five times for potato leafhoppers. The cages were held in a growth room at 22–27°C and 16:8 light–dark photoperiod. Samples of tissue were removed for analysis of amino acids at the end of the period of infestation. Because leafhoppers remove fluids as they feed, the percent moisture of the leaf tissue was compared between the treatments.

Artificial Defoliation. Thirty potato plants (cv. Kennebec, 5 weeks old) were placed at 22–27°C and 16:8 light–dark. To simulate the feeding behavior of the Colorado potato beetle, scissors were used to remove either 0, 25, or 50% of a leaflet. On the first day, the appropriate amount of tissue was cut from the top three leaves of each plant, on the second day from the next three leaves, and so on until there were no leaves left to cut (five days). This cutting pattern imitated the way potato beetles defoliate potato plants. Ten replicates of each treatment were used. Tissue samples were removed for analysis of amino acids at the end of five days.

Drought Stress. The effect of drought stress on the profile of amino acids in potato foliage was measured on 5-week-old potato plants (cv. Norchip) grown in a greenhouse where natural light was supplemented with sodium vapor lamps and the temperature ranged from 15°C (night) to 28°C (day). The variety Norchip was used because Kennebec seed was unavailable. Five plants were given 100 ml water/day, five 50 ml water/day and five were never watered. This experiment was arranged in a completely randomized design on a 1-m^2 area of bench surrounded by other potato plants. Randomization was necessary to account for variation in light intensity, temperature, and humidity. After two weeks, samples of tissue were removed for analysis of amino acids.

Sample Preparation and Analysis

Tissue samples were removed from plants with a standard single-hole punch at the end of each experiment and frozen at −70°C. Representative samples were obtained from between the major veins by punching one disk of tissue (0.28 cm^2) from every leaflet.

Tissue samples were thawed to room temperature. A subsample of 0.2 g wet weight was homogenized in ice-cold 10% sulfosalicylic acid containing 0.0003 N norleucine as the internal amino acid standard. Samples were pureed using an OMNI 2000 hand-held homogenizer in 5-ml centrifuge tubes embedded in crushed ice. The homogenate was centrifuged in 1.5-ml microfuge tubes in a bench-top centrifuge at 14000 *g* for 30 min. The supernatant was removed and stored frozen at −70°C for analysis. The samples were rendered volatile by preparing the *N*-heptaflourobutyral isobutyl derivatives according to the method of Lindqvist and Mäenpää (1982).

Samples were analyzed by gas–liquid chromatography (Hewlett Packard 5890, Avondale, Pennsylvania) by use of electron-capture detection with an HP 19091A-112, 5% phenylmethyl silicone-fused silica capillary column (25 m × 0.31 mm ID). A 50:1 sample splitter was used at the injection port (250°C) with a carrier gas flow rate of 1.4 ml/min and a detector temperature of 300°C. The initial phase of the oven temperature program was 100°C for 5 min. The temperature was then increased by 4°C/min to 280°C, which was maintained

for 10 min. Asparagine and aspartic acid, and glutamine and glutamic acid, were not separated, and they are reported as the sums of the two. Arginine was too volatile to identify reliably and was excluded from analyses.

Statistical Analysis

The amount of each amino acid in the samples was expressed as micromoles of amino acid per gram wet weight of sample for comparisons. Quantities were transformed by square roots to normalize the variance.

For each source of stress that was investigated, it was determined if the treatment groups differed with respect to the amount of amino acid using both univariate analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) (SAS Institute, 1988). The number of variables was reduced to leave enough degrees of freedom to estimate the error terms using stepwise discriminant analysis (STEPDISC) (SAS Institute, 1988). A canonical discriminant analysis (CANDISC) (SAS Institute, 1988) described the patterns of variation in amino acids among the treatment groups.

RESULTS AND DISCUSSION

Feeding by the Colorado potato beetle affected the amount of amino acids in potato foliage (Wilks' λ: $F = 2.85$; $df = 12,254.28$; $P = 0.0011$) (Figure 1). Feeding by beetles caused an increase in the amount of asparagine/aspartic acid and leucine, and a decrease in the amount of valine and histidine. The decrease in the amount of valine was the most important difference between the treatment groups because it had the highest canonical coefficient. Although multivariate methods showed that a change in several amino acids separated the degrees of defoliation by beetles, only the amount of histidine varied individually. These results appear contradictory, but multivariate methods can reveal differences among treatment groups not indicated by univariate methods if the variables are correlated (Tabachnick and Fidell, 1983). If all of the statistical data are considered, it can be concluded that defoliation by beetles does affect the profile of amino acids in potato foliage in a minor way.

Feeding by potato leafhoppers affected the amounts of amino acids in potato leaves (Wilks' λ: $F = 1.69$; $df = 15,66.65$; $P = 0.0329$) by causing an increase in the amounts of half of the amino acids (Figure 1). The increase in the amount of threonine was the most important difference between the treatment groups. There was no difference in the percent moisture of the leaves between the treatments ($F = 0.15$; $df = 3$; $P = 0.8609$), indicating that the changes in levels of amino acids were not an artifact of using wet weights. Schroeder et al. (1988) found that only the amount of proline (micromoles per gram of dry

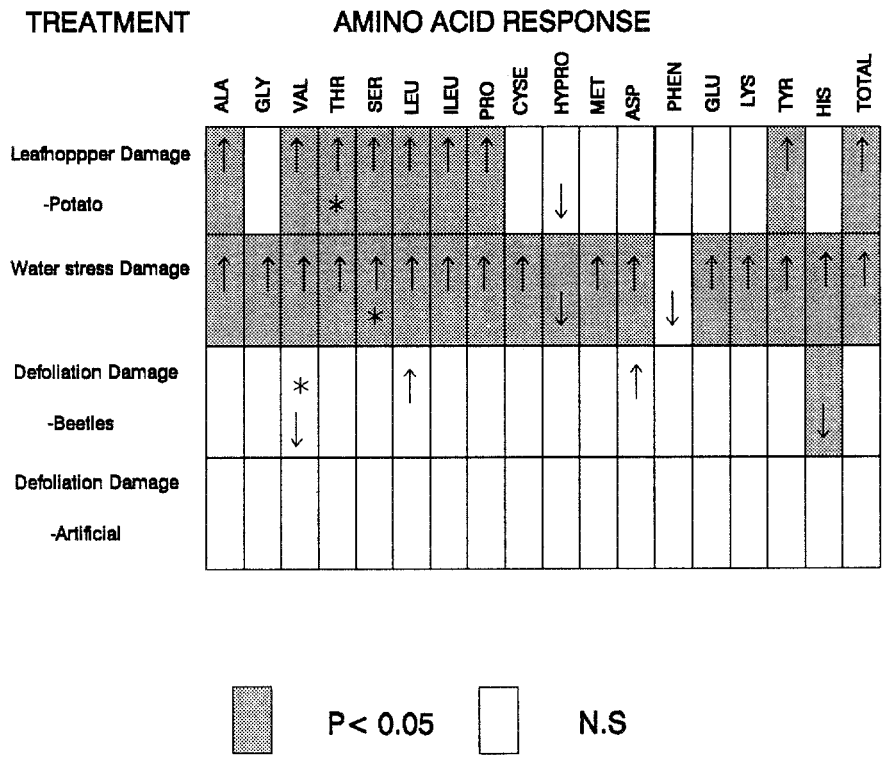

FIG. 1. A comparison of patterns of amino acid variation among the different experiments. A significant P value indicates that the amino acid increased or decreased as the level of damage increased for each type of treatment applied. ↑ or ↓ indicates whether the amount of amino acid increased or decreased based on canonical discriminant analysis and univariate means. * indicates amino acid chosen as the most important in separating the treatment groups by canonical discriminant analysis.

tissue) increased in response to feeding by leafhoppers in alfalfa and that the levels of crude protein decreased.

Different degrees of artificial defoliation had no effect on the amounts of amino acids in the leaves (Wilks' λ: $F = 1.00$; $df = 36,50$; $P = 0.4889$), and there was no significant variation in the amounts of any individual amino acids (Figure 1). Defoliation by potato beetles differs from artificial defoliation because feeding by beetles has a slight effect on the amino acid profile of the foliage, perhaps caused by salivary enzymes.

Drought stress significantly affected the amounts of amino acids in potato leaves (Wilks' λ: $F = 25.18$; $df = 4,62$; $P < 0.0001$). The treatments that received 50 and 100 ml water/day differed from the treatment that did not receive

water. The amounts of all amino acids increased except for hydroxy-L-proline and phenylalanine, which decreased in amount in response to drought stress (Figure 1). The most important factor in the separation of the treatment groups was the increase in the amount of serine. Because the percent moisture of the leaf tissue was not compared between the treatments, the increase in the amounts of amino acids could be a result of water loss from the leaf tissue, although the amounts of two amino acids did decrease.

The effect of drought stress on the profile of amino acids in different plants is well documented. Fukutoku and Yamada (1981) found that the amounts (micromoles per gram of dry weight) of isoleucine, leucine, valine, phenylalanine, glutamine, and histidine increased in drought-stressed soybean leaves. Proline and asparagine only accumulated under severe stress. Similarly, Sano and Kawashima (1982) found that the concentrations of most amino acids (micromoles per gram of fresh weight) increased while the concentrations of a few decreased in wilting tobacco leaves. Proline increased most dramatically. Hsiao (1973) and Waldren and Teare (1974) also found that the amount of proline increased in sorghum and soybean in response to severe drought stress. These results are similar to those found in this study where the concentrations of most amino acids, including proline, increased in response to drought stress.

Most authors cite an increase in the amount of proline in foliage as a symptom of stress. In this study, the amount of proline was found to increase in response to both drought stress and feeding by leafhoppers. Reinbothe and Miersch (1985) suggest that accumulated proline plays an important role in the balance of cell metabolism during stress. Proline is formed by de novo synthesis from both glutamate and arginine and may serve as a storage compound for reduced carbon and nitrogen (Hsiao, 1973; Boggess et al., 1976; Boggess and Stewart, 1976; Wrench et al., 1977). Levitt (1972) suggested that proline may act as an NH_3 storage compound and that other amino acids are converted to proline, which may accumulate without causing injury.

Increased amounts of amino acids other than proline may be caused by degradation of proteins. Levitt (1972) found that the rate of incorporation of ^{14}C-labeled serine into protein decreased and the transfer into other free amino acids increased in response to drought stress. Fukutoku and Yamada (1981b) observed an increase in amounts of amino acids usually found in the proteins of drought-stressed plants, indicating that degradation of proteins occurred.

In summary, damage caused by both leafhoppers and drought stress produce similar, although not identical, reactions in plants. This suggests that both types of damage could affect similar physiological processes, although comparisons using dry rather than wet weights of tissue should be done. Leafhoppers may cause hydrolysis of certain proteins in xylem and phloem cells with specific enzymes in their saliva, whereas drought stress may cause a more general and widespread degradation of protein through changes in membrane properties of

the cells (Cooke et al., 1980). Feeding by potato beetles had more effect than artificial defoliation on the amounts of amino acids possibly caused by salivary enzymes.

Acknowledgments—We would sincerely like to thank Margaret Carter for her invaluable technical assistance and advice with the gas chromatographic analyses. We would also like to thank the National Science and Engineering Research Council of Canada and the Ontario Ministry of Agriculture and Food for their support of this project. The data presented here are in partial fulfillment of the MSc degree for the senior author.

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DEFENSIVE SECRETION OF A CARABID BEETLE, *Helluomorphoides clairvillei*[1]

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Abstract—The defensive spray of a single female of the rare carabid beetle, *Helluomorphoides clairvillei* (subfamily Carabinae, supertribe Lebiitae, tribe Helluonini), was found to contain a mixture of compounds, including carboxylic acids (formic, acetic), aliphatic esters (principally nonyl acetate), and hydrocarbons (principally decane). In a single series of discharges, the beetle ejected a total of about 1% of its body weight as formic acid (1.1 mg). Our findings demonstrate that characterization of secretory components from even minimal samples of secretion should be possible in many cases.

Key Words—Coleoptera, Carabidae, beetles, *Helluomorphoides clairvillei*, defensive secretion, formic acid, acetates, formates, hydrocarbons.

INTRODUCTION

Information is now available on the chemistry of the defensive secretion of over 300 species of carabid beetles (Blum, 1981; Weatherston and Percy, 1978; Dettner, 1987). While early characterizations had been restricted largely to major components of the mixtures, improved instrumentation, particularly capillary gas chromatography, has made possible the characterization of minor components, even from very small samples of material. We here report on the composition of the defensive spray of a rare carabid beetle, *Helluomorphoides clairvillei*, that was available to us as a single specimen only. Some years ago,

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we investigated the secretions of two other species of *Helluomorphoides* and characterized only formic acid and nonyl acetate (Eisner et al., 1968). In *H. clairvillei* we were able to detect 26 components.

METHODS AND MATERIALS

The single female beetle (120.5 mg, 18 mm body length) was collected near Lake Placid, Highlands County, Florida, and maintained in the laboratory on water and freshly cut mealworms. The specimen is now deposited under lot label 1206 (T. Eisner) in the Cornell University insect collection.

Collection of Secretion. To obtain secretion for analysis, the beetle was induced to spray by pinching its legs with forceps while it was confined in a small vial. Two individual milkings were obtained in which the beetle was caused to eject several times in succession into vials (cold ether was added as solvent). After a period of three weeks, in which the beetle was kept undisturbed, it was again milked, but this time by being caused to spray only once into each of four vials, to which 100 μl of cold ether was then added.

Chemicals. Heptyl, octyl, nonyl, decyl, undecyl, and dodecyl acetates were purchased from Sigma Chemical Co. The formates were synthesized by heating the corresponding alcohols with 88% formic acid. The propionates and butyrates were made from the corresponding alcohols and acid anhydrides.

Derivatization. Pentafluorobenzyl esters of carboxylic acids and dimethyldisulfide (DMDS) derivatives of unsaturated compounds were prepared as previously described (Attygalle and Morgan, 1986).

Analytical Procedures. Gas chromatography was performed on a Hewlett-Packard (HP) 5890 instrument equipped with a splitless injector, a flame ionization detector (FID), and a HP 3396A integrator. The following 25-m $\times$ 0.22-mm fused-silica columns were used: DB-1; DB-Wax; Superox-FA.

Infrared and mass spectra were obtained with a HP 5890 gas chromatograph linked in series to a HP 5965A IR detector and a HP 5970 mass selective detector (MSD). Analyses were performed using a 25-m $\times$ 0.32-mm fused-silica column coated with SE-54. Mass spectra were also obtained on a HP 5890 gas chromatograph linked to a Finnigan ion trap detector (ITD).

For quantitative determinations of acidic components (ion-trap detection) and nonacidic components (flame-ionization detection), the external standards were, respectively, formic acid (Mallinckrodt) and decyl acetate (Sigma Chemical Co.).

RESULTS

The pungent odor of formic acid was easily recognized when the beetle was "milked." However, analytical determination of formic acid without prior derivatization is not entirely straightforward. Thus, the flame-ionization detector

(FID) most frequently used in GC hardly responds to formic acid. The ion-trap detector (ITD), on the other hand, is able to detect picogram amounts of underivatized formic acid. Using the ITD in conjunction with the Superox-FA capillary column, we were able to identify and quantify formic acid in the beetle's spray. A small amount of acetic acid was also detected. The presence of acetic acid was confirmed by conversion to its pentafluorobenzyl ester and observation of the mass spectrum of this derivative [*m/z* (%), 240(M^+, 11), 181(46), 161(16), 43(100)]. The mass spectrum and retention time of the ester corresponded to those obtained with an authentic sample of pentafluorobenzyl acetate. Table 1 shows the amount of formic and acetic acid present in the four consecutive discharges of the beetle. The total formic acid in the ejections amounted to 1.1 mg, or ca. 1% of the body mass of the beetle.

Figure 1 shows an FID gas chromatogram depicting the nonacidic components of the defensive secretion. Table 2 lists the compounds identified, together with the analytical data supporting the identifications. The proposed structures are based on the mass spectra obtained by GC-MS. In addition, the identifications of the major components were supported by infrared spectra obtained by GC-FTIR. Gas-phase FTIR spectra proved particularly helpful in recognizing acetate, formate, and hydrocarbon peaks. Thus, the absorption for carbonyl groups appeared characteristically at 1761–1762 cm^{-1} in the gas-phase FTIR spectra of the octyl, nonyl, decyl, and undecyl acetates, whereas that of formates appeared at 1740–1748 cm^{-1} (Table 2). A search for the ions *m/z* 61, 75, and 89 in the acquired mass spectral data, corresponding to the protonated carboxylic acids, readily identified those chromatographic peaks representing acetates, propionates, and butyrates, respectively. Although the signal for protonated formic acid at *m/z* 47 in the spectra of formates is not as strong as those of corresponding protonated acids in the spectra of other esters, it is highly characteristic and therefore analytically useful. The identifications of all the saturated ester peaks were confirmed by comparisons with authentic samples.

TABLE 1. MASS OF ACIDIC AND NONACIDIC COMPONENTS IN FOUR CONSECUTIVE DISCHARGES OF *H. clairvillei*

	Mass discharged [μg (% of total)]			
	1st spray	2nd spray	3rd spray	4th spray
HCOOH	44 (28)	201 (49)	853 (60)	35 (45)
CH_3COOH	2 (1)	5 (1)	15 (1)	1 (1)
Nonacidic components	112 (71)	205 (50)	564 (39)	41 (54)
Total	158	411	1432	77

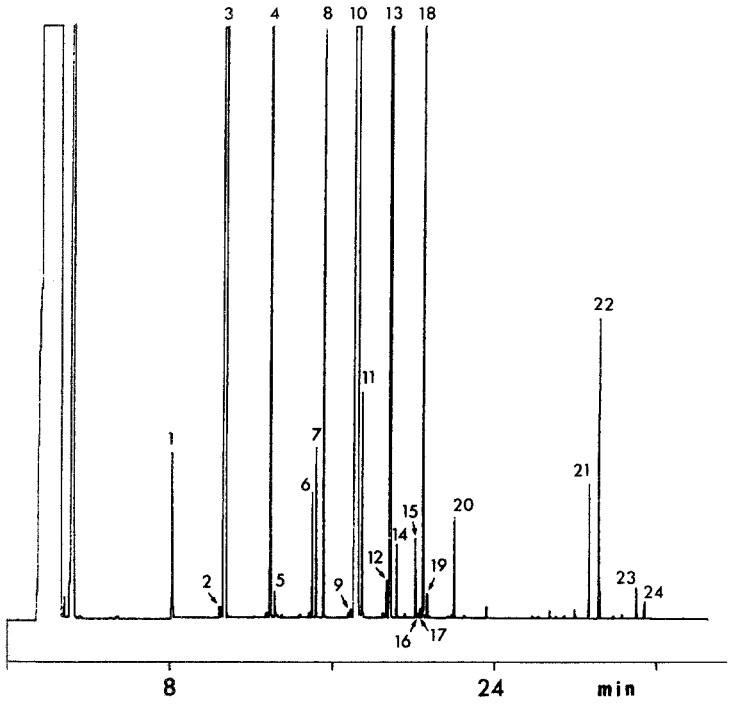

FIG. 1. FID gas chromatogram of *H. clairvillei* defensive secretion obtained on a 25-m × 0.22-mm fused-silica capillary coated with DB-5 (40°C for 4 min, then to 260°C at 8°C/min). Refer to Table 2 for peak identifications.

The double-bond positions of the unsaturated compounds, 1- and 3-decene, 3-nonen-1-yl acetate, and 4-undecen-1-yl acetate were determined by the DMDS derivatization method (Buser et al., 1983; Francis and Veland, 1981). The relevant mass spectral data are shown in Table 3. The mass spectrum of the DMDS derivative of the nonenyl acetate showed a molecular ion at *m/z* 278, and two intense ions at *m/z* 131 and 87, arising from a cleavage between the vicinal thiomethyl groups. The ion at *m/z* 131 represents $CH_3-(CH_2)_4-CH=S^+CH_3$, which arises from the distal end of the molecule, whereas the ion *m/z* 87 is from the functional-group-bearing end of the molecule after loss of acetic acid. Hence, the parent compound was identified as 3-nonen-1-yl acetate. Similarly, 4-undecen-1-yl acetate was identified on the basis of its derivative's characteristic mass spectrum (*m/z* 306, 145, and 101). Furthermore, after DMDS derivatization, two other chromatographic peaks representing a pair of isomers with molecular weight 234 were observed. These two peaks were identified as DMDS derivatives of 1-decene and 3-decene on the basis of their

TABLE 2. NONACIDIC COMPONENTS OF DEFENSIVE SPRAY OF *Helluomorphoides clairvillei* AND SPECTRAL EVIDENCE FOR STRUCTURE ASSIGNMENTS

No.[a]	Compound	Mean % by weight ± SD (N = 6 sprayings)[b]	Mass spectrum [m/z, %]	Gas-phase IR spectrum (resolution = 8 cm^{-1})
1	Nonane $[CH_3(CH_2)_7CH_3]$	0.54 ± 0.15	128(M^+)	
2	1-Decene + 3-decene[c]	0.05 ± 0.00	140(M^+)	
3	Decane $[CH_3(CH_2)_8CH_3]$	21.30 ± 5.30	142(M^+)	2933(s[d]), 2868(m), 1463(w)
4	Undecane $[CH_3(CH_2)_9CH_3]$	3.07 ± 0.73	156(M^+)	2933(s), 2867(m), 1463(w)
5	Heptyl acetate $[CH_3(CH_2)_6OCOCH_3]$	0.10 ± 0.03	98(M^+-60,5), 70(28), 61(17), 56(27), 55(26), 43(100).	
6	Dodecane $[CH_3(CH_2)_{10}CH_3]$	0.42 ± 0.12	170(M^+)	
7	Octyl acetate $[CH_3(CH_2)_7OCOCH_3]$	0.34 ± 0.05	112(M^+-60,3), 83(13), 70(16), 69(16), 61(71), 55(29), 43(100).	2936(s), 2873(m), 1762(s), 1458(w), 1370(w), 1232(s), 1041(w)
8	Nonyl formate $[CH_3(CH_2)_8OCOH]$	3.08 ± 1.02	126(M^+-46,6), 98(39), 97(39), 83(49), 70(74), 56(100), 55(98), 47(2), 41(84).	2934(s), 2868(m), 1748(s), 1466(w), 1168(s)
9	3-Nonen-1-yl acetate[c] $[CH_3(CH_2)_4CH{=}CH(CH_2)_2OCOCH_3]$	0.05 ± 0.00	124(M^+-60,11), 96(15), 95(15), 82(20), 67(50), 54(50), 43(100)	
10	Nonyl acetate $[CH_3(CH_2)_8OCOCH_3]$	57.62 ± 2.98	126(M^+-60,6), 98(17), 97(17), 84(18), 83(19), 70(28), 61(26), 56(38), 43(100).	2936(s), 2867(m), 1761(s), 1463(w), 1370(w), 1233(s), 1042(w)
11	Decyl formate $[CH_3(CH_2)_9OCOH]$	0.39 ± 0.16	140(M^+-46,12), 111(30), 97(51), 83(60), 70(95), 55(100), 47(6), 41(98).	2933(s), 2868(m), 1748(s), 1462(w), 1168(s)
12	Nonyl propionate $[CH_3(CH_2)_8OCOC_2H_5]$	0.08 ± 0.03	126(M^+-74,6), 97(30), 83(19), 75(45), 69(38), 57(100), 41(77)	
13	Decyl acetate $[CH_3(CH_2)_9OCOCH_3]$	6.50 ± 0.59	140(M^+-60,5), 112(12), 97(18), 83(25), 70(34), 61(30), 55(34), 43(100)	2935(s), 2866(m), 1761(s), 1464(w), 1370(w), 1233(s), 1045(w)
14	Undecyl formate $[CH_3(CH_2)_{10}OCOH]$	0.15 ± 0.03	154(M^+-46,5), 126(20), 111(20), 97(48), 83(70), 69(73), 55(73), 47(6), 41(100)	2933(s), 2868(m), 1740(s), 1168(s)
15	4-Undecen-1-yl acetate[c] $[CH_3(CH_2)_5CH{=}CH(CH_2)_3OCOCH_3]$	0.23 ± 0.03	152(M^+-60,23), 124(12), 109(17), 95(38), 81(56), 68(80), 61(1), 55(39), 43(100)	
16	Nonyl butyrate $[CH_3(CH_2)_8OCO(CH_2)_2CH_3]$	0.02 ± 0.00	126(M^+-88,10), 112(4), 111(5), 97(27), 89(63), 83(26), 71(65), 55(52), 41(100).	

TABLE 2. Continued

No.[a]	Compound	Mean % by weight ± SD (N = 6 sprayings)[b]	Mass spectrum [m/z, %]	Gas-phase IR spectrum (resolution = 8 cm^{-1})
17	Decyl propionate $[CH_3(CH_2)_9OCOC_2H_5]$	0.02 ± 0.00	140(M^+-74,6), 123(8), 111(9), 96(10), 84(9), 83(8), 75(68), 70(12), 57(100)	
18	Undecyl acetate $[CH_3(CH_2)_{10}OCOCH_3]$	2.44 ± 0.22	154(M^+-60,7), 126(9), 111(14), 97(23), 83(28), 69(35), 61(30), 55(39), 43(100)	2935(s), 2866(m), 1761(s), 1463(w), 1369(w), 1232(s), 1041(w)
19	Dodecyl formate $[CH_3(CH_2)_{11}OCOH]$	0.25 ± 0.25	168(M^+-46,2) 140(3), 125(7), 111(22), 97(23), 83(46), 69(54), 55(93), 47(2), 41(100)	
20	Dodecyl acetate $[CH_3(CH_2)_{11}OCOCH_3]$	0.20 ± 0.16	168(M^+-60,7), 140(9), 125(10), 111(16), 97(37), 83(42), 69(44), 61(32), 55(37), 43(100)	
21	Heneicosane $[CH_3(CH_2)_{19}CH_3]$	0.64 ± 0.55	296(M^+)	
22	9-methylheneicosane[e] $[CH_3(CH_2)_7CH(CH_3)(CH_2)_{11}CH_3]$	1.88 ± 1.80	310(M^+,1), 295(3), 281(2), 267(2), 266(2), 253(1), 239(2), 225(0.5), 224(2), 211(2), 197(12), 196(19), 184(2), 169(4), 168(7), 155(5), 141(15), 140(30), 127(7), 125(5), 113(12), 111(9), 99(21), 97(14), 85(63), 71(77), 57(100), 43(70)	
23	Tricosane $[CH_3(CH_2)_{21}CH_3]$	0.33 ± 0.31	324(M^+)	
24	9-Methyltricosane[e] $[CH_3(CH_2)_7CH(CH_3)(CH_2)_{13}CH_3]$	0.31 ± 0.32	338(M^+,0), 323(4), 295(5), 262(4), 225(11), 224(23), 197(6), 196(12), 182(4), 170(8), 169(6), 155(6), 141(12), 140(25), 127(16), 113(12), 111(16), 99(32), 85(86), 71(83), 57(100), 43(97)	

[a] Numbers refer to Figure 1.
[b] Including the two initial and four (consecutive) later sprayings.
[c] Configuration of the double bond was not determined.
[d] w = weak; m = medium; s = strong.
[e] Tentative structures; based on mass spectral data only.

TABLE 3. MASS SPECTRA OF DMDS DERIVATIVES OF UNSATURATED COMPOUNDS IN *H. clairvillei* DEFENSIVE SECRETION

Compound	Mass spectrum of DMDS derivative (*m/z*, %)
3-Nonen-1-yl acetate	278(M^+,35), 131(70), 87(54), 83(47), 61(66), 55(53), 43(100)
4-Undecen-1-yl acetate	306(M^+,54), 199(4), 145(90), 101(65), 97(41), 61(70), 55(74), 43(100)
1-Decene	234(M^+,38), 187(13), 173(100), 117(27), 83(38), 69(46), 61(96)
3-Decene	234(M^+,48), 145(100), 89(62), 59(82)

mass spectra (Table 3). The decenes are trace constituents in the secretion (0.05% combined). The capability of locating the double-bond positions in such minor constituents exemplifies the efficacy of the DMDS procedure as a potent microchemical method.

The beetle was dissected when it died. Its two defensive glands were identical in appearance to those depicted for other species of *Helluomorphoides* (Eisner et al., 1968).

DISCUSSION

Although aliphatic hydrocarbons are commonly found in arthropod defensive secretions, these hydrocarbons usually contain an odd number of carbon atoms; those with an even number are found only as trace constituents (Blum, 1981). Since hydrocarbons are considered to originate from decarboxylation of fatty acids, this distribution pattern generally reflects that of the fatty acids (Jackson and Blomquist, 1976). In this respect, the secretion of *H. clairvillei* is atypical in that the hydrocarbons show a Gaussian-like distribution with decane as the major representative (Table 2). Interestingly, a similar pattern is seen with the acetate esters in this secretion, with nonyl acetate as the major component.

Several high-molecular-weight hydrocarbons were also observed in this secretion (Figure 1). The four major constituents were identified as heneicosane, tricosane, 9-methylheneicosane, and 9-methyltricosane. However, the identification of the two branched hydrocarbons, based solely on mass spectral data, should be considered tentative since synthetic standards were not available for comparison.

We identified four formates in the secretion (Table 2). A wide variety of

formate esters have been found in the exocrine products of animals (Kuwahara et al., 1975, 1982; Weatherston et al., 1986). As in the present case, these formates are often found together with formic acid (Burger et al., 1981; Francke et al., 1985; Bagneres, 1991). To the best of our knowledge, the only formates known from carabid beetles are nonyl formate (Moore and Wallbank, 1968) and decyl formate (Moore, 1979; Kanehisa and Kawazu, 1985).

The total mass of secretory components recovered in the consecutive discharges of the beetle was not constant, indicating that there was variability in the amount of secretion ejected per discharge. There was also variability in the ratio of components in these sprayings. We attribute the latter variability, in part, to variable evaporative loss of formic acid from the samples during the collection procedure (the inevitable time-delay between milking of the beetle in the vial and addition of solvent to the vial was not kept constant). It is also possible that the two glands, which most probably did not contain the secretory components in the same ratios, and which could both have discharged in the sprayings, contributed unequally to the ejections. A further possibility (for which we have no direct evidence) is that the secretion is a coarsely distributed two-phase system rather than a one-phase system. If so, glandular output could be expected to be variable, even from consecutive discharges of the same gland.

Although the secretion of *H. clairvillei* here described is the most complex carabid defensive secretion reported so far, we do not believe such complexity to be exceptional. Nor is the production of a mixture of formic acid and a variety of lipophilic congeners in itself novel. In fact, the defensive use of a low-molecular-weight acid along with less polar compounds that help the acid penetrate an opponent's "armor" appears to have evolved independently several times (Eisner et al., 1961; 1972; Löfqvist, 1976; Moore and Wallbank, 1968; Moore, 1979; Weatherston et al., 1979). Nevertheless, it is worth noting that a combination of chromatographic, spectroscopic, and derivatization procedures has served to characterize a secretion consisting of over two dozen compounds obtained from the spray of a single insect, including compounds that constitute less than 0.05% of the mixture. In an entirely different context, a recent meticulous examination of the sexual pheromones of a brown alga led the authors to discover "a degree of elaboration not known before in algae," and they point out that "progress in analytical methods will make it necessary to re-evaluate earlier work, since at least some of the brown algal pheromone systems may be more complicated than was previously believed in respect to bouquet composition, relative biological activities, and enantiomer composition" (Müller et al., 1990). For chemotaxonomic purposes alone, analyses providing a high degree of chemical resolution are clearly more useful than those revealing only major components. Beyond this, the analysis of compounds from single specimens permits the examination of both quantitative and qualitative chemical differences between one individual and another. With pooled samples, a wealth of

potentially important information on variations of chemistry with sex, age, diet, season, geographical source, etc., is lost. For studies in chemical ecology, in contrast to explorations of "natural products" chemistry, analysis at the individual level would seem to offer special opportunities.

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INFLUENCE OF LIFE HISTORY DIFFERENCES OF TWO TACHINID PARASITOIDS OF *Helicoverpa zea* (BODDIE) (LEPIDOPTERA: NOCTUIDAE) ON THEIR INTERACTIONS WITH GLANDULAR TRICHOME/ METHYL KETONE-BASED INSECT RESISTANCE IN TOMATO

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Abstract—The effects of glandular trichome/methyl ketone (2-tridecanone and 2-undecanone) -based insect resistance in the wild tomato, *Lycopersicon hirsutum* f. *glabratum* C.H. Mull, accession PI 134417, on *Archytas marmoratus* (Townsend) and *Eucelatoria bryani* (Sabrosky) (Diptera: Tachinidae), both parasitoids of *Helicoverpa* (=*Heliothis*) *zea* (Boddie) (Lepidoptera: Noctuidae), were investigated in the laboratory. *A. marmoratus* deposits larvae (planidia) on the foliage of its host's food plant; planidia attach to passing hosts, penetrate the cuticle, and develop in the host pupae. *E. bryani* larviposits directly into its host; its larvae develop in the host larva. *A. marmoratus* planidia are killed by glandular trichomes of PI 134417 and also by trichomes of hybrid lines with no methyl ketones. The methyl ketones are toxic to planidia, but at least part of the effect is due to other factors, possibly physical entanglement. Both species can be affected indirectly by methyl ketones in the diet of the host. 2-Undecanone reduces the percentage of *A. marmoratus* larvae that reach pupation. This effect is evidently due to premature death and desiccation of the host pupa caused by 2-undecanone. 2-Tridecanone in host diets had no effect on *A. marmoratus*. In *E. bryani*, 2-tridecanone in the diet of the host reduced the number of parasitoids yielded by each parasitized host,

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although not the overall percentage of hosts parasitized. 2-Undecanone in the diet of the host had no effect on *E. bryani*.

Key Words—Parasitism, *Archytas marmoratus*, *Eucelatoria bryani*, Diptera, Tachinidae, tomato, *Lycopersicon hirsutum*, glandular trichomes, *Helicoverpa zea*, Lepidoptera, Noctuidae, trilevel interaction, 2-undecanone, 2-tridecanone.

INTRODUCTION

Host-plant resistance and biological control by parasitoids and predators are important tactics in many integrated pest management systems. These tactics are often cited as being compatible in that they combine the density-independent effects of host-plant resistance with the density-dependent effects of biological control (Bergman and Tingey, 1979; Duffey and Bloem, 1986). However, the same plant characteristics that condition resistance to a pest may also adversely affect beneficial insects, resulting in incompatibility of these tactics (Bergman and Tingey, 1979; Duffey and Bloem, 1986; Duffey et al., 1986; Obrycki, 1986; Price, 1986; van Emden, 1986). These adverse effects can be divided into the broad categories: (1) direct effects, usually resulting from contact between the parasitoid or predator and the host plant, in which the herbivore may not necessarily be involved; and (2) indirect effects, in which the herbivore is altered in some way that renders it less suitable as a host or prey.

In the literature on interactions between host-plant resistance and insect parasitoids and predators, few broad patterns are evident. Perhaps the most apparent pattern is the impediment of small natural enemies by plant trichomes. Glandular trichomes may entrap these insects, while nonglandular trichomes may impede their movement (reviewed by Obrycki, 1986). Otherwise, it would be difficult to predict how a given host-plant resistance characteristic might affect a given species of natural enemy. We hypothesize that one reason for the variation in these interactions is variation in the life histories of parasitoids and predators. The study reported herein was undertaken as a part of our effort to test this hypothesis by comparing the response to an insect-resistance factor by two species of parasitoids with different life histories.

The parasitoids selected for this study were two species of tachinid flies (Diptera), both of which attack the tomato fruitworm, *Helicoverpa* (=*Heliothis*) *zea* (Boddie) (Lepidoptera: Noctuidae): *Archytas marmoratus* (Townsend) and *Eucelatoria bryani* Sabrosky. *A. marmoratus* larviposits minute live maggots, or planidia, on its host's food plant. Planidia attach themselves to passing hosts and penetrate the cuticle. The parasitoid undergoes only limited development until the host pupates, when it develops rapidly and emerges from the host pupa as an adult (Hughes, 1975). No more than one fly emerges from a single host. *E. bryani*, in contrast, larviposits directly into its host. Its larvae develop rapidly,

killing the host larva after a few days. Mature larvae emerge from the dead host larva to pupate. A single host often yields several parasitoids, occasionally as many as 20 (Jackson et al., 1969; Bryan et al., 1972). The larvae of *A. marmoratus*, therefore, come into direct contact with the host's food plant, whereas those of *E. bryani* do not, as dead hosts would typically fall off the host plant. Hosts inside tomato fruit might not fall off, but fruit do not contain the resistance mechanisms examined in this study.

The host-plant resistance examined in this study was the methyl ketone (2-tridecanone and 2-undecanone)/glandular trichome-based resistance of the wild tomato, *Lycopersicon hirsutum* f. *glabratum* C.H. Mull, accession PI 134417. 2-Tridecanone, the 13-carbon methyl ketone, occurs in type VI (Luckwill, 1943) glandular trichomes on the foliage and is largely responsible for resistance to several insect pests of tomato, including the tobacco hornworm, *Manduca sexta* (L.) and Colorado potato beetle, *Leptinotarsa decemlineata* (Say), (Kennedy and Henderson, 1978; Kennedy and Yamamoto, 1979; Williams et al., 1980; Kennedy and Sorenson, 1985; Kennedy et al., 1985; Kennedy, 1986; Kennedy and Farrar, 1987). Neonate larvae of these insects typically die soon after being placed on resistant foliage. 2-Tridecanone confers a limited degree of resistance to *H. zea* as well; however, most of the resistance to *H. zea* is conferred by as yet undetermined factors in leaf tissues other than the trichomes (Farrar and Kennedy, 1987a). 2-Undecanone, the 11-carbon methyl ketone, also occurs in PI 134417 type VI trichomes, but is less abundant and less acutely toxic to neonate *H. zea* than 2-tridecanone (Dimock et al., 1982). However, in combination, 2-undecanone and 2-tridecanone are synergistic in their toxicity to several insects, including *H. zea* (Farrar and Kennedy, 1987b, 1988; Lin et al., 1987). 2-Undecanone, at levels commonly found in PI 134417 foliage, also causes extensive mortality of *H. zea* in the pupal stage if ingested by the fifth-instar larva (Farrar and Kennedy, 1987b, 1988).

In recent studies, the methyl ketone/glandular trichome-based resistance of PI 134417 has been shown to inhibit several species of insect parasitoids. These species include *Campoletis sonorensis* (Cameron) (Hymenoptera: Ichneumonidae), a larval parasitoid of *H. zea* (Kauffman and Kennedy, 1989a,c; Farrar and Kennedy, unpublished data), *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) egg parasitoids of *H. zea* and *M. sexta* (Kauffman and Kennedy, 1989b; Kashyap et al., 1991), and *Telenomus sphingis* (Ashmead) (Hymenoptera: Scelionidae), an egg parasitoid of *M. sexta* (Kashyap et al., 1991; Farrar and Kennedy, 1991). Farrar and Kennedy (unpublished data) also found reduced activity of an array of parasitoids on *M. sexta*-resistant plants in the field in North Carolina. That study did not include the tachinids studied here, as, in North Carolina, *E. bryani* does not occur naturally and *A. marmoratus* is not seen in high numbers until late in the growing season (Hughes and Rabb, 1976).

The study reported herein was designed to evaluate the effects of methyl

ketone/glandular trichome-based resistance of PI 134417 on *A. marmoratus* and *E. bryani* and to determine whether any differences between the two species were related to differences in their life histories.

METHODS AND MATERIALS

All insects were obtained from colonies maintained at North Carolina State University. Hosts [*H. zea* and *H. virescens* (F.)] were maintained on corn–soya–milk (CSM) diet (Burton, 1970). *A. marmoratus* was maintained on *H. zea* according to Hughes (1975) and Gross and Johnson (1985). *E. bryani* was maintained on *H. virescens* according to Bryan et al. (1969, 1972). All experiments, however, were done using *H. zea* as the host for both species.

All tomato plants were grown in a greenhouse using a commercial soil mix (Metro Mix 220) and were fertilized weekly (Peter's Professional 20-20-20 at 2.8 g/plant). Daily photoperiod was supplemented to 16 hr with metal halide lamps. The following plant lines were included in the study: PI 134417, the susceptible *L. esculentum* Mill. cultivar Better Boy, the F_1 hybrid (*L. esculentum* × PI 134417), and BC-2, a selection from an F_1 backcross family (F_1 × PI 134417). F_1 foliage has a density of type VI trichomes intermediate between those of PI 134417 and Better Boy, but no detectable methyl ketones (Kauffman and Kennedy, 1989b). BC-2 usually has a moderately high density of type VI trichomes and intermediate levels of methyl ketones (Kauffman and Kennedy, 1989b). The *L. esculentum* cultivar Walter was used to make the crosses since Better Boy, a hybrid cultivar, does not cross readily with PI 134417. Lepidopterous larvae respond similarly to Walter and Better Boy, and neither cultivar contains detectable levels of methyl ketones (Farrar and Kennedy, unpublished data).

A. marmoratus *Experiments.* Because *A. marmoratus* planidia spend a period of time on the surface of the host's food plant, experiments were conducted to evaluate the effects of the methyl ketones and/or glandular trichomes of PI 134417 on mortality of planidia on this foliage.

Planidia were picked up with a fine brush from the larviposition substrate (filter paper) and dropped onto foliage from a height of ca. 1 cm by flicking them off the brush with a second brush. In experiments using filter paper instead of foliage (see below), handling in this manner resulted in ca. 7% mortality of planidia. Care was taken not to touch the leaf with the brushes and thereby rupture trichome tips. Twenty-five to 35 planidia, constituting one observation, were placed on each leaflet. The petioles of the leaves were placed in flasks of water. The leaves were held at 25 ± 2°C for 24 hr, at which time the planidia were scored as alive or dead. Planidia that showed vigorous movement when gently prodded with a fine bristle were scored as alive, those that did not were

scored as dead. Ten replications of each plant line were done. Percentage mortality was calculated, normalized by arcsine $\sqrt{\%}$ transformation, and subjected to analysis of variance (ANOVA). Means were separated by the least significant difference (LSD) test.

To verify that effects seen in the above experiment (see below) were due to the glandular trichomes, the experiment was repeated including foliage from which the exudate of the glandular trichomes had been removed. Trichome exudate was removed by gently wiping the foliage, first with a dry cotton ball, then with a cotton ball saturated with a 0.1% aqueous solution of Triton X-155 (a nonphytotoxic emulsifying agent), then rinsing it, first in 0.1% Triton, then in clean water. Although it cannot be said with certainty that this method removes 100% of the trichome exudate, results of previously published studies (e.g., Farrar and Kennedy 1987a; Kennedy and Farrar, 1987) are consistent with removal of the vast majority of the exudate. Furthermore, microscopic examination of this foliage revealed no intact trichomes. This experiment was otherwise similar to the above experiment, except that BC-2 was omitted, since mortality on it was similar to that on PI 134417.

Every two weeks during the tests involving foliage, the density of type VI trichomes was measured for each plant line. In addition, the levels of 2-tridecanone and 2-undecanone were measured for the plant lines known to possess them, BC-2 and PI 134417. The other lines, F_1 and Better Boy, previously have been shown to possess no detectable levels of methyl ketones (Kauffman and Kennedy, 1989b; Farrar and Kennedy, unpublished). Four leaflets were collected for each sample. From each leaflet, a 5.8-mm-diam. disk was cut with a cork borer and type VI trichomes on one surface of each disk were counted. For PI 134417 and BC-2, 300 type VI trichome tips were collected with glass microcapillary tubes from each leaflet (total of 1200 tips per plant line). Counts and collections were done on the adaxial surface of two of the leaflets and from the abaxial surface of the other two leaflets. The samples were analyzed using a Hewlett-Packard 5880 gas chromatograph with a 15-m Durabond DB5 temperature-program analysis (100°C for 1 min, then to 275°C at 5°C/min, and held at 275°C for 5 min). 2-Tetradecanone (Wiley Organics, 4654 Kenny Rd., Columbus, Ohio 43220) was used as an internal standard. These data were used to calculate the concentration of 2-tridecanone and 2-undecanone in PI 134417 as weight per square centimeter of foliage.

To evaluate the toxicity of the methyl ketones, LC_{50} values (the concentration needed to kill 50% of the test insects) were determined for 2-tridecanone and 2-undecanone for *A. marmoratus* planidia. Standard solutions of a range of concentrations of purified ketones (2-tridecanone: Fluka Chemical Corp., Hauppauge, New York 11788; 2-undecanone: Pfaltz and Bauer, Inc., Stamford, Connecticut 06902) were prepared in acetone. Solutions were pipetted onto filter paper at a rate of 21 $\mu l/cm^2$ and the acetone allowed to evaporate. Rates of

0.21–1.05 $\mu g/cm^2$ and 1.05–15.12 $\mu g/cm^2$ were used for 2-tridecanone and 2-undecanone, respectively. Controls, treated with acetone only, were included in all tests. Filter papers were placed in the lids of inverted 5.5-cm disposable plastic Petri dishes and moistened with four drops of water per filter paper disk. Twenty-five to 30 planidia were dropped onto each filter paper disk as above. Dishes were sealed with Parafilm and held at 27°C for 24 hr, at which time the planidia were scored as dead or alive, as above. The data were adjusted for mortality in the controls (ca. 7%) using Abbott's (1925) formula, and probit analysis was used to calculate LC_{50} values.

H. zea larvae developing on tomato plants with methyl ketone-based resistance to *M. sexta* ingest methyl ketones during at least part of the larval stage. Therefore, there is the potential for indirect, or host-mediated, effects on the parasitoids. Experiments were conducted in which parasitoids were reared on *H. zea* larvae, which were in turn reared throughout the larval stage on diets containing 2-tridecanone and/or 2-undecanone. Diet (CSM) (Burton, 1970) was prepared in batches of 4.0 liters using a commercial blender. To make different treatments, 500 ml of warm (ca. 45°C) liquid diet was poured into a 1-liter blender. Measured amounts of the ketones were added and blended into the diet for a minimum of 1 min at high speed. Both ketones are liquids at 45°C. Diet was poured into 30-ml plastic cups (ca. 10 ml/cup) in which the larvae were reared. Control (untreated) diet was poured directly into cups. In all tests, concentrations of 0.3% and 0.06% (wet weight) were used for 2-tridecanone and 2-undecanone, respectively. These concentrations are representative of those found in PI 134417 foliage. *H. zea* shows some mortality in the early larval stage on diet with these concentrations of 2-tridecanone or 2-tridecanone plus 2-undecanone ($\bar{x}$ = 15 and 27%, respectively; Farrar and Kennedy, 1987b), but sufficient numbers of larvae can be reared on these diets.

To test for indirect effects on *A. marmoratus*, *H. zea* larvae were reared through the fifth instar on one of four diets: 2-tridecanone (0.3%), 2-undecanone (0.06%), 2-tridecanone plus 2-undecanone (0.3 + 0.06%), and control (untreated). Larvae were held at 27°C under a 16:8 light–dark photoperiod. On the first or second day of the fifth larval instar, using a small brush, three planidia were placed on the dorsal surface of each larva. Larvae were held until pupation on their respective diets. Uneaten diet was then removed from the cups. In *Helicoverpa* pupae parasitized by *A. marmoratus*, parasitoid larvae typically form respiratory funnels that are visible around the host's wing pads (Hughes, 1975). Pupae showing respiratory funnels were therefore classified as parasitized; those without, as unparasitized. Pupae were held through death, fly emergence, or moth emergence. Pupae that were classified as parasitized but that did not yield flies were dissected to determine if dead parasitoids were present. Thirty *H. zea* larvae per replicate were reared on each treatment, and the experiment was replicated three times. The following variables were calculated and

analyzed by chi-square tests: percentage parasitized pupae, percentage of total pupae yielding flies, percentage of parasitized pupae yielding flies, and percentage of dead parasitized pupae containing visible dead parasitoids. Developmental time from host pupation to fly emergence was analyzed by ANOVA with the LSD test to separate means.

The above experiment indicated that emergence of flies from parasitized hosts reared on diets with 2-undecanone was reduced (see below). 2-Undecanone causes extensive mortality of *H. zea* pupae (Farrar and Kennedy, 1987a, 1988). Therefore, we hypothesized that this reduction in fly emergence was due to death and desiccation of the host before the parasitoid could complete development. An experiment was thus conducted in which parasitized *H. zea* pupae were held at high or low relative humidity and fly emergence recorded. Larvae were reared on diet with 2-undecanone (0.06%) or control diet and infested with planidia as above (50 *H. zea* larvae per treatment). Unparasitized controls of both dietary treatments were also included. At pupation, pupae of each dietary treatment were randomly divided into two groups of 20–25 pupae. For the treatments that received planidia, only pupae with visible respiratory funnels were retained; other pupae were discarded. One group of pupae was placed in a 15-cm Petri dish lined with moist filter paper, covered with a plastic lid, and sealed with Parafilm (high relative humidity treatment). The other group was placed in a similar Petri dish but with dry filter paper and a screen wire lid (low relative humidity treatment). All Petri dishes were held in an environmental chamber at 27°C, 30% relative humidity, and a 16:8 light–dark photoperiod. After seven days, the plastic lids of the high relative humidity dishes were replaced with screen wire lids. (Preliminary tests indicated that high relative humidity for the entire developmental period reduced emergence of both adult flies and moths). Three replications were done. The percentage of *H. zea* pupae in which the parasitoid reached pupation, the percentage of *H. zea* pupae yielding flies, and the percentage of parasitoid puparia yielding adult flies were calculated. These variables were analyzed by chi-square tests for differences between dietary treatments within relative humidity treatments and for differences between relative humidity treatments within dietary treatments. The unparasitized controls were analyzed similarly for differences in percentage moth emergence.

E. bryani *Experiments.* A similar experiment was conducted to evaluate indirect effects on *E. bryani*. In this case, however, it was not possible to infest each larva with a constant number of parasitoids as it was with *A. marmoratus*, since *E. bryani* larviposits directly into its host. To equalize exposure to the parasitoids, ten fifth-instar larvae reared on each treatment were placed in a single cage (44 × 44 × 44-cm wire-screen cage with a cloth sleeve) with 25–30 flies for 3 hr. To separate larvae from different dietary treatments after exposure, each larva was marked with a felt tip pen. Marks were made on the

anterior, posterior, left middle or right middle portions of the thorax–abdomen. Preliminary tests indicated no effect of marking on parasitism or larval survival. Dietary treatments included 2-tridecanone (0.3%), 2-undecanone (0.06%), 2-tridecanone plus 2-undecanone (0.3 + 0.06%), and control (untreated). Thirty larvae were reared through the fifth instar on each diet. They were exposed to flies in three groups of 10 larvae per treatment on the first or second day of the fifth instar. Larvae were returned to the diet on which they were reared and held through host pupation or death. Uneaten diet was then removed, so that parasitoid larvae would not contact it upon emergence from the host. Parasitoid puparia were held for adult emergence. Three replications (over time, with different flies each time) were performed. Percentage of hosts parasitized (data pooled across replicates) was calculated and analyzed by chi-square tests. Numbers of puparia and flies per host (not including hosts which pupated), percentage of puparia yielding adult flies, and time to fly emergence were subjected to ANOVA with the LSD test to separate means.

RESULTS AND DISCUSSION

A. marmoratus *Experiments.* Plant line significantly affected the mortality of *A. marmoratus* planidia on foliage ($F = 65.83$, treatment $df = 3$, error $df = 33$, $P \leq 0.0001$). Mortality was high on PI 134417 and BC-2 and intermediate on F_1 (Table 1, Test 1). In the second experiment, there was a highly significant interaction between plant line and the presence or absence of trichome exudate ($F = 20.69$, interaction $df = 2$, error $df = 25$, $P \leq 0.0001$). Among treatments with intact trichomes, plant line again significantly affected mortality ($F = 43.30$, treatment $df = 2$, error $df = 25$, $P \leq 0.0001$), although mortality was higher on all lines than in the first experiment. In contrast, among treatments without trichome exudate, rates of mortality were almost identical on all plant lines, with no significant differences ($F = 0.07$, treatment $df = 2$, error $df = 26$, $P = 0.9333$). Within plant lines, there was a significant effect of trichome exudate removal for PI 134417 ($F = 154.58$, treatment $df = 1$, error $df = 16$, $P \leq 0.0001$) and for F_1 ($F = 72.13$, treatment $df = 1$, error $df = 16$, $P \leq 0.0001$), but not for Better Boy ($F = 1.86$, treatment $df = 1$, error $df = 17$, $P = 0.1900$). These results indicate that the glandular trichomes of PI 134417 cause mortality of *A. marmoratus* planidia. The trichomes of F_1 also caused mortality, but at lower levels. There appeared to be some mortality due to trichomes on Better Boy as well in the second experiment, although this effect was not significant.

Levels of methyl ketones, as measured by gas chromatography, were low, but within the range reported by Kauffman and Kennedy (1989b) (Table 1). Levels in BC-2 were higher than expected and similar to those in PI 134417. Numbers of trichomes per unit area were within the expected range.

TABLE 1. TRICHOME DENSITY, METHYL KETONE CONTENT, AND MORTALITY OF *A. marmoratus* PLANIDIA ON FOLIAGE OF *M. sexta*-RESISTANT AND -SUSCEPTIBLE TOMATOES

Plant line	Trichome density (#/mm^2)	2-Tridecanone (μg/cm^2)	2-Undecanone (μg/cm^2)	Mortality (%)[a] Test 1 Trichomes present N	Test 1 Trichomes present Mean	Test 2[b] Trichomes present N	Test 2[b] Trichomes present Mean	Test 2[b] Trichomes absent N	Test 2[b] Trichomes absent Mean
Better Boy	10.7			10	20.7 A	10	68.9 Aa	10	36.8 Aa
F1	14.2			10	69.7 B	10	83.0 Ba	10	35.1 Ab
BC-2	15.0	15.05	3.50	10	92.8 C				
PI 134417	17.9	14.83	3.36	10	90.1 C	10	95.8 Ca	10	36.8 Ab

[a] Means separation within columns by LSD; means with the same capital letter are not significantly different ($P > 0.05$).
[b] In test 2 only, means separation within rows by F test; means with the same lower case letter are not significantly different ($P > 0.05$).

In tests with purified chemicals, probit analysis yielded LC_{50} values of 0.676 μg/cm^2 (95% fiducial limits: 0.635–0.718 μg/cm^2) for 2-tridecanone and 8.914 μg/cm^2 (95% fiducial limits: 8.239–9.672 μg/cm^2) for 2-undecanone. These data indicate that levels of 2-tridecanone, at least, in PI 134417 and BC-2 foliage were sufficient to kill *A. marmoratus* planidia. However, elevated levels of mortality were also seen on F_1, which has an intermediate density of trichomes, but no methyl ketones (Kauffman and Kennedy 1989b, Farrar and Kennedy, unpublished). Therefore, factors in the trichomes other than, or in addition to, methyl ketones must be involved in planidial mortality. Physical entanglement of planidia in trichome exudate may be involved. Because PI 134417 and BC-2 represent both increased densities of trichomes and the presence of methyl ketones, it is not possible to separate these effects using data collected in this study. However, given that 2-tridecanone is highly toxic to the planidia, it is likely that both physical and chemical factors are involved.

Rearing *A. marmoratus* on *H. zea* fed diets with methyl ketones indicated an indirect, or host-mediated, effect of 2-undecanone, but not of 2-tridecanone. At the time of host pupation, there were no significant differences ($P > 0.05$) between treatments in percentage parasitized pupae (i.e., the percentage with visible respiratory tunnels) (Table 2). However, the number of hosts yielding

TABLE 2. RATES OF PARASITISM, SURVIVAL, AND DEVELOPMENT OF *A. marmoratus* ON *H. zea* REARED ON DIETS WITH OR WITHOUT METHYL KETONES

Parameter	Treatment	*N*	Mean
% of total pupae parasitized[a,b]	Control	75	67 A
	2-Tridecanone (0.3%)	73	67 A
	2-Undecanone (0.06%)	79	52 A
	2-Tridecanone (0.3%) + 2-undecanone (0.06%)	62	56 A
% of total pupae yielding adult flies[b]	Control	75	71 A
	2-Tridecanone (0.3%)	73	64 A
	2-Undecanone (0.06%)	79	33 B
	2-Tridecanone (0.3%) + 2-undecanone (0.06%)	62	31 B
% of parasitized pupae yielding adult flies[a,b]	Control	50	96 A
	2-Tridecanone (0.3%)	49	94 A
	2-Undecanone (0.06%)	41	50 B
	2-Tridecanone (0.3%) + 2-undecanone (0.06%)	35	51 B
% of dead parasitized pupae containing dead parasitoids[a,b]	Control	1	—[c]
	2-Tridecanone (0.3%)	1	—[c]
	2-Undecanone (0.06%)	14	100 A
	2-Tridecanone (0.3%) + 2-undecanone (0.06%)	17	88 A
Days from host pupation to fly emergence[d]	Control	53	15.0 A
	2-Tridecanone (0.3%)	47	15.5 A
	2-Undecanone (0.06%)	18	15.4 A
	2-Tridecanone (0.3%) + 2-undecanone (0.06%)	27	15.1 A

[a] Parasitized *H. zea* pupae were those that had visible respiratory funnels of *A. marmoratus* under or around the wing pads.
[b] Means separation for each variable by chi square; means with the same letter are not significantly different ($P > 0.05$).
[c] Only one dead parasitized host pupa in this treatment.
[d] Means separation for this variable by LSD; means with the same letter are not significantly different ($P > 0.05$).

adult flies, expressed either as a percentage of total hosts or as a percentage of parasitized hosts, was significantly ($P \leq 0.05$) reduced on treatments with 2-undecanone. There was no difference between diet with 2-undecanone only and that with both 2-undecanone and 2-tridecanone. Of the pupae reared on these two diet treatments, nearly all parasitized pupae that did not yield adult flies contained dead parasitoid larvae. There was no significant treatment effect on developmental time of those flies that reached the adult stage ($F = 2.30$, treatment $df = 3$, error $df = 139$, $P = 0.0796$). Although this probability value (0.0796) suggests a trend, developmental time on 2-tridecanone plus 2-undecanone, which could be expected to be the most toxic treatment, differed from that on the control by only 0.1 day in this case. In hosts reared on diets con-

taining 0.06% 2-undecanone, ca. 50% of *A. marmoratus* die between host pupation and parasitoid emergence. There were, however, no indications of any effects of any treatments on mortality of parasitoids between penetration of the host and host pupation.

Holding parasitized pupae at different relative humidities strongly influenced the effects of 2-undecanone on *A. marmoratus*. At low relative humidity (ca. 30%), 2-undecanone significantly decreased the percentage of hosts yielding fly puparia relative to the control ($\chi^2 = 9.5033$, $df = 1$, $P = 0.0021$) (Table 3); this effect is consistent with that in the above experiment. However, at high relative humidity (ca. 100%), 2-undecanone did not affect on the percentage of hosts yielding fly puparia ($\chi^2 = 0.0393$, $df = 1$, $P = 0.8429$). In puparia that were produced at low humidity, 2-undecanone did not affect the percentage yielding adult flies ($\chi^2 = 0.1886$, $df = 1$, $P = 0.6641$). However, at high relative humidity the percentage of puparia yielding flies was significantly higher for parasitoids from hosts reared on 2-undecanone ($\chi^2 = 9.8718$, $df = 1$, $P = 0.0017$). Differences in puparial numbers are reflected in the percentages of parasitized hosts yielding adult flies: this percentage was significantly lower on the 2-undecanone treatment of low relative humidity ($\chi^2 = 7.0528$, $df = 1$, $P = 0.0079$), but significantly higher on this treatment at high relative humidity ($\chi^2 = 7.2473$, $df = 1$, $P = 0.0069$). The percentage of unparasitized *H. zea* pupae yielding moths was significantly lower on diet with 2-undecanone at both high and low relative humidity, with no evidence of any effect of relative humidity; these results are consistent with those of Farrar and Kennedy (1987b, 1988).

The lack of an effect of 2-undecanone on the percentage of parasitized hosts yielding puparia at high relative humidity, in contrast to the pronounced effect of 2-undecanone at low relative humidity, supports our hypothesis that 2-undecanone affects *A. marmoratus* through premature death and desiccation of the host. The lack of an effect of 2-undecanone on the percentage of puparia yielding adult flies, at least at low relative humidity, shows that the effect is not a pupal toxicity effect like that which affects *H. zea*. We have no satisfactory explanation for the higher percentage of puparia yielding flies on the 2-undecanone treatment at high relative humidity. This percentage was, however, lower for both dietary treatments at high relative humidity than at low relative humidity. The high relative humidity treatments were held at ca. 100% relative humidity for seven days. Preliminary tests indicated that maintaining the level of relative humidity through the entire pupal period resulted in extensive mortality both of *A. marmoratus* and of unparasitized *H. zea*. In the first experiment with methyl ketone-treated diet, the developmental time of *A. marmoratus* on hosts reared on diet with 2-undecanone was slightly, although not significantly ($P > 0.05$), longer than on control hosts (Table 2). A slight developmental delay could have resulted in puparia from hosts reared on 2-undecanone passing the stage susceptible to excessive moisture after the seventh day, when they were allowed to dry out.

TABLE 3. EFFECTS OF 2-UNDECANONE (0.06%) IN HOST DIET ON EMERGENCE OF *A. marmoratus* ADULTS FROM PARASITIZED *H. zea* PUPAE, AND ON MOTH EMERGENCE FROM UNPARASITIZED PUPAE AT HIGH AND LOW RELATIVE HUMIDITY

Parameter	Diet	Approx. humidity 30% *N*	30% Mean	100% *N*	100% Mean	χ^2 for humidity	$P > \chi^2$ ($df = 1$)
% of parasitized pupae yielding fly puparia[a]	Control	65	76	63	75	0.0426	0.8365
	2-Undecanone	66	50	65	71	5.3896	0.0203
	χ^2 for diet		9.5033		0.0393		
	$P > \chi^2$ ($df = 1$)		0.0021		0.8429		
% of fly puparia yielding adult flies	Control	49	92	47	40	23.6724	0.0001
	2-Undecanone	33	91	46	74	2.1365	0.1438
	χ^2 for diet		0.1886		9.8718		
	$P > \chi^2$ ($df = 1$)		0.6641		0.0017		
% of parasitized pupae yielding adult flies[a]	Control	65	71	63	30	19.5408	0.0001
	2-Undecanone	66	45	65	52	0.2750	0.6000
	χ^2 for diet		7.0528		7.2473		
	$P > \chi^2$ ($df = 1$)		0.0079		0.0069		
% of unparasitized *H. zea* pupae yielding moths	Control	80	98	81	96	0.2147	0.6431
	2-Undecanone	75	36	74	34	0.0295	0.8636
	χ^2 for diet		65.3060		64.0410		
	$P > \chi^2$ ($df = 1$)		0.0001		0.0001		

[a] Parasitized pupae were those that had visible respiratory funnels of *A. marmoratus* under or around the wing pads.

In the field, *H. zea* pupates in the soil. The effect of 2-undecanone on *A. marmoratus* in the field would therefore probably depend on soil moisture. *A. marmoratus* might be killed under dry conditions but not under higher moisture conditions.

E. bryani *Experiments.* Results of the test for indirect effects on *E. bryani* differed markedly from those of the *A. marmoratus* experiments. 2-Undecanone had no significant effect on any parameter that was measured for *E. bryani* (Table 4). In contrast, 2-tridecanone, which had no effect on *A. marmoratus*, caused a decrease in the number of puparia per host for those hosts that yielded puparia. This effect did not, however, result in any significant differences in percentage parasitism (the percentage of hosts yielding one or more puparia). The percentage of puparia yielding adult flies was not affected ($F = 1.00$, treatment $df = 3$, error $df = 15$, $P = 0.4208$), nor was developmental time ($F = 0.01$, treatment $df = 3$, error $df = 910$, $P = 0.9994$).

Several ways exist in which the number of *E. bryani* puparia obtained from apparently parasitized host larvae is reduced. One way is superparasitism, in which there are more parasitoid larvae in the host than can develop on available

TABLE 4. RATES OF PARASITISM, SURVIVAL, AND DEVELOPMENT OF *E. bryani* ON *H. zea* REARED ON DIETS WITH OR WITHOUT METHYL KETONES

Parameter	Treatment	*N*	Mean
% of hosts yielding one or more puparia[a]	Control	77	60 A
	2-Tridecanone (0.3%)	78	54 A
	2-Undecanone (0.06%)	78	63 A
	2-Tridecanone (0.3%) + 2-undecanone (0.06%)	70	61 A
Number of puparia per host yielding one or more puparia[b]	Control	46	9.0 A
	2-Tridecanone (0.3%)	42	5.4 B
	2-Undecanone (0.06%)	49	8.1 A
	2-Tridecanone (0.3%) + 2-undecanone (0.06%)	43	4.7 B
% of puparia yielding adult flies[b]	Control	5	74 A
	2-Tridecanone (0.3%)	5	76 A
	2-Undecanone (0.06%)	5	73 A
	2-Tridecanone (0.3%) + 2-undecanone (0.06%)	5	81 A
Days from exposure of hosts to flies until fly emergence[b]	Control	298	12.3 A
	2-Tridecanone (0.3%)	174	12.2 A
	2-Undecanone (0.06%)	281	12.3 A
	2-Tridecanone (0.3%) + 2-undecanone (0.06%)	163	12.3 A

[a] Means separation for this variable by chi square; means with the same letter are not significantly different ($P > 0.05$).

[b] Means separation for these variables by LSD; means with the same letter are not significantly different ($P > 0.05$).

food. In this case, which is seen regularly in colony maintenance, the host dies within a few days after exposure to the flies and has the same general appearance as other parasitized hosts, but it yields no puparia. However, in the experiment only three larvae died prior to pupation but yielded no parasitoids. All of these larvae were reared on diet with 2-tridecanone. None were seen in the treatment that received both 2-tridecanone and 2-undecanone and that showed a similar reduction in the number of puparia per host. Therefore, it is unlikely that superparasitism was a factor in the effect of 2-tridecanone. Superparasitism would also require increased larviposition, which would not be expected in hosts reared on 2-tridecanone. Several other factors could be involved, however, including: reduction in the number of flies larvipositing in each host, reduction in the number of larvae inserted by each fly into each host, and mortality of parasitoid larvae in the host. However, our experimental design did not allow us to determine which of these factors was operating.

The methyl ketone/glandular trichome-based insect resistance of PI 134417 clearly adversely affects both *A. marmoratus* and *E. bryani*. These effects are different for each species, however, and at least a portion of these differences is apparently related to differences in life histories of the two tachinid species. *A. marmoratus* planidia are killed by the foliar trichomes. *E. bryani* larvae are not directly affected by foliar trichomes since they are deposited directly into the host. 2-Undecanone indirectly affects *A. marmoratus*, evidently by killing its host before the parasitoid can complete development. *E. bryani* escapes any such effects by completing development before the host pupates. Unlike *A. marmoratus*, *E. bryani* larvae are apparently susceptible to indirect effects of 2-tridecanone. This difference may be due to differential susceptibility to the toxic effects of 2-tridecanone, rather than any differences in life histories.

Previous studies have shown that variation in life histories and/or physical characteristics can also influence the effects of methyl ketone/glandular trichome-based resistance on other parasitoid species. Kashyap et al. (1991) found that the egg parasitoids *Telenomus sphingis* and *Trichogramma pretiosum* Riley both became entangled in trichome exudate. However, *T. sphingis*, the larger of the two species, can free itself through prolonged grooming, while *T. pretiosum* cannot free itself and eventually dies. In *Campoletis sonorensis*, rates of parasitism are reduced on PI 134417 (Farrar and Kennedy, unpublished data). Since *C. sonorensis* oviposits directly into its host, similar to *E. bryani*, this effect is not obviously related to the life history of *C. sonorensis*. However, *C. sonorensis* larvae emerge from the host on the foliage of the hosts' food plant, and, on PI 134417, are killed by the trichomes (Kauffman and Kennedy, 1989a,c). Species of parasitoids that do not emerge from the host on the foliage would avoid these effects.

In conclusion, the life history of a parasitoid can influence whether and how the parasitoid may be affected by a given host-plant resistance factor.

Variation in life histories, thus, may be one reason for the lack of general patterns in such interactions reported in the literature. It is therefore important to consider the life history of the insect when studying these interactions. So doing will increase the understanding of the nature of these interactions and better enable us to integrate biological control and host-plant resistance in pest-management systems.

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IDENTIFICATION OF TRAIL PHEROMONE PRECURSORS FROM SUBTERRANEAN TERMITE, *Coptotermes formosanus* SHIRAKI (ISOPTERA: RHINOTERMITIDAE)

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Abstract—Whole-body extracts of the termite, *Coptotermes formosanus* Shiraki served for examining the presence of trail pheromone precursor(s). Three trail pheromone precursor candidates, identified as dodecatrienyl stearate, dodecatrienyl oleate, and dodecatrienyl linoleate, were isolated using various chromatographic methods in conjunction with bioassay and by capillary GC-MS analyses.

Key Words—Subterranean termite, *Coptotermes formosanus*, Rhinotermitidae, Isoptera, trail pheromone precursor, dodecatrienyl fatty acid esters.

INTRODUCTION

Only (*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol (DTE-OH) has been identified as a trail pheromone of rhinotermitid termites. This compound was isolated from wood decayed by a brown rot fungus, *Gloeophyllum trabeum* (Matsumura et al., 1968, 1969) and proved identical with a trail pheromone of workers of *Reticulitermes virginicus* (Banks) on the basis of the biological activity (Tai et al., 1969).

We recently isolated the trail pheromone from other two rhinotermitid ter-

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mites, *Reticulitermes speratus* (Kolbe) (Tokoro et al., 1991) and *Coptotermes formosanus* Shiraki (Tokoro et al., 1989), and identified it as DTE-OH (Yamaoka et al., 1987; Tokoro et al., 1992a). Heterogeneric isolations suggest that DTE-OH may be a trail pheromone common to the family Rhinotermitidae.

Because the amount of the trail pheromone per individual was extraordinarily small (i.e., a worker termite could produce a 1.5-m long secretion of effective trail of the pheromone), we hypothesized that the pheromone might be stored in the termite body in the form of a precursor. Whole-body extracts of *R. speratus* workers were subjected to various chemical isolation procedures and bioassays to examine the presence of trail pheromone precursor (Tokoro et al., 1990). Fractions that mainly contained fatty acid esters were obtained from hexane extracts by means of silica-gel column chromatography. Trail-following activity of the fractions was activated by alkaline hydrolysis, while the original fractions did not show any conspicuous activity. Bioassay showed that the activity of hydrolyzed product was approximately 20 times higher than the original hexane extract. This suggests that the precursor candidate could be stored in termite bodies as an esterified form. Chemical analyses revealed that the complete structure of the hydrolyzed product was identical to DTE-OH (Tokoro et al., 1990).

Whole-body extracts of *C. formosanus* were also examined for the presence of a trail pheromone precursor. The hydrolyzed substance of several fractions prepared by silica-gel column chromatography showed high trail-following activity and was identified as DTE-OH (Tokoro et al., 1992b). The findings suggest that the trail pheromone of *C. formosanus* could be stored as with *R. speratus*.

The main purpose of the present investigation was to isolate and to identify the trail pheromone precursors of *C. formosanus*.

METHODS AND MATERIALS

Insects. Colonies of *C. formosanus* were collected in the city of Wakayama, Japan, and kept in the laboratory (Uji city) for about seven years with Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) as food source at 28 ± 2°C and ca. 80% relative humidity. Only undifferentiated pseudergates (workers) of *C. formosanus*, ca. 938 g and ca. 310,000 in number, were selected for the present experiment.

Chemicals. Synthetic (*Z,Z,E*)-3,6,8-dodecatrien-1-ol (DTE-OH) (Ikeda et al., 1984) was supplied by Dr. H. Yamamoto (Nagoya University, Japan). Standard dodecanoic fatty acid esters were prepared by an esterification reaction with *p*-toluenesulfonic acid as catalyst and purified by high-performance liquid chromatography (HPLC). All other reagents were purchased from Nacalai Tesque Inc. (NTI; Kyoto, Japan).

Confirmation of Precursor Candidate. Since precursor candidates would not always show trail-following activity, existence of precursor candidates during the purification procedures was examined after alkaline hydrolysis of isolates followed by termite bioassay. The trail-following activity was estimated by a modified open-field bioassay. When three or more of the nine workers followed the 15-cm trail treated with 2 μl test solution, the sample was designated positive. Tenfold dilution series and more highly diluted solutions of each sample were employed to determine the "minimum effective worker equivalent (MEWE)" [worker equivalent (WE)/15-cm trail]. Strength of trail-following activity was evaluated by MEWE. Details of the methods of hydrolysis and bioassay were described previously (Tokoro et al., 1989, 1990, 1991).

Extraction. The worker termites (ca. 938 g) had been fed with moistened filter paper as food source for about five days before extraction so that the existing intestinal materials were completely replaced with filter paper. The termites were then soaked in *n*-hexane (ca. 300 ml per 20,000 worker termites) for about 10 days without homogenization at $-20°C$.

Isolation. The hexane extracts were prepared by filtration and concentration by a rotary vacuum evaporator to obtain a yellowish crude lipid (ca. 71 g). A large portion of this crude lipid (ca. 68 g and ca. 300,000 worker equivalent) was first fractionated by silica-gel column chromatography with *n*-hexane (Hx)–ethyl acetate (EtOAc) and methyl alcohol (MeOH) eluants, and the cycle repeated once again (Figure 1).

The active fractions, from silica-gel column chromatography above, were further fractionated by HPLC with diethyl ether (Er)/Hx eluants (HPLC 1: 2.5% Er/Hx; HPLC 2: 0.5% Er/Hx; both flow rates were 1.0 ml/min). The HPLC was a model LC-9A (Shimadzu, Kyoto, Japan) pump connected in series to a model SPD-6AV (Shimadzu) UV-VIS spectrophotometric detector and to a model ERC-7511 (Erma, Tokyo, Japan) refractive index (RI) detector. The analytical column used was a Cosmosil 5SL (silica-gel, 25 cm $\times$ 4.6 cm ID, particle size 5 μm; NTI) with a Cosmosil 10SL (5 cm $\times$ 4.6 cm ID) guard column. The conjugated double bond of the active substance was detected by UV-VIS detector at 234 nm and additionally by the RI detector and fractionated at each peak for bioassay.

The final purification step was carried out by capillary gas chromatography (GC), which was conducted with a model GC-15A (Shimadzu), equipped with an FID detector. A fused silica wide-bore capillary column was a CBP1-W12-300 (12 m $\times$ 0.53 mm ID, Shimadzu) coated with methyl silicone (coating width: 3 μm). Analytical conditions were as follows: the oven temperature was kept at 180°C for the first minute and then raised at 20°C/min to 320°C and finally maintained at 320°C for 10 min; injection temperature was 320°C and detector temperature 350°C; the carrier gas was helium at a velocity of approximately 60 cm/sec. The outlet gas was fractionated for each peak, and the trail

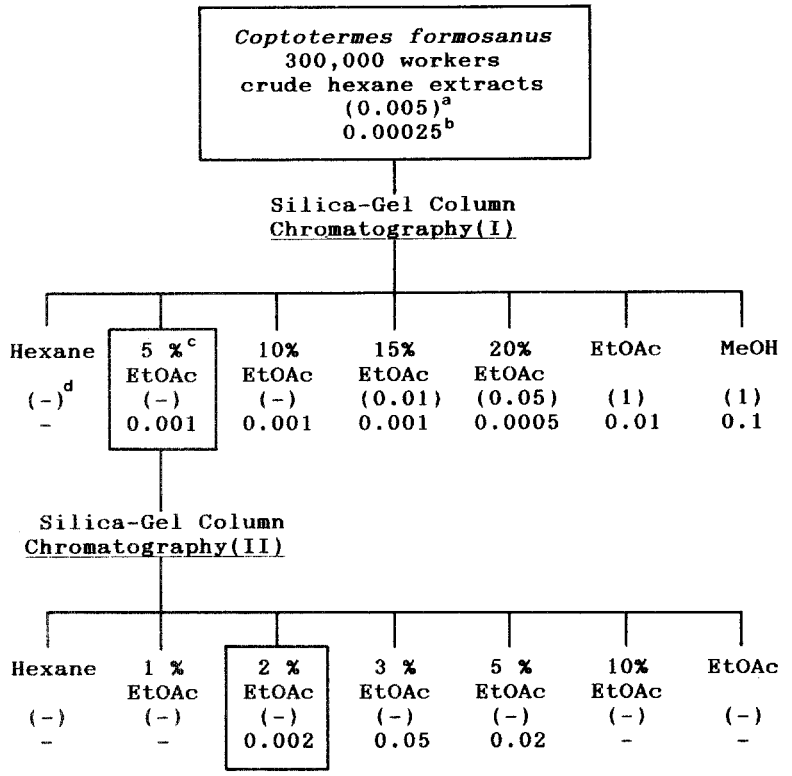

FIG. 1. Results of silica-gel column chromatography of the crude hexane extracts from *C. formosanus*. [a]The values in parentheses are the trail-following activity before hydrolysis. The activity was estimated by a modified open-field bioassay (Tokoro et al., 1991). When three or more of nine workers followed the 15-cm trail, the sample was designated positive. Tenfold dilution series and more highly diluted solutions of each sample were employed to determine the minimum effective worker equivalent (MEWE) (worker-equivalent/15-cm trail). [b]The values are the trail-following activity as MEWE (WE/15-cm trail) after alkaline (2% KOH/MeOH) hydrolysis. [c]Percentage by volume of ethyl acetate (EtOAc)/*n*-hexane (Hx) as eluants. [d]The signs indicate no trail following response or insufficient response (i.e., the estimated value was more than 10 WE/15-cm trail) for trail-following before (in parentheses) and after alkaline hydrolysis.

following activity after hydrolysis was determined. To obtain GC fractions for analysis, a glass tube cooled with Dry Ice in the middle portion was indirectly connected to the metal head of the FID detector with a Teflon tube. The glass tube was rapidly rinsed with diethyl ether to extract the effluent for the later evaluation of trail-following activity.

The active GC peak was analyzed by a capillary gas chromatograph–mass

spectrometer (GC-MS). The GC-MS was a model 5970 gas chromatograph (Hewlett-Packard (HP), combined with a model M-80B (Hitachi, Ibaraki, Japan) mass spectrometer equipped with a model 0101 on-line data system (Hitachi). A cross-linked methyl silicone fused silica capillary column (12.5 m × 0.2 mm ID, 0.2 μm, (HP) was used. The conditions were as follows: oven temperature of GC increased at a rate of 15°C/min over the range of 200°C (1 min) to 300°C (10 min); injection temperature was 300°C; and the carrier gas was helium at a velocity of approximately 60 cm/sec. The ion source temperature was 300°C, and the ionization voltage was 70 eV.

RESULTS AND DISCUSSION

Bioassay results of trail-following activity of the crude hexane extracts after hydrolysis indicated that a 0.00025 WE extract induced trail-following behavior over a 15-cm trail: the MEWE was identified as 0.00025 WE/15-cm trail. The activity was about 20 times higher than that of extract before hydrolysis (0.005 WE/15-cm trail) by MEWE. However, the mechanism of secretion of the pheromone (MacFarlane, 1983) was not clear in *C. formosanus*, since the hexane extract before hydrolysis indicated that a 0.005 WE extract induced trail-following over a 15-cm trail, and a 1 WE extract could induce a 30-m-long effective trail; a worker termite could produce a 30-m effective trail. While 0.00025 WE of the hydrolyzed hexane extract induced trail-following over a 15-cm trail, a worker termite could produce a 600-m effective trail. Therefore, there might be an appreciable quantity of trail pheromone stock as an esterified form in a *C. formosanus* worker body. If the termites produced the esterified pheromone by themselves, the storage system seemed to become more efficient. The results of a preliminary experiment clearly indicated that both pheromone and precursor candidates were stored in a sternal gland, as described elsewhere (Tokoro et al., 1992b).

Bioassay results on the hexane extracts after silica-gel column chromatographies are shown in Figure 1. The native trail pheromone was eluted mainly in the 20% EtOAc/Hx fraction, and the result was similar to that of a previous report (Tokoro et al., 1989). Active trail-following behavior was produced by several fractions after hydrolysis in this experiment (Tokoro et al., 1992b), although as for *R. speratus*, the fractions eluted with 5–10% EtOAc/Hx were activated (Tokoro et al., 1990). In the case of *C. formosanus*, the component of the fractions eluted with 5–10% EtOAc/Hx was activated by alkaline hydrolysis, and the component of the fractions that contained the native active substance was also activated. Each hydrolyzed product from the activated fractions was identical to DTE-OH (Tokoro et al., 1992b).

The active 5% EtOAc/Hx fraction was selected for further isolation. This

fraction contained the fatty-acid esters, including a vast quantity of triglycerides. The active component of the fraction was eluted with 2% EtOAc/Hx by the secondary silica-gel column chromatography (Figure 1).

The precursor candidates from the 2% EtOAc/Hx fraction were eluted by HPLC 1 in a peak at a retention time (R_t) of around 3 min. It seemed that the retention time of the precursor candidates was earlier than that of the triglycerides contained in the same fraction. On the secondary HPLC preparation (HPLC 2), the precursor candidates were eluted in peaks 1 (R_t 38–43 min) and 2 (R_t 47–53 min). The retention time of peak 1 was longer than that of lauryl laurate (R_t 22.3 min), and a little shorter than lauryl acetate (R_t 48.5 min). The retention time of peak 2 was close to that of lauryl acetate. Subsequently, both of the fractions were analyzed by capillary GC.

The GC retention times of the precursor candidates from both fractions (peaks 1 and 2) were about 10.5 min (peaks a and b, respectively). This retention time was a little longer than that of lauryl stearate (R_t 10.2), which had the same carbon number as the alcohol moiety of DTE-OH. The acid moiety of the precursor candidate, consequently, is expected to have 18 carbons if the precursor candidate is a stearate ester. However, the half-height width of peak a indicated that it consisted of multiple compounds.

Capillary GC-MS data of peaks a and b are shown in Figures 2 and 3. The components of peak a were separated into two component peaks by the total ion chromatogram (TIC) (peaks X and Y in Figure 2A). The electron impact (EI) mass spectrum of the peak X (scan 361; Figure 2B) showed a molecular ion at m/z 446 (ca. 6%). It also showed a diagnostic base peak ion at m/z 162 (M−284), which came from McLafferty rearrangement, that is, elimination of the fatty acid moiety of the esterified pheromone, as in the case of the DTE-acetate (Yamaoka et al., 1987). The prominent diagnostic fragment ions of DTE-OH, m/z 91, 105, 119, and 133 were observed (Tokoro et al., 1989). The present analysis indicated that the component of peak X corresponded to dodecatrienyl stearate.

The mass spectrum of the peak Y (scan 346; Figure 2C) showed a molecular ion at m/z 444 (ca. 1.4%) and a diagnostic base peak ion at m/z 162 (M−282) derived from McLafferty rearrangement, along with prominent diagnostic fragment ions of DTE-OH. These data indicated that peak Y was dodecatrienyl oleate.

The mass spectrum of peak Z (scan 353; Figure 3), which corresponded to peak b of the capillary GC chromatogram,showed a molecular ion at m/z 442 (ca. 1.6%) and a diagnostic base peak ion m/z 162 (M−280) and also prominent characteristic fragment ions of DTE-OH at m/z 91, 105, 119, and 133 were observed. These data indicated that the component of peak Z corresponded to dodecatrienyl linoleate.

Finally, stearic acid ester of DTE-OH (DTE-stearate), oleic acid ester of

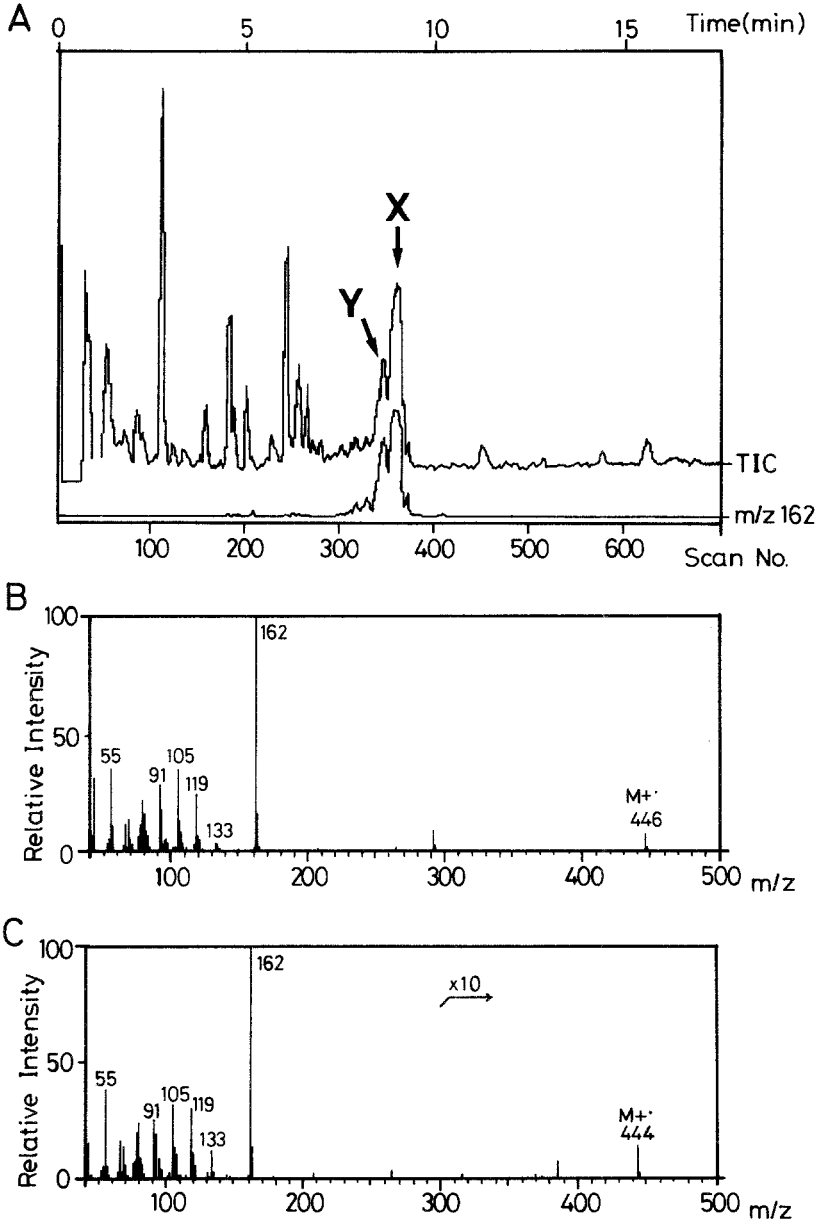

FIG. 2. Results of capillary GC-MS analyses. Total ion chromatogram (TIC) and mass chromatogram (*m/z* 162) of the active fraction (A). The arrows indicate the active peaks: scan 361 (peak X) and scan 346 (peak Y). Also shown are mass spectra of peaks X (B) and Y (C).

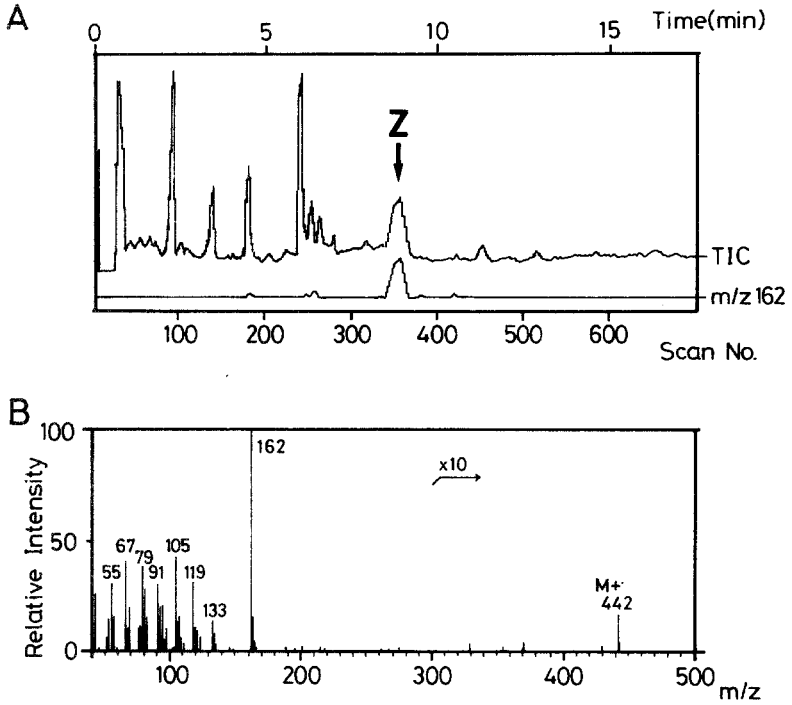

FIG. 3. Results of capillary GC-MS analysis. Total ion chromatogram (TIC) and mass chromatogram (*m/z* 162) of the active fraction (A). The arrow indicates the active peak: scan 353 (peak Z). Also shown is the mass spectrum of peak Z (B).

DTE-OH (DTE-oleate), and linoleic acid ester of DTE-OH (DTE-linoleate) were synthesized and subjected to capillary GC and capillary GC-MS analyses. The analyses demonstrated that the three peaks (X, Y, Z) were identical with the three compounds, respectively.

Thus, the precursors are stored as fatty acid esters in the *C. formosanus* bodies. Amounts of the isolated precursor candidates per individual worker termite estimated from GC analysis were ca. 100 pg for the DTE-stearate, ca. 25 pg for the DTE-oleate, and ca. 25 pg for the DTE-linoleate, approximately three times larger than that of the native pheromone isolated in a previous experiment (ca. 50 pg). Results of the bioassays after isolation indicated that the isolation procedure caused a decrease in activity (from 0.002 to 0.005 WE/15-cm trail) of the 2% EtOAc/Hx fraction (Figure 1). This may be due not only to unavoidable loss during the isolation but also dispersion of other active minor components esterified with DTE-OH.

It is still necessary to prove whether these candidates are true precursors

of the trail pheromone by other methodologies such as a tracer technique. If the precursor candidates are biosynthesized by *C. formosanus* themselves, the resultant decreased volatility would be advantageous for a longer storage. Yamaoka et al. (1985) demonstrated that the sex pheromone (bombykol) of the silkworm, *Bombyx mori*, was inactivated as bombykol linolenate bound to protein in the hemolymph. It is therefore possible that the precursor candidates bind with protein. Although further investigation is needed in order to account for the significance of esterification of the termite pheromone, it may be related to the transportation of the pheromone in the bodies if the precursors bind a protein. Therefore, it is of interest to determine the organ for biosynthesizing those precursor candidates, since the storing organ of those was already determined as the sternal gland (Tokoro et al., 1992b).

Based on the results of the first silica-gel column chromatography, it seems possible that there exist other kinds of precursor candidates of the trail pheromone in the hexane extracts, as described elsewhere (Tokoro et al., 1992b). It is necessary to demonstrate the existence of other precursor candidates and entirely reveal the supplementary system of the trail pheromone.

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ERRATUM

The following figure 1 continuation originally appeared on page 2534 of Journal of Chemical Ecology, Volume 17, Number 12 (December 1991) in the article, "Electroantennogram Responses of the Southern Pine Beetle Parasitoid *Dinotiscus dendroctoni* (Ashmead) (Hymenoptera: Pteromalidae) to Potential Semiochemicals" by S. M. Salom, G. Birgersson, T. L. Payne, and C. W. Berisford. The continuation of figure 1, "Mean percent EAGs ± SE from *Dinotiscus dendroctoni* to bark beetle pheromones and tree-produced volatiles, relative to EAG responses by each sex to the standard mixture of oxygenated monoterpenes. Each concentration was presented as 5 μl aliquots," appears here with its proper headings.

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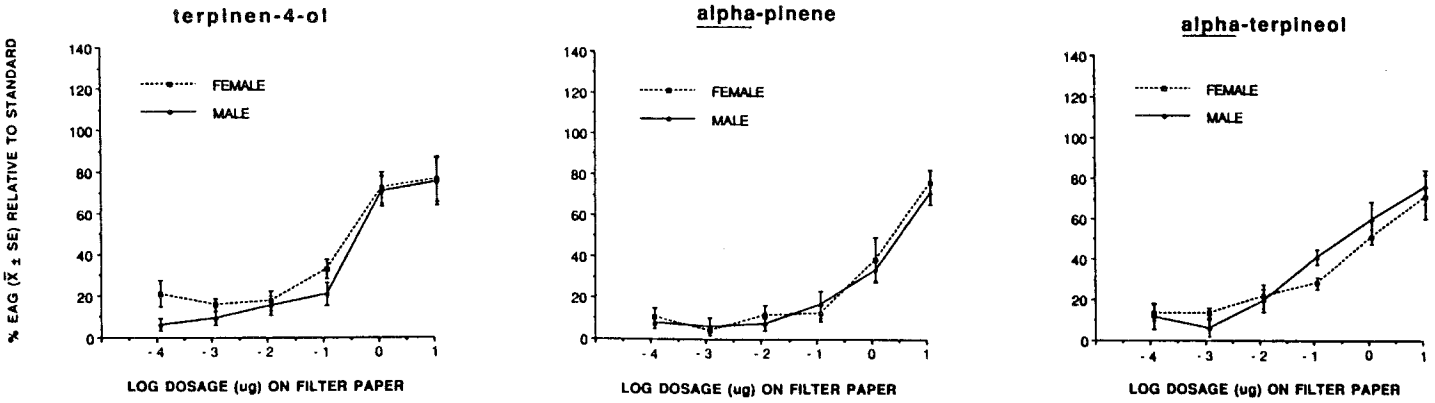

FIG. 1. Continued

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Book Review

Chemical Signals in Vertebrates 5. D.W. Macdonald, D. Müller-Schwarze, and S.E. Natynczuk. Oxford, U.K.: Oxford University Press, 1990. £55.00, 659 pp.

This book is yet another excellent contribution on chemical signals and vertebrate biology arising from the series of Chemical Signals in Vertebrates Conferences. The fifth conference was held in August 1988 at the Department of Zoology, University of Oxford, Oxford, United Kingdom.

This meeting was arranged to synthesize current technical and experimental progress, from an interdisciplinary perspective, in unraveling the fascinating but complex world of chemical signals in vertebrate biology. The book is divided into six sections: semiochemistry, neurophysiology and anatomy, reproduction and primers, social behavior, predators and prey, and applications.

The first section presents contributions ranging from Albone's general discussion of mammalian semiochemistry and the need for interdisciplinary collaboration of chemists and biologists to the specific dynamic solvent effect sampling technique described by Apps. Novotny et al. discuss the advantages of using current leads in rodent olfactory communication to acquire more general information on mammalian olfaction. Three papers discuss human olfactory communication, particularly the role of 16-androstene steroids and related compounds. A very interesting article on identification and practical use of host odor attractants for tse tse flies is given by Hall. This paper would have been more appropriate in the section on applications.

The second section has an elegant study by Keverne and Kaba, who discuss a neural mechanism for olfactory learning in mammals, with respect to pregnancy block in the mouse. A paper by Cocke and Thiessen outlines evidence that a chemical signal can induce significant alterations in the immune system in laboratory mice. This study provides considerable insight into the underlying mechanism of the actual process linking stress with immune suppression in rodents. Additional studies on physiological relationships to chemical signals include alarm pheromones in ostariophysan fishes and amniotic fluids in sheep. Reports on the anatomy of the vomeronasal organ of the prairie vole and Asian elephant reflects the breadth of interest in olfactory systems in mammals. This pattern is further supported by an introductory study of white eye secretion in mountain beaver and the evolutionary development of posterolateral gland development in microtine rodents.

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Section three includes excellent review papers on pheromonal contraception, male preferences for females of different reproductive conditions, fetal olfaction, responses of human neonates to odors of lactating females, and hormonal pheromones in goldfish. An interesting investigation of reproduction in meadow voles by Storey concluded that chemical cues were not sufficient for male-induced pregnancy disruption. This result contrasts with earlier work, which reported that chemical and physical cues were equally effective in the pine vole. Werner and Clarke discuss the relationship of olfactory signals to sexual development in field voles and the potential role in population regulation. The effects of female urinal and vaginal chemical signals in gerbils and hamsters, respectively, on male behavior are discussed in two additional papers. Individual recognition via body odor or chemical fingerprint in the reproductive behavior of mammals and its relationship to the major histocompatibility complex (MHC) is discussed by Brown et al. This intriguing subject is further discussed by Beauchamp et al. with respect to genetic determination of individual mouse odor and its relevance to individual and kin recognition. Additional contributions include the effects of mouse urine on development and behavior and the relationship of dominance status to the Bruce effect in house mice. With respect to large mammals, Marchinton et al. report on the behavioral components of scent marking in white-tailed deer.

The fourth section covers chemical signals and social behavior in a wide range of vertebrate species. Gosling reviews the role of scent marking of resource holders with respect to alternative mechanisms for advertising the costs of competition. An interesting study on an olfactory mechanism for female choice and residency assessment in the European rabbit is reported by Engel. Differential responses of farm cats to conspecific urine from different sources and the significance to group discrimination is discussed by Passanisi and Macdonald. Three papers report on various aspects of urine or scent marking in wolves and arctic foxes, including relationship to social status, reproductive state, and territorial demarcation. Primate social behavior is represented by studies of conspecific chemical signals in ringtailed lemurs, possibly related to the pattern of female dominance, and the scent marking behavior of wild marmosets having different functions depending on glandular origin.

Spatial distribution, territoriality, and scent marking are discussed for aardwolfs, European moles, pikas, and snakes. Social behavior and scent marking is reported for captive Virginia opossums. The complex network of olfactory communication in wild house mouse populations reported by Hurst suggests that mice urine-mark their physical environment both for their own orientation and to communicate chemically with each other. Aldhous reports on the role of olfactory cues in kin discrimination by mice. Sandnabba discusses the differences between aggressive and nonaggressive male mice in odor signals and

marking behavior, concluding that differences reduce fighting between potential rivals and communicate the aggressive status of a male to the female. Papers on conspecific odors in the rat and the semiochemical sources sampled by rats during social behavior complete this section.

The fifth section includes a comprehensive review of the responses of vertebrates to chemicals from predators by Weldon. Burghardt selectively reviews prey recognition and foraging in vertebrates with respect to diversity, ontogeny, and information transfer and uses snakes to demonstrate the complex interactions of prey cue recognition with various factors. Chemosensory orientation by eastern garter snakes with earthworm castings playing a major role in prey location is discussed by Gillingham et al. Cooper reviews the distribution and use of chemical cues in feeding among squamate families. Duvall and Chiszar describe a series of elegant experiments on the chemical ecology of vernal migration of prairie rattlesnakes. This combination of parallel field and laboratory analyses has yielded considerable insight into the dynamics of chemical foraging for prey by rattlesnakes. The ecological significance of identification of snake predators by eublepharid lizards is discussed by Dial. Robinson describes the avoidance responses of *Apodemus* and *Clethrionomys*, and the suppressed feeding behavior of European rabbits, to a synthetic anal-gland constituent from mink. These results are discussed in light of similar responses between predator-based odors and small mammal prey. Doving provides an intriguing assessment of chemical signals and migrating salmon, with particular emphasis on the importance of olfaction in selecting the appropriate layers of water to direct migration.

The sixth section, on applications, includes a comprehensive review of animal and plant odors for managing vertebrates by Müller-Schwarze. This discussion includes application of chemical signals to animal husbandry, biological pest control, wildlife management, and population monitoring. Abbott et al. report on the chemistry and behavior of a natural deer repellent from lion dung. Their short-term results on repellent efficacy are encouraging. However, their claim that this is the first multidisciplinary study in development of a predator-based repellent is clearly not true as there are several other interdisciplinary groups that have conducted research in this field. Signoret reports on the complex role of chemical communication in reproductive and social behaviors of domestic ungulates. A two-part sequence based on olfaction and sample contact is described by Rivard and Klemm for bull response to estrous pheromone in cattle. Sommerville et al. report on a dog discrimination bioassay for compounds in human sweat and a potential relationship to genotype. Bradshaw et al. discuss the potential use of anal sac volatiles from domestic dogs.

The editors should be congratulated on the considerable effort expended to organize and produce this excellent volume of papers. A suggestion for future volumes might be the addition of an abstract or summary in each paper as

required in contributions to scientific journals. I highly recommend this book for those working in this exciting and rapidly progressing field.

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THE RESPONSE OF MOUNTAIN PINE BEETLE (*Dendroctonus ponderosae*) TO LODGEPOLE PINE TREES BAITED WITH VERBENONE AND *exo*-BREVICOMIN

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Abstract—*exo*-brevicomin, a multifunctional pheromone of the mountain pine beetle, *Dendroctonus ponderosae*, was tested at release rates of 0.5 and 2.5 mg/day alone and in combination with the antiaggregation pheromone verbenone against unbaited controls. Significantly more lodgepole pine *Pinus contorta* var. *latifolia* trees were attacked, and at higher densities, with both release rates of *exo*-brevicomin than with all other treatments. Verbenone significantly reduced the response of mountain pine beetles to *exo*-brevicomin. Verbenone alone did not reduce the number of trees attacked by mountain pine beetle or the attack density when compared to the unbaited controls.

Key Words—*Dendroctonus ponderosae*, mountain pine beetle, Coleoptera, Scolytidae, pheromone, antiaggregation, verbenone, *exo*-brevicomin.

INTRODUCTION

The mountain pine beetle, *Dendroctonus ponderosae* Hopk., is currently the most destructive insect pest of mature forests in western North America (Gibson, 1989; Van Sickle, 1989). Commercially available aggregation semiochemicals are used to attract and concentrate mountain pine beetle populations into trees that are scheduled for subsequent direct control treatment (Borden and Lacey, 1985; McMullen et al., 1986). These aggregation semiochemicals include *trans*-verbenol, a pheromone produced by the female beetle that attracts mainly males (Pitman et al., 1968; Pitman, 1971; Conn et al., 1983; Borden et al., 1983),

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exo-brevicomin, a pheromone produced by the male beetle that attracts mainly females (Rudinsky et al., 1974; Conn et al., 1983), and the monoterpene myrcene, a kairomone that synergizes the insects response to their aggregation pheromones (Billings et al., 1976; Conn et al., 1983; Borden et al., 1983).

In recent years research on the mountain pine beetle has focused on antiaggregation pheromones. Verbenone (4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-one) has been identified in a number of beetles in the family Scolytidae and found to have antiaggregative properties. These species include *D. frontalis* Zimm., *D. brevicomis* LeC. (Renwick, 1967; Renwick and Vité, 1970; Hughes and Pitman, 1970; Payne et al., 1978; Bedard et al., 1980), *D. adjunctus* Bland. (Livingston et al., 1983), *Ips typographus* (L.) (Bakke, 1981; Schlyter et al., 1988), *I. paraconconfusus* Lanier (Byers and Wood, 1980), and *Tomicus piniperda* L. (Schlyter et al., 1988; Byers et al., 1989). In the mountain pine beetle, verbenone was identified in hindguts of emergent and feeding females (Pitman et al., 1969) and trapped in air passed over male–female pairs (Rudinsky et al., 1974).

Verbenone inhibited the response of mountain pine beetle to traps baited with attractants in field tests (Ryker and Yandell, 1983; Borden et al., 1987; Schmitz and McGregor, 1990). Lodgepole pine stands baited with verbenone alone or with verbenone and attractant bait had lower percentages of mass-attacked trees than blocks baited with attractants alone or unbaited blocks, although differences were not always statistically significant (Amman et al., 1989; Lindgren et al., 1989). In ponderosa pine, *P. ponderosae* Lawson, no significant difference in number of attacked trees was found between 2.5-acre plots treated with four densities of verbenone capsules and untreated plots. It was noted that some of the pine trees to which the verbenone capsules were attached were attacked by the mountain pine beetle (Bentz et al., 1989).

Although *exo*-brevicomin (*exo*-7-ethyl-5-methyl-6,8-dioxabiclyo[3.2.1]-octane) (Silverstein et al., 1968) has been found to be an aggregation pheromone for the mountain pine beetle, there is evidence that it has a multifunctional role in the insect's chemical ecology; at low release rates it induces an aggregative response and at high release rates it induces an antiaggregative response (Rudinsky et al., 1974). Experiments testing various release rates of *exo*-brevicomin as a pheromone for the mountain pine beetle on traps and trees with and without other attractants can generally be summarized as follows: at release rates of 0.04 mg/day and lower, no response by mountain pine beetle was found (Libbey et al., 1985); at 0.05 mg/day an aggregative response was found (Conn et al., 1983; Borden et al., 1987); rates of 4.0, 5.0, 10.0, and 12.0 mg/day resulted in antiaggregative responses (Ryker and Rudinsky, 1982; Libbey et al., 1985; Borden et al., 1987). At release rates between 0.05 and 4.0, results have not been as consistent. Lodgepole pine trees baited with *exo*-brevicomin released at 0.2 mg/day were more frequently attacked than unbaited trees, but the attraction

did not extend to the other pines within a radius of 10 m of the baited tree (Borden et al., 1990). Lodgepole pine trees baited with other semiochemicals plus *exo*-brevicomin released at 0.37 or 1.26 mg/day initially experienced higher attack densities than trees baited with the other semiochemicals alone, but final attack densities were not significantly different (Borden et al., 1983). McKnight (1979) found an antiaggregative response to *exo*-brevicomin released at 0.53 mg/day on western white pine, *P. monticola* Dougl., but on lodgepole pine he found no significant response at release rates from 0.53 to 2.28 mg/day in one experiment, while in a second experiment, also on lodgepole pine, there was a significant increase in the percentage of trees killed by the mountain pine beetle for the treatment including *exo*-brevicomin released at 0.53 mg/day. Borden et al. (1987) found an intermediate response between aggregation and antiaggregation when *exo*-brevicomin released at 0.5 mg/day was added to baited funnel traps.

endo- and *exo*-Brevicomin were tested in combination with verbenone as aggregation inhibitors for *D. frontalis*, and the brevicomin isomers and brevicomin–verbenone combination resulted in lower landing trap catches than verbenone alone (Richerson and Payne, 1979). Watterson et al. (1981) reduced *D. frontalis* emergent population density with a combination of *exo*- and *endo*-brevicomin and verbenone.

In 1989 we established an experiment with the following objectives: (1) to test the multifunctional role of *exo*-brevicomin and further refine the release rate that differentiates aggregative and antiaggregative responses; (2) to examine the response of the mountain pine beetle to combinations of the antiaggregative pheromone verbenone and the multifunctional pheromone *exo*-brevicomin; (3) to determine if individual trees can be protected from the mountain pine beetle by treatment with verbenone; and (4) to determine if trees in the immediate vicinity (5 m radius) of verbenone-baited trees can be protected from the mountain pine beetle.

METHODS AND MATERIALS

Lodgepole pine trees were baited with one of six treatments at each of 28 replications just east of Penticton, British Columbia, in late June 1989, prior to the main beetle flight period in late July–August. The six treatments were: (1) verbenone—released from a bubble cap at approximately 10 mg/day; (2) *exo*-brevicomin (high)—released from a 250-μl polyethylene Eppendorf tube at approximately 2.5 mg/day; (3) *exo*-brevicomin (low)—released from a 6-cm-long solid PVC release device (Flex Lure) at approximately 0.5 mg/day; (4) verbenone plus *exo*-brevicomin (high); (5) verbenone plus *exo*-brevicomin (low); and (6) unbaited control. Verbenone had optical purity of 84% and chemical

purity of 98%, *exo*-brevicomin was racemic with a chemical purity of 97% and 2% *endo*-brevicomin. All chemicals and release devices were supplied by Phero Tech Inc., Delta, British Columbia, Canada.

Baited trees were selected as larger diameter lodgepole pine with a mean diameter at breast height of 23.3 cm (±0.3 SE) and were spaced approximately 20 m apart. Baits were assigned randomly within each replication and stapled on the north side at a height of approximately 2 m on each tree. The replications ranged from 50 m to several kilometers apart and were in an area of active mountain pine beetle infestation. The locations of each replication were selected where the majority of trees were still alive but were of sufficient age and diameter to be susceptible to beetle attack (Safranyik et al., 1974). To verify that randomization had resulted in a similar number of unattacked lodgepole pine trees of suitable diameter for the mountain pine beetle to attack (Safranyik et al., 1974) in each treatment, an analysis of variance was conducted. The mean number of unattacked lodgepole pine 10 cm and larger was not significantly different among treatments ($F = 0.18$, $P = 0.97$).

In late September, following the beetle flight, baited trees and all trees within a radius of 5 m of each baited tree were checked for mountain pine beetle attacks. Tree species, diameter at breast height, and total number of attacks were recorded. If the number of attacks was too high to count, it was estimated using the method of Safranyik (1988) based on tree diameter, the attack height, and attack density in two 15-cm × 15-cm bark samples taken on opposite sides of the tree at breast height. The experiment was analyzed as a randomized block using the general linear models procedure in SAS (SAS Institute, Inc., 1985). Differences among means were tested using Duncan's multiple-range test. Data on number of attacks per baited tree and total attacks within a 5-m radius of the baited tree were transformed to $\log10(x + 1)$ prior to analysis.

A separate analysis was conducted on "mass-attacked" trees. Trees were classified as attacked (having one or more mountain pine beetle attacks on them) or mass attacked (having more than 31.3 attacks/m^2 at breast height); the attacked category included mass-attacked trees. Mass-attacked trees have been defined as those having more than five attacks in a 0.16-m^2 sample area at breast height (31.3/m^2), which is a beetle density sufficient to be lethal to the host tree (Lindgren et al., 1989).

RESULTS

Trees baited with both high and low release rates of *exo*-brevicomin were attacked more frequently than all other treatments (Table 1). There was no significant difference in the number of attacked trees between the two release rates of *exo*-brevicomin. The addition of verbenone significantly reduced the

TABLE 1. MEAN NUMBERS (SE) OF ATTACKED OR MASS-ATTACKED BAITED TREES FOR SIX SEMIOCHEMICAL TREATMENTS REPLICATED 28 TIMES

Treatment[a]	Attacked trees[b]	Mass-attacked trees[c]
exo-B. high	0.89a (0.06)	0.82a (0.07)
exo-B. low	0.86a (0.07)	0.68a (0.09)
Verb. + *exo*-B. high	0.39b (0.09)	0.11bc (0.06)
Verb. + *exo*-B. low	0.29bc (0.09)	0.21b (0.08)
Unbaited	0.18bc (0.07)	0.14bc (0.07)
Verbenone	0.10c (0.06)	0.00c (0.00)

[a] *exo*-B. = *exo*-brevicomin, high = 2.5 mg/day, low = 0.5 mg/day, Verb. = verbenone 10 mg/day.
[b] Means within a column followed by the same letter are not significantly different, ANOVA attacked $F_{5,135} = 21.0$, $P < 0.0001$; mass attacked $F_{5,135} = 25.5$, $P < 0.0001$; Duncan's test on means, $P = 0.05$.
[c] Trees having an attack density of more than $31.3/m^2$.

response of mountain pine beetle to *exo*-brevicomin so that it was not statistically different from the response to the unbaited (control) trees. The verbenone-baited trees were attacked less often than the unbaited trees but the difference was not statistically significant (Table 1). The treatment effect for mean number of mass-attacked trees was very similar to that for mean number of attacked trees except the position of the two verbenone plus *exo*-brevicomin treatments was reversed. None of the trees baited with verbenone was mass attacked (Table 1).

The mean numbers of trees attacked within a 5-m radius of the trees baited with *exo*-brevicomin were significantly higher than those of the other treatments (Table 2). There was no significant difference between the means of the two *exo*-brevicomin treatments. There also was no significant difference between the verbenone plus high *exo*-brevicomin treatment and the verbenone treatment, as was found for baited trees alone (Table 2). The numbers of trees that were mass attacked within 5 m of the baited trees showed the same treatment effects as for trees receiving any attack (Table 2).

The treatments had a similar effect on the number of mountain pine beetle attacks on baited trees as they did on the number of trees attacked. Trees baited with either high or low *exo*-brevicomin had significantly higher attack densities than trees in any of the other treatments (Table 3). Verbenone significantly reduced the attack density on trees baited with *exo*-brevicomin so that they were not statistically different from the unbaited trees. The attack density on the trees baited with verbenone was slightly lower but not significantly different from the control (Table 3).

The total number of attacks within a 5-m radius of baited trees reflected the same treatment preferences by the beetles as for the baited trees except that

TABLE 2. MEAN NUMBERS (SE) OF ATTACKED OR MASS-ATTACKED TREES WITHIN A 5-m RADIUS OF BAITED TREE FOR SIX SEMIOCHEMICAL TREATMENTS REPLICATED 28 TIMES

Treatment[a]	Attacked trees[b]	Mass-attacked trees[c]
exo-B. high	3.75a (0.56)	2.50a (0.43)
exo-B. low	3.75a (0.55)	2.46a (0.42)
Verb. + *exo*-B. high	1.21b (0.39)	0.61b (0.29)
Verb. + *exo*-B. low	1.18b (0.38)	0.61b (0.22)
Unbaited	0.89b (0.32)	0.50b (0.23)
Verbenone	0.71b (0.30)	0.46b (0.23)

[a] *exo*-B. = *exo*-brevicomin, high = 2.5 mg/day, low = 0.5 mg/day, Verb. = verbenone 10 mg/day.
[b] Means within a column followed by the same letter are not significantly different, ANOVA attacked $F_{5,135} = 15.5$, $P < 0.0001$; mass-attacked $F_{5,135} = 15.6$, $P < 0.0001$, Duncan's test on means, $P = 0.05$.
[c] Trees having an attack density of more than $31.3/m^2$.

TABLE 3. MEAN NUMBERS (SE) OF ATTACKS PER BAITED TREE AND TOTAL ATTACKS WITHIN A 5-m RADIUS OF BAITED TREE FOR SIX SEMIOCHEMICAL TREATMENTS REPLICATED 28 TIMES

Treatment[a]	Baited trees only[b]	Trees in 5-m plot
exo-B. high	291.0a (35.2)	587.2a (110.7)
exo-B. low	178.9a (33.6)	524.7a (92.5)
Verb. + *exo*-B. high	90.6b (45.9)	194.8b (87.6)
Verb. + *exo*-B. low	61.0b (29.7)	182.4b (76.7)
Unbaited	40.2b (20.7)	88.6b (37.9)
Verbenone	29.5b (29.5)	158.1b (73.2)

[a] *exo*-B. = *exo*-brevicomin, high = 2.5 mg/day, low = 0.5 mg/day, Verb. = verbenone 10 mg/day.
[b] Means within a column followed by the same letter are not significantly different, ANOVA baited tree $F_{5,135} = 27.2$, $P < 0.0001$; 5-m plot $F_{5,135} = 17.9$, $P < 0.0001$; Duncan's test on means, $P = 0.05$. Data transformed to $\log10(x + 1)$ for analysis.

trees in the unbaited treatment had fewer attacks than those near the verbenone treatment, but the difference was not statistically significant (Table 3).

DISCUSSION

exo-Brevicomin was attractive to mountain pine beetle at both release rates. Based on previous work by Borden et al. (1987) we expected some antiaggregative response at 2.5 mg/day. However, with the exception of one experiment

in which *exo*-brevicomin was released on western white pine (McKnight, 1979), the antiaggregative effects of *exo*-brevicomin have been demonstrated on traps, not live trees. In trees, any successful attacks by the beetles bring the complication of additional semiochemicals being released. If our release rates of *exo*-brevicomin were sufficient to induce an antiaggregative response from the mountain pine beetle, we would have expected a reduction in the number of attacks on the baited trees and an increase in attacks on the surrounding trees. It appears, however, that a release rate of 2.5 mg/day is too low to induce an antiaggregative response on live trees because even the baited trees were heavily attacked.

Our results clearly show that verbenone reduced the response of mountain pine beetle to trees baited with *exo*-brevicomin. Verbenone influenced not only the attack on the baited trees but on the trees within a 5-m radius of the baited tree. This supports results of previous studies using traps in which the response of mountain pine beetle to aggregation pheromones was reduced with the addition of verbenone (Ryker and Yandell, 1983; Borden et al., 1987; Schmitz and McGregor, 1990).

Our results provide some insight regarding the potential for protecting individual trees or small groups of trees with verbenone. The effect of verbenone on the mountain pine beetle cannot be described as repellent because the verbenone treatment did not result in significantly fewer attacked trees or lower attack densities on either the baited trees or trees within a 5-m radius of the baited trees when compared to the unbaited treatment. The effect was more that of neutralizing or masking the attractiveness of the aggregation pheromone *exo*-brevicomin to the mountain pine beetle.

Treating trees with verbenone would not appear to make the trees any less attractive to the beetle than an unbaited tree. It may, however, make them (and the trees within a 5-m radius) less attractive than a recently attacked tree and thereby reduce mass attack, hence tree mortality, in a treated stand. These results support previous findings that verbenone reduces the number of mass-attacked trees in stands (Amman et al., 1989; Lindgren et al., 1989). In our study no trees baited with verbenone were mass attacked.

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TECHNIQUE FOR USING MICROENCAPSULATED TERPENES IN LEPIDOPTERAN ARTIFICIAL DIETS

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Abstract—We have developed and tested a technique to microencapsulate terpene compounds by forming gelatin-walled microcapsules around the terpene essential oils. Eight terpenes that are common constituents of Douglas fir (*Pseudotsuga menziesii*) oleoresin were encapsulated, including five monoterpenes (α-pinene, β-pinene, camphene, myrcene, and limonene) and three oxygenated monoterpenes (bornyl acetate, linalool, and β-citronellol). The encapsulated terpenes were mixed into artificial diets to determine the effects they had on western spruce budworm (*Choristoneura occidentalis*) survival and reproduction, using a three-generation bioassay. We describe the technique and present data from preliminary bioassays with seven of the terpenes to demonstrate that *C. occidentalis* larvae would consume diets fortified with microencapsulated terpenes. The technique is useful because it helps to reduce the rate at which terpenes volatilize from the diets and it "packages" the terpenes in a manner more closely resembling the way they are present in the resin canals of plant foliage. Many scientists investigating the biological importance of terpenes in mediating plant–herbivore interactions may find that microencapsulated terpenes are useful in both laboratory diet studies and field and greenhouse experiments with plant material.

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Key Words—Artificial diets, *Choristoneura occidentalis*, Lepidoptera, Tortricidae, Douglas fir, microencapsulated terpenes, monoterpenes, multiple generation bioassay, nutritional ecology, oxygenated monoterpenes, *Pseudotsuga menziesii*, western spruce budworm.

INTRODUCTION

Defensive compounds, or allelochemicals, in host plants have been widely implicated as important traits influencing the plant's suitability as food for insect herbivores (Feeny, 1976; Rhoades and Cates, 1976; Rosenthal and Janzen, 1979; Hedin, 1983; Rhoades, 1983; Strong et al., 1984; Coley et al., 1985; Green and Hedin, 1986; Mattson et al., 1988). Several lines of evidence suggest terpenes are the most likely defensive chemicals in Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco (Coniferales: Pinaceae)] against the western spruce budworm [*Choristoneura occidentalis* Freeman (Lepidoptera: Tortricidae)] (Redak, 1982; Cates et al., 1983a,b, 1987; Perry and Pitman, 1983; Redak and Cates, 1984; Cates, 1985; Shepherd, 1985; Wagner and Tinus, 1985; Cates and Redak, 1986, 1988; Campbell, 1987; Hermann, 1987; Wulf and Cates, 1987; Wagner et al., 1989, 1990; Cates and Zou, 1990). *C. occidentalis* is a major defoliator of coniferous forests in western North America (Fellin and Dewey, 1982; Fellin et al., 1983; Harris et al., 1985; Sanders, et al., 1985; Wulf and Cates, 1987).

However, in order to evaluate the role of terpenes as potential resistance mechanisms in Douglas fir against western spruce budworm attack, we need to know which specific terpenes have the most significant effects on budworm survival and reproduction. This involves determining the response curves of the budworm to selected terpenes that occur in Douglas fir foliage to identify the minimum and maximum concentrations tolerated. Furthermore, we must know how different compounds in host foliage, such as various terpenes and/or nutrients, interact to influence the plant's suitability as food for the budworm (House, 1961; Mattson and Scriber, 1987).

Clancy (1991a) has developed a multiple generation bioassay to define the "nutritional niche" of *C. occidentalis*. Insects are reared for three successive generations on artificial diets that have prescribed levels of various host-plant nutrients or allelochemicals. Empirical data thus generated are used to estimate population growth and fitness, expressed as the number of F_1, F_2, and F_3 offspring produced. This provides a very complete evaluation of the effects that individual compounds (or combinations) have on budworm performance, and thus furnishes an indication of the biological significance of various natural products contained in plant tissue.

We wanted to develop a method that would enable terpenes to be incorporated into artificial diets to determine the effects of various terpenes on western spruce budworm fitness, using Clancy's (1991a) multiple generation bioassay. Two methods have been used in the past to add terpenes to lepidopteran artificial diets. Cates et al. (1987), Cates and Zou (1990), and Mattson et al. (1991) merely added purified terpene compounds to diets while they were being mixed in a blender. Another method, used by Isman and Rodriguez (1984), Rossiter et al. (1986), and Gunasena et al. (1988), involves dissolving the terpene and coating the solution onto a cellulose powder by evaporating the solvent, and then using the treated cellulose in the diet.

Several limitations are associated with adding terpenes to diets using these methods. First, the terpenes are not "packaged" the way they are in plant tissues. Terpene compounds are not evenly distributed throughout conifer needles; they are restricted to the resin canals. Second, all monoterpenes and many sesquiterpenes are volatile and evaporate into the air, especially at higher temperatures (Kramer and Kozlowski, 1979). Thus, diets prepared as described above will lose part of their terpene content over time through volatilization. Cates and Zou (1990) and Cates et al. (1987) compensated for this by mixing new diets every two or 10 days, respectively. Other researchers have just accepted the decline over time in the terpene content of the diets they are testing, or they have used a bioassay that only measures effects on performance for a short period (i.e., insects are fed the treatment diets for one instar versus the whole larval period). Additionally, the vapor pressure of the terpene in the container holding the diet and insects is too high when the terpene is not encapsulated.

We have developed and tested a technique that alleviates these problems by microencapsulating the terpene compounds with gelatin and then mixing the microcapsules of terpene into the diet. This is one of the techniques described by Usher et al. (1989) for dosing insects with compounds that normally deter feeding. We believe that many scientists investigating the biological importance of terpenes in mediating plant–herbivore interactions may find that microencapsulated terpenes are useful in experimentation.

The objectives of the studies reported here were to: (1) determine if various terpenes could be microencapsulated and incorporated into artificial diets; (2) establish if microencapsulation helped to reduce terpene volatilization from diets; (3) ascertain if dietary nitrogen was substantially increased by the addition of the microcapsules, since gelatin is a protein; and (4) conduct preliminary bioassays with seven of the eight terpenes we microencapsulated to demonstrate that *C. occidentalis* larvae would consume diets fortified with microencapsulated terpenes and show that this technique can be used to determine the effects of terpenes on budworm fitness. The results from subsequent, replicated bioassays with all eight microencapsulated terpenes will be reported separately (Karen M. Clancy, unpublished data).

METHODS AND MATERIALS

Microencapsulation of Terpenes and Incorporation into Diets. There are at least 30 different terpene compounds that occur in Douglas fir foliage (Cates and Redak, 1988; Wagner et al., 1989). A subset of eight was selected for evaluation of their biological importance to the budworm based on the following criteria: (1) quantitative abundance in Douglas fir foliage—we chose compounds that were typically present in concentrations of at least 20 μg/g dry mass of foliage; (2) indications from the literature that variation in foliar concentration of the terpene is associated with budworm defoliation, seasonal feeding patterns, survival, or growth; (3) availability of the terpene from chemical supply companies; and (4) formulation considerations—some of the terpenes we were interested in could not be microencapsulated successfully (e.g., camphor and anethole), and it was necessary to add isopropyl myristate (i.e., myristic acid, a C_{14} saturated essential fatty acid) to the camphene to microencapsulate it effectively. The problems encountered with encapsulating some of the terpenes are probably related to the physical properties of the compounds (e.g., solubility and surface tension). There were several other potentially interesting terpenes, such as caryophyllene, longifolene, and tricyclene, that we did not use because the quantity required for microencapsulation (at least 120 g) was too expensive.

The eight terpenes selected and the diet concentrations we tested were: α-pinene, β-pinene, camphene, and bornyl acetate at 100, 500, and 1500 ppm fresh mass, plus myrcene, limonene, linalool, and β-citronellol at 50, 150, and 500 ppm fresh mass in diets with 84.5% moisture. The concentrations were based on the reported ranges for the terpenes in Douglas fir foliage from trees in Arizona and Colorado (Wagner et al., 1989; Clancy, 1991b; Karen M. Clancy, unpublished data). Although there are many other terpenes that could also be important allelochemicals for the western spruce budworm, these eight provided a good start for investigating how the quantity and composition of oleoresin affects budworm performance.

The encapsulation process involved forming gelatin-walled microcapsules around the terpene essential oils by complex coacervation (Usher et al., 1989). Coacervates of gelatin and gum arabic were induced to deposit around the oil droplets in an emulsion, and the gelatin was set by cooling and chemical cross-linking with glutaraldehyde to harden the shell. The capsules were packaged as a water slurry. Diameters of the capsules ranged from about 30 to 80 μm, which corresponds well with the diameters of resin canals in Douglas fir needles (28–105 μm; Karen E. Burr and Karen M. Clancy, unpublished data).

The various microcapsule slurries were mixed into batches of warm, liquid artificial diet using an electric handheld whisk (i.e., a cooking appliance for mixing thick liquids). The diets were then poured into pans to cool and cut into cubes or were poured into 30-ml plastic cups. We did not observe any settling

of the microcapsules in the liquid diet; it appeared to be viscous enough to keep the small capsules suspended until the diet cooled and solidified. We also tried mixing the capsules into diets with a blender, but it was clear that many of the capsules ruptured. The experimental base diet to which the microencapsulated terpenes were added contained 1.25% nitrogen, 10.9% sugars, 84.5% moisture, and concentrations of most minerals that did not deviate substantially from the composition of Douglas fir foliage (Clancy, 1991a).

Samples of the diets were analyzed for terpene content by capillary column gas chromatograph (see Wagner et al., 1989, for details about the analysis). The terpenes were extracted from the diets using the following procedure: For the initial extraction, a 200-ml plastic cup about half full of diet cubes (about 35–65 g fresh mass of diet material) was brought to the Analytical Services Laboratory at Northern Arizona University within 24 hr of preparation. The diet cubes were weighed and placed in a flask with approximately 200 ml of chromatography-grade hexane, covered, and refrigerated (4°C) overnight. The following morning the flask was put in an ultrasonic bath for 10 min to rupture the microcapsules and release the terpene (We are not convinced this step was effective because subsequent extraction of terpenes from the microcapsule slurries indicated that terpene recovery was greater with longer extraction times. This implies the capsules were not ruptured in the ultrasonic bath. Thus, this step could probably be eliminated). The sample was then poured into a blender, and the volume was increased to 250 ml by adding more hexane. The diet–hexane matrix was blended at slow speed for 2–4 min to obtain a homogeneous slurry, then it was blended for 5 min at high speed to rupture the capsules. After blending, an aliquot was transferred to a 25-ml vial and stored in a freezer at −10°C. For the gas chromatography analysis, a 5-ml aliquot was removed, spiked with an internal standard, and filtered through a 0.45-μm membrane for analysis. The terpene concentration was calculated based on the fresh mass of the diet.

Terpene Volatilization from Diets. One of the advantages of adding microencapsulated terpenes to diets should be to slow the rate at which the terpenes evaporate into the air. We tested this by comparing terpene degradation over time for diets fortified with regular versus microencapsulated β-pinene. Four cups of diet cubes from both the regular and microencapsulated diets were analyzed separately for β-pinene content when the diets were initially prepared and after the diets had been incubated at 25°C for two and four weeks. The moisture content of the diets was determined at each sample time to enable us to convert the fresh mass concentrations to dry mass and thus eliminate variation due to moisture loss (the diets dry out over time). The data were analyzed with ANOVA to determine if β-pinene concentrations changed over time. Furthermore, when bioassays were conducted with the different microencapsulated terpenes, we quantified the dry mass terpene content of the diets when the

experiments were initiated and after the P_1 budworm larvae had fed on the diets for 35–39 days and were ready to be transferred to fresh diets.

Effect of Gelatin Microcapsules on Dietary Nitrogen. The gelatin material used to encapsulate the terpenes is a protein that could be utilized by the insects as a source of nitrogen. Although we suspected it was unlikely that the small amount of microcapsule material added would substantially change the N content of the diet, we conducted a study to test this possibility. We made 11-cup batches of the experimental base diet (see Clancy, 1991a, for recipe) with 0, 0.25, 1.00, 5.00, 20.00, and 50.00 ml of microencapsulated β-pinene added. This was representative of the amounts of the various microcapsule slurries that were used in the experiments (1.00–30.00 ml in the original recipes, 0.24–19.30 ml in the revised recipes). Four randomly selected cups of diet cubes from each batch were oven-dried (70°C) and analyzed separately for total Kjeldahl N (colorimetrically). The data were analyzed with ANOVA to determine if N content changed detectably with the addition of various amounts of microcapsules.

Effects of Microencapsulated Terpenes on Budworm Fitness. We determined the effects that various terpene compounds had on *C. occidentalis* survival and reproduction by rearing larvae for three consecutive generations on diets with different levels of the terpenes. The bioassay technique is described in detail in Clancy (1991a). The P_1 generation of the experiments started with egg masses from a laboratory colony of nondiapausing western spruce budworm. Survival rates were determined for all life stages, including survival from early to late instars, late instars to pupal stage, and pupal to adult stage. These measures were multiplied to estimate cohort survival to the adult stage. Egg masses produced by the adult moths were incubated to determine viability ($\geq 50\%$ of the eggs in the mass hatched). The bioassay for each treatment and generation started with 10 egg masses that usually produced 200–300 first-instar larvae. Up to 150 randomly selected late-instar larvae were transferred to fresh diets to complete development. Fifty to 120 larvae pupated and produced about 40–100 moths, which laid a total of 25–75 egg masses. Pupae were weighed and the average female pupal masses for each treatment and generation were used to predict female fecundity or the number of oocytes in the adult moth (Wagner et al., 1987).

The following population growth model was used to obtain a composite measure of how different levels of the terpenes affected budworm performance:

$$\begin{aligned}\text{number of } F_1 \text{ larvae} = {} & (\text{number of } P_1 \text{ larvae}) \\ & \times (P_1 \text{ percentage cohort survival to adult stage}) \\ & \times (P_1 \ \bar{X} \text{ female fecundity}) \\ & \times (F_1 \text{ percentage egg masses with viable eggs})\end{aligned}$$

The numbers of F_2 and F_3 larvae were calculated with data for the appropriate generation. The model estimated the number of first instars alive at the beginning of the F_1, F_2, and F_3 generations, assuming all the treatments had equal populations at the beginning of the P_1 generation (i.e., we assumed that all treatments started with a single P_1 larva).

RESULTS

Microencapsulation of Terpenes and Incorporation into Diets. Our initial assumption was that all the slurries of various microencapsulated terpenes would contain about the same amount of active ingredient. Consequently, standard recipes were developed for the different concentrations we wanted to test and experimental bioassays were started. However, analysis of the diets showed that except for myrcene, the terpene concentrations achieved were far from the targets, which were selected based on data from host trees. Levels of myrcene were within 4% of the target concentrations. For the remaining seven terpenes, variation from the target concentrations ranged from 13% to 455% ($\bar{X}$ = 185%, SD = 159%, N = 21). Nonetheless, the bioassays initiated with these original terpene formulations were completed to ensure that this technique could be used to determine the effects of terpenes on budworm fitness, and the results are reported here.

Subsequent analysis of the terpene microcapsule slurries indicated the active ingredient (by weight) ranged from 1.3% to 14.3% among different terpenes and between different batches of the same terpene. The diet recipes were revised by adjusting the amount of microcapsule slurry added, based on the percent active ingredient. Furthermore, test batches with an intermediate target concentration were made with each terpene and analyzed to compare the terpene concentration expected to the concentration achieved; additional adjustments to the recipes were made based on these results. New bioassay experiments were started once we were confident we could formulate diets with the desired levels of the various terpenes; results from replicated bioassays using the revised formulations plus the original myrcene formulation will be reported separately (Karen M. Clancy, unpublished data). The revised terpene formulations produced diets much closer to the target concentrations; the average deviation from the targets was 17% (SD = 14%, range 0–49%, N = 47). Furthermore, analysis of two separate batches of the same recipe demonstrated that the terpene concentrations achieved were duplicated within 20% on average (SD = 12%, range 2–44%, N = 23).

Terpene Volatilization from Diets. Diets with microencapsulated β-pinene maintained their terpene content over time better than diets with regular β-pinene (Table 1). The microencapsulated β-pinene diets lost (on average) 21% of their terpene content over four weeks (P = 0.138), whereas the regular β-pinene

TABLE 1. TERPENE DEGRADATION OVER TIME FOR DIETS FORTIFIED WITH MICROENCAPSULATED VERSUS REGULAR β-PINENE

Time at 25°C (weeks)	$\bar{X} \pm$ SE β-pinene concentration (ppm dry mass) ($N = 4$)	P value from ANOVA for changes in β-pinene concentration over time ($df = 2, 9$; $n = 12$)
Microencapsulated diets		0.138
0	4460 ± 420.5	
2	4233 ± 92.67	
4	3529 ± 314.1	
Regular diets		0.006
0	1534 ± 147.2	
2	960.3 ± 56.77	
4	713.8 ± 58.08	

diets lost 53% ($P = 0.006$). Although the decline in terpene concentration for the microencapsulated diets was not statistically detectable, these diets were much more variable regarding losses. This is probably because the microcapsules did not get as evenly distributed throughout the diet when they were whisked in as the regular terpenes did when they were blended in (although we did not notice any sticking or clumping due to whisking). Consequently, the power of the statistical test to detect real differences among the sample times was much lower for the microencapsulated diets compared to the regular diets.

Another potential caveat in interpreting the results in Table 1 relates to the different initial β-pinene concentrations achieved in the two diets. This was not intentional, but resulted from imprecise knowledge of the relationship between amounts of terpenes added and the resulting concentration in the diet. It is possible that the different initial terpene levels tested could influence the loss rates observed. Finally, it is likely that the lipids, phospholipids, and salts in the diet may alter the gelatin properties over time (E.A. Bernays, personal communication); this could account for the greater loss in microencapsulated β-pinene during weeks 2 through 4, than during the first two weeks.

Diets augmented with microencapsulated monoterpenes generally lost less of their terpene content over time than diets with oxygenated monoterpenes (Figures 1 and 2; data are for the revised terpene formulations). For the most part, there was some loss in concentrations of the monoterpenes α-pinene, β-pinene, camphene, myrcene, and limonene over a 35- to 39-day period, but the decline in concentrations of the oxygenated monoterpenes bornyl acetate, linalool, and β-citronellol over equivalent periods was much greater. We attribute the apparent increase in α-pinene over time to uneven distribution of the

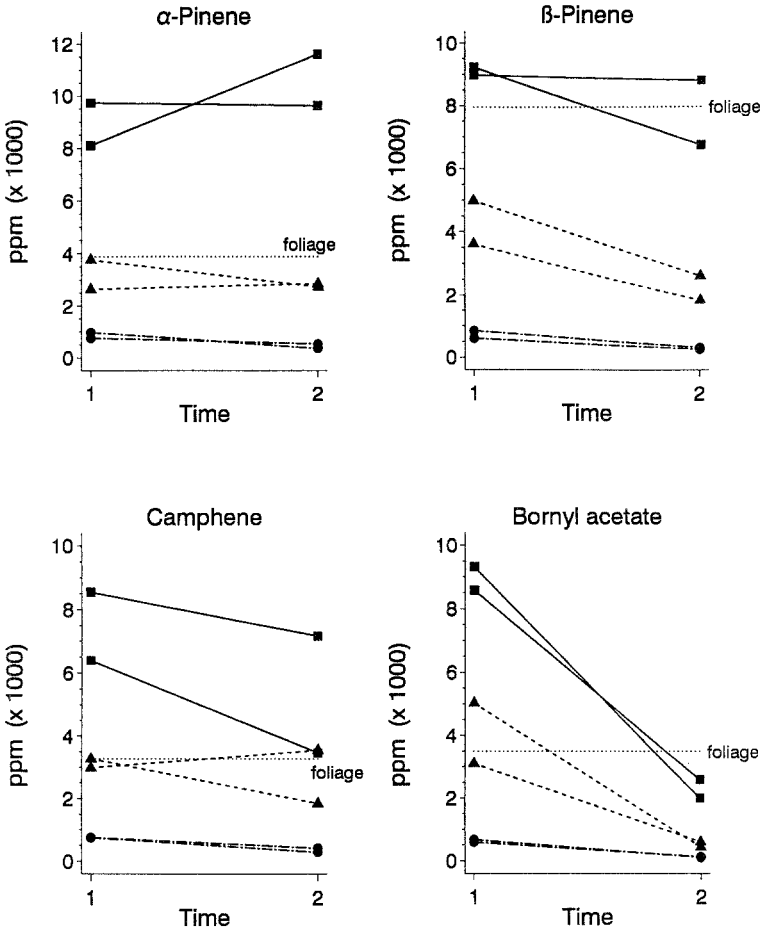

FIG. 1. Concentrations (ppm dry mass) of microencapsulated α-pinene, β-pinene, camphene, and bornyl acetate in experimental diets when they were initially prepared (time = 1) and after they had been incubated at 25°C for 35–39 days (time = 2; $\bar{X}$ = 36.5 days, SD = 1.23). Three levels of each terpene were tested: ■——■ = high target (9677 ppm); ▲----▲ = intermediate target (3226 ppm); ●–·–·–● = low target (645 ppm); these are equivalent to 1500, 500, and 100 ppm fresh mass for diets with 84.5% moisture. Each experiment was repeated twice. The dotted line labeled "foliage" shows the highest concentration of the terpene found in Douglas fir foliage to provide a reference point.

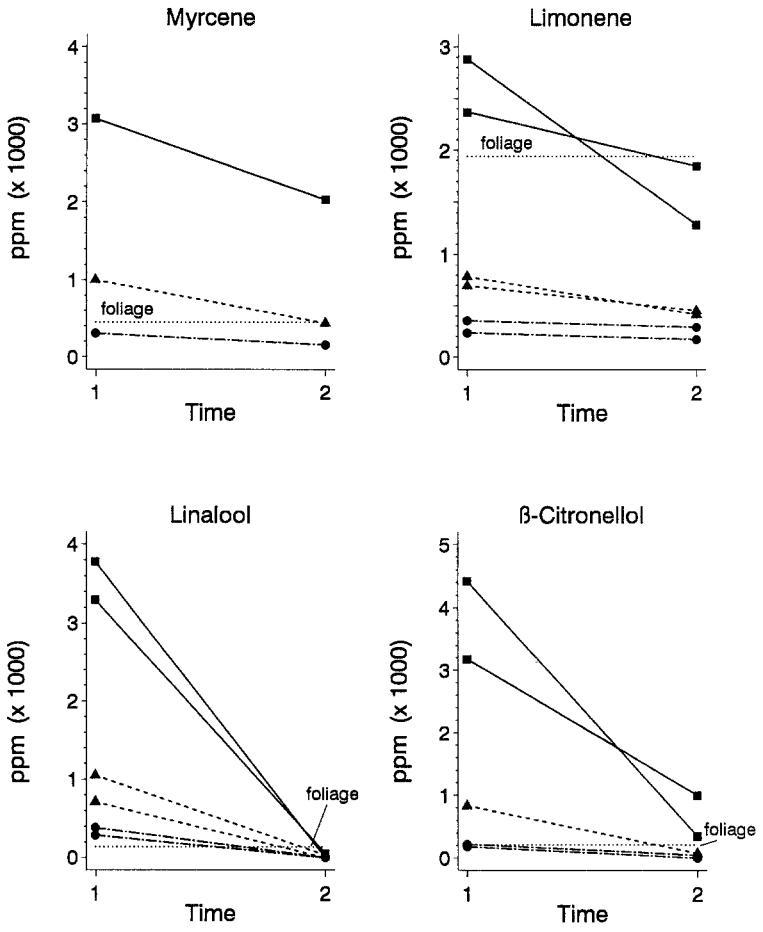

FIG. 2. As for Figure 1, but for microencapsulated myrcene (a single replication is shown), limonene, linalool, and β-citronellol. The high, medium, and low targets were 3226, 968, and 323 ppm, respectively, equivalent to 500, 150, and 50 ppm fresh mass for diets with 84.5% moisture.

microcapsules in the diet, plus possible errors in measuring dietary moisture content; we do not think the increase is significant.

Effects of Gelatin Microcapsules on Dietary N. The addition of microcapsules in amounts representative of those used in experiments did not cause a detectable increase in dietary N content ($P = 0.198$; Table 2). Although the diets with 50 ml of microencapsulated terpene appeared to have about 6% more N than the other diets (1.40% compared to 1.32%), the revised diet recipes

TABLE 2. NITROGEN CONCENTRATION FOR DIETS WITH VARIOUS AMOUNTS OF MICROENCAPSULATED β-PINENE ADDED

Amount of β-pinene added to diet (ml)	$\bar{X}$ ± SE nitrogen concentration (% dry mass) (N = 4)[a]
0	1.34 ± 0.0359
0.25	1.29 ± 0.0118
1.00	1.32 ± 0.0318
5.00	1.34 ± 0.0417
20.00	1.30 ± 0.0442
50.00	1.40 ± 0.0095

[a]The N levels were not different among the diets (P = 0.198; df = 5, 18; N = 24; P for Bartlett's test for homogeneity of variances = 0.142), indicating the gelatin in the microcapsules did not change the dietary N content.

contained about 20 ml of microencapsulated terpene at most. Thus, we conclude that such small amounts of the gelatin microcapsules added to the diets are unlikely to result in an increase in N that is biologically meaningful to the budworm.

The average N content of the experimental base diet was 1.25% (SD = 0.062, N = 68) (Clancy, 1991a). The test diets listed in Table 2 had an average N level of 1.33% (SD = 0.041, N = 6), which was higher than we expected based on past analyses, but not unreasonable considering normal batch-to-batch variation.

Effects of Microencapsulated Terpenes on Budworm Fitness. Data from the preliminary bioassays conducted with the original terpene formulations are presented in Figures 3–6. The higher concentrations tested generally far exceeded the upper limit reported for Douglas fir foliage, particularly for β-citronellol and linalool. Although the results are preliminary, they indicate that even very high concentrations of the monoterpenes β-pinene, α-pinene (Figure 3), and limonene (Figure 4) had very little effect on budworm population growth, but higher concentrations of camphene (Figure 5) had definite deleterious effects. Low concentrations of the oxygenated monoterpene bornyl acetate appeared to improve budworm fitness slightly, whereas high concentrations reduced fitness and very high concentrations (far exceeding levels in foliage) were clearly toxic (Figure 5). Likewise, extremely high concentrations of the other oxygenated monoterpenes, β-citronellol (Figure 4) and linalool (Figure 6), were deadly. Intermediate concentrations of β-citronellol had more severe impacts on the budworm than comparable levels of linalool.

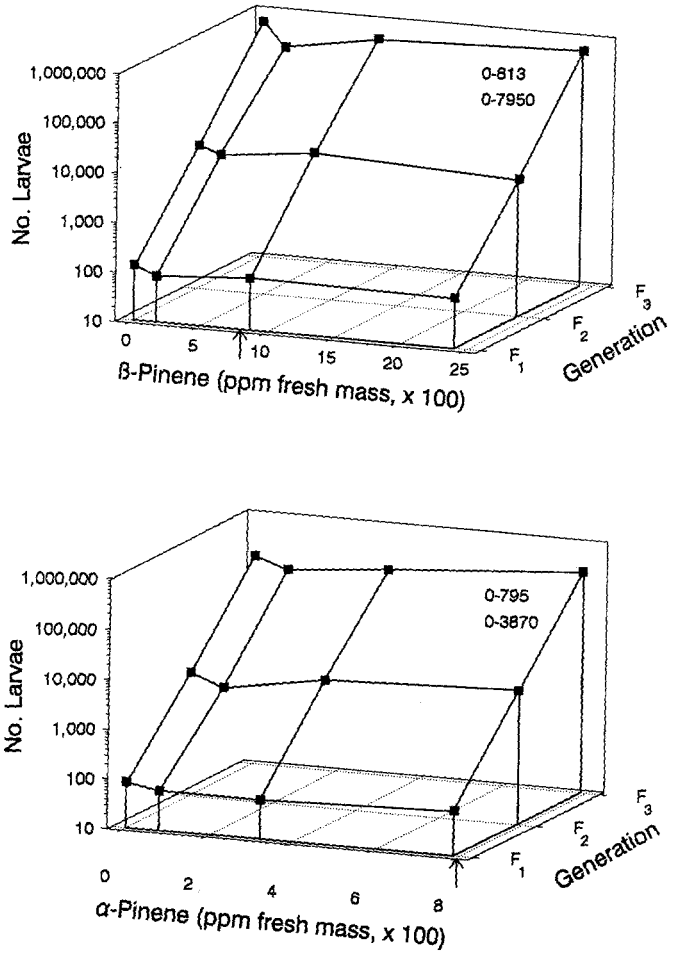

FIG. 3. Estimated western spruce budworm population growth (number of first instars alive at the beginning of the F_1, F_2, and F_3 generations) for diets with different levels of the monoterpenes β-pinene (top) and α-pinene (bottom), assuming all populations were equal at the beginning of the P_1 generation. See text for model of population growth used to calculate estimates. The numbers in the upper right corner list the range of terpene concentrations observed in foliage from Douglas fir trees in Arizona and Colorado; top numbers = ppm fresh mass, bottom numbers = ppm dry mass, and the arrows on the x axes indicate the highest fresh mass concentration.

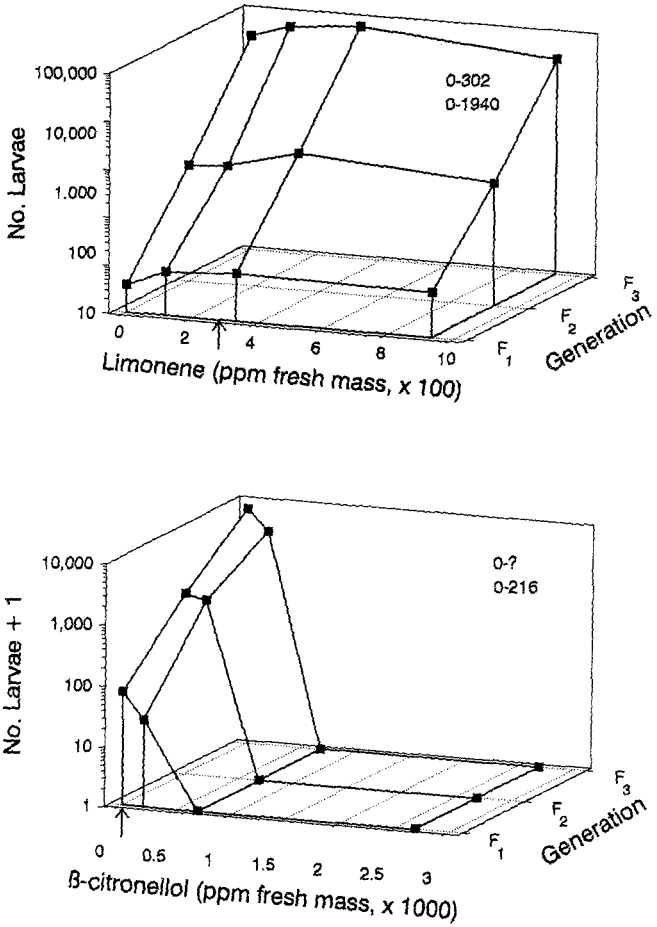

FIG. 4. As for Figure 3, but for the monoterpene limonene (top) and the oxygenated monoterpene β-citronellol (bottom).

DISCUSSION

Terpene compounds can be microencapsulated and mixed into lepidopteran artificial diets to determine the effects of terpenes on insect herbivore performance. This technique is useful because it packages the terpenes in a manner more closely resembling plant foliage and it reduces their volatilization rate from artificial diets. Although terpenes in conifer needles are not enclosed in protein shells, they are compartmentalized in resin canals rather than being distributed throughout the needle tissue. Preliminary bioassay experiments with diets containing seven terpenes that are common constituents of Douglas fir

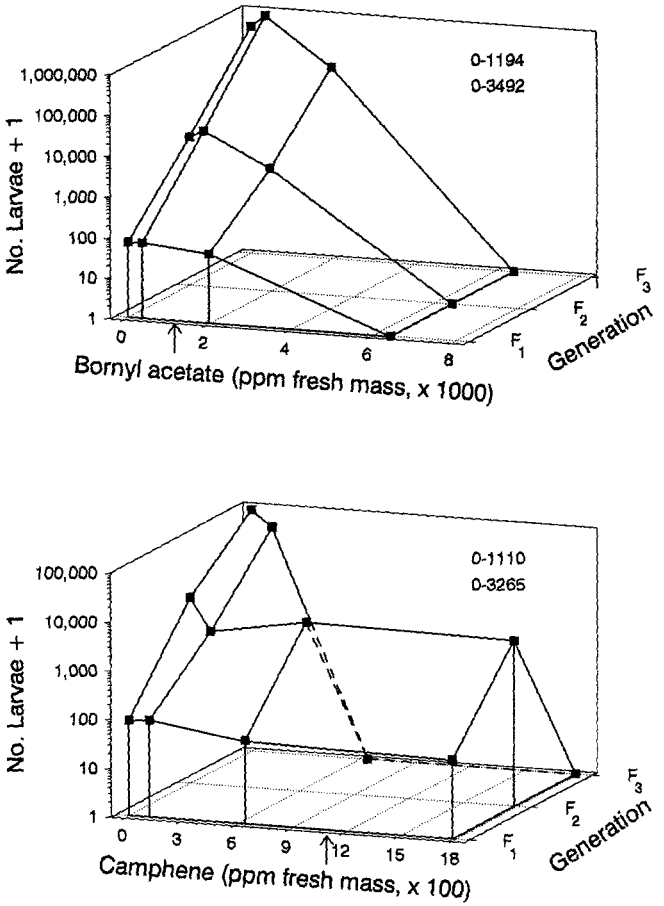

FIG. 5. As for Figure 3, but for the oxygenated monoterpene bornyl acetate (top) and the monoterpene camphene (bottom).

oleoresin indicated that even very high concentrations of β-pinene, α-pinene, and limonene had very little effect on western spruce budworm fitness, whereas relatively high levels of camphene, bornyl acetate, β-citronellol, and linalool had definite deleterious effects on budworm population growth.

Volatilization of terpene essential oils from diets is reduced because the terpene content of the microcapsules is not released until a larva bites the capsule open or ingests the capsule. Studies by Usher et al. (1989) have established that the gelatin microcapsule walls are broken down very rapidly after ingestion by armyworms [*Pseudaletia unipuncta* (Haworth) (Lepidoptera: Noctuidae)] and

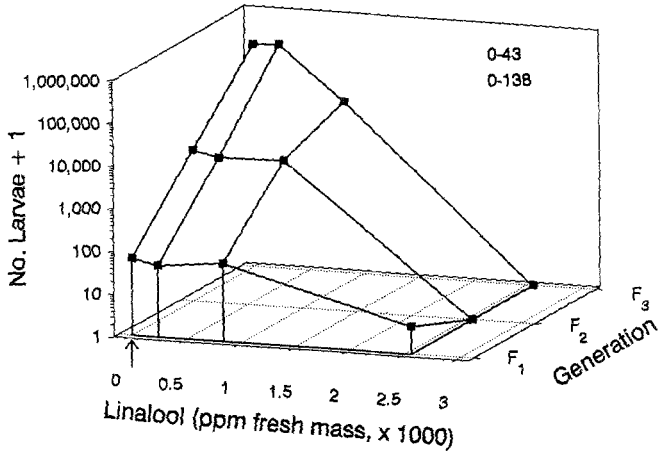

FIG. 6. As for Figure 3, but for the oxygenated monoterpene linalool.

grasshoppers [*Schistocerca americana* (Drury) (Orthoptera: Acrididae)], permitting release of the encapsulated compound in the gut.

Microencapsulation appeared to reduce volatilization of the monoterpenes tested more than the oxygenated monoterpenes (Figures 1 and 2). Our explanation for this is that the monoterpenes tended to encapsulate more efficiently than the oxygenated monoterpenes because they are less polar and thus less soluble in water. The encapsulation efficiency relates to the percentage of terpene compound residing within the microcapsule as opposed to being left to the exterior aqueous phase. In other words, a greater percentage of the oxygenated monoterpenes was unencapsulated in the aqueous phase. The unencapsulated terpene would tend to volatilize fairly rapidly (Table 1); we believe this accounts for the greater loss of terpene content in oxygenated monoterpene diets over time. In the future, it should be possible to compensate for solubility and achieve equal encapsulation efficiencies for all terpene compounds.

We predict that microencapsulated terpenes may have many useful applications not only in laboratory diet studies but also in field and greenhouse experiments with plant material. Materials can be added to the microcapsule slurry to adhere the capsules to surfaces. Thus, microencapsulated terpenes could be coated onto the surfaces of needles, leaves, twigs, etc., to provide plant material that is enhanced with particular terpene compounds or combinations. Usher et al. (1989, p. 121) pointed out that "Microcapsules adhering to leaf surfaces could be a relatively realistic dosing technique for plant secondary compounds, many of which are superficial or compartmentalized in leaves in nature."

There are some disadvantages associated with this technique: (1) not all

terpenes can be microencapsulated successfully; (2) the microencapsulation process requires a minimum of 120 g of terpene material; (3) the amount of active ingredient in the microcapsule slurry was quite variable among different batches because each compound has a different solubility in water, thus requiring more preliminary formulation work to produce the desired terpene concentrations; and (4) there is undoubtedly a less even distribution of the capsules whisked into the diet than if liquid terpenes were blended in. In spite of these limitations, we believe the technique is a much better way to incorporate terpenes into artificial diets than to merely blend terpenes into diets or to coat terpenes onto cellulose powder.

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FEEDING RESPONSE OF THE SILKWORM, *Bombyx mori*, TO UV IRRADIATION OF MULBERRY LEAVES

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Abstract—Ultraviolet irradiation of mulberry leaves caused a reduced feeding response in *Bombyx mori* larvae (ca. 22% reduction in consumption as compared with control). Sucrose content of the foliage decreased after exposure to UV irradiation (0.5 mW/cm^2) for 1 hr. Electrophysiological responses of the sensilla styloconica-I to sucrose concentration (ca. 0–80 mg/g) showed a reduced response to UV-irradiated foliage when compared with the control. From feeding, gustatory, and electrophysiological responses, as well as the measurement of sucrose contents of the leaves, we concluded that the reduced feeding response to UV-irradiated leaves is due to the reduced sucrose content of the mulberry leaves, thus reducing pleogostimulatory levels.

Key Words—UV irradiation, *Bombyx mori*, Lepidoptera, Bombycidae, mulberry leaf, sucrose, electrophysiology, sensilla responses.

INTRODUCTION

The mulberry, *Morus alba* (Moraceae), is the sole food plant for the silkworm, *Bombyx mori*, throughout its range. In the presence of laboratory ultraviolet (UV) irradiation, we found that *B. mori* larvae reduce their consumption rates

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(Yazawa et al., 1991a,b). It is well known that phytophagous insects reduce consumption rates of host plants in the presence of UV radiation (Berenbaum, 1978; Zangerl and Berenbaum, 1987). Zangerl and Berenbaum (1987) found that UV irradiation induces the biothynthesis of the furanocoumarin xanthotoxin, an antifeedant, in plants of the families Rutaceae and Umbelliferae. We also found changes in coumarin level in relation to UV radiation (Yazawa et al., 1991a,b) and UV radiation induced changes in sucrose content of mulberry leaves.

METHODS AND MATERIALS

Insects. Bombyx mori (N 601 × C601) were reared on an artificial diet containing chlorella (Yakuruto Co., Ltd., Tokyo). Male last-instar larvae were used in the feeding and electrophysiological experiments.

UV Radiation Sources and Levels. UV light (15W; National Electric Co., Ltd., Japan) levels of 0.2–0.6 mW/cm^2 for 1–60 min were applied to the picked plant leaves, *Morus alba.*

Feeding Response. The larvae used in the feeding experiment were newly molted fourth-instar larvae within several hours after ecdysis. The larvae were simply fed irradiated foliage for 1 hr. Six larvae were used in the experiments. The relative consumption rate of irradiated foliage and body weight were calculated by subtracting initial weight from body weight 1 hr after initiation of the feeding period.

Measurements of Sucrose Content. Two analytical methods for measurement of carbohydrates were employed. Method I (Figure 1) was used for getting precise data per one leaf or milligram of leaf; method II was used for obtaining a rapid estimate. The latter method consisted of directly extracting irradiated foliage with 80% hot EtOH (3 × 100 ml) and evaporating to dryness, after which 2 ml distilled water were added.

HPLC Analysis of Carbohydrates. Carbohydrate analysis was performed with high-performance liquid chromatography (HPLC) (Shimadzu LC-6A) with an IR detector. The column type was Shim-pack CLC-NH2 (Shimadzu Co. Ltd.) and was eluted with 70% acetonitrile at a flow rate of 1 ml/min. Authentic sucrose was also injected as a standard carbohydrate in the same manner.

Electrophysiological Response. The electrophysiological methods used in the experiments were the same as described previously (Ishikawa, 1963; Hirao and Arai, 1991). A head–thorax was amputated at the middle part of the thorax and secured with clay to a silver plate, which was used as the indifferent electrode. A glass capillary, which served as the stimulating–recording electrode, was applied externally over the tip of the hair. A silver wire inserted into the

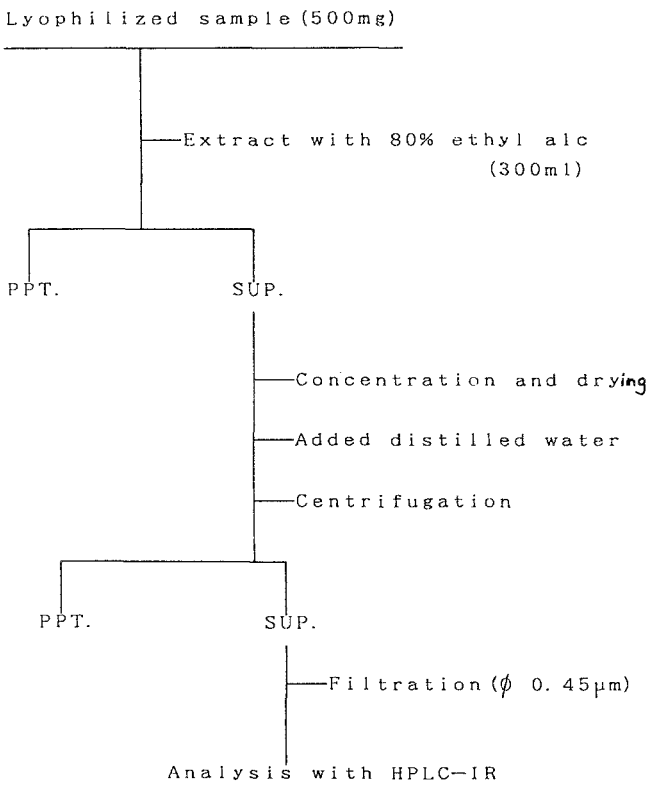

FIG. 1. Analytical methods of sucrose content of the mulberry leaves.

capillary was connected with a high-input impedance, cathode-follower preamplifier, followed by the main amplifier and oscilloscope. Recordings from sensilla styloconica-I and -II were carried out for 1 sec after 1 sec from the stimuli.

RESULTS

Feeding Response. As shown in Figure 2, the larvae consumed more non-treated mulberry leaves than UV-treated leaves. Exposure of more than 0.4 mW/cm^2 UV radiation for 1 min caused a reduction in body weight. With UV radiation for 30 min at more than 0.5 mW/cm^2, final body weight was reduced relative to initial body weight.

Sucrose Contents in Mulberry Leaves with Reference to UV Radiation. Typical chromatograms of sugar standards, as well as sugars obtained from

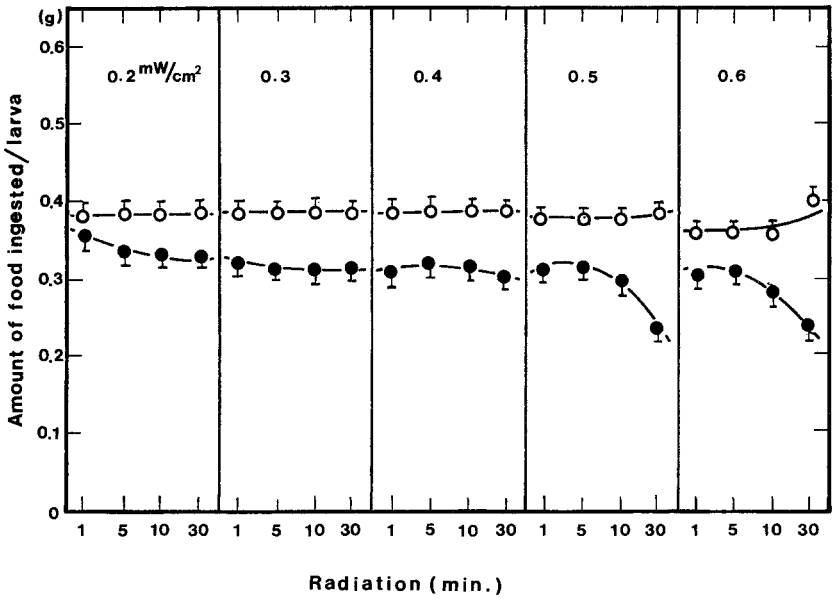

FIG. 2. Feeding response of *Bombyx mori* offered UV-irradiated leaves. Open and filled circles show amount of food ingested in the absence and presence of UV irradiation, respectively.

extracts from irradiated and nonirradiated leaves, are illustrated in Figure 3A–C, respectively. As shown in Figure 3B and C, four sugars (sucrose, glucose, maltose and fructose), were detected in the extraction with method II. After UV radiation (0.5 mW/cm^2) of the leaves, the sucrose peak became smaller (Figure 3B) compared to that of control (Figure 3C).

With UV treatment (0.5 mW/cm^2) for 0.5 hr on the mulberry leaves, total sugar content was reduced ca. 49% compared with the control (Figure 4A). Sucrose, glucose, maltose, and fructose were detected in the presence and absence of UV. In the presence of UV, the level of glucose, maltose, and fructose was not changed; only sucrose content was reduced when UV radiation was applied for 10 or 30 min (Figure 4A). Among total sugars, the sucrose content of the mulberry leaves was also reduced in proportion to UV intensity (Figure 4B). In the case of analytical method I, levels of sucrose per leaf (Figure 4B) were similar to those measured by analytical method II (Figure 4B). Analytical method I is more suitable for sucrose analysis, because it is conventional and produces reliable results.

Electrophysiological Response to Partially Purified Samples. Since the level of glucose and fructose did not change in the presence of UV, only sucrose, which shows high activity with sensilla styloconica-I (Ss-I), was checked elec-

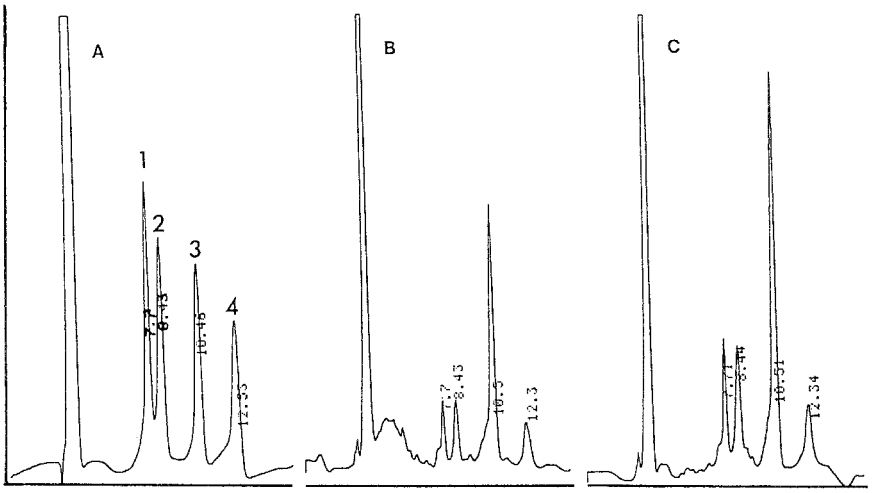

FIG. 3. The HPLC pattern of sugar analysis of the standard compounds (A), UV-exposed leaves (B), and control leaves (C). Ordinate: response; abscissa: retention time (min). 1, fructose; 2, glucose; 3, sucrose; 4, maltose.

trophysiologically. Electrophysiological response from Ss-I showed many impulses discharged in both the partially purified sample and the 1/10 dilution sample of the nonirradiated foliage, as compared with samples of irradiated foliage (Figure 5B and E). The number of impulses from Ss-II does not change with the presence and absence of UV. Figure 6 shows the relationship between actually detected sucrose concentrations and the number of impulses discharged from Ss-I. The number of impulses increased with increasing sucrose concentrations.

DISCUSSION

By our experimental methods, sucrose, glucose, fructose, and maltose were detected in mulberry leaves. There are other reports on the sucrose content of the mulberry leaf (Yazawa and Yamashita, 1974), since it is well known that the taste receptors of sensilla styloconica-I (Ss-I) and -II (Ss-II) in the larvae of *Bombyx mori* play important roles in feeding behavior. Hirao and Arai (1991) and Yazawa et al. (1991a,b) reported on the relation between sucrose and palatability of mulberry to *B. mori*, and they recorded impulses of the sugar receptor of Ss-I to *Morus alba* leaf extracts.

As shown in Figure 6, the number of impulses from Ss-I increased with increasing sucrose content of the leaves. The 25–33% decrease in sucrose con-

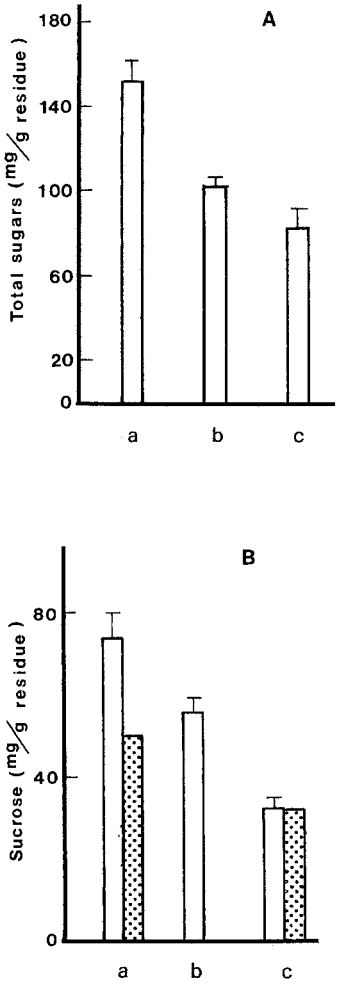

FIG. 4. The relationship between UV irradiation and total sugar (A, analytical method II) and sucrose (B, analytical methods I and II) content of the mulberry leaves. a, nontreatment; b, 10 min UV exposure; c, 30 min UV exposure. Vertical bar on each column indicates SD ± mean of three determinations. In Figure 4B, open column shows the data obtained by analytical method II; the filled column shows that of analytical method I.

centrations in the UV-treated foliage might account for the reduced feeding response. From the results of recording Ss-II (bitter sense) (Ishikawa, 1966; Hirao and Arai, 1991), we assume that bitter tasting substances are not synthesized in the mulberry leaves in the presence of UV radiation. Maltose analyzed from the mulberry leaves produced a similar response pattern to that of sucrose with respect to the Ls (sucrose) receptor on the Ss-I, but response of the receptor to maltose is low compared with sucrose (Ishikawa, 1963). In the present experiments, the Li (inositol) receptor, the activity of which is specifically high with respect to inositol, discharged many impulses in response to the sample of UV-treated and nontreated mulberry leaves. Mulberry leaves contain polyhydric

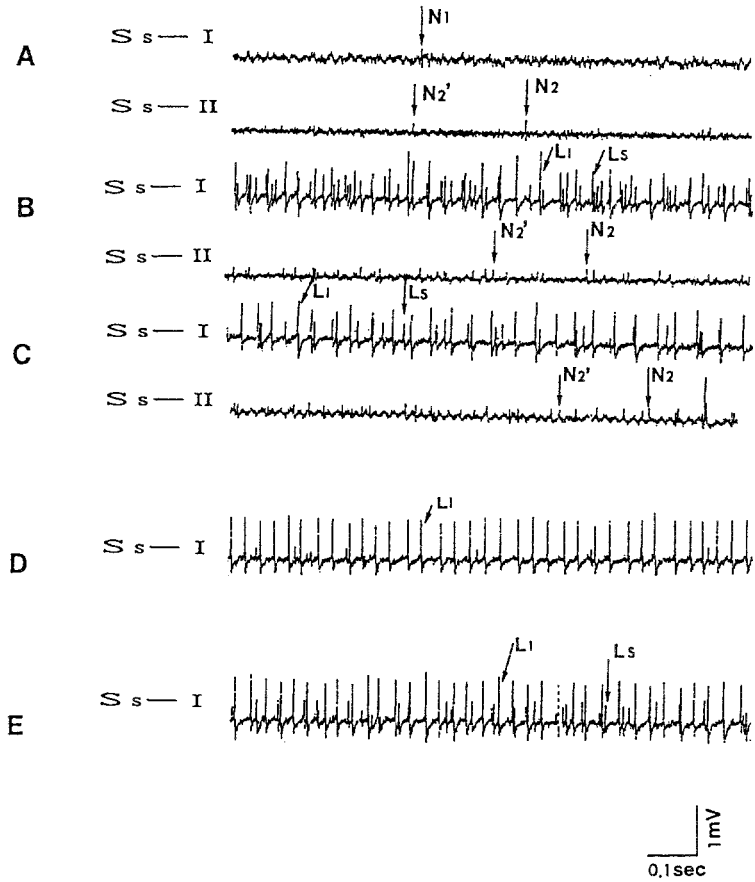

FIG. 5. The impulses discharged from Ss-I and Ss-II. (A) Response of 5 mM NaCl solution from Ss-I and -II; (B) response of control sample from Ss-I and -II; (C) response of sample with UV-treated leaves from Ss-I and -II; (D) response of sample with UV-treated leaves from Ss-I; (E) response of control sample from the Ss-I. N_1, N_2, N_2' = impulse of inorganic salt receptors; Li, Ls = impulses of inositol and sugar receptors, respectively.

alcohols, such as inositol (Naito and Hamamura, 1961). Therefore we must investigate the relationship between actual inositol content of mulberry leaves and the Li receptor activity.

The mechanism by which UV radiation affects the sucrose content of the leaf is not clear. Among four sugars in the leaves, only sucrose was reduced in the presence of UV; therefore, we presume that sucrose degradation is not induced by UV radiation. Probably, synthesis of sucrose was blocked through

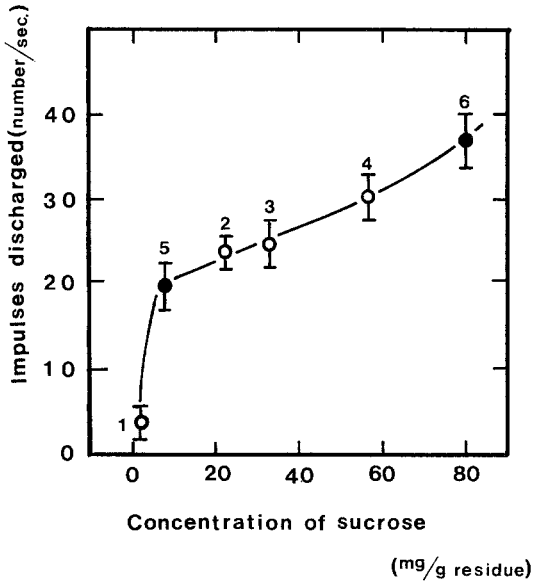

FIG. 6. The relationship between sucrose doses actually detected from the leaves and the number of impulses discharged. Open circles with numbers 1, 2, 3, and 4 indicate sucrose concentrations from sample during 60, 30, and 10 min UV irradiation, respectively. Closed circles with numbers 5 and 6 indicate sucrose concentrations from the control samples. Open and closed circles with numbers 1 and 5 are 1/10 dilution samples of open circle with number 3 and closed circle with number 6, respectively.

damage to the chloroplasts by UV irradiation. A similar reduction of sucrose contents by UV-B irradiation was previously reported by Takeuchi et al. (1989). Considering the role of sucrose as a phagostimulant, understanding the mechanism of sucrose reduction is of importance.

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AVOIDANCE OF ANTINUTRITIVE PLANT DEFENSE: ROLE OF MIDGUT pH IN COLORADO POTATO BEETLE[1]

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Abstract—The fate of the tomato foliar phenolic, chlorogenic acid, in the digestive systems of Colorado potato beetle *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) and *Helicoverpa zea* (Lepidoptera: Noctuidae) is compared. In larval *H. zea* and other lepidopteran species previously examined, approximately 35–50% of the ingested chlorogenic acid was oxidized in the digestive system by foliar phenolic oxidases (i.e., polyphenol oxidase and peroxidase) from the tomato plant. The oxidized form of chlorogenic acid, chlorogenoquinone, is a potent alkylator of dietary protein and can exert a strong antinutritive effect upon larvae through chemical degradation of essential amino acids. In contrast, in *L. decemlineata* less than 4% of the ingested dose of chlorogenic acid was bound to protein. In vitro experiments to determine the influence of pH on covalent binding of chlorogenic acid to protein showed that 30–45% less chlorogenic acid bound to protein at pHs representative of the beetle midgut (pH 5.5–6.5) than at a pH representing the lepidopteran midgut (pH 8.5). At an acidic pH, considerably more of the alkylatable functional groups of amino acids ($-NH_2$, $-SH$) are in the nonreactive, protonated state. Hence, polyphenol oxidases are unlikely to have significant antinutritive effects against the Colorado potato beetle and may not be a useful biochemical source of resistance against this insect. The influence of feeding by larval Colorado potato beetle on foliar polyphenol oxidase activity in tomato foliage and its possible significance to interspecific competition is also considered.

Key Words—*Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae, Colorado potato beetle, *Helicoverpa zea*, Lepidoptera, Noctuidae, phenolics, poly-

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phenol oxidase, peroxidase, chlorogenic acid, *Lycopersicon esculentum*, tomato, host-plant resistance, midgut pH.

INTRODUCTION

Feeding by the Colorado potato beetle, *Leptinotarsa decemlineata*, is normally restricted to solanaceous plants including potato, tomato, and eggplant (Hsiao, 1974, 1978; Hare, 1990). Because of the considerable damage this insect may cause to these crops, substantial efforts have been made to develop host-plant resistance to the beetle; however, commercially acceptable varieties have not been released (Hare, 1990). Several phytochemicals have been specifically implicated in resistance to the Colorado potato beetle (CPB), including steroidal glycoalkaloids (Hare, 1983, 1987; but see Barbour and Kennedy, 1991), methyl ketones (Kennedy and Sorenson, 1985) and the sesquiterpene, zingiberine (Carter et al., 1989). Although CPB causes a massive induction of serine protease inhibitors in tomato leaves during feeding (Green and Ryan, 1972), the beetle is insensitive to their adverse effects (Wolfson and Murdock, 1987) because it relies not upon serine proteases such as trypsin and chymotrypsin, but instead upon the thiol proteases, cathepsins B and H, for protein digestion (Murdock et al., 1987; Thie and Houseman, 1990).

Another important component of chemical defense in solanaceous plants is the enzymic oxidation of phenolics. In the wild potato species, *Solanum berthaultii*, type A glandular trichomes containing polyphenol oxidase are essential for resistance against CPB (Neal et al., 1989; Tingey, 1991). Moreover, type B trichomes containing sucrose esters increase the expression of resistance in the presence of type A trichomes (Neal et al., 1989; Pelletier and Smilowitz, 1990; Tingey, 1991). A significant amount of the total leaf polyphenol oxidase is associated with the chloroplast in potato and tomato. The majority of the work on phenolic oxidation in potato has focused on the entrapping properties of trichomes; little work has been done on the potential antinutritive properties of oxidized phenolics as a basis of resistance to CPB. Carter (1987) reported that resistance to CPB in tomato species was not associated with total foliar phenolic oxidation.

Our paper compares the ability of an antinutritive plant defense (i.e., polyphenol oxidase) to operate against two insects, CPB and *Helicoverpa* (=*Heliothis*) *zea*, with distinctly different gut physiologies, the former having an acidic midgut and the latter an alkaline midgut. In the highly polyphagous lepidopteran species, *H. zea* and *Spodoptera exigua*, phenolic oxidation exerts a strong antinutritive effect against larvae (Felton et al., 1989a,b). When larvae disrupt plant cellular compartments during feeding, the enzyme polyphenol oxidase mixes with phenolic substrates such as chlorogenic acid, leading to the rapid formation of orthoquinones (Felton et al., 1989a). The quinones readily

alkylate dietary protein, resulting in a substantial decline in the nutritive quality of the protein (Felton et al., 1989a,b; Duffey and Felton, 1989, 1991). The lepidopteran species tend to feed on fruiting structures, where polyphenol oxidase activity is markedly lower than in foliage, thereby avoiding a significant impact of phenolic oxidation upon food quality (Felton et al., 1989a). In contrast, CPB feeds primarily on foliage and hence is potentially exposed to high levels of phenolic oxidation products.

We report here an assessment of the potential for polyphenol oxidase to operate as an antinutritive source of resistance to CPB. The role of midgut pH in mediating the impact of phenolic oxidation on food quality is also considered.

METHODS AND MATERIALS

Insects and Plants. Eggs of CPB were obtained from a colony maintained on potato in the laboratory of Dr. George Kennedy, North Carolina State University. Emerging larvae were reared in environmental chambers on tomato, *Lycopersicon esculentum* (var. Castlemart) for one generation before experiments were begun. Eggs of *H. zea* were obtained from the Bioenvironmental Insect Control Laboratory, USDA (Stoneville, Mississippi). Plants used for feeding and induction assays were grown following Broadway et al. (1986).

Chemicals and Isotopes. Tritiated chlorogenic acid was prepared by tritium exchange by Research Products International Corp. (Gif-sur-Yvette, France) and purified using thin-layer chromatography conforming to Isman and Duffey (1983). The specific activity of the purified [^{3}H]chlorogenic acid was 153 mCi/mmol.

Enzyme Assays. Preparation of leaf tissue for enzyme analysis has been described previously (Felton and Duffey, 1990). Peroxidase and polyphenol oxidase activities were measured following Ryan et al. (1982) using guaiacol and chlorogenic acid as substrates, respectively. Leaf protein was measured using Coomassie blue G-250 dye following Jones et al. (1989).

Metabolism of Chlorogenic Acid in Larval Digestive System. Two-microliter aliquots of [^{3}H]chlorogenic acid containing ca. 205,000 dpm (ca. 215 ng) in 50% aqueous methanol were applied to the surfaces of tomato leaflets weighing ca. 50 mg. After the aqueous methanol evaporated, fourth-instar CPB or fifth-instar *H. zea* larvae that had been starved for 12 hr were placed on individual leaflets containing the radioisotope. Larvae completely ingesting the leaflet within 6 hr were used in the assay. Feces were collected as described (Felton et al., 1989a) from nine larvae per species and pooled in groups of three larvae for each replicate. A total of three replicates per species was analyzed by two-dimensional thin-layer chromatography (2% formic acid followed by *n*-butanol–ethanol–2% NH_4OH at 6:1:3 on cellulose) followed by liquid scintillation

counting for radioactivity associated with free chlorogenic acid, protein-bound chlorogenic acid, and polymeric chlorogenic acid (Felton et al., 1989a).

Oxidative Enzymes in Larval Midgut. To determine the presence of phenolic oxidizing enzyme activity in the beetle digestive system, five newly molted fourth-instar larvae were placed on an undamaged six- to seven-leaf stage tomato plant. Larvae were removed after 24 hr and held on ice for 30 min. Midguts were removed, and each midgut, containing lumen contents, was mechanically homogenized in ice-cold 1.15% KCl for 30 sec. Samples were centrifuged at 10,000*g* for 20 min; the supernatant was used immediately as the enzyme source for peroxidase and polyphenol oxidase. One leaflet was excised with a razor blade from each leaf of the tomato plant and assayed for peroxidase and polyphenol oxidase activities as described above. The experiment was replicated five times. Protein concentration was quantified using Coomassie brilliant blue G-250 following Jones et al. (1989).

Determination of pH in Larval Digestive System. Twenty fourth-instar CPB and 20 fifth-instar *H. zea* were placed individually in Petri dishes containing two tomato leaflets excised from six-leaf stage tomato plants. After 24 hr, regurgitate was individually collected from 10 larvae of each species for pH determination. Midgut luminal fluid was individually collected from the remaining 10 larvae per species following dissection of the digestive tract. The pH of the regurgitate and midgut fluid was determined with short-range pH paper.

Influence of pH on Polyphenol Oxidase and on Covalent Binding of Chlorogenic Acid to Protein. To determine the influence of pH on polyphenol oxidase activity and the subsequent binding of chlorogenic acid to protein, tomato polyphenol oxidase was partially purified by ammonium sulfate fractionation to a specific activity of 2300 units/mg protein (Felton and Duffey, 1991a). One unit of activity equals a change in OD_{470} of 0.001/min with chlorogenic acid as a substrate. A 1-ml solution containing 20 mg bovine serum albumin and 3.0 μmol chlorogenic acid was incubated with 30 μg polyphenol oxidase in 0.1 M sodium phosphate buffer at pH 5.5, 6.5, 7.5, or 8.5 for 60 min. Three replicates were performed at each pH. The polyphenol oxidase activity for each pH level was determined from 10-μl aliquots performed in triplicate (Ryan et al., 1982). Control treatments for each pH were treated identically except chlorogenic acid was omitted.

The solutions were then dialyzed (12,000 mol wt cutoff) at 1–4°C against 8 M urea for 24 hr to remove noncovalently bound chlorogenic acid (Barbeau and Kinsella, 1983), followed by dialysis against distilled H_2O for 24 hr, and finally desalted in a PD-10 Sephadex G-25M column (Pharmacia LKB, Uppsala, Sweden) according to the manufacturer's suggestions. The amount of chlorogenic acid covalently bound to the protein was determined from a standard curve using a SLM-Aminco 3000 Array spectrophotometer following Broadway et al.

(1986). The small amount of background absorbance in the respective control treatments was subtracted from each test protein.

Effect of Colorado Potato Beetle Feeding on Leaf Phenolic Oxidases. To determine the effect of CPB larval feeding on polyphenol oxidase activity, tomato plants (var. Castlemart) were grown in the greenhouse in fine mesh cages to exclude unwanted insects. When plants reached the six- to seven-leaf stage, one fourth-instar larva was placed on each plant on the lowest true leaf. No effort was made to restrict larval feeding to a particular leaf position. Control plants were treated identically throughout with the exception of not being infested with beetle larvae. Seven plants per treatment were maintained in the cages during the experiment. After seven days, plants were transported to the laboratory where six leaflets (one terminal leaflet per leaf) per plant were removed for analyses. Leaflets included both damaged and undamaged samples. Leaflets for each replicate plant were pooled, homogenized, and assayed in triplicate for polyphenol oxidase and peroxidase as described above.

RESULTS

Metabolism of Chlorogenic Acid in Larval Digestive System. The majority of excreted chlorogenic acid in the CPB was not covalently bound to protein but remained in unbound, monomeric form (Table 1). Less than 4% of excreted chlorogenic acid was covalently bound to protein and/or amino acids, but a significant amount (ca. 16%) of excreted chlorogenic acid was polymerized. In contrast, considerably more excreted chlorogenic acid was bound to protein and/or amino acids in larval *H. zea*: nearly 35% was covalently bound, and approximately 14% was polymerized. Polymerized chlorogenic acid remained at or

TABLE 1. FATE OF EXCRETED [^{3}H]-CHLOROGENIC ACID IN FECES OF COLORADO POTATO BEETLE *Leptinotarsa decemlineata* AND TOMATO FRUITWORM *Helicoverpa zea* LARVAE

Chemical	Percent of total excreted radioactivity[a]	
	CPB	*H. zea*
Chlorogenic acid (unbound)	82.9 (±12.2) A	34.1 (±7.5) B
Chlorogenic acid–protein conjugate	3.5 (±0.9) A	34.8 (±7.6) B
Chlorogenic acid (polymer)	16.3 (±4.4) A	13.7 (±2.6) A

[a]Means in rows followed by a different letter are statistically different at $P < 0.05$ by ANOVA and separation of means by 95% confidence limits. Each value represents the mean of three replicates. Numbers in parentheses represent 95% confidence limits.

near the origin in the TLC systems tested. Chlorogenic acid chemically oxidized with 0.1 N NaOH or with ferrous sulfate at pH 8.0 behaved similarly.

Oxidative Enzymes in Larval Midgut. Substantial polyphenol oxidase activity was present in the midgut contents in CPB larvae feeding on tomato foliage (Table 2). Activity was approximately 36% of that found in tomato foliage used as the food source. It is unknown if the midgut contains endogenous polyphenol oxidase or is merely derived from ingested foliage; nevertheless, ingested polyphenol oxidase activity is reduced in the gut of CPB. Peroxidase activity in the midgut contents was nearly 12× in excess of that present in ingested leaflets (Table 2). The enriched peroxidative activity of the midgut with respect to the food plant implies the presence of endogenous peroxidase.

pH of Larval Digestive System. The pH of the CPB regurgitate ranged from 5.5 to 6.0, whereas the regurgitate from *H. zea* was 7.0–7.4. The beetle's midgut fluid was acidic at pH 6.0–6.5, but the caterpillar's midgut was alkaline, ranging from 8.0 to 8.7.

Influence of pH on Polyphenol Oxidase Activity and Covalent Binding of Chlorogenic Acid to Protein. Both polyphenol oxidase from tomato foliage and covalent binding of chlorogenic acid to protein were significantly affected by pH (Figure 1). Optimal oxidase activity occurred at pH 6.5 within the pH range of the beetle midgut lumen, but at pH 8.5, representative of the lepidopteran midgut, polyphenol oxidase activity was nearly 50% less. However, maximal covalent binding was observed at the alkaline pH 8.5. At pHs occurring in the digestive system of CPB (i.e., 5.5–6.5), ca. 25–40% less covalent binding occurred than at pH 8.5 (Figure 1).

Effect of Colorado Potato Beetle Feeding on Leaf Phenolic Oxidases. Feeding by CPB for 48 hr on tomato foliage did not significantly ($P > 0.05$) affect foliar peroxidase activity (Figure 2). However, polyphenol oxidase activity in foliage increased by 92% when feeding damage by CPB occurred (Figure 2; $P < 0.01$).

TABLE 2. PHENOLIC OXIDIZING ENZYMES[a] IN TOMATO FOLIAGE AND MIDGUT OF COLORADO POTATO BEETLE

Source	Polyphenol oxidase	Peroxidase
Tomato foliage	3.940 (±0.122)	7.481 (±0.078)
Beetle midgut[b]	1.432 (±0.050)	86.420 (±5.94)

[a] Enzyme activities expressed as change in OD_{470}/min/mg protein. Numbers in parentheses represent 95% confidence limits of the mean with five replications per tissue. Polyphenol oxidase measured with chlorogenic acid as a substrate and peroxidase with guaiacol.

[b] Midgut measurements made with midgut wall and lumen contents.

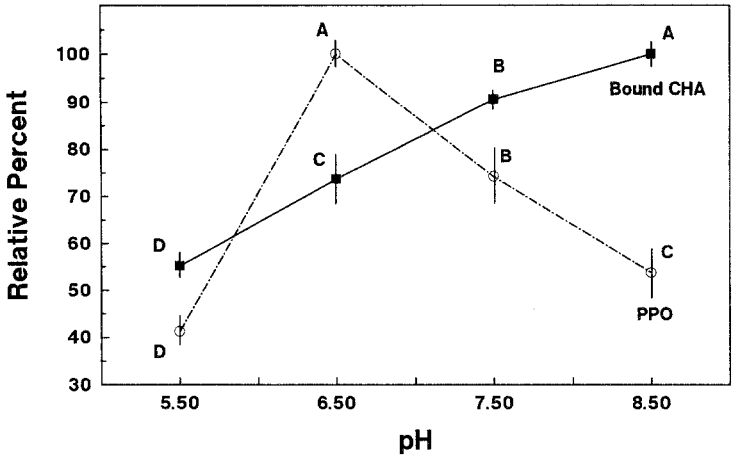

FIG. 1. Influence of pH on tomato foliar polyphenol oxidase (PPO) activity and on the covalent binding of chlorogenic acid (CHA) to protein. Each point represents the mean of three replicates. Error bars indicate 95% confidence limits. Means on each line not followed by the same letter are significantly different at $P < 0.05$ by 95% confidence limits. The absolute values for 100% relative polyphenol oxidase activity and 100% covalent binding are respectively, 2300 units/mg protein/min and 144.4 nmol chlorogenic acid bound/mg protein.

DISCUSSION

The fate of the foliar phenolic, chlorogenic acid, in the digestive system of the Colorado potato beetle was strikingly different from its fate in lepidopteran species (Felton et al., 1989a; Felton and Duffey, 1991a). In the Colorado potato beetle, the majority of ingested chlorogenic acid remained unoxidized in the beetle's digestive system (ca. 80%); however, a substantial amount (16%) was oxidatively polymerized. A small amount of chlorogenic acid (<4%) was covalently bound to amino acids and/or proteins in the beetle digestive system. In contrast, in the lepidopteran species *H. zea*, *Spodoptera exigua*, *Manduca sexta*, and *Trichoplusia ni*, approximately 35–50% of excreted chlorogenic acid was covalently bound to protein or amino acids (Felton et al., 1989a; Felton and Duffey, 1991a, unpublished data).

The differences in phenolic–protein binding among these insects are largely explicable by differences in midgut pH. The midgut luminal pH in the lepidopteran species was moderately to highly alkaline (i.e., pH 8.0–11.0; Felton et al., 1989a; Felton and Duffey, 1991a; Dow, 1984), but the pH of the digestive fluids of the beetle was mildly acidic (i.e., pH 5.5–6.5; see also Wolfson and Murdock, 1987). In an acidic midgut, chlorogenic acid is less likely to be covalently bound to amino acids and proteins for two reasons. First, the nonen-

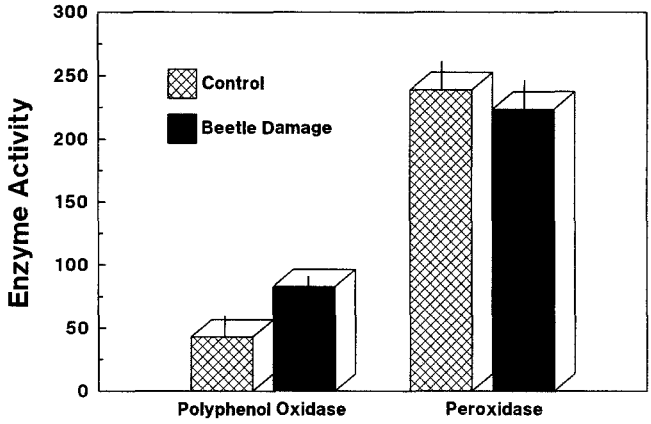

FIG. 2. Effect of foliar feeding by Colorado potato beetle larvae on activities of tomato polyphenol oxidase and peroxidase. Enzyme activity expressed in units of change in OD_{470}/min/gm foliage with guaiacol as substrate for peroxidase and chlorogenic acid as substrate for polyphenol oxidase. Controls represent undamaged plants. Error bars indicate 95% confidence limits. Each treatment was replicated seven times. Activities of enzymes were determined after seven days feeding by one fourth-instar larva. Polyphenol oxidase activity was significantly higher ($P < 0.01$) in plants damaged by beetle larvae. No significant differences were observed in peroxidase activity.

zymic oxidation (i.e., autooxidation) of chlorogenic acid is minimized at acidic pH (Barbeau and Kinsella, 1983; Cilliers and Singleton, 1989). Second, the chemical reactivity of the nucleophilic functional groups of amino acids (−SH and −NH or $-NH_2$) is strongly influenced by pH. In alkaline conditions, many amino acid side chains are unprotonated and, consequently, more prone to nucleophilic attack by electrophiles (e.g., quinones, epoxides); on the other hand in acidic pH (<6.5), the side chains become protonated and hence less susceptible to alkylation (Pierpoint, 1983; Barbeau and Kinsella, 1983). For instance, the pK_as of the reactive −SH, −NH, and $-NH_2$ groups of cysteine, histidine, and lysine, are respectively 8.1, 8.8, and 9.0. Consequently, at acidic pH, few of these groups are unprotonated and the oxidative polymerization of phenolics is favored, thereby minimizing the antinutritive effects of phenolics on protein. The phenolic polymer of chlorogenic acid possesses negligible toxicity to lepidopteran species (Felton et al., 1989a); however, its toxicity to CPB has not been determined.

The oxidative polymerization of phenolics by insect peroxidase and phenol oxidases has been hypothesized to be a detoxicative process in certain insects (e.g., aphids; Peng and Miles, 1988). It is conceivable that in insect species possessing an acidic midgut, peroxidase and polyphenol oxidase activities (Table

2) contribute to detoxification of chlorogenic acid. However, it must also be noted that the midgut of the CPB presumably possesses a negative oxidation potential because of the necessity for thiol digestive proteases to be in a reducing environment (Murdock et al., 1987; Thie and Houseman, 1990). A reducing environment minimizes phenolic oxidation (Appel and Martin, 1990) and may partially account for our observations that substantially less total phenolic oxidation (i.e., polymer formation and protein-bound phenolic) occurs in CPB compared to the lepidopteran species, despite the fact that foliar polyphenol oxidases in tomato are highly active at the pH of the CPB midgut (Felton et al., 1989a).

The relatively large differences in the pH of the digestive system between the leaf-feeding beetle, CPB (mildly acidic, ca. 5.5–6.5), and the leaf-feeding lepidopterans, *Manduca sexta* and *Trichoplusia ni* (highly alkaline, >9.5) may represent two divergent physiological mechanisms for tolerating phenolics and their oxidation products. We have hypothesized that, although quinones are more reactive with amino acids in the highly alkaline midgut, the alkalinity would allow for greater solubilization of plant protein, thus compensating for any nutritional loss in essential amino acids due to conjugation of phenolics with amino acids (Felton and Duffey, 1991a). This appends the earlier interpretation of gut alkalinity as an adaptive mechanism to circumvent tannin toxicity through inhibition of hydrogen bond complexation (Berenbaum, 1980). Alternatively, an acidic midgut impedes the covalent conjugation of amino acids with the electrophilic quinones, thus preventing the loss of essential amino acids. Therefore, it follows that insects with mildly alkaline digestive systems (i.e., pH 8–9; *H. zea* and *Spodoptera exigua*) are predicted to be most susceptible to the antinutritive effects of phenolic oxidation. Hence, we predict that host-plant resistance based upon the antinutritive properties of polyphenol oxidase will not be particularly effective against insects possessing acidic midguts (e.g., many beetles and grasshoppers).

The ability of an acidic midgut to inhibit phenolic-protein covalent interactions cannot fully account for the unexpectedly small amount of phenolic–protein conjugate observed in feces of the CPB (i.e., <4%; Table 1) as compared to the 55% observed in vitro (Figure 1). We are aware that there are other physiological and/or biochemical properties of the CPB digestive system that probably contribute to minimizing phenolic–protein binding and/or phenolic toxicity. In fact, a host of other mechanisms are known to be involved in detoxifying phenolics in herbivorous insects; the sheer number of detoxification mechanisms implicates phenolics as important chemical mediators in plant–herbivore associations. These mechanisms involve a combination of physiological, biochemical, and chemical processes (Table 3). For instance, phenolics may be maintained in their reduced state via quinone reductase, which was reported in several lepidopterans including *H. zea* (Yu, 1987; Felton and Duffey, 1992).

TABLE 3. SUMMARY OF PHYSIOLOGICAL AND BIOCHEMICAL MECHANISMS OF INSECT HERBIVORES FOR TOLERATING TOXICITY FROM PHENOLICS AND QUINONES

Mechanism	Author and citation
Enzymatic mechanisms	
Quinone reductase	Yu (1987), Felton and Duffey (1992)
Ascorbate reducing enzymes	Felton and Duffey (1992)
Superoxide dismutase	Ahmad and Pardini (1990)
Catalase	Ahmad and Pardini (1990), Pritsos et al. (1988), Felton and Duffey, (1991b)
Glutathione transferase	Weinhold et al., (1990)
Esterases	Lindroth and Hemming (1990)
Reduced β-glucosidase activity	Lindroth (1988)
Glutathione reductase	Ahmad and Pardini (1990), Pritsos et al. (1988), Felton and Duffey (1992)
Nonenzymatic mechanisms	
Reducing conditions in gut	Appel and Martin (1990)
Gut surfactants	Felton and Duffey (1991a)
Alkaline midgut	Felton and Duffey (1991a)
Acidic midgut	This investigation
Ascorbic acid	Felton and Duffey (1992)
Glutathione	unpublished data

The widely occurring antioxidant enzymes catalase, superoxide dismutase, and glutathione reductase have received considerable attention as protective mechanisms against oxidative damage by phenolics (e.g., Ahmad and Pardini, 1990; Pritsos et al., 1988; Felton and Duffey, 1991b). Glutathione transferases can facilitate the conjugation and elimination of phenolic oxidation products (Weinhold et al., 1990). Lindroth (1988) reported on the role of diminished β-glucosidase activity in Lepidoptera as an adaptation to minimize the enzymatic conversion of phenolic glycosides to the more toxic aglycone. Moreover, esterases are also important in the detoxification of some phenolics (Lindroth and Hemming, 1990).

Reducing agents such as ascorbate and glutathione may provide additional protection by reducing oxidized phenolics (i.e., quinones). We have also proposed that ascorbate functions in an enzymatic reducing system utilizing the enzymes ascorbate free radical reductase and dehydroascorbate reductase to maintain the redox properties of ascorbate (Felton and Duffey, 1992). The reducing conditions found in the midgut of *Manduca sexta* were also postulated to be a protective mechanism against phenolic oxidation (Appel and Martin, 1990). Gut surfactants, such as lysolecithin, may directly inhibit polyphenol oxidase activity (Felton and Duffey, 1991a). Furthermore, the metal-binding protein,

ferritin, in the midgut of Lepidoptera may sequester iron and prevent its participation as a catalyst in phenolic autooxidation (Nichol and Locke, 1989).

If CPB successfully avoids the antinutritive or toxic effects of phenolic oxidation as contended, then the induction or amplification of polyphenol oxidase activity by CPB feeding (Table 2) would seem to have limited defensive value against CPB. This may be analogous to the induction of serine protease inhibitors by CPB damage, which do not target the digestive processes of CPB (Ryan et al., 1986; Wolfson and Murdock, 1987). The induction of these defenses, however, may indirectly alter interspecific competition. The fitness of other herbivores, which are susceptible to protease inhibitors and/or phenolic oxidation (e.g., *H. zea*; Felton et al., 1989a,b), may be compromised when utilizing host plants previously or concurrently damaged by CPB. This form of competition mediated through host-plant chemistry may be comparable to the induction of cucurbitacins in *Cucurbita* reported by Tallamy and Krischik (1989), wherein the cucurbitacins were effective defenses against generalist herbivores but were ineffective against specialist herbivores because these chemicals acted as feeding stimulants. Damage caused by early-season leaf-chewing guilds in *Quercus emoryi* affected the performance of a late-season, leaf-mining guild via chemical interferences (Faeth, 1986). Furthermore, interspecific competition between spider mites and a vascular wilt fungus in cotton is presumably chemically mediated (Karban et al., 1987). Karban and English-Loeb (1990) also showed that a "vaccination" of Willamette spider mites on grapevines prevented large population densities of Pacific spider mites from occurring. Our data provide further evidence to support the contention that the future emphasis in insect–plant interactions should be on interactive networks of species rather than simply upon species pairs.

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COMPUTER TRACKING AS A BEHAVIORAL GC DETECTOR: NEMATODE RESPONSES TO VAPOR OF HOST ROOTS

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Abstract—A computer tracking system previously developed for studying the behavioral responses of nematodes to volatile stimuli was modified so that the effluent of a gas chromatograph passed over the nematodes while their behavior was analyzed. Since the behavior of several hundred nematodes can be analyzed simultaneously, this technique has the potential to provide a sensitive assay for behaviorally active fractions from the GC. This potential was tested with infective juveniles of the root-knot nematode *Meloidogyne incognita* and vapor from the roots of host tomato plants. Direct injection of 1 ml of vapor led to reproducible responses at times corresponding to nitrogen plus oxygen elution and to carbon dioxide elution. Experiments with pure gases demonstrated responses to oxygen and to carbon dioxide. Attempts were made to find less volatile and less active components by pulling large volumes of vapor through a trap of Tenax GC and subsequently eluting them onto the GC. This approach did not lead to the detection of other activities. We conclude that, other than carbon dioxide, there are no volatile stimuli released from host roots in effective quantities.

Key Words—Root-knot nematode, *Meloidogyne incognita*, root vapor, bioassay, gas chromatography, chemotaxis, tomato, carbon dioxide, oxygen.

INTRODUCTION

We have previously developed a technique that permits the rapid acquisition of behavioral information on a group of animals (Dusenbery, 1985a). This technique employs a video camera interfaced to a microcomputer and a program

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that keeps track of the position of up to several hundred individuals simultaneously. The technique was developed to study the responses of nematodes to volatile stimuli that were carried in a stream of air flowing over them (Dusenbery, 1985b; Pline and Dusenbery, 1987). In this paper, we report on experiments in which this system was connected to a gas chromatograph (GC) so that the effluent of the GC was carried in the airstream over the nematodes. This technique is analogous to the use of insect antennae for monitoring GC effluents (Moorhouse et al., 1969; Arn et al., 1975). The system was used to look for volatile stimuli that plant-parasitic nematodes might employ to locate the roots of host plants.

Nematodes are economically important pests in agriculture, and identification of the stimuli they use to move to the roots of host plants might provide better ways of controlling them (Dusenbery, 1987b). However, relatively little information is available on the mechanisms by which they locate host plants. The common assumption is that chemotaxis to chemicals released by the roots plays an important role (Steiner, 1925; Prot, 1980). One of the most widespread nematode pests is the root-knot nematode *Meloidogyne incognita*. This investigation is part of a series on the sensory behavior of infective juveniles of this species (Diez and Dusenbery, 1989a,b; Dusenbery, 1987a, 1988a–c, 1989; Goode and Dusenbery, 1985; Pline and Dusenbery, 1987; Pline et al., 1988).

METHODS AND MATERIALS

Nematodes and Host Plants. The nematodes were grown on tomato plants (Rutgers cultivar) as previously described (Goode and Dusenbery, 1985; Diez and Dusenbery, 1989b). Plants used for a source of chemical stimuli were similar tomato plants that had grown six to eight weeks.

Computer Tracking System. The computer tracking system was similar to that previously described (Pline and Dusenbery, 1987; Dusenbery, 1992). An IBM XT contained an Imaging Technology video camera interface and to it was added a MetraByte DASCON-1 multifunction board that contains a four-channel, 12-bit analog to digital (A/D) converter that was used to obtain a record of the GC oven temperature and signal from the GC detector.

Gas Chromatograph. The GC was an HP 5890. For some experiments it was fitted with a cryogenic cooler for the oven. The column was 1/8-in. stainless steel, 6 ft long, and packed with Tenax GC. This large-bore column was used to increase the flow rate and amount of material eluted. The response of the nematodes was not sufficiently fast that the brief peaks characteristic of higher-resolution columns were expected to be advantageous. To further broaden the peaks to match the response of the nematodes, the rate of flow was reduced to about 0.1 ml/sec (helium). When natural samples were studied, the oven tem-

perature was increased after injection in order to maintain approximately constant peak width across a broad range of retention times. In order to separate oxygen and nitrogen from carbon dioxide, the temperature program was started at about 3°C.

A thermal conductivity detector (TCD) was used in order not to destroy the chemicals eluting from the column. The effluent of the detector was injected into the center of a parallel flow of hydrated air, which was 10 times larger in volume flow rate than the effluent flow of the column (Figure 1). This combined flow was carried through 157 cm of 0.06-cm internal diameter Teflon tubing to the chamber containing the nematodes. Within this length of tubing lateral diffusion should be sufficient to provide good mixing. In some experiments, a splitter was used to bypass the TCD in case biologically active chemicals were altered in the hot detector, but no evidence of alteration was found.

Traps. Traps for volatile chemicals were made from 5 cm of 1/8-in. stainless steel tubing fitted with a stainless steel frit at each end and filled with 28 mg of Tenax GC. Before use the trap was purged by passing helium at 7 ml/min at a temperature of 290°C overnight. The trap was loaded by pulling air through it at a rate of about 1 ml/min for several hours. The trap was then immediately connected at the head of the GC column (Mieure and Dietrich, 1973) and flow and a temperature program started.

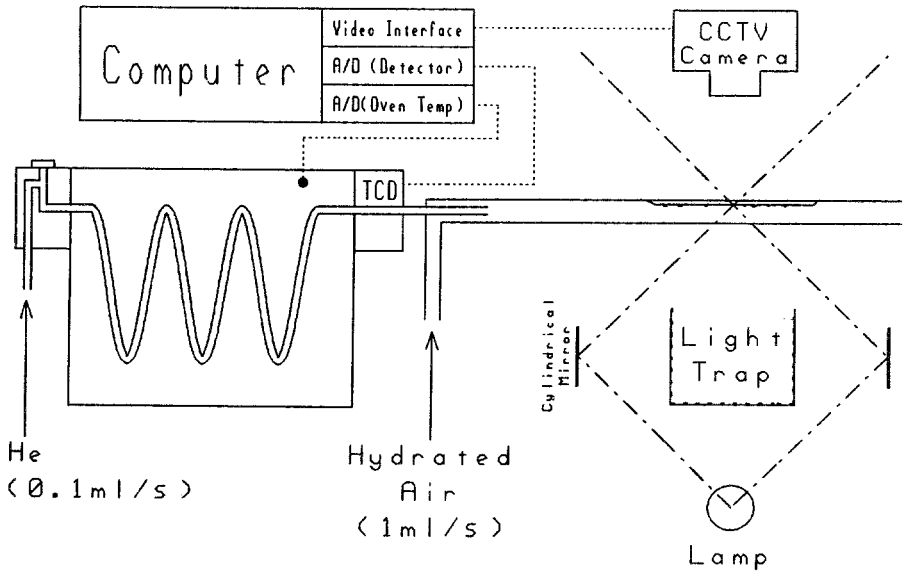

FIG. 1. Schematic diagram of the system. The nematodes are located on the right, under the camera.

RESULTS

The simplest experiments involved the direct injection of a sample of root vapor into the GC. In most experiments, a 1-ml sample was taken with a gas-tight syringe from a plastic bag in which the roots of a tomato plant and 100 ml of water had been sealed for 24 hr (Figure 2). With these samples, three TCD peaks were apparent. The first contained both O_2 and N_2, the second CO_2, and the third H_2O. The average rate of movement of the nematodes usually showed several peaks that appeared to be above background, but not much. Repetition of the experiment revealed that the only reproducible responses were those that lagged slightly behind the first and second TCD peaks. Carbon dioxide is known to be a powerful stimulus under these conditions (Pline and Dusenbery, 1987), and the nematodes respond to it when CO_2 is injected into the GC (data not shown). This accounts for the second response peak.

In order to identify which gases were responsible for the first response peak, samples of the pure gases were injected onto the GC (Figure 3). Nitrogen produced no responses. Oxygen usually produced a clear response peak that lagged slightly the TCD peak. This supports the reasonable hypothesis that oxygen was the stimulus for the first response peak from root vapor samples.

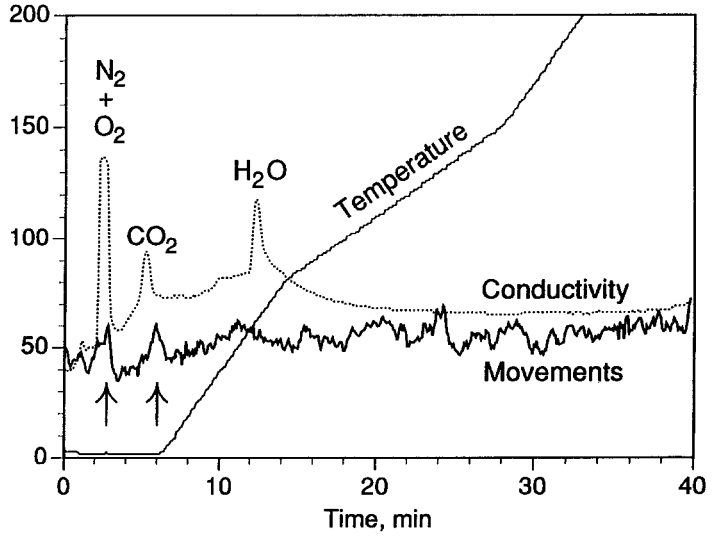

FIG. 2. Direct injection of root vapor into GC. A 1-ml sample was injected at time zero. The plot shows the oven temperature, the relative output of the TCD, and the average rate of movement of the nematodes. The latter has been smoothed with a running average of three samples. The two arrows indicate the response peaks that were found to be reproducible in repeated experiments.

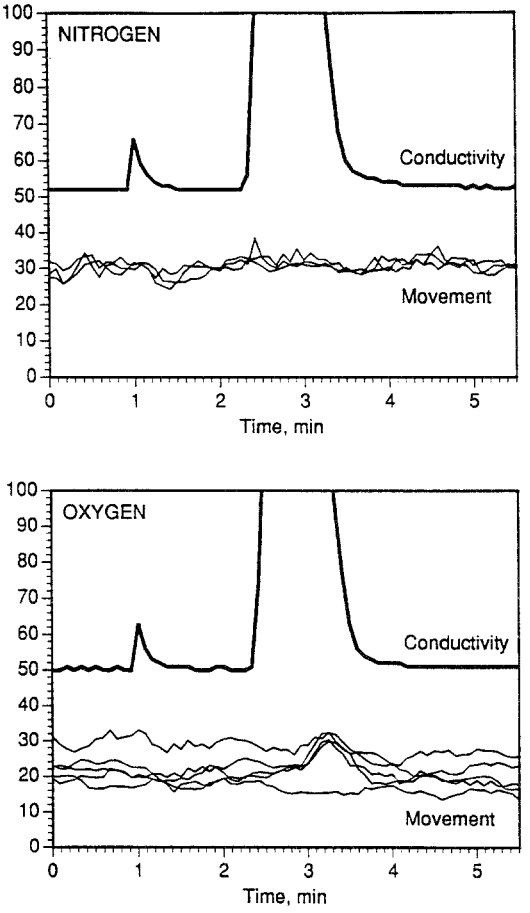

FIG. 3. Responses to pure gases. The upper graph is for the injection of 1 ml of nitrogen; the lower is for oxygen. The column temperature was a constant 0°C. The heavy lines show the output of the TCD. The thin lines show typical responses in repeated experiments. No response is apparent for the three repeats of nitrogen injection. Among the five repeats of oxygen injection, three show very clear responses, one is probable, and one shows no evidence of a response. This variability is typical of what was found in experiments not shown.

Direct injection resulted in a significant dilution ($\approx$50-fold) of the chemicals in the sample. Consequently, there remained a possibility that there were other constituents in the vapor that were diluted to below threshold in the apparatus. In order to search for these, approximately 10 liters of air were pulled through a Tenax GC trap, which can be assumed to trap most chemicals of

interest (e.g., Buttery and Ling, 1984; Sakaki et al., 1984). The trap was then attached at the head of the GC column and trapped chemicals eluted during the programmed temperature increase.

Figure 4 shows typical results for a root vapor sample and a control in which only ambient air was pulled through the trap. For both samples, the thermal conductivity detector shows a large peak of water eluting around 5 min (and atypically broad). The only other peaks detected are several eluting at 30–45 min and present only in the root vapor sample. These peaks occur at a time when 6- to 10-carbon *n*-alkanes would elute. In both samples, the nematode

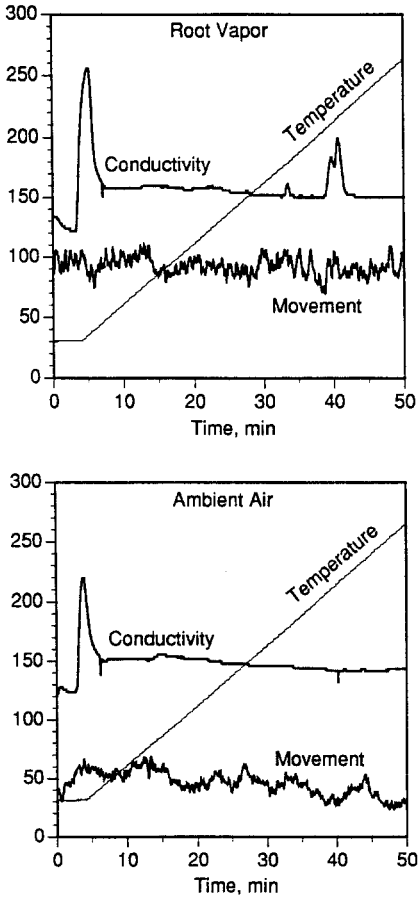

Fig. 4. Eluting Tenax traps on the GC. Typical results for traps that had been charged with root vapor (above) or ambient air (below). The large TCD peak at about 5 min is due to water eluting from the GC.

movements show no clear responses and none of the possible responses were reproduced in repeated experiments.

In other experiments (not shown), samples were obtained by inserting a tube into the center of a 15-cm pot containing a tomato plant and pulling out a sample of the airspace from the undisturbed roots and soil. This type of sampling did not lead to the discovery of any additional stimuli.

DISCUSSION

These results demonstrate that computer tracking has the potential to act as a useful detector of behaviorally active chemicals as they elute from a gas chromatograph. The technique could presumably be adapted to work with a wide range of other kinds of animals.

The results further indicate that there are not any strong volatile stimuli released by the roots of host plants other than carbon dioxide, which was previously shown to be a powerful stimulus to this (Pline and Dusenbery, 1987) and other (Klingler, 1961) nematodes. There is clear evidence from other work (Diez and Dusenbery, 1989b) that there exist nonvolatile repellents released by the roots. It is possible that unstable volatile compounds are released but decompose before detection with our methods. However, it is doubtful that any such chemicals could persist long enough in the soil to act very far from the roots. Roots absorb oxygen and experiments not shown suggest the nematodes are attracted to oxygen. Consequently, the response to oxygen should hinder host finding if it plays any significant role.

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MELIACEAE AND RUTACEAE LIMONOIDS AS TERMITE ANTIFEEDANTS EVALUATED USING *Reticulitermes speratus* KOLBE (ISOPTERA: RHINOTERMITIDAE)

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Abstract—Four limonoids were evaluated by a no-choice bioassay method as termite antifeedants using *Reticulitermes speratus* Kolbe. Obacunone showed the highest degree of antifeeding potency (PC_{95} = 1133 ppm w/w), followed by nomilin (PC_{95} = 4475 ppm), azadirachtin (PC_{95} = 65,293 ppm), and limonin (with PC_{95} estimates beyond the bioassay limits), which was the least potent. None of the four limonoids exhibited any evidence of acute toxicity towards *R. speratus*, although termites fed on obacunone and nomilin at concentrations higher than their PC_{95} estimates tend to die faster than those in the unfed treatment. Obacunone and nomilin also showed a drastic antifeedant consumption (AC) reduction effect at concentrations higher than 510 and 1360 ppm, respectively. With azadirachtin and limonin, such an effect was not observed. The present data suggest that both obacunone and nomilin have an absolute antifeeding activity towards *R. speratus*.

Key Words—*Reticulitermes speratus*, Isoptera, Rhinotermitidae, termite,

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antifeedant, obacunone, nomilin, azadirachtin, limonin, protection concentration.

INTRODUCTION

Substances currently used as wood preservatives, such as arsenic oxide–metal salt mixtures, pentachlorophenol, coal tar creosote, and soil-borne termiticides, are broad-spectrum toxicants. Although they have provided effective control against termites, their extensive and sometimes indiscriminate use has led to serious social and environmental repercussions. Tough legal restrictions have been imposed on their sale and use, and some have been removed from the market (Anonymous, 1986). This has prompted researchers to identify alternative antitermite compounds that are selective and more acceptable environmentally.

A number of new synthetic chemicals, particularly those with delayed toxicity, have been evaluated as baited insecticides against termites (Jones, 1984; Su et al., 1985, 1987; Scheffrahn and Su, 1987; Su and Scheffrahn, 1988; Logan and Abood, 1990). However, one of the chemicals, hydramethylnon, could not successfully control *Coptotermes formosanus* Shiraki in a field trial (Su et al., 1982).

Natural products have also been scrutinized for the same purpose. Work is still scarce, but several extracts of hardwoods are already known to be active (Carter and Mauldin, 1981; Carter et al., 1983). Our present interest on natural products is focused on limonoids, a group of bitter tetranortriterpenoids found predominantly in Meliaceae and Rutaceae plants (Klocke and Kubo, 1982), one of the most promising groups of the natural products for insect control (Klocke et al., 1989; Champagne et al., 1989). Recently, Rutaceae limonoids (obacunone, nomilin, and limonin), which are known to be active against several insect pests (Klocke and Kubo, 1982; Randall and Bentley, 1986; Randall et al., 1987; Champagne et al., 1989), were observed to have potential as antifeedants against termites (Kawaguchi et al., 1989; Serit et al., 1991). Although work on the pesticidal properties of Meliaceae limonoids are voluminous, their antitermite activity has remained largely unknown.

Among the Meliaceae limonoids, azadirachtin is the most suitable candidate for this study. Azadirachtin was first identified as a potent feeding deterrent for the desert locust, *Schistocerca gregaria* (Butterworth and Morgan, 1868, 1971), and has subsequently been reported to have antifeeding activity against nearly 200 species of insects (Jacobson, 1986; Saxena, 1989). In fact, Kubo and Klocke (1982) considered azadirachtin to be one of the most potent insect control agents isolated from botanical sources. In view of the potential future role of limonoids in termite control and the present scarcity of biological data related to their antitermite activity, we compared the antifeeding potency of azadirachtin, oba-

cunone, nomilin, and limonin using *Reticulitermes speratus* Kolbe, a termite of economic importance in Japan.

METHODS AND MATERIALS

Termite. The *R. speratus* colony used in this study was collected in Mie Prefecture, Japan, and maintained in well-humidified chambers at 23°C. The termites used were at least at their third instar (pseudergates), based on their relative sizes. Their weights were determined from a random sample of 50 such individuals as 1.85 ± 0.02 mg (mean ± SE).

Limonoids. The four limonoids are shown in Figure 1. Rutaceae limonoids (i.e., obacunone, nomilin, and limonin) were purified from *Citrus natsudaidai* seeds, as previously described (Serit et al., 1991), while the Meliaceae limonoid (azadirachtin A) was obtained from Daicel Chemical Industries Ltd., Himeji City, Japan, as 98% pure.

No-Choice Bioassay Method. The bottom of transparent plastic cups (6 cm rim diam., 4.5 cm bottom diam., and 3.5 cm height) was layered with 6 g of

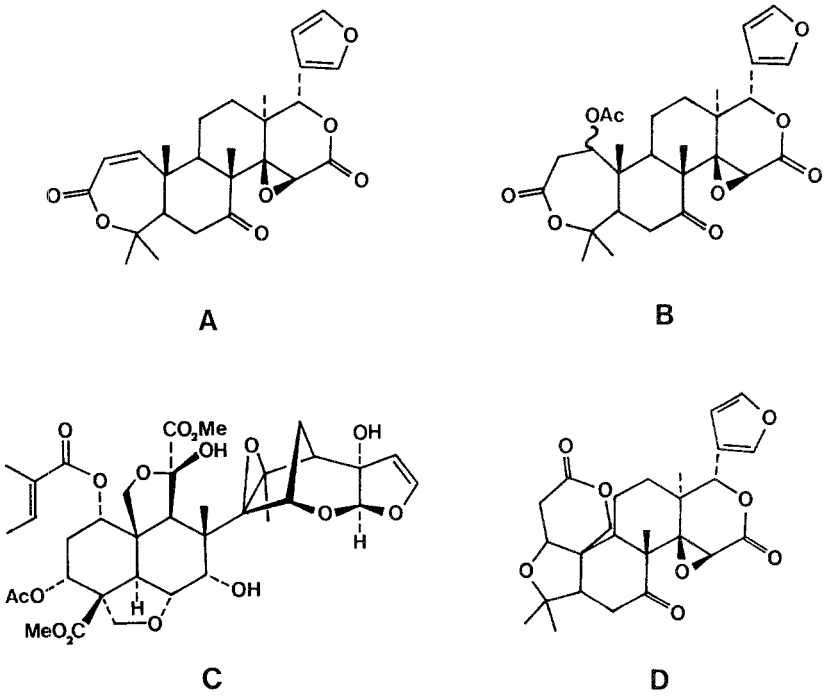

FIG. 1. Structures of obacunone (A), nomilin (B), azadirachtin (C), and limonin (D).

sea sand (Nacalai Tesque Inc., Kyoto, Japan), and moistened with 400 μl of microfiltered water. The sand was subsequently remoistened with 200 μl of water every 10 days during the bioassay.

Paper disks (Toyo Roshi Kaisha Ltd., Tokyo, Japan; 8 mm diam. and 1.5 mm thickness) were oven-dried (40°C, 48 hr). The required amount of each limonoid in 60 μl of an appropriate solvent (i.e., methanol for azadirachtin, and acetone for obacunone, nomilin, and limonin) was applied to the oven-dried paper disks; control and blank disks received only the solvent. Concentration ranges were as follows: azadirachtin, 680–13,600 ppm; obacunone, 340–1700 ppm; nomilin, 680–10,200 ppm; and limonin, 1700–68,000 ppm. The disks were desiccated (room temperature, 48 hr), individually weighed (initial weight, IW), and placed on the sand with 20 termites, except for the control disks (no termites). In order to check *R. speratus* survival under complete starvation, each bioassay was run with an unfed treatment. Survival in each cup was recorded every five days for 25 days. On day 25, disks were collected, oven-dried (40°C, 48 hr), desiccated (room temperature, 48 hr), and individually weighed (final weight, FW). Each bioassay was conducted in three sequential runs of five replicates each.

Calculation and Data Analysis. Despite the drying and desiccation, the control disks were still observed to increase by an average of 1.38% in weight as a result of the water addition during the bioassay. Therefore, a correction procedure was necessary. Disk weight loss, which was the estimate of the amount of food consumed (C), was calculated as $IW_s - [(FW_s \times IW_c)/FW_c]$, where subscript c indicates control disks, and subscript s the treated and blank disks.

Percentage feeding reduction (% FR) was calculated as $(1 - C_t/C_b) \times 100\%$, where subscripts b and t indicate blank and treated disks, respectively. The data were analyzed using probit analysis (Finney, 1971) to estimate the 50% and 90% protection concentrations (or PC_{50} and PC_{95}, respectively) for each limonoid, but comparison of their antifeeding potency was based on their PC_{95} estimates, as suggested by Klocke and Kubo (1982).

Amount of limonoid lost per disk at each concentration was used as the estimate the antifeedant consumption (AC). Assuming an even distribution, AC was calculated as $(C_t/IW_t) \times$ concentration (Lewis and van Emden, 1986). The means were compared using a Student's t test for unpaired data.

RESULTS

Antifeeding Potency. After 25 days, termites consumed a mean ± SE of 19.36 ± 0.82 mg of the blank disks (initial weight = 29.41 ± 0.69 mg). At the highest concentrations tested, azadirachtin (13,600 ppm) and limonin (68,000 ppm, maximum disk capacity for limonin) could only induce an average feeding

reduction of 80.70 and 50.40%, respectively. On the other hand, it required only 850 ppm of obacunone or 3400 ppm of nomilin to reduce feeding by 89.20 and 89.84%, respectively.

The most potent antifeedant against *R. speratus* was obacunone (PC_{95} = 1133 ppm), followed in decreasing order of potency by nomilin (PC_{95} = 4475 ppm), and azadirachtin (PC_{95} = 65,293 ppm); limonin, with PC_{95} estimates beyond the bioassay limits, was the least potent (Table 1). Regression slopes were steep for obacunone (5.33) and nomilin (3.21), but gradual for azadirachtin (1.11) and limonin (0.48).

Effect on Survival. Unfed termites reached an average survival of 0.3% on day 25, compared with 95.0% survival in the blank treatment (Table 2). At their highest concentrations tested, azadirachtin and limonin could only reduce survival on day 25 to 40.3 and 56.7%, respectively. With obacunone and nomilin, it required only 1190 and 4250 ppm, respectively, to cause a survival similar (i.e., 0.5 and 0.7%, respectively) to those of the unfed treatment. The result also shows that none of the four limonoids exhibited any evidence of acute toxicity, although *R. speratus* fed on obacunone or nomilin at concentrations higher than their PC_{95} (or higher than 1190 and 4250 ppm, respectively) had a tendency to die faster than the unfed termites.

Antifeedant Consumption. For potent antifeedants such as obacunone and nomilin, maximum antifeedant consumptions (ACs) were observed at 510 ppm (6.97 $\pm$ 0.69 μg) and 1360 ppm (13.87 $\pm$ 0.70 μg), respectively (Figure 2A and B). At higher concentrations, obacunone (510–850 ppm) and nomilin (1360–3400 ppm) had a drastic reduction effect on ACs. Further increase in obacunone

TABLE 1. PROTECTION CONCENTRATION (PC) ESTIMATES FOR VARIOUS LIMONOIDS AGAINST *Reticulitermes speratus*[a]

Limonoids	Probit line[b]	PC_{50}	PC_{95}
Obacunone	$Y = 5.33X - 9.67$	571 (507–643)	1,133 (996–1,289)
Nomilin	$Y = 3.21X - 5.08$	1,387 (1,296–1,484)	4,475 (4,088–4,898)
Azadirachtin	$Y = 1.11X + 1.32$	2,157 (2,003–2,324)	65,293 (53,112–80,269)
Limonin	$Y = 0.48X + 2.77$	47,791 (28,928–60,374)	[c]

[a] PC values are amount of antifeedant in ppm (w/w), with their 95% fiducial limits in the parenthesis.

[b] Probit line equation of percent feeding reduction (Y) and $\log_{10}$ concentration (X); all correlation coefficients of the probit lines were significant at $P < 0.001$.

[c] Beyond the bioassay limits.

TABLE 2. *R. speratus* SURVIVAL FED ON LIMONOIDS AT DIFFERENT CONCENTRATIONS

Antifeedant	Concentration[a] (ppm)	Survival (%)[b] 5 days	10 days	15 days	20 days	25 days
Unfed termites		87.3	47.7	18.3	1.3	0.3
Blank		100.0	99.7	98.7	97.0	95.0
Obacunone	340	98.0	93.0	92.0	91.3	89.3
	510	95.0	84.3	80.3	78.3	74.0
	680	98.3	68.0	57.3	45.3	38.3
	1,020	83.3	24.3	16.0	4.7	3.0
	1,360	91.0	27.0	10.7	1.3	0.3
	1,700	92.0	31.3	10.3	1.7	0.3
Nomilin	680	96.3	93.7	93.3	92.0	86.7
	1,700	88.3	67.0	54.7	43.0	25.7
	3,400	84.0	39.0	21.7	8.0	1.0
	5,100	83.7	26.0	12.0	1.3	0.7
	6,800	81.0	31.0	5.3	0.7	0.3
	10,200	73.3	13.3	2.7	0.3	0
Azadirachtin	680	100.0	89.7	86.3	81.0	81.0
	1,700	94.3	88.3	73.3	72.7	64.3
	3,400	95.0	87.3	73.3	66.3	54.7
	5,100	97.0	86.7	72.7	68.0	64.3
	6,800	88.3	83.7	74.3	57.3	49.0
	13,600	85.3	79.7	67.0	57.7	40.3
Limonin	1,700	100.0	99.3	98.7	96.0	88.7
	3,400	98.0	94.3	92.7	84.3	74.7
	6,800	98.0	96.3	93.3	83.7	73.0
	13,600	99.0	96.7	92.0	80.3	68.7
	40,800	98.3	94.0	89.7	75.3	59.0
	68,000	98.0	92.3	88.7	72.3	56.7

[a] Only six concentrations are presented in this study.
[b] Averages of 15 replicates in three sequential runs of five each.

and nomilin concentrations caused ACs to fluctuate between 0.30 ± 0.20 and 2.04 ± 0.61 μg (for obacunone) and 4.36 ± 0.70 and 6.86 ± 0.82 μg (for nomilin).

In contrast, for the weak termite antifeedants, azadirachtin and limonin, maximum ACs were not observed at any of the concentrations tested. In fact, their ACs increased steadily from 7.71 ± 0.39 μg at 680 ppm to 56.85 ± 3.01 μg at 13,600 ppm for azadirachtin, and from 27.44 ± 0.75 μg at 1700 ppm to 666.62 ± 11.39 μg at 68,000 ppm for limonin (Figure 2C and D).

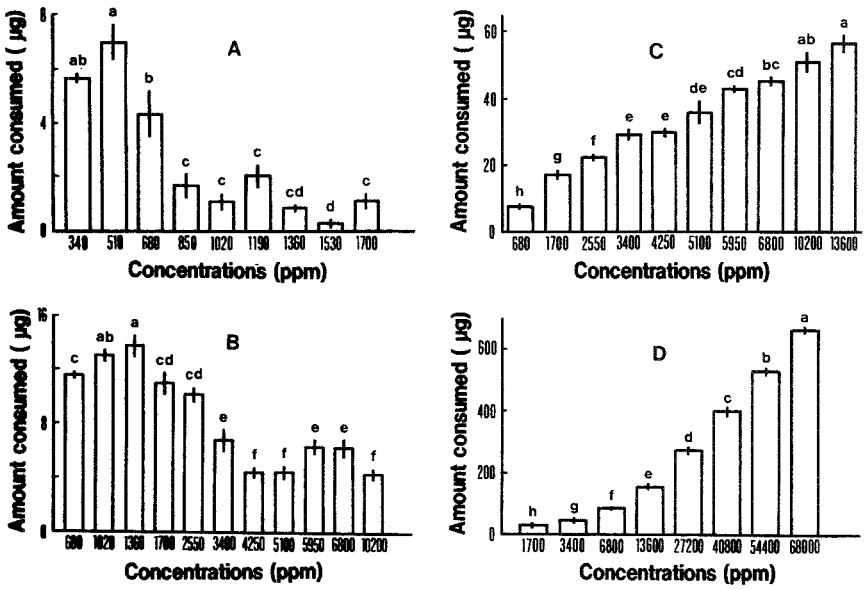

FIG. 2. Antifeedant consumption curves for obacunone (A), nomilin (B), azadirachtin (C), and limonin (D): means ± SE (vertical lines) with at least one similar letter are not significantly different at $P < 0.05$.

DISCUSSION

Unlike the usual pest insect control programs, the main goal of termite control is to protect specific properties (Spear, 1970). This is where antifeedants could effectively be used to provide selective protection.

When dealing with termites infesting building structures and furniture, frequent treatments would not only be expensive but impractical. If antifeedants are to be used, those with absolute activity are preferable as they would ensure a more lasting protection. One efficient way to identify such antifeedants is to expose them to insects until starvation, and the insects should remain deterred if their activity is absolute (Klocke et al., 1989). Based on this criterion, the antifeeding activity of obacunone and nomilin against *R. speratus* seems to be more absolute than relative. This was clearly evident from their effect on *R. speratus* survival (Table 2). Azadirachtin and limonin, at the concentration ranges tested, appeared to act as relative antifeedants, in which starving termites started to feed on the treated disks and survived.

There are at least seven isomeric azadirachtins available from natural sources, of which azadirachtin A is the most common (Rembold, 1989). Although azadirachtin has been reported potent as an antifeedant against nearly

200 insect species (Jacobson, 1986; Saxena, 1989), it was earlier reported to have no effect on feeding and survival of the termite *R. santonensis* and to be only moderately active against *Pieris brassicae* larvae (Butterworth and Morgan, 1971). The present finding also shows that azadirachtin (as represented by azadirachtin A) could only exhibit a weak antifeeding potency against *R. speratus* and apparently no acute toxicity. Based on their PC_{95} ratios, obacunone and nomilin were, respectively, about 58- and 15-fold more potent than azadirachtin. There is still insufficient evidence to infer a structure–activity relationship, but modifications of the A-ring and its side chains from the simple structures in obacunone and nomilin to those in azadirachtin and limonin might have been a cause of the activity loss.

Azadirachtin, obacunone, and nomilin were equally potent as antifeedants against *Heliothis zea* (PC_{95} = 6 μg/disk), but differed against *Spodoptera frugiperda*, with azadirachtin sixfold stronger. However, azadirachtin was toxic to both insects (less than 10 ppm), while obacunone and nomilin were not toxic at concentrations up to 2500 ppm (Klocke and Kubo, 1982). Obacunone and nomilin had been observed to be inactive against *Peridroma saucia* at 0.5 μmol/g diet (Champagne et al., 1989). Limonin, which has only been reported active (96% feeding reduction = 100 μg/disk) against *Leptinotarsa decemlineata* (Randall et al., 1987), is generally considered a weak antifeedant (Klocke and Kubo, 1982; Randall et al., 1986).

It has been emphasized that neem products such as azadirachtin-enriched pesticides (Koul et al., 1990) have a relatively short residual effect of about five to seven days under tropical conditions (Schmutterer, 1988, 1990). This poor stability of azadirachtin and its apparent lack of absolute antifeeding activity against termites are presently thought to be the main factors reducing its antifeeding activity in this long-term bioassay system, and thus limiting its practicality in termite control.

AC curves showed at least two major differences between the potent (Figure 2A and B) and weak antifeedants (Figure 2C and D). First, maximum ACs for obacunone and nomilin were observed at concentrations corresponding to 50% feeding reduction or PC_{50} (Table 1 and Figure 2). PC_{50} for azadirachtin and limonin were, respectively, 2157 and 41,791 ppm, but maximum ACs were not observed at these concentrations. Secondly, ACs for obacunone and nomilin dropped sharply at concentrations higher than 510 and 1360 ppm, respectively, indicating that feeding in this instance was affected by the limonoid concentrations rather than by the total amounts consumed. At concentrations higher than the corresponding 90% feeding reduction (i.e., obacunone > 850 ppm or nomilin > 3400 ppm), ACs fluctuated but at lower levels. In contrast, within the concentration ranges tested, ACs for azadirachtin and limonin increased with concentration, which might be characteristic of weak or relative antifeedants. As feeding was still observed at 300 μg/disk (or approximately 10,200 ppm) of

obacunone (Serit et al., 1991), initial feeding seems necessary for the antifeedants to later affect feeding.

Among the four limonoids tested, only obacunone and nomilin exhibit potential for use as antifeedants against termites. There are at least three advantages of using obacunone and nomilin in termite control. First, Rutaceae limonoids are readily available in large quantity as the unwanted by-products of the citrus processing industry. For instance, a theoretical calculation indicates the potential availability of natural limonoids extracted strictly from grapefruit seeds to be over 300 metric tons per annum (Klocke and Kubo, 1982). Meliaceae limonoids, such as azadirachtin, on the other hand, are both limited in abundance and too complicated for large-scale synthesis. At its effective concentrations, azadirachtin may be impractical for use against termites. Limonin is not at all practical. Second, obacunone and nomilin have yet another desirable effect on termites in that when consumed at high concentrations they could appreciably effect the survival of the termite intestinal microflora, resulting in a faster rate of mortality (Serit et al., 1991). Third, obacunone and nomilin are not toxic, even to termites. They are apparently harmless to humans as they are also present in citrus juices, peels, and seeds (Maier et al., 1977).

We have virtually relied upon toxic chlorinated hydrocarbon for the control of subterranean termites. Antifeedants that could effectively be used to protect selected properties will fit well into any integrated pest management system for termites. More importantly, their use will gradually reduce our dependence on the toxic chemicals, and thus offers a real hope for a new generation of insect control chemicals that will not pollute our environment, poison our food, or harm nonpest species (Ley and Toogood, 1990). The present activity and safety of obacunone and nomilin therefore warrant further investigation of Rutaceae limonoids as antifeedants against termites.

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INFLUENCE OF DIFFERENT HABITATS AND MATING ON OLFACTORY BEHAVIOR OF ONION FLIES SEEKING OVIPOSITIONAL HOSTS

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Abstract—Using traps baited with natural and synthetic onion volatiles, we examined the effects of different habitats and mating on the olfactory behavior of laboratory-reared and wild onion flies. Rankings of olfactory treatments as host-finding stimuli for females were dependent on their mating status and the habitat in which they were foraging. In habitats devoid of hosts, traps baited with individual alkyl sulfides were as effective as 4-day-old chopped onions and more effective than 1-day-old onions in eliciting host-finding behavior in laboratory-reared unmated females (LUF) and laboratory-reared mated females (LMF). However, upwind dispersal and percent recapture were always significantly greater in LUF. In one experiment, Pr_2S_2 was 19 times more attractive to LMF in a fallow field, as than it was in an onion field. Reduced effectiveness of alkyl sulfides as host-finding stimuli in onion fields probably results in part because they are less findable, but more importantly because of a change in searching behavior after females have mated. Evidence to support the latter contention is that traps baited with alkyl sulfides and onions were equally findable by unmated females in both habitats. The behavior of LMF was identical to that of wild females, whereas the behavior of LUF was identical to wild males. The hypothesis that olfactory host-finding behavior in onion flies is modified by the resource level was upheld. Alkyl sulfides appear to be the primary, and possibly the only, chemical effectors of host-finding at the patch level of resource distribution, whereas the complex blend emitted by aged, chopped, or damaged onions appears to be acting at the final level of host-finding, while egg-laying females are moving between adjacent hosts in search of an optimal oviposition site.

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INTRODUCTION

While foraging for oviposition sites, herbivorous insects may encounter host-plant resources in a hierarchical sequence: habitat, patch, and resource item (Hassel and Southwood, 1978). This hierarchy of resource levels probably selects for a hierarchical host-selection response pattern, especially with regard to the sorts of stimuli and sensory systems insects use to recognize resources (Prokopy, 1986; Harris and Miller, 1988).

Insects that use olfaction to locate host plants will likely encounter variations in the quantity and quality of chemical information as a function of resource level. For example, plant odor concentrations may range from minute when the insect is several meters downwind of the source, to high when the insect alights on the plant. Likewise, while plant odors are usually released as complex blends, the number of constituents that an insect might detect simultaneously should decrease as a function of downwind distance from the source (Visser, 1986), especially if the chemical mixture is composed of different classes of compounds with different molecular weights. Thus, perceived plant odor composition might be expected to vary at different levels of resource distribution.

The anthomyiid fly, *Delia antiqua* (Meigen), is an oligophagous herbivore that infests a small number of domesticated *Allium* spp. (Perron et al., 1958, 1960). The olfactory basis for host recognition and oviposition by female onion flies after alighting on or near a plant was shown to depend on the presence of propylthiol chemicals specific to plants in the *Allium* genus (Bernhard, 1970; Vernon et al., 1978). However, whether they were tested at optimal release rates (Dindonis and Miller, 1981a) or as simple mixtures (Vernon et al., 1981), alkyl sulfides such as *n*-dipropyl disulfide (Pr_2S_2) have never proven to be potent baits for onion flies in onion fields (Miller et al., 1984). Consequently, the most effective host-finding stimulus for the female onion fly is thought to be a "complex blend" of volatiles commonly emitted by aged, microbially and/or insect-infested onions (Ikeshoji et al., 1980; Dindonis and Miller, 1981b; Vernon et al., 1981; Hausmann and Miller, 1989). To date, the hypothesis that this complex blend of volatiles is the most effective host-finding stimulus for onion flies at all levels of resource distribution has not been tested experimentally.

Contrary to the above findings, synthetic Pr_2S_2 has proved to be a potent host-finding stimulant for laboratory-reared onion flies foraging in habitats devoid of host plants (Judd and Borden, 1988, 1989). Pr_2S_2 induces significant upwind dispersal in unmated, but not mated female onion flies located at least 100 m downwind, and on average ca. 65% of the released insects were recaptured with

Pr_2S_2 baits. Apparent differences in the response of onion flies to Pr_2S_2 while foraging in and outside onion fields may be evidence that resource level, mating, and/or laboratory rearing influence olfactory behavior of onion flies seeking ovipositional hosts, either individually or collectively.

We tested four hypotheses with regard to olfactory behavior of female onion flies: (1) individual propylthiol compounds are as effective host-finding stimuli as aged, microbially infested onions; (2) olfactory responses are independent of mating status; (3) olfactory responses are independent of habitat; and (4) the olfactory behavior of laboratory-reared onion flies is equivalent to wild onion flies.

METHODS AND MATERIALS

Experimental Insects. Natural populations of onion flies are very low or at least very transient outside commercial onion fields (Martinson et al., 1989), therefore it is very difficult to examine the behavior of wild onion flies in different habitats. For that reason, and in order to control the sex, age, mating status, and hunger of onion flies responding to host-plant volatiles, all studies were conducted with laboratory-reared and released flies. When these studies were conducted, our culture of flies had been reared on onion in the laboratory for three years. However, simultaneous comparisons of the behavior of laboratory-reared and wild onion flies allowed us to test for effects of laboratory culturing on behavior.

Rearing and release of onion flies were as described by Judd and Borden (1988). Larvae were reared on onions and stored as pupae until required for experiments. Emergent adults were maintained at 22°C under a 16:8 hr light–dark photoperiod, in an environmental chamber where they fed ad libitum on Ticheler's (1971) diet. At least 24 hr before being released flies were dusted lightly with one of several colored fluorescent powders (DayGlo Color Corp., Cleveland, Ohio).

Experiment 1: Trap Catches with Different Host-Plant Olfactory Stimuli. Trap catches provide a relative measure of the effectiveness of different olfactory baits as host-finding stimuli (Dindonis and Miller, 1981a). Therefore, five *Allium*-related baits were compared as host-finding stimuli: 1- and 4-day-old, chopped onion tissue and three synthetic, but naturally occurring, alkyl sulfides (Pierce et al., 1978), Pr_2S_2, methyl-propyl-disulfide ($MPrS_2$) and propenyl-propyl-disulfide ($PPrS_2$).

Onions of an unknown cultivar were chopped (mean particle weight ca. 100 mg) and 100 g was placed 2 cm deep into plastic Petri dishes (2 cm high × 9 cm diameter). Lids were placed over the Petri dishes to reduce desiccation, and they were held at 22°C and 65% relative humidity in an environmental

chamber for either one or four days until required for testing. Pr_2S_2 was purchased (Eastman Kodak, Rochester, New York, 98% pure by gas chromatography) and $MPrS_2$ and $PPrS_2$ were synthesized as described by Pierce et al. (1978). Each alkyl sulfide was suspended in paraffin oil (1% by volume) and dispensed individually into plastic Petri dishes (25 mm high × 60 mm diameter) in 5-ml aliquots. Dishes containing sulfides were set inside 9-cm-diameter Petri dishes.

All 9-cm Petri dishes holding baits were placed in the center of 20 × 20-cm white, horizontal, sticky traps to capture alighting flies (Judd and Borden, 1988). In order to allow volatile emission and prevent entry of flies into baits, 9-cm Petri dish lids were supported ca. 5 mm above all baits on three nails, which also held the sticky square in position. An unbaited 9-cm Petri dish and lid on a white, sticky trap served as a control to measure visual responses to traps.

To ensure that released insects were distributed homogeneously, equal numbers of flies were released from 49 equally spaced, intersecting points of a 60 × 60 m grid. Six replicates of the six treatments (unbaited control, Pr_2S_2, $MPrS_2$, $PPrS_2$, and 1- and 4-day-old chopped onion) were arranged within the 60 × 60-m release area using a 6 × 6 Latin-square randomization (Cochran and Cox, 1957), with rows and traps within rows 10 m apart. This experimental design ensured that each trap was surrounded by four groups of released flies, each at a distance of ca. 7 m. Baits were placed in traps ca. 1 hr before release of flies at 2000–2200 hr Pacific daylight time (PDT). This experiment was concluded after 24 hr.

Experiment 2: Upwind Dispersal in Response to Host-Plant Olfactory Stimuli. In the previous experiment, trap catches were employed as a measure of the "effectiveness" of olfactory stimuli in eliciting host-finding behavior. However, trap catches reveal very little about the underlying behavioral mechanisms that the captured insects used to find the volatile source (Kennedy, 1978). For example, trap catches do not reveal whether onion flies are stimulated to fly towards the trap from some distance or are simply arrested after randomly encountering the trap.

A circular trapping design (Judd and Borden, 1988, 1989), in which onion flies are released from the center of a circular array of traps, provides information on the direction of movement before capture. Used in conjunction with continuous wind measurement, this circular design can be used to assess the effectiveness of different olfactory stimuli in eliciting upwind dispersal and long-range host finding in released onion flies.

Two treatments, 4-day-old chopped onion tissue and Pr_2S_2, both as previously described,were compared for their ability to elicit upwind dispersal in female onion flies. A third, unbaited control treatment served to measure dis-

persal in the absence of olfactory stimuli and visual response to traps. Individual treatments were compared in three separate but simultaneous circular releases. Each treatment circle (10 m radius) consisted of eight traps, one in each major and minor cardinal direction. Individual trap circles were set 50–100 m apart. Treatment baits and flies were placed in the field between 2000 and 2200 hr PDT. This experiment was concluded at 1200 hr PDT the following day. Wind direction was measured continuously throughout this experiment with a SIAP mechanical anemometer (Judd and Borden, 1988).

Influence of Resource Level on Olfactory Behavior. Searching for onions in a fallow field is considered equivalent to searching at the habitat level of resource distribution, while searching for individual onions in an onion field is probably equivalent to within-patch search (Judd and Borden, 1988; Harris and Miller, 1988). To test the hypothesis (Judd and Borden, 1988, 1989) that response to host-plant olfactory stimuli is dependent on resource level, we conducted experiments 1 and 2 (as described above) in two sites simultaneously: a 5-ha fallow, bare-soil field and a 0.5-ha field of 10- to 25-cm high onions (var. Autumn Spice). A significant treatment × site interaction would indicate that olfactory behavior is dependent on resource level.

Influence of Mating on Olfactory Behavior. Female onion flies reach reproductive maturity at ca. 8–10 days of age and will not usually oviposit until they have mated (Theunisson, 1976). As a result, mated, gravid females are probably more stimulated to oviposit than similarly aged unmated females. To test the hypothesis (Judd and Borden, 1988) that olfactory behavior of female onion flies is dependent of their mating status, we released both mated and unmated, 10-day-old, gravid females simultaneously in each habitat, in both experiments 1 and 2 described above. In experiment 1 (grid-type release) we released 245 unmated and 343 mated females in the fallow field, and 490 females of each type in the onion field. In experiment 2 (circular release) we released 50 females of each mating status from the center of each treatment circle in each habitat. A significant treatment × fly interaction in these experiments would indicate that mating influences olfactory behavior.

Statistical Analyses. To make valid comparisons between the response of mated and unmated females or laboratory-reared females and wild flies, we had to convert all trap-catch data to percentages (treatment catch/total catch on all treatments), because actual trap catches reflect differences in population size as well as behavior. Transforming catches to percentages serves to rank treatments within a fly class, which allows us to compare across fly classes independent of differences in the population size of each class of flies. Percentages were transformed (arcsine $\sqrt{p}$), and analyzed by an ANOVA appropriate for Latin squares (Cochran and Cox, 1957). Treatment means were compared using the Student-

Newman-Keuls (SNK) multiple-range test (Zar, 1984). Factorial ANOVAs (treatment, habitat, fly class) were used to test for the effects of olfactory stimuli, resource level, mating, and laboratory-rearing on olfactory behavior.

In experiment 2, contingency tables and χ^2 tests were used to test the hypotheses that trap catches with different olfactory treatments were independent of resource level and mating, and to test for differences in the behavior of laboratory-reared and wild onion flies. Directional-flight data from experiment 2 (circular release) were analyzed using a Rayleigh test (Batschelet, 1981; Judd and Borden, 1988).

RESULTS

Trap Catches with Different Host-Plant Olfactory Stimuli. The response of laboratory-reared unmated and mated females to olfactory baits in fallow versus onion fields was analyzed with a three-way factorial ANOVA (treatment, fly class, habitat). This analysis revealed a significant treatment effect ($F_{5,120} = 16.93$, $P < 0.001$). However, treatment rankings are dependent on mating status and habitat, as the treatment × fly class ($F_{5,120} = 3.75$) and treatment × habitat ($F_{5,120} = 5.70$) interactions were both highly significant ($P < 0.005$). Therefore, statements about the effectiveness of various olfactory baits as host-finding stimuli must be restricted to a particular fly class or habitat.

The ranking of olfactory baits as host-finding stimuli for unmated and mated females in the fallow field was very similar. Traps baited with individual sulfides caught as many unmated and mated flies as did 4-day-old onion and more than 1-day-old onion (Table 1). However, there was a marked change in the behavior of mated females across habitats. For example, unmated females were equally responsive to Pr_2S_2, $MPrS_2$, and 4-day-old onion in both habitats, whereas mated females exhibited a significant response to 4-day-old onion over all of the alkyl sulfides tested in the onion field (Table 1). Comparing mean percent catches with Pr_2S_2 and 4-day-old onion baits across rows in Table 1 clearly illustrates how the relative responsiveness of mated females to alkyl sulfides is reduced by about threefold in onion fields, while their responsiveness to 4-day-old onion is increased by about twofold.

The olfactory response of laboratory-reared unmated and mated females (LUF and LMF, respectively) and wild female (WF) and male (WM) onion flies in an onion field (Table 2) was compared using a two-way ANOVA (treatment, fly class). This analysis revealed a significant treatment effect ($F_{5,110} = 21.06$, $P < 0.0001$), but a nonsignificant ($F_{3,110} = 0.001$, $P > 0.05$) fly effect. The fly × treatment interaction was also not significant ($F_{15,110} = 1.62$, $P = 0.079$). However, data in Table 2 indicate some differences and similarities in behavior based on mating status and sex. For example, while LMF and WF

TABLE 1. MEAN PERCENTAGE OF LABORATORY-REARED UNMATED AND MATED FEMALE (LUF, LMF) ONION FLIES RECAPTURED ON TRAPS BAITED WITH INDIVIDUAL, SYNTHETIC DISULFIDES OR AGED CHOPPED ONION IN DIFFERENT HABITATS (SIX REPLICATES/TREATMENT)

Treatments	LUF[a]		LMF[a]	
	Fallow field (N = 245)	Onion field (N = 490)	Fallow field (N = 343)	Onion field (N = 490)
Methyl-propyl-disulfide	5.9a	4.0ab	3.6a	1.3b
Propenyl-propyl-disulfide	2.8bc	2.2bc	3.3a	2.2b
Dipropyl-disulfide	$3.5bc_1$	$3.3ab_1$	$2.8a_1$	$0.9b_2$
4-day-old onion	$3.7ab_2$	$5.4a_2$	$3.7a_2$	$8.9a_1$
1-day-old onion	1.3cd	1.6bc	1.9b	2.8b
Unbaited control	0.2d	0.8c	0.8b	0.5b
Total catch (n)	171	258	254	137
Recaptured (%, n/N)[b]	70_1	53_2	74_1	28_3

[a] Percentages within a column = means of number recaptured per treatment and replicate divided by total number recaptured (n). Column means followed by the same letter or row means subscripted by the same number are not significantly different (SNK test, $\alpha = 0.05$).

[b] Percentages within this row subscripted by the same letter are not significantly different ($\alpha = 0.05$) using a modified SNK test for independent binomial data (Zar, 1984).

TABLE 2. MEAN PERCENTAGE OF LABORATORY-REARED AND WILD ONION FLIES CAPTURED ON TRAPS BAITED WITH INDIVIDUAL, SYNTHETIC DISULFIDES OR AGED CHOPPED ONION IN AN ONION FIELD (SIX REPLICATES/TREATMENT)

Treatment	Mean percentage capture[a]			
	LUF	LMF	WF	WM
Methyl-propyl-disulfide	4.0ab	1.3b	1.6b	2.7ab
Propenyl-propyl-disulfide	2.2bc	2.2b	1.8b	2.4ab
Dipropyl-disulfide	$3.3ab_1$	$0.9b_2$	$1.8b_2$	$2.9ab_1$
4-day-old onion	$5.4a_2$	$8.9a_1$	$7.1a_1$	$5.3a_2$
1-day-old onion	1.6bc	2.8b	3.5b	1.7b
Unbaited control	0.8c	0.5b	0.8b	1.5b
Total catch (n)	258	137	140	118

[a] Laboratory-reared unmated females (LUF), laboratory-reared mated females (LMF), wild females (WF), and wild males (WM). Percentages within a column = means of number caught per treatment and replicate divided by total number caught (n). Column means followed by the same letter or row means subscripted by the same number are not significantly different (SNK test, $\alpha = 0.05$).

rank olfactory stimuli identically, both behave differently than LUF or WM (Table 2). LMF and WF were most responsive to 4-day-old onion, while LUF and WM discriminated very little between 4-day-old onion and alkyl sulfides (Table 2). These differences and similarities are seen most clearly when mean responses to 4-day-old onion and Pr_2S_2 are compared within rows across columns of Table 2.

Trap Catches with Host-Plant Olfactory Stimuli in Circular Arrays. As in the previous experiment (Table 1), the olfactory behavior of LUF and LMF was similar in a fallow field but significantly different in the onion field when the circular trapping array was employed (Table 3). LMF were equally responsive to onion and Pr_2S_2 baits in the fallow field (38 vs 38%), but aged-onion baits were 18-fold more attractive than Pr_2S_2 baits in the onion field (36 vs 2%). Comparing female recapture within a habitat clearly indicates that LUF are more likely to be caught on traps baited with real and artificial host odors than are LMF, especially when a circular trapping arrangement is used, thus corroborating findings of Judd and Borden (1988).

Upwind Dispersal in Response to Host-Plant Olfactory Stimuli. As in all previous experiments of this type (Judd and Borden, 1988, 1989), dispersal of LUF in fallow fields was significantly directional in the presence of host-plant odor and nondirectional in its absence (Table 3). Pr_2S_2 and 4-day-old onion

TABLE 3. COMPARISON OF PERCENT RECAPTURE, MEAN FLIGHT DIRECTION, AND FLIGHT DIRECTEDNESS OF LABORATORY-REARED FEMALE ONION FLIES EXPOSED TO DIFFERENT OLFACTORY STIMULI IN FALLOW FIELD (FF) AND ONION FIELD (OF)

Treatment	Mating status	Percent recapture[a]		Directedness of flight ($\bar{R}$)[b]		Mean flight direction ($\bar{\phi}$)[c]	
		FF	OF	FF	OF	FF	OF
4-day-old onion	unmated	$68a_1$	$64a_1$	0.33*	0.58**	22°	97°
	mated	$38b_1$	$36b_1$	0.29 NS	0.36 NS	38°	167°
Dipropyl-disulfide	unmated	$60a_1$	$32a_2$	0.32*	0.74**	22°	80°
	mated	$38b_1$	$2b_2$	0.25 NS	NC	12°	NC
Unbaited control	unmated	$8a_2$	$22a_1$	0.12 NS	0.71**	167°	86°
	mated	$14a_1$	$2b_1$	0.14 NS	NC	182°	NC

[a] Paired percentages within a column followed by different letters or within a row subscripted by different numbers are significantly different ($P < 0.05$) by a χ^2 test on actual counts.
[b] Statistical significance of $\bar{R}$ (Rayleigh test [Batschelet, 1981]) indicated by: NS = $P > 0.05$; * = $P < 0.05$; ** = $P < 0.005$. NC indicates values not calculated due to small sample size.
[c] Mean wind direction was 37°.

treatments elicited similar mean dispersal directions ($\bar{\phi}$) and directedness ($\bar{R}$), indicating similar effectiveness as host-finding stimuli. Random dispersal of LMF in the presence of Pr_2S_2 (Table 3) is consistent with our previous studies (Judd and Borden, 1988); however, lack of directed dispersal by LMF exposed to 4-day-old onion baits is demonstrated for the first time (Table 3).

Although dispersal of onion flies within onion fields was directional, it appeared to be independent of trap-released volatiles because dispersal by LUF was significantly directional even in the unbaited control treatment (Table 3). The mean dispersal directions for LUF tended to be crosswind to upwind (Table 3). Conversely, dispersal by LMF in the circle of 4-day-old onion baits was not significantly directional and the mean direction of dispersal was slightly downwind (Table 3). Unfortunately, directional statistics could not be calculated for dispersal of LMF in the Pr_2S_2 and unbaited control treatment circles, as recapture rates were too low.

Catches of Laboratory-Reared and Wild Onion Flies in Circular Assays. The total relative catch of LUF, LMF, WF, and WM on 4-day-old onion, Pr_2S_2, and unbaited traps for the circular experiment conducted in the onion field were compared using contingency tables and χ^2 tests. A 3 × 4 contingency table (three treatments, four fly classes) was highly significant ($\chi^2 = 37.79$, $df = 6$, $P < 0.001$), indicating that responses to olfactory treatments were dependent on fly class. Table 4 summarizes the results of all possible pairwise comparisons using 2 × 3 contingency tables. The experimentwise error rate for these com-

TABLE 4. NUMBER AND PERCENTAGE OF LABORATORY-REARED AND WILD ONION FLIES CAUGHT ON TRAPS ARRANGED IN CIRCULAR ARRAYS IN ONION FIELD

Treatment	Number (and percentage) of flies of each type caught/treatment[a]				Contingency tables tested[b]	Statistical significance level[c]
	LUF	LMF	WF	WM		
4-day-old onion	32	18	172	39	LUF vs LMF	0.025
	(54.2)	(90)	(75.1)	(46.9)	LUF vs WF	0.005
Dipropyl-disulfide	16	1	43	23	LUF vs WM	NS
	(27.1)	(5)	(18.8)	(27.7)	LMF vs WF	NS
Unbaited control	11	1	14	21	LMF vs WM	0.005
	(18.7)	(5)	(6.1)	(25.4)	WF vs WM	0.001

[a] Percentages in a column = numbers caught per treatment divided by total number caught on all treatments. LUF = laboratory-reared unmated females; LMF = laboratory-reared mated females; WF = wild females; WM = wild males.

[b] 2 × 3 contingency tables (two fly classes × three treatments) tested the hypothesis that response to treatments was independent of fly classification.

[c] Significance of χ^2 statistic for contingency tables: NS = $P > 0.05$.

parisons was held below 5% by adjusting alpha of individual comparisons. These analyses corroborated previous observations (Table 2) that LMF behave the same as WF, but different from LUF and WM, while LUF behave the same as WM.

DISCUSSION

Despite ample evidence that plant-odor blends mediate host selection, most available data are related to behaviors that occur after an insect has found the resource, e.g., amounts of foliage eaten or numbers of eggs laid. Very little direct evidence bears on the role of odor mixtures in distant host-plant location (May and Ahmad, 1983), and even less about the effects of resource level on insects' response to host-plant olfactory stimuli (Prokopy, 1986; Bell, 1990).

The view that a complex odor blend associated with damaged onions is the most effective olfactory stimulus for onion flies seeking ovipositional hosts has arisen out of research conducted in agricultural crops (Dindonis and Miller, 1980a, 1981b; Ishikawa et al., 1981; Vernon et al., 1981; Miller et al., 1984). To a host-seeking onion fly, onion fields probably represent the patch level of resource distribution (Judd and Borden, 1988; Harris and Miller, 1988). Our results indicate that olfactory behavior of onion flies, like visual behavior (Judd and Borden, 1991), is quite different when they are foraging for patches of onions as opposed to individual host plants in a patch of onions. Thus, discussion of host-finding behavior of onion flies should consider resource levels.

Whether the "effectiveness" of olfactory treatments as host-finding stimuli is based on rates of recapture or stimulation of upwind dispersal, when tested in a fallow field, individual alkyl sulfides were as effective as the complex blend of alkyl sulfides, aldehydes, and alcohols emitted by 4-day-old onion (Ikeshoji et al., 1980; Vernon et al., 1981). In contrast, the complex odor blend from damaged onions was clearly the most effective host-finding stimulus in onion fields, but only for mated, gravid, and presumably ovipositing females.

These results do not necessarily disprove the hypothesis that a complex blend of volatiles is the most effective host-finding stimulus for onion flies foraging outside onion fields, because a valid comparison of various volatiles as host-finding stimuli requires that each be tested at an optimal concentration and release rate. As the concentrations and release rates of volatiles from the 4-day-old onion tissue are unknown and variable, we cannot say categorically that a complex, aged-onion blend would not be a superior host-finding stimulus to Pr_2S_2 if it were released at higher concentrations. In fact, Miller et al. (1984) showed that catches of onion flies increased with increasing amounts of aged onion. However, the latter results should be interpreted with caution as increased catches with larger baits may simply reflect traps that are sampling a larger area

and population. Hence, increased catches with a higher release-rate bait does not identify a bait that is inherently more attractive unless the populations and active spaces are controlled to the extent they were in our experiments. A true test of the complex blend hypothesis (Dindonis and Miller, 1981b) must await identification of the active constituents of the aged-onion odor blend, as previously identified chemicals (Ikeshoji et al., 1980; Ishikawa et al., 1983) have not proven themselves to be as effective as aged onion (Miller et al., 1984; Hausmann and Miller, 1989).

In spite of the caveat above, what remains important from the perspective of this paper is that an amount of aged onion that was more effective than alkyl sulfides in an onion field was not more effective in a fallow field. Our results indicate that a simple, but distinctive chemical signal such as Pr_2S_2, even when released at a relatively low rate (6–9 μg/min) , acted as effectively as a complex odor signal when onion flies were foraging outside an onion patch. Additionally, our finding that each synthetic disulfide bait caught more onion flies than freshly chopped onion outside an onion field, combined with the observation that freshly cut onion emits almost exclusively alkyl disulfides (Vernon et al., 1981), suggests that the release rate of alkyl disulfides may be more important than the complexity of the signal when onion flies are foraging at the habitat level of resource distribution. One might infer from these results that the probability of an onion fly locating a new patch of onions is dependent on patch size or density, rather than the quality of individual host plants within the patch. In contrast, assessment of host-plant quality after finding a patch likely depends on more complex chemical signals, hence, the strong response of ovipositing females to 4-day-old onion in an onion field.

The question of why mated females are more responsive to aged-onion baits than alkyl sulfides at the patch level of foraging has both proximate and ultimate answers. One proximate explanation may be that alkyl sulfide baits are simply less findable in an onion field. Note that the proportion of mated females recaptured with Pr_2S_2 decreased substantially in the onion field. It was this reduction in response to Pr_2S_2 by mated females, more than an increase in response to aged onion that produced the significant treatment differences in nearly all statistical tests performed. The low release rate from sulfide baits, relative to the concentration of natural sulfides surrounding many thousand of plants, may make sulfide baits indiscernible, i.e., their plumes may be masked in the same way insects' pheromones may be masked in a crop permeated with pheromone. Likewise, onion flies somewhat habituated by natural sulfides in an onion field may be less likely to respond to a point source of sulfides. Yet, if these were the only explanations, why did LUF appear capable of finding the alkyl sulfides in both habitats?

An alternative proximate explanation for the different olfactory response pattern in onion fields may be that compounds from aged-onion baits are inter-

acting with volatiles released from onions growing in the field in a way that enhances mated female response. The fact that traps releasing specific host-plant volatiles often capture more insects when placed in a crop of host plants, as opposed to a fallow field, is sometimes cited as evidence that mixtures of volatiles are the most effective host-finding stimuli for phytophagous insects (Geurin et al., 1983). The inference is that volatiles tested in the host crop are synergyzed by crop-released volatiles to provide a superior stimulus (Finch, 1980). If chemical interaction of this type were responsible for enhancing mated female response to aged-onion odor in an onion field, we would expect the ratio of female recaptures with onion-baited traps in the onion field and the fallow field to be greater than 1 : 1. In fact, the ratios for both unmated and mated females' response to aged onion in different habitats were 1 : 1. Therefore, chemical synergysm of this type does not seem adequate to explain our results.

The most probable ultimate cause for the response of mated females to odor from damaged onions in an onion field is that this odor identifies host plants that may maximize a female's reproductive success and decrease developmental time of her offspring (Gorlenko et al., 1956; Zurlini and Robinson, 1978; Schneider et al., 1983). Such behavior would be consistent with predictions from optimal foraging models (Pyke et al., 1977; Jaenike, 1978) that suggest the most suitable resources should always be consumed when encountered and, hence, responded to when detected. Likewise, the fact that unmated females do not oviposit may explain in part why they are less discriminating than ovipositing females. Our results support the hypothesis (Judd and Borden, 1991) that the olfactory behavior of females, in particular their ranking of host stimuli, changes as a function of mating status and resource level.

How ovipositing females might locate these optimal resources while foraging among individual onion plants is not entirely clear from our studies. It has been suggested that onion flies respond anemotactically to damaged onions from several meters downwind (Dindonis and Miller, 1980b), independent of reproductive development (Miller and Strickler, 1984). While unmated females clearly disperse upwind in response to simple synthetic and complex natural host-plant chemical signals, we were unable to find any evidence of anemotactic responses by mated females in fallow areas or onion fields, in this or previous studies (Judd and Borden, 1988). Another interesting observation from these studies was that recapture of mated females was always significantly less than that of unmated females regardless of the type of olfactory stimulus involved. These differences were most apparent in the circular trapping arrays. Perhaps the mode of search used by females changes with mating status. Movement of unmated females may be more free ranging and directional than that of mated females, allowing them to locate upwind baits more easily in the experimental setting we have employed. As movement by mated females appears to be random with respect to the mean wind direction, perhaps odor from decomposing onions

influences behavior at short-range, by arresting movement through increased turning or stimulating landing.

Although complex blends can increase the host-finding responses in several species, including the Colorado potato beetle, *Leptinotarsa decemlineata* L., apple maggot, *Rhagoletis pomonella* (Walsh), and carrot rust fly, *Psila rosae* (F.), to our knowledge the role of, or necessity for, complex blends in distant host-plant patch-finding has not been demonstrated for any oligophagous species (Visser and Avé, 1978; Fein et al., 1982; Geurin et al., 1983). It seems predictable, however, that when olfaction is an important component of distant host-plant recognition, insects will utilize a simple, but distinctive chemical message. In some cases this chemical message may also be the predominant host volatile (Miller and Borden, 1990). This prediction seems likely in light of the fact that the integrity of a complex chemical signal may not be maintained several meters downwind in a mixed plant community (Wilson, 1968; Visser, 1986). Of course the validity of this hypothesis will depend on the chemical class, structure, and molecular weight of those chemicals making up the blend. The more diverse these chemicals are, the more likely this hypothesis will hold true. In the case of onion fly, it remains to be shown exactly what chemicals are responsible for the response to damaged onions.

Our results support the hypothesis that the predominant propylthiol volatiles from onions, such as Pr_2S_2, are probably the primary chemical effectors of long-range host finding at the patch level of resource distribution for onion flies. The complex odor blend associated with damaged onions appears to be acting at the final level of the host-finding sequence, when egg-laying females are moving between adjacent host plants in search of an optimal oviposition site.

Hypothetical Optimal Host-Finding Strategy for Onion Flies. It is now possible to construct a plausible sequence of events for an optimal host-finding strategy in *D. antiqua* (Judd, 1986). For onion flies that do not locate onions by chance during random dispersal after emergence (Martinson et al., 1989), long-range host finding of new patches (fields) of onions probably occurs through the detection and upwind response to volatile host alkyl sulfides (Judd and Borden, 1988, 1989). Visual stimuli come into play at short range and are probably used to delineate patch boundaries when flies approach the source of olfactory stimuli (Judd and Borden, 1991). Once a female onion fly crosses the boundary into a patch of onions, as she would on the border of an onion field, she must locate individual plants on which to lay her eggs. Healthy onions are often the site of oviposition early in the season; however, if some plants are already mechanically damaged, diseased, or infested with insects, location of these plants would be enhanced by the release of metabolites resulting from microbial degradation of onion tissue (Miller et al., 1984). Within a patch, damaged or decomposing onions are probably located through odor-mediated

arrestment or landing responses rather than long-range directed movements. Near this odor source (ca. 1 m), the decision to alight on a potential host will be determined by the size, shape, and spectral reflectance of the stem (Harris and Miller, 1988; Judd and Borden, 1991).

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AGGREGATED OVIPOSITION IN *Delia antiqua* (MEIGEN): A CASE FOR MEDIATION BY SEMIOCHEMICALS

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Abstract—Experiments conducted in the laboratory tested the hypotheses that aggregated oviposition by onion maggot flies, *Delia antiqua* (Meigen), is caused by stimuli associated with ovipositing females, newly laid eggs, or both. Using a paired oviposition station bioassay that eliminated visual stimuli associated with the treatment under study, 67% of the eggs laid by caged females were in response to the odor of females already ovipositing on an onion slice, as opposed to 33% of the eggs laid in response to an onion slice alone. When newly laid eggs were transferred to onion slices and held for either 24 or 48 hr before being bioassayed against similarly aged untreated onions, 74% and 97% of the eggs were laid at the egg-treated onion stations, respectively. Similar results were achieved when an aqueous wash of newly laid eggs was applied to the onion slice. When the egg wash was processed through a bacterial filter or when eggs were present but not in contact with onions, all response was eliminated. These results implicate microorganisms transmitted on the egg surface in creating an attraction for ovipositing females. Heptane extracts of ovipositor tips from mated, ovipositing females induced 72% of the test females to oviposit near points at which extracts were applied to the oviposition station floor. A behavioral sequence for an optimal host-selection strategy is hypothesized, whereby host-seeking female onion flies respond to host-derived alkyl sulfides at long range and metabolic by-products of microbially infested hosts and visual cues at short range (ca. 1 m), with final selection of oviposition sites potentially reinforced by contact with an aggregation pheromone released or left on the substrate by ovipositing females.

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INTRODUCTION

Insects may lay eggs in different patterns, from highly dispersed to highly aggregated. Presumably, the distribution pattern of the eggs imparts some selective advantage to the individuals that use it (Stamp, 1980). For example, solitary eggs may escape predation or parasitism or may afford the resultant larvae exclusive access to a limited resource. Conversely, laying eggs in groups or masses may reduce desiccation, facilitate parental care, help overcome resistant hosts, or allow optimal use of a host with a patchy distribution. Thus, there is apparently some optimal density of eggs per unit of resource that insects can actively regulate by altering oviposition behavior (Peters and Barbosa, 1977; Prokopy, 1981).

Variable oviposition patterns occur in the dipteran family Anthomyiidae. For example, a montane *Delia* sp. lays solitary eggs, or at the most, two eggs, in the ovaries of *Polymonium foliosissimum* Gray, and employs an epideictic (marking) pheromone (Prokopy, 1981) to deter further oviposition at the original oviposition site (Zimmerman, 1979, 1980, 1982). Similarly, the sorghum shootfly, *Atherigona soccata* Rondani, usually lays one egg per sorghum plant (Ogwaro, 1978) and deposits a water-soluble marking pheromone with the glue used to cement eggs to leaves (Raina, 1981). A forest nursery pest, *Delia anthracina* (Czerny), lays from one to three eggs on cones of spruces, a behavior consistent with the limited carrying capacity of the cones and the ability of a single larva to consume about 55% of the potential seeds in a cone (Hedlin, 1973).

In contrast to these cases of solitary oviposition, there is growing evidence that the onion maggot, *Delia antiqua* (Meigen), lays its eggs in a highly aggregated manner. Using egg traps to monitor oviposition patterns in the field, Loosjes (1976) harvested hundreds of eggs, representing the oviposition of several females in individual traps. Robinson and Zurlini (1979) observed "social facilitation" of oviposition in mass-rearing cages, and Harris and Miller (1983) found that female *D. antiqua* tended to oviposit on treatments where other females were already ovipositing. Likewise, in a two-choice bioassay, with identical olfactory stimuli, Vernon (1979) found oviposition to be characteristically aggregated at one stimulus or the other. We have often observed female *D. antiqua* ovipositing in groups around a single oviposition station, while equivalent stations remain unoccupied. As they probe the substrate, many females in these groups are observed dragging their extended ovipositors, in a behavior

consistent with the deposition of a pheromone (Figure 1). Pheromone-mediated group oviposition has been demonstrated in several insect genera, including *Andrena* and *Trogoderma* (Brossut, 1975), *Schistocerca* (Norris, 1963), *Musca* (Bay and Pitts, 1977), and *Lucilia* (Barton Browne, 1958; Barton Browne et al., 1969).

Our objectives were to investigate three hypotheses that could explain the group oviposition behavior of *D. antiqua* and consequent aggregation of newly laid eggs, as seen in the laboratory: (1) there is a "female factor," e.g., a pheromone released by ovipositing females, that causes other gravid females to aggregate in the same location; (2) there is an "egg factor," e.g., a volatile semiochemical associated with newly laid eggs, either produced by the eggs themselves or by associated microorganisms that promotes aggregation; or (3) aggregation is induced by a combination of female and egg factors.

METHODS AND MATERIALS

Design and Experimental Use of Oviposition Station Bioassay. Experiments were conducted with an oviposition station bioassay (Vernon et al., 1977), modified so that all possible contact with a host- or insect-associated stimulus

FIG. 1. Female *D. antiqua* in typical ovipositor-dragging posture, suggesting potential for deposition of a pheromone.

was eliminated. This was done by locating such stimuli above a wire-mesh screen barrier, set 5 cm above the floor of the station arena inside an inverted cup (Figure 2). Although potential auditory stimuli were not altogether eliminated if live insects were enclosed in the upper half of this apparatus, it was designed to eliminate visual and tactile cues associated with the treatment flies while focusing primarily on olfactory stimuli.

All experiments were conducted with a laboratory colony of onion flies that was started in 1978 and infused in 1982 with several thousand flies obtained from Agriculture Canada laboratory in London, Ontario (Harris et al., 1984). As assays were conducted over several years, these studies were conducted with flies that had been reared on onion for one to four years in our laboratory (Judd and Borden, 1988). In each experiment, a replicate consisted of 15 females and five males allowed to move freely, ingest food and water, mate, and oviposit within a 25 × 25 × 45-cm, wooden-frame cage with screened walls and top, and Plexiglas front. Water and food (Ticheler, 1971) were available ad libitum throughout each experiment. Two paired oviposition stations were randomly assigned to positions diagonally separated by 10 cm on the floor of each cage. All test flies were between 14 and 28 days old and had previously oviposited on a sliced onion. In each replicate, females were given 24 hr at 22°C and 60% relative humidity under a 16:8 hr light–dark photoregime to oviposit at the station of choice except where noted below. Stations were then removed and the eggs counted.

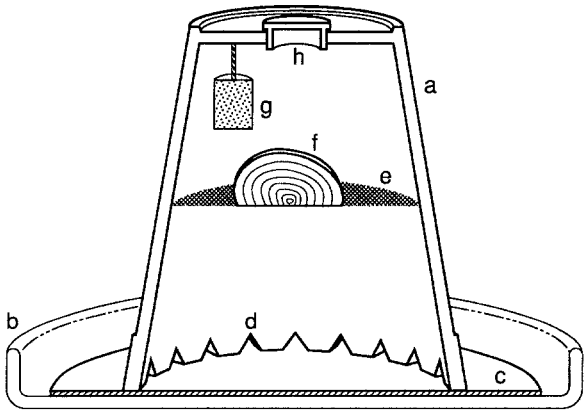

FIG. 2. Cut-away diagram of oviposition station (Vernon et al., 1977) modified to enclose hidden stimuli above screen within inverted 250 ml styrofoam cup, while permitting odors to be released through oviposition grooves around rim of cup. (a) inverted cup, (b) Petri dish, (c) filter paper, (d) oviposition apertures, (e) wire screen, (f) onion slice, (g) moist cotton wick, and (h) removable snap cap.

In all experiments, an onion slice was a common stimulus in both the treatment and control stations. The stimulus or treatment of main interest was then superimposed on the background onion stimulus in one of the stations.

Experiments with Adult Flies as Stimuli. Three preliminary experiments were conducted to test conclusively whether aggregated oviposition by *D. antiqua* occurs and to implicate whether or not it is mediated by semiochemicals.

The treatment stimuli, consisting of 10 mated, ovipositing females (experiment 1), 10 virgin females (experiment 2), and 10 virgin males (experiment 3) were placed above the screen in the upper half of one of the oviposition stations. Mated females had been exposed to males for seven days and were confirmed to have mated by dissection of spermathecae from a subsample of females. There were 20 replicates in experiment 1, and 10 in experiments 2 and 3. Mated females confined within the oviposition station (experiment 1) were free to oviposit above the screen and usually did so on the onion. Occasionally a few eggs would fall through the screen, but these could be identified because they were near the center of the filter paper where responding flies outside the inverted cup could not have reached.

Experiments with Eggs as Stimuli. The previous experiments did not separate stimuli associated with either the flies or their eggs. Therefore, three experiments (experiments 4–6) tested the hypothesis that there is an egg-associated stimulus that promotes aggregated oviposition by *D. antiqua*. Three hundred newly laid *D. antiqua* eggs collected within 24 hr of oviposition were the experimental stimulus in all three experiments. In experiment 4, eggs were placed on the center of the filter paper floor under the inverted cup (Figure 2). Thus, eggs were separated from both the onion slice and the ovipositing females outside the station. In experiments 5 and 6 eggs were placed directly on the onion slice for periods of 24 and 48 hr, respectively, before being tested. There were six replicates in experiment 4, and 10 in experiments 5 and 6.

Experiments with Egg Wash Water as Stimulus. If aggregated oviposition is induced by factors associated with *D. antiqua* eggs, these factors may be biotic (e.g., microbes on the egg surface) or abiotic (e.g., chemicals on the egg surface). Two experiments were designed to discriminate between these two hypotheses.

To collect eggs for this experiment, a special oviposition station was constructed out of glass, which in turn was wrapped in aluminum foil and autoclaved. A sterile wax wick loaded with 1 μl of dipropyl disulfide (Vernon et al., 1977) was placed inside the sterilized glass oviposition station, and the entire apparatus was placed in a rearing cage with several hundred gravid females. Several hundred 1-day-old eggs were harvested from beneath this glass oviposition station by gently washing eggs attached to the inside lip of the inverted cup with 50 ml of autoclaved, distilled water. Eggs adhering to the filter paper under the glass cup were removed with a sterile brush. The eggs and wash water

were agitated gently for ca. 5 min. Half of the resulting egg wash water was passed through a 20-μm Millipore filter (Millipore Corp., Bedford, Massachusetts) to remove all free-living microorganisms. This entire egg collection procedure was performed in a laminar airflow hood to minimize external contamination.

In experiment 7, ovipositing females were given a paired choice between oviposition stations baited with an onion slice over which 10 μl of unfiltered egg wash water was pipetted and a similar onion slice treated with 10 μl of autoclaved, distilled water. In experiment 8, paired onion slices were treated with either 10 μl of filtered egg wash water or 10 μl of autoclaved, distilled water. Experiments 7 and 8 were replicated 10 times.

Experiment with Ovipositor Extract. A final experiment tested the hypothesis that aggregated oviposition was stimulated by a pheromone produced in the ovipositor and released during oviposition. Extruded ovipositor tips, comprising three to four distal segments, were excised from mated, gravid females that had begun to oviposit. Ten ovipositors were immediately immersed in 25 μl of heptane held on ice. Four sites on the filter paper ca. 5 mm outside the apertures in the rim of the inverted oviposition cup (Figure 2), were spotted with 2.5-μl aliquots of extract, each containing one female ovipositor tip equivalent. In this way ovipositing females could contact the extract, just as they would contact a pheromone deposited onto a substrate during or after oviposition (Figure 1). Each control station received an equivalent amount of solvent. After 1 hr, the number of females ovipositing on each treatment was recorded and the eggs counted. This experiment was replicated 12 times.

We limited this assay to 1 hr because we had observed that when an oviposition station became crowded as female aggregations formed, an occasional female would wander to the adjacent station and the presence of the onion stimulus would often cause them to oviposit. This secondary oviposition often caused another aggregation to form, which, if left to run several hours, might obscure initial treatment differences.

Statistical Analyses. In all experiments, the numbers of eggs laid or females ovipositing per treatment were converted to percentages of the total number of eggs laid or females ovipositing on both treatments. Percentages were transformed (arcsine $\sqrt{p}$) and compared using paired t tests (Zar, 1984).

RESULTS AND DISCUSSION

Experiments with Adult Flies as Stimuli. The first three experiments demonstrated conclusively that only odors associated with mated, ovipositing females induced aggregated oviposition by *D. antiqua* (Table 1). Sixty-seven percent of the eggs laid in experiment 1 were at oviposition stations containing mated

TABLE 1. OVIPOSITION RESPONSE BY *D. antiqua* FEMALES IN 8 EXPERIMENTS WHEN EXPOSED TO CHOICE OF OVIPOSITION STATIONS WITH HIDDEN STIMULI OFFERING ODOR OF ONION ALONE (CONTROL IN ALL 8 EXPERIMENTS), OR ONION TREATED IN VARIOUS WAYS

Type of stimuli and exp. no.	No. of replicates	Treatment superimposed on onion odor[a]	Mean (± SE) percentage of eggs laid per stimulus[b]	
			Treatment plus onion	Onion alone
Flies				
1	20	10 mated females	66.8 ± 4.3a	33.2 ± 4.3b
2	10	10 virgin females	49.6 ± 11.2a	50.4 ± 11.2a
3	10	10 males	35.1 ± 9.3a	64.9 ± 9.3a
Eggs				
4	6	300 eggs on paper	40.1 ± 11.4a	59.1 ± 11.4a
5	10	300 eggs on onion 24 hr	73.6 ± 13.9a	26.4 ± 13.4b
6	10	300 eggs on onion 48 hr	96.6 ± 2.3a	3.4 ± 2.3b
Egg wash water				
7	10	10 μl unfiltered	77.7 ± 7.6a	22.3 ± 7.6b
8	10	10 μl filtered	41.9 ± 5.8a	58.1 ± 5.8a

[a] Times for eggs on onions refer to time when the bioassay began.
[b] Means within a row followed by different letters are significantly different ($P < 0.05$), paired t test.

females ovipositing on onion. Neither nonovipositing, virgin females (experiment 2) nor males (experiment 3) induced aggregations (Table 1). These results uphold the hypothesis that aggregated oviposition is induced by an olfactory stimulus associated with egg-laying females. However, they do not discriminate between possible egg or fly factors.

Experiments with Eggs as Stimuli. When eggs were present but not in contact with the onion slice (experiment 4), there was no induced aggregation (Table 1). However, when eggs were in contact with onion slices for 24 hr before being assayed (experiment 5), oviposition became markedly aggregated at stations containing this stimulus (Table 1). Aggregation was even more intense when eggs had been in contact with onions for 48 hr (experiment 6, Table 1). Because none of the eggs placed on the onion slices had hatched, even after 48 hr, the induced aggregation could not have been caused by larval activity. Similarly, because eggs kept separated from onion were unable to induce aggregation, any stimulus directly associated with the odor of eggs or an accessory product coating their surface can be ruled out.

However, if some factor on the eggs, either biotic or abiotic, were able to break down the onion tissue when eggs were in direct contact with it, the induced

aggregation might then have been caused by an odor released by contaminated onion tissue, or by active biotic factors on that tissue, or both. The activity of microorganisms would be consistent with numerous reports of microbially induced attraction and oviposition of *D. antiqua* (Ikeshoji et al., 1980; Hough et al., 1981; Miller et al., 1984; Hausmann and Miller, 1989a,b). The next series of experiments was designed to discriminate between possible biotic and abiotic factors on the eggs by separating from the eggs any microorganisms that were resident on their surface.

Experiments with Egg Wash Water as Stimulus. Filtering an aqueous egg wash through a bacterial filter (experiment 8) completely eliminated the aggregated oviposition induced by onion slices treated with unfiltered egg wash (experiment 7, Table 1). As the filtered egg wash water would contain no microorganisms, but would contain any water-soluble chemicals associated with the eggs, biotic, not abiotic, factors are implicated as the agents of increased oviposition when eggs contact onions.

Microorganisms have been implicated several times in enhancing attraction of *D. antiqua* to onions, and several species of bacteria have been isolated from either *D. antiqua* adults or infested onions (Gorlenko et al., 1956; Ikeshoji et al., 1980; Hough et al., 1982; Miller et al., 1984; Hausmann and Miller, 1989a). Microbe-infected nonhosts (e.g., potato) are only marginally more attractive than uninfected nonhosts to *D. antiqua* (Hausmann and Miller, 1989a). Thus, an interaction between bacteria and onions enhances attraction. The likely basis for this interaction is the release of a metabolite not found or present in very small amounts in noninfected onions. Several compounds, including ethyl acetate, tetramethylpyrazine and *n*-heptanal (Ikeshoji et al., 1980); 2-phenylethanol (Ishikawa et al., 1983); and *n*-valeric acid (Ishikawa et al., 1984), have been implicated in this enhanced attraction. However, none appears to account for the greatly increased activity associated with microbial infection. While a mixture of 2-phenylethanol and *n*-valeric acid was up to five times more attractive than decomposing onion pulp (Ishikawa et al., 1984), the onion pulp was preincubated at 25°C for eight days, and Miller et al. (1984) found a sharp decline in the attractiveness of chopped onion after four days. Therefore, a classical isolation and identification procedure (Silverstein, 1970) will probably be required to yield the most active constituents.

Merez (1984) concluded that *D. antiqua* deposits eggs lacking bacteria, whereas Gorlenko et al. (1956) reported isolating *Erwinia carotovora* from all lifestages of *D. antiqua*, including eggs. It is interesting that Hausmann and Miller (1989a) reported that onion tissue inoculated with *E. carotovora* var. *carotovora* was more attractive and stimulated more oviposition than axenic onion tissue or onion tissue inoculated with other bacteria. However, the work of Schneider et al. (1983) has clearly established that onion maggots are capable of developing on onion tissue in the absence of microbes and, thus, onion

maggot larvae can function as true herbivores. Therefore, the important questions with regard to the role of microbes in onion fly biology are: do onion maggots ever feed on onion in the absence of bacteria under natural conditions, and if they do not, then what benefit do the bacteria provide?

The answer to the first question is probably that most larvae feed in the presence of bacteria because larvae will inoculate the onion as they begin to feed or bacteria in the soil will inadvertently enter through feeding sites, but certainly some may not be exposed to bacteria. The answer to the second question is that bacterial contaminants of onion may predispose healthy onions to more successful attack by *D. antiqua* (Hausmann and Miller, 1989b), provide or make available certain nutrients that enhance larval survival (Eymann and Friend, 1985), and they may be responsible for the decrease in development time when *D. antiqua* feeds on decomposing onion (Gorlenko et al., 1956; Schneider et al., 1983; Zurlini and Robinson, 1978).

Although an association between onion flies and bacteria is not necessarily requisite (sensu Schneider et al., 1983) and association between *E. carotovora*, onion maggot eggs, and onions infested with onion maggot larvae may be coincidental, it seems plausible that microbes transmitted from female *D. antiqua* to eggs and larvae might have a greater probability of finding their way into damaged onion tissue at the exclusion of other bacteria. Such an association would be similar to the relationship between scolytid beetles and microorganisms that subdue resistant hosts and/or provide nutrients for invading beetles (Whitney, 1982).

We do not know how newly hatched *D. antiqua* larvae might acquire microorganisms from the egg surface, but it is possible that they either ingest the spent chorion after hatching or acquire microorganisms through mechanical contact. In either case, it may not be necessary that eggs come directly in contact with onions in nature. Positive proof of trans-ovum transmission and induced attraction mechanisms would demand isolation and identification of microorganisms, not from infected host plants, but from gravid females and newly laid eggs, and inoculation of uninfected onion tissue with pure microbial cultures. Hough et al. (1982) have shown that a mixture of several microbial species from male and female onion flies is capable of enhancing oviposition when it is placed on onion tissue. Possibly, many species of microbes can fulfill this role, and it will be of interest to establish the range of species isolated from different onion maggot populations.

Experiment with Ovipositor Extract. When females seeking an oviposition site were given a choice of substrates treated with either a heptane extract of ovipositors or a control substrate treated with heptane only, 72% chose the extract-treated substrate (Figure 3). This choice was reflected almost identically in the percentages of eggs laid at the two stations. This induced aggregation (Figure 3) could not have been due to microbial activity, because any microbes

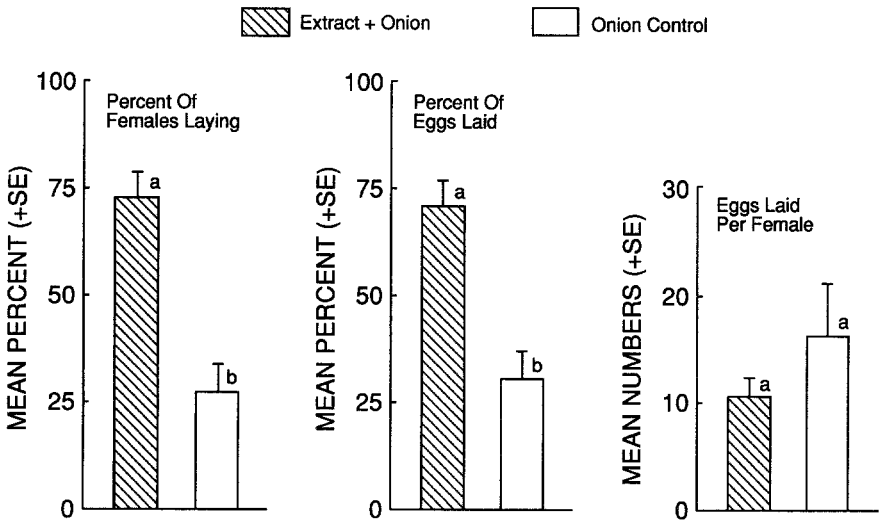

FIG. 3. Oviposition responses in 1 hr by *D. antiqua* females offered a choice of an experimental oviposition station in which the substrate at the sites of oviposition was treated with a heptane extract of ovipositors from egg-laying females, or a control station in which the substrate was treated with heptane only ($N = 12$). Paired bars superscripted by different letters are significantly different, paired t test, $P < 0.05$.

would have been killed in the heptane solvent, and contact of microbes with onion was precluded (Table 1). In a further experiment (data not shown), extracts of virgin females did not stimulate aggregated oviposition.

Oviposition, Marking, or Aggregation Pheromone? Our data uphold the hypothesis that a female factor, a pheromone, is in part responsible for the group oviposition observed in *D. antiqua* under laboratory conditions. The ovipositor extract is probably not an oviposition stimulant in the strictest definition (although more oviposition bouts could be generated), because once females made a choice, they each apparently laid the same number of eggs, regardless of the station they chose (Figure 3).

It is possible that the female-produced pheromone extracted from *D. antiqua* is similar to the marking pheromones found in other anthomyiids (Zimmerman, 1979, 1980, 1982; Raina, 1981). In each of these species, females deposit a pheromone on the host during oviposition, and they search each new host, presumably for the presence of pheromone, before making a decision not to oviposit on already infested hosts. Similar searching behavior could readily precede a positive decision by *D. antiqua* to oviposit if a pheromone were being deposited on onion stems during or after oviposition. While we have extracted a pheromone from egg-laying females, attempts to isolate the same pheromone

from substrates onto which *D. antiqua* had oviposited were unsuccessful (G.J.R. Judd, unpublished data). Therefore, we have no evidence that the chemical we have extracted is actually being deposited on the substrate during oviposition.

When the ovipositor extract was superimposed upon an oviposition stimulant, it induced females to form groups while laying eggs. However, these results do not necessarily prove that such a pheromone evolved as an oviposition aggregation pheromone. Aggregations of both males and virgin females might also have been induced had we tested them. A pheromone that induces aggregations of both sexes has been extracted from the cuticular lipids of female *L. cuprina* (Emmens, 1981).

Group Oviposition and Implications for Ovipositional Bioassays. Laboratory studies examining oviposition in the onion fly often report (Harris and Miller, 1983, 1984) that treatment variances are heterogenous. This heterogeneity is undoubtedly due in large part to group oviposition (Harris and Miller, 1983). While our results provide ample explanation for this variance, and heteroscedastic variance presents problems for statistical analysis (Zar, 1984), group oviposition and the mechanisms causing it have more important implications for the interpretation of group ovipositional bioassays, including our own.

While eggs laid on a treatment by the first female are the result of that treatment's stimulatory capacity, subsequent eggs are possibly laid in response to deposition of microbes and pheromone by "pioneer" females. Because the effects of bacterial contaminants are stronger than the effects of the "aggregation pheromone," assays running 24 hr or longer, in which females or bacteria carried by females can potentially contact onion tissue, will be particularly sensitive to a grouping effect. However, deposition of a pheromone, even an ephemeral one having perhaps 30–60 min activity, would be expected to influence subsequent oviposition to some extent. As each ovipositing female deposits more pheromone, the running time for activity of the pheromone would be reset to zero and oviposition of each subsequent female might be affected for at least an additional 30–60 min period. In this way the influence of the pheromone could be extended throughout an assay running several hours. Therefore, the egg-laying behaviors of all females, except the very first one, may not be independent of one another. A lack of independence would invalidate many statistical tests (Zar, 1984). At the very least, differences between treatments may be magnified by grouping, making certain treatments appear more stimulatory than they might otherwise. We acknowledge that our own experiments are subject to these same grouping effects. For example, the activity of the "pheromone extract" we applied to an oviposition station is probably magnified by the deposition of pheromone from the responding females, and deposition of a pheromone might have increased oviposition at stations baited with microbially infested onion tissue.

Based on our findings, we recommend caution in evaluating group ovipo-

sition assays and suggest that at the very least a comparison should be made between the results from group and single female assays. While certainly not the only solution, ovipositional bioassays with individual flies and replacement of treatments after each oviposition bout would eliminate the problems we have highlighted. Replacement of the treatment between oviposition bouts is recommended to reduce the effect a female might have on her own subsequent behavior.

Natural Implications of Aggregated Oviposition. Extrapolating field behavior of animals from laboratory observations is always difficult. Undoubtedly our two-choice bioassay procedure with stimuli in close proximity provides an experimental setting that maximizes detection of differences between treatments. The obvious shortcoming of this approach is that the importance of a stimulus could be overestimated because the magnitude of the difference is often equated with biological significance. By the same token, the biological significance of an insect's behavior or an observed phenomenon, particularly among agricultural pests, may not always appear relevant if we mistakenly believe that the behavior necessarily evolved in the current agricultural context. These considerations should be kept in mind when evaluating the importance of onion flies as a source of microbes in decomposing onion or an aggregation pheromone as it relates to field situations.

Insects may achieve a selective advantage by aggregating in areas that, among other things, provide food, shelter, or the opportunity to mate and reproduce (Borden, 1985). Egg clustering may also increase the survival of eggs and reduce desiccation in dry environments by decreasing the amount of egg surface exposed to ambient conditions (Stamp, 1980). In their natural habitats, many *Allium* species grow in dry conditions and have very patchy distributions (Stearn, 1944a,b); any onion fly able to find this unevenly distributed resource efficiently would gain a considerable selective advantage. Moreover, if the potential resource were resistant to infestation, as are healthy, mature, wild or cultivated *Allium* spp. (Loosjes, 1976; Zurlini and Robinson, 1978; Finch and Eckenrode, 1985; Hausmann and Miller, 1989b), an advantage would be imparted by exploiting the activity of conspecifics to increase resource suitability, e.g., through preconditioning by previous maggot feeding and the activity of associated microorganisms (Hausmann and Miller, 1989b). Response to microbially infested onions might in itself produce aggregations of egg-laying females (Judd and Borden, 1992) or at very least increase chance encounters under low population densities, as these hosts represent optimal oviposition sites (Zurlini and Robinson, 1978).

A behavioral sequence for an optimal host-selection strategy is hypothesized (Judd, 1986), whereby host-seeking female onion flies respond to host-derived alkyl sulfides at long range (Judd and Borden, 1989) and metabolic by-products of microbially infested hosts and visual cues at short range (ca. 1 m)

(Judd and Borden, 1991). Once located, oviposition at these optimal sites is induced by alkyl disulfides (Vernon et al., 1977), synergized by visual stimuli (Harris and Miller, 1983, 1984) and microbially produced metabolites (Hausmann and Miller, 1989a,b), and perhaps reinforced, if only ephemerally, by the detection of and response to an aggregation pheromone.

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INTRODUCTION OF METHYL GROUPS TO ACETATE SUBSTITUTED CHAIN OF (*Z*)-5-DECENYL ACETATE, A PHEROMONE COMPONENT OF TURNIP MOTH, *Agrotis segetum*[1]: SYNTHESIS, SINGLE-SENSILLUM RECORDINGS, AND STRUCTURE-ACTIVITY RELATIONSHIPS

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Abstract—Methyl-substituted analogs of (*Z*)-5-decenyl acetate, a pheromone component of the turnip moth, *Agrotis segetum*, have been synthesized and studied by electrophysiological single-sensillum recordings and molecular mechanics calculations [MM2(85)]. The analogs are monomethyl substituted in the 2, 3, 4, and 5 positions and geminally dimethyl substituted in the 2, 3, and 4 positions. The methyl groups have been employed as space probes to study the degree of steric complementarity between the acetate-substituted alkyl chain of the pheromone component and its receptor. The electrophysiological activities, interpreted in terms of a receptor interaction model, indicate significant steric repulsive interactions between the introduced methyl groups and the receptor. This implies a high degree of complementarity between the acetate-substituted alkyl chain of the natural pheromone component and its receptor.

Key Words—Lepidoptera, Noctuidae, *Agrotis segetum*, (*Z*)-5-decenyl acetate, pheromone analog, methyl substitution, structure–activity, single-sensillum recordings, receptor interaction, conformational analysis, molecular mechanics.

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[1]Schiff., Lepidoptera: Noctuidae.

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INTRODUCTION

Moth sex pheromone components interact with their receptors in a highly selective way (Priesner, 1979). Furthermore, the very small amount of substrate needed to activate the receptors demonstrates that the natural pheromone component has an extremely high affinity for the receptor tuned to this component. These characteristics of the receptor suggest a high complementarity between the natural substrate and the receptor cavity. The degree of complementarity between the pheromone component and its receptor can be studied by introducing space-demanding substituents to various positions of the parent molecule and investigating the effects of such substituents on the biological activity.

In a previous structure–activity study we systematically introduced alkyl groups to the terminal chain of (*Z*)-5-decenyl acetate (**1,** Figure 1), a pheromone component of the turnip moth, *Agrotis segetum*, and studied the electrophysiological single-cell responses of these analogs (Jönsson et al., 1991). By introducing alkyl substituents as space probes, we were able to characterize the degree of complementarity between the substrate and its receptor with respect to the terminal (nonpolar) chain of **1.** We concluded that there is a very high degree of complementarity between the receptor and the terminal chain of the pheromone component **1.**

In the present study, we have further systematically varied the structure of **1,** this time in the chain between the double bond and the acetate group, by introducing methyl groups in the 2, 3, 4, and 5 positions and geminal dimethyl groups in the 2, 3, and 4 positions. The compounds studied are shown in Figure 1. These compounds were synthesized, and the receptor responses were determined by the electrophysiological single-sensillum (single-cell) technique.

Previous studies on alkyl-substituted pheromone components have concentrated on substitutions in the terminal alkyl chain (Priesner et al., 1977; Bestmann et al., 1979, 1980; Schwarz et al., 1989; Jönsson et al., 1991). To our knowledge, alkyl derivatives of moth sex pheromone components of the monoene acetate type, substituted in the chain connecting the acetate group and the double bond, have not been previously studied.

The main aim of the present study is to characterize the degree of complementarity between the acetate-substituted alkyl chain of the pheromone component and the receptor by using methyl groups at various positions along the chain as probes for repulsive steric interactions with the receptor. In conjunction with our previous study (Jönsson et al., 1991), the results of such a study should make it possible to obtain a detailed picture of the degree of complementarity between the entire alkyl part of the pheromone component and its receptor.

A repulsive interaction between the introduced substituent and the receptor will show up as a decrease of the electrophysiological activity. However, an observed activity decrease may also be due to a high conformational energy

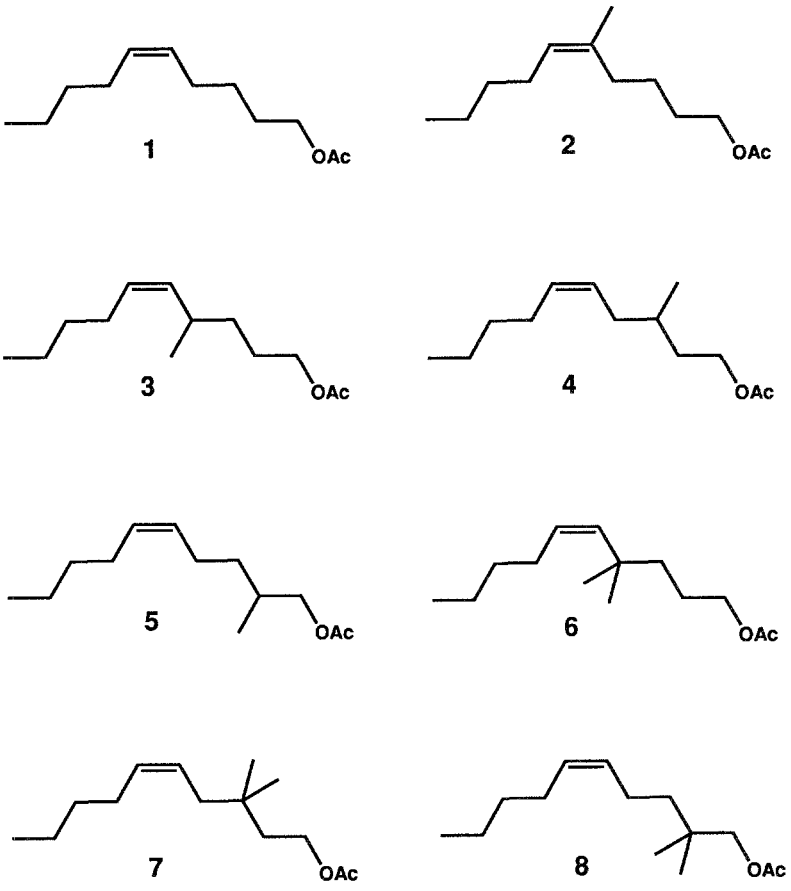


FIG. 1. Compounds studied.

required by the analog to acquire its biologically active conformation. In order to separate these effects, we have, as in our previous work (Jönsson et al., 1991), calculated conformational energies by the molecular mechanics method MM2(85) and used our previously developed receptor interaction model (Liljefors et al., 1987) to calculate the required conformational energies.

METHODS AND MATERIALS

Synthesis. All reagents were obtained from commercial suppliers and were used without further purification. Diethyl ether and tetrahydrofuran (THF) were distilled from dark blue solutions of sodium benzophenone radical anion. *N,N'*-

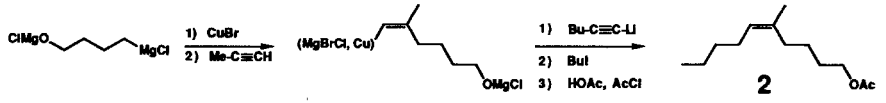
Dimethyl-*N,N'*-propylene urea (DMPU) was used as cosolvent as a substitute for the carcinogenic HMPA. It was stored under argon over 4A molecular sieves. The crude pheromone products were purified by flash chromatography with TLC–silica gel 60H and with petroleum ether–ethyl acetate (20:1) as eluent. Geometric isomers were separated by argentation chromatography (Houx et al., 1974). All products were further purified by preparative GLC using a 6-m × 4-mm 15% OV-351 column and/or by high-performance liquid chromatography (HPLC) using a semipreparative straight-phase column (Kromasil, particle size 10 μm, 250 × 10 mm ID) and a differential refractometer as monitor.

Analytical GLC was carried out on a Varian 3400 capillary GLC fitted with a DB-Wax or a OV-240-OH column. The purity of the isolated final products was >99% and no trace (<0.05%) of the natural pheromone component **1** could be detected. [^{1}H]- and [^{13}C]NMR spectra were recorded in $CDCl_3$ solution on a Varian XL-300 or a Jeol JNM-PMX60 spectrometer. Chemical shifts (δ) are given downfield relative to TMS. The $CHCl_3$ signal was used as an internal reference (7.26 ppm). High-resolution mass spectra were obtained using a Jeol JMS-SX 102 spectrometer.

The synthetic schemes for the preparation of compounds **2–8** are shown in Schemes 1–6.

(Z)-*5-Decenyl Acetate* ***(1).*** This compound was prepared as previously described (Olsson et al., 1983).

5-Methyl-(Z)-5-decenyl Acetate ***(2).*** 5-Methyl-(Z)-5-decenol was prepared according to Gardette et al. (1985) from ω-magnesium-protected 4-hydroxybutyl magnesium chloride (30 mmol). The crude alcohol was dissolved in dry pyridine (7.5 ml) and acetic anhydride (5 ml) and the mixture was stirred for 4 hr at room temperature. It was then poured into water and extracted with pentane,



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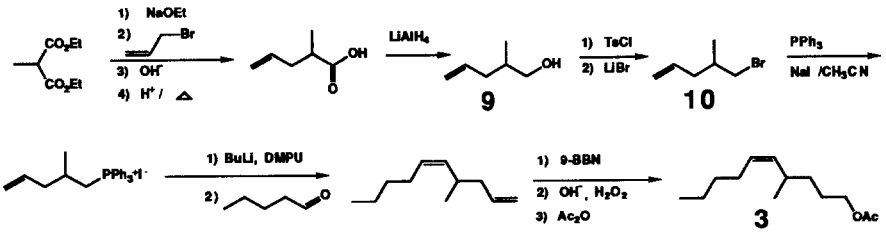

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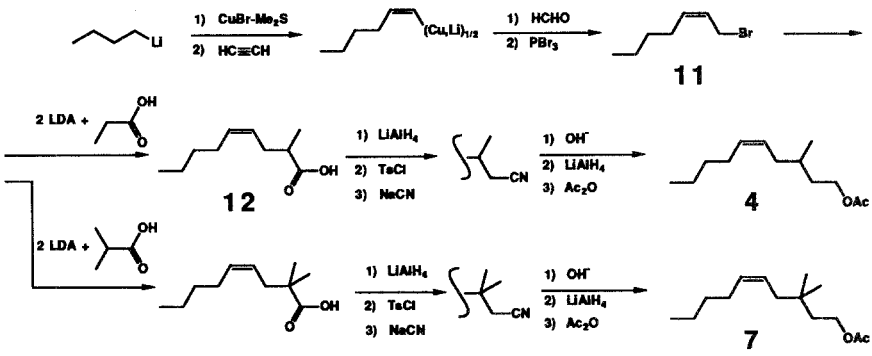

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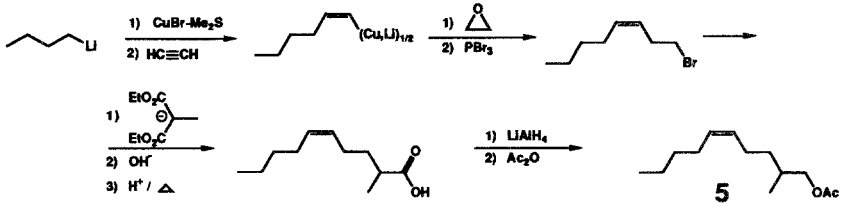

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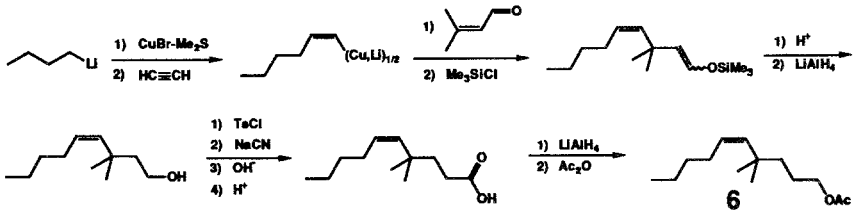

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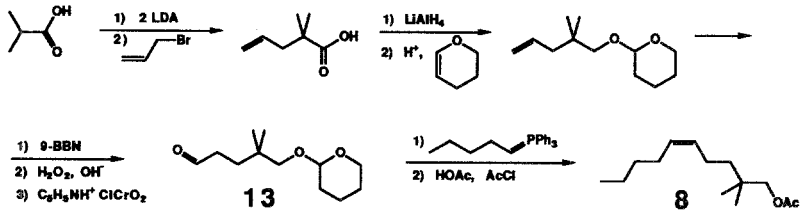

SCHEME 6.

washed with 1 M HCl, and $NaHCO_3$, and finally dried over $MgSO_4$. The solvents were removed and the crude product purified by flash chromatography to give 3.5 g (55% yield) of the trisubstituted olefin. A small amount of disubstituted olefin deriving from acetylene contaminated propyne was carefully removed in the final purification. Analysis by GLC (column DB-Wax 100–215°C at 2°C/min) revealed that the final product contains 0.8% of the *E* isomer. δ_H (300 MHz) 0.88 (t, 3H, CH_3CH_2), 1.23–1.33 (m, 4H, CH_3CH_2, $CH_2CC=$), 1.36–1.48 (m, 2H, $=CCCH_2$), 1.55–1.65 (m, 2H, CH_2CO), 1.65–1.67 (m, 3H, $=CCH_3$), 1.92–2.08 (m, 4H, $CH_2C=$), 2.03 (s, 3H, $COCH_3$), 4.06 (t, 2H, CH_2O), 5.10–5.17 (m, 1H, CH=C); δ_C (75.4 MHz) 14.0, 21.0, 22.4, 23.3, 24.3, 27.5, 28.4, 31.2, 32.3, 64.5, 125.9, 134.5, 171.2; high resolution CI mass spectrum, for $C_{13}H_{25}O_2$ ($M+H^+$), calcd. 213.1854, found 213.1855.

2-Methyl-4-pentenol ***(9).*** 2-Methyl-4-pentenoic acid (11.4 g, 0.1 mol), freshly prepared according to Ställberg-Stenhagen (1946), was dissolved in ether (100 ml) and slowly added to a stirred slurry of lithium aluminum hydride (6.9 g, 0.18 mol) in ether (500 ml) at −10°C and stirred overnight at room temperature. The mixture was then cooled to −20°C and the excess hydride was destroyed (7 ml H_2O), 7 ml 15% NaOH, 21 ml H_2O). The resulting white slurry was filtered and the solution dried and concentrated to give a colorless liquid (10.0 g). The purity was 90% by GLC. δ_H(60 MHz) 0.9 (d, 3H, CH_3), 1.2–2.3 (m, 3H, CH, CH_2), 2.5 (s, 1H, OH), 3.5 (d, 2H, CH_2O), 4.8–6.2 (m, 3H, $CH_2=CH$).

2-Methyl-4-pentenyl Bromide ***(10).*** 2-Methyl-4-pentenol **(9)** (10 g, 0.1 mol) was dissolved in dry pyridine (20 ml) at 0°C. *p*-Toluenesulphonyl chloride (22.9 g, 0.12 mol) was added and the mixture stirred for 6 hr. It was then poured into dilute HCl and ice, extracted with ether and washed with water and $NaHCO_3$. The extract was dried, concentrated, and the residual oil dissolved in acetone (200 ml) with anhydrous lithium bromide (13 g, 0.15 mol). The mixture was refluxed (16 hr), poured into water, and extracted with pentane. The extracts were washed with water, dried ($MgSO_4$), and concentrated to give a colorless oil (12.1 g, 74%), which was employed for the synthesis of **3,** described below, without further purification.

4-Methyl-(Z)-5-decenyl Acetate ***(3).*** 2-Methyl-4-pentenyl bromide **(10)** was treated with triphenylphosphine (19.4 g, 74 mmol) and sodium iodide (27 g, 180 mmol) in acetonitrile (100 ml) under reflux (72 hr). The main part of the solvent was removed by rotary evaporation, CH_2Cl_2 (25 ml) was added and the residual slurry filtered. The solution was treated with ether and a semisolid oil precipitated. The supernatant solution was removed and the residual solvents removed under reduced pressure to give 31.0 g of a sticky product. NMR spectrum showed signals at 7.3 and 7.8 ppm, indicating unreacted triphenylphosphine (attempts to purify the product by crystallization were not successful).

The crude phosphonium salt (11.8 g, 25 mmol) and dry THF (50 ml) was cooled to 0°C under nitrogen. *n*-Butyllithium (25 mmol) was injected, and the resulting dark red solution was stirred for 1 hr. It was then cooled to −72°C, DMPU (8 ml) was added, and after 15 min freshly distilled valeraldehyde (2.7 ml, 25 mmol). The mixture was stirred for 1 hr and then for another 1 hr without external cooling. Water was added and the solution was extracted with pentane, dried, concentrated, and chromatographed (silica gel, pentane as eluant) to give 1.25 g of the alkadiene (33% yield from the bromide). δ_H (60 MHz) 0.8–1.1 (m, 6H, CH_3), 1.2–1.5 (m, 4H, CH_2), 1.6–2.2 (m, 5H, allylic), 4.8–5.5 (m, 5H, vinylic).

The diene (8.2 mmol) was dissolved in THF (10 ml) and cooled in an ice bath under nitrogen. 9-Borabicyclo[3.3.1]nonane (9-BBN, 16.4 ml, 0.5 M in THF) was injected, the solution stirred at room temperature overnight, and then recooled. A mixture of 3 N NaOH (2.8 ml) and 30% H_2O_2 (3.3 ml) was added dropwise, stirred for 60 min at 35°C, water (10 ml) was added, and the mixture extracted with ether. The ether phase was washed (H_2O), dried ($MgSO_4$), and concentrated. The crude product was acetylated and purified as described for compound **2** to give 1.23 g (70% yield). Analysis of the product by GLC indicated the presence of 4.9% of the *E* isomer, which was removed by argentation column chromatography to give the final product. δ_H (300 MHz) 0.89 (t, 3H, $\mathbf{CH_3}CH_2$), 0.94 (d, 3H, $\mathbf{CH_3}CH$), 1.14–1.42 (m, 6H, $CH_3\mathbf{CH_2}$, CH_2CC=, =$CCCH_2$), 1.48–1.69 (m, 2H, CH_2CO), 1.92–2.11 (m, 2H, CH_2C=), 2.04 (s, 3H, $COCH_3$), 2.36–2.51 (m, 1H, =CCH) 4.03 (t, 2H, CH_2O), 5.09 (m, 1H, J = 10.9 Hz, =$\mathbf{CH}$CH), 5.32 (m, 1H, J = 10.9 Hz, $CH_2\mathbf{CH}$=); δ_C (75.4 MHz) 14.0, 21.0, 21.4, 22.3, 26.7, 27.2, 31.4, 32.1, 33.6, 64.7, 129.0, 135.5, 171.2; high resolution CI mass spectrum, for $C_{13}H_{25}O_2$ ($M+H^+$), calcd. 213.1854, found 213.1853.

(Z)-2-Heptenyl Bromide (11). (Z)-2-Heptenol was prepared according to Alexakis et al. (1980) from butyllithium (244 ml, 2 M in hexane, 0.5 mol). The crude alcohol was diluted with ether (425 ml), pyridine (5.8 ml) was added, then PBr_3 (19 ml, 0.2 mol), dropwise. After stirring overnight, the mixture was poured into ice water, washed with $NaHCO_3$ and water, and then dried over Na_2SO_4. Fractional distillation afforded pure **11** (48.7 g, 55% yield): bp 62–65°C/14 mm. δ_H (60 MHz) 0.9 (t, 3H, CH_3), 1.1–1.7 (m, 4H, CH_2), 1.9–2.3 (m, 2H, CH_2C=), 4.0 (d, 2H, CH_2Br), 5.3–5.9 (m, 2H, CH=CH).

3-Methyl-(Z)-5-decenyl Acetate (4). 2-Methyl-(Z)-4-nonenoic acid (**12**) was prepared by a modification of a method of Pfeffer et al. (1972). To a stirred solution of diisopropylamine (13.5 ml, 0.1 mol) in dry THF (70 ml) at −5°C under nitrogen was added *n*-butyllithium in hexane (48.5 ml 2 M, 0.1 mol) and after 10 min propionic acid (2.6 ml, 35 mmol). DMPU (4.8 ml, 40 mmol) was added to the milky white solution and the temperature allowed to rise to room temperature. After 30 min of stirring, the cloudy solution was cooled to 0°C

and (Z)-2-heptenyl bromide **(11)** (6.2 g, 35 mmol) added rapidly. The reaction mixture was stirred for 2 hr at room temperature and then neutralized with ice cold 10% HCl. The aqueous layer was extracted with hexane, and the combined organic phases washed with 10% HCl, water, and dried (Na_2SO_4). Removal of the solvent yielded the crude acid **12,** which was reduced with lithium aluminum hydride as described above for **9,** chromatographed (silica gel, petroleum ether–ether 6:1) to give pure 2-methyl-(Z)-4-nonenol (1.75 g, 32%). TLC: R_f 0.17 (hexane–ethyl acetate 10:1); δ_H (60 MHz) 3.5 (d, 2H, CH_2O).

p-Toluenesulphonyl chloride (2.6 g, 14 mmol) was added to a stirred ice-cooled solution of 2-methyl-(Z)-4-nonenol (11.2 mmol) in dry pyridine (6 ml) and stirred for 4 hr. It was then poured into dilute HCl and ice, extracted with ether, and washed with water and $NaHCO_3$. The ether solution was dried ($MgSO_4$) and concentrated in vacuo to give the crude tosylate quantitatively. TLC: R_f 0.36 (hexane–ethyl acetate 10:1).

This was dissolved in DMSO (12 ml) and NaCN (1 g) was added. The mixture was stirred and heated at 60°C overnight, poured into ice-water, and extracted with ether. The ether extract was washed with water and brine, dried ($MgSO_4$), and concentrated to give the nitrile. TLC: R_f 0.47 (hexane–ethyl acetate 10:1); δ_H (60 MHz) 2.3 (d, 2H, CH_2CN).

A solution of the crude nitrile and NaOH (8 g) in 60% ethanol (35 ml) was heated under reflux for 30 hr. The mixture was stripped from ethanol by rotary evaporation, diluted with water, and acidified with HCl and thereafter extracted with ether. The ether solution was washed with water and brine, dried, and concentrated to give the acid. TLC: R_f 0.06 (hexane–ethyl acetate 10:1).

The crude acid was then reduced with lithium aluminum hydride as for **9** to give 3-methyl-(Z)-5-decenol [TLC: R_f 0.17 (hexane–ethyl acetate 10:1); δ_H (60 MHz) 3.5 (t, 2H, CH_2O)], which was acetylated as described for **2** to give the final product in an overall yield of 69% from 2-methyl-(Z)-4-nonenol. δ_H (300 MHz) 0.89 (t, 3H, CH_3CH_2), 0.91 (d, 3H, $=CCCCH_3$), 1.28–1.49 (m, 5H, CH_3CH_2, $CH_2CC=$, $=CCCH$), 1.55–1.75 (m, 2H, CH_2CO), 1.87–2.09 (m, 4H, $CH_2C=$), 2.04 (s, 3H, $COCH_3$), 4.03–4.17 (m, 2H, CH_2O), 5.29–5.47 (m, 2H, J = 11.0 Hz, $CH=C$); δ_C (75.4 MHz) 14.0, 19.4, 21.0, 22.4, 27.0, 30.5, 31.9, 34.3, 35.1, 63.0, 127.5, 131.3, 171.2 high resolution CI mass spectrum, for $C_{13}H_{25}O_2$ ($M+H^+$), calcd. 213.1854, found 213.1849.

2-Methyl-(Z)-5-decenyl Acetate ***(5).*** (Z)-3-Octenyl bromide was prepared from lithium (Z)-dihexenyl cuprate (0.12 mol) and ethylene oxide (12.5 ml, 0.25 mol) to furnish (Z)-3-octenol (Alexakis et al., 1980), which was converted to the bromide in the same manner as described for the preparation of **11.** Distillation afforded 21.5 g, 45% yield; bp 72–79°C/14 mm. δ_H (60 MHz) 0.9 (t, 3H, CH_3), 1.1–1.7 (m, 4H, CH_2), 1.9–2.7 (m, 4H, $CH_2C=$), 3.2 (d, 2H, CH_2Br), 5.0–5.7 (m, 2H, $CH=CH$).

Alkylation of ethyl methylmalonate (6.0 ml, 35 mmol) using sodium, dry

ethanol, and the prepared (Z)-3-octenyl bromide, with subsequent hydrolysis and decarboxylation, was carried out as described for compound **9.** The prepared acid was then reduced with lithium aluminum hydride and acetylated to yield the final product (2.6 g, 35%). δ_H (300 MHz) 0.89 (t, 3H, CH_3CH_2), 0.93 (d, 3H, CH_3CCO), 1.12–1.48 (m, 6H, CH_3CH_2, $CH_2CC=$, $=CCCH_2$), 1.73–1.87 (m, 1H, CHCO), 1.98–2.12 (m, 4H, $CH_2C=$), 2.05 (s, 3H, $COCH_3$), 3.83–3.98 (m, 2H, CH_2O), 5.27–5.41 (m, 2H, J = 10.8 Hz, CH=C); δ_C (75.4 MHz) 14.0, 16.7, 20.9, 22.3, 24.5, 26.9, 31.9, 32.1, 33.4, 69.3, 129.2, 130.3, 171.2; high resolution CI mass spectrum, for $C_{13}H_{25}O_2$ ($M+H^+$), calcd. 213.1854, found 213.1849.

4,4-Dimethyl-(Z)-5-decenyl Acetate ***(6).*** Neat 3-methyl-2-butenal (3.9 ml, 40 mmol) was injected into a solution of lithium (Z)-dihexenyl cuprate (25 mmol) at −72°C. To this reaction mixture after 30 min of stirring at −50°C, was successively added trimethyl silyl chloride (15 ml, 116 mmol), triethylamine (17.8 ml, 128 mmol), and DMPU (6.3 ml, 52 mmol) (Chuit et al., 1981). The temperature was allowed to rise to 0°C and 4% HCl was added to neutralize the mixture. The ether solution was washed with sat. $NaHCO_3$, NaCl, dried ($MgSO_4$), concentrated, and distilled to give 4.5 g of a colorless oil (47% yield); bp 105–120°C/14 mm. This product was dissolved in THF (30 ml) and added to a solution of oxalic acid (1 g) in water (30 ml). The mixture was stirred 1 hr at room temperature. The aqueous layer was extracted once with ether and the combined organic phases were washed with $NaHCO_3$ and dried. The solvent was evaporated and the residue chromatographed (silica gel, petroleum ether–ethyl acetate 20:1) to furnish 1.95 g (62%) of the pure aldehyde. According to the previously described reaction sequence for compound **4,** the aldehyde was reduced with $LiAlH_4$ to the alcohol, tosylated, and converted to the nitrile; then hydrolyzed, reduced, and acetylated to give 1.8 g of **6.** Overall yield from the aldehyde 72%. δ_H (300 MHz) 0.90 (t, 3H, CH_3CH_2), 1.09 (s, 6H, $=CCCH_3$), 1.29–1.41 (m, 6H, CH_3CH_2, $CH_2CC=$, $=CCCH_2$), 1.58–1.64 (m, 2H, CH_2CO), 2.04 (s, 3H, $COCH_3$), 2.10–2.17 (m, 2H, $CH_2C=$), 4.03 (t, 2H, CH_2O), 5.14–5.26 (m, 2H, J = 12.1 Hz, CH=C); δ_C (75.4 MHz) 14.0, 21.0, 22.4, 24.3, 28.1, 29.0, 32.4, 36.0, 40.0, 65.3, 130.1, 137.7, 171.2; high resolution CI mass spectrum, for $C_{14}H_{27}O_2$ ($M+H^+$), calcd. 227.2011, found 227.1999.

3,3-Dimethyl-(Z)-5-decenyl Acetate ***(7).*** This compound was prepared via the same sequence of reactions as described for compound **4.** Addition of isobutyric acid in place of propionic acid to lithium diisopropylamide resulted in a clear homogeneous solution, which was warmed at 50°C for 2 hr before alkylation. No DMPU was added as cosolvent. 2,2-Dimethyl-(Z)-4-nonenoic acid was obtained in 72% yield. Due to steric hindrance of the neopentyl system, the displacement of tosylate by cyanide required extended reaction time (24 hr) and increased temperature (120–130°C). Overall yield from (Z)-4-nonenoic acid

was 42%. δ_H (300 MHz) 0.89 (t, 3H, CH_3CH_2), 0.91 (s, 6H, $=CCCCH_3$), 1.25–1.37 (m, 4H, CH_3CH_2, $CH_2CC=$), 1.54–1.62 (m, 2H, CH_2CO), 1.95–2.04 (m, 4H, $CH_2C=$), 2.03 (s, 3H, $COCH_3$), 4.11 (t, 2H, CH_2O), 5.34–5.51 (m, 2H, J = 11.1 Hz, CH=C); δ_C (75.4 MHz) 14.0, 21.1, 22.4, 27.0, 31.8, 32.9, 39.5, 39.6, 61.9, 125.4, 132.1, 171.2; high resolution CI mass spectrum, for $C_{14}H_{27}O_2$ ($M+H^+$), calcd. 227.2011, found 227.2016.

2,2-Dimethyl-(Z)-5-decenyl Acetate ***(8).*** Following the method described above for the preparation of **7,** the dilithio anion of isobutyric acid (0.14 mol) was alkylated with allyl bromide to give 17.6 g as residue. The crude acid was reduced with lithium aluminum hydride to furnish 13.7 g (86% yield) of the alcohol (purity 88% by GLC). The crude alcohol was thereafter dissolved in ether (100 ml) containing *p*-toluenesulfonyl chloride (0.1 g), and dihydropyran (18 ml, 0.2 mol) was added dropwise to the solution. The mixture was stirred overnight, then washed with K_2CO_3, dried over K_2CO_3, and concentrated. Distillation of the residue in base-washed equipment afforded 18.9 g product, bp 54–62°C/1 mm. The protected alkenol (5.0 g, 25 mmol) was oxidized with 9-BBN as described for compound **3.** It was then oxidized using pyridinium chlorochromate (10.8 g, 50 mmol) buffered with NaOAc (10.6 g) to give 2.5 g of pure aldehyde **13** (47% from the alkenol).

Triphenylphosphine (26.2 g, 0.1 mol) and pentyl bromide (13.7 ml, 0.11 mol) in acetonitrile (100 ml) were heated under reflux for 30 hr. The main part of the solvent was removed by rotary evaporation and dry ether was slowly added to the mixture. The precipitated white crystals were collected on a filter, washed with dry ether, and dried in vacuo over P_2O_5 to give 41.3 g of the phosphonium salt. Part of this (4.13 g, 10 mmol) was treated with *n*-butyllithium and thereafter with the aldehyde **13** in a Wittig condensation reaction as described for compound **3.** The crude product was thereafter acetylated with a mixture of acetic acid and acetyl chloride at 50°C overnight. It was then poured into icewater, extracted with pentane, and washed with $NaHCO_3$. The solvent was concentrated and flash chromatographed to give 1.3 g (63%) of the product, which was purified by argentation column chromatography to give an isomerically pure final product. δ_H (300 MHz) 0.90 (t, 3H, CH_3CH_2), 0.92 (s, 6H, CH_3CCO), 1.26–1.35 (m, 6H, CH_3CH_2, $CH_2CC=$, $=CCCH_2$), 1.94–2.06 (m, 4H, $CH_2C=$), 2.06 (s, 3H, $COCH_3$), 3.80 (s, 2H, CH_2O), 5.27–5.39 (m, 2H, J = 10.8 Hz, CH=C); δ_C (75.4 MHz) 14.0, 20.9, 21.8, 22.3, 24.2, 26.7, 26.8, 31.9, 33.7, 39.1, 72.4, 129.7, 129.9, 171.2; high resolution CI mass spectrum, for $C_{14}H_{27}O_2$ ($M+H^+$), calcd. 227.2011, found 227.2021.

Electrophysiology. Electrophysiological activities were determined by the single sensillum (single-cell) technique (Kaissling, 1974). Olfactory receptor cells specifically tuned to (Z)-5-decenyl acetate **(1)** are present in antennal sensilla type SW1 of *Agrotis segetum* (Hallberg, 1981; Löfstedt et al., 1982, van der Pers and Löfstedt, 1983). The method used was modified according to van

der Pers and Den Otter (1978) and has been described previously (Liljefors et al., 1987; Bengtsson et al., 1990). Dose–response curves were constructed using five different stimulus amounts. The amounts used for the pheromone component **1** were 10^{-4}–1 μg and for the analogs 10^{-3}–100 μg, in decadic steps. For each stimulus amount, 10 replicates were recorded and the mean value of the number of action potentials generated during 1 sec from the onset of stimulation was calculated and employed in the construction of the dose–response curves. Errors are expressed as standard errors of mean (SEM). The electrophysiological activity of each compound in relation to that of **1** is expressed as the reciprocal of the relative quantities required to elicit the same response from the receptor cell as the natural pheromone component **1.**

Corrections for differences in volatility have been made using relative vapor pressures, as previously described (Liljefors et al., 1985; Bengtsson et al., 1990). The correction factors for compounds **2–8** are based on vapor pressure data for saturated hydrocarbons (pentane–tridecane) and their methyl substituted analogs (Weast, 1971). The correction factors used are 2.2 for the monomethyl analogs and 4.6 for the dimethyl analogs.

Molecular Mechanics Calculations. The calculations of energy-minimized geometries and conformational energies were done using the molecular mechanics program MM2(85) developed by Allinger and coworkers (Burkert and Allinger, 1982). Starting structures for MM2(85) were obtained from the molecular modeling system MIMIC (Liljefors, 1983; von der Lieth et al., 1984).

RESULTS

Chemicals. The methyl substituted pheromone analogs **2–8** were prepared by the sequences of reactions shown in Schemes 1–6. The trisubstituted olefin analog **2** (Scheme 1) was obtained by carbocupration of propyne, followed by alkylation of the intermediate alkenyl cuprate, a method reported to give very high isomeric purity (Gardette et al., 1985) and this was also found in our case. The olefin analogs **4–7** were prepared analogously from carbocupration of acetylene to form olefinic intermediates and products with excellent isomeric purity. The α-dimethyl-substituted analog **6** (α with respect to the double bond) was thus prepared by conjugate addition of the intermediate alkenyl cuprate to 3-methyl-2-butenal and subsequent chain elongation and acetylation (Scheme 5). The β-substituted analogs **4** and **7** were obtained by treating the alkenyl cuprate with formaldehyde (Scheme 3). This allylic alcohol precursor was transformed to the corresponding bromide and reacted with the dianions of propionic acid and isobutyric acid, respectively. The alkylated products were chain elongated and acetylated similarly to compound **6.** Due to elimination of the homoallyllic system, the use of the corresponding reaction sequence to introduce a

methyl group in the γ-position was less successful. An attempt to prepare compound **5** by malonic ester synthesis furnished the mono-substituted analog in acceptable yield (Scheme 4). Compounds **3** (Scheme 2) and **8** (Scheme 6) were prepared by Wittig reactions in THF with DMPU as cosolvent. GLC analysis of the products showed the presence of 7–10% of the *E* isomers, which were removed by argentation chromatography.

Receptor Cell Responses. Electrophysiological single-cell activities for compounds **1–8** are shown in Figure 2. The relative activities shown in Figure 2 include corrections for differences in volatility (see Methods and Materials).

All methyl-substituted analogs display a significantly reduced activity compared to the activity of the natural pheromone component **1.** However, depending on the position of the introduced methyl substituent(s), the decrease in activity differs.

Methyl substitution in the 5 position at the double bond (compound **2**) reduces the electrophysiological activity by a factor of 600 compared to that of **1.** Monomethyl or dimethyl substitution in the 4-position, (compounds **3** and **6**) gives virtually inactive compounds. In position 3 (compound **4**), monomethyl substitution leads to a moderate drop of activity by a factor of 22, which makes this compound the most active one of the analogs in the present series. However, geminal dimethyl substitution in this position (compound **7**) reduces the activity considerably. Compound **7** is 1100 times less active than the natural component

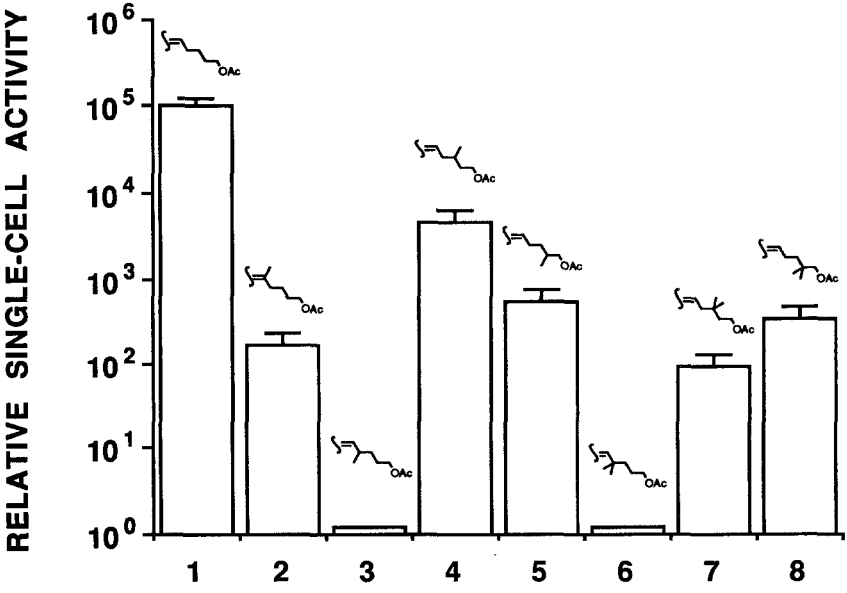

FIG. 2. Relative single-cell activities (+SEM) for compounds **1–8.**

1. In contrast to the large activity difference between monomethyl and dimethyl substitution in position 3, the corresponding substitutions in position 2 give very similar electrophysiological activities. Compounds **5** and **8** are less active than **1** by a factor of 180 and 288, respectively.

Comparing the effects on the electrophysiological activity of mono- and dimethyl substitution in positions 2 and 3, monomethyl substitution in position 3 leads to the more active compound **(4),** while the 2-dimethyl substituted compound **8** is more active than the 3-dimethyl substituted one **(7).**

Conformational Energies. The calculated thermodynamically most stable conformers of compounds **2–8** are shown in Figure 3. These are the lowest-energy conformers found by energy minimizations [MM2(85)] of a large number of trial conformers for each compound. With the exception of compound **6,** attractive van der Waals (dispersion) forces between the terminal alkyl chain and the acetate-substituted chain of the pheromone analogs stabilize a "coiled" conformer in which the two chains are in an essentially parallel arrangement.

In our previous conformational analysis of the pheromone component **1,** we found two *cis*-oid and one *trans*-oid conformation with all-anti alkyl chains to be the thermodynamically preferred conformations of this compound (Liljefors et al., 1987). One of these conformers correspond to the deduced biologically active conformation of **1** shown in Figure 4. As the type of "coiled" lowest-energy conformation found for most of the methyl-substituted compounds studied in the present work (Figure 3) was not included in our previous conformational analysis of **1,** we have reinvestigated the conformational properties of **1,** including conformers of the "coiled" type. In spite of two gauche arrangements about adjacent bonds in **1** (C-2—C-3 and C-3—C-4), we also found that for **1,** the "coiled" type of conformer was lower in energy than any of the conformers we have found previously. This conformer of **1,** shown in Figure 3, is calculated to be 1.1 kcal/mol lower in energy than the *cis*-oid and *trans*-oid conformers described above. In order to investigate if this finding requires revision of our previous structure–activity analysis of analogs of **1,** we have recalculated all previously reported conformational energies. The result of this revised conformational analysis is that all previously obtained structure–activity results are virtually unchanged by the inclusion of conformers of the "coiled" type. In particular, the correlation obtained between conformational energies and electrophysiological activities (Liljefors et al., 1987; Bengtsson et al., 1987) is still valid with an energy of ca. 2 kcal/mol, corresponding to a decrease of the electrophysiological activity by a factor of 10. The conformational energies reported in our study on alkyl substitution in the terminal chain of **1** (Jönsson et al., 1991) are also essentially unaffected by the inclusion of "coiled" conformers. It should be noted that the "coiled" conformer of **1** is not a likely candidate for the biologically active conformation. As described above, the "coiled" conformer has gauche arrangements about the C-2—C-3 and

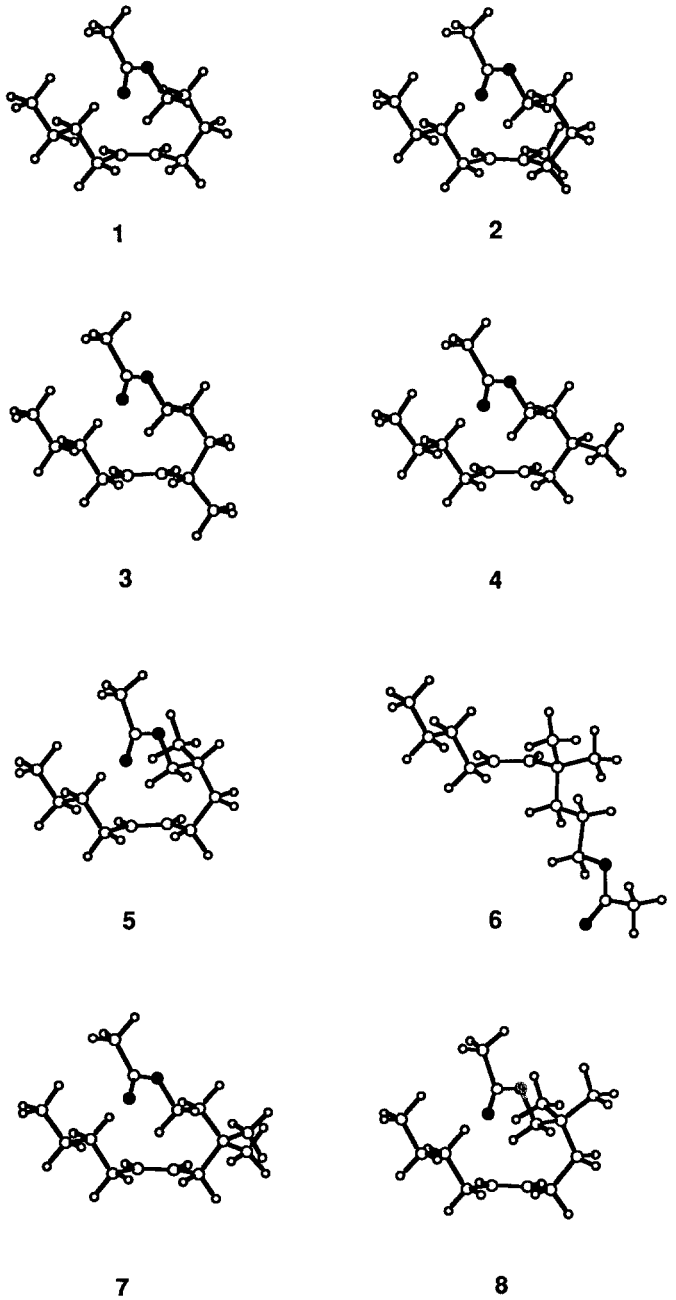

FIG. 3. Calculated [MM2(85)] lowest energy conformers for compounds **1–8.**

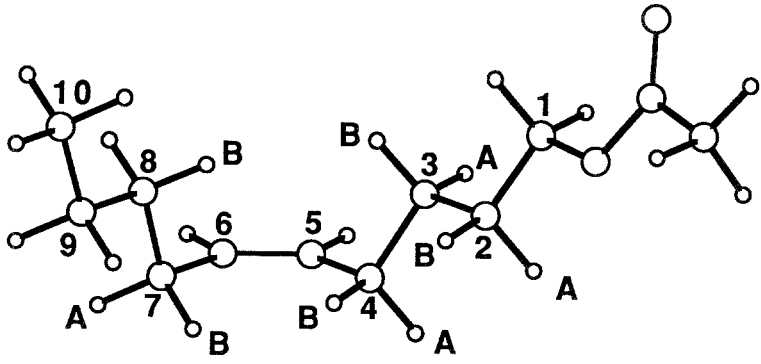

FIG. 4. The proposed biologically active conformation of compound **1** and the possible methyl group directions in methyl-substituted analogs of **1**.

C-3—C-4 bonds, which is not compatible with the quite high activities of (*Z*)-5,(*E*)-2 and (*Z*)-5,(*E*)-3 analogs of **1**, in which the corresponding bonds are locked in an anti arrangement (Bengtsson et al., 1987).

In order to calculate the energy required for each of compounds **2–8** to acquire the biologically active conformation, according to our receptor interaction model (Liljefors et al., 1987), a second series of calculations was performed in which each methyl-substituted molecule was superimposed on the proposed biologically active conformation of **1** (Figure 4) and energy minimized. During the minimization, the positions of the acetate group and the terminal methyl group were held fixed at the positions of the corresponding parts of **1**, while the double bond of the analogs was allowed to move in a plane containing the double bond of **1**. A more detailed description of the computational procedure is given by Liljefors et al. (1987). For each compound, the difference between the energy calculated in this way and the energy of the thermodynamically preferred conformer yields the energy required by an analog in order to optimally mimic the biologically active conformation of **1**. The calculated energies correspond to internal energies or entalphies. We assume that the entropic factor is similar for the different compounds in the present series.

The calculated energies required by each compound to acquire its biologically active conformation (in relation to the energy required by the natural component **1**), are shown in Table 1. For compound **2**, rearrangement of the compound from its thermodynamically preferred conformation to the biologically active one, as described above, is calculated to require only a small amount of energy, 0.2 kcal/mol in excess of the energy required by **1**.

A methyl substituent in positions 2, 3, and 4 (compounds **3–5**) may adopt two different directions, denoted A and B in Figure 4. In the B direction, the substituent is oriented towards the terminal chain and may suffer from repulsive

TABLE 1. CALCULATED CONFORMATIONAL ENERGY DIFFERENCES (kcal/mol) BETWEEN PROPOSED BIOLOGICALLY ACTIVE CONFORMATION AND LOWEST ENERGY CONFORMATION OF COMPOUNDS **1–8**[a]

Compound	A	A + B	B
1		0.0[b]	
2		0.2	
3	1.0		5.0
4	1.7		1.5
5	1.3		1.0
6		3.2	
7		2.4	
8		0.2	

[a]The energy values are given in relation to that of **1**. A and B denote alkyl group directions according to Figure 4. The energies may be used to estimate the expected decrease in the electrophysiological response due to a "pure" conformational effect by using the relation that ca. 2 kcal/mol corresponds to a decrease in the response by a factor of 10 (Liljefors et al., 1987; Bengtsson et al., 1987).
[b]The A/B designation is not applicable in this case.

steric interactions with this chain, while in the A direction it is directed away from the terminal chain. Monosubstitution in the A and B directions corresponds to different enantiomers. A relatively small energy is calculated for a 4-methyl substituent (compound **3**) in the A direction (1.0 kcal/mol, Table 1), while in the biologically active conformation of the compound with a methyl group in the B direction causes severe repulsive interactions with the terminal alkyl segment leading to a high energy to acquire this conformation (5.0 kcal/mol, Table 1). This repulsive interaction is less pronounced in positions 3 and 2. A methyl group in these positions (compounds **4** and **5**) gives similar calculated energies (1.0–1.7 kcal/mol) irrespective of its direction. Geminal dimethyl substitution in the 4, 3, and 2 positions results in a successive decrease of the calculated energy, 3.2, 2.4, and 0.2 kcal/mol, respectively.

DISCUSSION

Methyl substitution of the pheromone component **1** changes the hydrophobicity, shape, and conformational properties of the parent compound. The increased hydrophobic character of the methyl substituted analogs is expected to give a higher affinity to a nonpolar receptor cavity. Due to the additive nature of this physicochemical property, the hydrophobic effects can be predicted to be essentially identical for all monomethyl analogs and for all dimethyl analogs (Leo et al., 1971; Hansch and Leo, 1979). The increased molecular size due to

the methyl substituent may cause steric interference between the analog and a complementary receptor site and consequently a decrease in the biological activity due to repulsive steric interactions. This effect may give information on the dimension of different parts of the receptor cavity. A methyl substituent may also alter the conformation of the molecule and affect the energy required to bring an analog into the biologically active conformation. In terms of our conformational energy model (Liljefors et al., 1987; Bengtsson et al., 1987), an increase of this energy by ca. 2 kcal/mol corresponds to a decrease of the electrophysiological activity by a factor of 10. We have used this model to investigate if a decrease in the observed activity may be due to unfavorable conformational energies or to steric repulsive interactions with the receptor.

A methyl group in the 5 position, compound **2,** decreases the electrophysiological activity by a factor of 600 compared to the natural substrate **1** (Figure 2). This activity decrease may be compared to that due to the corresponding substitution in the other double bond position, position 6, which results in an activity drop of only 10 times (Jönsson et al., 1991). The calculated conformational energies for both these analogs to acquire the active conformation are small (Table 1) (Jönsson et al., 1991). This implies that the observed activity decrease is most probably caused by steric repulsive interactions between the methyl substituent and the receptor. The steric repulsions are clearly more pronounced in the 5 position than in the 6 position. These results demonstrate a high complementarity, in particular at position 6, between the "backside" of the *Z* double bond of natural substrate and the receptor.

Geminal dimethyl substitution in the 4 position (compound **6**) results in complete inactivity (Figure 2), which was previously also found for geminal dimethyl substitution in the 7 position (Jönsson et al., 1991). Although the conformational energy required to bring compound **6** into its active conformation is 3.2 kcal/mol higher than is required by **1** (Table 1), the main reason for the inactivity of **6** is concluded to be repulsive steric interactions with the receptor. It is clear that the extra volumes produced by the methyl groups of **6,** as well as the methyl groups in the 7-dimethyl compound (Jönsson et al., 1991) are detrimental to a productive receptor interaction. It is likely that at least some of these methyl groups at each of the positions interfere with the interaction between the *Z* double bond and its receptor site. Monomethyl substitution in the 4-position (compound **3**) gives an essentially inactive compound, which should be compared to the moderate activity decrease by a factor of 45 due to a methyl group in the analogous 7 position (Jönsson et al., 1991). The calculated conformational energy penalty for a methyl group in the A direction is low (Table 1), but it is significantly higher (5.0 kcal/mol) for a methyl group in the B direction. The high calculated energy for a B substituent partly explains the low activity, but probably repulsive interactions are involved as well. The low calculated energy for an A-oriented substituent clearly indicates that an A substituent destructively

interacts with the receptor. An investigation of the pure enantiomers of compound **3** is necessary for a final analysis. However, it is clear that the steric requirements of the receptor cavity are even more strict in the area corresponding to position 4 of the substrate than in the area corresponding to the other allylic position (position 7). The discussion above and our previous study (Jönsson et al., 1991) lead to the prediction that while one of the enantiomers of the 7-methyl compound may be significantly more active than the other, both enantiomers of 4-methyl-substituted **1** should be essentially inactive.

In the 3 position, geminal dimethyl substitution (compound **7**) reduces the biological activity considerably, 1100 times, compared to 95 times for the same substitution in the corresponding position in the terminal chain, position 8 (Jönsson et al., 1991). The calculated conformational energy for **7** is 2.4 kcal/mol (Table 1). In terms of our model, this corresponds to an activity decrease by approximately a factor of 10, due to the conformational effect. This indicates that there is substantial repulsion between the methyl group and the receptor in at least one of the A and B directions of **7** (Figure 4). Monomethyl substitution in the 3 position (compound **4**), decreases the activity only 22 times, which is similar to the activity decrease by a factor of 45 due to a methyl group in the 8 position (Jönsson et al., 1991). The two possible methyl group directions of **4** give very similar conformational energies (Table 1). Considering the low activity of the dimethyl-substituted compound **7** and the quite high activity of the monomethyl-substituted compound **4,** this implies that in the 3 position only one of the substituent directions, A or B, sterically interferes with the receptor. Since the B direction is "shielded" by the terminal alkyl chain (Figure 4), the destructive methyl group position is most probably the A position.

Dimethyl substitution in the 2 position (compound **8**) causes a reduction of the electrophysiological activity, compared to that of **1,** by a factor of 288 (Figure 2). The calculated conformational energy effect on the activity is negligible (Table 1). The corresponding monomethyl-substituted compound **5** displays an activity that is only slightly higher than that of compound **8** (Figure 2). The calculated conformational energy penalties for **5** are low for both substituent directions (Table 1) and should not cause a significant reduction of the activity. The observed activity decreases for compounds **5** and **8,** and the calculated low conformational energy effects indicate that there are repulsive steric interactions with the receptor in at least one of the A and B directions. A study of the enantiomers of **5** is required for a final analysis. Methyl substituents in the 2 position may interfere with binding sites for the ester oxygen of the acetate group. It has been shown that the presence of this oxygen atom is necessary for a productive receptor interaction (Liljefors et al., 1984).

Since the observed activity of **5** is lower than that of **4** (Figure 2) and since the calculated conformational energy penalties are almost identical for these compounds (Table 1), the combined repulsive interactions in the A and B direc-

tions are more pronounced for a methyl substituent in the 2 position than in the 3 position. This would be expected to result in the 3,3-dimethyl compound **7** being more active than the corresponding 2,2-dimethyl-substituted compound **8.** However, this is not the case. Compound **8** is found to be somewhat more active than compound **7** (Figure 2). This observation may be explained by the 2.2 kcal/mol higher conformational energy penalty for **7** than for **8,** which counteracts the less severe repulsions with the receptor for compound **7.**

CONCLUSIONS

In a previous paper (Jönsson et al., 1991), we concluded that there is a high steric complementarity between the receptor and the terminal alkyl part (n-chain) of the pheromone component **1.** The receptor tuned to this compound clearly does not tolerate the extra bulk of a methyl group in positions 6–9 of the natural substrate. The study reported above shows that a methyl substituent in at least one of the A and B directions (Figure 4) in position 2–4, in the alkyl chain connecting the *Z* double bond and the acetate group (m-chain), causes repulsive steric interactions with the receptor. This indicates that there is a high degree of steric complementarity also between the receptor and the acetate-substituted alkyl chain of **1.** It is noteworthy that geminal dimethyl substitution in the m-chain leads to a higher decrease of the electrophysiological activity than dimethyl substitution in the corresponding positions (with respect to the double bond) in the n-chain.

Methyl substitution at either double bond position causes repulsive steric interactions with the receptor. The activity decrease is most pronounced in the 5 position, to which the m-chain is connected.

Our studies on methyl-substituted analogs of **1** indicate that the entire hydrocarbon part of the compound interacts with the receptor in a highly complementary way. This finding rationalizes the very high selectivity of the pheromone component receptor. Furthermore, the high complementarity implies that all methylene units in the pheromone are involved in the binding of the pheromone component and thus contribute to the affinity of the molecule. This rationalizes the observed high affinity of the pheromone component for its receptor.

Acknowledgments—This work was financially supported by the Swedish Natural Science Research Council, which is gratefully acknowledged.

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MULTIVARIATE STUDY OF MOOSE BROWSING IN RELATION TO PHENOL PATTERN IN PINE NEEDLES

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Abstract—Extracts of needles from Scots pine, *Pinus sylvestris* L., grown on fertile (A) or poor (B) soil, and from lodgepole pine, *P. contorta* Doug. (C), have been analyzed with HPLC and UV detection. By application of multivariate analysis, the phenol pattern was shown to vary between species and between habitats. Within groups A and B, moose food choice was highly correlated to phenol pattern. Concentrations of most of the analyzed compounds were internally correlated. Information related to pine growth rate could also be extracted from the phenolic pattern for A, B, and C. Moose food choice was not correlated to pine growth rate.

Key Words—Phenols, moose browsing, *Alces alces*, multivariate analysis, chemical defense, pine, *Pinus sylvestris* L., *P. contorta* Doug., herbivory.

INTRODUCTION

Trees of the genus *Pinus* are an important food source for moose, *Alces alces*. Moose exhibit both species and individual tree preferences. Preferential browsing of pines growing side by side by moose has been reported. Some individual trees were completely destroyed, whereas others were untouched (Haukioja et al., 1983; Danell et al., 1990).

Evidence suggests that plants have a chemical defense against herbivores (Feeny, 1976; Bryant and Kuropat, 1980; Bryant et al., 1984). The relationship between moose browsing and chemical characteristics of pine trees has hardly been studied. Löyttyniemi and Hiltonen (1978) and Löyttyniemi (1981) found

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no correlation between moose browsing and contents of monoterpenes or nutrients. Niemelä and Danell (1988), investigating a number of morphological and chemical parameters (including weight of the ether-soluble fraction, fiber, ash, metals and nitrogen content), found the amount of available twig biomass, but not the chemical parameters, to be correlated to moose browsing.

Recently, Danell et al. (1990) studied the correlation between moose browsing and individual terpenes from Scots pine needles. They found a negative correlation between pinifolic acid content and consumption. A similar repellency was observed with another compound, angelicoidenol-2-*O*-β-D-glucoside, isolated from Scots pine (Sunnerheim-Sjöberg, 1991).

Plant phenols possess defense functions against herbivores (Feeny, 1976; Harborne, 1985; Rhodes, 1985; Haslam, 1988). Tannins and total phenol concentration have been intensively studied with regard to these aspects, and several authors have reported correlations with feeding behavior (Häggman and Rousi, 1985; Marks et al., 1988). However, the mechanism for defense is not understood and, in all probability, the role of phenols in plant defense is a complex one (Harborne, 1985).

Several naturally occurring phenolic compounds have been reported to act as defensive substances against herbivores. For example, the phenolic stilbene pinosylvin and its monomethyl ether—both isolated from green alder (*Alnus crispa*)—are highly repellent to snowshoe-hares (*Lepus americanus*) (Bryant et al., 1983; Clausen et al., 1986). Platyphylloside [5-hydroxy-1,7-bis-(4-hydroxyphenyl)heptan-3-one-5-*O*-D-glucoside], isolated from silver birch (*Betula pendula*) was shown to reduce digestibility in vitro in goat rumen liquor, suggesting a defensive role for this phenol against ruminants (Sunnerheim et al., 1988). A more general discussion of phenols as defense substances in birch, willow, and poplar has been presented by Palo (1984).

The aim of this work was to investigate whether there are significant correlations between the phenol pattern in pine needles and moose browsing on pine. Needle extracts from pines exposed to moose living in the wild have been studied using high-performance liquid chromatography (HPLC) with UV detection. Previous reports from our research group (Popoff and Theander, 1975, 1976, 1977; Theander, 1982; Lundgren et al., 1982; Lundgren, 1987) have shown that the vast majority of pine needle constituents analyzed by this method are phenolic glycosides. Since chemical defense might result from a combination of several compounds, and since the chromatographic patterns from pine needles are complex, multivariate analysis was used. This method offers the possibility of analyzing large amounts of data. The plant material used is the same as in the investigation of Niemelä and Danell (1988).

In view of the role of phenols as growth regulators (Kefeli and Kutacek, 1976, 1979; Jacobs and Rubery, 1988) and the findings that soil nutritional factors affect phenol content (Barry and Manley, 1986; Briggs, 1990), the phenol

data in this investigation were also examined to see if phenol pattern was correlated with pine growth rate.

METHODS AND MATERIALS

Plant Material

Two Scots pine (*Pinus sylvestris*) phenotypes from fertile (A) and poor (B) soils, with low and high productivity, respectively, and lodgepole pine (*Pinus contorta*) (C) from an intermediate habitat were used. The pines were all growing in stands with no overstory, outside Umeå in coastal northern Sweden (around 63°45′N, 20°0′E). Fifty unbrowsed, 2-m-high, single-stemmed pines of each type were cut, and morphological data of each tree were noted. The trees were cut in late autumn, when the ground was frozen.

From each of the test pines, twig samples (twig diameter < 6 mm) were cut and stored at -20°C before extraction.

The trees were then exposed to wild-living moose: three pines, one of each type, were placed at each of 50 stations. The pines were inserted into the ground and appeared similar to growing trees.

For a more detailed description of the plant material, including the various growth parameters and the determination of moose consumption, see Niemelä and Danell (1988).

Extraction

Needles (0.50 g) were extracted in a mixer (Ultra-turrax) with 2×5.0 ml 95% aqueous ethanol for 2 and 0.5 min, respectively. The same procedure was repeated but with 80% aqueous ethanol. The combined extracts were glass-filtered and evaporated to dryness. Water (5.0 ml) was added, and the solution was extracted with 3×5.0 ml petroleum ether. The aqueous phase was centrifuged and the supernatant was transferred to a measuring flask. Water was added to 5.00 ml. The extracts were stored at -20°C before HPLC analysis.

Chemical Analysis

HPLC was performed on a Spectra Physics SP 8100 HPLC system equipped with a SP 8840 variable UV detector and using an analytical column (Chrompack CP-TM-spher C18, 4.6 mm $\times$ 250 mm). Mobile phase: 0–50 min, 10–80% aqueous methanol; 50–60 min, 80% aqueous methanol. Flow rate 1.5 ml/min. Oven temperature: 40°C. Detection wavelength: 280 nm. Chromatographic data, measured as peak areas, were automatically transferred to a computer. All samples were prepared and analyzed in random order to minimize systematic errors.

Statistical Analysis

Principal component analysis (PCA) (Wold et al., 1984; Jolliffe, 1986) was performed to study the main tendencies of systematic variation between the species and phenotypes. The relations between the block of dependent variables (growth rate and moose consumption) and independent variables (chromatographic data) were studied using the method of partial least-squares regression (PLSR) (Geladi and Kowalski, 1986; Martens and Naes, 1989) on latent variables.

Input Data: Independent variables. The "phenol patterns" are described by the automatically integrated chromatographic peaks from substances absorbing UV light of 280 nm.

Input Data: Dependent Variables. Relative consumption is: (biomass removed − biomass left on ground)/(available biomass). Consumption choice is: (relative consumption from one tree)/(total relative consumption at the test station). Growth rate was estimated by dividing the height of the tree by its age. For further details about the plant material and the moose browsing data, see Niemelä and Danell (1988).

The number of objects in the PLSR consumption models: A, 33 objects; B, 30 objects; C, 24 objects; and in the growth rate models: A, 45 objects; B, 39 objects; C, 24 objects.

Principal Component Analysis (PCA). PCA is used for reduction of the dimensionality of a data set, which consists of a large number of intercorrelated variables, while retaining as much as possible of the original variation as a new set of variables, the principal components. These are uncorrelated, and ordered so that the first few components retain most of the variation present in all of the original variables.

PCA is here used for an overview of the data set and for the identification of subgroups. If subgroups are present, better calibrations are sometimes obtained with separate models for each group.

Partial Least-Squares Regression (PLSR). In PLSR the linear relation between dependent variable(s) and the set of independent variables is calculated. [For example: consumption choice = f(phenolic pattern) (Geladi and Kowalski, 1986; Martens and Naes, 1989).]

Terms and Definitions

The data within brackets refer to a particular example; namely areas of chromatographic peaks from extracts of Scots pine needles in relation to consumption choice.

Variables. Measured quantities (here: areas of 55 chromatographic peaks give 55 variables). Each variable represents one dimension in a multidimensional space.

Objects. Each object represents one point in a multidimensional space. (Here: 33 pine individuals).

Principal Component 1 (PC1). A line of best fit to the points in the multidimensional space (here: a straight line passing as close as possible to all the 33 experimental points in 55 dimensions).

Principal Component 2 (PC2). A new line—orthogonal to PC1 and least-squares adapted to the residuals (here: the residuals are the distances from each of the 33 points to PC1 expressed as coordinates of the 55 variables).

PLSR Factor. The orthogonal two-dimensional coordinate system that describes the relation between the dependent (here: consumption choice) and the independent variables (here: chromatographic data). The PLSR factors have similar functions for PLSR as principal components have for PCA.

Validation

Prediction Model. For each object, the dependent variable, y_{pred}, (here: consumption choice) is calculated (from its chromatographic data) based on a PLSR model (Martens and Naes, 1989, Chapter 3, p. 5).

Standard Error of Prediction (SEP).

$$SEP = \sqrt{\frac{\sum (y_{\text{pred}} - y_{\text{obs}})^2}{n}}$$

where y_{pred} = from PLSR model predicted value, y_{obs} = the corresponding observed value, and n = number of predicted objects. For PCA and PLSR calculations, the SIRIUS software was used (Kvalheim and Karstang, 1988).

Cross Validation (CV). The principal component/PLSR factor is calculated for some of the objects and the rest is predicted by the model. This is repeated for all objects (Wold et al., 1987; Martens and Naes, 1989).

Cross-Validation Ratio (CVr). Ratio between predicted and present residual standard deviation. CV ratio < 1 is a criterion for a significant principal component/PLSR factor.

RESULTS AND DISCUSSION

Chromatographic Pattern and Principal Component Analysis

Typical chromatograms from the HPLC analysis are given in Figure 1. The principal component analysis showed that the phenol pattern in *P. contorta* differed significantly from *P. sylvestris* (Figure 2). Component one (PC1) mainly separated the two species, while PC2 mainly separated the two habitats of *P. sylvestris*. Differentiation of the two species could be made by visual inspection of the chromatograms (Figure 1). PCA applied to the samples of Scots pine

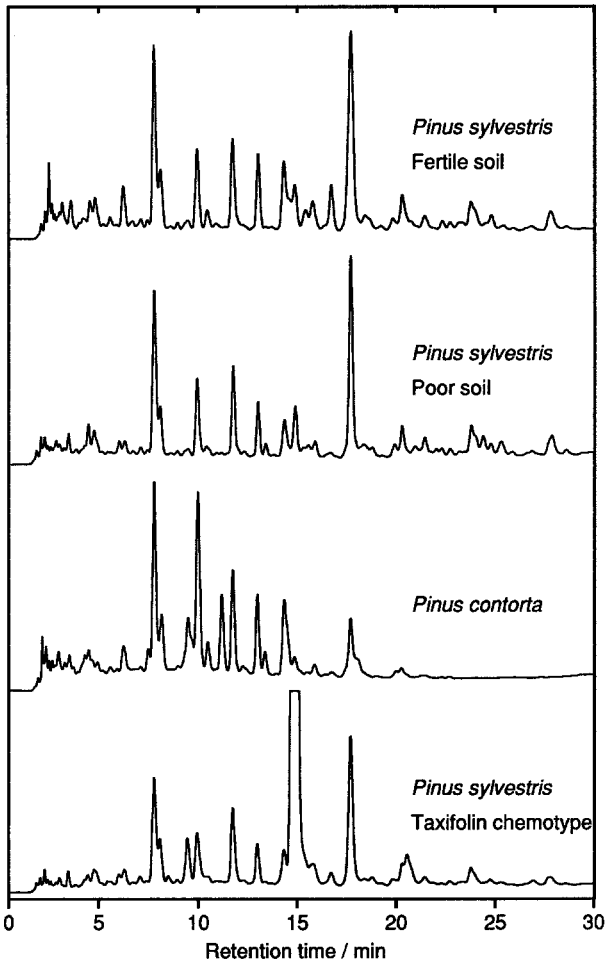

FIG. 1. Representative chromatograms of extracts from *P. sylvestris* in fertile soil (A), *P. sylvestris* in poor soil (B), *P. contorta* (C), and from a high-taxifolin chemotype of *P. sylvestris*.

showed that the major variation in phenol composition was related to the contrast between phenotypes (Figure 3). These divisions indicated that the three subgroups should be analyzed separately in the following PLSR-model calculations.

Among Scots pines, some clones (seven of 49 for A and five of 49 for B) contain taxifolin-3′-*O*-β-D-glucoside in high concentration—3% of needle dry matter—and other clones lack this compound (Sunnerheim-Sjöberg et al., 1991). Figure 1 shows an example of a chromatogram from a taxifolin-glucoside-rich

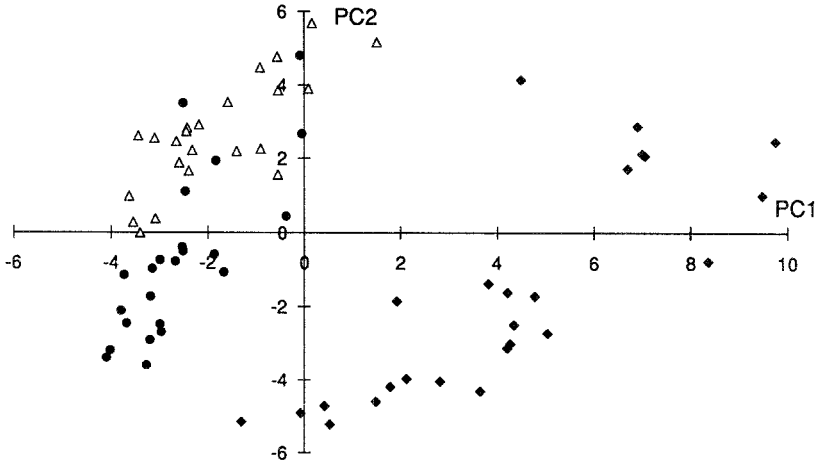

FIG. 2. PCA of A, B, and C, two principal components. Objects: Δ = *Pinus sylvestris*, fertile soil (A); • = *P. sylvestris*, poor soil (B); ♦ = *P. contorta* (C).

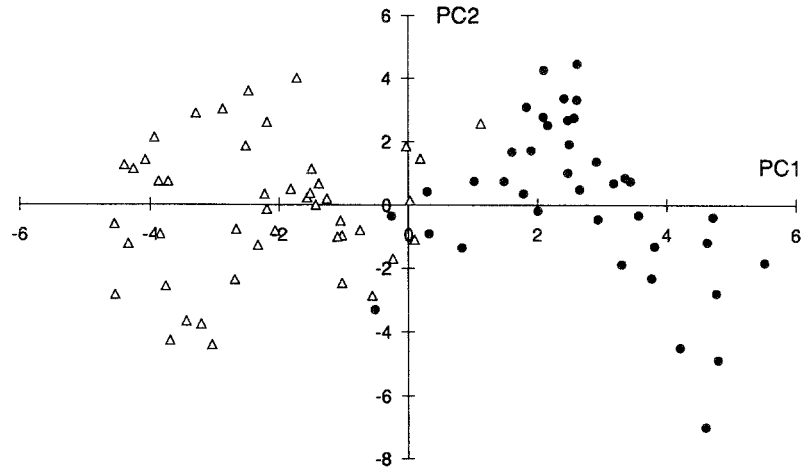

FIG. 3. PCA of A and B, two principal components. Objects: Δ = *Pinus sylvestris*, fertile soil (A); • = *P. sylvestris*, poor soil (B).

pine. The large peak corresponding to taxifolin-glucoside hides some adjacent peaks, leading to a decrease in information from these variables. However, exclusion of the taxifolin-glucoside-rich pines from the PLSR models had only a minor influence on the predictions. The other peaks in the chromatograms were not identified. The areas of 47 of 55 chromatographic peaks were internally

correlated, i.e., the peak areas were dependent on other peak areas in the chromatogram.

Partial Least-Squares Regression Models

Relative Consumption. Since available biomass differed between A, B, and C (203, 158, and 134 g dry matter/tree, respectively), relative consumption (consumption/biomass available) was used as the dependent variable in the PLSR models. Relative consumption was correlated with the chromatographic pattern for A. One significant factor was obtained. Figure 4 shows how a PLSR model predicted the relative consumption for A. Observed relative consumption ranged from 10 to 98% and SEP was 17% (Table 1). Trees from stations where all three trees were browsed by moose were included in the model, resulting in 33 objects. When the unbrowsed trees were also included in the model, the explained variance decreased. This indicated that the moose had to taste the twig—odor was not sufficient—to determine palatability. No significant correlations were found for B and C.

Consumption Choice. If one pine at a station was intensively browsed, the other trees were also heavily browsed (Niemelä and Danell, 1988). Consumption differences might be affected by the surroundings; topography and vegetation

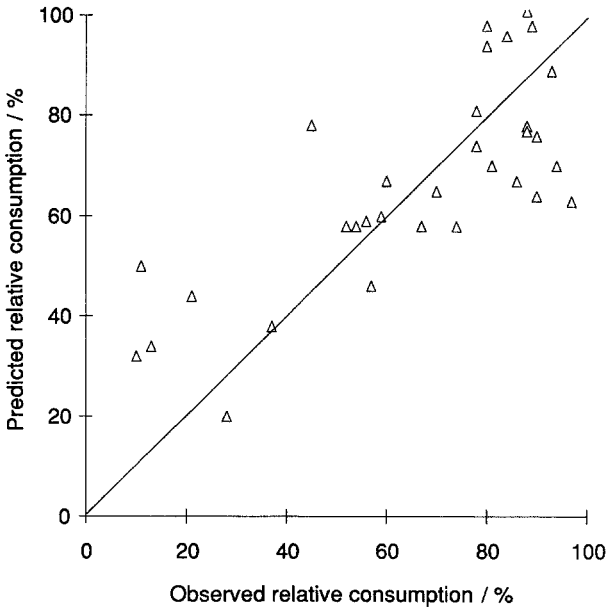

FIG. 4. Predicted versus observed relative consumption for *P. sylvestris*, fertile soil (A); one-factor model.

TABLE 1. VALIDATION RESULTS FROM PLSR MODELS WITH CHROMATOGRAPHIC DATA AS INDEPENDENT VARIABLES AND DIFFERENT DEPENDENT VARIABLES[a]

Dependent variable	Indep. variable	Factor 1		Factor 2		Factor 3		SEP	Figure
		CVr	EV	CVr	EV	CVr	EV		
Relative consumption	A_{Ph}	0.93	56%					17%	4
Consumption choice	A_{Ph}	0.97	55%	0.94	78%			4.5%	5
Consumption choice	B_{Ph}	0.98	35%	0.97	48%	0.99	62%	4.6%	6

[a] A_{Ph} and B_{Ph} are phenol composition for *Pinus sylvestris* fertile and poor soils, respectively, and EV is the explained variance.

are not identical at all stations and the number of moose were not identical. Moreover, the moose might have been more hungry at some stations than others. Therefore, "consumption choice" was probably a more relevant variable than "relative consumption" for quantifying feeding behavior. If "consumption choice" was used as a dependent variable, significant models were obtained for both Scots pine phenotypes. Observed consumption choice varied from 6 to 48% for A and from 16 to 55% for B. Standard errors of predictions were 4.5 and 4.6%, respectively, and explained variances were 78 and 62% for A and B, respectively, indicating that phenol composition was very well correlated to food choice (Figures 5 and 6, Table 1).

Two PLSR-factors were obtained for A and three for B, which might point to several independent defense systems. A PLSR model including both Scots pine habitats was not significant, which might point to different chemical defenses in trees from soils of different nutritional status or differences in phenol pattern due to pine growth rate overrode the variation in consumption choice.

The age of the trees from fertile soil varied from 6 to 8 years. For B, pines older than 11 and younger than 9 years were excluded from the model, leaving 30 objects in the model. When all ages, 8–15 years, were included, the models were not significant. Since trees of 2 m height were selected, the older trees among the pines investigated had a slower growth rate. Growth rate is correlated to phenol pattern (see below), and this interference on the consumption choice models is reduced by studying a more narrow range of ages. Thus, within an area, and among pines of similar growth rate, moose food choice was well predicted by the PLSR models based on the chemical composition of the needles.

For *P. contorta*, no significant PLSR model was obtained with consumption choice as the dependent variable. The variations in age (7–12 years), growth

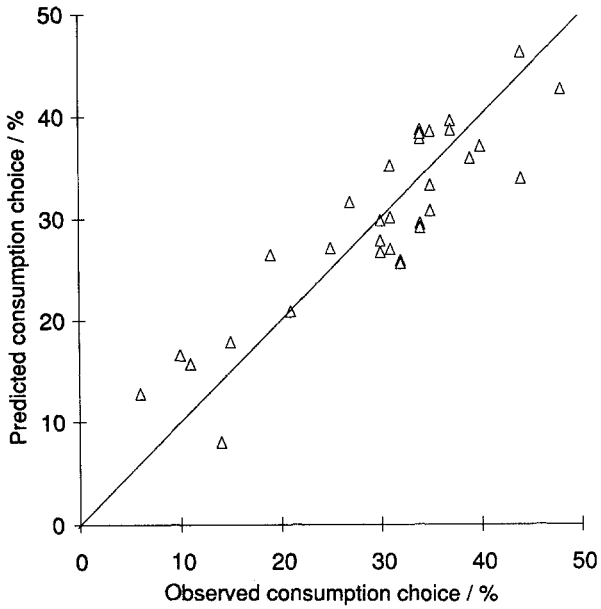

FIG. 5. Predicted versus observed consumption choice for *P. sylvestris*, fertile soil (A); two-factor model.

rate, and phenol composition (Figure 2) were larger compared to *P. sylvestris* and might account for the lack of correlation.

Are Phenols Repellents?

None of the 55 variables alone could be held responsible for the moose food choice. Only one variable was correlated to consumption choice (CV ratio 0.90), but the relation was valid only for fertile soil phenotypes and the explained variance was low (22%). However, the composition of the phenolic compounds in the needles was strongly correlated with moose food choice. The models show only correlations and tell us nothing about phenols as repellents. Phenols might lack effect and may only be markers for active compounds not detected here, or they may be active in combination with other compounds—both phenolic and nonphenolic. Nevertheless, the model can predict moose food choice from the phenol data.

Pine Growth Rate. Figure 3 shows that different soil fertilities could be distinguished by means of the phenol composition in the pine needles. This observation supports the theories of Bryant et al. (1983) and Coley et al. (1985) that the evolutionary response to resource limitation is an inherently slow growth and that the composition of secondary compounds differs between plants in

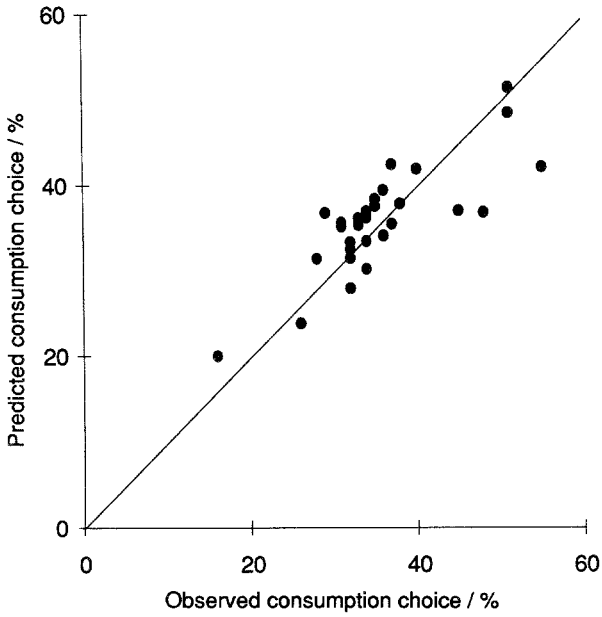

FIG. 6. Predicted versus observed consumption choice for *P. sylvestris*, poor soil (B); three-factor model.

different habitats. It might also reflect an effect of soil nutritional status on phenol content (Rhodes, 1985; Barry and Manley, 1986; Briggs, 1990).

However, even within the three stands, A, B, and C, pine growth rate was correlated to phenol pattern. These results support the findings that certain phenols are growth regulators (Kefeli and Kutacek, 1976, 1979; Jacobs and Rubery, 1988; Kuiters, 1989). The models used 34–51% of the variance in the phenol pattern to explain growth rate, giving a standard error of prediction of 1.6–3 cm/year. Thus, the phenol composition in the needles was less correlated to pine growth rate than to twig consumption by moose, but the consumption choice models were only valid for pines of similar growth rate.

Consumption versus Growth Rate

Bryant and Kuropat (1980) and Danell and Huss-Danell (1985) noted a correlation between plant growth rate and herbivore preference for different species. The present investigation of browsing preference for individual trees of the same species showed no such correlation. Neither relative consumption nor consumption choice was correlated to pine growth rate, although both consumption and growth rate were correlated to phenolic pattern. The variables

important for the different models were not identical; i.e., the growth rate and the consumption models were influenced by different phenols.

CONCLUSIONS

- Moose food choice was related to the phenol composition of the needles of Scots pine among pines of similar growth rate.
- Palatability was probably determined by a complex covariation of a number of compounds in the needles.
- Food choice was not correlated to pine growth rate.
- Within the same location, pine growth rate was correlated to phenol pattern for both Scots and lodgepole pines.
- The phenol composition of the needles was different for trees of different species and for trees from soils of different fertilities.
- Concentrations of most of the individual phenols were internally correlated.

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6,10,14-TRIMETHYLPENTADECAN-2-ONE: A BERMUDA GRASS PHAGOSTIMULANT TO FALL ARMYWORM (LEPIDOPTERA: NOCTUIDAE)

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Abstract—A phagostimulant, 6,10,14-trimethylpentadecan-2-one (phytone), was isolated and identified from Bermuda grass, *Cynodon dactylon* (L.). The phagostimulant activity of this isoprenoid ketone was established from bioassays of fall armyworm larvae, *Spodoptera frugiperda* (J.E. Smith). Larvae displayed increased body mass accumulation as well as preference to diet supplemented with this molecule. Neonate larvae fed diet supplemented with chromatographic isolates of phytone-containing fractions from six Bermuda grass cultivars showed a 10–40% increase in body mass accumulation as compared with controls. This variation in larval body mass accumulation seems attributable to a differential concentration of phytone in the cultivars, which ranged from 0.5 to 43 ppm. Additionally, first-instar larvae responded preferentially to diet pellets topically treated with phytone in concentrations as low as 0.1 ppm.

Key Words—Allelochemistry, Bermuda grass, *Cynodon dactylon*, phagostimulant, *Spodoptera frugiperda*, Lepidoptera, Noctuidae, fall armyworm, 6,10,14-trimethylpentadecan-2-one.

INTRODUCTION

Host selection by phytophagous insects is mediated principally by secondary plant metabolites (Fraenkel, 1959; Thorsteinson, 1960). Both larval and adult insect herbivores are known to be responsive to a variety of plant chemical stimuli (Visser, 1986). Phagostimulants and deterrents are thought to provide

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the bulk of information that govern choice of food plant and host suitability (Dethier, 1947; Schoonhoven, 1968). Given the crucial role of these molecules in modifying insect behavior and physiology, manipulation of crop plants with defined allelochemic profiles has been recognized as affording a promising mode of insect pest management (Smith, 1990). This approach seems inherently plausible with crops of low economic returns such as forages (Bernays and Barbehenn, 1987).

A major perennial source of nutritious forage is Bermuda grass, *Cynodon dactylon* (L.), which enjoys a cosmopolitan distribution with 4 million ha under cultivation in the United States alone. One of its major insect herbivores, the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), has systematically eroded the value of this crop over the last decade (Mitchell, 1979; Sparks, 1979; Hogg et al., 1982; Johnson, 1987). In addition, given the fall armyworm's migratory behavior, Bermuda grass serves as an important reservoir for population outbreaks in grain crops such as corn. In an effort to develop resistant cultivars, conventional breeding programs and tissue-culture-derived lines have yielded several Bermuda grass variants to which fall armyworm has shown variable feeding preference (Lueck et al., 1968; Lynch et al., 1983; Quisenberry and Wilson, 1985; Jamjanya and Quisenberry, 1988; Croughan and Quisenberry, 1989). Additional studies (Quisenberry et al., 1988) have implicated allelochemics such as feeding deterrents and/or phagostimulants as the basis of fall armyworm variable response to Bermuda grass cultivars.

Phagostimulants have been implicated in insect host plant preference and suitability (Soni and Finch, 1979; Saxena and Prabha, 1975; Jones and Coaker, 1978; Mori, 1982; Stadler and Buser, 1984; Blust and Hopkins, 1987). Their relationship to resistance to insect herbivory has been implicated in several studies (Guerren and Ryan, 1984; Khan et al., 1987). In this study, we report the isolation and identification of 6,10,14-trimethylpentadecan-2-one as a Bermuda grass phagostimulant and the quantitative variability in six cultivars and somaclones.

METHODS AND MATERIALS

Chemicals. All chemicals used in these experiments were of the highest purity commercially available, particularly distilled-in-glass solvents (Burdick and Jackson Co.).

Insect Culture. Fall armyworm (rice strain; Pashley, 1986) was cultured on a modified meridic diet (Quisenberry and Whitford, 1988) according to procedures by Perkins (1979) at 27.6 $\pm$ 0.5°C, 16:8-hr photoperiod, >50% relative humidity.

Phagostimulant Isolation Protocols. Six Bermuda grass cultivars were

selected for this experiment on the basis of differential feeding responses observed with the fall armyworm (Quisenberry and Wilson, 1985; Jamjanya and Quisenberry, 1988; Croughan and Quisenberry, 1989). These included Grazer, Tifton 78, Alicia, OSU 71 × 6-7, and two Brazos somaclones regenerated from tissue-culture-derived lines (R1 and R2). The grasses were oven dried (60°C) and milled (Wiley Mill, with a 1-mm sieve) prior to extraction. Crude extracts were obtained by sequential Soxhlet extraction with hexane, ether, and methanol typically with 40 g of each grass. Based on preliminary observations that suggested phagostimulant activity in the ether crude extract, this fraction was concentrated and the active component isolated by a bioassay-guided fractionation scheme. Initially, the crude extract was separated by adsorption silica gel (180 g) flash column chromatography (Still et al., 1978). The following solvents were used: hexane, a gradient (10–100% in 10% increments of CH_2Cl_2) of CH_2Cl_2–hexane, EtOAc, and MeOH. An active phagostimulatory fraction collected at 50% CH_2Cl_2 was separated into several components by reverse-phase preparative thin-layer and high-pressure liquid chromatography (RPTLC and HPLC). RPTLC was carried out on 1000 μm, 20 × 20 cm, Whatman PLKC 18F plates with MeOH. The active fraction was isolated at $R_f = 0.44$ and further purified by HPLC (Waters Delta Prep HPLC). HPLC was performed on a Waters 100 Å Delta Pak C-18 preparative column with a solvent flow rate of 80 ml/min (210 psi) monitored at 254 nm. Phagostimulant activity was observed to a peak $R_t = 2.3$ min. All fractions were concentrated under reduced pressure with a rotary evaporator.

Fractions from each cultivar were subjected to analyses by gas chromatography (GC) with flame ionization detectors (Waters Dimension 1 gas chromatograph). The conditions for GC analyses with a 30-m DB-5 megabore capillary column included a temperature program of 40°C/1 min, 20°C/min to 285°C (hold) with a He flow rate of 6 ml/min at a detector sensitivity of 16×10^{-12}. All samples were additionally analyzed on a more polar capillary column (DB-225) to confirm sample separation and identity.

The active HPLC isolate was subjected to GC-mass spectrometry (70 eV, Hewlett Packard 5890 Series 2 mass spectrometer) for structural identification. The mass fragmentation pattern was indicative of a saturated ketone (*m/e* 58, C_3H_6O) and was unambiguously identical to published spectra of 6,10,14-trimethylpentadecan-2-one (Ikan et al., 1973).

An authentic sample of this material was prepared by oxidative cleavage of 3,7,11,15-tetramethyl-2-hexadecen-1-ol with $NaIO_4/KMnO_4$ (Lemieux and von Rudloff, 1955). Phytone was purified by phase separation of the acidified product into ether, which was then concentrated and separated by flash silica gel 60 column chromatography using a CH_2Cl_2–hexane gradient. Phytone was eluted at 50% CH_2Cl_2–hexane, 60% yield (TLC $R_f = 0.7$, silica gel with 10% EtOAc–CH_2Cl_2). The structure was confirmed through GC-MS, which yielded

an identical spectra to that published as well as that found in Bermuda grass. In addition, capillary GC of coinjected active isolate from Bermuda grass and synthetic phytone yielded a single peak at 13.00 min. Final identification of this molecule was based on the identity of authentic samples with unknowns both in terms of GC retention times and MS fragmentation patterns.

Bioassays. Fractionated material from each cultivar, HPLC isolate, or synthetic phytone were adsorbed onto cellulose followed by solvent removal under reduced pressure with a rotary evaporator. Solvent-treated cellulose served as the control. The treated cellulose was then mixed into the modified meridic diet to effect a final concentration equivalent to a gram of tissue extracted for silica gel column and HPLC fractions. Phytone was impregnated onto cellulose to effect a final concentration of 1 and 10 ppm in the diet. A larva was incubated in a polystyrene cup (29.7 ml) with 1.5 g of diet and each treatment had 16 replicates. Bioassays were conducted with freshly eclosed fall armyworm larvae (1 mg initial weight) at 26.7 $\pm$ 0.5°C, 16:8-hr photoperiod, >50% relative humidity. Larvae were weighed subsequently at four, six, and eight days post-treatment.

Choice Tests. First-instar fall armyworm larvae were tested for their response to phytone in a choice assay at concentrations of 0.01, 1, and 10 ppm. The assay consisted of placing diet pellets with and without phytone equidistant from each other in a divided Petri plate (15 $\times$ 1.5 cm) and releasing a larva in the center of the dish. Pellets without phytone were solvent-treated and served as controls. The plates were sealed with parafilm and incubated under identical conditions as described for bioassays. Twenty replicates were tested simultaneously for each treatment. The larvae were then observed as to their feeding preference at 48 hr.

Statistics. Bioassay data were analyzed as a randomized block design using PC-SAS (SAS Institute, 1985). Significant treatment means were separated using Duncan's multiple-range test (Duncan, 1955). Choice test data were analyzed using Student's *t* test (Steel and Torrie, 1980).

RESULTS

Silica gel adsorption chromatography of crude Soxhlet-extracted Bermuda grass cultivars yielded a fraction (50% CH_2Cl_2–hexane) that stimulated a significant increase in fall armyworm larval body weight accumulation (Table 1). However, there were significant differences observed in response among the cultivars and somaclones tested. Larval weight accumulation for established cultivars Grazer, Tifton 78, Alicia, and OSU 71 $\times$ 6-7 displayed a 30–40% increase in comparison to somaclones derived from Brazos (10–15%) by day 8.

Further fractionation of this active phagostimulatory isolate from Grazer

by preparative reverse-phase TLC and HPLC yielded a single peak that induced a 33% increased larval weight accumulation (Table 1). This response was identical to larvae fed diet treated with synthetic phytone. Capillary GLC analyses of the active silica gel fractions from each cultivar with respect to the molecule identified from the HPLC isolate (phytone) revealed a variability of the concentration of the molecule ranging from 0.15 to 43 ppm (Table 2, Figure 1).

TABLE 1. FALL ARMYWORM LARVAL MASS ACCUMULATION IN RESPONSE TO PHAGOSTIMULANT FRACTIONS FROM CULTIVARS AND SOMACLONES OF BERMUDA GRASS

Treatments	Larval weight (mg) $\bar{X}$ (± SE)[a]		
	Day 4	Day 6	Day 8
Control	21(2)f	136(17)bcd	258(24)d
Grazer	83(8)a	192(12)a	365(21)b
Tifton 78	48(2)cd	159(9)ab	355(21)bc
Alicia	23(4)ef	136(9)bcd	337(23)bc
OSU 71 × 6-7	67(8)b	180(17)a	366(22)b
Brazos (R1)	38(2)de	124(10)cd	297(23)bcd
Brazos (R2)	35(3)def	120(10)d	285(25)cd
HPLC Isolate[b]	60(5)bc	161(11)ab	344(24)bc
Phytone			
1 ppm	54(3)bc	158(8)ab	354(22)bc
10 ppm	58(3)bc	166(5)ab	461(18)a

[a]Means within a column followed by the same letter are not significantly different ($P = 0.05$, $df = 144$, Duncan's multiple-range test) (Duncan, 1955).
[b]HPLC isolate of phytone was at 1 ppm of diet.

TABLE 2. CONCENTRATIONS OF PHAGOSTIMULANT PHYTONE IN BERMUDA GRASS CULTIVARS AND SOMACLONES

Bermuda grass cv.	Phytone concentration (ppm)[a]
Grazer	43
Tifton 78	25
OSU 71 × 6-7	3
Alicia	1.5
Brazos R1	0.3
Brazos R2	0.15

[a]Concentration of phytone was determined for each Bermuda grass cultivar and somaclone using fatty acid ester standards. Phytone was identified from each cultivar by its identity of GLC R_t values and mass spectral fragmentation pattern with an authentic sample.

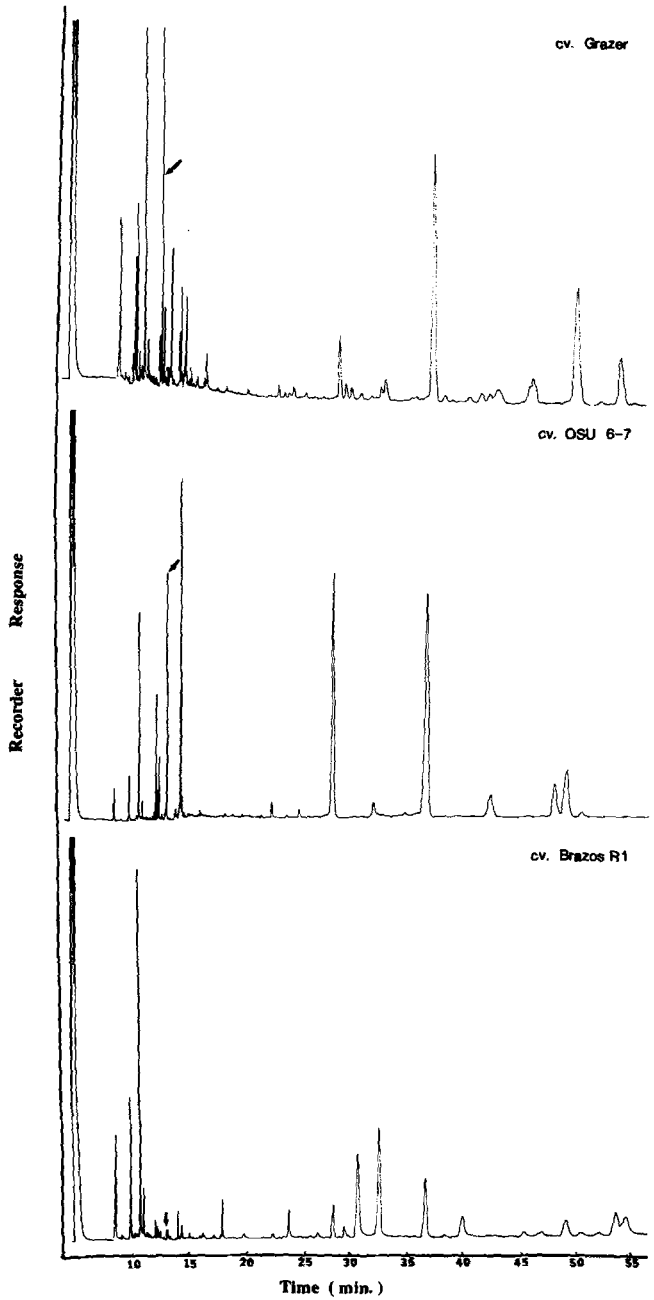

FIG. 1. Capillary GLC analyses of phagostimulant flash column adsorption chromatography fraction from three Bermuda grass variants. Variability in the concentration of phytone (R_t = 13.00 min, indicated by arrows) from equivalent masses of sample (50 mg).

GC-MS of the phagostimulant isolate indicated a long-chain saturated ketone with an *m/e* of 268 (Figure 2). The mass spectral pattern of this molecule was identical to 6,10,14-trimethylpentadecan-2-one (phytone) (Ikan et al., 1973). Oxidative cleavage of the allylic alcohol [3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol)] yielded an authentic preparation of phytone with an identical mass spectra and GC retention time (13.00 min) in comparison with the phagostimulant isolate from Bermuda grass.

Bioassays of fall armyworm larvae in choice tests elicited a preferential

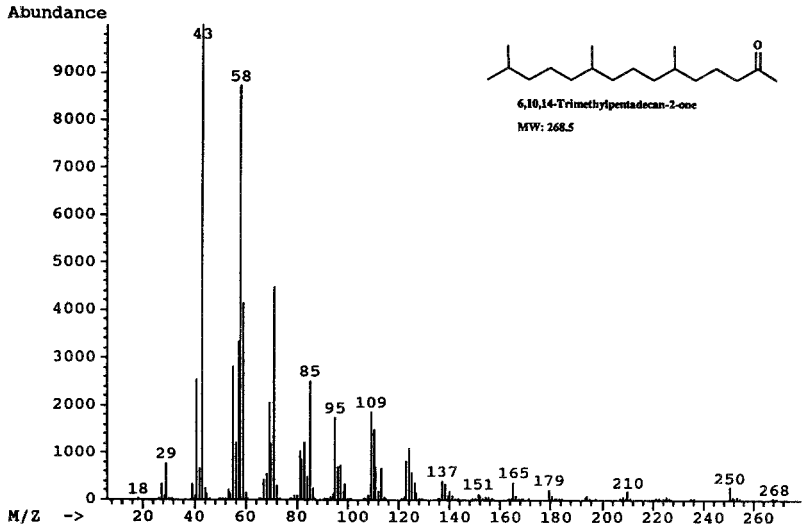

FIG. 2. Electron impact mass spectra of the fall armyworm phagostimulant, phytone, isolated from Bermuda grass.

TABLE 3. RESPONSE OF FALL ARMYWORM FIRST-INSTAR LARVAE IN CHOICE ASSAY WITH BERMUDA GRASS PHAGOSTIMULANT, PHYTONE

Phytone conc. (ppm)	Larvae (%)[a]	
	Treated	Untreated
0.01	75a	25b
0.1	90a	10b
1.0	90a	10b

[a] Phytone was applied topically to diet pellets and larvae were monitored 48 hr postreatment. Means within a row followed by the same letter are not significantly different ($P = 0.01$, $df = 19$, Student's t test) (Steel and Torrie, 1980.)

response from diet pellets treated topically with phytone (Table 3). That is, larvae consistently preferred diet to which phytone was topically applied.

DISCUSSION

A phagostimulant, 6,10,14-trimethylpentadecan-2-one, was isolated and identified from Bermuda grass. This molecule, at concentrations as low as 1 ppm, was found to influence larval food choice as well as larval growth patterns, responses typical of phagostimulants (Mori, 1982). Indeed, choice assays with and without this ketone indicate a preference for the former by the fall armyworm larvae. In addition, meridic diet supplemented with a fraction from six Bermuda grass variants containing phytone at variable concentrations led to significant gain in larval weights that was cultivar dependent. Brazos somaclones, with phytone concentrations less than 1 ppm, stimulated larval weight gains that were not significantly different from controls. Cultivars such as Grazer, Tifton 78, Alicia, and OSU 71 × 6-7, with phytone concentrations ranging from 1.5 to 43 ppm, did display significant weight gain increases. Given the fact that these body mass increases among the cultivars were not significantly different, it would seem that phagostimulation of fall armyworm larvae by phytone is more of a threshold effect. That is, an optimal concentration of 1 ppm is sufficient to stimulate an excess larval feeding response, although larvae are capable of detecting phytone in concentrations as low as 0.01 ppm in choice tests.

While these studies support the idea that phytone in Bermuda grass influences host selection and suitability by fall armyworm, the degree of resistance seems driven by a quantitative variation of this phagostimulant with respect to feeding deterrents. Bioassays of total and fractionated extracts from several Bermuda grass variants by Quisenberry et al. (1988) suggest the occurrence of allelochemics with variable antibiotic properties to fall armyworm (Mohamed and Quisenberry, unpublished data). Our results are consistent with current thought on the relationship of different of secondary plant metabolites and their the final feeding response of insect herbivores. That is, host choice by insects is determined by a balance of chemosensory information from negative and positive stimuli (Jermy, 1966; Schoonhoven, 1968; Dethier, 1982).

Phytone has been documented previously in marine algae (Rao and Pullaiah, 1982) and lavender oil (Kaiser and Lamparsky, 1983). This ketone also was reported as a major component of the male crab, *Carcinus maenas*, androgenic glands, functioning as an inhibitor of ovarian vitellogenesis (Ferezou et al., 1976).

The occurrence of a somewhat volatile phagostimulant in fall armyworm preferred host, Bermuda grass, may account for the highly mobile nature of this insect. It is possible that migration patterns of both adult and larval stages may

be influenced by phytone. Volatile phagostimulants have been proposed for use in crop management schemes, a notable success is the control of corn rootworms (*Diabrotica barberi* Smith & Lawrence and *D. undecimpunctata howardi* Barber) with curcurbacitin baits treated with insecticides (Gould, 1991). In this vein, phytone may be potentially useful in a trap crop, as a general attractant, or a synergist to fall armyworm sex pheromone.

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ALLELOPATHY OF YELLOW FIELDCRESS (*Rorippa sylvestris*): IDENTIFICATION AND CHARACTERIZATION OF PHYTOTOXIC CONSTITUENTS

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Abstract—Both the neutral and acidic fractions of the acetone extract of yellow fieldcress (Kireha-inugarashi, *Rorippa sylvestris* Besser) inhibited lettuce seed germination. Salicylic, *p*-hydroxybenzoic, vanillic, and syringic acid were identified in the acidic fraction. In the neutral fraction, hirsutin (8-methylsulfinyloctyl isothiocyanate), 4-methoxyindole-3-acetonitrile, and pyrocatechol were identified. Bioassay using a root exudate recirculating system showed *R. sylvestris* during flowering inhibited the lettuce seedling growth. Hirsutin (13 μg/plant/day) and pyrocatechol (9.3 μg/plant/day) were the major compounds released into the rhizosphere. Several combinations of pyrocatechol, *p*-hydroxybenzoic acid, vanillic acid, and hirsutin reduced lettuce seedling growth. These compounds seemed to be allelochemicals.

Key Words—Allelopathy, *Rorippa sylvestris*, weeds, salicylic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, hirsutin, 4-methoxyindole-3-acetonitrile, pyrocatechol, root exudate.

INTRODUCTION

A cruciferous weed species, *Rorippa sylvestris* Besser (yellow fieldcress, Kireha-inugarashi in Japanese) has been naturalized in Hokkaido since the 1950s and is considered to be one of the worst weeds in wet fields and pastures (Morita, 1981). The weed has strong propagative power and invades the territories of other plant species. It may be allelopathic against neighboring plants.

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R. sylvestris contains hirsutin in the roots, which inhibits lettuce growth (Kawabata et al., 1989). Isothiocyanates in cruciferous plants play an important role as inhibitors of plant growth in addition to their antimicrobial activity (Harborne, 1988). These sulfur-containing compounds are usually found in macerated tissues due to mixing of the precursor glucosinolates and the hydrolytic enzyme thioglucosidase (Olsen and Sørensen, 1981).

Allelopathic inhibition of seed germination and plant growth typically occurs by the joint action of several allelochemicals. Additive or synergistic effects have been reported with combinations of monoterpenes, organic acids, and several classes of phenolic compounds (Einhellig, 1987; Williams and Hoagland, 1982).

Many studies of possible allelopathic effects on germination and growth have been dealt with using crude plant extracts or soil leachates, which make it difficult to discern the active principle in plant–plant interactions. For this reason, we used a bioassay with a root exudate recirculating system to explore the inhibitory constituents in the rhizosphere of *R. sylvestris* against lettuce growth. Inhibitory activities of pure compounds, singly and in combination, against lettuce seedling growth were also tested.

METHODS AND MATERIALS

Plant Collection and Extraction. Fresh roots of *Rorippa sylvestris* Besser (yellow fieldcress) were collected on the Hokkaido University campus in August 1988. The fresh roots (5.4 kg) were extracted with acetone for one month at room temperature. The filtered extract was concentrated under reduced pressure, and the aqueous concentrate was acidified with HCl (1 N), followed by extraction with ethyl acetate. The ethyl acetate extract was further divided into an acidic and a neutral fraction. From the aqueous residue, a basic fraction was obtained in the usual way. The basic fraction did not show any significant activity against lettuce.

Bioassay of Inhibitory Activity against Lettuce. A lettuce (*Lactuca sativa* L.) seed germination bioassay was used to examine the inhibitory activity of each extract, chromatographic fraction, or isolated compound. An appropriate amount of each of the samples was dissolved in 0.5 ml of acetone, and the solution was poured into a 6-cm Petri dish with two sheets of filter paper (No. 3, 5.5 cm, Toyo Roshi Co., Ltd.) placed on the bottom. Acetone was evaporated and 2 ml of water containing 100 ppm (w/v) Tween-80 was poured into the Petri dish. The dishes were covered and kept in the dark for 24 hr at room temperature to allow samples to dissolve completely and avoid light reaction. Thirty lettuce seeds were placed on the filter paper and incubated at 23°C in the dark. Germination percentages were recorded after 24 hr incubation. A

control with water containing 100 ppm (w/v) Tween-80 was included in each experiment. Each experiment was duplicated.

A lettuce seedling growth bioassay was used to determine the activity of the isolated compounds. The compound was dissolved in 150 μl of acetone and mixed into 15 ml of hot agar solution (0.35% w/v) in a Petri dish. The agar solution was cooled and 20 germinated lettuce seedlings (24 hr after germination) were placed in each dish. The dishes were covered and kept in a growth chamber (23°C, 12 hr dark and 12 hr light). The hypocotyl and root lengths for each seedling were measured at the end of one week. Mean hypocotyl and root lengths were determined for 20 seedlings (two replications). A control was included in each experiment.

Fractionation and Identification of Acetone Extract. The acidic fraction was subjected to silica gel column chromatography using hexane, ethyl acetate, and methanol as eluents. The hexane–ethyl acetate (1:1, v/v) eluate inhibited lettuce seed germination by 79% at 200 ppm (w/v). Further purification of this fraction by silicagel preparative thin-layer chromatography (TLC) using chloroform–methanol (2:1, v/v) as a mobile phase yielded compound I. Compound I was characterized by EI-MS, [^{1}H]NMR, and IR spectra, which were identical with those of authentic salicylic acid.

The hexane–ethyl acetate (2:3,v/v) eluate inhibited lettuce seed germination by 32% at 200 ppm (w/v). Compounds II, III, and IV were isolated from this eluate by silica gel preparative TLC. Compound II had a mp of 220–221°C and EI-MS, [^{1}H]NMR, UV, and IR spectra that were identical with those of authentic *p*-hydroxybenzoic acid. Compound III had a mp of 215°C and EI-MS, [^{1}H]NMR, UV, and IR spectra that were identical with those of authentic vanillic acid. Compound IV (colorless needles, 2.0 mg) had EI-MS, [^{1}H]NMR, and IR spectra that were identical with those of authentic syringic acid.

The neutral fraction including phenolics, which contained hirsutin (compound VII) (Kawabata et al., 1989), inhibited lettuce seed germination by 90% at 1000 ppm. Compound V (buff-colored needles, 45.9 mg) was isolated by silica gel preparative TLC (R_f = 0.78, developed with $CHCl_3$–MeOH, 20:1, v/v) and had a mp of 143°C. FD-MS *m/z*: 186 (M^+, 100%), EI-HR-MS *m/z*: M^+, 186.0797 ($C_{11}H_{10}N_2O$ requires 186.0793); EI-MS *m/z* (%): 186 (M^+, 90), 171 (100), 155 (8), 143 (13), 130 (4), 116 (13), 101 (4), 89 (12), 75 (4), 63 (7), 51 (4). The [^{1}H]NMR, [^{13}C]NMR, UV, and IR spectra were identical with those of authentic 4-methoxyindole-3-acetonitrile. Compound VI (colorless needles, 9.7 mg) was also isolated by silica gel preparative TLC (R_f = 0.46, developed with $CHCl_3$–MeOH, 10:1, v/v) and had a mp of 103.5°C. Compound VI had FD-MS, EI-MS, [^{1}H]NMR, UV, and IR spectra, which were identical with those of authentic pyrocatechol.

Bioassay Using Root Exudate Recirculating System. A bioassay using a root exudate recirculating system was performed following the method of

Stevens and Tang (1985). Donor pots containing *R. sylvestris* and acceptor pots containing lettuce seedlings were made from 1-gal brown glass bottles with the bottoms cut off. Both donor and acceptor pots contained coarse grade silica sand as the medium, with a 10-cm layer of small rock (2 cm size) under the sand. The sand in acceptor pots was topped with 1 cm of vermiculite to maintain moisture. Nutrient solution (Tadano and Tanaka, 1976) from the donor pot was lifted through 8-mm-diameter glass tubing to the acceptor pot using an air pump. Containers and glass tubings were wrapped with aluminum foil to exclude light. The solution was circulated at a rate of about 0.8 liters/hr. In the resin control pot, hydrophobic allelochemical compounds were removed from the recirculating solution using an XAD-4 column (18 × 120-mm glass column packed with 10 g Amberlite XAD-4 polymeric adsorbent resin). Treatments consisted of *R. sylvestris* as a donor species, *R. sylvestris* with a column containing XAD-4 (resin control), and a donor pot without the weeds (pot control). *R. sylvestris* plants (fresh weight ca. 50 g) were established in donor pots, and 30 lettuce seedlings (24 hr after germination) were planted in acceptor pots. Nutrient levels were maintained, and pH kept at 5.5 with H_2SO_4. Recirculating solution was not changed during the experiment except for replenishing water and nutrient solution. Lettuce seedlings were removed 14 days after beginning the bioassay, and plant heights, root lengths, and fresh weights of aerial parts and subterranean parts (two replications) were determined.

Quantification of Allelochemical Candidates in Recirculating Solution. Hydrophobic substances (100 μg) trapped with XAD-4 resin were eluted with acetone. Residual water was removed azeotropically by several additions of methanol. After 200 μl of pyridine and 100 μl of *N,O*-bis(trimethylsilyl)acetamide were added to the sample, the glass tube containing the reaction mixture was sealed with a polyethylene stopper and heated in an oil-bath at 80°C for 30 min (Schulz and Herrmann, 1980). 1-Octanol (15 μl) was then added to the sample as an internal standard and the trimethylsilylated sample was analyzed with gas chromatography [1.5% Silicone OV-17 on Uniport HP (80–100 mesh), 1 m].

Hirsutin was determined by adding 50 μl of methylene chloride and 3 ml of 20% ammoniacal ethanol (1 part concentrated NH_4OH and 4 parts anhydrous ethanol) to a portion (1 mg) of the total substances trapped with XAD-4 resin and heating the mixture for 2 hr at 50°C in a water bath (Wetter and Young, 1976). After cooling, the optical density was measured at 230, 259, and 304 nm (λ_{max} values of hirsutin–phenylthiourea). The blank was 50 μl of methylene chloride in 3 ml of 20% ammoniacal ethanol. On TLC chromatogram, hirsutin was found to be only one isothiocyanate.

Bioassay of Combined Compounds. Six combinations of two compounds (pyrocatechol plus *p*-hydroxybenzoic acid, pyrocatechol plus vanillic acid, pyrocatechol plus hirsutin, *p*-hydroxybenzoic acid plus vanillic acid,

p-hydroxybenzoic acid plus hirsutin, and vanillic acid plus hirsutin) with the concentration of each component 0.9×10^{-4} M, four combinations of three compounds (pyrocatechol plus *p*-hydroxybenzoic acid plus vanillic acid, pyrocatechol plus *p*-hydroxybenzoic acid plus hirsutin, pyrocatechol plus vanillic acid plus hirsutin, and *p*-hydroxybenzoic acid plus vanillic acid plus hirsutin) with the concentration of each component 0.6×10^{-4} M, and all compounds combined (pyrocatechol plus *p*-hydroxybenzoic acid plus vanillic acid plus hirsutin) with the concentration of each component 0.45×10^{-4} M were tested to determine their effects on lettuce seed germination. This total concentration (1.8×10^{-4} M) used in the bioassay represents a reasonable biological test level for the comparison of naturally occurring phytotoxic chemicals.

Analysis of Data. Differences among means were calculated using Duncan's multiple range test at the 5% level of probability. This allowed the effect of each compound, combined compounds, and the recirculating solution, including substances trapped with XAD-4 resin, to be examined.

RESULTS AND DISCUSSION

Both the neutral and acidic fractions of the acetone extracts inhibited lettuce seed germination (data not shown). Salicylic, *p*-hydroxybenzoic, vanillic, and syringic acid were isolated from the acidic fraction (Figure 1). At 200 ppm,

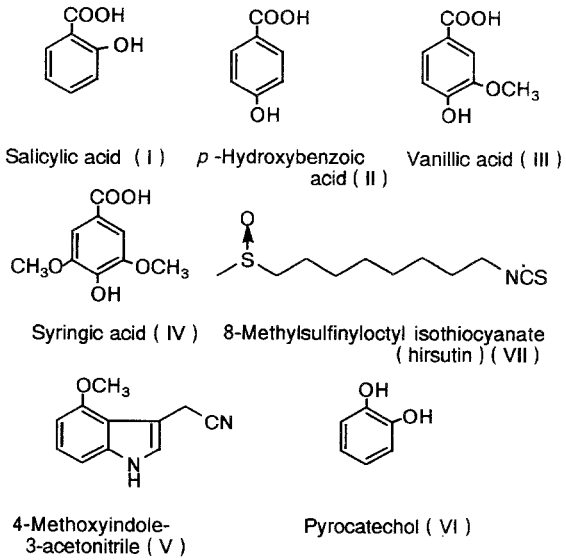

FIG. 1. Chemical structures of allelochemical candidates in the text.

these phenolic acids inhibited germination at 100, 100, 93, and 57%, respectively. Many phenolic compounds are inhibitory to seed germination or plant growth (Kuiters, 1989). In some instances, these compounds affect fundamental plant processes such as hormonal balance, protein synthesis, photosynthesis, chlorophyll production, plant–water relations and membrane permeability (Einhellig, 1986).

In addition to hirsutin, 4-methoxyindole-3-acetonitrile and pyrocatechol were isolated from the neutral fraction (Figure 1). 4-Methoxyindole-3-acetonitrile and pyrocatechol inhibited the germination at 200 ppm (w/v) by 12% and 100%, respectively. 4-Methoxyindole-3-acetonitrile had been isolated from the clubroots of Chinese cabbage (*Brassica pekinensis*) (Nomoto and Tamura, 1970), but this is the first report of this compound in *R. sylvestris*.

We have examined the effect of removing the hydrophobic substances using XAD-4 resin from the root exudates of *R. sylvestris* on lettuce seedling growth. Differences between the resin control and the pot control (no *R. sylvestris*) were presumed to be the results of inhibition by hydrophilic root exudates, while differences between the resin control and *R. sylvestris* were presumed to be caused by hydrophobic root exudates. Differences between *R. sylvestris* and the pot control represent the total allelopathic effect.

Root exudates inhibited lettuce seedling growth in June, July and August (Table 1). These were the flowering stages of *R. sylvestris*. However, for March

TABLE 1. INHIBITION OF LETTUCE GROWTH FROM EXPOSURE TO YELLOW FIELDCRESS ROOT EXUDATES

	Lettuce growth[a]								
	Aerial parts				Subterranean parts				
	Plant height		Fresh weight		Root length		Fresh weight		Germination inhibitory activity[c]
Period	vs Cont.	XAD-4[b]	vs Cont.	XAD-4[b]	vs Cont.	XAD-4[b]	vs Cont.	XAD-4[b]	
March	NS		NS		NS		NS		−
June	**		**		**		**		++
July	**	**	**	**	*	*	**		++
August	**	**	**	**	*	NS	NS	NS	+
September	*		*		NS		NS		−
November	NS		NS		NS		NS		−

[a] Significance levels: **, 0.01 level of probability; *, 0.05 level of probability; NS, not significant.
[b] Difference between yellow fieldcress and resin control (effect of hydrophobic root exudates).
[c] Lettuce germination inhibitory activity of hydrophobic substances in recirculating solution (400 ppm, 24 hr). Inhibitory activity: ++, 100%; +, ≥70%; −, <70%.

and November bioassays, lettuce seedling growth was not inhibited (Table 1). These periods were the vegetative growing stages of *R. sylvestris*. Aerial parts of the lettuce plant were more sensitive than the subterranean parts to the inhibition (Table 1). These responses were probably caused by the accumulation of toxic root exudates, as nutrient solution was not changed during the experiments.

Hydrophobic root exudates (XAD-4 versus control) inhibited growth of acceptor lettuce seedlings in the July and August bioassays (Table 1). The rate of hirsutin, pyrocatechol, vanillic acid, and *p*-hydroxybenzoic acid released from *R. sylvestris* roots in July and August was 13.0, 9.3, 1.1, and 0.7 μg/plant/day, respectively (data not shown). Recently, we (Mizutani and Yamane, 1991) reported that the content of precursor glucosinolates (glucohirsutin and deoxyglucohirsutin) increased during the vegetative growing stage and the flowering stage of *R. sylvestris*, suggesting that the content of glucosinolates may relate to the content of hydrophobic root exudate (hirsutin) and the bioassay results on this report. Deoxyhirsutin, which has no activity against lettuce, is another possible isothiocyanate, but we could not detect deoxyhirsutin in the hydrophobic root exudate.

Tang and Takenaka (1983) reported that for 2-month-old *Carica papaya* L. the rate of benzyl isothiocyanate release from the root system was 2–3 μg/tree/day in a continuous root exudate trapping system. This herbicidal secondary metabolite was released from the undisturbed plant root system.

Einhellig (1987) reported that allelopathic inhibition of germination and plant growth typically occurs from the joint action of several allelochemicals. Thus, the potential impact of an allelochemical on plant growth should be evaluated with regard to both the presence of associated allelopathic compounds and the influence of other chemical and physical conditions in the environment.

Several combinations of pyrocatechol, *p*-hydroxybenzoic acid, vanillic acid, and hirsutin reduced lettuce seedling growth (Table 2). Pyrocatechol plus *p*-hydroxybenzoicacid, pyrocatechol plus vanillic acid, pyrocatechol plus hirsutin, and *p*-hydroxybenzoic acid plus hirsutin significantly reduced lettuce hypocotyl elongation. From the combination effect (total inhibition rate of the single test divided by constituent number of combination), pyrocatechol plus *p*-hydroxybenzoic acid significantly reduced hypocotyl elongation, while the other combinations produced slight or insignificant effects.

On the other hand, several combinations showed significant effects on root elongation of lettuce seedlings. The combinations of pyrocatechol plus *p*-hydroxybenzoic acid and pyrocatechol plus vanillic acid, pyrocatechol plus *p*-hydroxybenzoic acid plus vanillic acid, and the combination of all four compounds increased root elongation. Among these, the combination of pyrocatechol plus *p*-hydroxybenzoic acid at 230% over the control was the most

TABLE 2. EFFECT OF COMBINATIONS OF POTENTIAL ALLELOPATHIC COMPOUNDS ON LETTUCE GROWTH

Combination[a]				Length (%)[b]	
Pyrocatechol	*p*-Hydroxybenzoic acid	Vanillic acid	Hirsutin	Hypocotyl	Root
+	−	−	−	58 e	70 e
−	+	−	−	96 a	54 f
−	−	+	−	89 b	50 f
−	−	−	+	44 h	66 e
+	+	−	−	51 fg	230 a
+	−	+	−	54 efg	170 b
+	−	−	+	49 g	69 e
−	+	+	−	90 b	52 f
−	+	−	+	53 efg	70 e
−	−	+	+	56 ef	63 e
+	+	+	−	63 d	180 b
+	+	−	+	56 ef	93 d
+	−	+	+	55 ef	97 d
−	+	+	+	69 c	90 d
+	+	+	+	65 d	110 c

[a] The pluses in each row indicate which chemicals were combined, of the four listed at the top. See text.

[b] Percent lengths (percent of the control growth) for a single test or a combination test, followed by the same letter are not significantly different at the 0.05 level of probability as determined by Duncan's multiple range test.

effective. These data suggest an auxin effect observed in the lettuce root growth bioassay at low concentrations of phytotoxic compounds (Manners and Galitz, 1985).

Our investigation clearly shows several phenolic compounds and hirsutin to be potent phytotoxins to developing lettuce seedlings and suggests that these compounds are allelochemical compounds in plant–plant interactions.

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FLORAL VOLATILES OF *Tanacetum vulgare* L. ATTRACTIVE TO *Lobesia botrana* DEN. ET SCHIFF. FEMALES

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Abstract—The European grapevine moth (EGVM), *Lobesia botrana*, is a major pest of grapes in Europe. Females are attracted to a nonhost plant: tansy (*Tanacetum vulgare* L.), which is a common weed in Slovakian vineyards. A steam distillate extract of tansy flowers was analyzed by means of a GC-EAG technique to screen constituents detected by the olfactory receptors of EGVM females. From more than 200 GC peaks, nine peaks corresponding to monoterpenoids released an EAG response in more than 70% of the females (N = 15): *p*-cymene, *d*-limonene, α-thujene, α-thujone, β-thujone, thujyl alcohol, terpinene-4-ol, (*Z*)-verbenol, and piperitone. The steam distillate of tansy as well as a synthetic blend of identified compounds released consistent attraction in a field cage. The use of nonhost plants and host plant odors in integrated pest management is discussed.

Key Words—*Lobesia botrana*, *Vitis vinifera*, *Tanacetum vulgare*, tansy,

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grapevine, Lepidoptera, Tortricidae, Compositae, plant–insect relationships, olfaction, allelochemicals, terpenoids, coupling GC-EAG.

INTRODUCTION

The role of host plant odors mediating different behaviors has received much attention for monophagous and polyphagous insects. It has been suggested that host-specific molecules as well as more commonly occurring compounds are used by phytophagous insects to identify their host plants (Finch, 1980; Visser, 1986; Masson and Mustaparta, 1990).

The European grapevine moth (EGVM), *Lobesiabotrana* Den. et Schiff., represents one of the most important pests of vineyards in Europe. This insect is considered a polyphagous insect restricted to a narrow range of plants belonging to different families (Stoeva, 1982). Olfaction seems to play an important role in its colonization patterns. The attraction of adults has been observed in response to fermented wine and fruit odors (Feytaud and Bos, 1914; Roussel and Vonderheyden, 1964). In nature, EGVM females are strongly attracted to tansy, *Tanacetum vulgare* L. (Gabel, 1992). This attraction appears to be sexually selective, since males are seldom observed on this plant, and has been attributed to volatiles emitted by the flowers (Gabel et al., 1991). Tansy is not considered to be a host plant of the EGVM nor a rendezvous site for mating (Gabel, 1992).

Tansy is a wild species of Compositae growing in temperate regions. In Slovakian vineyards, the absence of herbicide usage favors the natural occurrence of this plant within vineyard rows and at the borders. More than 30 chemotypes of tansy have been reported worldwide according to the major constituents belonging to monoterpenes and sesquiterpenes (Nano et al., 1979; Holopainen et al., 1987).

In this paper, we report on the detection of tansy flower volatiles by EGVM females and the attractiveness of a reduced mixture of these volatiles. We also discuss the potential use of tansy and nonhost plant odors in integrated control programs.

METHODS AND MATERIALS

Plant Extract. Flowers of tansy (without the pedicel) were collected along vineyard borders and stored at $-15°C$. Tansy flowers were steam distilled using a distillation–extraction device modified after Likens and Nickerson (Pharmacopea Bohemoslovaca, 1987). Flowers (440 g) were extracted in 1 liter of

distilled water. The solvent-free fraction corresponding to the first 2 hr of distillation was collected and used for the experiments.

Insect Material. A laboratory strain of EGVM from INRA Pont-de-la-Maye, annually infused with wild insects from Bordeaux vineyards (France), was reared on a semisynthetic diet under controlled conditions (16:8 hr light–dark; 22°C ± 1°C; 60% relative humidity). Two-day-old, mated females were used for electrophysiological recordings.

Gas Chromatography–Electroantennography Coupling. A gas chromatograph (GC) was equipped with an on-column injector, a 5-m-long deactivated fused silica megabore precolumn, a 30-m-long fused silica megabore DB-5 column (5% phenyl-, 95% methyl-pyloxane), a deactivated Y-shaped fused silica outlet splitter heated at a constant temperature (250°C), and a flame ionization detector (FID) (split ratio = $\frac{2}{3}$ towards the antenna) (see Thiéry et al., 1990, for details). The carrier gas was helium. The temperature was raised linearly at 5°C/min from 40°C up to 250°C. Molecules eluted from the column were blown into a humidified stream of purified air: air speed = 20 cm/sec, flow diameter = 0.8 cm. The relative concentration of each constituent was expressed as a percentage of the sum of all the peaks after subtraction of the solvent peak area.

Electroantennograms (EAG) were recorded from 15 females. Isolated heads of EGVM were mounted between the recording electrode covering the tip of the antenna and the indifferent electrode inserted in the clypeus. EAG and GC signals were stored on a microcomputer using custom software. GC peaks that elicited an EAG response in more than four individuals were subsequently chemically identified.

Identification. Identification of chemicals was performed by GC–mass spectrometry (GC: HP 5890II; MS: HP5971A). Identifications were based on electron impact ionization (EI, 70 eV). The mass spectra obtained were compared to spectra from the NBS Database and confirmed by injection of standard chemicals when available.

Bioassay. The attractiveness of the steam distillate and of a blend of the chemicals identified through the GC-EAG analysis were tested in a field cage with an artificial population of EGVM. A row of vine grapes (Cabernet Sauvignon cultivar) was covered under a tunnel made of P17 tissue (polypropylene, 0.17 mm thickness, SODOCA S.A.R.L., France) (length = 12 m, height = 2.5 m, width = 3 m). Five traps designed for EGVM females (Gabel, 1990) were used: one control, two baited with tansy extract, and two baited with the synthetic blend. The traps were placed evenly within the row, 1 m above the ground. Exchangeable trapping plates were coated with Bird Tanglefoot glue (Tanglefoot Co., Michigan). Males (N = 738) and mated females (N = 592) were released in the cage. The number of insects recaptured was counted each morning during five consecutive days. The odors were released from a glass

TABLE 1. MOLECULES RELEASING EAG IN MORE THAN 11 OF 15 *Lobesia botrana* FEMALES[a]

Molecule	Amount (%)	EAG response (in mV ± SD)
p-Cymene	0.27	0.16 ± 0.05
d-Limonene	0.03	0.19 ± 0.08
α-Thujene	0.28	0.20 ± 0.08
α-Thujone	1.05	0.33 ± 0.16
β-Thujone	78.34	0.67 ± 0.28
Thujyl alcohol	0.64	
Terpinen-4-ol	0.75	0.29 ± 0.07
(*Z*)-Verbenol	4.33	0.30 ± 0.10
Piperitone	6.35	0.25 ± 0.10

[a]Identifications by GC-MS (electron impact ionization), mass spectra compared to reference spectra (NBS Database) and confirmed by injection of standards.

tube (1 cm diam.) filled with either the extract of tansy (1 ml) or a blend of monoterpenes (1 ml) in the proportions expressed in Table 1. The monoterpenes used in the synthetic blend were of GC standard quality: *p*-cymene, thujyl alcohol, piperitone, terpinen-4-ol (INRA, Dijon, France), *d*-limonene and (*Z*)-verbenol (Fluka), isomers of thujone (Pharmaceutical Faculty, Bratislava, CSFR). α-Thujene was not available.

RESULTS AND DISCUSSION

Steam distillation of tansy flowers yielded an extract in which over 200 peaks were detected by the GC analysis. The GC-EAG method was used as a screening procedure to locate the most active fractions and compounds of this complex blend. Only 24 GC peaks released measurable EAG responses in 27% of the females. Nine peaks elicited responses in 70% of the females (Figure 1). These peaks are monoterpenes: *p*-cymene, *d*-limonene, α-thujene, α-thujone, a blend of β-thujone and thujyl alcohol, terpinen-4-ol, (*Z*)-verbenol, and piperitone.

EAG responses closely matched the corresponding GC peaks (Figure 1). This relationship was true for the major peak (β-thujone) where the EAG response was maintained until end of the peak tail (Figure 1). Responses were observed during the release of thujyl alcohol, which occurred while the antenna was still recovering from the stimulation of β-thujone. Improved separation or classical EAG studies are needed to confirm if thujyl alcohol stimulates the olfactory receptors of EGVM females. Both major and minor compounds released EAG

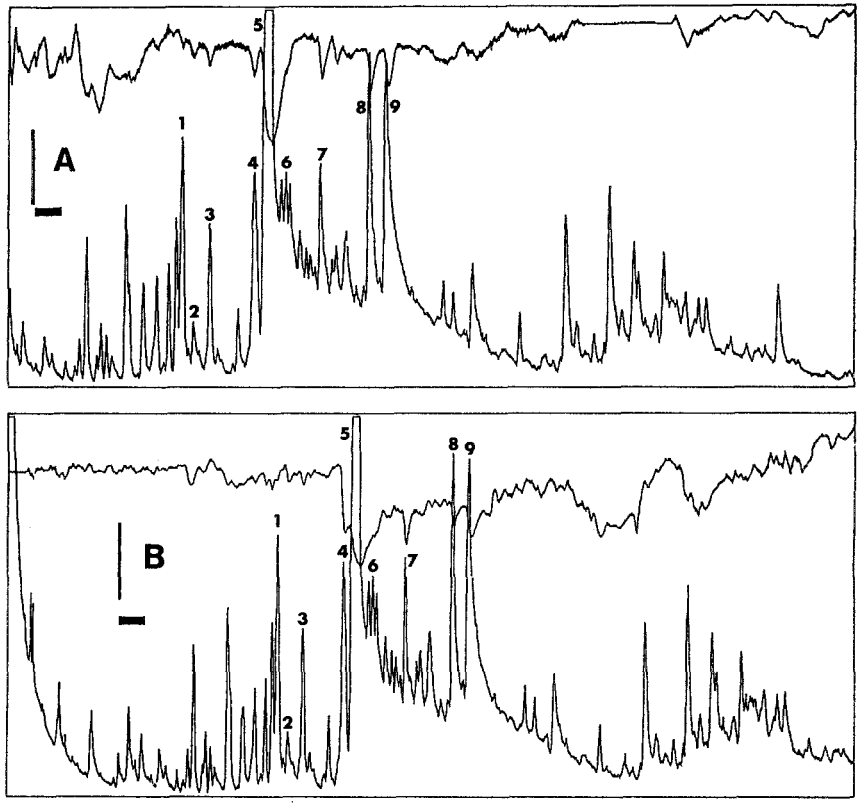

FIG. 1. GC-EAG recordings of two females of *Lobesia botrana* in response to tansy extract. Upper trace = EAG, lower trace = FID signal. 1 = *p*-cymene, 2 = *d*-limonene, 3 = α-thujene, 4 = α-thujone, 5 = β-thujone, 6 = thujylalcohol, 7 = terpinen-4-ol, 8 = (Z)-verbenol, 9 = piperitone. Horizontal bar = 100 sec; vertical bars—A: 1 mV, B: 2 mV.

responses in EGVM females (Table 1). The detection of chemicals at low concentrations within plant extracts suggests an important biological activity of these substances.

The biological activity of eight of the nine monoterpenes [*p*-cymene, *d*-limonene, α-thujone, a blend of β-thujone and thujyl alcohol, terpinen-4-ol, (Z)-verbenol, and piperitone] identified in the tansy flowers were compared to the steam distillate (Figure 2). A total of 28.4% of the released females were recaptured in the traps (2.6% in the control, 15.5% with the steam distillate, and 10.3% with the synthetic blend). Low temperature (13.5°C) and high wind speed (15 m/sec) reduced the effectiveness of the traps during the first night. We assumed that tansy odors were effective only against females. For this reason

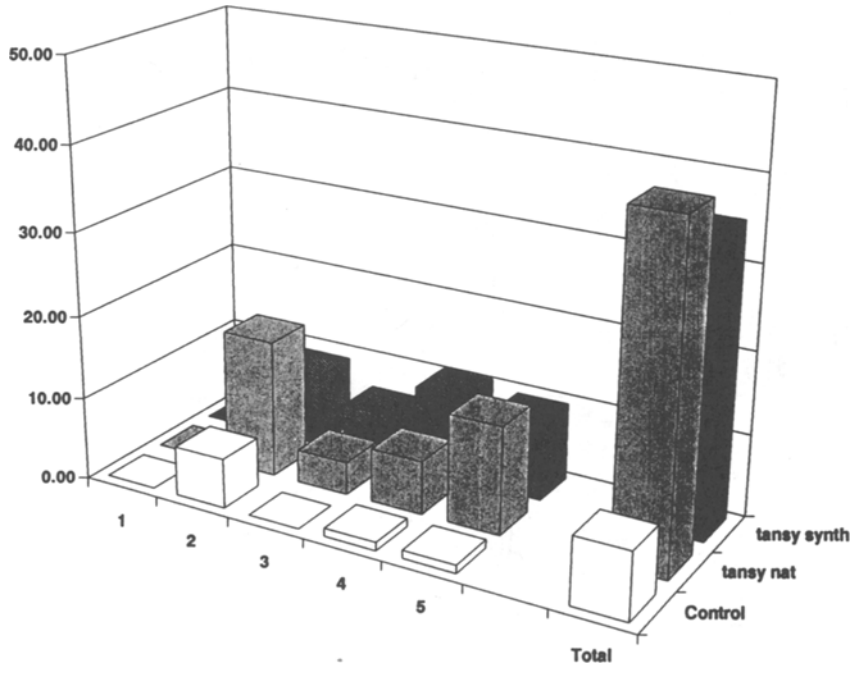

FIG. 2. Daily number of *Lobesia botrana* females caught in two different odorant traps (5 days): white bars = control, grey bars = tansy steam distillate, striped bars = synthetic blend.

we have used a type of trap especially designed to catch females (Gabel, 1990). However, males (22%) were also recaptured, possibly attracted by sexual pheromones emitted from the trapped females (Gabel and Stockel, 1988). Catching females with tansy extracts confirmed previous reports on the attractiveness of natural populations of EGVM females by tansy plants (Gabel, 1992). At the concentration used, our synthetic blend was almost as attractive as the steam distillate. α-Thujene was lacking in the synthetic blend used. The role of this chemical as well as different concentrations of the reduced blend have now to be tested.

Terpenic compounds have been tested as possible trapping agents against EGVM by Stellwaag and Götz (1937), but their results were inconclusive mainly for methodological reasons. Except for thujone, most of the monoterpenes used in our trapping experiments are present in flower or leaf extracts of grapevines (Schreier et al., 1976). These data suggest that such molecules are involved in olfactory recognition of plants by EGVM females.

Other volatile compounds, such as general green leaf volatiles, might con-

tribute to the olfactory recognition in the EGVM. Chemical analysis of various grapevine cultivars reported that green odor volatiles were emitted from leaves and immature grapes [e.g., 1-hexanol, (*E*)-2-hexen-1-ol, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal] (Kepner and Webb, 1956; Wildenradt et al., 1975; Schreier et al., 1976; Shimizu and Watanabe, 1981). Such chemicals are produced at low concentration in Compositae flowers (Rudlof, 1963; Etiévant et al., 1984; Flath et al., 1985; Souleles and Stamatakou, 1991). In our experiments, we could not demonstrate whether the females detect these substances. Other distillation methods should be used to extract and concentrate such chemicals.

The attraction of EGVM females towards a nonhost plant on which oviposition and feeding was never observed raises an exciting ecological question. Olfactory detection of major terpenoids of tansy suggests that females might search for tansy-specific chemicals. However, the exact role of tansy in the biology of EGVM is not yet understood. Tansy flowers and nectar might provide a food source needed by females for egg maturation (Stoeva, 1982). Tansy has long been used for various pharmaceutical and toxicological purposes (Chandler et al., 1982; Dembitskii et al., 1984). It is therefore possible that the occurrence of females on tansy flowers is related to pharmacophagy as demonstrated in the Lepidoptera (Boppré, 1984).

Nonhost plant odors can interfere with the perception of the particular blend used by an herbivore and modify its orientation (Thiéry and Visser, 1986, 1987). Tansy has been reported to alter colonizing patterns of different insect species. Observations made on the flea beetle, *Phyllotreta cruciferae*, indicated that tansy inhibits adult colonization (Latheef and Ortiz, 1984). Essential oils of tansy have also been proposed as repellents against aphids in orchards (Dembitskii et al., 1984) and Colorado potato beetle (Schearer, 1984). In the cabbage worm, *Pieris rapae*, tansy increases the number of eggs laid on crop plants, whereas in the cabbage looper, *Trichoplusia ni*, the effect was the reverse (Latheef and Ortiz, 1983). In the case of EGVM, the odor produced by tansy could be used to (1) interfere with the orientation of females to their host, (2) lure females outside the vineyards, (3) monitor the population dynamics, or (4) directly control the population by mass trapping. The reduced blend of monoterpenes that evoked significant electrophysiological and behavioral activity for the EGVM, must now be tested for its behavior-evoking activity in larger scale experiments in vineyards.

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SEASONAL VARIATION OF LIPIDS IN FEMORAL GLAND SECRETIONS OF MALE GREEN IGUANAS (*Iguana iguana*)

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Abstract—Femoral gland secretions were collected from 21 captive adult male green iguanas (*Iguana iguana*) in Orotina, Costa Rica, and San Diego, California, during the breeding (November) and nonbreeding (March) seasons. Lipids were extracted with methylene chloride, weighed, separated by thin-layer chromatography, and analyzed by gas chromatography–mass spectrometry. Free and esterified C_{14}–C_{26} fatty acids, 5β-cholestan-3α-ol (epicoprostanol), cholest-5-en-3β-ol (cholesterol), cholest-5-en-24-methyl-3β-ol (campesterol), cholesta-5,22-dien-24b-ethyl-3β-ol (stigmasterol), cholesta-8,24(5α)-dien-4,4,14α-trimethyl-3β-ol (lanosterol), cholest-5-en-24-ethyl-3β-ol (β-sitosterol), and two uncharacterized sterols were indicated. More

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lipids were recovered from femoral gland secretions obtained during the breeding than the nonbreeding months, indicating that secretion deposits may be more detectable during the mating season.

Key Words—Femoral glands, lipids, fatty acids, sterols, pheromones, *Iguana iguana*.

INTRODUCTION

Femoral glands are secretory structures located on the ventral surface of the hindlegs of many lizards (Cole, 1966). These glands are most active during the breeding season and usually are better developed in males than in females. Femoral gland secretions, which contain lipids and proteins (Gasc et al., 1969; Weldon et al., 1990; Alberts, 1990, 1991), elicit increased tongue-flicking in iguanid lizards (Duvall, 1986; Alberts, 1989), and may function in conspecific recognition and home-range marking (Alberts, 1992). The location of the glands on the underside of the hindlegs probably facilitates deposition of secretions onto the substrate during locomotion (Fergusson et al., 1985).

A number of studies indicate that femoral gland activity is modulated by androgenic hormones. In several lizard families, increased femoral gland size and productivity are positively associated with testicular activity (Regamey, 1935; Atland, 1941; Van Wyk, 1990), whereas castration results in glandular atrophy (Matthey, 1929; Regamey, 1932; Padoa, 1933). Fergusson et al. (1985) demonstrated that exogenous testosterone stimulates femoral gland activity in the ornate dragon, *Amphibolurus fordi*. In male green iguanas, *Iguana iguana*, femoral gland productivity is positively correlated with endogenous plasma testosterone levels throughout the year (Alberts et al., 1992). Regulation of secretory activity by androgens is commonly observed in mammalian scent glands known to produce pheromones (Ebling, 1977).

The green iguana is a large lizard inhabiting trees along rivers and streams in neotropical lowland forests. In the wild, large dominant males defend territories, usually occupied by several females and subordinate males (Dugan, 1982; Rodda, 1992). Courtship begins at the end of the rainy season in October and November and is followed by mating at the beginning of the dry season (Rand and Greene, 1982). The presence of a well-developed olfactory epithelium and vomeronasal organ (Gabe and Saint Girons, 1976; Schwenk, 1992) indicates that green iguanas probably detect volatile and nonvolatile chemicals. Tongue-flicking, which could deliver odorants to the vomeronasal organ or facilitate gustatory reception, occurs at a high frequency in both wild and captive green iguanas (Burghardt et al., 1986).

Adult male iguanas possess prominent femoral glands, while those of females are greatly reduced (Lazell, 1973; Rodda, 1991). Behavioral and phys-

iological studies with captive green iguanas indicate that femoral gland secretions may play an important role in establishing and maintaining dominance relationships among males during the breeding season (Alberts et al., 1992). We report here on seasonal differences in the lipid composition of femoral gland secretions in male green iguanas. In addition, we examine individual variation in lipid composition among males.

METHODS AND MATERIALS

Femoral gland secretions were collected from 15 adult male green iguanas at the Fundacion Pro Iguana Verde in Orotina, Costa Rica, in November 1989 and March 1990 and from six adult male green iguanas in a captive colony at the Zoological Society of San Diego, California, in December 1989. The Costa Rica iguanas originally came from a wild population in Panama and the San Diego iguanas from a wild population in Belize. Samples were obtained by applying gentle manual pressure around the openings of four femoral pores (Figure 1) and removing secretion plugs with small forceps. Samples were placed in glass vials containing several milliliters of methylene chloride. Secretion plugs were macerated with a metal spatula, filtered to remove insoluble components, and dried under N_2. The lipid residue was allowed to desiccate

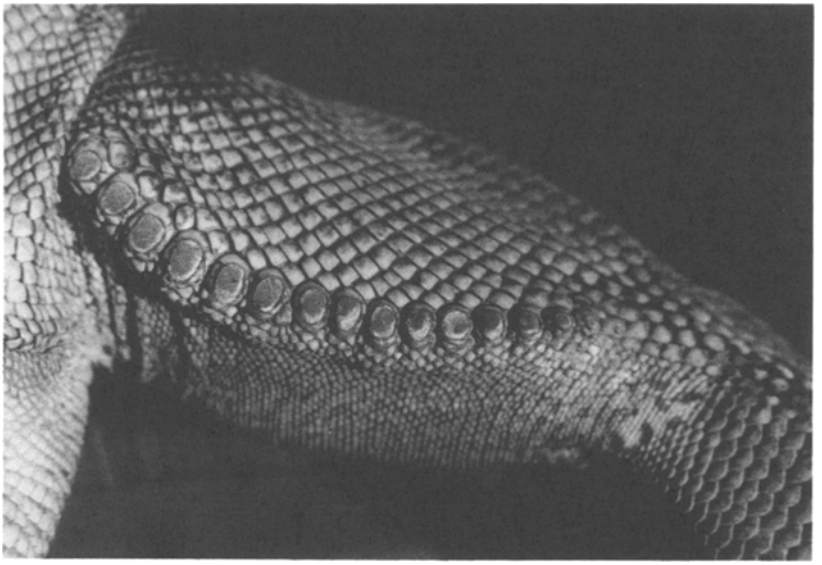

FIG. 1. A row of femoral glands on the ventral surface of the hindleg of an adult male green iguana. The diameter of the largest femoral pore is approximately 4.5 mm.

overnight, and then was weighed and redissolved in hexane. All samples were stored at −80°C. Samples collected from the 15 males in the Costa Rica colony were pooled for each of the two collections.

Aliquots of each pooled sample from the breeding and nonbreeding seasons (400 μg each) were applied to 0.25-mm preparative thin-layer chromatography (TLC) plates of silica gel 60G and resolved by successive development in hexane, toluene, and hexane–diethyl ether–acetic acid (80:20:2, v/v). Mixed nonpolar lipid standards (Nu-Chek-Prep, Elysian, Minnesota) were developed in an adjacent lane. Fractions from the region to which each of the five lipid standards migrated (cholesterol, R_f 0.25; oleic acid, R_f 0.43; triolein, R_f 0.60; methyl oleate, R_f 0.70; cholesteryl oleate, R_f 0.83) were eluted from each plate with diethyl ether. The six samples from the San Diego colony were not fractionated by TLC due to the limited material available from individual males.

All fractions eluted from TLC plates, except the sterol fraction, were converted to methyl esters by transesterification prior to analysis by gas chromatography–mass spectrometry (GC-MS) (Long et al., 1988). The sterol and steryl ester fractions were converted to trimethylsilyl derivatives by adding 200 μl Tri-Sil silylating agent (Pierce, Rockford, Illinois) and allowing them to equilibrate at room temperature for at least 30 min. The unfractionated pooled secretions from Costa Rica and the six individual samples from San Diego were transesterified and then trimethylsilylated prior to GC-MS analysis. Two microliters of each sample were injected into a Hewlett-Packard model 5995C GC-MS system with ultrapure helium as the carrier gas (DB-5 column, 30:1 split injection). The temperature program for the column was: T_{initial} = 180°C/1.0 min, programmed at 5°C/min, T_{final} = 350°C/0 min.

The mass spectrum of each compound evident on the gas chromatograms was obtained. The retention index of each compound was calculated based on retention time relative to that of the normal hydrocarbon series C_{15}–C_{25}, C_{28}, C_{32}, C_{36}. Lipids were identified by comparison of retention indices and mass spectra of derivatives with those of authentic standards and published data in the National Bureau of Standards mass spectral data base.

RESULTS AND DISCUSSION

Our results confirm the presence of a diversity of lipids in the femoral gland secretions of male green iguanas (Weldon et al., 1990), and document several previously unreported compounds (Table 1). Saturated C_{14}–C_{26} and unsaturated C_{16}–C_{24} fatty acids were identified. Hexadecanoic acid was the most abundant fatty acid, followed by C_{20}, C_{18} and its unsaturated homologs, and C_{22}. Saturated C_{15}–C_{25} fatty acids were detected in trace amounts. A series of eight sterols was also evident, six of which were identified: 5β-cholestan-3α-ol (epicoprostanol),

TABLE 1. MOLECULAR WEIGHT (MW) AND RETENTION INDEX (RI) OF DERIVATIVES OF LIPIDS IDENTIFIED IN FEMORAL GLAND SECRETIONS OF ADULT MALE GREEN IGUANAS DURING BREEDING (BR) AND NONBREEDING (NB) SEASONS

Compound	MW	RI	BR (%)	NB (%)
Tetradecanoic acid	256	1720	0.58	0.86
Pentadecanoic acid	270	1820	0.15	0.20
Hexadecenoic acid	282	1900	1.25	1.51
Hexadecanoic acid	284	1926	18.22	17.55
Heptadecanoic acid	298	2022	0.44	0.26
Octadecadienoic acid	308	2094	5.58	4.60
Octadecenoic acid	310	2102	6.73	5.29
Octadecanoic acid	312	2124	9.23	8.05
Nonadecanoic acid	326	2221	0.46	1.15
Eicosenoic acid	338	2300	1.30	1.38
Eicosanoic acid	340	2327	10.86	11.46
Heneicosanoic acid	354	2424	0.27	0.19
Docosadienoic acid	364	2476	0.24	0.18
Docosenoic acid	366	2495	2.54	0.87
Docosanoic acid	368	2526	6.42	5.04
Tricosanoic acid	382	2628	0.13	0.08
Tetracosenoic acid	394	2700	0.67	0.78
Tetracosanoic acid	396	2730	2.67	2.72
Pentacosanoic acid	410	2829	0.15	<0.01
Hexacosanoic acid	424	2934	0.30	0.44
Epicoprostanol	388	3098	2.33	1.38
Unknown sterol I	384	3160	1.28	1.42
Cholesterol	386	3192	6.54	8.39
Unknown sterol II	402	3201	0.83	0.94
Campesterol	400	3305	7.41	6.08
Stigmasterol	412	3332	1.25	0.86
Lanosterol	426	3393	11.06	17.24
β-Sitosterol	414	3408	1.11	1.08

cholest-5-en-3β-ol (cholesterol), cholest-5-en-24-methyl-3β-ol (campesterol), cholesta-5,22-dien-24b-ethyl-3β-ol (stigmasterol), cholesta-8,24(5α)-dien-4,4,14α-trimethyl-3β-ol (lanosterol), and cholest-5-en-24-ethyl-3β-ol (β-sitosterol). Unknown sterol I (retention index = 3160) had a molecular weight two mass units less than cholesterol, and its mass spectrum shared many characteristics with that of cholesterol. This suggests that it is similar in structure to cholesterol but contains an additional unsaturation site. Unknown sterol II (retention index 3201) exhibited a structure similar to and a molecular weight 14 mass units greater than epicoprostanol, suggesting that it differs from epicoprostanol by possessing an additional methyl group.

The most abundant sterol in the secretions during both the breeding and nonbreeding seasons was lanosterol, followed by cholesterol and campesterol. The remaining five sterols were detected at much lower concentrations. Some of these sterols may be dietary in origin. Like many iguanine lizards, green iguanas are herbivorous throughout their lives (Van Devender, 1982). Campesterol, stigmasterol, β-sitosterol, and their respective esters, which together represented approximately 10% of total lipids by weight, typically occur in plants (Goodwin, 1985).

Seasonal variation in the lipid composition of green iguana femoral gland secretions was evident. During the breeding season, the mean percentage by weight of lipids in the secretions (35.10% $\pm$ 0.016) significantly exceeded that during the nonbreeding season (13.40% $\pm$ 0.007; Paired t test = 13.23, df = 14, P = 0.0001, arcsine square-root transformation). Although the overall relative abundances of fatty acid components showed little variation between the breeding and nonbreeding seasons (Table 1), there was considerable variation in the proportion of these acids in different lipid classes (Table 2). Most of the unsaturated acids in the free fatty acid, methyl ester, and triglyceride fractions occurred at a higher concentration during the breeding than the nonbreeding

TABLE 2. RELATIVE ABUNDANCES OF C_{14}–C_{26} SATURATED AND UNSATURATED FATTY ACIDS IN TLC FRACTIONS OF FEMORAL GLAND SECRETIONS FROM FREE FATTY ACID (FA), METHYL ESTER (ME), TRIGLYCERIDE (TR), AND STERYL ESTER (SE) REGIONS DURING BREEDING AND NONBREEDING SEASONS

	FA fraction		ME fraction		TR fraction		SE fraction	
	BR	NB	BR	NB	BR	NB	BR	NB
Saturated acids								
C_{14}	5.6	8.1	4.1	7.5	1.9	5.8	7.4	3.6
C_{16}	20.6	29.0	26.1	25.5	37.5	33.7	47.0	32.7
C_{18}	6.4	10.6	12.6	14.0	14.2	15.8	13.8	37.1
C_{20}	4.5	18.1	18.2	25.2	17.5	25.1	8.6	1.5
C_{22}	1.3	5.8	6.3	8.5	4.4	7.5	4.2	<0.01
C_{24}	2.0	2.1	2.1	3.6	0.9	2.3	3.1	<0.01
C_{26}	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	2.0	<0.01
Unsaturated acids								
C_{16}	16.6	<0.01	5.3	<0.01	3.3	<0.01	<0.01	<0.01
C_{18}	14.4	9.8	18.7	15.7	20.3	9.8	13.9	25.1
C_{20}	7.5	4.3	2.5	<0.01	<0.01	<0.01	<0.01	<0.01
C_{22}	11.4	7.0	3.0	<0.01	<0.01	<0.01	<0.01	<0.01
C_{24}	9.7	5.2	1.1	<0.01	<0.01	<0.01	<0.01	<0.01

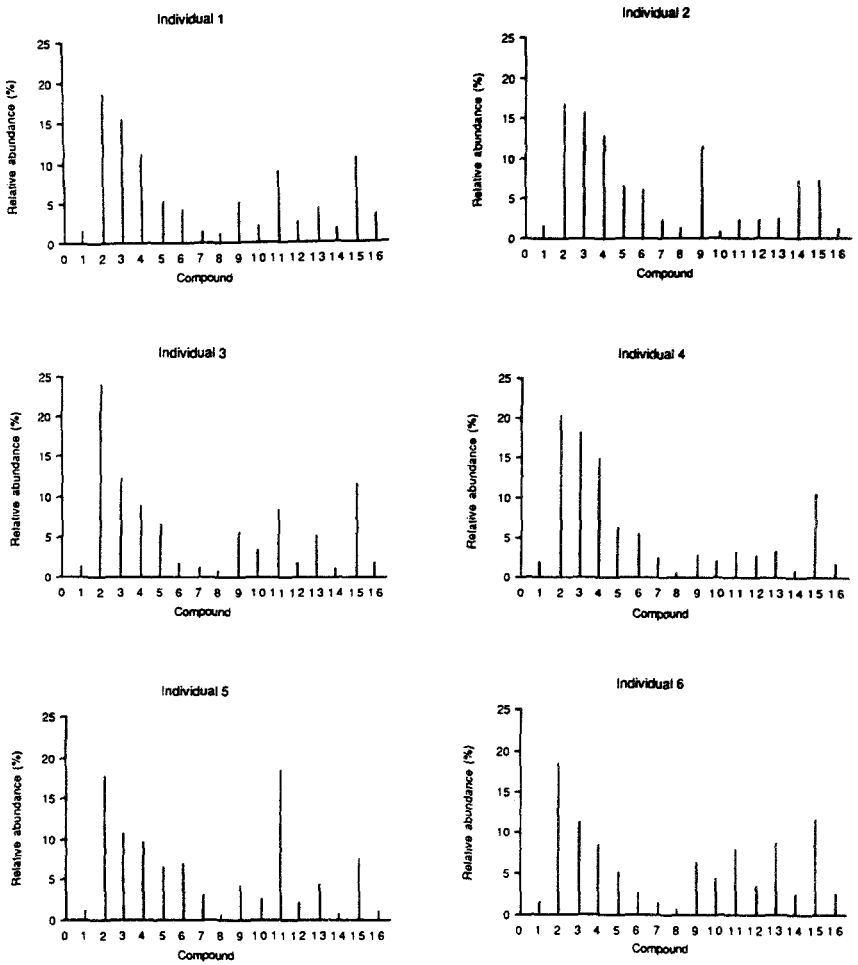

FIG. 2. Relative abundances of the following lipid compounds in the femoral gland secretions of six adult male green iguanas: 1 = tetradecanoic acid, 2 = hexadecanoic acid, 3 = octadecanoic acid, 4 = octadecenoic acid, 5 = octadecadienoic acid, 6 = eicosanoic acid, 7 = docosanoic acid, 8 = tetracosanoic acid, 9 = epicoprostanol, 10 = unknown sterol I, 11 = cholesterol, 12 = unknown sterol II, 13 = campesterol, 14 = stigmasterol, 15 = lanosterol, 16 = β-sitosterol.

season. Both the increased lipid content and greater unsaturation evident during the breeding season probably increase the volatility of femoral gland deposits. Higher volatility of secretions during the mating period may enhance the detectability of the secretions at the time of year when they are presumed to have their greatest utility.

Little individual variation was detected in the relative abundances of fatty acid components (Figure 2). The same eight acids were present in similar relative concentrations for the six individuals examined. Slightly more variation was evident among the sterol components, but cholesterol and lanosterol generally were the most abundant of these compounds. These results contrast with those reported for some mammals, in which individual variation in the lipid composition of glandular secretions, especially in fatty acid components, has been demonstrated and is hypothesized to function in individual recognition (e.g., Gorman, 1976).

Weldon et al. (1990) failed to detect differences in the femoral gland lipids of juvenile and adult male green iguanas, suggesting a lack of ontogenetic variability in lipid composition. Significant variation in the lipid composition of male and female secretions was not detected in the two lizards in which it has been examined, *Dipsosaurus dorsalis* (Alberts, 1990) and *Amphibolurus fordi* (Cogger, 1978). These studies suggest that, at least for these species, insufficient intraspecific variation exists for femoral gland lipids to permit individual recognition.

Results for femoral gland lipids of iguanas differ from those for femoral gland proteins, which exhibit individual variation, but no detectable seasonal variation (Alberts, Phillips, and Werner, unpublished data). It is possible that the lipids in green iguana femoral gland secretions permit initial detection of secretion deposits in the environment through the release of volatile compounds. After secretion deposits are localized, low volatility proteins may provide more information on the status and identity of the signaler. Secretions from more individuals need to be examined, and behavioral tests conducted to determine the degree to which chemical variation in different secretion components is perceived and utilized in social behavior.

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FORMONONETIN 7-*O*-GLUCOSIDE (ONONIN), AN ADDITIONAL GROWTH INHIBITOR IN SOILS ASSOCIATED WITH THE WEED, *Pluchea lanceolata* (DC) C.B. CLARKE (ASTERACEAE)

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Abstract—Formononetin 7-*O*-glucoside (ononin), an isoflavonoid, was isolated from the soils of cultivated areas associated with the perennial weed, *Pluchea lanceolata*. Aqueous solutions of this compound inhibited significantly root and shoot growth of mustard at 1×10^{-4} M, 5×10^{-4} M, and 1×10^{-3}M. The level of inhibition was similar to that of hesperidin and taxifolin 3-arabinoside, as reported earlier. The potential allelopathic effect of this compound under field conditions is discussed.

Key Words—Allelopathy, Asteraceae, formononetin 7-*O*-glucoside, *Pluchea lanceolata*, weed.

INTRODUCTION

Our earlier observations established the presence of potential toxins in *Pluchea lanceolata* (DC) C.B. Clarke (Inderjit and Dakshini, 1990) and in its associated soils (Inderjit and Dakshini, 1991). Here, we describe a new potential toxin, the isoflavonoid, formononetin 7-*O*-glucoside (ononin) in soils associated with the weed. To learn whether or not this compound, like hesperidin and taxifolin 3-arabinoside reported earlier (Inderjit and Dakshini, 1991), was toxic, formononetin was tested against mustard seeds.

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METHODS AND MATERIALS

Soils next to and away from (as control) the weed *Pluchea lanceolata* were collected from cultivated fields around the metropolitan city of Delhi. These soils, sandy loam, were air-dried at room temperature (25 ± 5°C), sieved (2 mm sieve), and stored in paper bags for 16 weeks prior to chemical analysis (Allen, 1989), identification, and characterization of phenolic constituents (Harborne, 1973). The chemical characteristics of associated soils were as follows: pH = 7.72 ± 0.23; electrical conductivity = 146.35 ± 76.15 μmho/cm; exchangeable PO_4 = 3.50 ± 2.06 (mg/100 g); Zn = 0.106 ± 0.093 (mg/100 g); Na = 63.6 ± 34.5 (mg/100 g); K = 21.32 ± 15.17 (mg/100 g); Mg = 23.32 ± 15.17 (mg/100g); Ca = 146.02 ± 138 (mg/100 g).

Extraction, Purification, and Identification of Phenolic Compounds. Twenty grams of soil was shaken for 1 hr with 100 ml of double-distilled water (DDW) at room temperature (25 ± 5°C). The soil extracts were then filtered and evaporated to dryness under vacuum. The residues were dissolved in 10 ml of methanol and chromatographed (Whatman No. 3, 46 × 57 cm), one dimension in butanol–acetic acid–water (BAW, 4:1:5, upper phase) following Harborne (1973). The developed chromatograms were then scanned under UV($-NH_3$) and UV($+NH_3$) and bands marked. Each of these bands was eluted in 10 ml of methanol, concentrated under vacuum, and again chromatographed on Whatman No. 3 in four solvent systems: BAW, DDW, 15% acetic acid, and forestal (conc. HCl–acetic acid–water, 3:30:10). After confirming the purity of these compounds, each was again concentrated and chromatographed on Whatman No. 3 and washed repeatedly with DDW through descending chromatography. Finally, these compounds were eluted from chromatograms with methanol and used for spectral analysis, acid hydrolysis, and acid–base shifts (NaOH, $AlCl_3$, $AlCl_3$–HCl, NaOAc, NaOAc–H_3BO_3) of parent as well as hydrolyzed fractions. R_f (×100) values (Whatman No. 1) and fluorescence (UV, UV+NH_3) of compounds in the four solvents were recorded. The aqueous layer of the hydrolyzed fraction was used for identification of the sugar moiety (Harborne, 1973). Additionally, the aglycone portion was cochromatographed with the authentic samples and the spectral data matched with that of authentic compounds (Mabry et al., 1970).

Mass Spectroscopy. Mass spectral value of the isolated compound was analyzed through a mass spectrometer (Jeol JMX 303), operated at an accelerator voltage of 3 kV and an ion source temperature at 280°C with scan speed of 100 m/e/sec.

Seed Germination and Growth Experiments. To evaluate the allelopathic potential of the isolated compound, germination and growth experiments were performed with various concentrations of aqueous solutions of the compound. *Brassica juncea* cv. PR 45, a common cultivated crop, was selected to assess

the allelopathic potential of the compound. Fifty seeds ($N = 3$) were sown in 15-cm-diam Petri plates on one layer of filter paper (Whatman No. 1), placed on a cotton pad, moistened with 60 ml of DDW (as control), or test solutions ranging from 1×10^{-4} M to 1×10^{-3} M (S1, S2, and S3). Germination percentages were calculated when no further germination occurred. Root and shoot length were recorded on the sixth day.

RESULTS AND DISCUSSION

The analysis resulted in the extraction of six compounds. Of these, two were common to the control as well as the weed-associated soils. Three of these compounds were taxifolin 3-arabinoside and hesperidin (Inderjit and Dakshini, 1991) and a phenolic acid. The fluorescence, R_f ($\times 100$) values, and absorption spectral data (Table 1); mass spectra showing presence of parent ion at 286.26; as well as glucose as sugar moiety confirm that a fourth compound isolated from associated soils of the weed *Pluchea lanceloata* is formononetin 7-*O*-glucoside (ononin).

Percent seed germination of mustard was not affected significantly by any of the formononetin test solutions (Table 2). However, root and shoot length were inhibited significantly ($P < 0.01$) by all concentrations except root length with S1 (Table 2). An increase in percent inhibition in root length by 18.11% and in shoot by 15.99% was recorded in S3 as compared to S1, showing that inhibition was concentration-dependent.

Formononetin 7-*O*-glucoside (ononin) has been reported in root extracts of *Baptisia australis*, *Glycyrrhiza uralensis*, *Hedysarum polybotrys*, *Ononis spinosa*, and *Trifolium pratense* and in leaves of *Cicer arietinum*, *Glycine max*, and *Trifolium polyphyllum* (Dewick, 1988). However, their availability in the soil and their allelopathic potential have not been determined. Chang et al. (1969) reported the presence of an isoflavonoid in red clover, but its presence in red clover-infested soils was not observed. They found that red clover soil sickness was due to various phenolic acids that were degradation products of isoflavonoids. However, in the present investigation the isoflavonoid formononetin 7-*O*-glucoside detected from the weed-infested soils remained persistent in saline–alkaline soils. Further, it remained stable after being stored for four months, thus suggesting that it is more potent allelopathically by itself as compared to its degraded constituents. The persistence of the isoflavonoid in the soil could be due to its antimicrobial activities (Kramer et al., 1984). However, whether formononetin 7-*O*-glucoside also possesses such characteristics and thus maintains toxic potential, needs further investigation. The mechanism(s) whereby isoflavonoid formononetin 7-*O*-glucoside interferes with the seedling growth of mustard is not clear. However, several suggestions on the effects of isoflavonoids

TABLE 1. FLUORESCENCE, R_f ($\times 100$) VALUES, AND SPECTRAL CHARACTERISTICS OF AUTHENTIC AND ISOLATED ISOFLAVANOID, FORMONONETIN 7-*O*-GLUCOSIDE FROM ASSOCIATED SOILS OF THE WEED, *Pluchea lanceolata*

	Fluorescence[a]		R_f ($\times 100$)[b]				Spectral characteristics					
Source	UV	UV + NH_3	DDW	BAW	HOAc	Forestal	Methanol	NaOH	$AlCl_3$	$AlCl_3$–HCl	NaOAc	NaOAc–H_3BO_3
Authentic[c]	FB	FB	ND	ND	74	ND	258	258	259	257	257	255
							301sh[e]	301sh	300sh	301sh	304sh	302sh
Isolated[d]	B	FB	41	88	71	77	255	255	256	254	256	255
							301sh	301sh	302sh	302sh	302sh	302sh

[a] Fluorescence key: B, blue; FB, fluorescent blue.
[b] Solvent key: DDW, double distilled water; BAW, butanol–acetic acid–water (4:1:5, upper phase); HOAc, 15% acetic acid; forestal, conc. HCl–acetic acid–water (3:30:10). ND, data not available.
[c] Spectral data from Mabry et al. (1970).
[d] Spectral values for the isoflavanoid isolated from the roots and associated soils of the weed.
[e] sh, shoulder.

TABLE 2. EFFECT OF THREE DIFFERENT CONCENTRATIONS (S1, S2, AND S3)[a] OF ISOFLAVANOID, FORMONONETIN ON SEED GERMINATION AND SEEDLING GROWTH OF MUSTARD

Treatment	Seed germination (%)	Seedling length[b] (cm)		Inhibition[c] (%)	
		Root	Shoot	Root	Shoot
Control[d]	88	8.39 ± 2.36	3.94 ± 0.88		
S1	78	7.28ns[e] ± 3.39	2.81*** ± 0.89	13.23	28.68
S2	78	7.14* ± 3.51	2.55*** ± 0.70	14.89	35.28
S3	74	5.76** ± 2.93	2.18*** ± 0.76	31.34	44.67

[a]S1, 10^{-4} M; S2, 5×10^{-4} M; and S3, 10^{-3} M.
[b]Seedling length on sixth day of sowing.
[c]Percent inhibition in comparison to control.
[d]Double-distilled water served as control.
[e]ns, not significant; *$P < 0.09$; **$P < 0.001$; ***$P < 0.00001$.

on physiological processes have been suggested by Koeppe and Miller (1974), Lang and Racker (1974), and Rice (1984). The fact that this isoflavonoid is released into the soil and inhibits the seedling growth of mustard shows that it is a potent allelopathic compound. Furthermore, its rapid leaching with water and persistence in the soil during storage ensure its presence in the soil prior to the sowing of seeds in the fields. The significance of such a release is relevant since this weed is perennial and maintains continuous availability of the compound along with the other allelochemicals. The present study not only establishes the allelopathic potential of the isoflavonoid formononetin 7-*O*-glucoside but also of the weed *Pluchea lanceolata* as it maintains a pool of not only formononetin, but also hesperidin and taxifolin (Inderjit and Dakshini, 1991) as three potential allelochemicals in the soil around its root zone.

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TANNIC ACID INHIBITION OF AMINO ACID AND SUGAR ABSORPTION BY MOUSE AND VOLE INTESTINE: TESTS FOLLOWING ACUTE AND SUBCHRONIC EXPOSURE

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Abstract—The acute effects of tannin (tannic acid; TA) on nutrient absorption were studied by measuring sugar and amino acid uptake across the brush border (luminal membrane) of intact intestine in the presence and absence of TA. Incubation of tissue for 4–9 min in TA solution (1 mg/ml) caused a reduction in passive influx of L-glucose in voles and mice and a reduction in carrier-mediated influx of D-glucose and total influx of L-proline in mice, but not voles. In subchronic experiments, mice and voles were fed for 7–14 days a diet with 4% TA, but there was no significant effect on intestinal brush border uptake of L-glucose, D-glucose, or L-proline (or three other amino acids tested in voles). In a synthesis of our study with others in the literature, three inferences are made from the patterns of effects across solutes, time scales of exposure, and species. First, the transport inhibitory effects following acute exposure are probably mediated by two processes: increased resistance to passive flux across an effective unstirred layer juxtaposed to the brush border membrane, perhaps due to tannin–mucin binding, and reduced Na^+-coupled nutrient uptake across the intestinal brush border. Second, there is a species sensitivity difference in TA's effect on the second process. Third, the negative effects observed at the acute time scale in vitro do not necessarily occur in animals eating TA subchronically because little TA reaches the luminal membrane, or if it does its effects are quickly reversed when the tissue is removed and washed with solution free of TA.

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Key Words—Dietary tannins, tannic acid, digestion, absorption, small intestine, brush border membrane, unstirred layer, mucus, *Microtus*, *Mus*.

INTRODUCTION

Several plant secondary metabolites (PSMs) exhibit characteristics in vitro that suggest important effects on nutrient absorption in vivo. For example, lectins bind specific sugar residues located on the surface of cells, which can cause histological damage (Pusztai, 1979a,b) and impair food digestion (Banwell et al., 1983) and nutrient absorption (Jaffe and Camejo, 1961).

Tannins and phenolics are among the most widespread of the PSMs. Because they complex with natural polymers (for review, see Bernays et al., 1989), it has been suggested that they can act directly on gut enzymes and possibly tan the luminal membrane, thereby blocking nutrient absorption (Swain, 1979). Ingestion of certain varieties of the field bean (*Vicia faba*) containing polyphenolics is associated with reduced sugar and amino acid absorption in chickens (Santidrian et al., 1981). Extracts from the field bean containing polyphenolics reduced D-glucose absorption in vivo in rat ileum (Motilva et al., 1983).

The hydrolyzable tannin, tannic acid, has been the most studied polyphenolic. In rats it was found to erode the mucosal lining of the gastrointestinal tract (Mitjavila et al., 1977) and inhibit enterocyte metabolism (Mitjavila et al., 1968). In isolated rat brush border membrane, tannic acid inhibits sucrase activity (Welsch et al., 1989a) and D-glucose transport (Welsch et al., 1989b).

In this study, we tested for effects of acute and subchronic exposure of tannic acid on intestinal nutrient absorption in laboratory mice and a wild herbivore, the prairie vole (*Microtus ochragaster*). We chose tannic acid because it is an effective protein-binding agent (Bernays et al., 1989) and is more inhibitory to glucose transport than other phenolics that have been tested (Welsch et al., 1989b). We were interested in four issues.

First, we wondered whether the past findings of reduced glucose absorption in response to acute exposure to tannins applied under in vivo conditions. No studies in mammals (to our knowledge) have tested for effects of subchronic ingestion of dietary tannic acid on intestinal absorption, which is the most natural manner of exposure of the enterocytes to a secondary metabolite.

Second, we were interested in tannic acid's possible effects on amino acid absorption. This is practically unstudied even though tannins are known to reduce apparent protein digestion in some cases (Mole and Waterman, 1987).

Third, intestinal nutrient uptake into enterocytes occurs across several barriers by several processes, and we sought to determine which barriers and processes were affected by tannic acid. The barriers are either an unstirred water layer or the mucous coat (Smithson et al., 1981), which are juxtaposed to the

intestinal brush border membrane barrier. The processes include passive and carrier-mediated transport, the latter sometimes Na^+-dependent (hence active). To discriminate among these barriers and processes we used several solutes as probes (see below).

Finally, we considered it important to test another species besides laboratory rats or mice. The generality of tannic acid effects is uncertain, and other studies have documented traits in some animals that ameliorate the potential effects of tannins in the gut (e.g., high gut pH, presence of surfactants, secretion of parotid proteins; reviewed in Bernays et al., 1989).

METHODS AND MATERIALS

Animals. Prairie voles (*Microtus ochrogaster*) were obtained from an outbred colony maintained at the University of Illinois, Urbana. The original stock for the colony was live-trapped in nearby oldfield habitats. Laboratory mice (*Mus musculus*) 8–10 weeks old, were obtained from Sprague-Dawley, Indianapolis, Indiana. Both species were housed in plastic shoebox cages, maintained on a 15:9 hr light–dark regime at 21 ± 2°C, and fed and watered ad libitum. Mice and voles in the acute studies ate standard rodent chow (Purina Formulab), supplemented with alfalfa pellets (Purina Lab Rabbit Chow) in the case of the voles. The tannic acid content of these diets was negligible (unpublished observations). In subchronic studies, both species were fed ad libitum experimental diets with or without tannic acid (TA) (and lacking other PSMs that might be in rodent or rabbit pellets) for 7–14 days. Seven days is adequate for mice to acclimate to TA-containing diets (Freeland et al., 1985).

Experimental Diets. The experimental diets (Table 1) were based on formulations designed specifically for voles (Lindroth et al., 1984), but they were also satisfactory for mice if the fiber content was lowered (personal observation). Therefore, fiber level was set at 15% (Table 1), although in one study with voles it was 40%.

The level of TA incorporated into the diet, 4% (Sigma Chemical Co., St. Louis, Missouri), was chosen because it was enough to cause reduced apparent digestive efficiency but not so much as to cause mass loss and thus nonspecific decline in intestinal structure and function. At 4% there was no significant decrease in daily food intake of either species and both species ate enough to maintain body mass (personal observation; below), whereas at 6% TA mass gain was reduced in voles (Lindroth and Batzli, 1984). Furthermore, apparent protein digestibility was reduced at 4% TA in the mice (Meyer, 1989) and somewhere between 3% and 6% in the voles (Lindroth and Batzli, 1984). The added TA displaced an equal volume of sucrose and corn starch.

TABLE 1. COMPOSITION OF SYNTHETIC DIETS

Component	Components (g/kg diet)
Casein[a]	115.0
DL-Methionine[a]	5.0
Corn starch[a]	252.4
Sucrose[a]	207.0
Fiber[b]	150.0
Corn oil[a]	72.2
Vitamin[a,c]	16.6
Mineral mix[a,d]	16.6
$CaCO_3$[e]	9.4
$Ca(H_2PO_4)_2 \cdot H_2O$[d]	5.8
Tannic acid[d,f]	***

[a] Ingredients from Teklad, Inc., Madison, Wisconsin.
[b] Purified cellulose (solka-floc).
[c] Vitamin mix catalog No. 40060.
[d] Rodent mineral mix catalog No. 79055; custom mineral mix for lagomorphs = 11.94 g $KHCO_3$, 8.55 g K_2HPO_4, 5.0 g NaCl, 3.32 g MgO, 0.9 g $FEC_6H_5O_7$, 0.1 g $MnSO_4 \cdot H_2O$, 0.06 g $ZnCO_3$, 0.04 g $CuSO_4$, 0.004 g $CoCl_2 \cdot 6H_2O$, 0.004 g KIO_3.
[e] Ingredients from Sigma Chemical, St. Louis, Missouri.
[f] In experimental diets, tannic acid (40 g/kg) replaced equal mass of sucrose.

Uptake Measurements. In both mice and voles we measured the uptake of two sugars (L-glucose and D-glucose) that have different utility in discriminating changes in nutrient transport processes. We considered L-glucose at trace concentration a probe for changes in epithelium that affect primarily passive transport (Karasov and Diamond, 1983). These might include change in surface area, permeability of the brush border membrane (bbm) to hydrophilic organic solutes, and resistance of the mucous or unstirred layer adjacent to the membrane to passive solute flux. The mucous/unstirred layer and the brush border membrane constitute two series resistances to solute uptake (see Karasov and Diamond, 1983, and references therein).

We considered D-glucose at saturating concentration a probe for changes in epithelium that affect either the stereospecific glucose transporter or the electrochemical gradient that energizes the Na-coupled transport. This is so for two reasons: (1) because our technique for measuring D-glucose uptake, using L-glucose as a simultaneous marker for adherent glucose and passive glucose uptake (Karasov and Diamond, 1983), yields a measure of the mediated, stereospecific transport of glucose, and (2) because when the membrane transporters are saturated the membrane becomes the primary resistance to glucose uptake and the relative importance of the mucous/unstirred layer diminishes (Karasov and Diamond, 1983).

We also measured the total uptake of L-proline (Pro) in both mice and voles, and three additional solutes [L-leucine (Leu), L-lysine (Lys), and L-aspartate (Asp)] in voles. These four amino acids are transported by the four Na^+-dependent amino acid transporters in vertebrate intestinal brush border membrane (Karasov, 1988). Total uptake of amino acids includes carrier-mediated and nonmediated (passive) pathways. In some cases the uptake of the amino acids was partitioned into its Na-dependent and Na-independent components using Na-free solutions (below).

The preparation for measuring solute uptake across the brush border (not transepithelial flux) was an excised, everted sleeve of intestine 1-cm long, cut from the proximal, middle, and distal small intestine, and secured to a rod with the intestinal mucosa pointing outwards (Karasov and Diamond, 1983). Measurements were begun around noon to reduce effects of any diurnal rhythm in uptake. Rodents were anesthetized (ca. 0.1 ml sodium pentabarbital) and the small intestine isolated and perfused with ice cold Ringer solution. Solution composition (in mM) was 128 NaCl, 20 $NaHCO_3$, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, and 1.2 $MgSO_4$, gassed with 5% CO_2–95% O_2 to yield pH 7.3–7.4, osmolarity 290 mosm.

In experiments testing for an effect of acute exposure, TA was added at a concentration of 1 mg/ml (i.e., 0.1%) because this concentration has been shown to reduce glucose uptake in isolated bbm vesicles (Welsch et al., 1989b) and in perfused intestine (Mitjavila et al., 1970) and because higher concentrations are difficult to solubilize (personal observation). In comparison, during the subchronic feeding trials actual luminal concentration was probably ≤ 8 mg TA/ml because the diet concentration was 40 mg TA/g food and the ratio of food to water in the intestinal lumen was 0.201 ± 0.031 g/ml ($N = 4$) (hence $40 \times 0.201 = 8$). The actual luminal TA concentration, however, may be lower due to the hydrolysis of TA to gallic acid and glucose in the acidic stomach or binding to dietary or endogenous protein (below). In these acute experiments 10 mM HEPES was used as the buffer, and KOH was added to yield a final pH of 7.4. In the control solutions (also buffered with HEPES and KOH), mannitol (ca. 10 mM) was added instead of TA and both solutions had a final osmotic pressure of 290 mosm.

Sleeves were kept in ice cold mammalian Ringer bubbled with 5% CO_2–95% O_2 until an uptake measurement was made. After a preincubation for 5 min in Ringer at 37°C with or without 0.1% TA, the sleeve was incubated for 1–4 min at 37°C in a stirred (1200 rpm) solution containing radioactive tracers with or without 0.1% TA. In those solutions that contained 0.1% TA, bubbling with O_2 probably favored oxidation of TA; a color change to yellow was observed. However, in rats both oxidized and reduced forms of TA inhibit D-glucose transport to the same extent (Welsch et al., 1989b).

Incubation times to yield unidirectional uptake rates were chosen as 1 min

for D-glucose, 4 min for L-glucose, and 2 min for amino acids (L-proline, Pro; L-leucine, Leu; L-lysine, Lys; L-aspartate, Asp) based on our previous studies with mice (Karasov and Diamond, 1983) and results of validation experiments with voles described in the Appendix.

In studies of passive uptake of L-[1-^{3}H[N]]glucose (New England Nuclear), [*carboxyl*-^{14}C]inulin (ICN) was used to correct for L-glucose in adherent fluid. In studies of carrier-mediated uptake of D-[6-^{3}H]glucose (20–40 Ci/mmol; Amersham), L-[1-^{14}C]glucose (30–40 mCi/mmol; Amersham) at trace concentration was used to correct simultaneously for D-glucose in adherent fluid and for D-glucose taken up passively. In studies of total uptake (i.e., sum of carrier-mediated and passive uptake) of L-[2,3-^{3}H]Pro (American Radiolable), L-[4,5-^{3}H]Leu (ICN), L-[G-^{3}H]Lys (ICN) and L-[2,3-^{3}H]Asp (ICN), [*carboxyl*-^{14}C]inulin (ICN) was used to correct for amino acid in fluid adhering to the tissue.

Following incubations, tissues were rinsed or blotted (Karasov and Diamond, 1983), and 1-cm lengths of tissue were removed from the rods for weighing, solubilization, and scintillation counting (Karasov and Diamond, 1983). Other details about measurement of uptake in voles are provided in the Appendix.

Carrier-mediated D-glucose uptake was measured at 50 mM, a saturating concentration in vertebrate intestine studied with the everted sleeve preparation (Karasov, 1988). Uptake at 25 mM Pro was measured in mice because at this concentration the Pro uptake is 75% Na^+-dependent and nearly maximal (the transporter is nearly saturated; Karasov et al., 1986). This concentration was also used in voles, but because Na^+-dependence was lower (below), we also used a concentration of 0.01 mM Pro at which more of the uptake was Na^+-dependent. Pro uptake kinetics had not previously been characterized in voles, and so this was studied in the voles also.

Morphometric Measurements. To test for changes in intestinal gross morphology that may have resulted from TA diet, we measured three intestinal parameters in all rodents: (1) intestinal length (to the nearest 0.5 cm) from pylorus to the ileal–cecal junction, (2) intestinal wet mass per centimeter ($\pm$0.1 mg), and (3) proportion that mass of scraped mucosa bears to mass of whole intestinal thickness, measured by cutting a 2-cm piece of intestine and scraping off the mucosa with a glass slide and determining its dry mass ($\pm$0.1 mg) and that of the remaining tissue. We report most nutrient uptake rates normalized to 1-cm length of intestine, which we consider physiologically most meaningful. However, for studying treatment effects on uptake rates using adjacent tissues from within an animal, we also normalized uptake rates to milligrams of wet mass as recommended by Karasov (1988). To estimate the summed uptake rate of the entire length of the small intestine for each solute, we interpolated rates

linearly between successive positions along the intestine and then summed over the small intestine length.

Statistical Analysis and Presentation of Data. Results are given as means ± SE (N = number of rodents unless indicated otherwise). For comparisons of single measures between control and experimental tissues from within individual voles, paired t tests were performed. For comparisons of single measures between voles, Student's t test was used. Simultaneous tests for effects of intestinal position on parameters within voles, and diet effects on parameters between voles, were determined by a repeated measures ANOVA followed by Tukey's honest significant difference (HSD) test. A one-tailed test was used for testing the a priori hypothesis that incubation in the presence of TA reduces nutrient uptake. The $P < 0.05$ level was considered significant.

For fitting uptake rates to kinetic models to derive kinetic parameters, and for comparing kinetics of uptake rates in different diet groups, we used the procedures recommended by Motulsky and Ransmas (1987). Kinetic models included: (1) simple diffusion; $J = P[S]$ where J = uptake, $[S]$ = substrate concentration, P = permeability coefficient; and (2) diffusion plus one mediated system; $J = P[S] + J_{max}[S]/(K_m + [S])$ where J_{max} = maximal uptake rate, K_m = the transport constant such that $K_m = [S]$ when $J = J_{max}/2$.

RESULTS

Tests for Acute Effects of 0.1% Tannic Acid Solution on Nutrient Uptake

Exposure of mouse intestinal sleeves to 0.1% TA solution caused reduction of total Pro uptake and passive and carrier-mediated glucose uptake (Table 2). Reductions were greater and statistically significant when 1- or 2-min uptake incubations in the presence of TA were preceded by 5-min preincubations in the presence of TA. This suggests a time dependence to the uptake inhibition by TA in mice.

In contrast, in voles total Pro uptake and carrier-mediated D-glucose uptake were not significantly reduced but appeared to increase (Table 2). As in mice, however, passive glucose uptake, measured with L-glucose, was significantly lower in tissues exposed to TA.

We were concerned that the ca. 40% reductions in L-glucose uptake in both species might be artifacts of the overestimation of adherent L-[1-^{14}C]glucose due either to inulin–TA–membrane binding or inulin influx across disrupted membranes, rather than to decreased L-glucose uptake per se. Thus, we also compared inulin and L-glucose spaces, which are simply the amount of radioactivity per unit tissue, normalized to the amount in the incubation solution and

TABLE 2. ACUTE EFFECT OF TANNIC ACID (TA, 1 mg/ml) ON SOLUTE UPTAKE IN VITRO BY MIDINTESTINE[a]

Solute	Uptake normalized	Control (nmol/min)	Incubate with TA (% of control)	Preincubate and incubate with TA (% of control)
Mice				
25 mM L-proline	per mg intestine	4.91 ± 0.13 (6)	92.1 ± 5.1 (6)	90.6 ± 4.8*[b] (6)
	per cm intestine	204 ± 14	97.3 ± 2.9	93.5 ± 8.4
50 mM D-glucose	per mg intestine	6.93 ± 0.43 (6)	98.6 ± 3.7 (6)	79.4 ± 6.2**[b] (6)
	per cm intestine	393 ± 34	96.0 ± 4.1	82.6 ± 6.0**[b]
1 mM L-glucose	per mg intestine	0.055 ± 0.002 (6)	74.9 ± 7.2* (6)	57.3 ± 2.9**** (6)
	per cm intestine	1.69 ± 0.10	79.3 ± 8.0*	66.7 ± 4.0***
Voles				
0.01 mM L-proline	per mg intestine	0.00284 ± 0.0005 (10)	101.2 ± 7.1 (9)	155.8 ± 18.3* (5)
	per cm intestine	0.117 ± 0.015	102 ± 4	162.2 ± 20.9*
25 mM L-proline	per mg intestine	4.35 ± 0.21 (6)	98.5 ± 5.0 (6)	—
	per cm intestine	191 ± 20	90.4 ± 5.5	—
50 mM D-glucose	per mg intestine	4.69 ± 0.44 (12)	100.8 ± 3.2 (11)	107.8 ± 11.6 (5)
	per cm intestine	211 ± 15	100.7 ± 2.8	107.3 ± 9.4
1 mM L-glucose	per mg intestine	0.039 ± 0.003 (14)	75.4 ± 5.8*** (6)	64.2 ± 14.2**** (8)
	per cm intestine	1.59 ± 0.19	71.1 ± 5.7**	58.3 ± 9.3***

[a]Values are means ± SE (N = number of tissues for control, and number of paired comparisons in other cases). Within each row, values that are significantly different from their respective controls are signified with asterisks. Paired t test; *$P < 0.05$; **$P < 0.025$; ***$P < 0.01$; ****$P < 0.001$.

[b]Signifies an *a priori* test, hence one-tailed. Control tissues were adjacent sleeves from the same animals, and comparisons were made of uptake when normalized to both tissue mass and tissue length. Uptake of L-glucose was measured at tracer concentration and normalized to a concentration of 1 mM.

thus having units of microliters per centimeter (Karasov and Diamond, 1983). L-Glucose space of tissues incubated in the presence of TA did appear to be lower (voles, $P = 0.009$; mice, $P = 0.135$; paired comparisons among adjacent tissues within individuals) whereas inulin space in the same tissues was similar in voles ($P = 0.35$) and nonsignificantly higher in mice ($P = 0.115$). Thus, in mice, but not in voles, this artifact might have occurred and contributed to a seemingly larger decrease in L-glucose uptake than actually occurred.

Tests for Effect of Subchronic Intake of 4% Tannic Acid Diet on Body Mass and Gut Structure

Body mass was not significantly different between mice fed TA and control diets (respectively, 22.9 ± 1.6 g vs. 25.7 ± 2.0, $N = 7$ in each case; $P > 0.3$) nor between voles fed the two diets (respectively, 35.3 ± 1.8 g vs. 35.5 ± 1.6, $N = 15$ in each case; $P > 0.9$). Small intestine length also did not

differ significantly with diet in either mice (respectively, 41 ± 1 cm vs. 41 ± 1; $P > 0.8$) or voles (respectively, 28.1 ± 0.6 cm vs. 27.1 ± 0.7; $P = 0.28$). In both species, diet had no significant effect on intestinal mass at any of the three positions tested nor on the percent of intestine mass that was mucosa (Figure 1).

Tests for Effect of Subchronic Intake of 4% Tannic Acid Diet on Nutrient Uptake in Mice

There was no significant effect of diet on either Pro or D-glucose uptake at any position in the small intestine of mice (Figure 2), or summed uptake over the entire length of small intestine for either Pro ($P > 0.5$) or D-glucose ($P = 0.28$). L-Glucose uptake normalized to 1 mM also did not differ significantly between control mice (0.38 ± 0.09 nmol/min/cm, $N = 6$) and TA mice (0.36 ± 0.06, $N = 6$) ($P > 0.8$). Thus, in contrast to the findings in the acute

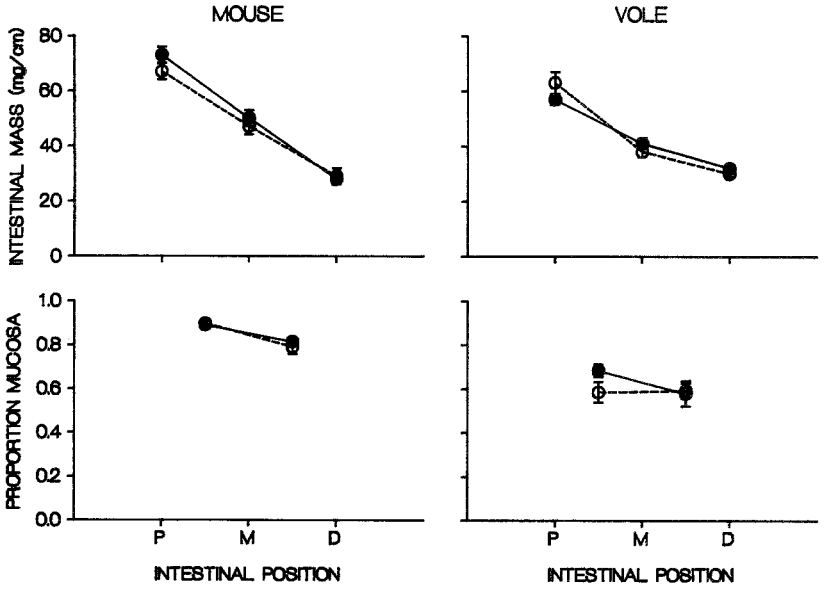

FIG. 1. Intestine wet mass per centimeter (top figures) and proportion of that mass that was mucosa (bottom figures) in mice (left) and voles (right) fed control diet (open symbols) or tannic acid diet (closed symbols). In mice the effect of diet was not significant for either gut mass per centimeter ($P > 0.4$; repeated measures ANOVA) or the proportion of the mass that was mucosa ($P > 0.6$). In voles also the effect of diet was not significant for either gut mass per centimeter ($P > 0.9$) or mucosa mass ($P > 0.4$). In both species gut mass decreased significantly in the distal direction ($P < 0.001$ in both cases), as did mucosa mass in mice ($P < 0.001$) but not voles ($P = 0.17$).

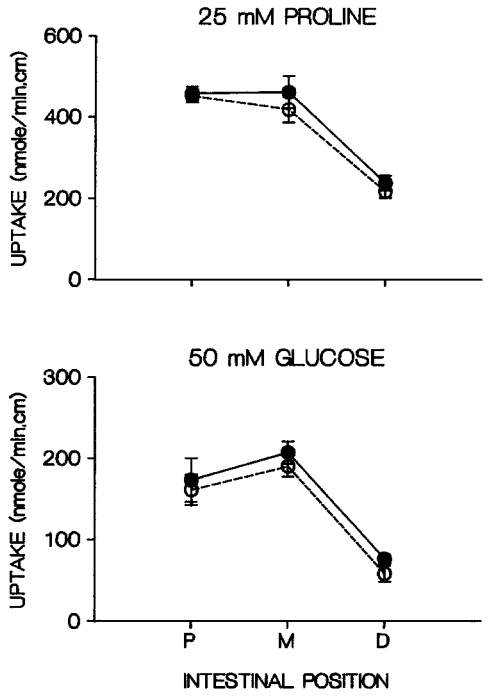

FIG. 2. Total uptake of L-proline per centimeter (top) and carrier-mediated D-glucose uptake (bottom) at three intestinal positions in mice fed control diet (open symbols) or tannic acid diet (closed symbols). P, proximal; M, mid; D, distal. Values are means ± SE ($N = 7$ in each case). Diet had no significant effect on uptake of either L-proline or D-glucose ($P > 0.4$ in each case; repeated measures ANOVA).

experiments, subchronic exposure to TA had no significant effect on passive or mediated uptake of sugar or total uptake of Pro in mice.

Tests for Effect of Subchronic Intake of 4% Tannic Acid Diet on Nutrient Uptake in Voles

Kinetics of Pro Uptake in Midgut. There was no significant effect of diet on Na^+-independent Pro uptake ($F_{2,62} = 0.10$, $P > 0.3$; Figure 3) or total uptake measured in the presence of Na^+ ($F_{2,62} = 1.184$, $P > 0.25$; Figure 3). Na^+-independent uptake was no better fit to a model of diffusion plus carrier-mediated uptake than to a simple diffusion (i.e., linear) model ($F_{2,27} = 0.45$, $P > 0.3$). Therefore, we took the slope derived from the data of both diet groups, 2.7 ± 0.3 μl/min/cm ($N = 11$ voles), as a measure of the apparent passive permeability coefficient (P*) to Pro. Uptake in the presence of Na^+ was better fit to a model of nonsaturable (i.e., passive) plus a carrier-mediated pro-

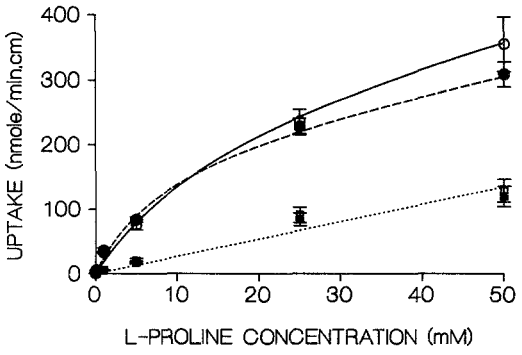

FIG. 3. Total uptake (± SE) of L-proline in the absence (squares) and presence (circles) of Na^+, as a function of concentration in midgut of voles fed for 14 days the control diet (open; $N = 7$ voles) or the tannic acid diet (closed; $N = 7$ voles). Line of dots is linear least-squares fit of Na^+-independent uptake. Solid and dashed curves are nonlinear least-squares fits of uptake in the presence of Na^+ to the equation: uptake = P* × concn + ([J_{max} × concn]/K_m^* + concn]), where P* is the slope of the Na^+-independent line. There was no significant effect of diet (see text). Fitted kinetic parameters for the control voles were P* = 2.5 ± 0.2 μl/min/cm, J_{max} = 212 ± 15 nmol/min/cm, K_m^* = 8.7 ± 2.1 mM; for tannic acid voles, P* = 2.7 ± 0.2 μl/min/cm, J_{max} = 302 ± 59, K_m^* = 18 ± 10.

cess than to one of simple diffusion alone ($F_{1,64} = 45.5$, $P < 0.001$) and could not be computer fit to a model with two carrier-mediated processes. When kinetics of Pro uptake were evaluated for each individual vole and then compared by diet, there was still no significant diet effect on P* ($P > 0.7$), J_{max} ($P = 0.14$), or apparent K_m ($P = 0.12$).

Amino Acid Uptake as a Function of Intestinal Position (Experiments with 40% Solka Floc Diet). There was no significant effect of diet on total Pro uptake at any region of the intestine (Figures 4A and B) whether measured at 25 mM ($P = 0.647$, repeated measures ANOVA) or 0.01 mM ($P = 0.513$) (Figure 4). Neither was there a significant effect on summed Pro uptake at 25 mM ($P > 0.5$). The proportion of total Pro uptake that was Na^+-independent at 0.01 mM was 33.8 ± 7.2% in control voles and 28.5 ± 8.7% in TA voles ($P > 0.5$).

Total uptake of 0.01 mM Leu also was not significantly affected by diet at any intestinal position ($P > 0.5$; Figure 4C). Lys uptake at 0.01 mM in midgut of control voles (545 ± 74 pmol/cm/min, $N = 8$) and TA voles (613 ± 73, $N = 8$) did not differ significantly ($P > 0.5$). Asp uptake at 0.01 mM also did not differ significantly ($P > 0.5$) in control voles (329 ± 50 pmol/cm/min, $N = 8$) and TA voles (379 ± 61, $N = 8$).

Glucose Uptake. Carrier-mediated D-glucose uptake was measured in voles fed diet with and without TA for seven days (Figure 4D). Repeated-measures

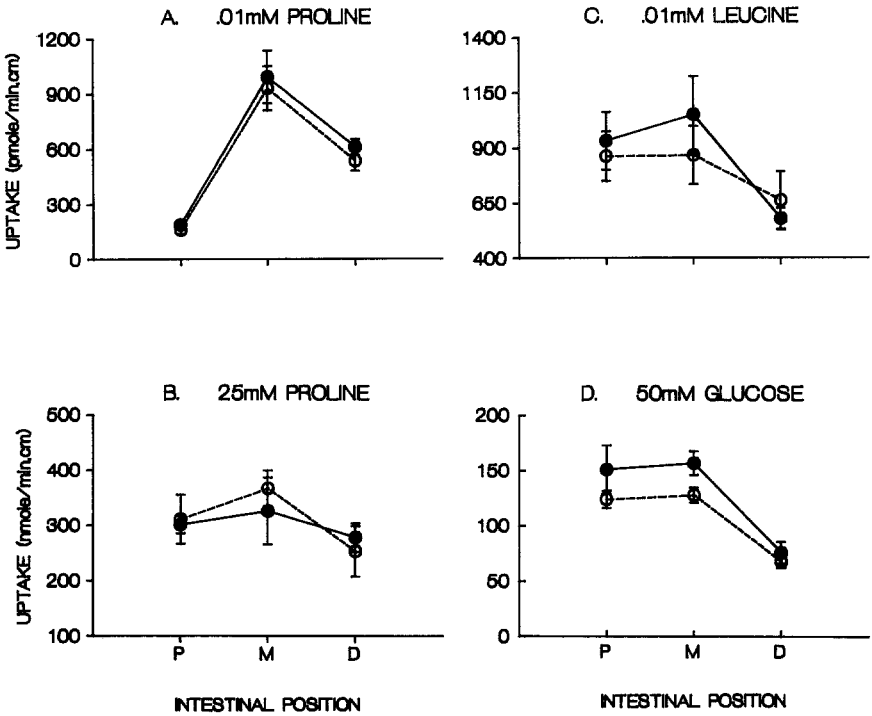

FIG. 4. Total uptake of L-proline per centimeter at 0.01 mM (A) and 25 mM (B), total uptake of L-leucine at 0.01 mM (C), and carrier-mediated D-glucose uptake (D) at three intestinal positions in voles fed control diet (open symbols) or tannic acid diet (closed symbols) seven days. P, proximal; M, mid; D, distal. Values are means $\pm$ SE ($N \geq 5$ voles in each case, with the exception of controls in B where $N = 4$). See text for statistical comparisons.

ANOVA indicated that the former group had significantly higher uptake ($P = 0.049$), but comparisons (Tukey) at each position were not significant for the proximal ($P = 0.265$), middle ($P = 0.055$), and distal ($P = 0.589$) intestine. Summed D-glucose uptake was 23% higher in TA-fed voles (3.88 ± 0.63 μmol/min, $N = 6$) than in controls (3.15 ± 0.15, $N = 6$) ($P = 0.034$) even though intestinal lengths were similar (respectively, 28.6 ± 0.5 cm vs. 28.2 ± 0.9; $P > 0.5$). In the midgut, L-glucose uptake, normalized to 1 mM, did not differ significantly between control voles (1.12 ± 0.10 μl/min/cm, $N = 6$) and TA voles (1.22 ± 0.06, $N = 6$) ($P > 0.4$).

Overall, in voles (as in mice) subchronic exposure to TA had no significant effect on total uptake of amino acids. Carrier-mediated glucose uptake was significantly higher in TA fed voles, but passive glucose uptake was unaltered.

DISCUSSION

Our study indicates a species dependence in the sensitivity of intestinal nutrient absorption to acute exposure to TA. Furthermore, even in a species acutely sensitive (e.g., mice), there are not necessarily parallel decreases in amino acid and sugar absorption in response to subchronic TA exposure, at least at the dietary concentration used in this study. Indeed, the only hint of an effect from subchronic exposure was in the opposite direction—a possible increase in carrier-mediated glucose uptake by vole intestine (Figure 4D).

In the following discussion we attempt to synthesize our results with others in the literature. We first consider the observed acute effects in relation to possible mechanisms of action. Then, we discuss the relation between responses at the acute and subchronic time scales, especially in relation to the broader nutritional picture.

Acute Effects of Tannic Acid on Intestinal Nutrient Absorption. In mice, D-glucose absorption was decreased following exposure to 0.1% TA, whether measured in vivo in intestine perfused with TA (Mitjavila et al., 1970) or in vitro in intact intestine (this study; Table 3). Additionally, mouse intestine

TABLE 3. EFFECTS OF TANNIC ACID ON INTESTINAL NUTRIENT ABSORPTION

Exposure	Nutrient absorption	Species	Preparation	Effect	Reference
Acute (conc. in solution)					
1 mg/ml	D-glucose	Mouse	In vivo, perfusion	−	Mitjavila et al. (1970)
1 mg/ml	D-glucose	Mouse	In vitro, tissue	−	This study
1 mg/ml	D-glucose	Rat	In vitro, bbmv	−	Welsch et al. (1989a,b)
1 mg/ml	D-glucose	Vole	In vitro, tissue	+	This study
1 mg/ml	L-glucose	Mouse	In vitro, tissue	−	This study
1 mg/ml	L-glucose	Vole	In vitro, tissue	−	This study
1 mg/ml	L-glucose	Rat	In vitro, bbmv	0	Welsch et al. (1989a,b)
1 mg/ml	L-proline	Mouse	In vitro, tissue	−	This study
1 mg/ml	L-proline	Vole	In vitro, tissue	0	This study
1 mg/ml	L-methionine	Mouse	In vivo, perfusion	−	Mitjavila et al. (1970)
1 mg/ml	Butyrate	Mouse	In vivo, perfusion	−	Mitjavila et al. (1970)
Chronic (% of diet)					
2.5% of diet	D-galactose	Chicks	In vivo, perfusion	−	Marzo et al. (1987)
4% of diet	D-glucose	Mice	In vitro, tissue	0	This study
4% of diet	D-glucose	Vole	In vitro, tissue	+	This study
4% of diet	L-glucose	Mice	In vitro, tissue	0	This study
4% of diet	L-glucose	Vole	In vitro, tissue	0	This study
4% of diet	L-proline	Mice	In vitro, tissue	0	This study
4% of diet	Pro, Leu, Lys, Asp	Vole	In vitro, tissue	0	This study

absorption rates of methionine (Met) and butyrate were reduced when measured in vivo (Mitjavila et al., 1970), and Pro and L-glucose uptake by intact intestine was reduced (this study; Table 3). At first glance, this uniform pattern across solutes suggests a nonspecific effect of TA, perhaps tanning of either the luminal membrane (Swain, 1979) or of mucin (composed of mucoproteins, glycoproteins, and glycolipids) in the adjacent mucous coat (Mitjavila et al., 1968), hence partially blocking nutrient absorption across either an effective unstirred layer or the brush border membrane (bbm).

Other observations indicate that different, more specific mechanisms are involved. First, in both voles and rats changes in D-glucose (or Pro) uptake were not paralleled by changes in L-glucose uptake (Table 3). Second, in our experiments, which were performed using a saturating concentration of D-glucose as a probe and L-glucose as a marker simultaneously, the significant decrease in stereospecific glucose uptake (Table 2) indicates an effect in addition to that measured solely with L-glucose, an effect on the stereospecific glucose transporter or the electrochemical gradient that energizes the Na^+-coupled transport. Because Pro uptake was also decreased in the mice and is highly (ca. 75%) Na^+-dependent, we suspect an effect on the electrochemical gradient rather than a very specific effect on the glucose transporter.

There is also other evidence suggesting that in rats a more specific effect of TA is on Na^+-coupled transport of sugar and amino acids. Studies with brush border membrane vesicles (bbmv) indicated that uptake of D-glucose was reduced following exposure to TA, but because L-glucose transport was not altered, a change in bbm permeability to passive glucose flux was probably not the cause. Instead, measurements under conditions of Na^+ equilibrium suggested that TA may reduce glucose uptake by altering the Na^+ electrochemical gradient (Welsch et al., 1989b). Welsch et al. (1989b) suggested that the Na^+ gradient might be dissipated by membrane Na permeability changes, but it is also possible that basolateral extrusion of Na (which sets up the gradient) might be inhibited, as suggested by decreased respiration of enterocytes (Mitjavila et al., 1968, 1977) and inhibition of Na,K-ATPase at the basolateral membrane (Lacombe et al., 1976).

There is another aspect of the data on mouse intestine that seems consistent with the hypothesis that, in mice, an effect of TA is reduction of Na^+-coupled transport of nutrients. Within the in vivo study (Mitjavila et al., 1970), and in our in vitro study, glucose absorption was inhibited by TA to a greater extent than was amino acid absorption, and butyrate absorption was inhibited least. This order coincides with the extent to which their absorption is Na^+-dependent. In mouse intestine D-glucose absorption is at least 75% Na^+-dependent (Karasov and Diamond, 1983), Pro and Met absorption are 33–67% Na-dependent at the concentrations tested (Karasov et al., 1986), and butyrate absorption is probably primarily passive (hence, Na^+-independent).

Thus, the most unifying mechanistic explanation of the negative impact of acute TA exposure on intestinal nutrient absorption (Table 3) is that there are two effects. One involves inhibition of passive flux, and it is most apparent in measurement of flux of solutes whose transport is primarily passive (such as L-glucose, butyrate). Because it was not observed in the rat bbmv preparation, which lacks the mucous coat (Smithson et al., 1981), it probably involves an increase in resistance to solute flux due to fixation of TA by mucin (Mitjavila et al., 1968). The other effect involves inhibition of Na^+-coupled transport, which is most apparent in measurement of flux of solutes whose transport includes this pathway (such as D-glucose, amino acids). Both effects appear to occur rapidly following exposure of intestine to TA, because all of the acute effects documented in Table 3 occurred following exposures of ≤ 30 min.

The reason why the apparent effect on Na-coupled transport is not observed in vole intestine is unknown. One interesting idea is that a higher level of mucin, and hence a greater TA fixation capacity, might act as a protective barrier against the toxic effect of TA on enterocytes. Studies with bbmv (e.g., Welsch et al., 1989b) can help discriminate between hypotheses involving extracellular and membrane events.

Relationship between Effects of Acute and Subchronic Exposure to Tannic Acid. Whatever the explanation for reduced nutrient absorption following acute exposure to TA, it is remarkable that subchronic ingestion of TA had no significant effect on nutrient absorption in mice or voles (Figures 2–4). The answer to this paradox may be that little TA reaches the luminal membrane, or that if it does, its effects are quickly reversed when the tissue is washed with solution free of TA.

Factors that could minimize contact between ingested TA and the intestinal luminal membrane include acid hydrolysis to glucose and gallic acid, and preintestinal binding into insoluble protein–tannin complexes. Some TA might be hydrolyzed in the acid environment of the stomach (although we do not know of any tests of this), and the gallic acid moiety appears to have a negligible effect on glucose absorption (Welsch et al., 1989b) or fecal N excretion (Glick and Joslyn, 1970). The parotid glands of mice increase in mass by 700% after four days on tannin-containing diets (Freeland et al., 1985) and secrete copious quantities of proline-rich proteins with a strong binding affinity for tannins (Mehansho et al., 1983, 1985). Whether this occurs in voles is unknown, but the fact that they cannot subsist on quebracho tannin diet (Lindroth and Batzli, 1984; Meyer, 1989) whereas mice can (Freeland et al., 1985; Meyer, 1989) suggests that they lack this parotid adaptation or some other protective trait.

It is also possible that even if TA reaches the luminal membrane and elicits its toxic effects, those effects are quickly reversed once the TA is flushed away when the tissue is washed with solution free of TA (or by a TA-free meal in vivo). In this regard it is instructive to note that when rat intestine was perfused

with TA, the inhibition of absorption of glucose, Met, and butyrate was reversed following perfusion with tannin-free solution (Mitjavila et al., 1970).

Overview. The effects of TA and other tannins on intestinal nutrient absorption, and on digestion in general, appear to us complex. This is partly because our results and analysis indicate that when tannins interact with intestinal tissue they may reduce nutrient uptake by enterocytes by at least two mechanisms—binding with mucin, which may reduce passive flux across an effective unstirred layer juxtaposed to the brush border membrane, and toxic effects on enterocyte function, which reduces Na^+-coupled nutrient transport across the brush border membrane.

Whether these effects actually occur in vivo may depend on the amount of tannin reaching the luminal membrane. This amount may be reduced by pregastric binding into insoluble complexes, gastric hydrolysis, and intestinal hypersecretion of mucin. Thus, effects on digestion at the whole-animal level are not easily predicted from studies conducted at the in vitro level.

Finally, an added complication is that there appear to be notable differences between species in physiological response to tannins at the tissue and whole-animal level. Mouse intestinal tissue appears to be more sensitive to TA than vole intestine. Chickens may be even more sensitive to TA than either mice or voles, because ingestion of TA-containing diet is associated with reduced intestinal sugar absorption and reduced growth (Marzo et al., 1987).

These species differences in TA and tannin effects on nutrient absorption and nutrition preclude broad generalizations about nutritional or ecological effects of tannins, but they do offer a comparative approach for the study of the mechanisms of action of tannins at the cellular, tissue, and whole-animal level.

APPENDIX: SELECTION OF INCUBATION TIME

We previously found that 2-min and 1-min incubations yielded measures of unidirectional uptake of amino acids and glucose, respectively, in mice (Karasov and Diamond, 1983; Karasov et al., 1986). We performed the following tests to verify whether this was also the case for voles.

We incubated tissues for various times in Ringer solution containing tracer inulin and Pro or Leu (50 mM) and determined tissue uptake after blotting the tissue. Adjacent tissues from the same voles were incubated to minimize interindividual variation; one piece incubated for 2 min was a control and the adjacent piece was incubated for 1 or 4 min.

Pro uptake proved to vary linearly with time to at least 2 min, as evidenced by uptakes at 2 min that were 1.78 ± 0.11 times uptakes at 1 min ($N = 6$ pairs of tissues; not significantly different from expected value of 2.0, $t_5 = 2.0$, $P > 0.1$). Uptakes at 4 min, however, were significantly less than double the

uptakes at 2 min (ratio = 1.74 ± 0.1, $N = 6$ pairs of tissues, $t_5 = 2.6$, $P < 0.05$). A smaller number of comparisons for Leu uptake suggested a similar pattern for that solute: ratio 2 min/1 min = 1.763 ± 0.16 ($N = 3$, $P > 0.2$ for difference from 2.0); ratio 4 min/2 min = 1.43 ± 0.13 ($N = 4$, $P < 0.025$ for difference from 2.0). Inulin space, which was used to correct measured amino acid uptake for amino acid in the adherent fluid, was similar at 1 min (0.36 ± 0.02 μl/mg, $N = 7$ tissues), 2 min (0.39 ± 0.02, $N = 8$), and 4 min (0.40 ± 0.02, $N = 8$). Thus, the inulin space had equilibrated by 1 min.

For D-glucose, 1 -min incubations apparently underestimated unidirectional uptake because uptake for tissues incubated for 1 min were 1.634 ± 0.063 times uptakes at 0.5 min ($N = 6$ pairs of tissues, $t_5 = 5.81$, $P < 0.005$ for difference from 2.0). Uptakes at 2 min were 1.655 ± 0.147 times uptakes at 1 min ($N = 7$, $t_6 = 2.347$, $P = 0.06$).

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ACTIVITY OF PERFLUOROBUTYL-CONTAINING COMPONENTS IN PHEROMONE BLEND OF CABBAGE LOOPER MOTH, *Trichoplusia ni*

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Abstract—Substitution of nine hydrogens by nine fluorines in the butyl terminus of (Z)-7-dodecenyl acetate, the major component of the pheromone blend of the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae), produces a highly volatile analog that is significantly less active as a replacement for the natural pheromone component, either alone or substituted in the six-component pheromone blend of this species. When 9,9,10,10,11,11,12,12,12-nonafluoro-(Z)-7-dodecenyl acetate (nf-Z7-12:OAc) was tested in a wind tunnel as a single component or as part of the six-component blend, males exhibited preflight activation, but they did not orient in the pheromone plume and fly upwind over a five-decade range of doses at levels comparable with those of the natural component and blend. Addition of large amounts of nf-Z7-12:OAc to the optimal dose of the natural blend also did not effect flight arrestment as when increased amounts of Z7-12:OAc were added. The nonafluoro (nf) analog of (Z)-9-tetradecenyl acetate, a minor component in the *T. ni* blend, did not affect male behavior when substituted in the six-component blend, and substitution of both nf analogs in the natural blend produced the same results as observed for blends containing nf-Z7-12:OAc. The lack of an effect with substitution of nf-Z9-14:OAc is discussed in relation to the reported redundancy in the action of minor components in *T. ni*.

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Key Words—(*Z*)-7-Dodecenyl acetate, (*Z*)-9-tetradecenyl acetate, pheromone blend, *Trichoplusia ni*, flight behavior, flight tunnel, pheromone analog, perfluoroalkyl substitution, Lepidoptera, Noctuidae.

INTRODUCTION

Partial substitution of fluorine for hydrogen in pheromone components can lead to analogs with varying amounts of activity on pheromone perception by male moths. For example, three different modifications of the major component of the European grape berry moth *Eupoecilia ambiguella* pheromone, Z9–12 : OAc, were examined in field-trapping and flight-tunnel assays. An allylic difluoro analog was as attractive as the parent compound, but the trifluoromethyl and the pentafluoroethyl analogs were inactive by themselves. When large amounts of pentafluoroethyl analog were added to the main component, Z9–12 : OAc, it appeared to mimic the effect of dodecyl acetate in increasing trap catch (Rauscher et al., 1984; Sun and Prestwich, 1990; Bengtsson et al., 1990). A variety of perfluoroalkyl analogs have been examined using single-cell and electroantennogram methods with *Heliothis zea* (Z9–14 : Al and Z11–16 : Al), the southwestern corn borer *Diatraea grandiosella* (Z9–16 : Al and Z11–16 : Al), and with the cabbage looper *Trichoplusia ni* (Z9–14 : OAc and Z7–12 : OAc). In each of the analogs, the alkyl chain on the alkene was replaced by a completely fluorinated alkyl chain of the same length (Prestwich et al., 1990). In each of the three insect sensory systems, the perfluoroalkyl-containing pheromone analogs were recognized by the same cell that responded to the parent alkyl compound; however, an analog concentration 1000–10,000 times higher was required for equal output.

Orientation of male moths to the natural pheromone blend, to partial blends, and to single components has been extensively evaluated for *T. ni* (Linn et al., 1984, 1985). Moreover, many fundamental studies on the electrophysiology of pheromone perception by single olfactory sensilla have been performed with *T. ni* (Prestwich et al., 1990; see Todd et al., 1991). For these reasons, we chose to compare the effects of the electrophysiologically active nonafluoro-Z9–14 : OAc (nf-Z9–14 : OAc) and nf-Z7–12 : OAc analogs of the natural components on moth behavior.

METHODS AND MATERIALS

Insects. Moths were obtained from a laboratory colony maintained for the past two years from field-collected pupae in Geneva, New York. The moths were reared on a semisynthetic medium (Shorey and Hale, 1965), on a 16 : 8 hr light–dark photoperiod at 25°C, 50–60% relative humidity, unless otherwise

noted. Males were separated from females as pupae. Adult males were segregated daily by age, provided with 5% sugar water, and kept under similar conditions to those during rearing.

Chemicals. The *T. ni* sex pheromone is a six-component mixture comprised of 12:OAc (6.8%), Z5–12:OAc (7.6), Z7–12:OAc (100), 11–12:OAc (2.3), Z7–14:OAc (0.9), and Z9–14:OAc (0.6) (Bjostad et al., 1984). A standard solution of this blend was prepared in hexane and checked on capillary GLC (45-m Carbowax 20 M column) to ensure chemical purity of individual compounds (>99%), with a detection limit of 0.1%. Three similar solutions were prepared at the same time: one with the nf-Z7–12:OAc substituted for Z7–12:OAc, one with nf-Z9–14:OAc instead of Z9–14:OAc, and one with both analogs instead of the parent compounds. In addition, standards of Z7–12:OAc and nf-Z7–12:OAc were prepared. The nonafluoro analogs (Figure 1) were synthesized as described previously (Prestwich et al., 1990). Materials were purified on silica gel and evaporatively distilled prior to use. Capillary GLC (DB-5, 0.21 mm × 30 m) verified 99.9% homogeneity for materials for bioassay.

Dilution series of the four blends, the Z7–12:OAc, and nf-Z7–12:OAc solutions were then prepared and were applied in 100-μl amounts to red rubber septa (5 × 9 mm, A.H. Thomas Company) to achieve the desired doses.

Procedure for Testing Insects. The flight behavior of individual males was observed in the flight tunnel of Miller and Roelofs (1978). General flight-tunnel conditions were 0.3 lux scotophase, 50–55 cm/sec air velocity, 23°C, 40–70% relative humidity. Males of the appropriate age (3–4 days old) were taken to the room housing the flight tunnel 2 hr prior to onset of scotophase, placed individually in 10 × 5-cm screen cages provided with a water-soaked dental wick, and then onto a platform adjacent to the tunnel under the same light intensity and temperature as experienced in the tunnel itself, to acclimate to

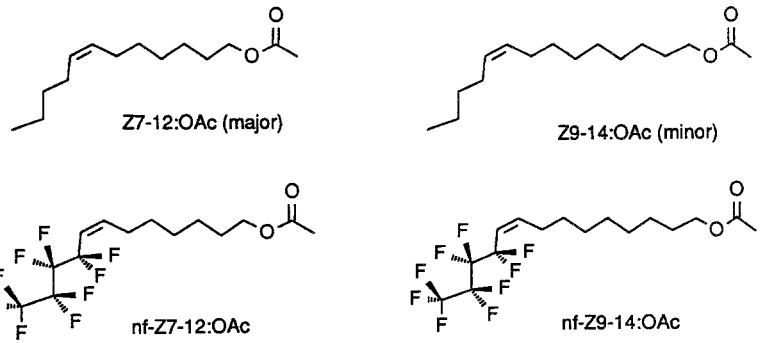

FIG. 1. Structures of major and minor pheromone components and their nf analogs.

tunnel conditions. Response to pheromone was observed by placing the cage containing a male in the odor stream at a distance of 1.5-m downwind from the source. Males were allowed 60 sec to respond and were scored for three key behaviors typically exhibited in the flight tunnel: taking flight, initiation of upwind flight, and source contact (Linn et al., 1984). In addition to the behaviors themselves, the time taken to execute each behavior was recorded, using a mini-computer. Males were tested once and then discarded.

Males were first tested to a dose-series of the Z7–12:OAc and nf-Z7–12:OAc components alone. Each test day 10 different males were tested at each of five doses (0.01, 0.1, 1, 10, and 100 μg), with replicates run until 50 males were tested with each compound–dose combination. Males were then tested with the four blend combinations. Each test day five different males were tested with each blend at the five doses indicated above. Replicates were run until 50 males were tested with each blend–dose combination. Finally, males were tested with a series of 10-μg sources of the natural blend containing excess amounts of either Z7–12:OAc or the nf-Z7–12:OAc component. Each test day 10 males were tested with each of eight combinations (see Figure 5), with replicates run until 50 males were tested with each treatment. For all tests, comparisons were made at each dose between the natural component or blend and the nonafluoro-containing analogs or blends, using the method of adjusted significance levels for proportions (Ryan, 1960).

RESULTS AND DISCUSSION

The number of source contacts following upwind flight over a 1.5-m distance by male *T. ni* to Z7–12:OAc alone, the natural six-component blend, nf-Z7–12:OAc, and blends with the substituted nf analogs are shown in Figure 2. Several features are noteworthy. First, the dose–response curve for the natural blend peaks at a lower loading than does Z7–12:OAc alone. This result was expected based on previous flight-tunnel studies with *T. ni*. In two studies (Linn et al., 1984, 1985) it was shown that response thresholds for the natural six-component blend are significantly lower than for the major component alone.

Second, with nf-Z7–12:OAc, either alone or as a substitute for Z7–12:OAc in the blend, males did not fly upwind and did not make source contact over a series of doses at which Z7–12:OAc or the six-component blend elicited responses for this species. This lack of response also was not entirely unexpected based on the results of electrophysiological measurements. In a previous study with *T. ni* (Prestwich et al., 1990) it was shown that single sensilla responded in a dose-dependent and analog-specific fashion to nf-Z7–12:OAc and nf-Z7–12:OH. However, in these single-sensilla studies there was a significant shift in the dose–response curve with these compounds, with a threshold at 10 μg

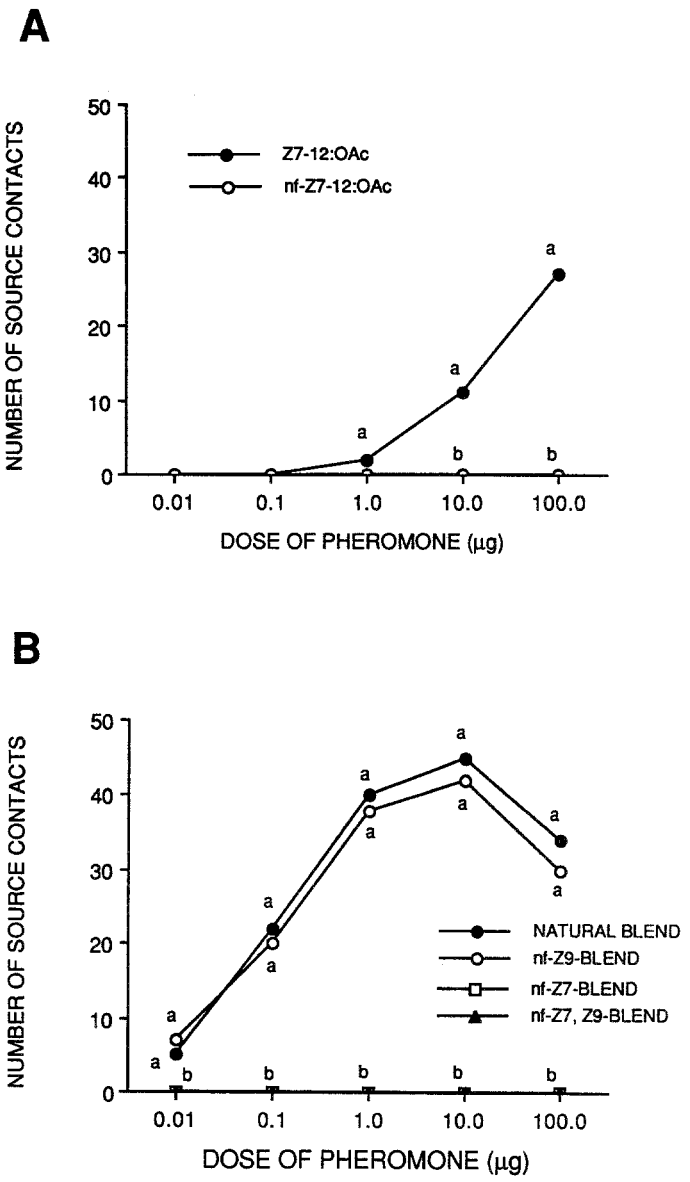

FIG. 2. Number of source contacts by male *T. ni* to a dose series of the Z7–12:OAc or the nf-Z7–12:OAc component alone (A) and to the natural blend or blends containing substituted nf-Z9–14:OAc, nf-Z7–12:OAc, or both nf-analogs (B). $N = 50$ for each source and dose combination. Small letters indicate significant differences ($P < 0.05$) between treatments at any given dose, according to the method for adjusted significance levels (Ryan, 1960).

for the nf-Z7–12:OAc component compared with 0.02 μg for Z7–12:OAc. Thus, one interpretation is that the nf-Z7–12:OAc component results in a weaker interaction with receptor sites for Z7–12:OAc, requiring greater amounts of the material for a given level of neural stimulation to occur.

Third, substitution of the nf analog of Z9–14:OAc, a minor component, to the six-component blend, also had no effect on male response, and once again this was expected on the basis of previous studies. In Prestwich et al. (1990), electrophysiological measurements showed that, as with the nf-Z7–12:OAc component, sensillum cells specific for Z9–14:OAc responded to the nf analog, but at a significantly higher dose–response range. Since there is such a small amount of this component in the blend to begin with, a weaker interaction with the nf-Z9–14:OAc component would render it almost undetectable when present at normal levels that elicit maximal response from males. This would suggest that substitution of nf-Z9–14:OAc in the blend effectively results in males being exposed to a five-component blend, a situation that with many other species would result in a lowered response to pheromone. However, in Linn et al. (1984) it was reported that, whereas the major component Z7–12:OAc was required in the blend for any male response to occur, the minor components could be selectively removed from the blend without affecting maximal response to the blend. This redundancy has been supported recently by electrophysiological studies at the antennal level by Todd et al. (1991). Of particular interest with respect to the present study was the observation by Linn et al. (1984) that, whereas the Z7- and Z9–14:OAc components could be selectively removed without affecting behavior, removal of both compounds resulted in a four-component blend with significantly lower activity. Thus we would expect no effect on male response levels by removal of Z9–14:OAc, such as in the present case with substitution of the much weaker nf-Z9–14:OAc, but we would predict that substitution of both nf-Z7- and nf-Z9–14:OAc in the blend would result in a significant decrease in upwind flights and source contacts.

The data presented in Figure 2 are for source contact, the terminal behavior in the response sequence observed in the flight tunnel. To establish whether there was any effect of the nf analogs on male response, more detailed behavioral profiles for the 1-, 10-, and 100-μg doses from Figure 2 are shown in Figure 3. With the single components (Figure 3A) or the blends (Figure 3B) there was an increase in the number of males taking flight with increased dose, although there were no significant differences in the number of males taking flight to the nf-containing vs. natural treatments at the two higher dose levels. The values for taking flight to treatments containing substituted nf analogs were significantly greater than spontaneous levels of flight activity observed, indicating that males were able to detect the treatments containing the nf-Z7–12:OAc. However, at all three doses, although there was an increase in the number of males flying upwind and making source contact to the sources containing Z7–12:OAc alone

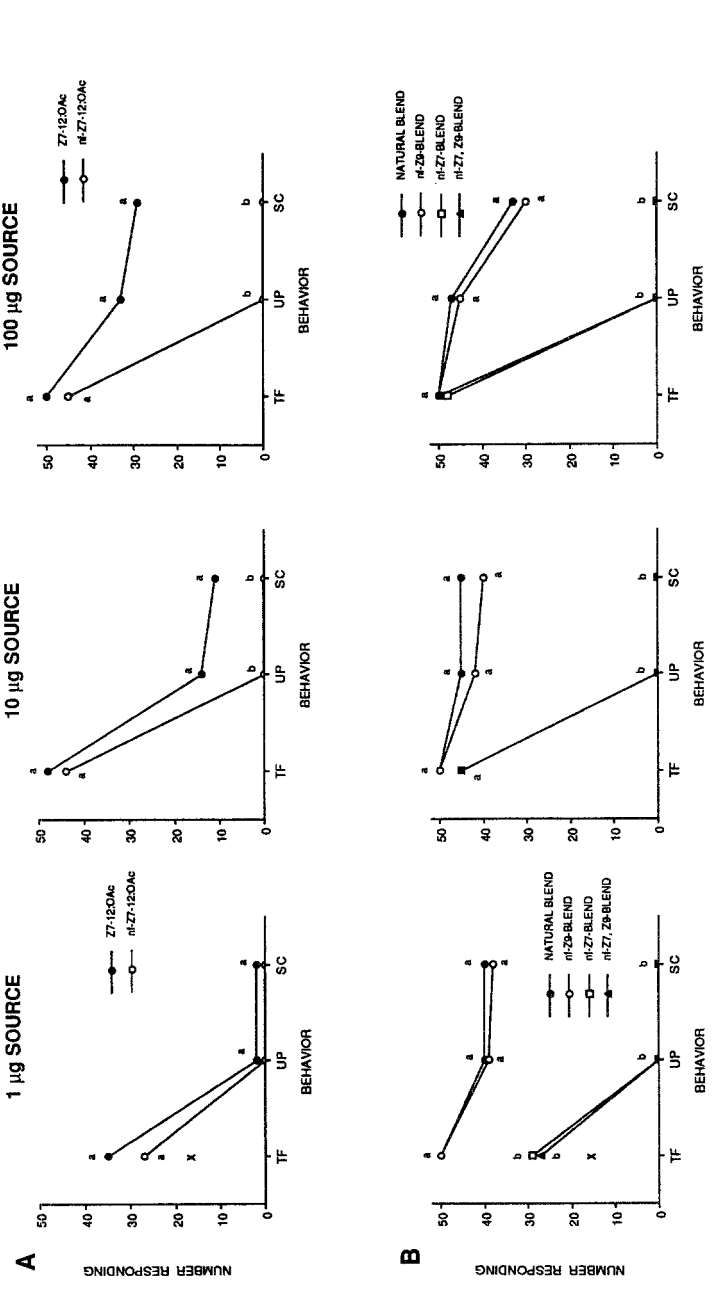

FIG. 3. Number of male *T. ni* exhibiting three behaviors in the upwind flight sequence (TF = taking flight, UP = upwind flight, SC = source contact) to the 1-, 10-, and 100-μg sources of *Z*7–12 : OAc or nf-*Z*7–12 : OAc alone (A) and to the natural blend or blends containing nf-*Z*9–14 : OAc, nf-*Z*7–12 : OAc, or both nf analogs (B). $N = 50$ for each source and dose combination. Small letters indicate significant differences ($P < 0.05$) between treatments at any given dose, according to the method for adjusted significance levels (Ryan, 1960). The X mark for TF at 1 μg indicates the level of nonpheromone stimulated spontaneous flight.

or the natural blend, these behaviors did not occur with substitution of the nf-Z7–12:OAc analog. In fact, in no case did a male attempt to orient in the odor plume to a treatment when substituting nf-Z7–12:OAc for the natural compound.

We also noted that, whereas there was no difference in the number of males taking flight to natural vs. nf-containing treatments, there was a marked difference in the temporal aspects of the response pattern, with males responding to nf-containing sources taking significantly longer to do so compared with the natural components or blends (data are shown in for the 1-μg source in Figure 4). This phenomenon of an increased latency to response, as well as an increased level of pre-upwind-flight activities, has also been observed for the flight-tunnel responses of the European grape berry moth to a perfluoroethyl analog of Z9–12:OAc (Bengtsson et al., 1990).

The above results taken together suggest that the nf-Z7–12:OAc compound has an extremely weak interaction with receptor sites for Z7–12:OAc, resulting in a weaker stimulus discharge of the antennal neurons. That this is the case, as opposed to an hypothesis that the nf analog blocks sensory processing, is demonstrated by the last two experiments on arrestment and habituation. Figure 5 shows that addition of 10 μg of Z7–12:OAc to the source containing 10 μg of the blend did not affect male behavior, but when 100 or 1000 μg of Z7–12:OAc was added, male response decreased. This is as expected from the dose–response curve in Figure 1 showing that with a 100-μg source of the natural blend, male source contacts decreased as a result of increased levels of arrested

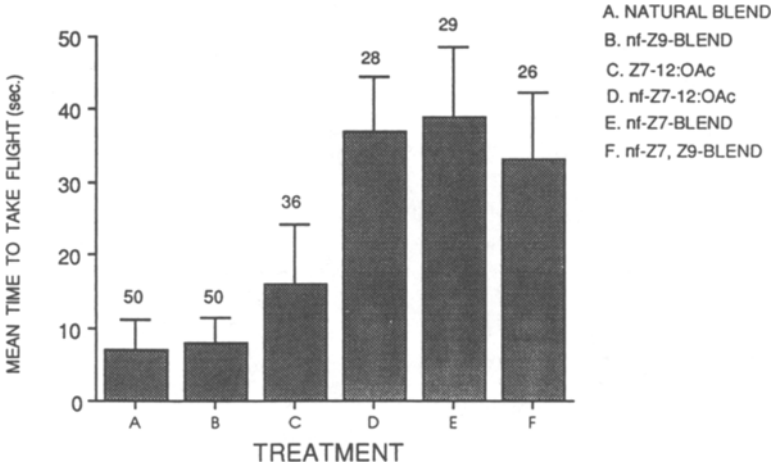

FIG. 4. Time taken for male *T. ni* to take flight to the series of 1-μg sources. Values are means $\pm$ SD, with *N* for each treatment indicated above each bar.

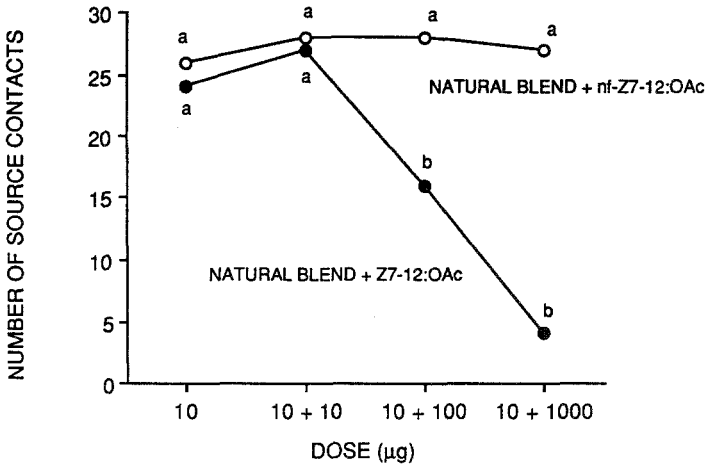

FIG. 5. Number of source contacts by male *T. ni* to a 10-μg dose of the natural blend, with added amounts (10, 100, or 1000 μg) of nf-Z7–12:OAc or Z7–12:OAc. $N = 50$ for each source and dose combination. Small letters indicate significant differences ($P < 0.05$) between treatments at any given dose, according to the method for adjusted significance levels (Ryan, 1960).

upwind flight to this high dose (see Figure 3B, 100 μg; also Baker, 1985). However, addition of 1–1000 μg nf-Z7–12:OAc to the natural 10-μg blend source did not have an affect on male response to the 10-μg source. Once again, these data support the hypothesis that the nf-Z7–12:OAc compound is significantly less active as a replacement for the natural pheromone component.

In an experiment relating to habituation, males were first exposed to a 100-μg source of nf-Z7–12:OAc alone for 2 min. During this time they were not allowed to initiate flight from the release cage. They were then removed from the odor stream in the tunnel and retested with a 10-μg source of the blend 3 min later. A control group that was not exposed to nf-Z7–12:OAc was also tested. With the 100-μg nf-Z7–12:OAc source all of the males tested ($N = 25$) became active in their release cages and exhibited sustained activity over the 2-min period. When tested with the natural 10-μg blend source 3 min later, however, 25 males activated, 25 took flight, and 23 reached the source. Of the control group, 25 activated and took flight, with 21 reaching the source. These results confirm that although males appear able to detect the presence of nf-Z7–12:OAc, as evidenced by their activity during the 2-min exposure, the analog does not affect male perception of the natural pheromone signal. The results of our experiments support the notion that nf-Z7–12:OAc in *T. ni* is more "slippery" in its interaction with antennal receptor sites compared with the natural

components (Prestwich et al., 1990; Prestwich, 1991) and had no effect on male behavior when added to the natural pheromone blend.

Synergism, here meaning an increase in trap capture, had been reported previously by Bengtsson et al. (1990) in field responses of the European grapeberry moth to lures containing a fixed amount of Z9–12:OAc and increasing doses of the perfluoroethyl analog, 11,11,12,12,12,-pentafluoro-Z9–12:OAc (pf-Z9–12:OAc). pf-Z9–12:OAc alone elicited no upwind flight or source contacts with the lure; only activation of preflight activities was noted. In addition, this compound was inactive in the field. However, when added to 100 μg Z9–12:OAc, quantities of 100, 500, and 2000 μg pf-Z9–12:OAc effected trap catch increases of two-, four-, and sevenfold. Interestingly, this effect on trap capture with Z9–12:OAc also occurs when 12:OAc is added (a fivefold increase in amount of 12:OAc added to Z9–12:OAc increases trap catch by a factor of 2–7; Rauscher et al., 1984), which suggests the possibility that the pentafluoroethyl analog is mimicking the effect of the saturated compound.

Both behavioral and electrophysiological results with perfluorinated analogs of moth pheromone components appear to support the importance of the hydrophobicity of the terminal alkyl group in pheromone-receptor protein interactions (Prestwich, 1991). When terminal butyl or hexyl groups were replaced with perfluoro groups, male antennal olfactory receptor neurons from four moth species (*T. ni*, *H. zea*, *D. grandiosella*, and *Agrotis segetum*) showed 100- to 10,000-fold lower activities relative to the parent compounds (Prestwich et al., 1990; Wu et al., 1991). Negligible poststimulus firing was also observed. With respect to effects on behavior, in *E. ambiguella*, pf-Z9–12:OAc was inactive when tested alone, but increased trap catches when added to the natural pheromone component. However, in the case of *T. ni* and *A. segetum*, when nf-Z7–12:OAc (*T. ni*) or nf-Z5–10:OAc (*A. segetum*) were substituted in the six- and three-component pheromone blends, male response was the same as that observed to respective blends missing the Z7–12:OAc or Z5–10:OAc components. In the case of *T. ni*, this resulted in no upwind flights, whereas in *A. segetum* upwind flight levels and source contacts were significantly reduced, but not eliminated, since males in this species are able to fly upwind to the remaining two-component blend of Z7–12:OAc and Z9–14:OAc.

Overall, the replacement of terminal alkyl groups with perfluoroalkyl groups in these pheromone components produced compounds with increased volatility, displaced electrophysiological dose–response profiles, and significantly lowered biological activity (Prestwich et al., 1990). Because of diminished receptor cell sensitivity, it has been proposed that the binding of the fluorinated analogs to putative receptors is reduced as a result of a less favorable interaction between the hydrophobic protein binding site and the more rigid and more polar lipophobic perfluoroalkyl moiety (Prestwich, 1991). In contrast to reports for lepidopteran pheromone systems, however, the Rothamsted group has observed that

a heptadecafluoro compound, in which the *n*-octyl group in a mosquito oviposition attractant pheromone was replaced by a perfluorooctyl group, retained high biological activity in the field and in the laboratory (Dawson et al., 1990).

The current model of pheromone perception (see Prestwich, 1991) implicates at least three types of proteins that recognize and bind to the pheromone molecules (Vogt et al., 1990; Breer et al., 1990). Thus, the ultimate electrical signal produced by the pheromone–sensillum interaction is potentially modulated by three protein–ligand interactions: pheromone–PBP (pheromone binding protein), pheromone–SE (sensillar enzyme), and pheromone–RP (receptor protein). At each level, the perfluoroalkyl components may compete with pheromone molecules for the binding sites. This complex interplay allows for rapid signal transduction and stimulus degradation, and varying fluorinated analogs can affect these equilibria differently.

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DIEL PERIODICITY AND INFLUENCE OF AGE AND MATING ON SEX PHEROMONE TITER IN GYPSY MOTH, *Lymantria dispar* (L.)

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Abstract—The diel periodicity of sex pheromone titer from pheromone glands of female *Lymantria dispar* is described. On the day of emergence (day 0), pheromone titer was generally low; means ranged from 1 to 4 ng *cis*-7,8-epoxy-2-methyloctadecane during photophase and gradually increased to 8.4 ng over the course of scotophase. For day-1, -2, and -3 females, the diel fluctuations of titer were more pronounced. Lowest titers (5–9 ng) occurred 0–4 hr after lights-on, and peak titers (19–32 ng) were found 0–4 hr before lights-off. Comparison of the average daily titer among the different age groups (data pooled over six time points at 4-hr intervals) indicated that significantly less pheromone was extracted from glands of day-0 (4.5 ng) than day-1 (12.4 ng), day-2 (15.4 ng), or day-3 females (13.5 ng). No significant differences were found among the three older ages. Females *in copula* exhibited a rapid reduction in titer within the first 0.5 hr of mating initiation (7.6 ng vs. 19.5 ng from virgin females of similar age). After the second 0.5 hr, the reduction in titer was not nearly as marked, falling only to 4.5 ng. Twenty-four hours after mating, titer fell below the limits of detection (0.5 ng). All extracts from pheromone glands of virgin or mated females contained <1.0 ng of the putative pheromone precursor, 2-methyl-*cis*-7-octadecene.

Key Words—*Lymantria dispar*, Lepidoptera, Lymantriidae, pheromone gland titer, diel periodicity, age effect, mating effect, *cis*-7,8-epoxy-2-methyloctadecane, 2-methyl-*cis*-7-octadecene.

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INTRODUCTION

In the female gypsy moth, *Lymantria dispar* (L.), the rate of pheromone emission from naturally calling females exhibits a characteristic diel periodicity (Charlton and Cardé, 1982). At 24°C, pheromone release rates were at their lowest during the interval shortly before and after lights-on. During photophase, emissions gradually increased, peaked during late photophase–early scotophase, then decreased over the course of scotophase. This pattern, however, was not mirrored by the periodicity of calling behavior. At 24°C, the percent female calling fell to its lowest (45%) during the interval shortly before and after lights-on; at all other times, 100% of the females exhibited calling behavior (Charlton and Cardé, 1982). To understand how emission rates fluctuate during periods when there are no overt changes in the level of calling, a description of the diel periodicity of pheromone titer in the gland is necessary.

Other factors, such as age and mating, also influence pheromone production. Early field studies demonstrated that traps baited with gland extracts from newly eclosed gypsy moth females caught few males, whereas those baited with gland extracts from older females caught large numbers (Collins and Potts, 1932). Quantification of disparlure at 4 hr after lights-on has shown that the titer of pheromone increased with age, peaked four days after the day of emergence, and then fell back to low levels in females six days after the day of emergence (Giebultowicz et al., 1990). After a single mating, gypsy moth females ceased to call, and generally failed to attract additional males before ovipositing a single large egg mass (Doane, 1968). Traps baited with mated females (Forbush and Fernald, 1896; Cardé et al., 1973) or with extracts from mated female abdominal tips (Collins and Potts, 1932) also caught few males compared to traps baited with virgin females. Direct analysis of pheromone titer by GLC has shown that 24 hr after mating pheromone titer declined to very low levels and remained low for the next several days (Giebultowicz et al., 1990).

To further understand the biology of pheromone production in *L. dispar*, we report on the diel periodicity of the titer of the sex pheromone, *cis*-7,8-epoxy-2-methyloctadecane, as well as for the presumed pheromone precursor, 2-methyl-*cis*-7-octadecene. We also show how age affects the average daily pheromone titer and evaluate the short-term changes that occur in pheromone titer after mating.

METHODS AND MATERIALS

Moths. Gypsy moth egg masses from the New Jersey standard strain were supplied by the USDA-APHIS Methods Center, Otis Air National Guard Base, Massachusetts. Larvae were reared on a modified wheat germ diet (Bell et al., 1981) in an environmental chamber held at 24 ± 2°C, 50% relative humidity,

16 hr:8 hr light–dark cycle. Under these conditions, development to adult eclosion required approximately 6.5 weeks. Eclosion was strictly diurnal with 24.8% of the females emerging 0–4 hr after lights-on, 50.4% 4–8 hr after lights-on, 23.6% 8–12 hr after lights-on, and 1.2% 12–16 hr after lights-on. These values concur with previous reports of eclosion periodicity (ODell, 1978; Ma et al., 1982). Adult females were held in paper containers (110 mm diam. × 80 mm tall) with transparent plastic lids. The age in days was based on the age in hours from the time of eclosion. Day 0 refers to females 0–24 hr old; day 1, 24–48 hr old; day 2, 48–72 hr old; and day 3, 72–96 hr old.

Gland Extractions and Calling Behavior Observations. The female gypsy moth pheromone, commonly referred to as disparlure, has been identified as (7*R*,8*S*)-*cis*-epoxy-2-methyloctadecane (Bierl et al., 1970; Iwaki et al., 1974; Hansen, 1984). To assess glandular titers of the pheromone and the putative precursor, 2-methyl-*cis*-7-octadecene, the abdominal tip, which included sclerite IX, the pheromone gland (Hollander et al., 1982), and part of sclerite VIII, was excised from individual females and extracted in 150 μl hexane for 1 hr in a conical glass tube. A known amount (25 ng) of internal standard, *cis*-9,10-epoxy-eicosane, was added to the hexane immediately before the gland was extracted. Each tube was placed in a vial containing a reservoir of 100 μl hexane. Vial caps were Teflon-lined and samples were stored at −15°C until analysis.

Efficiency of pheromone extraction was determined by extracting the same gland ($N = 5$) sequentially for 1 min, 1 hr, and 24 hr. For each extraction period, the gland was immersed in 150 μl hexane with 25 ng of the internal standard. Gland transfers between tubes were done with a hooked insect pin. The first extraction period (1 min) yielded 83.2% (±2.6 SD) of the total extractable pheromone with the remaining 16.8% (±2.6 SD) of the pheromone obtained in the next extraction period (1 hr). Extraction of the same glands for an additional 24 hr failed to yield any more pheromone (<0.5 ng) and sporadically contained extraneous compounds that interfered with reliable quantification of pheromone. Therefore, 1 hr was chosen for the length of gland extractions.

To establish the diel periodicity of titer, gland extractions were performed every 4 hr beginning at lights-on for day-0, -1, -2, and day-3 females. Adult females were held in paper containers in groups of five, and observations of female calling behavior were made immediately (within 10 min) before the pheromone glands were excised and extracted. A female was scored positively for calling according to the criteria reported by Tang et al. (1987). All extractions and calling behavior observations were made under the same light and temperature conditions as the rearing regime. During scotophase, illumination was provided by a flashlight covered with a Kodak red filter. Determinations were not made for day-4 females or older since a female's average life-span is only six days (equivalent to our day-5 females) (Forbush and Fernald, 1896).

The effect of age on pheromone titer was evaluated by comparing the

average daily pheromone titer of day-0, -1, -2, and day-3 females. The average daily titer for each female age was calculated by combining the data from the six extraction times at 4-hr intervals beginning at lights-on.

To evaluate the influence of mating on pheromone titer, individual males (24–48 hr old) were placed with individual calling females (48–52 hr old) 8 hr after lights-on, and each male–female pair was housed in separate paper containers (110 mm diam. × 80 mm tall) with transparent plastic lids. This time, 8 hr after lights-on was chosen since it fell within the period (1100–1500 hr) of maximal male attraction to females (Cardé et al., 1974) and because it occurs just before the period of peak pheromone titer in day-2 females. Mating ensued within a few minutes, and those pairs that were not forcibly separated remained in copula for 58.7 min (±13.3 SD). This length of time agrees well with previous reports of copulation duration (Forbush and Fernald, 1896; Doane, 1968; Loerch and Cameron, 1984). Pheromone titers were determined immediately before copulation, and 0.5, 1.0, and 24 hr after mating initiation. Forcible separation of the mating pairs was necessary for all the 0.5-hr pheromone titer determinations and for one of the 1-hr determinations.

Sample Analysis. FID-GLC analysis was used to quantify amounts of pheromone present in ovipositor extracts. Samples were concentrated to ca. 0.8 μl under filtered N_2 and injected on a 2-m × 2-mm-ID glass column packed with SP-2100 (3% loading on 100/120 Supelcoport; N_2 carrier flow 30 ml/min). Peaks were identified by retention time comparison with synthetic compounds, and areas were quantified using an HP-3390 integrator. Under isothermal operation at 175°C, the olefin, disparlure, and the internal standard eluted at 4.4, 8.3, and 13.6 min, respectively. The lower analytical limit of the system was ca. 0.5 ng. Mass spectral analysis was performed on a few samples to confirm the identities of disparlure and the olefin in the gland extracts.

RESULTS

The effect of time of day on female pheromone titer is shown in Figure 1. Despite the inherent variability found in the data, daily trends were observable. On the day of emergence (day 0), pheromone titers were generally low during photophase (mean titers ranged from 1 to 4 ng), then gradually increased to 8.4 ng during the latter half of scotophase. For day-1 females, the lowest titer (5.1 ng) was found 4 hr after the onset of photophase, and the peak titer (19.0 ng) was observed at lights-off. For day-2 and day-3 females, the lowest titers occurred at lights-on (7.4 ng and 5.3 ng, respectively), and the peak titers were found 4 hr before lights-off (22.7 ng and 26.0 ng, respectively). Excluding the day of emergence, it appeared that daily pheromone titer in the gypsy moth did exhibit a patterned fluctuation. The diel periodicity of titer was characterized by low

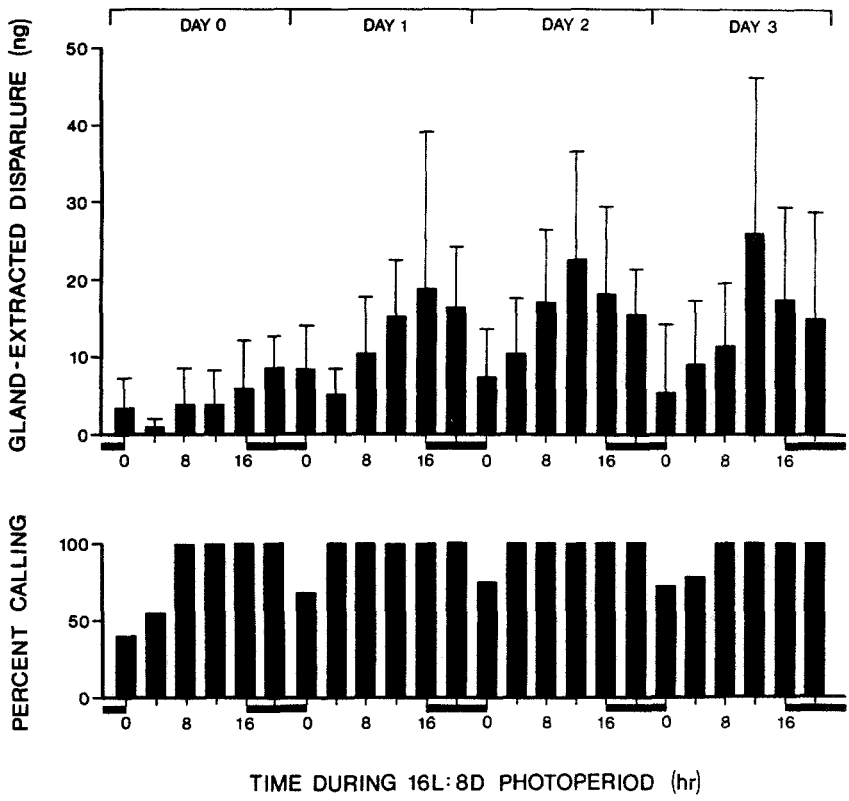

FIG. 1. Diel periodicity of sex pheromone titer (upper graph) and percent female calling immediately before each pheromone extraction time (lower graph). Mean values for each extraction period were obtained from 8 to 12 females, and vertical bars denote standard deviations from the mean. Glands were extracted individually for 1 hr in 150 μl hexane containing 25 ng internal standard, and analyzed by GLC-FID. Day 0 refers to females 0–24 hr old, day 1 to females 24–48 hr old, etc. Scotophase is indicated by dark horizontal bars.

titers at or just after the onset of photophase and peak titers at or just before the onset of scotophase.

Values for pheromone titer and percent female calling were also obtained for females within 0.5 hr of eclosion, while the wings were still undergoing expansion and hardening (data not graphed). For all these females, the pheromone titers were below the limits of detection (<0.5 ng), and none had initiated calling ($N = 5$). During this period, the ovipositor was protruded with the pheromone gland partially exposed, but there was not the rhythmic protrusion and retraction of the ovipositor that is characteristic of calling (Tang et al.,

1987). By 2–3 hr after the time of eclosion, pheromone titer increased to 1.2 ng ± 0.7 (SD, $N = 5$), and three of the five females had already initiated calling behavior.

Comparison of the diel periodicity curves for calling and pheromone titer (Figure 1) showed that females called almost continuously and that periods of 100% calling were often not indicative of periods of maximal pheromone titer. Typically, titer began to rise with the onset of 100% female calling. Once the peak titer was reached, titer began to decrease while calling activity continued at the 100% level. Periods of lowest calling activity, however, were either coincident or within 4 hr of the time of lowest pheromone titer. For example, on day 0 and day 1, lowest percent female calling was observed at lights-on, and lowest pheromone titer was observed 4 hr after lights-on. On day 2 and day 3, the period of lowest titer occurred at lights-on and coincided with the period of lowest calling activity.

The influence of age on the average daily pheromone titer is shown in Table 1. Day-0 females had significantly less pheromone in the gland (4.5 ng) than day-1 (12.4 ng), day-2 (15.4 ng), or day-3 females (13.5 ng). The average daily titer of the older three age groups, however, showed no significant differences.

Following copulation, a rapid decline in pheromone titer was observed (Table 2). Titer from females 0.5 hr after mating initiation was 7.6 ng compared to 19.5 ng from virgin females of similar age. The initial rapid rate of pheromone decrease, however, did not continue during the following 0.5 hr. Pheromone titer from these females (i.e., 1.0 hr after mating initiation) only fell an additional 3.1 ng to 4.5 ng. By 24 hr, titer from mated females had fallen below the limits of detection (0.5 ng). In addition, these latter females each laid large

TABLE 1. EFFECT OF FEMALE AGE ON AVERAGE DAILY PHEROMONE TITER[a]

Female age[b]	N	Pheromone titer (ng/gland) mean (SD)[c]
Day 0	60	4.5 a (4.7)
Day 1	59	12.4 b (11.1)
Day 2	60	15.4 b (10.7)
Day 3	60	13.5 b (13.3)

[a] Glands were extracted individually in 150 μl hexane containing 25 ng internal standard, and analyzed by GLC-FID.

[b] Day 0 refers to females 0–24 hr old, day 1 to females 24–48 hr old, etc.

[c] Means followed by different letters were significantly different ($P < 0.05$) by the Student-Newman-Keuls multiple-range test after square root transformation of the data.

TABLE 2. EFFECT OF MATING ON PHEROMONE TITER[a]

Female status[b]	N	Time of extraction after mating initiation (hr)	Pheromone (ng/gland) mean (SD)[c]
Virgin	23		19.5 a (12.8)
Mated	8	0.5	7.6 b (4.8)
	10	1.0	4.5 b (3.2)
	7	24.0	<0.5 c (0.7)

[a] Female glands were extracted individually in 150 μl hexane containing 25 ng internal standard, and analyzed by GLC-FID.

[b] Single males (24–48 hr old) were placed with individual calling females (48–52 hr old) 8 hr after lights-on. Glands from virgin females were extracted immediately, otherwise mating ensued within a few minutes, and glands were extracted at the times indicated. Forcible separation of mating pairs was necessary for all of the 0.5-hr determinations and for one of the 1-hr determinations.

[c] Means followed by different letters were significantly different ($P < 0.05$) by the Student-Newman-Keuls multiple-range test after logarithmic transformation of the data.

discrete egg masses by the time of pheromone extraction. Two females, however, were observed to mate normally but did not exhibit the decline in titer 24 hr later. They deposited partial egg masses with the majority of the eggs retained. Pheromone titers from these two females (8.9 ng and 26.1 ng) were not included in the data of Table 2.

Titer of the putative pheromone precursor, 2-methyl-*cis*-7-octadecene, from individual virgin or mated female gland extracts never exceeded 1.0 ng, and was typically below the limits of detection (0.5 ng).

DISCUSSION

The diel periodicity of sex pheromone titer in the gypsy moth was characterized by low titers at or just after the onset of photophase and peak titers at or just before the onset of scotophase. A similar pattern also has been observed for the diel periodicity of pheromone emissions; pheromone release rates from calling females were lowest just after the onset of photophase and highest just before the onset of scotophase (Charlton and Cardé, 1982). Since females call continuously except at lights-on, it appears that the amount of pheromone emitted relates directly to the amount of pheromone present in and/or on the gland. When pheromone titer was at its peak (19–32 ng), peak emission rates of 14–15 ng/hr (Charlton and Cardé, 1982; Tang et al., 1987) were observed, and 100% of the females called; when pheromone titer was at its lowest (5–9 ng), lowest emission rates (2–5 ng/hr) were recorded (Charlton and Cardé, 1982),

and percent female calling was also at its lowest (40–60%; Charlton and Cardé, 1982).

Other species that exhibit congruence between pheromone titer and emission periodicities from calling females include *Heliothis subflexa* and *H. virescens*. For both of these species, peak titers of the major pheromone component, (*Z*)-11-hexadecenal, were exhibited during periods of peak emissions and peak percent female calling, and lowest titers were found during periods of lowest emissions and lowest percent female calling (Heath et al., 1991). Quantifying pheromone release rates from *H. virescens* glands that were forcibly extruded, Pope et al. (1982) also concluded that the amount of pheromone emitted was dependent on the amount of pheromone in and/or on the gland.

In certain other moth species, an asynchronous relationship between pheromone emission and titer periodicities has been observed. In *Trichoplusia ni*, peak titer of the major pheromone component, (*Z*)-7-dodecenyl acetate, in and/or on the gland occurred at lights-off and always preceded the peak in pheromone emissions, which occurred 2–4 hr later (emission data was from forcibly extruded glands) (Hunt and Haynes, 1990). Furthermore, the peak in pheromone emission rate was observed when percent female calling was on the rise, and lowest pheromone titers were observed during periods of maximal percent female calling (Hunt and Haynes, 1990). Data from naturally calling females also indicated that during the period the pheromone gland was everted, pheromone release declined exponentially, with highest emission rates observed during the first 5 min of gland eversion (Bjostad et al., 1980). In *Holomelina lamae*, similar trends also have been noted. Quantities of the major pheromone component, 2-methylheptadecane, released from calling females were highest just after the onset of calling, and emission rates decreased as calling continued (Schal et al., 1987). Titer was also observed to decrease suddenly with the onset of female calling (Schal and Cardé, 1986).

By comparing the rates of pheromone release with the rates of pheromone biosynthesis, explanations can be proposed to account for the different relationships observed. For example, in the gypsy moth, *H. subflexa*, or *H. virescens*, the concurrent rise and fall in titer and emission periodicities would be possible if the rate of pheromone emission were always less than, but varied in proportion with the rate of pheromone biosynthesis. In *T. ni* or *H. lamae*, pheromone emission rates would have to exceed rates of pheromone biosynthesis to account for the observed periodicities. The latter hypothesis has also been proposed by Ono et al. (1990) to explain the asynchronous relationship observed for periodicities of calling and pheromone titer in the potato tuber moth. Data describing the *in vivo* rates of pheromone biosynthesis at different times of the day should determine the validity of these hypotheses.

In addition to quantifying the amount of disparlure in virgin and mated female gland extracts, we also examined the levels of the olefin, 2-methyl-*cis*-

7-octadecene. The olefin has been interpreted as an intermediate in the biosynthetic pathway of disparlure (Kasang et al., 1974) and is an inhibitor of male attraction to disparlure (Cardé et al., 1973). The levels of olefin found in our 1-hr gland extracts were always <1.0 ng and often fell below the limits of detection (0.5 ng). Previous reports (Bierl et al., 1972) have suggested that gland titers of the olefin exceeded disparlure titers because, during the course of isolating and identifying disparlure, it was found that epoxidation of active fractions containing both the olefin and disparlure led to a 10-fold increase in male activity. However, Bierl et al. (1970) reported that they extracted 78,000 female abdominal tips and that half this extract contained ca. 15 μg of the olefin (purification losses were not reported), or 0.4 ng per female, which is similar to what we found.

Our results showed that gypsy moth pheromone titers were influenced by age. The average daily titer was low on the day of eclosion (day 0), increased on day 1, and then remained unchanged on day 2 and day 3. This differs from the effects of age reported by Giebultowicz et al. (1990), who found that titer was low on the day of eclosion, increased until reaching a peak in 5-day-old females (equivalent to our day-4 females), and then declined over the next two days. Experimental differences can be held partially accountable. We determined the effects of age on the average daily titer, i.e., an average that was calculated from the titers obtained from six time points taken every 4 hr, whereas Giebultowicz et al. (1990) determined the effects of age on the mean titer obtained from one time point 4 hr after lights-on, which our data indicated fell during the period of minimal titer. Direct comparison of titers obtained at 4 hr after lights-on shows that for days 0, 1, and 2, the two sets of data are comparable. For day-3 females, however, the pheromone titer observed by Giebultowicz et al. (1990) at 4 hr after lights-on was 33 ng, as opposed to 10 ng found in our data for the same time. In a later paper, Giebultowicz et al. (1991) reported titers from virgin day-2 females of 5–6 ng for glands taken 10–12 hr after lights-on. In our data, 10–12 hr after lights-on corresponded to the period of peak titer, which we found to be 23 ng in day-2 females. Since insect rearing conditions and diet were basically the same in our experiments and those of Giebultowicz et al. (1990, 1991), differences in the data could be due to differences in insect handling and gland extraction techniques or to the insects themselves.

Mating in the gypsy moth precipitated a rapid decline in pheromone titer that was evident within 0.5 hr of mating initiation while females were still *in copula* (virgin female titer was 19.5 ng versus 7.6 ng from females that were mated for 0.5 hr). After the second 0.5 hr, the reduction in titer was not nearly as marked, falling only to 4.5 ng, and by 24 hr, pheromone titer was below the limits of detection (0.5 ng). These data suggest that two separate physiological mechanisms were involved in controlling the decline in titer after mating. This is consistent with the hypothesis of Giebultowicz et al. (1991) that the transition

from a virgin to a mated state is under two-step regulation. Giebultowicz et al. (1991) found that mechanical disturbance of the bursa copulatrix by insertion of the male genitalia during mating resulted in a transient suppression of calling behavior and a transient decline in pheromone titer. Permanent suppression of calling and pheromone production, however, only occurred when sperm was present in the spermatheca (Giebultowicz et al., 1991). In the brown-banded cockroach, termination of calling behavior in mated females was also found to be under a similar two-step regulation, i.e., placement of the spermatophore in the bursa caused an immediate cessation of calling, and continued suppression of calling was caused by the presence of sperm in the spermatheca (Smith and Schal, 1990). Insufficient sperm in the spermatheca may also explain why two of the gypsy moth females that we observed to mate normally did not exhibit a decline in pheromone titer 24 hr later (8.9 ng and 26.1 ng), nor did they deposit complete egg masses.

The nervous system appears to play a major role in the reproductive behavior of the female gypsy moth. In virgin females, calling behavior, pheromone production, and pheromone emission all depend on the CNS acting through the ventral nerve cord (Hollander and Yin, 1982; Tang et al., 1987) without any apparent endocrine or neuroendocrine involvement (Hollander and Yin, 1985; Tang et al., 1987; Raina et al., 1989). Neural innervation during mechanical disturbance of the bursa was also essential to effect the transition from a virgin to a mated state (Giebultowicz et al., 1991). Although it is still not clear whether sperm in the spermatheca is detected neurally or chemically, mechano-like sensilla in the spermatheca of *Plodia interpunctella* have been observed (Lum and Arbogast, 1980). It seems plausible, therefore, that during the transition from a virgin to a mated state, the CNS of female gypsy moths could be under neural inhibition induced by sensory feedback from the bursa and the spermatheca, thereby suppressing calling behavior, pheromone release, and pheromone production.

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EFFECT OF KAIROMONES FROM EGG AND FEMALE ADULT STAGES OF *Ostrinia nubilalis* (HÜBNER) (LEPIDOPTERA, PYRALIDAE) ON *Trichogramma brassicae* Bezdenko (HYMENOPTERA, TRICHOGRAMMATIDAE) FEMALE KINESIS

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Abstract—Volatile chemicals emanating from the different developmental stages of *Ostrinia nubilalis* Hübner (Lepidoptera, Pyralidae) increase the mobility of *Trichogramma brassicae* Bezdenko (Hymenoptera, Trichogrammatidae) in a linear airflow olfactometer. In this paper, we have demonstrated that airborne chemicals from egg masses and virgin females during calling activity stimulate an intensive search behavior by *Trichogramma* females. On the other hand, emanations from mated females with extruded abdominal tips do not incite the parasitoid's movement. For the moment we cannot elucidate, with these bioassays, the real role of these kairomones as attractants, guides, stimulants, or retainers.

Key Words—*Trichogramma brassicae*, Hymenoptera, Trichogrammatidae, kairomone, pheromone, *Ostrinia nubilalis*, Lepidoptera, Pyralidae, biological control, European corn borer, parasitoid.

INTRODUCTION

The process of host selection by a parasitoid was described by Vinson (1985) as seven successive steps: habitat preference, potential host community location, host location, host examination, ovipositor probing, ovipositor drilling, and

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oviposition. The success of each step depends on many environmental and host factors that involve both chemical and physical cues, indicating that the process is not absolutely random. Chemicals appear to be major stimuli in the host selection process. Their importance is noted at each step of the process. Physical factors start to play a more important role beginning with the host examination step (Vinson, 1985). Such semiochemicals are called kairomones (Brown et al., 1970) and have a role in host-seeking, recognition, and oviposition behaviors.

Allelochemical sources involved in the host selection process are varied. Considering all the host–parasitoid couples investigated, kairomones can be produced by different host developmental stages. Eggs are the target of oophagous parasitoids and can be a direct source of kairomones. For example, the eggs of several Noctuidae species are attractive for several *Trichogramma* spp. females (Nordlund et al., 1977a, 1981; Ferreira et al., 1979; Ferreira-Anunciada and Pintureau, 1981; Gross et al., 1981; Bourarach and Hawlitzky, 1984). Surface chemical compounds of *Ostrinia nubilalis* egg masses are attractive for *T. brassicae* [synonymy with *T. maidis* (Pintureau, 1990) confused with *T. evanescens*] and stimulate their probing behavior (Renou et al., 1989). Noldus and van Lenteren (1985b) have shown that cabbage leaves treated with extracts of *Pieris brassicae* eggs are explored for a longer time by *T. evanescens* females than control leaves. This stage is also a source of kairomone for other oophagous (Strand and Vinson, 1982b, 1983a,b; Bin and Vinson, 1985) and egg-larval parasitoid species (Kainoh et al., 1982; Strand and Vinson, 1982a). Egg chemicals appear to act by short-range attraction and/or by contact (Renou et al., 1989). While the parasitoid already stands within a host community, such chemical cues might intervene at location and examination levels.

The kairomonal activity of eggs may be associated with an oviposition-deterring pheromone that could act by contact (Nordlund et al., 1977b; Prokopy and Webster, 1978; Noldus and van Lenteren, 1985b). Components released at the time of oviposition could interact with those of the eggs. Thus, colleterial glands (Ferreira et al., 1979) and accessory glands (Nordlund et al., 1977b, 1987) are active in the behavior of several *Trichogramma* species. For different larval parasitoid species, host larval instar and their products are a source of semiochemicals (Weseloh, 1974; Wilson et al., 1974; Gross et al., 1975; Thompson et al., 1983). Moreover, the presence of adults, particularly females, may stimulate the parasitoid's searching behavior. In oophagous insects, it was thus shown that *T. evanescens* females explored a longer time on cabbage leaves on which *Mamestra brassicae* adults have stayed than on control leaves (Smits, 1982). *Spodoptera littoralis* and *Helicoverpa armigera* females (Lepidoptera, Noctuidae) elicit behavioral responses for two Trichogrammatidae species, *Trichogrammatoidea lutea* and *Trichogramma rhenana* (Bourarach and Hawlitzky, 1984). On the other hand, oophagous and egg-larval parasitoids can be attracted by the host's sex pheromone (Noldus and van Lenteren, 1985a; Noldus et al.,

1988; Isidoro and Bin, 1988). Lewis et al. (1982) and Nordlund et al. (1983) have shown that volatile chemicals released from host abdominal tips and excretions stimulate the parasitoid's searching behavior. Moreover, these chemicals increase parasitism in the field. Likewise, this activity is found in the synthetic sex pheromone of *Heliothis zea* and *Spodoptera frugiperda*.

Scales lost by *H. zea* females at the time of oviposition have also been shown to be a source of kairomones (Lewis et al., 1971, 1972, 1975a,b; Jones et al., 1973). Such kairomones act by contact (Beevers et al., 1981), not by olfaction. Several contact kairomones have been identified for other host–parasitoid couples such as *Heliothis zea–T. pretiosum* (Gueldner et al., 1984), *Mamestra brassicae–T. evanescens* (Smits, 1982), *Pieris brassicae* and *P. rapae* for *T. evanescens* (Noldus and van Lenteren, 1985b) and also for *O. nubilalis–T. nubilale* (Shu and Jones, 1989; Shu et al., 1990). Moth scales are also a stimulus for predators (Lewis et al., 1977). Such scale-contained semiochemicals induce an arrestment response of parasitoids that is expressed by an increased klinokinesis, thus involving an increased searching behavior of parasitoid on treated areas.

In the past few years, efforts have been made in France by INRA, in association with a private company, UNCAA (Union Nationale des Coopératives Agricoles d'Approvisionnement), to elaborate and develop biological control measures against the European corn borer (ECB) *Ostrinia nubilalis* Hübner (Lepidoptera, Pyralidae) by using an oophagous insect, *Trichogramma brassicae* Bezdenko (Hymenoptera, Trichogrammatidae). In this paper we report studies on the source of volatile chemical cues used by *T. brassicae* to locate their host *O. nubilalis*, particularly in view of a possible application of semiochemicals to enhance the efficiency of parasitoids in biological control programs. We have examined the responses of *T. brassicae* to egg masses, to virgin females in different situations linked to their pheromonal cycle, and to mated females of the host, *O. nubilalis*.

METHODS AND MATERIALS

Parasitoids

A *T. brassicae* strain was used. These wasps were taken from an industrial culture, originating from material collected yearly on *O. nubilalis* egg masses in the field by UNCAA.

The parasitoids were reared on eggs of the Mediterranean flour moth, *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae) and held in diapause to await field utilization. We used *Trichogramma* from the first generation after diapause. Adult wasps were maintained on honey, in glass tubes at 25 $\pm$ 1°C, with 70% relative humidity, and a photoperiod of 16:8 hr light–dark. For these experi-

ments, only 48-hr-old mated female wasps were used (Smits, 1982); these females had no previous oviposition experience.

Moths

Adult *O. nubilalis* were reared on an artificial diet according to a modified procedure of Poitout and Bues (1970), held at 25 ± 1°C, 80% relative humidity, and a 16:8 hr light–dark photoperiod. They have been laboratory reared for a maximum of eight generations. Each year, the strain was renewed by collecting wild larvae in autumn, near Houdan (Yvelines, France).

Odor Source

Host Egg Masses. Host egg masses less than 24 hr old were provided from laboratory cultures. The oviposition substrate consisted of wax paper covering the open top of the cage containing the adults. These egg masses were easily collected by brushing off the wax paper. An increasing number of egg masses (1, 3, and 5 egg masses of medium-size, 15–20 eggs) was used to determine whether there was a concentration threshold for parasitoid females.

Virgin Female Host Adults. Stock colony pupae were collected, sexed, and separated. They were placed under a 16:8 hr light–dark photoperiod with two different times of photoperiod initiation: (1) lights on at 6 AM, or (2) lights on at 4 PM to facilitate daytime experiments. In addition, a continuous photophase to inhibit normal sex pheromone synthesis was used (Skopik et al., 1981; Subchev, 1983). Relative humidity is a critical condition; thus, it was held constant at 90–100%. In all experiments, females were 3-day-old virgins when pheromonal emission was observed to be maximum (personal observations).

In the first experiment, moths were used in the third scotophase after emergence and during their calling activity (the last 2 hr of scotophase). During this period, extrusion of the abdominal tip continued for several hours. For these experiments, female abdominal tips were extruded artificially by pressure with tweezers to allow continual sex pheromone emission, even in the light. Pheromone emission was demonstrated by the characteristic behavior of males.

Mated Female Host Adults. One female and two males (all 2 days old) were placed in a 110-mm-diam. × 80-mm-high plastic container with a layer of filter paper and placed in a climate room at 25 ± 1°C, 90–100% relative humidity, and a 16:8 hr light–dark photoperiod. Mating occurred during the first night and egg laying during the following one. After each test, the females were dissected in order to verify the presence of the spermatophore (Drecktrah and Brindley, 1967; Showers et al., 1974).

Bioassays

Bioassays were performed in a linear airflow olfactometer (Renou et al., 1989) (Figure 1). Ten *Trichogramma* females were introduced through the entry tube downwind of the odor source. The test insects were exposed to odors from different developmental host stages and to control air. A new sample odor source was used for each replication. The airflow was regulated and measured by flowmeters at 1.8 liters/hr. For each test, we calculated a moving index minute by minute (Renou et al., 1989). The different experiments consisted of series of at least 30 observations. When testing egg masses, we used a brush to place them inside the glass tube, and experiments were conducted for 15 min. For adults, the test odor source contained one female in a glass vial for 20 min.

Statistical Analysis

Student's *t* test was used for statistical comparisons between treatment and control. We calculated the difference between means every minute, at a significance level of 95%; significant differences are indicated by an asterisk in Figure 2.

RESULTS

Odor Source: Egg Masses

We wanted to determine whether volatile chemicals emanating from this target stage have an effect on parasitoid behavior. When examining female *Trichogramma* movement, we observed enhanced parasitoid mobility in the presence of three egg masses in comparison with the control (Figure 2, treatment B); no difference in mobility was observed in the presence of one and five egg masses (Figure 2, treatments A and C).

Odor Source: Virgin Females

We examined the responses of *Trichogramma* females exposed to emanations of *O. nubilalis* calling virgin females during the last 2 hr of scotophase. The test odor source was then constituted by sex pheromone blend and female-related odors. The results for calling virgin females (Figure 2, treatment D) show a significant difference from those for controls after 6 min. The *Trichogramma* came more quickly to the area around the odor source than in the control tube.

Virgin females were then tested before their calling period during scotophase to determine whether the responses shown in the preceding test were due to factors associated with calling behavior in interspecific relationships. This

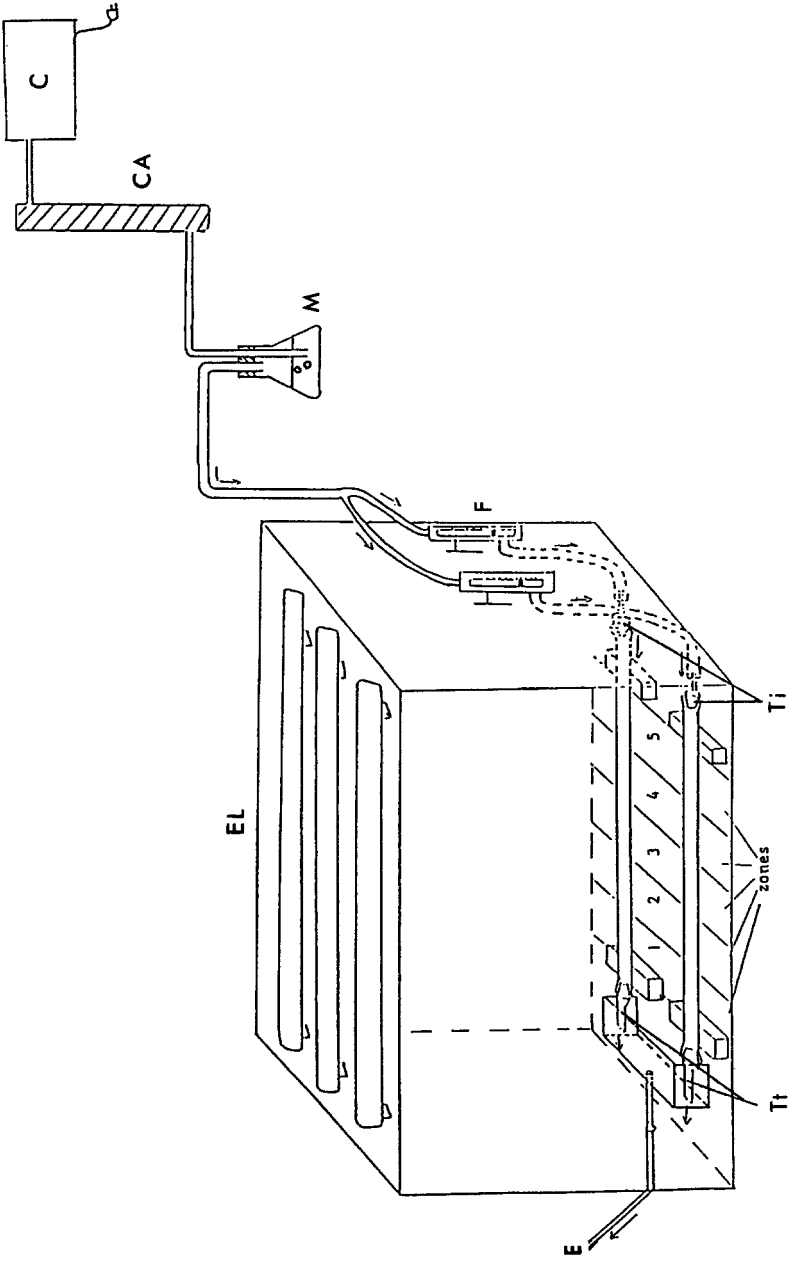

FIG. 1. Schematic representation of the linear airflow olfactometer set-up and peripheral equipment. C: compressor; CA: column of activated carbon for air purifying; M: moistening; E: exhaust; EL: electric lighting with 3 neon tubes; F: flowmeters; Ti: tips with odor source in the tested arm or nothing for the control; Tt: tips with 10 *Trichogramma* females in each. Arrows indicate the direction of the air flow.

treatment		min1	min2	min3	min4	min5	min6	min7	min8	min9	min10	min11	min12	min13	min14	min15	min16	min17	min18	min19	min20
A	O	3.83	6.10	8.70	10.26	11.96	12.53	13.33	14.36	15.5	15.36	16.36	16.33	17.06	17.33	18.56					
	C	3.23	6.13	8.83	10.86	11.50	13.13	14.13	15.13	16.3	16.43	17.33	17.13	17.9	17.96	18.7					
B	O	3.50	6.46	10.20	13.33	16.33	19.10	21.36	22.46	23.6	25.73	27.26	29.33 *	30.16 *	31.16 *	32.36 *					
	C	2.70	5.70	8.80	11.73	14.13	16.13	18.73	20.26	21.43	22.76	23.4	24.63	26.1	26.53	27.0					
C	O	4.93	8.50	13.30	17.66	20.73	23.50	26.36	28.20	29.8	30.83	31.7	33.1	34.33	35.53	36.1					
	C	5.83	10.06	14.33	18.90	22.50	25.50	27.63	28.36	29.56	31.03	31.4	32.03	32.83	33.23	34.0					
D	O	6.06	10.50	14.80	18.16	20.83	22.40	24.53 *	26.50 *	28.33 *	29.3 *	30.06 *	31.73 *	32.43 *	33.6 *	33.6 *	34.06 *	34.7 *	35.43 *	35.96 *	36.06 *
	C	5.03	8.93	12.20	14.80	16.83	19.33	21.06	22.86	24.33	25.36	26.3	27.2	27.13	27.96	29.23	29.33	29.83	30.03	31.3	31.1
E	O	3.23	6.26	9.30	12.0	14.63	16.73	18.93	20.13	22.06	22.56	23.0	24.13	25.63	26.3	27.5	28.5	29.2	29.13	30.16	30.36
	C	2.96	5.73	9.46	13.03	15.30	17.13	19.0	20.86	23.1	24.26	25.23	25.8	26.76	27.5	27.96	28.46	29.16	30.1	30.0	29.73
F	O	4.46	7.26	9.90	12.23	14.36	16.30	18.60	20.30	20.96	22.03	23.5	24.43 *	25.1 *	25.73 *	26.9 *	28.0 *	28.43 *	28.83 *	29.33 *	29.33 *
	C	3.23	5.96	8.93	11.83	13.26	14.56	17.06	18.13	19.2	19.76	20.83	21.43	21.96	22.43	22.96	23.7	24.03	24.6	24.96	24.86
G	O	3.23	6.26	9.30	12.0	14.63	16.73	18.93	20.13	22.06	22.56	23.0	24.13	25.63	26.3	27.5	28.5	29.2	29.13	30.16	30.36
	C	2.96	5.73	9.46	13.03	15.30	17.13	19.0	20.86	23.1	24.26	25.23	25.8	26.76	27.5	27.96	28.46	29.16	30.1	30.0	29.73
H	O	3.23	4.86	7.03	9.10	11.4	13.66	15.86	17.60	18.9	20.16	21.53	22.66	23.36	23.83	24.53	24.6	25.5	25.9	26.36	27.16
	C	2.70	4.86	6.96	9.20	11.40	13.20	14.90	17.23	18.16	19.7	21.16	21.7	22.36	22.53	22.43	23.4	23.3	23.86	24.23	25.3
I	O	4.33	7.76	10.93	13.80	16.43	18.86	20.46	22.16	23.03	24.2	24.9	26.13	27.36	27.56	28.5	29.06	29.56	29.76	30.06	30.1
	C	4.70	8.0	11.20	14.56	17.20	19.40	21.06	23.30	24.76	26.46	27.96	28.66	28.93	30.3	30.6	30.33	30.8	30.9	31.2	31.93
J	O	4.23	7.16	10.1 *	12.6 *	15.06 *	17.0	18.56	19.86	21.53	23.03	23.7	24.86	25.26	25.96	26.7	27.5	28.06	28.26	29.23	29.76
	C	3.0	5.26	7.3	9.4	11.5	14.1	15.23	17.06	19.3	20.93	21.7	22.53	23.16	23.6	24.66	25.33	25.86	25.56	25.93	26.46
K	O	4.56	7.46	10.3	12.36	14.8	17.16	18.23	20.0	21.6	22.13	22.83	23.3 *	24.26 *	25.2 *	25.3 *	25.43 *	25.8 *	26.86	27.23	28.0
	C	4.66	8.10	11.13	13.26	16.03	18.46	20.43	22.16	23.73	24.7	25.43	26.6	27.46	28.53	28.46	29.33	29.13	29.23	29.86	29.9
L	O	2.66	4.83	7.23	9.36	11.03	13.03	14.70	16.06	17.43	18.9	20.1	20.8	21.83	22.06	23.23	23.83	24.86	24.96	25.6	26.23
	C	2.5	4.56	6.4	8.33	10.56	12.36	13.53	14.36	15.56	16.36	17.66	18.6	18.9	20.13	21.03	21.83	22.83	23.36	24.23	24.8
M	O	3.1	5.76	8.0	10.46	12.93	14.86	16.93	19.16	19.6	21.0	22.26	22.8	23.96	25.76	25.56	26.0	27.0	27.36	26.76	27.23
	C	2.06	5.0	7.5	10.26	12.9	15.86	17.56	18.63	20.36	21.4	21.73	22.86	23.5	24.6	25.7	26.26	26.83	26.83	27.83	28.63

FIG. 2. Responses of *Trichogramma brassicae* to different treatments with host odor sources in the linear olfactometer. min1 to min20: the numbers represent the moving index calculated each minute in the olfactometer; O = odor test; C = control test; * = significant difference between the odor test and the control. The odor sources are (A) one egg mass; (B) three egg masses; (C) five egg masses; (D) host virgin females in calling activity; (E) virgin females in scotophase just before the calling period without extruded pheromonal gland; (F) virgin females in photophase with extruded pheromonal gland; (G) virgin females in photophase without extruded pheromonal gland; (H) virgin females reared under continuous photophase with extruded pheromonal gland; (I) virgin females reared under continuous photophase without extruded pheromonal gland; (J) mated females before their first oviposition without extruded abdominal tips; (K) mated female before their first oviposition with extruded abdominal tips; (L) mated females after their first oviposition without extruded abdominal tips; (M) mated females after their first oviposition with extruded abdominal tips.

test consisted of exposing the parasitoids to emanations from virgin females during the scotophase, immediately before the onset of calling activity and without extrusion of the pheromonal gland, in order to determine whether the volatile chemicals shown in the first test were due to passive diffusion through the pheromonal gland. The results (Figure 2, treatment E) showed no significant difference over 20 min.

In the third experiment, parasitoids were exposed to virgin females of *O. nubilalis* during the photophase (after eight of the 16 hr of light), some with extruded abdominal tips and others without. The emanations from virgin females with extruded pheromonal gland increased movement of the *Trichogramma*; a significant difference was found between treatment and control (Figure 2, treatment F), whereas virgin noncalling female odors do not induce any particular behavior in the parasitoids compared to the control (Figure 2, treatment G).

The previous tests suggested that substances involved in increased parasitoid movement are related to the synthesis of the female sex pheromonal blend. To examine this phenomenon further, female pupae were reared under continuous light until their utilization three days after emergence. The absence of photoperiodicity disturbs the synthesis of sex pheromones, making females ineffective in attracting males (personal observation).

Light-exposed *O. nubilalis* females with and without extruded abdominal tips showed no significant differences between the treatments and the control (Figure 2, treatments H and I).

Odor Source: Mated Females

A male may transmit substances other than the reproductive spermatophore during mating. This may have an effect on the behavior of the parasitoids, and this hypothesis may also apply to females which, in laying their first eggs, release substances that may then stimulate the search behavior of the parasitoids. In both cases, we followed the same procedure as in the tests described above, i.e., with and without extrusion of the abdominal tip. All experiments were carried out in the middle (after 8 hr) of the light period (of 16 hr).

Emanations from mated females before their first oviposition, without extruded abdominal tips, cause a slight modification in *Trichogramma* activity (Figure 2, treatment J). The difference from the controls was only significant during the first few minutes (minutes 3–5) of a 20-min test. In contrast to the other tests, *Trichogramma* females were less responsive to mated females with an extruded abdominal tip, suggesting some sort of restraint (Figure 2, treatment K).

The response of female *Trichogramma* to host females tested after their first oviposition, with and without extrusion of their abdominal tips, caused no modification in the movement of the parasitoids (Figure 2, treatments L and

M). It thus appears that females loose the above-described properties once they have laid their eggs.

DISCUSSION

It is known that many insects exhibit positive anemotropism. The tendency to walk upwind in the control ("unscented") current suggests that *Trichogramma*, too, follow this rule. The volatile compounds emanating from the egg masses induce a modification in search behavior of *Trichogramma*, as Renou et al. (1989) demonstrated using extracts. However, these seem to be very concentration-sensitive. Under our experimental design, the parasitoids responded significantly only to the emanations from a group of three egg masses. It appears that the emanations from a single egg mass are not sufficient to lead to an increase in the movement activity of *Trichogramma*, which may be included under parasitoid search behavior. On the other hand, it appears that the emanations from five egg masses were too strong, suggesting that this concentration, perhaps by saturating the olfactory receptors, inhibits the behavioral response. Thus, these parasitoids probably have a minimum and maximum concentration threshold, which may explain the lack of response obtained by Kaiser et al. (1989), who presented 50 egg masses to the parasitoids.

There is a particular event in the life of female moths, namely the calling activity, which permits sexual recognition. It has already been shown that sex pheromones are kairomones for other species of *Trichogramma* (Lewis et al., 1982; Noldus et al., 1988). With regard to the adult stage of the host, comparison of the 1st and 2nd experiments confirms that virgin females liberate a kairomone by extrusion of their pheromonal gland. Thus, there exists a mixture of one or more molecules responsible for activating the displacement activity of *Trichogramma*. A similar mechanism has been demonstrated for *Trichogramma pretiosum* moving to the sex pheromone emitted by *Heliothis zea*, which it parasitizes (Noldus et al., 1988). Likewise, females in photophase (third experiment) induce an increase in oriented movement by *Trichogramma* when the abdominal tip is extruded. The behavioral response is less marked than for the emanations from a calling virgin, which would suggest that the molecule(s) is(are) in the process of being synthesized during photophase.

The fourth experiment suggests that, because of the 16:8 hr light–dark photoperiod, these kairomonal molecules are synthesized in the gland with the sex pheromone. Abnormal conditions, such as continuous photophase (Subchev, 1983, 1985), disturb the normal synthesis of the sex pheromone, making it ineffectual on the behavior of the males of their species and also on that of their parasitoids.

We have thus demonstrated a kairomone that is synthesized during pho-

tophase up until its liberation during the last 2 hr of scotophase. This kairomone is synthesized along with the sex pheromone of which it is a part, but which does not play any role in attracting the male since the synthetic pheromone does not modify the search behavior of *Trichogramma* [Kaiser et al., 1989; and Renou, who tested four different doses of synthetic pheromone: 1, 10, 100, and 1000 ng (personal communication)].

In addition, emanations from mated females without extruded pheromonal gland cause a slight activation of parasitoid movement, whereas mobility drops once the abdominal tip is extruded. In that case, the *Trichogramma* appear to be restrained and are not stimulated to move upwind toward the odor source. It is necessary to observe the oviposition behavior of the corn borer to interpret this phenomenon. This will be examined in the future. We may assume that after laying its first egg mass, the female may liberate an inhibitory substance around the deposited eggs. While deterring the female *Trichogramma*, these chemical signals, combined with those that attract, may favor parasitism through a better exploration of the substrate. This negative signal has been shown only in mated females, and they seem to loose these kairomonal properties after laying their first egg masses.

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STUDIES ON *Paecilomyces marquandii* FROM NEMATODE SUPPRESSIVE CHINAMPA SOILS

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Abstract—Two applications of isolates of *Paecilomyces marquandii* from suppressive chinampa soils or P. lilacinus from Peru, fungi that parasitize nematode eggs, generally gave better control of tomato root-knot due to *Meloidogyne incognita* than did a single application. The effects on root galling by each of the *Paecilomyces* isolates varied between experiments; however, the ovicidal potential of the three isolates did not differ significantly. Proteins specific for each of the isolates were demonstrated by SDS gel electrophoresis. The results indicate that *P. marquandii* is one of the natural soil organisms that contribute to nematode suppression in the chinampa agricultural soils.

Key Words—Nematode suppressive soil, *Paecilomyces marquandii*, *P. lilacinus*, *Meloidogyne incognita*, biological control.

INTRODUCTION

Paecilomyces lilacinus, a fungus parasite of nematode eggs, has been extensively investigated as a plant nematode biological control agent. Jatala (1985) reviewed studies that found consistent and efficient control of root-knot nematode (*Meloidogyne* spp.) on many crops by a strain of *P. lilacinus* from the International Potato Center (IPC), Peru. Cabanillas et al. (1989) tested 13 iso-

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lates of *P. lilacinus* including the IPC isolate against *M. incognita* on tomato and concluded that mixtures of isolates gave the best control. In contrast, *P. lilacinus* did not effectively suppress *M. incognita* in 20 naturally infested tomato fields in California (Gaspard et al., 1990a,b). No control was obtained in studies of the IPC *P. lilacinus* isolate against *M. javanica* on tobacco in microplots (Hewlett et al., 1988). These studies represent a cross section of the many investigations that have yielded conflicting results on the efficacy of *P. lilacinus* in controlling plant nematodes.

Recently, the natural suppression of root pathogenic fungi (Lumsden et al., 1987) and of plant parasitic nematodes (Zuckerman et al., 1989) in the chinampa agricultural soils of central Mexico has been reported. The latter study involved the isolation and characterization of microbes that displayed antinematodal properties but did not include an assay for ovicidal fungi. Further examination of the chinampa soil revealed the widespread presence of another species of *Paecilomyces*, *P. marquandii*. *P. marquandii* has been reported from eggs of *Heterodera glycines* (Carris and Glawe, 1989), but to our knowledge the potential of this fungus as a nematode biocontrol agent has not been studied.

A primary objective of the current work was to examine the chinampa soils for the presence of nematophagous fungi, thereby adding to the characterization of nematode suppressiveness displayed by these soils.

This paper reports on studies of two isolates of *P. marquandii* from these soils and includes comparison of these cultures with the IPC isolate of *P. lilacinus*.

METHODS AND MATERIALS

Organisms. The nematode species for these trials was *Meloidogyne incognita* Race 3 and the tomato variety was *Lycopersicon esculentum* cv. Rutgers. The *P. lilacinus* culture originated from the IPC, Peru, and was obtained directly from Dr. N. Acosta, University of Puerto Rico, Mayaguez, Puerto Rico. The *P. marquandii* isolates, designated PM 1 and PM 2, were recovered from 50% of 20 samples taken from each of two locations of chinampa soil near Mexico City, D.F. Fungus cultures were maintained on potato dextrose agar at 25°C.

Greenhouse Trials. The experiments reported here consisted of three greenhouse trials, which encompassed 90 pots. For each trial, 2- to 3-week-old tomato seedlings grown in steam-sterilized potting soil were transferred individually to 5-in. pots containing one part sterilized soil to one part sand (1 kg/pot).

For experiments 1 and 2, fungus isolates were grown on steam-sterilized bran, sterilized for 20 min on two consecutive days. *Paecilomyces* spp. were grown for seven days on potato dextrose agar (PDA) at 25°C in the dark. Plates were flooded with sterile distilled water and scraped with a spatula to obtain a

spore suspension. Each 25 g of bran was inoculated with 1 ml of a 1×10^6 spores/ml suspension and incubated at 25°C in the dark for 10 days. To determine initial inoculum densities, 1 g of infested bran was shaken vigorously in 100 ml of sterile water and a dilution series to 10^{-10} prepared. One milliliter of each dilution was added to chitin–rose bengal agar (Godoy et al., 1982), the plates incubated for seven days at 25°C in the dark, and colonies counted. Controls for these experiments were sterilized bran inoculated with sterile water. Each treatment was replicated five times.

Where *Paecilomyces* spp. were applied as a drench (Experiments 2 and 3), the inoculum was grown on potato dextrose broth (PDB) (23 g/liter). Each flask containing 50 ml of media was inoculated with a 5-mm plug of fungus and incubated at room temperature for 10 days on a rotary shaker. Each pot received 50 ml of fungus culture and controls received 50 ml of PDB. Each treatment was replicated five times.

Seven days following the initial application of *Paecilomyces* spp., 5000–10,000 *M. incognita* eggs extracted and collected by the method of Hussey and Barker (1973) were added to each pot where appropriate. Greenhouse temperatures were between 24°C and 30°C. The duration of each experiment was 49 days.

One day prior to harvest of each experiment, a 5-mm soil core was taken from those pots that had received *Paecilomyces*. A 1-g subsample was added to 100 ml of sterile water and stirred vigorously for 10 min. One-milliliter aliquots were withdrawn while the suspension was stirring, added to Petri plates, and covered with liquefied chitin–rose bengal agar. Each plate was swirled to distribute the soil suspension, sealed with parafilm, and incubated at 25°C in the dark. Each combination was replicated three times.

Data were recorded on fresh and dry weights of the plants, and galling was assessed using a gall index as follows: 1 = 0–25% of roots galled; 2 = 25–50% galled; 3 = 50–75% galled; 4 = 75–100% galled. Data were also taken on the number of galls per root system. The data were analyzed by ANOVA supplemented by paired *t* tests.

Protein Analyses. To determine if the two *P. merquandii* isolates could be differentiated, proteins from the three fungus isolates were compared by SDS gel electrophoresis. The isolates were first grown in shake culture in potato dextrose broth for seven days at room temperature (22–25°C). Preparation of samples proceeded in the cold. Each culture was processed by passage through Whatman No. 1 filter paper, washed three times with distilled water containing 1 mm phenylmethylsulfonyl fluoride (PMSF), an equal volume of distilled water–PMSF added, and the resultant mixture processed in a Waring blender. This mixture was then passed through a Kontes "mini-bomb" cell disruption chamber using nitrogen at 100 kg/cm^2. The resultant homogenate was centrifuged at 38,720 *g* for 10 min at 4°C, the supernatant removed, 10% mercaptoethanol–

SDS added, and the sample stored frozen prior to electrophoresis. The procedure was replicated three times.

Proteins were separated by SDS gel electrophoresis on 7.5% polyacrylamide gels using a Hoefer Scientific SE600 vertical slab unit with discontinuous buffer, based on the method of Laemmli (1970). Molecular weight markers (1450–480,000) were purchased from Schwarz/Mann Biotech (Cleveland, Ohio 44128). Gels were stained with silver nitrate (Merril et al., 1980). The procedures were modifications of those described by Coles et al. (1989) and were repeated until reproducible protein spectra were attained for each isolate.

Culture Studies. In studies to compare the cultural characteristics of the three isolates, fungus cultures were grown at 25°C on potato dextrose agar. A sterilized cork borer was used to obtain uniform 5-mm fungus plugs to initiate cultures. Colony diameters were measured every two to three days and differences in colony appearance recorded.

RESULTS

Greenhouse Trials. A single application of each of the isolates gave little control of tomato root-knot (Table 1). The three trials in which the pathogenicity of the three *Paecilomyces* isolates to *M. incognita* eggs were compared gave

TABLE 1. EFFECTS OF SINGLE APPLICATION OF *Paecilomyces marquandii* ISOLATES AND *P. lilacinus* ON ROOT-KNOT OF TOMATO[a]

Treatment	Fresh weight (g/plant)		Gall index[b]	Control (%)[c]
	Top	Root		
Untreated control (25 g bran)	88.8 ± 11.1	19.5 ± 2.6		
Infested control (25 g bran)	86.5 ± 6.6	19.0 ± 1.2	2.0 ± 1.1	
PL + Mi	94.4 ± 14.6	20.1 ± 2.9	3.0 ± 0.71	
PM 1 + Mi	82.3 ± 11.2	17.1 ± 2.6	2.2 ± 0.71	15
PM 2 + Mi	88.8 ± 10.7	18.5 ± 2.1	2.0 ± 1.3	8
PM 1 + PM 2 + Mi	92.1 ± 10.4	18.9 ± 1.3	2.4 ± 1.6	
PM 1 + PL + Mi	94.2 ± 19.4	18.8 ± 3.7	2.7 ± 2.4	
PM 2 + PL + Mi	96.8 ± 10.6	20.7 ± 2.8	3.0 ± 0.71	

[a] Each treatment replicated five times. The data presented are the average of five replications ± the standard error. Fungus inoculum was added to each pot in 25 g bran. Inoculum density was 5000 *M. incognita* eggs/plant. There were no significant differences in plant weight or gall index. PL = *Paecilomyces lilacinus*; PM 1 = *Paecilomyces marquandii* isolate 1; PM 2 = *P. marquandii* isolate 2; Mi = *Meloidogyne incognita*.

[b] Gall index: 1 = 0–25% roots galled; 2 = 26–50%; 3 = 51–75%; 4 = 76–100%.

[c] Control (%) = 100 − (galls/treatment/galls/infested control).

variable results (Tables 2 and 3). Where intense root-knot pressure existed, significant reductions in galling generally occurred (Table 3). Tables 2 and 3 show variations in the efficacy of PM 2, specifically 50.3% control in one trial and 10.0% control in the second. However, a consistent trend was observed when data on the reduction of galling for all trials was averaged. These figures show a reduction in galling of 40% by the IPC *Paecilomyces*, 39% by PM 1

TABLE 2. EFFECTS OF TWO APPLICATIONS OF *Paecilomyces marquandii* ISOLATES AND *P. lilacinus* ON ROOT-KNOT OF TOMATO[a]

Treatment	Fresh weight (g/plant)		Gall index[b]	Control (%)[c]
	Top	Root		
Untreated control (25 g bran)	74.8 ± 13.67	14.6 ± 3.1		
Infested control (25 g bran)	68.0 ± 10.16	17.4 ± 1.3	1.6 ± 0.55	
PL + Mi	69.7 ± 16.25	15.3 ± 2.6	1.2 ± 0.45	31.5
PM 1 + Mi	72.3 ± 19.14	14.9 ± 4.5	1.4 ± 0.54	33.7
PM 2 + Mi	79.3 ± 21.48	16.7 ± 4.8	1.0 ± 0.0	50.3

[a]Each treatment replicated five times. The data are presented as in Table 1. Inoculum density was 5000 *M. incognita* eggs/plant. There were no significant differences in plant weight on gall index. Two applications of fungus. In the first application the inoculum was added in 25 g bran. In the second application, the inoculum was added as a 50 ml drench.
[b]Gall index and abbreviations, see Table 1.
[c]Control (%), see Table 1.

TABLE 3. TRIAL TO EVALUATE EFFECTS OF TWO DRENCH APPLICATIONS OF *Paecilomyces* ON ROOT-KNOT OF TOMATO[a]

Treatment	Fresh weight (g/plant)		Gall index[b]	Control (%)[c]
	Top	Root		
Untreated control (+50 ml PDB)	38.6 ± 4.0	6.7 ± 0.81		
Infested control (+50 ml PDB)	39.3 ± 4.7	6.7 ± 0.86	4.0 ± 0.89a	
PL + Mi	39.4 ± 9.1	7.0 ± 2.4	3.4 ± 0.89b	34.0
PM 1 + Mi	37.1 ± 7.1	6.5 ± 1.5	3.0 ± 0.71b	31.0
PM 2 + Mi	43.7 ± 9.1	7.1 ± 1.5	4.0 ± 0.71a	10.0

[a]Each treatment replicated five times. The data are presented as in Table 1. Gall indices followed by different letters are significantly different at the 5% level. Two applications each of 50 ml drench. Inoculum density was 10,000 eggs/pot.
[b]Gall index and abbreviations, see Table 1. Data followed by different letters differ significantly at the $P < 0.05$ level.
[c]Control (%), see Table 1.

and 36% by PM 2. We therefore conclude that these three isolates do not differ in pathogenicity to *M. incognita* eggs. All *Paecilomyces* isolates were successfully reisolated from treated soil after seven weeks (Table 4).

Plant growth was generally slightly better where the fungus–bran combinations were used. However, these differences were neither consistent nor statistically significant when compared to growth of the uninfected controls to which bran was added.

Culture Studies. The *P. marquandii* isolates each initially developed as white surface mycelium, with the underside of the culture exhibiting a bright, diffusible yellow pigment. Bright, purple spores were formed at 10–14 days. Growth rates of the isolates were significantly different, with *P. lilacinus* growing faster than the *P. marquandii* strains.

Under the same conditions, *P. lilacinus* formed a white surface mycelium with the underside of the culture tan to light brown. Brown to light purple spores formed on the leading edge of the mycelium at 10–14 days. *P. lilacinus* grew about twice as fast as did the two *P. marquandii* isolates.

Protein Analyses. The three isolates were differentiated on the basis of their protein spectra. The salient differences, shown on a representative gel, were four proteins (Figure 1C, protein cluster around second arrow from the top in high concentrations, which ranged from 166 to 250 kDa) present in *P. lilacinus* but not in *P. marquandii* isolates. PM 2 displayed a 470-kDa protein (Figure 1B indicated by second arrow from the top) at high concentration that was not present in PM 1. Two proteins (6 and 10 kDa, Figure 1B) present at lower concentrations in PM 2 were absent from PM 1. Several proteins common to *P. lilacinus* and PM 2 were not observed from PM 1 (e.g., 10-and 48-kDa proteins, Figure 1B,C).

DISCUSSION

Chinampa agricultural soils contain endemic populations of microorganisms that reduce population levels of plant-parasitic nematodes and decrease damage from these plant pests. In previous studies (Zuckerman et al., 1989), the level of nematode suppression was estimated at approximately 80%. The initial screen of chinampa soil in our laboratory yielded three fungal and six bacterial isolates that exhibited antinematodal activity (Zuckerman et al., 1989). Several additional microbes with similar properties were isolated in subsequent studies of suppressive soils (Zuckerman, Dicklow, and Marban-Mendoza, unpublished data).

In the current study, control by two applications of *Paecilomyces* averaged 30–40%. Based on these results, we estimate that root-knot control by *P. marquandii* in the field would reach 10–15%, since higher levels of control are

TABLE 4. INITIAL AND FINAL DENSITIES OF THREE *Paecilomyces* ISOLATES

Isolates	Initial inoculum density (CFU/g)[a]	Inoculum density at seven weeks (CFU/g soil)
P. lilacinus	1×10^8	1.96×10^3
P. marquandii isolate 1	9.9×10^9	1.18×10^3
P. marquandii isolate 2	5.2×10^{10}	1.12×10^3

[a] CFU = colony-forming units.

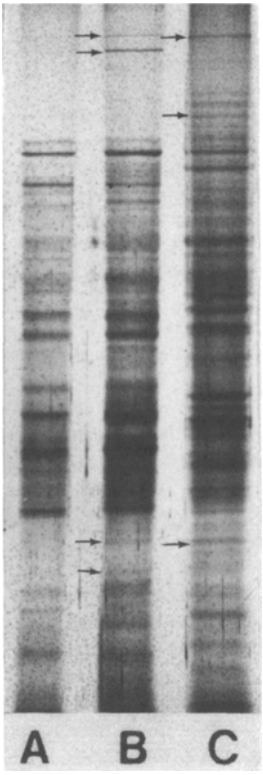

FIG. 1. Protein spectra of the (A) *Paecilomyces marquandii* isolate 1, (B) *Paecilomyces marquandii* isolate 2; and (C) *Paecilomyces* IPC isolate. Proteins specific for each isolate or common to two isolates but absent from the other as described in the text are indicated by arrows.

consistently attained in the greenhouse. Our interpretation of the current results is that *P. marquandii* plays a small, but consistent and important role as one of the natural biosphere colonists that contribute to the suppression of nematode damage in the chinampa soils.

Two applications of the *P. marquandii* isolates and the *P. lilacinus* isolate gave better root-knot control than did one application. The three isolates appeared to display equal ovicidal activity. Gel electrophoresis discriminated between *P. lilacinus* and the *P. marquandii* isolates and also showed specific differences in the two *P. marquandii* isolates. The faster growth rate of *P. lilacinus* suggests that this isolate might more readily colonize the plant rhizosphere than does *P. marquandii*.

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DISCRIMINANT ANALYSIS OF CUTICULAR HYDROCARBONS OF SOCIAL WASP *Polistes exclamans* VIERECK AND SURFACE HYDROCARBONS OF ITS NEST PAPER AND PEDICEL

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Abstract—The cuticular lipids were analyzed from individual *Polistes exclamans* workers collected from 10 nests. Surface lipids were also recovered from the paper and pedicels of these nests. Twenty-two hydrocarbons were identified in these lipid fractions. The major components of the wasp cuticular lipids were *n*-heptacosane, *n*-nonacosane, methylhentriacontane, and methyltritriacontane. Discriminant analysis of the hydrocarbon profiles of the adult wasps showed that the wasps group together according to their respective colonies. Several colonies from the same geographical location clustered more closely together than colonies from diverse locations. The nest papers and pedicels did not group with the wasps from their respective colonies, but the nest papers clustered together separately, as did the nest pedicels.

Key Words—*Polistes exclamans*, Hymenoptera, Vespidae, cuticular hydrocarbons, discriminant analysis, nest pedicel, nestmate recognition, social wasps.

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INTRODUCTION

Insect cuticular hydrocarbons provide a hydrophobic barrier that protects individuals from desiccation (Blomquist and Dillwith, 1985; Hadley, 1985; Lockey, 1988). For many insect species, cuticular hydrocarbons have been shown to serve additional functions such as sex pheromones or recognition factors (Howard and Blomquist, 1982; Blomquist and Dillwith, 1985; Espelie et al., 1991).

Kin recognition cues for social insects are thought to be chemicals located in the cuticle that can be detected by other members of the colony (Wilson, 1971; Hölldobler and Michener, 1980; Fletcher and Michener, 1987). Studies with ants and termites have indicated that cuticular hydrocarbons play an important role in the process of kin recognition (Howard et al., 1982; Brill et al., 1986; Obin, 1986; Bonavita-Cougourdan et al., 1987; Vander Meer et al., 1989a; Bagnères et al., 1991). Several species of insects that are able to integrate into ant or termite colonies have cuticular hydrocarbon patterns very similar to those of their hosts (Howard et al., 1980; Vander Meer and Wojcik, 1982; Espelie and Hermann, 1988; Vander Meer et al., 1989b). These foreign insects are thought to be utilizing chemical mimicry in order to overcome normal nestmate recognition, thus supporting the possibility that cuticular hydrocarbons play a major role in kin recognition.

For several species of social wasps the nest has been clearly shown to be critical for the ontogeny of nestmate recognition ability (Shellman and Gamboa, 1982; Pfennig et al., 1983; Ryan et al., 1985; Gamboa et al., 1986; Venkataraman et al., 1988). When workers emerge, they come in contact with the nest and, with as little as 1 hr exposure to the nest, they are later able to discriminate between nestmates and nonnestmates (Pfennig et al., 1983). During the early stages of the colony development, when there are few adults on the nest, it would be especially advantageous for the emerging workers not to have to rely on the presence of the queen or workers to learn or acquire the nest odor (Gamboa et al., 1986).

Hydrocarbons have been found on the surface of the nests of several species of social wasps that are the same as those found on the cuticle of the respective wasps (Espelie and Hermann, 1988, 1990; Espelie et al., 1990). These nest paper hydrocarbons are necessary for *Polistes metricus* workers to be able to recognize their nest (Espelie et al., 1990). It has also been shown that these hydrocarbons must be on the nest in order for newly emerged *P. metricus* wasps to later recognize their nestmates (Singer and Espelie, 1992).

Although the importance of the nest for nestmate recognition has been established for several *Polistes* species, the role of cuticular and nest paper hydrocarbons has been examined only with *P. metricus*. In the present paper, we characterize the hydrocarbons extracted from the cuticle of *Polistes excla-*

mans workers and show with discriminant analysis that wasps from the same colony have a very similar profile of cuticular lipids.

METHODS AND MATERIALS

Collection. Ten colonies of *Polistes exclamans* were collected from sites in Clarke, Elbert, and Jackson counties in northeastern Georgia in June and July 1990 and immediately brought to the laboratory. Wasps were separated from their nests and frozen at −20°C. Workers were selected randomly from the wasps of each colony and extracted individually in redistilled hexane at room temperature for 60 sec (Blomquist et al., 1987). Pedicels were severed from the nest and extracted in hexane for 2 min at room temperature. Surface lipids were recovered from the nest paper by directing a stream of hexane at the outer surface of the nest and collecting the hexane in a beaker beneath the nest (Espelie et al., 1990).

Chemical Analysis. The hexane extracts were concentrated to dryness under a stream of nitrogen. The samples were resuspended in hexane (50–500 μl) and aliquots (usually 0.1–1%) were analyzed by combined gas chromatography–mass spectrometry. A Hewlett Packard 5890A gas chromatograph was equipped with a 25-m cross-linked methyl silicone capillary column with helium as the carrier gas. The oven temperature was kept at 55°C for 3 min after injection (splitless) and then the temperature was increased at a rate of 15°/min to a final temperature of 305°C. The column was connected to a Hewlett Packard 5970 mass selective detector, and mass spectra were recorded at 1.3-sec intervals at 70 eV. Data were analyzed with a Hewlett Packard Chem Station, and individual components were characterized by their mass spectra. Equivalent chain lengths were determined using standard *n*-alkanes (Sigma Chemical Co.) (Nelson, 1978; Blomquist et al., 1987), and quantitation was based upon peak areas of total ion chromatograms (Espelie and Bernays, 1989).

Discriminant Analysis. Results from the GC-MS analysis of the surface hydrocarbons from each wasp, nest paper, and nest pedicel were loaded according to percent composition onto an 84 × 22 matrix (Lotus 123). These data were transferred to a flat ASCII file on a Sun Microsystems 4/280 server running SunOS 4.0.3 (UNIX) prior to initial processing to produce submatrices and vectors representing wasps, nest paper, nest pedicels, and row/column labels, respectively. The smaller data subsets were analyzed by discriminant and hierarchical cluster analyses using the 2.2 version of the S-plus statistical software package (Statistical Sciences, Inc., 1989, 1990). Discriminant analyses were performed on the hydrocarbon profiles of the wasps only from colonies 0–9 and

on profiles of the wasps, nest papers, and nest pedicels of colonies 0–9 (Lachenbruch, 1975). Hierarchical cluster analyses, using average-linkage cluster formation on Euclidean distance between centroids of points, examined the hydrocarbon profiles of wasps, nest paper, and nest pedicels from each colony (Pielou, 1984; Becker et al., 1988). The data set was reduced from 22 to nine hydrocarbons for the analyses that included extracts of the nest papers and pedicels. The nine hydrocarbons in the reduced data set were common to the profiles of wasps, nest papers, and pedicels.

RESULTS

The cuticular hydrocarbons of *P. exclamans* workers were found by GC-MS analysis to be a mixture of *n*-alkanes, alkenes, methylalkanes, and dimethylalkanes. The major *n*-alkane was *n*-nonacosane, while the dominant methyl-branched hydrocarbons were a series of positional isomers of methylheptacosane, methylnonacosane, and methylhentriacontane (Table 1). The cuticular hydrocarbon compositions of the *Polistes exclamans* workers from the 10 nests (colonies 0–9) were very similar (Figure 1). Workers from nest 0 and nest 6 differed from one another primarily in the proportions of the methylheptacosanes and methylnonacosanes (Table 1).

The results of the GC-MS analyses of the cuticular hydrocarbons of the 50 wasps from the 10 colonies were analyzed by discriminant analysis in an effort to identify similarities between the cuticular hydrocarbon profiles of individual wasps from each nest. A plot of the first and second discriminant variables from this analysis resulted in a pattern where the five wasps from each colony clustered together in distinct groups (Figure 2). Colonies 0, 1, 2, and 3 were collected from widely separated geographic locations (>5 km). Colony 4 was collected at the same site as colony 3; the two nests were separated by 10 m. The hydrocarbon patterns for the wasps from colonies 0–4 are separated by the first discriminant variable (Figure 2). Colonies 5, 6, 7, 8, and 9 were collected from the same location and were located within 10 m of each other (>10 km from colonies 0–4). In the discriminant analysis, the hydrocarbon profiles of the workers from nests 5–9 are clustered in five groups, which are very close together. The individual wasps are clustered according to colony, but these clusters are not well separated by the first discriminant variable. The linear coefficients of the first discriminant variable are listed in Table 2. The components that were weighted most heavily in the analysis were heptacosene, *n*-heptacosane, *n*-octacosane, and dimethyltritriacontane.

The nest papers and pedicels of *P. exclamans* nests had surface hydrocarbon profiles that were very similar to those of the wasps (Table 1). However, several hydrocarbons that were minor components in the cuticular hydrocarbon profiles

TABLE 1. HYDROCARBON COMPOSITION (%) OF CUTICULAR LIPIDS OF WASPS AND EXTRACTS OF SURFACE OF NEST PAPER AND SURFACE OF NEST PEDICEL FROM TWO *Polistes exclamans* NESTS

			Nest 0			Nest 6		
Peak[a]	ECL[b]	Component[c]	Wasp	Paper	Pedicel	Wasp	Paper	Pedicel
1	22.9	Tricosene	0.00	0.00	0.00	0.00	0.00	0.00
2	23.0	*n*-Tricosane	0.00	0.29	0.00	0.00	0.00	0.00
3	24.9	Pentacosene	0.00	0.00	0.00	0.00	0.00	0.00
4	25.0	*n*-Pentacosane	0.61	0.45	0.00	0.19	0.00	0.00
5	26.9	Heptacosene	0.00	0.09	0.00	0.00	0.00	0.00
6	27.0	*n*-Heptacosane	2.74	5.52	2.37	2.37	2.51	0.95
7	27.3	11- and 13-Methylheptacosane	3.23	4.55	6.80	0.69	0.00	0.00
8	27.4	7-Methylheptacosane	0.78	0.00	0.00	1.04	0.00	0.00
9	27.7	3-Methylheptacosane	3.15	5.92	11.08	2.62	2.91	2.70
10	28.0	*n*-Octacosane	0.95	1.33	0.00	0.92	0.00	0.00
11	28.9	Nonacosene	0.68	0.86	0.41	0.77	0.00	0.00
12	29.0	*n*-Nonacosane	12.50	11.30	3.15	11.33	19.93	6.13
13	29.3	13- and 15-Methylnonacosane	22.41	19.42	30.40	24.23	19.50	31.58
14	29.5	5-Methylnonacosane	0.66	1.32	1.96	1.32	1.16	1.63
15	29.6	11,15- and 13,17-Dimethylnonacosane	1.15	2.98	9.67	1.10	3.21	5.87
16	29.7	3-Methylnonacosane	10.79	11.82	10.04	15.81	30.74	18.96
17	30.0	*n*-Triacontane	2.34	2.07	1.39	2.36	0.00	1.59
18	31.0	*n*-Hentriacontane	0.88	0.91	0.00	0.59	0.00	0.00
19	31.3	13- and 15-Methylhentriacontane	20.66	13.94	12.61	20.03	13.10	22.57
20	31.6	11,15- and 13,17-Dimethylhentriacontane	4.59	3.77	1.72	5.67	4.21	5.36
21	33.3	13- and 15-Methyltritriacontane	3.53	2.48	0.94	4.09	0.00	2.65
22	33.6	11,15- and 13,17-Dimethyltritriacontane	1.29	0.43	0.00	0.51	0.00	0.00

[a]Peak numbers correspond to those in Figure 1.
[b]Equivalent chain length.
[c]Components are only listed if their presence was confirmed by mass spectral analysis.

of the wasps were not present in the extracts of the nest papers and pedicels. Consequently, a reduced data set (using the nine most dominant hydrocarbons) was utilized for a discriminant analysis of the hydrocarbon profiles of the wasps, nest papers, and pedicels. A plot of the first and second discriminant variables from this analysis resulted in clustering of wasps according to colony, but there was no association of the wasps with their respective nest paper or pedicel

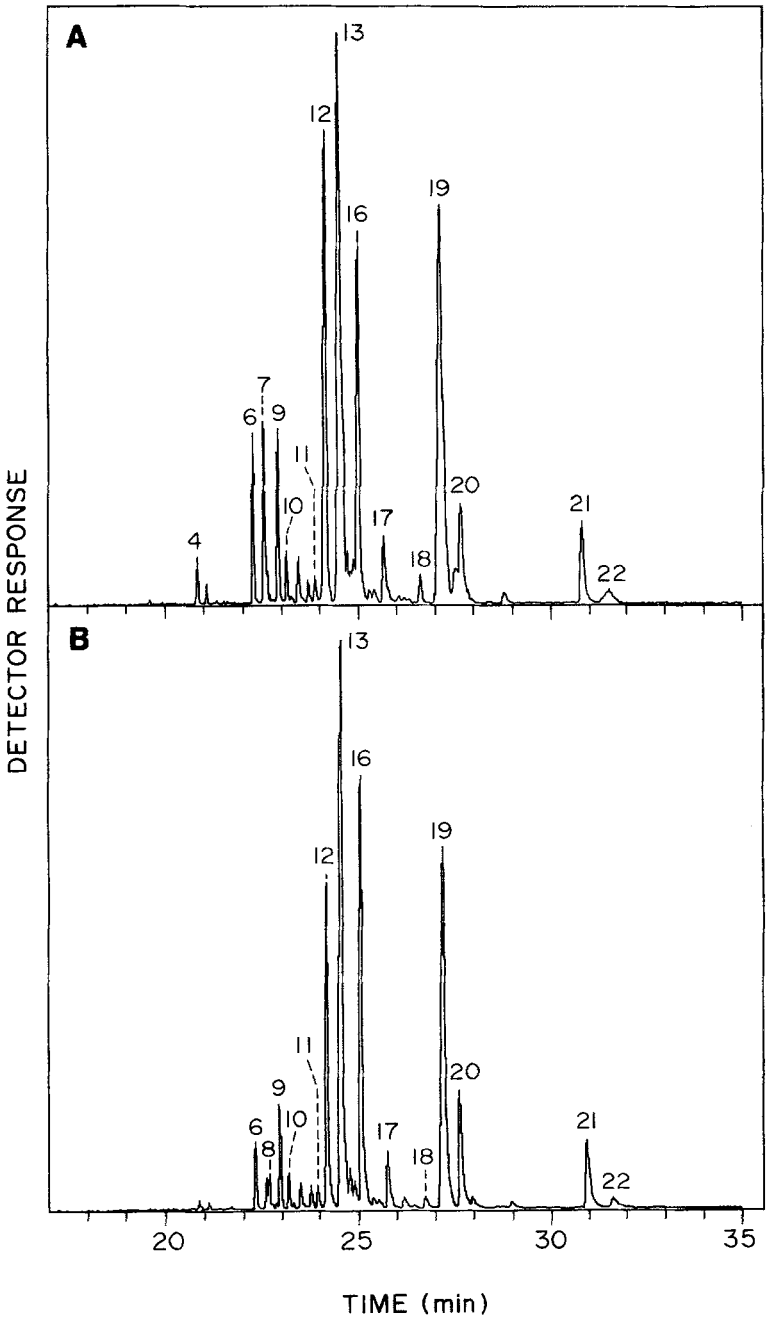

FIG. 1. Total ion chromatograms of the cuticular hydrocarbons of individual *Polistes exclamans* workers from (A) nest 0; and (B) nest 6. Numbered components are identified in Table 1.

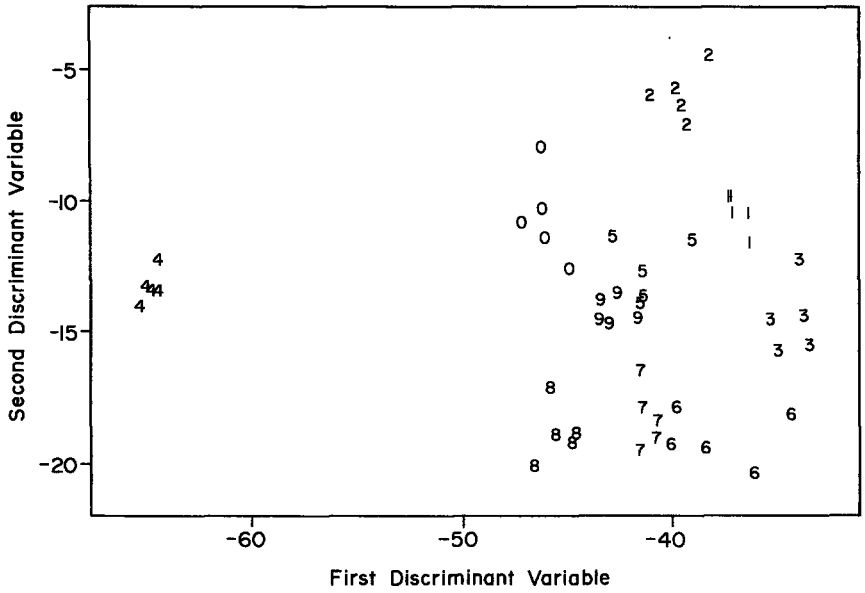

FIG. 2. Plot of the first two discriminant variables from a discriminant analysis of the cuticular hydrocarbons from workers from 10 colonies (0–9) of *Polistes exclamans*. Each number represents an individual wasp. Analysis was done on the complete data set (Table 2).

(Figure 3). The hydrocarbon profiles of the pedicels and the nest paper formed separate clusters. When nest paper profiles from two colonies plotted close to each other (e.g., 4 and 5), the hydrocarbon profiles of the nest pedicels from these two colonies did not plot close together. The hydrocarbons that were weighted most heavily in this analysis of wasps, nest paper, and nest pedicels were *n*-nonacosane and 3-methylnonacosane (Table 2).

A hierarchical cluster analysis was performed on the reduced data sets of hydrocarbon profiles for the wasps, nest papers, and nest pedicels (Figure 4). Again, most of the wasps clustered together according to colony. The nest paper and nest pedicel profiles clustered at either extreme of the dendrogram, indicating that they are very dissimilar to the wasps from their respective colonies. That is, nest paper profiles and nest pedicel profiles formed groups that were largely distinct.

DISCUSSION

The cuticular hydrocarbon composition of *P. exclamans* workers is similar to the compositions recently reported for workers of *P. annularis* (Espelie and Hermann, 1990) and *P. metricus* (Espelie et al., 1990). The major hydrocarbons

TABLE 2. FIRST LINEAR COEFFICIENTS FOR DISCRIMINANT ANALYSES

Peak	Component[a]	First linear coefficients	
		Figure 2	Figures 3 and 4
1	$C_{23:1}$	−5.1195	
2	n-C_{23}	0.6789	
3	$C_{25:1}$	−4.4480	
4	n-C_{25}	−8.1508	
5	$C_{27:1}$	25.1742	
6	n-C_{27}	6.4316	−0.1418
7	13-Methyl-C_{27}	−2.2580	−1.1920
8	7-Methyl-C_{27}	−2.6737	
9	3-Methyl-C_{27}	−4.1727	−0.3301
10	n-C_{28}	3.2724	
11	$C_{29:1}$	−7.0478	
12	n-C_{29}	−1.9051	0.6749
13	13-Methyl-C_{29}	0.7705	−0.0979
14	5-Methyl-C_{29}	0.4209	
15	Dimethyl-C_{29}	−3.8420	
16	3-Methyl-C_{29}	0.1375	0.3692
17	n-C_{30}	0.6942	
18	n-C_{31}	0.8175	
19	13-Methyl-C_{31}	−1.8284	−0.2076
20	Dimethyl-C_{31}	0.9354	−0.6031
21	Methyl-C_{33}	0.3865	0.0702
22	Dimethyl-C_{33}	3.3526	

[a] Full names of components are listed in Table 1.

of *P. exclamans*, *n*-nonacosane and mid-chain methyl-branched positional isomers of methylnonacosane and methylhentriacontane (Table 1), were present in the cuticular lipids of both *P. annularis* and *P. metricus*. However, *P. annularis* and *P. metricus* workers had much higher proportions of dimethylbranched hydrocarbons (30% and 17%, respectively) than did *P. exclamans* (7%). *P. exclamans* and *P. annularis*, which are in the subgenus *Aphanilopterus*, had higher amounts of terminally methyl-branched hydrocarbons (15% and 29%, respectively) than did *P. metricus* (0.3%), which is in the subgenus *Fuscopolistes*.

Surface hydrocarbons may play an important role in nestmate recognition for *P. exclamans*. The similarity in hydrocarbon profiles of wasps within colonies (Figure 2) supports this possibility. Although the wasps from the same colony were clustered according to their hydrocarbon profiles, they did not have profiles that were similar to the hydrocarbons on the surface of their respective nests (Figure 3). It may not be necessary for a wasp to have the same surface

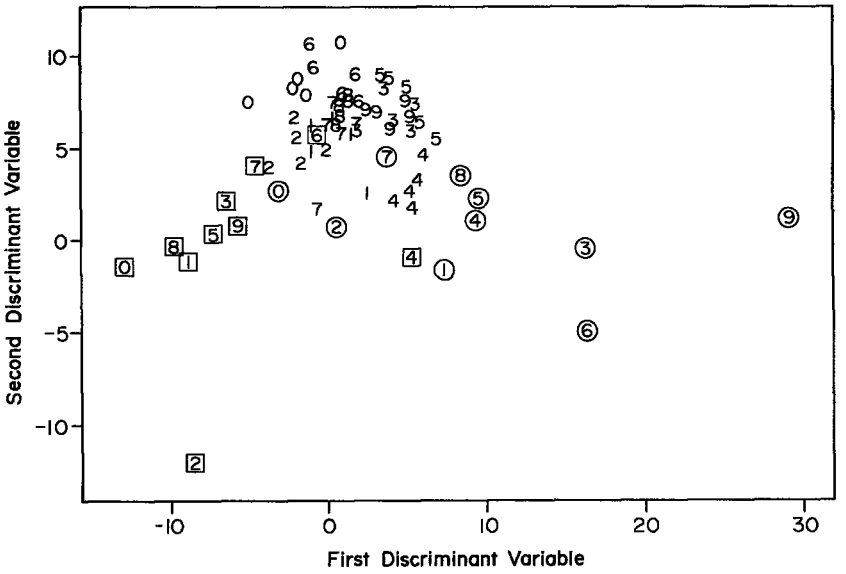

FIG. 3. Plot of the first two discriminant variables from a discriminant analysis of the hydrocarbons from workers (isolated numbers), nest paper (circles), and nest pedicel (squares) from 10 colonies (0–9) of *Polistes exclamans*. Analysis was done on the reduced data set (Table 2).

chemistry as its nest paper in order for that wasp to recognize the nest and other wasps from its colony. It is possible that the wasps, or wasps from some of the colonies, may utilize only some of the surface hydrocarbons for nest and nestmate recognition. Some wasps might recognize their nestmates primarily by the presence of the methyl-branched hydrocarbons (or the *n*-alkanes) rather than the mixture of both. With this possibility in mind, it is interesting that the cuticular lipids of the social wasps species that have been analyzed are a mixture of types of hydrocarbons, rather than merely a mixture of homologs with varying chain lengths (Espelie and Hermann, 1988, 1990; Espelie et al., 1990; Butts et al., 1991). Another possibility is that the mechanism of recognition in *P. exclamans* differs from that in *P. fuscatus* and *P. metricus*. For example, the nest may not be intimately involved in the development of recognition ability.

The similarity in the hydrocarbon profiles of the nest pedicel extracts (Figures 3 and 4) is intriguing. The hydrocarbon composition of the pedicels from 13 nests of *P. metricus* were also found to be very similar (Espelie et al., 1990). The pedicel and paper of *P. annularis* (Espelie and Himmelsbach, 1990) and *P. metricus* (Singer et al., 1992) nests have been shown to contain a protein that has a silklike amino acid composition. The pedicel has approximately six times as much protein as the nest paper. This higher protein content may cause

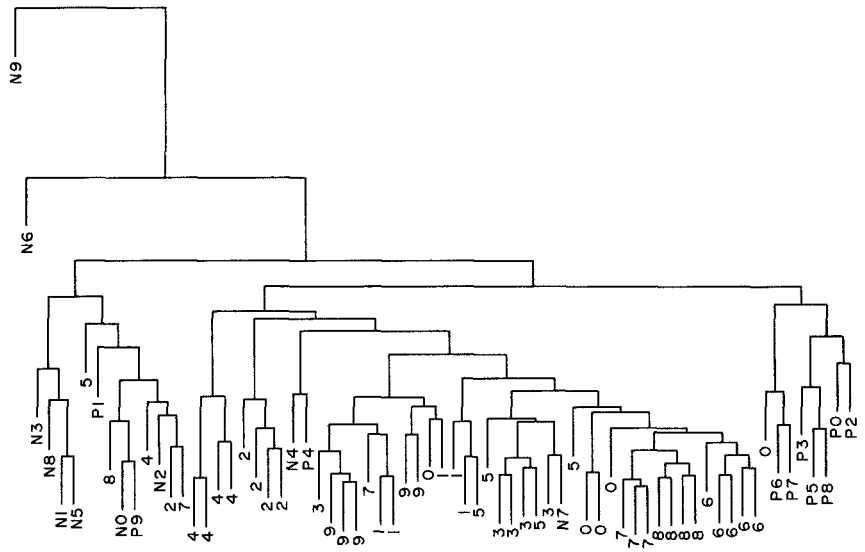

FIG. 4. Hierarchical clustering of the hydrocarbon profiles from individual workers (isolated numbers), nest paper (N), and nest pedicel (P) from 10 colonies (0–9) of *Polistes exclamans*. Analysis was done on the reduced data set (Table 2).

the selective retention of some of the surface hydrocarbons and result in the hydrocarbon profiles of the pedicel being different from those of the nest paper.

Many *Polistes* species are strongly philopatric and usually return to nest within 10 m of where their parental nests were built the previous year (Klahn, 1979; Strassmann, 1981; Reeve, 1991). Consequently, *Polistes* colonies are frequently found in aggregations. Workers are very likely to encounter members of other colonies, including related conspecifics. Willer and Hermann (1989) found that it was common for *P. exclamans* workers to switch from one nest to another at a study site that had several nests in close proximity. It was recently shown that *P. exclamans* workers are more tolerant of nonnestmates from nearby nests than they are of nonnestmates from distant nests (Pfennig, 1990). *P. exclamans* have a lower affinity for their nest than other *Polistes* species. In laboratory tests where *P. exclamans* workers were placed in a chamber with their own nest and another nest, they were found to be on their own nest 9% of the time, the other nest 3% of the time, and no nest 85% of the observed time (Singer and Espelie, unpublished results). In a similar test, the observed locations for *P. metricus* workers were 66, 3, and 31% for their own nest, the other nest, and no nest, respectively (Espelie et al., 1990). Wasps from the five colonies (5–9) that were collected from the same location in this study have similar hydrocarbon profiles (Figure 2). If variations in surface hydrocarbons

are important for kin recognition, it may be difficult for wasps with such similar profiles to discriminate between nestmates and nonnestmates from nearby nests. Behavior tests, such as those done with *P. metricus* workers (Singer and Espelie, 1992), will help to determine the role that cuticular hydrocarbons and nest paper hydrocarbons play in nestmate recognition for *P. exclamans*.

Acknowledgments—We thank Kimberly Fitzgerald for excellent technical assistance and the residents of northeastern Georgia who allowed us to collect wasp colonies from their property. This work was supported, in part, by Sigma Xi Grants-in-Aid to T.L.S. and by funds allocated by the University of Georgia Agricultural Experiment Station.

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D-(+)-PINITOL, AN OVIPOSITION STIMULANT FOR THE PIPEVINE SWALLOWTAIL BUTTERFLY, *Battus philenor*

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Abstract—Oviposition by females of the pipevine swallowtail butterfly, *Battus philenor*, was stimulated by contact with alcoholic extracts of host foliage. D-(+)-Pinitol was isolated and identified from leaf material of one host species, *Aristolochia macrophylla* (Aristolochiaceae). In combination with chloroform-soluble components of host leaf material, this compound was comparable to the parent extract in stimulating oviposition.

Key Words—*Battus philenor*, Papilionidae, Lepidoptera, *Aristolochia macrophylla*, Aristolochiaceae, learning, D-pinitol, sequoyitol, *myo*-inositol, cyclitols, oviposition stimulant.

INTRODUCTION

Plant secondary chemicals are thought to pose both toxicological and behavioral barriers to evolutionary changes in host use by phytophagous insects (Fraenkel, 1959; Ehrlich and Raven, 1964; Feeny, 1987). Arguing that secondary chemicals represent principally behavioral barriers, Dethier (1941) and Jermy (1976,

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1984) proposed that colonization of a novel plant by an insect species will be more probable if that plant contains compounds similar to those that the insects already use as cues in host recognition.

Dethier (1941) suggested that evolutionary shifts across plant families by swallowtail butterflies (family Papilionidae) were facilitated by essential oils shared by these families, compounds to which he found that the larvae were attracted (see also Takaishi et al., 1969; Saxena and Prabha, 1975; Khattar and Saxena, 1978). While the significance of essential oils as host-selection cues by papilionid larvae remains somewhat unclear (D.R. Papaj, K. Hale, D. Miller, and P. Feeny, unpublished), Feeny (1987) suggested that evolutionary host shifts were as likely to be regulated by the behavior of the adult female as by that of the larvae. Adult females choose the plant on which eggs will hatch and are known to oviposit "mistakenly" on nonhost plants from time to time. Plants on which mistakes are made may occasionally be incorporated into the host range of an insect population, perhaps leading eventually to the formation of new insect species (cf. Bush, 1975).

If inclusion of a given novel plant into a species' host range depends on how often eggs are mistakenly laid on the plant and if mistakes occur more frequently on plants containing chemicals similar to those that females use to select hosts than on chemically dissimilar plants, we might anticipate that related insect species will tend to exploit host species that are phytochemically, although not necessarily taxonomically, related. Exactly this pattern is observed in some taxa of butterflies (Ehrlich and Raven, 1964; Feeny et al., 1983), although little is yet known about which particular compounds are important in mediating host recognition.

Our paper is part of a broader effort by this and other laboratories to characterize the oviposition stimulants of the Papilionidae (Ichinosé and Honda, 1978; Saxena and Goyal, 1978; Feeny et al., 1983, 1988; Nishida, 1977; Ohsugi et al., 1985; Nishida et al., 1987; Nishida and Fukami, 1989; Honda, 1986, 1990). Comparative studies of different species in a family for which both larval food plants (Scriber, 1984) and systematics (Miller, 1987) are particularly well known should permit clarification of the roles played by plant chemistry in the evolution of phytophagous insects (Feeny, 1987, 1991). Here we report the isolation and identification of an oviposition stimulant for the pipevine swallowtail butterfly, *Battus philenor*, from the foliage of one of its host species, *Aristolochia macrophylla*. The pipevine swallowtail is found across much of the continental United States and Mexico (Tyler, 1975). Over its entire range, adults oviposit and larvae feed exclusively on plants in the genus *Aristolochia* in the family Aristolochiaceae (Opler and Krizek, 1984). A previous study (Papaj, 1986a) indicated that methanolic extracts of *A. macrophylla* leaves elicited oviposition by pipevine swallowtail females on artificial substrates treated with those extracts.

METHODS AND MATERIALS

Insects. Females of *B. philenor* used in bioassays comprised three types: those caught in the wild, those reared from eggs of individuals collected in the wild, and those reared from larvae collected in the wild. All adults and larvae were collected at two sites: Terrapin Mountain Overlook and Thunder Ridge Lookout, along the Blue Ridge Parkway in Jefferson County, Virginia. Individuals at these sites feed and oviposit exclusively on *A. macrophylla* (Papaj, 1986b).

Adults were stored in glassine envelopes at ca. 5–15°C until return to our laboratory at Cornell University. In the laboratory, individuals were stored in envelopes at 4°C on alternate days. On the days on which they were brought to room temperature and used in assays, they were fed a solution of 15% honey in water. All individuals were permitted to lay eggs on either *A. tagala* or *A. macrophylla* at least once every three days. Larvae collected in the field were stored at ca. 5–15°C until return to our laboratory at Cornell University where they were placed in cages located in a greenhouse. Larvae were fed mainly on cuttings of *A. macrophylla* collected from residential sites in Ithaca, New York, but also on live *A. macrophylla* and *A. tagala* plants cultured in the greenhouse. Larvae hatching from eggs laid by wild females held captive in the laboratory were reared under the same conditions.

Plants. A. macrophylla was collected from either of the general localities: (1) Jefferson County, Virginia, at various montane sites along the Blue Ridge Parkway, (2) Tompkins County, New York, at various sites in residential areas principally within the Ithaca city limits. Leaves were transported in a cooler to the laboratory at Duke or Cornell Universities, where they were extracted immediately. Representative plant specimens have been deposited in the Bailey Hortorium, Cornell University.

Bioassay Procedure. Bioassay of adult female response to plant extracts (concentrations expressed as gle, gram leaf equivalent, fresh weight) followed Feeny et al. (1988) with some modifications. A quantity (gle) of test extract was applied in a thin line across a 2.54-cm-wide strip of Whatman No. 1 cellulose. Solvent was blown off with nitrogen. The test strip was then hung in a cage and misted with water. A female was placed on the strip and permitted to walk up the strip. A positive response was recorded if the female curled her abdomen and extended her ovipositor upon tarsal contact with the treated zone. If the female did not curl her abdomen or extend her ovipositor within 15 sec, a negative response was recorded.

Females were usually pretested by placing them on damp paper in the absence of an extract or solvent. Any female that responded positively to the water blank was discarded. Similarly any female that extended her proboscis in response to the contact with a test strip (as if to drink) was also discarded. While

individuals were commonly retested in successive assays, females used in one assay were not reused for at least 30 min. Butterflies tested at shorter intervals exhibited a carry-over effect, tending to respond positively even to solvent if the response to the previous fraction had been positive.

Extraction and Fractionation of Plant Material. Leaves of *A. macrophylla*, in batches of approximately 300 g (fresh weight), were macerated in hot 95% EtOH (~2.5 liters), boiled for 5 min, and filtered. EtOH was removed from the filtrate by evaporation under reduced pressure at <40°C, and the resulting aqueous suspension was centrifuged to remove chlorophyll. The dark brown aqueous fraction (300 ml) was then extracted with $CHCl_3$ (3 × 300 ml) to yield the $CHCl_3$ fraction (Figure 1). The remaining aqueous fraction (post-$CHCl_3$ aqueous fraction) was further fractionated into the EtOAc fraction and the post-

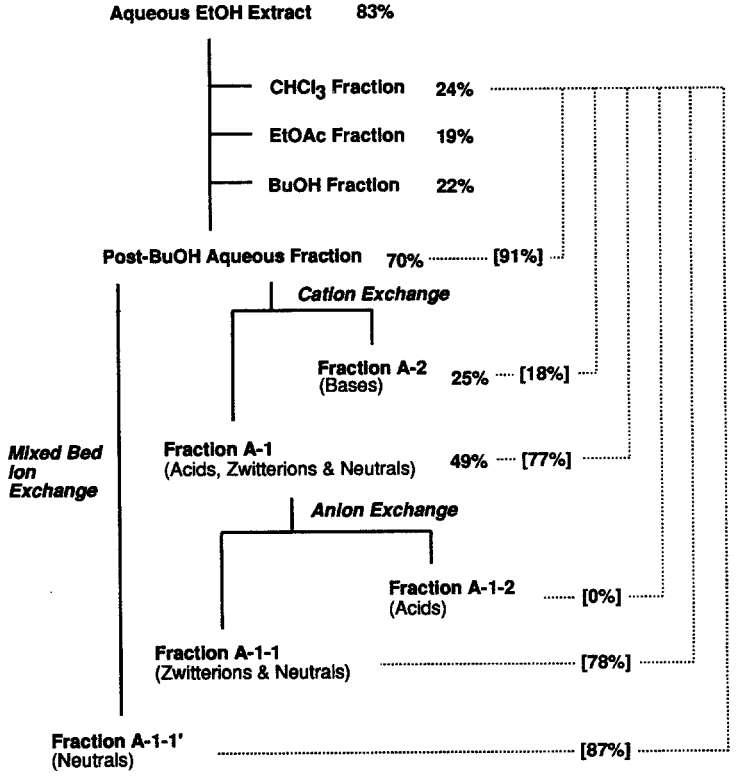

FIG. 1. Extraction scheme for isolation of oviposition stimulants for *Battus philenor* from leaves of *Aristolochia macrophylla*. Bioassay responses, as percentage of females giving a positive response, are shown for several fractions and (in brackets) combinations of fractions (see Tables 1 and 2 for relevant doses).

EtOAc fraction by extraction with EtOAc (3 × 300 ml). The post-EtOAc fraction was extracted with *n*-BuOH (3 × 300 ml) to obtain the BuOH fraction and the post-BuOH aqueous fraction (Figure 1). All the organic and aqueous fractions were concentrated under reduced pressure at <40°C. The organic fractions were evaporated to dryness and taken up in MeOH.

Instrumentation. HPLC fractionations were performed on a Waters 600 Multisolvent Delivery System equipped with a Waters model U6K LC injector, a Waters 490 Programmable Wavelength Detector, a Waters R 401 Differential Refractometer, and a Waters 730 Data Module.

Gas chromatography was carried out on a Perkin Elmer model 3920 GC, equipped with a FID detector and a glass column of 3% OV-101 (180 × 0.6 cm OD and 0.2 cm ID) supported on Chromosorb W-HP (mesh range 100–120). The flow rate of the carrier gas, N_2, was maintained at 40 ml/min. and the injector and detector temperatures at 280°C. EI mass spectra of TMSi derivatives were recorded on a Finnigan model 3300 Mass Spectrometer (70 eV) equipped with a wide-bore capillary column (Supelco) coated with SPB-1.

Analysis of Active Material. For the separation of bases from polar neutrals, acids, and zwitterions present in the post-BuOH aqueous fraction, the latter was subjected to cation exchange chromatography on Amberlite CG-50 (Sigma). The fraction (5 gle in 1.1 ml water) was loaded on a column (10 × 1.1 cm) of the resin packed in water. Two eluates (200 ml each) were collected using deionized water and 2 N HCl, respectively. The aqueous and acid eluates were concentrated by evaporation under reduced pressure to yield fractions A-1 (containing polar neutrals, acids and zwitterions) and A-2 (containing bases), respectively (Figure 1).

Further fractionation of A-1 was carried out by anion exchange chromatography. A-1 (5 gle) was applied to a column (10 × 1.1 cm) of Rexyn 203 (OH) resin (Fisher Scientific) prewashed with water (approx. 400 ml). Elution was achieved with water (100 ml), followed by 2 N HCl (100 ml). Both eluates were evaporated under reduced pressure. The aqueous fraction A-1-1 consisted of neutrals and zwitterions, whereas the second fraction A-1-2 contained acids (Figure 1).

For more rapid separation of polar neutral compounds from the post -BuOH aqueous fraction, the latter (20 gle) was passed through a column (17.5 × 1.1 cm) of Rexyn I-300 (H/OH) resin (Fisher Scientific). This mixed-bed resin is a strong cation and anion exchanger and therefore removes zwitterions and acidic and basic compounds from a mixture. The neutral compounds were eluted with water (200 ml). The aqueous fraction A-1-1′ (Figure 1) was concentrated by evaporation under reduced pressure at <40°C.

HPLC Fractionation of A-1-1′. The active fraction A-1-1′ was separated further into fractions 1–5 by HPLC on a YMC PAK-24 (25 × 1.0 cm) NH_2 partition column (YMC Inc.). The column was eluted with an isocratic system

of acetonitrile–water (75:25) at 3.0 ml/min. Separation was monitored by UV (195 and 200 nm) and RI detectors.

Preparation of Trimethyl Silyl Derivatives. HPLC fractions (each 0.4 gle in 100 μl water) were transferred to Reacti-vials (Pierce, 1 ml) and dried under a stream of N_2. The trimethyl silyl (TMSi) derivatives were prepared using Sil-Prep (Alltech Associates, 50 μl/sample). Each reaction mixture was stirred vigorously for 5 min on a Vortex mixer and then heated at ca. 120°C in a mineral oil bath for approximately 20 min with occasional shaking. The TMSi derivatives of standard samples of D-(+)-pinitol (100 μg), D-(+)-glucose (80 μg), sequoyitol (100 μg), sucrose (80 μg), and *myo*-inositol (100 μg) were prepared similarly. The sources of the standard compounds were: D-(+)-pinitol—a gift from Karen Lee Koster, Cornell University; D-(+)-glucose and *myo*-inositol—Sigma, sequoyitol—Roth; and sucrose—Empire Food Inc.

The TMSi derivatives were analyzed by GC using the following program: (1) 100°C for 4 min, (2) 100–305°C at 4°C/min, (3) hold at 305°C for 20 min.

Quantitative Analysis of A-1-1'. Fractions 1, 2, and 4 were reacted to form TMSi derivatives as above. Concentrations were determined by reference to standard GC chromatograms of TMSi derivatives of D-(+)-pinitol , sequoyitol, *myo*-inositol, and D-(+)-glucose.

Preparation of Acetyl Derivatives. Fraction 1 (0.2 gle) was dissolved in dry pyridine (100 μl) and dry acetic anhydride (100 μl) and kept overnight at room temp. The reaction mixture was diluted with ice cold water (200 μl) and evaporated to dryness. The gummy residue was taken up in water (200 μl) and extracted with $CHCl_3$ (3 × 200 μl). The $CHCl_3$ fraction was evaporated to dryness and redissolved in $CHCl_3$ (100 μl). A standard sample of D-(+)-pinitol (100 μg) was acetylated in the same manner. GC analyses of the acetates of fraction 1 and D-(+)-pinitol (2 μl each) were performed using the following program: (1) 100°C for 4 min, (2) 100–305°C at 8°C/min, (3) hold at 305°C for 5 min.

RESULTS

Ethanolic extracts of both young and mature *A. macrophylla* leaves were active in eliciting oviposition responses (Table 1), verifying earlier findings with methanolic extracts prepared in a similar manner (Papaj, 1986a). Bioassays indicated that activity was retained primarily in the aqueous layer after successive extractions with organic solvents (Table 1). Organic fractions, when bioassayed alone, were not active. The activity of the post-BuOH aqueous fraction, followed over a range of concentrations, increased steadily to a maximum at 1 gle and then decreased (Figure 2). Addition of the $CHCl_3$ fraction to the post-BuOH aqueous fraction, both at 0.1 gle, appeared to enhance activity, although the increase was not statistically significant (Table 1).

TABLE 1. CONTACT OVIPOSITION RESPONSES OF *Battus philenor* FEMALES TO EXTRACTS AND FRACTIONS OF EXTRACTS OF *Aristolochia macrophylla* LEAVES[a]

Extract	*N*	Response (%)
Young July leaves (EtOH)	30	**80**
Young September leaves (MeOH)	30	**83**
Mature July leaves (MeOH)	15	**67**
Solvent control: MeOH	30	10
$CHCl_3$ fraction (in MeOH)	17	24
Post-$CHCl_3$ aqueous fraction	16	**69**
Solvent control: MeOH	16	19
EtOAc fraction (in MeOH)	31	19
Post-EtOAc aqueous fraction	31	**65**
Solvent control: MeOH	32	3
BuOH fraction (in MeOH)	32	22
Post-BuOH aqueous fraction	30	**70**
Solvent control: MeOH	35	11
Post-BuOH aqueous fraction	16	**63**[b]
Post-BuOH aqueous fraction + $CHCl_3$ fraction (in MeOH)	23	**91**[b]
Solvent control: 50% aq. MeOH	25	8

[a] All doses were 0.1gle. All fractions from young leaf extracts. Results in boldface within a block differed significantly from respective solvent controls (χ^2 test, corrected for continuity, $P < 0.05$).
[b] Responses not statistically different from each other ($P = 0.074$).

The post-BuOH aqueous fraction was separated by weak cation-exchange chromatography into two fractions: (1) A-1, containing zwitterions and acidic and polar neutral compounds, and (2) A-2, containing basic compounds (Figure 1). When A-1 and A-2 were tested individually at 0.5 gle, activity was found only in A-1 (Table 2). The activity of A-1 appeared to be enhanced when bioassayed in combination with the $CHCl_3$ fraction while A-2 remained inactive (Table 2).

Fraction A-1 was partitioned on a weak anion-exchange resin. This procedure resulted in the separation of zwitterions and neutral compounds (fraction A-1-1), eluted with water, from acidic compounds (fraction A-1-2), eluted with HCl (Figure 1). Separate bioassays of A-1-1 and A-1-2 in combination with the $CHCl_3$ fraction indicated that activity resided solely in fraction A-1-1 (Table 2). The activity of A-1-1 could have been due to neutral compounds, zwitterions, or a combination of both. To resolve this question, a mixture of neutral compounds alone was isolated from the post-BuOH aqueous fraction by passing the latter through a mixed bed of strong cation- and anion-exchange resin. The neutral compounds were eluted with water to yield fraction A-1-1′ (Figure 1).

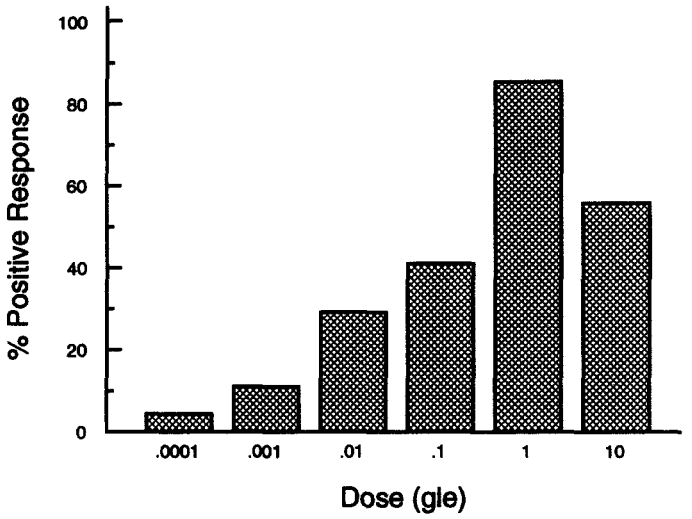

FIG. 2. Relationship of oviposition response of *Battus philenor* females to concentration of post-BuOH aqueous fraction from leaves of *Aristolochia macrophylla*. At least 15 females were tested at each concentration.

Fraction A-1-1′ alone, unlike the post-BuOH aqueous fraction, was inactive. However, when tested in combination with the $CHCl_3$ fraction, A-1-1′ was more active than the $CHCl_3$ fraction alone (Table 2). When both were tested in combination with the $CHCl_3$, A-1-1′ was as active as the post-BuOH aqueous fraction (Table 2).

Fraction A-1-1′ was separated by HPLC into five fractions, corresponding to four major peaks on the RI trace (fractions 1–4, respectively; Figure 3) and a combination of all pre- and postpeak eluents (fraction 5). When the fractions were tested in combination with the $CHCl_3$ fraction, only fraction 1 exhibited significant activity (Table 3).

Compounds present in fractions 1–4 were identified on the basis of their HPLC retention times, GC retention times, and mass spectra of their TMSi derivatives in comparison with those of standard compounds (Table 4) (Binder and Haddon, 1984; Sherman et al., 1970). Fraction 1 was identified as pinitol, fraction 2 as a mixture of sequoyitol and D-(+)-glucose, fraction 3 as sucrose, containing traces of glucose, and fraction 4 as *myo*-inositol (Figure 4). The identity of fraction 1 was confirmed by comparison of the GC retention time of its acetate with that of the penta acetate of D-(+)-pinitol (R_t = 14 min.).

Sequoyitol (0.5 gle), *myo*-inositol (0.6 gle), and D-(+)-pinitol (0.25 gle) were bioassayed in combination with the $CHCl_3$ fraction. Only D-(+)-pinitol was found to be active, evoking a 100% response (Table 3). Thus, the primary

TABLE 2. CONTACT OVIPOSITION RESPONSES OF *Battus philenor* FEMALES TO ION-EXCHANGE FRACTIONS OF POST-BuOH AQUEOUS FRACTION OF *Aristolochia macrophylla* EXTRACTS[a]

Fraction	Dose (gle)	*N*	Response (%)
Cation-exchange separation			
Post-BuOH aqueous fraction	0.5	35	**60**
Fraction A-1 (acids + neutrals)	0.5	35	**49**
Fraction A-2 (bases)	0.5	24	25
Control: MeOH/H_2O		25	8
Post-BuOH aqueous fraction + $CHCl_3$ fraction	0.5/0.2	17	**88**
Fraction A-1 (acids + neutrals) + $CHCl_3$ fraction	0.5/0.2	17	**77**
Fraction A-2 (bases) + $CHCl_3$ fraction	0.5/0.2	17	18
Control: $CHCl_3$ fraction	0.2	18	17
Anion-exchange separation			
Post-BuOH aqueous fraction + $CHCl_3$ fraction	0.5/0.2	31	**87**
Fraction A-1-1 (neutrals + zwitterions) + $CHCl_3$ fraction	0.5/0.2	37	**78**
Fraction A-1-2 (acids) + $CHCl_3$ fraction	0.5/0.2	15	0
Control: H_2O		37	8
Mixed-bed Ion-Exchange Separation			
Fraction A-1-1′ (neutrals)	0.5	20	0
Fraction A-1-1′ (neutrals) + $CHCl_3$ fraction	0.5/0.2	20	**60**
Control: $CHCl_3$ fraction	0.2	20	15
Fraction A-1-1′ (neutrals) + $CHCl_3$ fraction	0.5/0.2	36	**72**
Control: $CHCl_3$ fraction	0.2	20	30
Post-BuOH aqueous fraction + $CHCl_3$ fraction	0.5/0.2	30	**87**
Fraction A-1-1′ (neutrals) + $CHCl_3$ fraction	0.5/0.2	15	**87**
Control: H_2O		26	0

[a] Results in boldface within a block differed significantly from respective controls (χ^2 test, corrected for continuity, $P < 0.05$).

active component of the post-BuOH aqueous fraction is D-(+)-pinitol, the activity of which is dependent on combination with one or more components of the $CHCl_3$ fraction.

DISCUSSION

D-(+)-Pinitol (5-*O*-methyl-D-*chiro*-inositol) has been reported previously by Plouvier (1956) from the leaves of three species of *Aristolochia*, including *A. macrophylla* Lam. (=*A. sipho* L'Her.; cf. Bailey, 1949). This compound is

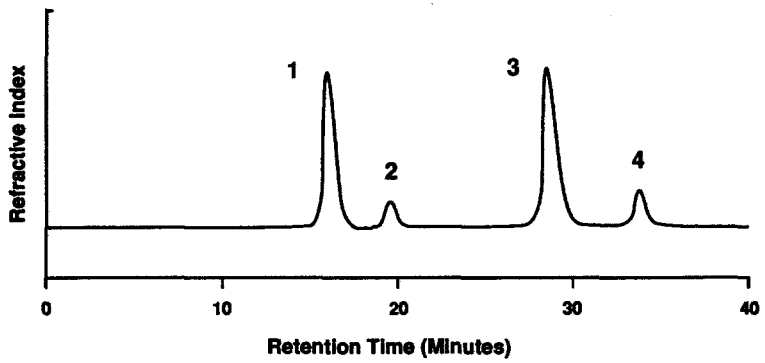

FIG. 3. HPLC trace of fraction A-1-1′, monitored with a RI detector, showing retention times of polar neutral components. Compounds were identified as follows: 1 = D-(+)-pinitol, 2 = D-(+)-glucose and sequoyitol, 3 = sucrose, 4 = *myo*-inositol.

TABLE 3. CONTACT OVIPOSITION RESPONSES OF *Battus philenor* FEMALES TO COMPONENTS OF FRACTION A-1-1′ (NEUTRALS), SEPARATED BY HPLC, AND TO CYCLITOL STANDARDS[a]

Column fraction or pure compound	Dose (gle)	*N*	Response (%)
Fraction A-1-1′ + $CHCl_3$ fraction	0.5/0.03	16	**75**
Fraction 1 + $CHCl_3$ fraction	0.6/0.03	14	**86**[b]
Fraction 5 + $CHCl_3$ fraction	0.6/0.03	18	56
$CHCl_3$ fraction	0.03	12	42[b]
Solvent control: H_2O		20	25
Fraction 2 + $CHCl_3$ fraction	0.5/0.03	12	33
Fraction 3 + $CHCl_3$ fraction	0.6/0.03	9	22
Fraction 4 + $CHCl_3$ fraction	0.6/0.03	12	33
$CHCl_3$ fraction	0.03	14	14
Solvent control: H_2O		18	33
Fraction 1 + $CHCl_3$ fraction	0.5/0.2	12	**91**
D-(+)-Pinitol (500 μg) + $CHCl_3$ fraction	0.25/0.2	10	**100**
Control: $CHCl_3$ fraction	0.2	13	38
myo-Inositol (200 μg) + $CHCl_3$ fraction	0.6/0.2	12	20
Sequoyitol (200 μg) + $CHCl_3$ fraction	0.5/0.2	12	58
$CHCl_3$ fraction	0.2	12	25
Solvent control: H_2O/MeOH		17	37

[a] Each block represents a different bioassay. Results in boldface within a block differed significantly from respective controls (χ^2 test, corrected for continuity, $P < 0.05$).
[b] Results differed significantly from each other (χ^2 test, $P < 0.05$).

TABLE 4. IDENTIFICATION AND QUANTIFICATION OF COMPONENTS OF FRACTION A-1-1′ (NEUTRALS), SEPARATED BY HPLC ON AMINO COLUMN

HPLC fraction	R_t HPLC (min)	TMSi Derivative R_t (GC) (min)	TMSi Derivative Mass spectral data[a] [m/z, (rel. abund.)]	Compound	Concentration (μg/gle)
1	16.5	30.5	555[M+1]$^+$(0.6), 554[M]$^+$(0.2), 540(0.9), 539(0.3), 507(1), 449(10), 433(11), 375(3), 374(5), 343(3)	D-(+)-Pinitol	2000
2	19.8	34.4	555[M+1]$^+$(8), 554[M]$^+$(5), 540(1), 539(0.8), 507(3), 449(7), 433(20), 375(3), 374(2), 343(1)	Sequoyitol	380
		32.1/35.5	435[M−105]$^+$(5), 361(1), 345(3), 319(1), 305(2), 217(17), 204(100)	D-(+)-Glucose	370
3	28.6	53.7		Sucrose	
		32.1/35.5		D-(+)-Glucose (trace)	
4	33.5	38.6	613[M+1]$^+$(3), 597(0.4), 522(0.8), 507(4), 432(5)	*myo*-Inositol	340

[a]Prevalence of [M+1]$^+$ ions probably resulted from high sample pressure in the EI source (McLafferty, 1957; Silverstein et al., 1974).

one of the most widespread and abundant cyclitols in the plant kingdom (Plouvier, 1963; Kindl and Hoffmann-Ostenhof, 1966; Dittrich and Korak, 1984); however, it cannot by itself explain the extraordinary specificity to members of the genus *Aristolochia* that is characteristic of ovipositing pipevine swallowtails. Such specificity must be attributable to one or more compounds in the active chloroform fraction that interacts synergistically with D-(+)-pinitol. Nishida et al. (1987) recently identified contact oviposition stimulants for another *Aristolochia*-feeding swallowtail butterfly, *Atrophaneura alcinous* (recently reclassified as *Parides alcinous*; cf. Miller, 1987). They found that a mixture of sequoyitol (an isomer of D-(+)-pinitol) and aristolochic acids, isolated from *Aristolochia debilis*, elicited oviposition as effectively as did the parent extract. Since aristolochic acids are restricted in distribution to the family Aristolochiaceae (Mix et al., 1983; Chen and Zhu, 1987), while sequoyitol is widely distributed (Plouvier, 1963; Kindl and Hoffmann-Ostenhof, 1966), Nishida et al. (1987) attributed the oviposition specificity of *P. alcinous* females to the presence of the aristolochic acids. Current work in our laboratory suggests that aristolochic acids, which occur in the chloroform fraction of *A. macrophylla*

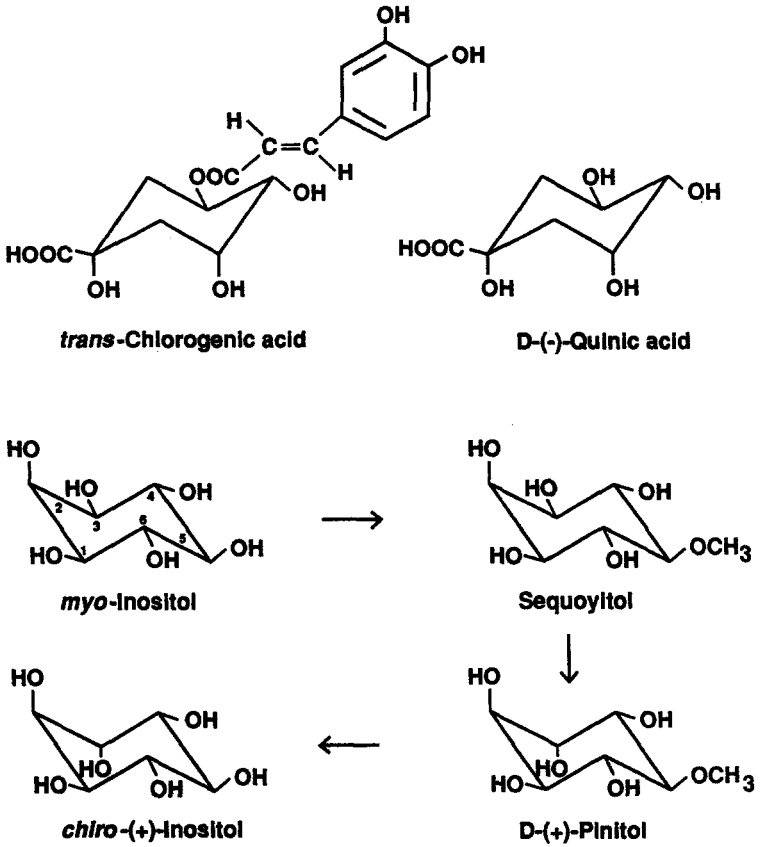

FIG. 4. Cyclitols and structurally similar compounds known to serve as larval feeding or oviposition stimulants for swallowtail butterflies. Inositol structures are portrayed in their preferred conformations. Arrows indicate typical biosynthetic relationships of D-(+)-pinitol to related inositols.

extracts, likewise contribute to host recognition by ovipositing pipevine swallowtails (K. Sachdev-Gupta, P. Feeny, and M. Carter, unpublished results).

Although we found D-(+)-pinitol to be the major methyl inositol in *A. macrophylla* leaves, traces of sequoyitol were also present. At similar doses in terms of gram leaf equivalents, sequoyitol was less stimulatory to *B. philenor* females than was D-(+)-pinitol (Table 3). While we do not yet know whether *P. alcinous* females would respond to pinitol, it is possible that these two species, representing the two subtribes (Battiti and Troiditi, respectively) of the tribe Troidini (Miller, 1987), have diverged in their relative sensitivity to the two isomers.

Inositols have frequently been shown to play a role in host recognition by insect larvae or adults. Hamamura et al. (1962) showed that *myo*-inositol, isolated from mulberry leaves, acts as a cofactor in stimulating feeding by silkworm larvae, *Bombyx mori*. More recently, both *myo*-inositol and D-pinitol, from the host plant *Lespedeza cuneata*, have been shown to act as biting stimulants (Numata et al., 1979) and arrestants (Numata et al., 1985) for larvae of the yellow butterfly, *Eurema hecabe mandarina*. Among swallowtails, (+)-*chiro*-inositol is one of several oviposition stimulants for *Papilio xuthus* (Ohsugi, et al., 1991). Curiously, while *myo*-inositol and *chiro* -inositol failed to enhance oviposition responses of a related *Citrus*-feeding species, *P. protenor*, this species responded strongly to D-(−)-quinic acid (Honda, 1990), which is similar in structure to the inositols (Figure 4). Both *P. protenor* (Honda, 1990) and *P. polyxenes* (Feeny et al., 1988) respond strongly to *trans*-chlorogenic acid, which is a cinnamic acid ester of quinic acid. The frequency with which cyclitols, or molecules of similar structure (Figure 4), have been reported as synergists in host recognition suggests that they may play a fundamental role in chemoreception, perhaps as primers for responses to other plant compounds of less general distribution. Even quite simple carbohydrates have enormous potential for encoding biological information and are implicated increasingly in cell–cell recognition (e.g., Sharon and Lis, 1989). In animal cells, at least, *myo*-inositol gives rise to a variety of inositol phosphates that play fundamental signaling roles by modifying cellular calcium dynamics in response to external stimuli (Berridge and Irvine, 1989).

A major biosynthetic pathway to D-pinitol in plants, established particularly in gymnosperms, originates from *myo*-inositol, itself formed from D-glucose-6-phosphate (Kindl and Hoffmann-Ostenhof, 1966). Methylation of *myo*-inositol to sequoyitol is followed by epimerization of sequoyitol to D-(+)-pinitol (Figure 4) (Kindl and Hoffmann-Ostenhof, 1966). Our finding of small amounts of *myo*-inositol and sequoyitol, in addition to D-(+)-pinitol, is consistent with the occurrence of this pathway in *A. macrophylla*. D- *chiro*-(+)-Inositol is a well-known demethylation product of D-(+)-pinitol (Kindl and Hoffmann-Ostenhof, 1966; Dittrich and Brandl, 1987). In several leguminous plants, D-pinitol is formed by epimerization of D-ononitol, itself arising by methylation of *myo*-inositol (Dittrich and Brandl, 1987). The frequent reports of insect responses to D-(+)-pinitol, sequoyitol, *myo*-inositol and D-*chiro*-(+)-inositol, rather than to other isomers of simple or methyl inositols, presumably reflect the widespread occurrence of these biosynthetic pathways, and hence their metabolites, in plants. The extent and significance of specificity by particular insect species for one inositol rather than another remains to be established.

The physiological or ecological functions, if any, of D-pinitol in plants remain obscure (Gardner et al., 1984). An early report of toxicity to *Heliothis zea* larvae, suggested as a cause of resistance to insects in certain soybean

varieties (Dreyer et al., 1979), was not confirmed by subsequent studies (Gardner et al., 1984). D-Pinitol is not toxic to mice (Tarazona and Sanz, 1990). Although the compound was reported as a feeding inhibitor for *H. zea* larvae (Reese et al., 1982), it had no effect on the feeding behavior of the pea aphid, *Acyrthosiphon pisum* (Campbell and Binder, 1984). On the basis of the evidence at hand, it seems unlikely that D-pinitol serves a significant defensive role against herbivores.

We close by noting that D-pinitol's impact on host selection by *B. philenor* very likely goes well beyond the stimulatory effect elicited upon tarsal contact. In addition to contact phytochemicals, *B. philenor* females also use leaf shape as a cue by which to find host plants (Rausher, 1980), orientation responses that have to be learned (Papaj, 1986a). While the stimulus modified by experience is a visual one, the experience itself is of a chemical nature: females permitted to contact nonhost plants treated with ethanolic host extracts learn the shape of the nonhost leaves (Papaj, 1986b). Contact phytochemicals thus act both as releasers for egg deposition behavior and as unconditioned stimuli (i.e., "rewards") with which previously neutral leaf-shape stimuli (i.e., conditioned stimuli) are positively associated.

We eagerly await the opportunity to examine whether contact with pinitol, alone or in combination with chloroform-soluble components, is required in order for pipevine swallowtail butterflies to learn the leaf shape of their host plant. Associative learning of visual cues upon contact with phytochemical stimuli has been reported for one other butterfly, *Pieris rapae* (Traynier, 1984, 1986, 1987). In *P. rapae*, approach-and-alighting responses to color are altered by prior contact with a single compound, sinigrin, a glucosinolate commonly found in the insect's cruciferous hosts. Sinigrin was shown previously to elicit egg deposition upon contact by female *P. rapae* (David and Gardiner, 1962; Renwick and Radke, 1983). We predict that, in contrast to Traynier's findings with *P. rapae*, at least three chemical components will be required to induce a preference for leaf shape in *B. philenor* butterflies. One of these components is likely to be D-(+)-pinitol.

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FATE OF IRIDOID GLYCOSIDES IN DIFFERENT LIFE STAGES OF THE BUCKEYE, *Junonia coenia* (LEPIDOPTERA: NYMPHALIDAE)

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Abstract—The buckeye butterfly, *Junonia coenia* (Lepidoptera: Nymphalidae), specializes on plants that contain iridoid glycosides. To determine the fate of these compounds in larvae, pupae, and adults of this species, we reared larvae on artificial diets with and without iridoid glycosides, and on leaves of a host plant, *Plantago lanceolata* (Plantaginaceae). Quantification by gas chromatography showed that newly molted third-, fourth-, and fifth-instar larvae reared on leaves of *P. lanceolata* contained means of 5.13, 2.88, and 6.83% dry weight iridoid glycoside. In contrast, the mean iridoid glycoside concentration of actively feeding fifth-instar larvae was 0.28% dry weight, that of pupae was 0.19% dry weight iridoids, and adults contained no detectable iridoids. Feeding experiments suggested that this reduction in actively feeding larvae was due to the metabolism of iridoid glycosides. *P. lanceolata* leaves in these experiments contained a mean of 1.00% dry weight iridoid glycoside, with a 2:1 ratio of aucubin to catalpol. Calculation of iridoid consumption and utilization indices showed that larvae fed artificial diets consumed, digested, and sequestered aucubin and catalpol in similar ways. When these indices were calculated for larvae fed leaves of *P. lanceolata*, catalpol was sequestered twice as efficiently as aucubin.

Key Words—*Junonia coenia*, Lepidoptera, Nymphalidae, *Plantago lanceo-*

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lata, chemical defense, evolution of unpalatability, iridoid glycosides, aucubin, catalpol, ecological chemistry, sequestration.

INTRODUCTION

Chemicals used in defense by insects may be synthesized de novo or acquired from larval or adult food resources (Blum, 1981; Brower, 1984; Brattsten, 1986). For those insects that sequester chemical defenses from larval host plants, the amount of defensive chemicals that they contain (and therefore, their protection from potential predators) is a function of plant chemical content, the insect's ability to select plants or plant parts high in useful defensive chemicals, and insect sequestrative efficiency (Brower and Brower, 1964; Bowers, 1988a). However, not all insects feeding on plant species with potential defensive compounds sequester these compounds for defense; in some cases the compounds are metabolized or excreted (Brattsten, 1979, 1986; Duffey, 1980; Blum, 1983). In addition, not all compounds that are sequestered contribute to an insect's unpalatability (Brower, 1984).

Iridoid glycosides are sequestered by insect species in four orders: Lepidoptera, Hymenoptera, Homoptera, and Coleoptera (Bowers, 1988a, 1992; Nishida and Fukami, 1989; Rimpler, 1991). The Lepidoptera have been particularly studied in this regard. For example, checkerspot butterflies of the genus *Euphydryas* may contain as much as 9% dry weight iridoid glycosides (Gardner and Stermitz, 1988), although iridoid glycoside concentration varies immensely among individuals from a single population (Gardner and Stermitz, 1988; Bowers, 1992). These butterflies are generally unpalatable to birds (Bowers, 1980, 1981; Bowers and Farley, 1990), but chemical content and degree of unpalatability may vary depending on the larval host plant (Bowers, 1980; Belofsky et al., 1989; Bowers and Farley, 1990).

The buckeye butterfly, *Junonia coenia* Hübner (Nymphalidae), specializes on plants that contain iridoid glycosides (Bowers, 1984). Two iridoid glycosides, aucubin and catalpol, that are found in many of the host plants of *J. coenia*, serve as both feeding and oviposition stimulants (Bowers, 1984; Pereyra and Bowers, 1988). Adult buckeye butterflies are palatable to gray jays, *Perisoreus canadensis* (Corvidae) (Bowers and Farley, 1990), and exhibit a pattern and coloration typical of palatable lepidopterans: the underside is quite cryptic, whereas the upperside has bright eyespots that may startle predators (Cott, 1940; Edmunds, 1974). The adults were also readily eaten by a captive big brown bat (*Eptesicus fuscus*), although the larvae were rejected (Bowers, personal observation). Buckeye larvae also appear to be unpalatable to ants (Dyer and de la Fuente, personal observation). The coloration of buckeye larvae is not easily classified as cryptic or warning: last-instar larvae are black with spines, blue and yellow spots, and an orange head. They may be quite apparent when feeding

on the host plant, but difficult to find in low vegetation (Camara, personal observation; Bowers, personal observation). Qualitative analyses of buckeye tissue by thin-layer chromatography had indicated that two iridoid glycosides, aucubin and catalpol, were present in larvae and pupae, but were absent or in undetectable amounts in the adult (Bowers and Puttick, 1986).

To determine the fate of iridoid glycosides in different life stages of *J. coenia*, we performed a series of chemical assays and a feeding experiment designed to answer the following questions: (1) What are typical host-plant iridoid glycoside concentrations to which larvae of the buckeye are exposed? (2) What happens to aucubin and catalpol in larvae, pupae, and adults?

METHODS AND MATERIALS

Study Organisms. Junonia coenia (Nymphalidae) were from a laboratory culture originally started with eggs obtained from a culture maintained at Duke University. Larvae were reared on garden- and greenhouse-grown *Plantago lanceolata* (Plantaginaceae) leaves for approximately seven generations prior to the feeding experiment in March 1988. *P. lanceolata* is commonly used as a host plant by *J. coenia* (Bowers, 1984; Scott, 1986) and contains two iridoid glycosides, aucubin and catalpol (Duff et al., 1965; Bobbitt and Segebarth, 1969).

Iridoid Glycoside Content of P. lanceolata *Leaves.* In the feeding experiments to determine nutritional and iridoid indices (described below), half of each leaf fed to the larvae was saved for iridoid glycoside analysis. The iridoid glycoside content of those leaves was used to calculate the iridoid indices and to provide a measure of the range of iridoid glycoside concentrations to which larvae were exposed in the leaf diet.

The iridoid glycoside contents (aucubin and catalpol) of plants were determined by gas chromatography using the methods of Gardner and Stermitz (1988). Using gas chromatography, we were able to detect levels as low as 0.001 mg of iridoid glycoside per sample. Phenyl-β-D-glucose was used as an internal standard. Purified aucubin was obtained from W. Berkowitz and catalpol was extracted from *Catalpa speciosa* (Bignoniaceae). Gas chromatography showed that both these compounds were at least 99% pure.

Iridoid Glycoside Content of Larvae, Pupae, and Adult J. coenia. Newly molted third- ($N = 5$ groups of five larvae), fourth- ($N = 5$ groups of two larvae) and fifth-instar larvae ($N = 16$), pupae ($N = 14$), and adults ($N = 10$) were taken from the laboratory culture maintained on *P. lanceolata* leaves to be analyzed for iridoid glycosides. Each insect was weighed on a Mettler HK60 microbalance to the nearest 0.01 mg. Because the larvae were newly molted, they had no food material in the gut. We were therefore analyzing larval iridoid

content. However, caterpillars may be attacked by potential predators while they are feeding, at which time the gut contents may also be important in determining their unpalatability and thus acceptability to predators (Brower, 1984). We therefore also analyzed actively feeding fifth-instar larvae ($N = 15$). In addition, a total of six actively feeding fifth-instar larvae were analyzed for iridoid glycoside content one, two, and three days after molting ($N = 2$ per day). Individuals were frozen for 48 hr and were then dried for at least 48 hr at 50°C. Extraction and gas chromatographic analysis were as described above for plants.

Quantification of Fate of Ingested Iridoid Glycosides in J. coenia. Because the above assays showed a dramatic decrease in levels of iridoid glycosides in actively feeding fifth-instar larvae, we designed a feeding experiment to answer the following questions: (1) What happens to iridoid glycosides in the fifth (final) instar? (2) Does presence of iridoid glycosides in the diet affect feeding and food utilization efficiency? (3) How efficiently do fifth-instar *J. coenia* larvae sequester aucubin compared to catalpol?

Newly hatched first-instar *J. coenia* larvae were transferred in groups of 20 to Petri dishes and fed a standard artificial diet (Nijhout, unpublished data) containing no iridoid glycosides or plant material, for the first four instars (approximately two weeks). Immediately after molting to the fifth instar, larvae were assigned to one of four experimental diets ($N = 15$/diet): (1) diet containing 2% dry weight catalpol (488.0 mg) (diet + CAT); (2) diet containing 2% dry weight aucubin (488.0 mg) (diet + AUC); (3) diet with no iridoids (diet); and (4) *P. lanceolata* leaves (leaves). Each newly molted fifth-instar caterpillar was weighed at the beginning of the experiment. Larvae were fed ad libitum until they were found spinning a silken pad for pupation. All food was weighed prior to feeding, and any leftover food was recovered, dried, and weighed.

Ten additional newly molted fifth-instar larvae were weighed, frozen, and dried at 50°C to constant weight in order to obtain a wet weight/dry weight conversion factor. Ten pieces of each artificial diet were separately weighed, dried at 50°C, and reweighed, in order to calculate a conversion factor to be used to determine the dry weight of diet eaten by each larva (Waldbauer, 1968).

Because individual *P. lanceolata* leaves on a given plant have variable concentrations of iridoid glycosides (Bowers, Collinge, Fajer, unpublished data), larvae on the leaves diet were fed in the following manner: individual leaves were cut down the midvein; one half was weighed and fed to the larva, and the other half was weighed and dried at 50°C for subsequent iridoid glycoside analysis. In this way, we could estimate the total amount of iridoid glycosides fed to each larva.

Larvae used in the feeding experiment were collected either when they were hanging to pupate or after they had pupated and were frozen and dried as above. All larval frass was collected from each caterpillar in the feeding experiment, dried, and weighed prior to iridoid glycoside analysis. Extraction and

quantification of iridoid glycosides from leaves, larvae, pupae, and frass were done as described above.

Standard nutritional indices were calculated for each caterpillar (Waldbauer, 1968), and the results from the iridoid glycoside analyses were then used to calculate an iridoid glycoside budget for each individual larva (see Roeske et al., 1976, for cardenolide indices). We compared the amount of iridoid glycoside ingested, the amount sequestered by the larva, the amount eliminated in the frass, and the amount lost, or metabolized. We then calculated iridoid utilization indices (Table 1), which were based on our chemical analyses and the standard nutritional indices. For the two artificial diets containing iridoids, the dry weight of diet eaten by each caterpillar was multiplied by the concentration of iridoid glycoside in the diet (2%) to yield the "iridoid ingested" (Table 1). For those larvae fed leaves, the concentrations of both aucubin and catalpol in the remaining leaf halves (i.e., those not fed to the larvae), as determined by gas chromatography, were multiplied by the dry weight eaten by each caterpillar to yield iridoid ingested (Table 1). Iridoid gained (Table 1) was the amount of iridoid present in larvae or pupae fed each of the diets (determined by gas chromatography). Iridoid in frass (Table 1) was obtained by analyzing all of the frass from each individual larva. For those larvae fed leaves, aucubin and catalpol indices were calculated separately.

Statistical Analyses. Nutritional and iridoid indices are proportions, and they were therefore transformed [arcsine (square root)] prior to analyses. Nutritional indices for larvae in the four treatments were compared by one-way ANOVA. Orthogonal contrasts compared the diet vs. diet + CAT and diet + AUC treatments, and the leaves diet vs. all artificial diets. Iridoid indices were

TABLE 1. DEFINITIONS OF AND FORMULAS USED FOR CALCULATING IRIDOID INDICES; MODIFIED FROM NUTRITIONAL INDICES OF WALDBAUER (1968)

$$\text{Relative consumption rate } (RCR_I) = \frac{\text{iridoid ingested}}{\text{mean larval weight}}$$

$$\text{Relative growth rate } (RGR_I) = \frac{\text{iridoid gained}}{\text{mean larval weight}}$$

$$\text{Approximate digestibility } (AD_I) = \frac{\text{iridoid ingested} - \text{iridoid in frass}}{\text{iridoid ingested}}$$

$$\text{Efficiency of conversion of ingested iridoid } (ECI_I) = \frac{\text{iridoid in insect}}{\text{iridoid ingested}}$$

$$\text{Efficiency of conversion of digested iridoid } (ECD_I) = \frac{\text{iridoid gained}}{\text{iridoid ingested} - \text{iridoid in frass}}$$

analyzed by one-way ANOVA first by comparing larvae fed diet + CAT to those fed diet + AUC, and second by comparing aucubin and catalpol indices for larvae fed *P. lanceolata* leaves.

RESULTS

Iridoid Glycoside Content of Plantago lanceolata. Analyses of leaves of *P. lanceolata* used in the feeding experiments ($N = 15$) showed that total iridoid glycoside content ranged from 0.69 to 1.17% dry weight ($\bar{X} = 1.00 \pm 0.04$ SE). Although variable, the concentration of aucubin ($\bar{X} = 0.66 \pm 0.03$ SE) was approximately twice that of catalpol ($\bar{X} = 0.34 \pm 0.02$ SE).

Iridoid Glycoside Content of Larvae, Pupae, and Adult J. coenia. Newly molted third-, fourth-, and fifth-instar larvae of *J. coenia* maintained on *P. lanceolata* leaves contained relatively high amounts of both aucubin and catalpol, the two iridoid glycosides present in their food plant (Table 2). We found that both the amount and the concentration of iridoid glycosides in actively feeding fifth-instar larvae were less than one sixth of that in newly molted fifth-instar larvae (Table 2). The iridoid glycoside content of pupae was about the same as that of the actively feeding larvae, although the concentration was reduced (Table 2). Adults did not contain any detectable iridoid glycosides (Table 2). Fifth-instar larvae one and two days postmolt contained no detectable iridoid glycosides ($N = 2$ for each day), but larvae collected three days after molting contained very small amounts of iridoid glycoside: 0.03 mg aucubin in one larva (0.05% dry weight) and 0.02 mg aucubin and 0.02 mg catalpol in the other (0.07% dry weight).

Quantification of Fate of Ingested Iridoid Glycosides in J. coenia. Larvae fed *P. lanceolata* leaves in the feeding experiment consumed food at a faster rate (RCR) and grew slightly faster (RGR) than those larvae fed artificial diets (Table 3). However, the leaves were less digestible than the artificial diets (AD, Table 3), probably because of their high fiber content. Larvae fed leaves were also much less efficient at converting leaf material to biomass (ECI and ECD, Table 3). There were no differences among larvae fed artificial diets in four of the five nutritional indices calculated; the one exception was that the plain artificial diet was slightly more digestible than diets containing either aucubin or catalpol (AD, Table 3).

Although fifth-instar larvae fed *P. lanceolata* leaves ingested over 3 mg iridoid glycoside,less than 2% of that was detectable in the pupae, and less than 0.1% in the frass (Table 4). Thus the drop in iridoid glycoside content of actively feeding fifth-instar larvae (Table 2) appears to be due to metabolism of the iridoid glycosides and not to their gross elimination in the frass.

There were no significant differences among larvae in the iridoid utilization

TABLE 2. SEQUESTRATION OF AUCUBIN AND CATALPOL BY VARIOUS LIFE STAGES OF *J. coenia* REARED ON *P. lanceolata* LEAVES

			Iridoid glycoside content					
			Aucubin		Catalpol		Total	
Life stage	*N*	Weight (mg, mean ± SE)	Amount (mg, mean ± SE)	Dry weight (%, mean ± SE)	Amount (mg, mean ± SE)	Dry weight (%, mean ± SE)	Amount (mg, mean ± SE)	Dry weight (%, mean ± SE)
Newly molted 3rd[a]	5	2.31 ± 0.14	0.08 ± 0.01	3.58 ± 0.34	0.03 ± 0.01	1.55 ± 0.32	0.12 ± 0.01	5.13 ± 0.60
Newly molted 4th[b]	5	4.82 ± 0.52	0.08 ± 0.02	1.89 ± 0.47	0.04 ± 0.01	1.00 ± 0.27	0.13 ± 0.02	2.88 ± 0.73
Newly molted 5th	16	13.89 ± 1.09	0.48 ± 0.05	3.79 ± 0.47	0.39 ± 0.04	3.04 ± 0.31	0.87 ± 0.08	6.83 ± 0.71
Feeding 5th	15	79.83 ± 6.36	0.08 ± 0.03	0.15 ± 0.07	0.06 ± 0.02	0.12 ± 0.05	0.14 ± 0.05	0.28 ± 0.12
Pupa	14	91.75 ± 3.13	0.06 ± 0.01	0.09 ± 0.02	0.10 ± 0.02	0.12 ± 0.03	0.17 ± 0.03	0.19 ± 0.04
Adult	10	37.46 ± 3.20	0	0	0	0	0	0

[a] Larvae were combined into five groups of five larvae each for chemical analysis; weights, amounts, and concentrations are given on an individual basis.
[b] Larvae were combined into five groups of two larvae each for chemical analysis; weights, amounts, and concentrations are given on an individual basis.

TABLE 3. STANDARD NUTRITIONAL INDICES [MEAN (SE)] FOR FIFTH-INSTAR *J. coenia* LARVAE ON FOUR DIETS USED IN FEEDING EXPERIMENTS

Diet[a]	RCR	RGR	AD	ECI	ECD
Diet	0.70	0.25	0.44[b]	0.36	0.83
	(0.02)	(0.01)	(0.02)	(0.01)	(0.04)
Diet + CAT	0.70	0.25	0.40	0.35	0.90
	(0.02)	(0.01)	(0.01)	(0.01)	(0.03)
Diet + AUC	0.69	0.24	0.40	0.35	0.89
	(0.02)	(0.01)	(0.01)	(0.01)	(0.04)
Leaves	1.38[c]	0.26[c]	0.32[c]	0.19[c]	0.60[c]
	(0.04)	(0.01)	(0.02)	(0.01)	(0.04)

[a]Diet = artificial diet with no additives, Diet + CAT = diet + 2% catalpol, Diet + AUC = diet + 2% aucubin, Leaves = *Plantago lanceolata* leaves.
[b]$P < 0.05$, orthogonal contrast: artificial diet with no additives vs. artificial diets with iridoids.
[c]$P < 0.05$, orthogonal contrast: *P. lanceolata* leaves vs. all artificial diets.

TABLE 4. FATE OF INGESTED IRIDOID GLYCOSIDES [MEAN (SE)] IN HANGING FIFTH-INSTAR LARVAE AND PUPAE OF THE BUCKEYE, *Junonia coeania*, FED ON *P. lanceolata* LEAVES ($N = 15$)

Iridoid	Amount ingested (mg)	Amount in body (mg)	Amount in frass (mg)
Aucubin	2.21(0.15)	0.03(0.01)	0.002(0.002)
Catalpol	1.13(0.08)	0.03(0.01)	none detected
Total	3.34(0.12)	0.06(0.02)	0.001(0.001)

indices on artificial diets containing aucubin or catalpol (Table 5). When the iridoid glycosides were presented to the larvae singly in artificial diets, the two compounds were consumed (RCR_I), digested (AD_I) and sequestered (ECI_I and ECD_I) in similar ways (Table 5). However, those larvae fed *P. lanceolata* leaves, which contained both aucubin and catalpol, utilized the two compounds in slightly different ways (Table 6). The relative consumption rate (RCR_I) of aucubin was significantly greater than that of catalpol (Table 6), because the *P. lanceolata* leaves fed to the larvae contained more aucubin than catalpol. There were no differences in the relative growth rates (RGR_I) of larvae for aucubin vs. catalpol. Neither of the iridoids was excreted, but there was differential sequestration of the two compounds by the *J. coenia* larvae. The larvae were

TABLE 5. IRIDOID UTILIZATION INDICES [MEAN (SE)] FOR *J. coenia* LARVAE: LARVAE FED ARTIFICIAL DIETS CONTAINING AUCUBIN OR CATALPOL[a]

Index	Diet + AUC	Diet + CAT	*P*
RCR_I	0.012(0.001)	0.013(0.001)	NS
RGR_I	0.010(0.002)	0.014(0.003)	NS
AD_I	0.731(0.029)	0.763(0.024)	NS
ECI_I	0.008(0.002)	0.011(0.002)	NS
ECD_I	0.011(0.003)	0.014(0.003)	NS

[a]See Table 1 for Definitions.

TABLE 6. IRIDOID UTILIZATION INDICES [MEAN (SE)] FOR *J. coenia* LARVAE: LARVAE FED *P. lanceolata* LEAVES

Index	Aucubin	Catalpol	*P*
RCR_I	0.009(0.000)	0.005(0.001)	<0.05
RGR_I	0.166(0.032)	0.150(0.028)	NS
ECI_I	0.019(0.003)	0.038(0.007)	<0.01
ECD_I	0.019(0.003)	0.038(0.007)	<0.01

[a]Indices were calculated for aucubin and catalpol separately. ECI_I and ECD_I were equal for each compound because none of the larvae excreted either compound.

twice as efficient at sequestering catalpol than aucubin (ECI_I and ECD_I, Table 6).

DISCUSSION

Our assays indicate that newly molted, last-instar *J. coenia* larvae contain a mean of as much as 6.8% dry weight iridoid glycosides. Iridoid glycosides, especially catalpol, appear to be responsible for the unpalatability of larvae and adults of members of the genus *Euphydryas* (Bowers and Puttick, 1986; Stermitz et al., 1986; Belofsky et al., 1989; Bowers, 1992) and are therefore likely to render buckeye larvae distasteful as well.

Unpalatable insects often have characteristics that advertise their unpalatability to potential predators. In addition to feeding on one or more plant species that contain potential defensive compounds, they are usually warningly colored, often gregarious, and may have other behaviors that enhance their apparency to potential predators (Brower, 1984). Larvae of *J. coenia* are solitary and colored black with spines and blue and yellow spots. Black is often considered a warning

color (Cott, 1940; Sillen-Tullberg, 1988; Sillen-Tullberg and Leimar, 1988), but *J. coenia* larvae may be difficult to find in the field. The coloration of this species is not easily categorized as cryptic or warning and larvae of *J. coenia* do not appear to fit the pattern of characteristics that are typical of unpalatable insects. Nonetheless, we found that these larvae contained iridoid glycoside concentrations at levels at or above those that make checkerspot butterflies unpalatable (Bowers and Farley, 1990). In addition, ants and a captive bat avoided *J. coenia* larvae, but did eat the adults (Dyer, de la Fuente, and Bowers, unpublished observations), suggesting that the presence of iridoid glycosides in buckeye larvae make them unpalatable to putative predators.

Sillen-Tullberg (1988) and Sillen-Tullberg and Leimar (1988) suggested that unpalatability may evolve before gregariousness, and such may be the case with *J. coenia*. As Sillen-Tullberg (1988, p. 303) has pointed out, warning coloration is not a necessary companion of unpalatability, and the coloration of buckeye larvae may be aposematic under some circumstances and cryptic under others (Papageorgis, 1975; Jarvi et al., 1981). Thus buckeye larvae may be unpalatable, but may not yet have evolved gregariousness.

Our chemical analyses showed that total iridoid glycoside contents of actively feeding fifth-instar larvae were one sixth that of unfed, newly molted fifth-instar larvae (decreasing from 0.87 mg in newly molted fifth instars to 0.14 mg in actively feeding fifth-instar larvae). In addition, the larvae were not excreting the intact compounds in the frass, which suggests that they were metabolizing them. For example, iridoid glycosides may be hydrolyzed by β-glucosidases in the caterpillar's gut (Spencer, 1987). Alternatively, the compounds may be bound with carrier molecules that render them inaccessible to our extraction methods (Haunerland and Bowers, 1986; Wilkinson, 1986; Wink, 1988). The loss of iridoid glycosides appears to commence with feeding in fifth-instar larvae, since actively feeding larvae at one, two, and three days after molt had low to unmeasurable amounts of iridoid glycosides. If the iridoid glycosides were attached to carrier molecules, then they were irrevocably bound or eventually degraded, because we never detected intact iridoid glycosides in adults. Since intact iridoid glycosides are known to confer unpalatability, either of these fates might result in palatable individuals.

Our results showed a dramatic decrease in iridoid glycoside content in *J. coenia* pupae compared to newly molted last-instar larvae. Chemical analyses of other insects feeding on plants containing iridoid glycosides have revealed similar patterns. For example, *Ceratomia catalpae* (Sphingidae), which feeds only on species of *Catalpa* (Bignoniaceae), has gregarious, warningly colored larvae and a cryptic adult. Larvae may contain as much as 15% dry weight iridoid glycosides, and larval hemolymph may contain 50% dry weight iridoids (Bowers, Collinge, and Lawrence, unpublished data). Pupae are relatively low

in iridoid glycosides (<0.3% dry weight) and adults are devoid of these compounds (Bowers, Collinge, and Lawrence, unpublished data).

Other species that sequester iridoid glycosides show similar patterns of a decrease in iridoid glycoside content from larva to pupa to adult. Larvae of *Euphydryas anicia*, which sequester iridoids and retain them through to the adult stage, had significantly higher concentrations (mean = 11.5% dry weight, N = 6) of iridoid glycosides than adults (mean = 2.3% dry weight, N = 6) (L'Empereur and Stermitz, 1990). Analysis of larvae, pupae, and adults of an undescribed species of *Lepipolys* (Noctuidae) showed that larvae sequester the iridoid glycoside antirrhinoside from their host plants *Maurandya antirrhiniflora* and *Antirrhinum majus* (Scrophulariaceae) (Boros et al., 1991). Larvae contain antirrhinoside in amounts ranging from 3.2% to 11.2% dry weight (N = 11), pupae contain 0–0.6% dry weight (N = 3), and adults contain no detectable antirrhinoside (N = 9) (Boros et al., 1991).

Pupal meconium of monarchs, *Danaus plexippus* (Nymphalidae) (Nishio, 1980), *E. anicia* (L'Empereur and Stermitz, 1990), *E. phaeton*, *C. catalpae*, *J. coenia* (Bowers and Puttick, 1986), two species of *Lepolys* (Noctuidae) and *Meris paradoxa* (Geometridae) contain relatively large amounts of ingested cardenolides (monarchs) or iridoids (other species). These data suggest that it may be physiologically difficult to retain the large amounts of phytochemicals in the larvae through to the pupal and adult stages (see Bowers, 1992, for a more detailed discussion).

Junonia coenia larvae were more efficient at sequestering catalpol than aucubin (Tables 2 and 6). Despite our finding that the concentration of aucubin in *P. lanceolata* was about twice that of catalpol, larvae contained approximately equal amounts of the two compounds, even in the last instar and pupa. Alternatively, *J. coenia* larvae may metabolize aucubin into catalpol. However, the gas chromatographic analyses showed only aucubin in larvae fed on artificial diets with aucubin, and only catalpol in those fed diets with catalpol, which contradicts this alternative hypothesis. Thus, catalpol appears to be more efficiently sequestered from leaves than aucubin in *J. coenia*, as has been found for *E. phaeton* (Nymphalidae) (Belofsky et al., 1989). Interestingly, catalpol appears to be responsible for the unpalatability of *Euphydryas* species (Bowers, 1980, 1988b).

Insect sequestration of defensive chemicals from host plants may involve physiologically expensive processes. For example, passage of the compounds from the gut into hemolymph and thence to the site of sequestration may require special carrier molecules (Duffey, 1980; Haunerland and Bowers, 1986; Wilkinson, 1986; Wink, 1988), as well as the construction of specialized glands or organs for storage (Duffey, 1980; Scudder et al., 1986). In addition, sequestration may require physiological or morphological adaptations for prevention of

autotoxicity to the insect (Scudder et al., 1986; Cavin and Bradley, 1988). Such a cost might be detected as a negative correlation of insect chemical content with fitness-related parameters such as size, weight, growth rate, or fecundity (Brower and Moffitt, 1974; Cohen, 1985).

Data relevant to this question are few. Cohen (1985) found a negative correlation between cardenolide concentration and adult size and weight in the monarch, *Danaus plexippus* (Nymphalidae), but no such correlation in the closely related queen, *D. gillippus*. Examination of *D. plexippus* larvae showed no evidence of any negative correlation of host-plant cardenolide content and larval weight (Erickson, 1973; Seiber et al., 1980; Cohen, 1985). In a study of chemical defense in larvae of several species of chrysomelids, daily removal of defensive secretions resulted in decreased weight gain compared to a control group, but only in some of the species tested (Rowell-Rahier and Pasteels, 1986). In another system, analysis of the data of Gardner and Stermitz (1988) on iridoid glycoside content of *Euphydryas anicia* (Nymphalidae) butterflies showed no correlation between adult weight and iridoid glycoside content for males and a positive correlation for females (Bowers, 1988a).

To evaluate the potential of a cost associated with sequestering iridoid glycosides in *J. coenia*, we compared biomass and iridoid glycoside content and concentration of newly molted fifth-instar larvae and pupae. There were significant negative correlations between biomass and iridoid glycoside concentration for both these stages (larvae: $Y = 13.263 - 0.463X$, $r^2 = 0.511$, $P < 0.002$; pupae: $Y = 0.020 - 0.007X$, $r^2 = 0.342$, $P < 0.03$); but not for biomass and iridoid glycoside content (larvae: $Y = 1.314 - 0.032X$, $r^2 = 0.170$, $P = 0.11$; pupae; $Y = 0.602 - 0.005X$, $r^2 = 0.245$, $P = 0.072$).

Indication of a physiological cost to sequestration of chemical compounds is typically revealed by a negative correlation of chemical concentration (percent dry weight) with body weight, growth rate, or fecundity (e.g., Brower and Glazier, 1975; Cohen, 1985). Alternatively, amount rather than concentration can be used to determine whether or not a cost exists. In this paper, we have presented the data in both ways, and found a significant negative relationship of larval weight with concentration (percent dry weight) in both larvae and pupae, but no significant negative relationship of body weight with chemical content (milligrams). Both amount and concentration are probably important in calculating physiological costs. One milligram of iridoid glycoside in a 10-mg caterpillar is very different from 1 mg in a 500-mg caterpillar because the concentrations are different: although the amount of biochemical machinery needed to sequester or detoxify 1 mg may be the same in caterpillars of both sizes, the proportion of the insect's total resources used may differ greatly. The best demonstration of a cost would thus be a negative correlation of both amount and concentration with body weight, fecundity, or some other performance variable related to an individual's fitness.

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INVESTIGATION OF SEX PHEROMONE COMPONENTS OF FEMALE ASIAN CORN BORER, *Ostrinia furnacalis* (Hübner) (LEPIDOPTERA: PYRALIDAE) IN TAIWAN

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Abstract—(*E*)-12-Tetradecenyl acetate (*E*12–14:OAc), (*Z*)-12-tetradecenyl acetate (*Z*12–14:OAc), and tetradecanyl acetate (14:OAc) were extracted and identified as major chemical components from female tips of the Asian corn borer, *Ostrinia furnacalis* (Hübner) (Lepidoptera: Pyralidae) in Taiwan, with a combined wash ratio of 48:37:15 and an individual female ratio of 45:39:16. The average amount of *E*12–, *Z*12–14:OAc and 14:OAc in each female gland was 6.6 ± 4.6, 5.8 ± 3.5, and 2.4 ± 1.7 ng/female, respectively. The mixture of these three synthetic chemicals not only gave strong activities in male antennae but also could catch significantly more males than virgin females in field-trapping tests. The field test also showed no significant difference in trapping ability among the three-component Taiwan formulations, the two-component Taiwan formulation (*E*12–14:OAc and *Z*12–14:OAc, 53:47), and the Japan formulation (*Z*12–14:OAc and *E*12–14:OAc, 3:2).

Key Words—Sex pheromone, *Ostrinia furnacalis*, *E*12–14:OAc, *Z*12–14:OAc, 14:OAc.

INTRODUCTION

The Asian corn borer, *Ostrinia furnacalis* (Hübner), is a serious pest of corn in Taiwan all year around. Although the males was reported to be attracted by a large quantity of the synthetic mixture of (*Z*)-12-tetradecenyl acetate (*Z*12–14:OAc) and (*E*)-12-tetradecenyl acetate (*E*12–14:OAc) in the fields, the

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attractiveness was much less than the virgin females (Chow et al., 1984). In Japan, mainland China, and the Philippines, a 3:2 (Ando et al., 1980a), 47:53 (Cheng et al., 1981), and ca. 1:1 (Klun et al., 1980) ratio of these geometric isomers (*Z* to *E*) had been reported in the Asian corn borer, respectively. The Asian corn borer might have different sex pheromone ratios in different areas of Asia. In the present study, the chemical constituents of the sex pheromone of the Asian corn borer in Taiwan were investigated, and the trapping ability of different ratios was also compared, in order to provide a better attractant for the existing mass trapping practices in corn fields.

METHODS AND MATERIALS

Insects. Female pupae of the Asian corn borer, *Ostrinia furnacalis*, used in this study were collected from the corn field in Nankang, Taipei, Taiwan. Female pupae were maintained in environmental chambers at 23–26°C under a 14:10 hr light–dark photoperiod. Emerged moths were fed with 10% aqueous sucrose solution.

Preparation of Pheromone Gland Extract. Ovipositors from 100 2- to 4-day-old virgin females were excised during the calling period in the late scotophase and immersed in 5 μl hexane per female for 10 min. The extracts were stored under 0°C for structure identification.

Chemical Analysis. Gas chromatography (GC) of the extract was performed on three capillary columns. A Shimadzu 14-A GC equipped with a flame ionization detector was used with a 30-m × 0.25-mm (ID) fused silica capillary column of Carbowax 20 M phase, a 30-m × 0.25-mm fused silica capillary column of DB-17 phase, and a 30-m × 0.25-mm fused silica capillary column of DB-225 phase. Chromatographic conditions: nitrogen carrier 0.5 kg/cm^2, column temperature was isothermal at 190°C.

Gas chromatographic–mass spectrometric (GC-MS) analysis was conducted using a Finnigan Mat Incos 50 spectrometer. The GC-MS data were obtained using a 30-m × 0.25-mm column of DB-1701 phase. The column temperature was run from 120 to 260°C at 3°C/min and held at 260°C for 5 min using helium as carrier gas. Electron impact (EI) mass spectra were collected at 70 eV with separator and source at 180°C.

The chemical components were identified by comparison of their mass spectra with those of authentic standards. Subsequently, the identification was verified by comparison of the GC retention time of the natural product to an authentic standard under the same GC condition as described above. Standards of *E*12–14:OAc and *Z*12–14:OAc were kindly supplied by Dr. J.A. Klun (USDA, Agricultural Research Service, Insect Chemical Ecology Laboratory, BARC-East, Beltsville, Maryland), and 14:OAc was purchased from Sigma Chemical Co. (St. Louis, Missouri).

Pheromone Titer Determination. For pheromone titer determination, ovipositor extracts were prepared from 15 individual 2- to 4-day-old virgin females. Each ovipositor was excised during the calling period in the late scotophase and immersed in 5 μl hexane for 10 min. The extracts were subsequently analyzed for *E*12–14:OAc, *Z*12–14:OAc and 14:OAc with the external standard method of quantitative analysis.

EAG Analysis. The procedure of EAG bioassay experiments was based on Ando et al. (1980a,b). In our preliminary test, the antennae of the male Asian corn borer were found to be rather strongly elicited by (*Z*)-11-tetradecenyl acetate (*Z*11–14:OAc), so the sensitivity of each antenna used in EAG analysis was checked by application of 10 μg *Z*11–14:OAc as standard compound. Each EAG test was replicated by the use of at least eight different antennae.

Field Trapping I. Field trapping with virgin females and synthetic chemicals [96% purity, which was synthesized and supplied by the Taiwan Agricultural Chemicals and Toxic Substances Research Institute (TACTSRI)] with the blend of *E*12–14:OAc/*Z*12–14:OAc/14:OAc = 48:37:15 (combined wash ratio) was carried out at three corn fields in Taipei, during January 1–25, 1991. One and 5 mg synthetic chemicals were dissolved in hexane and loaded in plastic tubes the day before the field test. The plastic tube was hung on the inside top of a wing-shaped sticky trap. Blank traps and traps baited with three 2- to 3-day-old virgin females per trap were used for comparison. Virgin females came from field-collected matured larvae. Females were changed and a trap record was made every two days. Traps were placed 10 m apart and 0.8–1 m above ground, and randomized nightly. A total of three replicates was made in this field test.

Field Trapping II. The ratio of *E*11–14:OAc, *Z*11–14:OAc, and 14:OAc was slightly different between the combined wash (48:37:15) (Figure 1) and individual females (45:39:16) (Table 1), and 14:OAc was reported not to be a sex pheromone in previous studies (Ando et al., 1980; Klun et al., 1980; Cheng et al., 1981). Therefore, a comparison of male field responses to the above three-component Taiwan formulations, two-component Taiwan formulation (*E*12–14:OAc/*Z*12–14:OAc = 53:47), and the Japan formulation (*Z*12–14:OAc/*E*12–14:OAc = 3:2) was carried out in the corn field during October 18–December 3, 1991, according to the above method. A trap record was made every four days.

Duncan's new multiple-range test (Steel and Torrie, 1980) was used to analyze the results.

RESULTS AND DISCUSSION

Chemical Analysis. GC analysis showed that a total of three major peaks was found in the gland extract (Figure 1). Previously reported sex pheromone components for the Asian corn borer included *E*12–14:OAc and *Z*12–14:OAc

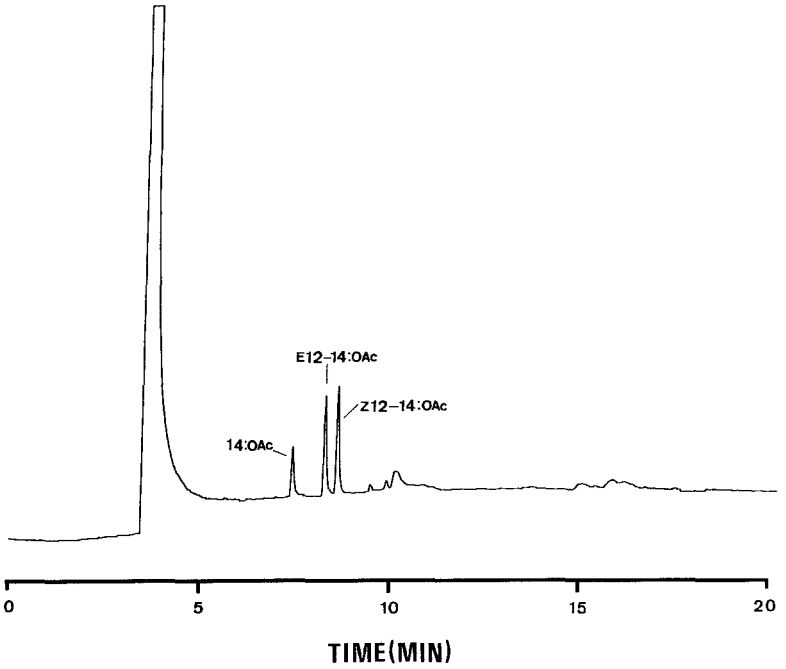

FIG. 1. Gas chromatogram of the combined wash female crude extract. The gas chromatography was performed on a 35-m × 0.25-mm Carbowax 20 M column, temperature was isothermal at 190°C.

TABLE 1. AVERAGE QUANTITY AND PERCENTAGE OF *E*12–14:OAc, *Z*12–14:OAc AND 14:OAc PRODUCED BY INDIVIDUAL 2- TO 4-DAY-OLD *Ostrinia furnacalis* VIRGIN FEMALES ($N = 15$)

Component	Mean quantity (ng/female)	Percentage
*E*12–14:OAc	6.6 ± 4.6	45
*Z*12–14:OAc	5.8 ± 3.5	39
14:OAc	2.4 ± 1.7	16

(Ando et al., 1980b; Klun et al., 1980). These two compounds and other related compounds were prepared for comparison, and it was found that synthetic *E*12–14:OAc, *Z*12–14:OAc, and 14:OAc had the same retention times (Table 2) and mass spectra as the three major peaks from ovipositors extract. The mass spectra of these three components of female tips do give a fragmentation ion at *m/z* 61, indicating that these three components are acetates. The first peak had

TABLE 2. RETENTION TIMES OF 14:OAc, *E*12–14:OAc, *Z*12–14:OAc AND PHEROMONE GLAND EXTRACT OF FEMALE *Ostrinia furnacalis* ON THREE GAS CHROMATOGRAPHIC COLUMNS

Column type	Retention time (min)			
	14:OAc	*E*12–14:OAc	*Z*12–14:OAc	Extract
Carbowax 20 M	7.49	8.32	8.64	7.47 8.30 8.62
DB-17	9.34	9.69	9.94	9.34 9.69 9.94
DB-225	11.85	12.88	13.68	11.83 12.82 13.64

a fragment ion of *m/z* 196 ($M^+ - 60$), indicating it is a 14:OAc; the second and the third peaks had a fragment ion of *m/z* 194 ($M^+ - 60$), indicating that these components are *E*12–14:OAc and *Z*12–14:OAc, respectively.

In the pooled extract, the concentration of the compounds identified as *E*12–14:OAc, *Z*12–14:OAc, and 14:OAc were determined to be in a ratio of 48:37:15, respectively, using the external standard method of quantitative analysis.

Pheromone Titer Determination. The average pheromone titer of individual 2- to 4-day-old virgin females was 14.8 ± 9.3 ng/female, with *E*12–14:OAc 6.6 ± 4.6 ng/female, *Z*12–14:OAc 5.8 ± 3.5 ng/female, and 14:OAc 2.4 ± 1.7 ng/female, with a ratio of 45:39:16 (Table 1). If we only considered *E*12–14:OAc and *Z*12–14:OAc, then the ratio between these two compounds should be 53:47. In fact, this two-component Taiwan formulation has the same blend ratio as that in the mainland China (Cheng et al., 1981). The field attractiveness of the two-component and three-component blend ratios was described in field trapping II.

EAG Analysis. The result of EAG analysis was shown in Figure 2. This assay revealed that all three singly tested compounds, 14:OAc, *E*12–14:OAc, and *Z*12–14:OAc, could elicit strong antennal responses in the male Asian corn borer, with the greatest antennal response elicited by *E*12–14:OAc and the mixture of the three compounds.

Field Trapping I. The result of field trapping of wild males with the synthetic sex pheromone (combined wash ratio, 48:37:15) and virgin females is shown in Table 3. The attractiveness of 1 mg and 5 mg synthetic compounds

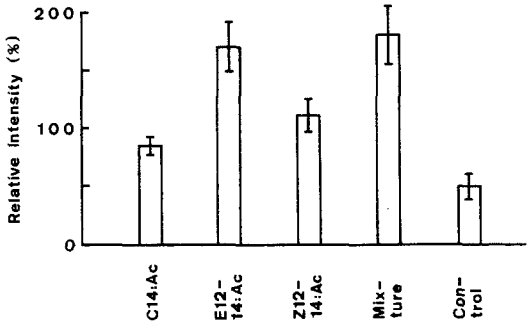

FIG. 2. EAG response of male *Ostrinia furnacalis* to 10 μg of 14:OAc, *E*12–14:OAc, Z12–14:OAc, and the mixture of *E*12–14:OAc,Z12–14:OAc, and 14:OAc (48:37:15). The response of each test chemical is expressed as percent of the response to the standard compound, 10 μg of Z11–14:OAc.

TABLE 3. MEAN NUMBER OF MALE *Ostrinia furnacalis* ATTRACTED TO SYNTHETIC SEX PHEROMONE AND VIRGIN FEMALES IN THREE CORN FIELDS DURING JANUARY 4–18, 1991[a]

*E*12-14:OAc/Z12-14:OAc/14:OAc (48:37:15)			
1 mg	5 mg	3 virgin females	Control
85.0 ± 15.1a[b]	84.0 ± 20.6a	14.0 ± 8.8b	0b

[a]The lure type was a plastic tube.
[b]Means followed by different letters are significantly different. ($P < 0.05$, Duncan's new multiple-range test).

was significantly higher than the virgin females ($P < 0.05$). There was no significant difference in the attractiveness between 1 mg and 5 mg synthetic chemicals.

Field Trapping II. No significant difference in males response was found among the three-component Taiwan formulations, the two-component Taiwan formulation, and the Japan formulation (Table 4).

The results of the present study showed that 14:OAc, although found in the gland wash of the Asian corn borer, did not enhance or suppress male responses by adding to or subtracting from trap capture with *E*/Z-12 mixture. The same results were also reported in other studies of corn borer populations in the United States (Klun and Junk, 1977) and Canada (Struble et al., 1987) and recently in the Asian corn borer (Zhao et al., 1990). On the other hand,

TABLE 4. MEAN NUMBER (MALES/TRAP/4 DAYS) OF MALE *Ostrinia furnacalis* ATTRACTED TO DIFFERENT SEX PHEROMONE BLEND RATIOS OF FORMULATION I, II, III, AND IV DURING OCTOBER 18–DECEMBER 3, 1991[a]

I	II	III	IV	Control
3.8 ± 4.3a[b]	4.4 ± 3.6a	6.0 ± 6.2a	9.0 ± 5.8a	0.0 ± 0.0b

[a]The lure type was a plastic tube. I: three-component Taiwan formulation (*E*12–14:OAc/Z12–14:OAc/14:OAc = 48:37:15, combined wash ratio). II: three-component Taiwan formulation (*E*12–14:OAc/Z12–14:OAc/14:OAc = 45:39:16, individual female ratio). III: two-component Taiwan formulation (*E*12–14:OAc/Z12–14:OAc = 53:47). IV: Japan formulation (*Z*12–14:OAc/*E*12–14:OAc = 3:2).

[b]Means followed by same letters are not significantly different. ($P < 0.05$, Duncan's new multiple-range test).

the result of male field responses to a range of pheromone ratios may suggest that the majority of males were not precisely tuned, or some behavioral morphs existed in the population, responding to different isomer ratios as in *Agrotis segetum* (Hansson et al., 1990). Therefore, individual variation in the sex pheromone system of the Asian corn borer in Taiwan should be further investigated.

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HYDROXAMIC ACID GLUCOSIDES IN HONEYDEW OF APHIDS FEEDING ON WHEAT

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Abstract—DIMBOA glucoside (2-O-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one), the main hydroxamic acid (Hx) in intact wheat plants, was detected in the honeydew of *Rhopalosiphum padi* feeding on seedlings of six wheat cultivars that differed in their concentration of Hx, suggesting that the chemical circulates in the phloem. Neither the aglucone (DIMBOA) nor its main breakdown product were found in any of the honeydew samples. Honeydew production by aphids caged on seedlings of the wheat cultivars and DIMBOA glucoside concentrations in the honeydew followed biphasic curves when plotted against Hx concentration, suggesting passive ingestion of the chemical from the phloem at low Hx concentrations and limited ingestion due to feeding deterrency by Hx in mesophyll cells at high Hx concentrations. The presence of plant toxins such as Hx glucosides in the phloem sap, the main ingesta of aphids, and in the mesophyll cells, has major implications for plant defense, through a feeding deterrent effect during stylet penetration, and deterrency (antixenosis) along with antibiosis during feeding.

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Key Words—*Rhopalosiphum padi*, Homoptera, Aphididae, wheat, maize, DIMBOA, hydroxamic acids, aphid honeydew.

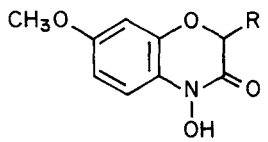
INTRODUCTION

Hydroxamic acids (Hx) are present in a number of Gramineae (Niemeyer, 1988a,b; Zuñiga et al., 1983; Copaja et al., 1991a,b; Barría et al., 1991; Niemeyer et al., 1991) including wheat. Hx exist in the intact plant as glucosides from which the more toxic aglucone is released upon disruption of the plant tissue and contact with a β-glucosidase (Hofman and Hofmanova, 1969; Cuevas et al., 1992). The most common Hx in maize and wheat tissue is 2-O-β-D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA glucoside) (Niemeyer, 1988a) (Scheme 1).

Hydroxamic acids are believed to play a role in the resistance of the plants to pests and diseases (Niemeyer, 1988a). Thus, a number of workers have reported negative correlations between the Hx content of maize and wheat and the performance of insect herbivores feeding on the plants. Most of the work has been concerned with the effects of Hx on the European corn borer (Klun and Robinson, 1969; Campos et al., 1989), the western corn rootworm (Xie et al., 1990), and cereal aphids (Long et al., 1977; Argandoña et al., 1980; Beck et al., 1983; Bohidar et al., 1986; Thackray et al., 1990; Givovich and Niemeyer, 1991).

The concentration of hydroxamic acids is higher in the vascular bundles than in the mesophyll (Argandoña and Corcuera, 1985; Argandoña et al., 1987). However, there is no clear evidence that Hx circulate in the phloem. Thus, although Niemeyer et al. (1989b) found DIMBOA in the bodies of aphids feeding on wheat, Molyneux et al. (1990) failed to find 6-methoxybenzoxazolin-2-one (MBOA, the main breakdown product of DIMBOA) in the honeydew of *Schizaphis graminum* feeding on wheat.

The aim of the experiments reported here was to determine whether DIMBOA and/or its glucoside could be detected in the honeydew of aphids feeding on wheat.



DIMBOA R = OH

DIMBOA glucoside R = O-β-D-glucopyranosyl

SCHEME 1.

METHODS AND MATERIALS

Determination of Hx and DIMBOA in Plant Material. High-performance liquid chromatography (HPLC) was employed for DIMBOA analysis in wheat seedlings. The method was essentially that described by Niemeyer et al. (1989a).

Determination of DIMBOA and Its Glucoside in Aphid Honeydew. Aphid honeydew was analyzed for DIMBOA glucoside, DIMBOA, and MBOA using HPLC. A standard of DIMBOA glucoside was obtained as described by Lyons et al. (1988).

Insect Material. Aphids were reared on oats (cv. Nahuen), a plant lacking Hx, in a chamber at 22°C with a 6° range.

Presence of DIMBOA Glucoside in Honeydew of R. padi. The wheat cultivars employed were chosen to contain a wide range of Hx concentrations. Experiments were carried out with seedlings at the one-leaf stage, when Hx levels are highest (Argandoña et al., 1980). Oats (cv. Nahuen) were used as a control without Hx. Twenty adult aphids were placed in a clip cage (2 cm diameter) fitted with a disk of aluminum foil at the bottom and clipped to the abaxial surface of a leaf of a wheat or oat seedling. After 36 hr, the cage was detached from the plant and the aluminum disk weighed. After washing successively with methanol and water and drying, the disk was weighed again. The difference between these measurements was attributed to honeydew produced. The methanol–water washings were concentrated to dryness, redissolved in 200 μl of water, and analyzed by HPLC. Three sets of experiments, each consisting of seven clip cages were performed.

RESULTS

Presence of DIMBOA Glucoside in Honeydew of R. padi *Feeding on Wheat.* DIMBOA glucoside was found in the honeydew of aphids caged on all six wheat cultivars. Neither aglucone nor MBOA were found in the honeydew samples analyzed (detection limit = 0.1 mmol/kg fresh weight).

DIMBOA glucoside concentrations in the honeydew of aphids feeding on wheat are shown in the lower curve of Figure 1. Rates of honeydew production are shown in relation to whole-leaf DIMBOA concentration in the upper curve of Figure 1. Honeydew production in oats, a plant lacking Hx, was 5.35 $\pm$ 0.16 μg/aphid/36 hr.

DISCUSSION

R. padi is mainly a phloem feeder (Pollard, 1973). Hydroxamic acids are known to occur in mesophyll cells of wheat plants (Argandoña et al., 1987). The present finding of DIMBOA glucoside in aphid honeydew suggests that the

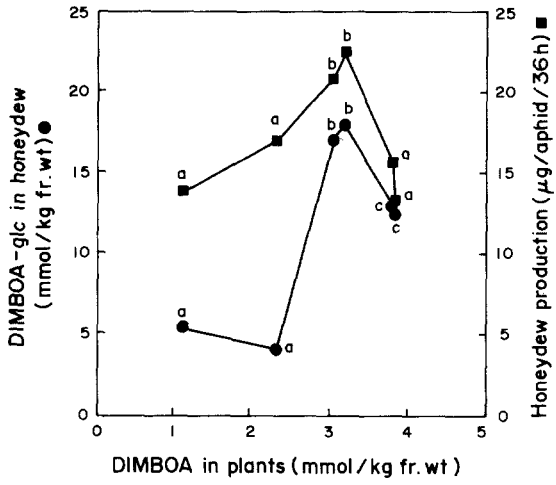


FIG. 1. Mean concentration of DIMBOA glucoside in honeydew (●) and mean honeydew production (■) of *R. padi*, as a function of DIMBOA concentration in wheat seedlings where they fed for 36 hr. Points in each curve followed by different letters are significantly different ($P < 0.01$).

chemicals are also present in wheat phloem. Further evidence for the presence of DIMBOA glucoside in phloem is provided by the failure to detect the aglucone in any of the honeydew samples: if prolonged ingestion had occurred from mesophyll cells ruptured during stylet penetration, then contact with β-glucosidase would be expected and DIMBOA should have been found in the honeydew. The lack of any DIMBOA in the samples also suggests the absence of a suitable β-glucosidase in the digestive tract of *R. padi* or the presence of efficient detoxifying glucosyltransferases. The failure of Molyneux et al. (1990) to find MBOA in the honeydew of *Schizaphis graminum* feeding on a DIMBOA-containing wheat is consistent with these results, since the glucoside does not produce the aglucone breakdown product MBOA under the conditions they used. The results are also consistent with electronic monitoring studies of aphid feeding behavior, which suggest the presence of hydroxamic acids in the phloem sap of the same wheat cultivars employed in this study, albeit in concentrations below those producing feeding deterrency (Givovich and Niemeyer, 1991).

The relationship between glucoside concentration present in honeydew and whole-leaf DIMBOA concentration in wheat followed a biphasic pattern (lower curve in Figure 1). This suggests passive ingestion from phloem sap at low leaf concentrations, and a combination of feeding deterrency, possibly during stylet penetration of the mesophyll, and ingestion from tissues with lower or non-existent Hx glucoside concentrations at high whole-leaf concentrations. Elec-

tronic monitoring of aphid feeding behavior showed that ingestion from xylem, a tissue where Hx have not been detected (Argandoña and Corcuera, 1985), increased as whole-leaf Hx concentration increased (Givovich and Niemeyer, 1991).

A biphasic curve can also be seen in the relationship between honeydew production and whole-leaf concentration of DIMBOA in the wheat cultivars (upper curve in Figure 1). The negative correlation between honeydew production and DIMBOA concentration at the high DIMBOA range may be attributed to feeding deterrency by DIMBOA. The positive correlation obtained at the low DIMBOA range may be attributed to a higher ingestion rate (and consequently, higher excretion rate) in order to compensate for the toxic effects of increasing Hx concentration of the ingesta. These effects may be related to the capacity of Hx to inhibit mitochondrial energy-linked reactions (Niemeyer et al., 1986) and digestive enzymes (Cuevas et al., 1990). Interestingly, honeydew production in oats, a plant lacking Hx, is comparable to that in the lower-DIMBOA-containing wheats.

These results suggest that Hx may be playing different roles in resistance to aphids depending on where the toxin is located in the plant. A high mesophyll concentration of Hx may provide a level of feeding deterrency during stylet penetration, while a high phloem sap concentration may also provide deterrency combined with antibiosis. Further research should determine if DIMBOA glucoside can be found directly in phloem sap.

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PHENYLALKENALS IN PONERINE (*Leptogenys* SP.) AND MYRMICINE (*Pogonomyrmex* SP.) ANTS

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Abstract—Cephalic extracts of two unrelated species of ants, *Leptogenys processionalis* and *Pogonomyrmex rugosus*, have been found to contain 2-phenylpropenal and 2-phenyl-2-butenal, while two other species related to the first, *L. chinensis* and *L. kitteli*, lacked either. *L. kitteli* also produced a tetrasubstituted pyrazine found previously only in two New Zealand ants in the genus *Mesoponera*. The chemical reactivity of the phenylalkenals suggests their function in repelling attack by predators.

Key Words—Phenylalkenals, *Leptogenys* spp., *Pogonomyrmex rugosus*, Hymenoptera, Formicidae, 2-phenylpropenal, 2-phenyl-2-butenal, 2,5-dimethyl-3-(1-methyl)butylpyrazine, 2,5-dimethyl-3-isopentylpyrazine, 2,5-dimethyl-3-isopentenyl-6-isopentylpyrazine, 4-methyl-3-heptanone, 5-methyl-3-hexanone, Maillard reaction, benzaldehyde, chemoreceptors, defensive allomones.

INTRODUCTION

A large variety of natural products have been identified as exocrine products of ants as abundant testimony to the biosynthetic virtuosity of these hymenopterans. Species in the subfamily Ponerinae are particularly distinctive, producing a vari-

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ety of pheromones and allomones belonging to diverse chemical classes (Blum, 1981). For example, ponerines generate alkyl sulfides (Casnati et al., 1967), pyrazines (Wheeler and Blum, 1973), aryl esters (Duffield and Blum, 1975), and aliphatic ketones (Duffield and Blum, 1973). These compounds are all mandibular gland products that function as either alarm pheromones, defensive allomones, or both. The already demonstrated chemical versatility of the ponerine mandibular gland makes it seem certain that analyses of additional species will yield further natural product surprises.

In the present report, we describe structures of two phenylalkenals as cephalic products of the ponerine ant *Leptogenys processionalis*. In addition, these compounds have been identified in an unrelated species, *Pogonomyrmex rugosus*, a graminivorous myrmicine that has been previously demonstrated to produce aliphatic ketones and alcohols in the mandibular glands (McGurk et al., 1966). These phenylalkenals appear to have escaped earlier detection. Two other species of *Leptogenys* were also analyzed and found to have a chemistry very different from that of *L. processionalis*. One of these species, *L. kitteli*, was distinctive in producing a tetrasubstituted pyrazine that has been detected previously only in two ponerine species of New Zealand ants in the genus *Mesoponera* (Fales et al., 1988).

METHODS AND MATERIALS

Both *L. processionalis* and *L. chinensis* were collected in Bangalore, India, and *L. kitteli* was collected in Sabah, Borneo. The harvester ant *P. rugosus* was collected in Tucson, Arizona.

Either heads or whole bodies of the *Leptogenys* species and heads of *P. rugosus* were placed in methylene chloride and the extracts sent to NIH for analysis. After concentration, these extracts were used directly for GC-MS analyses.

2-Phenylpropenal was synthesized according to established procedures (Crossland, 1981).

GC-MS was run on an LKB-2091 mass spectrometer operating at 70 eV with a source temperature of 270°C and an ionizing current of 20 μA. The GC column was a 30-m capillary coated with OV-1 methyl silicone and was programmed from 50 to 300°C at 10°C/min.

RESULTS

L. processionalis. The cephalic secretion is dominated by five compounds (Figure 1, peaks 1–5). Eluting later, and not shown on this chromatogram, are a more abundant series of fatty acids and hydrocarbons. Peaks 1, 2, and 4 were

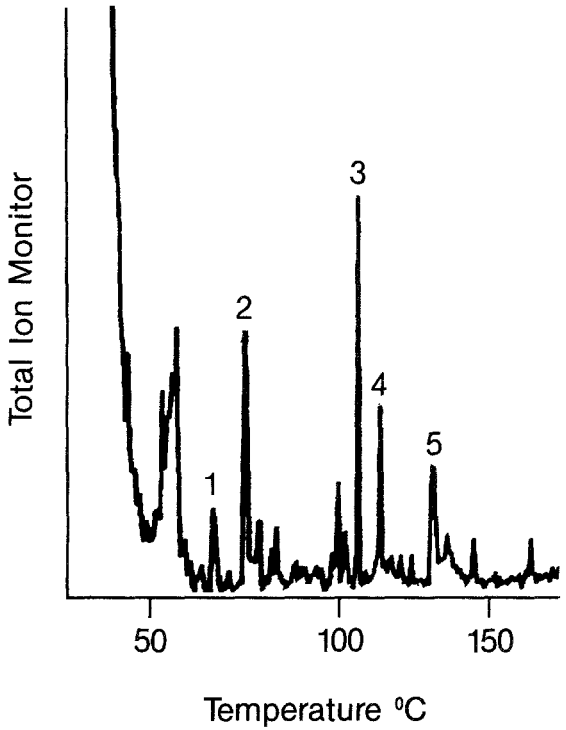

FIG. 1. Cephalic extract of *L. processionalis*. Chromatographic conditions in Methods and Materials. Peak 1: benzaldehyde, 2: phenol, 3: 2-phenylpropenal, 4: naphthalene (an artifact?), 5: 2-phenyl-2-butenal.

easily identified as benzaldehyde, phenol, and naphthalene (an artifact?) by comparison with the NIST/EPA/MSDC (1991) mass spectral data base. Peak 5 was similarly identified as 2-phenyl-2-butenal [*m/z*, (relative intensity): 146 ($M^{+}\cdot$, 77), 145 (12), 131 (9), 128 (10), 118 (17), 117 (100, $M^{+}\cdot - CHO$), 116 (24), 115 (81), 103 (9), 102 (11), 91 (54), 89 (13), 78 (17), 77 (14), 65 (13), 63 (22), 62 (9), 58 (8), 54 (8), 52 (8), 51 (22), 50 (11)]. Peak 3 showed a similar pattern shifted 14 amu lower [*m/z* (relative intensity): 132 (42, $M^{+}\cdot$), 131 (3), 104 (52), 103 (100, $M^{+}\cdot - CHO$), 102 (12), 90 (3), 78 (13), 77 (50), 76 (7), 75 (6), 74 (7), 65 (3), 63 (8), 62 (6), 61 (3), 55 (3), 54 (3), 52 (7), 51 (31), 50 (16)], and the lower homolog, 2-phenylpropenal, was a putative structure. The structure of this compound was confirmed by comparison (MS and GC retention times) with a synthetic sample prepared according to the literature (Crossland, 1981).

Since aromatic exocrine components have not been encountered frequently as cephalic natural products of ants, the blend of compounds generated by *L.*

processionalis is particularly distinctive. Benzaldehyde has been detected in a myrmicine ant in the genus *Veromessor* (Blum et al., 1969), whereas phenol has been identified in a myrmicine species in the genus *Monomorium* (Blum et al., 1985).

Extracts of bodies of the same species contained a compound tentatively identified as 2,5-dimethyl-3-(1-methyl)butylpyrazine (GC not shown) by its mass spectrum, which is identical to that described by Wheeler and Blum (1973). However, other substitution patterns on the pyrazine ring have also been detected in ants (Wheeler and Blum, 1973), and decisive assignment of the 2,5-dimethyl structure must await comparison with authentic samples (GC) or possibly GC-IR. This ant is thus provided with a component that belongs to a class of natural products characteristic of ponerine ants. Species in a diversity of ponerine genera produce pyrazines in their mandibular glands (Longhurst et al., 1978), and we assume that this is the case here as well.

L. chinensis. Cephalic extracts of this ant contained none of the aromatic constituents identified in *L. processionalis*. On the other hand, *L. chinensis* workers apparently do synthesize a pyrazine (GC not shown) that is isomeric with the one produced by *L. processionalis*. This compound, again tentatively identified by the identity of its mass spectrum with that of Wheeler and Blum (1973) as 2,5-dimethyl-3-isopentylpyrazine, is a widely occurring natural product in the mandibular gland secretions of ponerine ants (Wheeler and Blum, 1973; Brown and Moore, 1979).

L. kitteli. The cephalic secretion of this species contains a compound again tentatively identified by its mass spectrum as 2,5-dimethyl-3-isopentylpyrazine (Figure 2, peak 1), the same pyrazine produced by *L. chinensis*. However, the secretion of *L. kitteli* is distinguished by the presence of a second, unusual, tetrasubstituted pyrazine, 2,5-dimethyl-3-isopentenyl-6-isopentylpyrazine (Figure 2, peak 2). This compound, which is the major product in the exudate, has been previously identified only as a mandibular gland product in two species of New Zealand ponerine ants in the genus *Mesoponera* (Fales et al., 1988).

None of the aromatic constituents identified as cephalic products of *L. processionalis* were detected as natural products of *L. kitteli*.

Pogonomyrmex rugosus. As is the case with *L. processionalis*, the cephalic secretion of *P. rugosus* contains a diversity of aromatic constituents (Figure 3). Both species share benzaldehyde (peak 3) as well as the newly discovered phenylalkenals [peaks 5 and 6 (identified by subtraction of the spectrum of the coeluting phenylacetaldehyde)], but they differ considerably in other particulars. Thus, the exudate of *P. rugosus* is thoroughly dominated by 4-methyl-3-heptanone (Figure 3, peak 2), an ethyl ketone that is characteristic of species in this genus (McGurk et al., 1966). Traces of 5-methyl-3-hexanone (Figure 3, peak 1) were also detected, both ketones being identified through comparison with literature spectra (NIST/EPA/MSDC 1990). On the other hand, no alka-

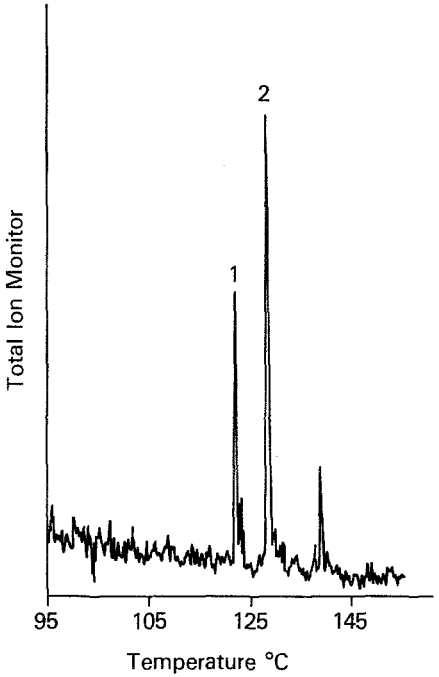

FIG. 2. Cephalic extract of *L. kitteli*. Peak 1: 2,5-dimethyl-3-isopentylpyrazine, 2: 2,5-dimethyl-3-isopentenyl-6-isopentylpyrazine.

nones were detected as cephalic products of *L. processionalis*. Similarly, constituents such as phenylacetaldehyde and phenylacetic acid (peaks 4 and 7) constitute two of the five aromatic compounds that characterize the exudate of *P. rugosus;* these compounds were not detectable as cephalic products of *L. processionalis*.

DISCUSSION

Pyrazines have been demonstrated to be characteristic mandibular gland products of species in a wide variety of ponerine genera (Wheeler and Blum, 1973; Longhurst et al., 1978; Brown and Moore, 1979). In all cases these compounds have been demonstrated to function as alarm pheromones that can attract excited workers to the source, which may be stung by these aggressive ponerines. Because of the painful stings possessed by the pyrazine-producing ponerines, it will not prove surprising if these heterocycles function as an early-warning system for predators, enabling them to avoid a thoroughly unpleasant

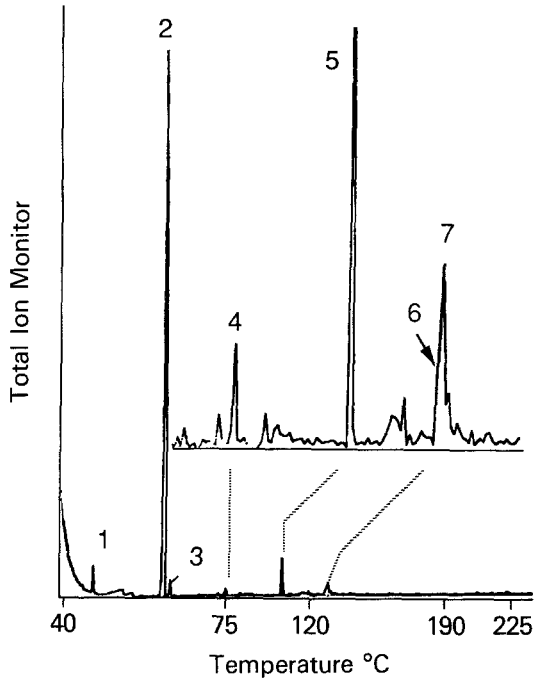

FIG. 3. Cephalic extract of *P. rugosus*. Peak 1: 5-methyl-3-hexanone, 2: 4-methyl-3-heptanone, 3: benzaldehyde, 4: phenylacetaldehyde, 5: 2-phenylpropenal, 6: 2-phenyl-2-butenal, 7: phenylacetic acid.

experience with the algogenic ponerines. The same can certainly be said for species in the myrmicine genus *Pogonomyrmex*. Rothschild (1989) have suggested that pyrazines are components of a generalized warning system in which toxic insects are easily recognized as unpalatable or dangerous prey. Furthermore, these pyrazine-producing ponerines, which share common traits such as similar appearance, exaggerated attack behavior, and painful stings, may well constitute a mimicry complex in which species in different genera may advertise their dangerous qualities with similar pyrazine signals.

The aromatic constituents generated by *L. processionalis* and *P. rugosus* in their cephalic secretions are qualitatively exceptional in terms of the exudates of ants. Aromatic aldehydes are rarely encountered as ant natural products. Even compounds such as benzaldehyde are uncommon exocrine products of ants and, for that matter, insects in general. Phenylacetic acid, a product of *P. rugosus*, has been reported to be an antibiotic produced in thoracic glands of myrmicine ants (Schildknecht and Koob, 1970).

2-Phenylpropenal has also been identified in both wings and bodies of both

sexes of an adult butterfly *Pieris rapae* (Honda and Kawatoko, 1976); its function in this insect is not known. However, the chemical reactivity of these phenylalkenals suggests that they might function as excellent defensive allomones to blunt the attacks of predators by jamming their olfactory chemoreceptors. For example, these electrophilic substances could be readily attacked by nucleophilic sites (NH and SH) in olfactory proteins, rendering a smell-oriented predator incapable of hunting with facility. It is not unlikely that the phenylalkenals, along with the other aromatic components, form part of the chemical arsenal utilized by these ants in adversarial contexts.

It has not escaped our attention that the phenylalkenals are well-known products of protein degradation (List and Freund, 1967) where they appear to derive from a Maillard reaction between glucose and phenylalanine (Baltes and Mevissen, 1988). The possibility that this reaction could take place from similar components in the injection port of the gas chromatograph was minimized by examining the extracts of *L. processionalis* after silylation with bis(trimethylsilyl)trifluoroacetamide, a reagent that should prevent such reactions by converting the intermediates to unreactive derivatives. The same phenylalkenals were, in fact, still detected along with other unidentified silylated products.

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CALLING BEHAVIOR AND PHEROMONE TITER IN THE SMALLER TEA TORTRIX MOTH, *Adoxophyes* SP. (LEPIDOPTERA: TORTRICIDAE)

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Abstract—The calling behavior and pheromone titer in the female smaller tea tortrix moth, *Adoxophyes* sp., were investigated under a 14:10-hr light–dark photoperiod. Quantitative gas chromatographic analysis of ovipositor extract for (*Z*)-11-tetradecenyl acetate (Z11–14:Ac) and (*Z*)-9-tetradecenyl acetate (Z9–14:Ac), the major pheromone components of this species, obtained on the third day postemergence, indicated that extractable amounts of sex pheromone were present throughout the period of observation. Maximal pheromone titer and calling activity was reached at 8 and 10 hr after onset of scotophase, respectively. The ratio of Z11–14:Ac to Z9–14:Ac through the 24-hr period varied significantly. The significance of the sex pheromone component ratio variation on the attraction of males was tested in a field experiment. The ratio of males trapped by the most attractive blend versus the least attractive one was 2.16.

Key Words—Calling behavior, pheromone titer, *Adoxophyes* sp., Lepidoptera, Tortricidae, (*Z*)-11-tetradecenyl acetate, (*Z*)-9-tetradecenyl acetate.

INTRODUCTION

In moths, the daily rhythm of sexual activity such as sex pheromone production and its subsequent release during the calling period usually depend upon exogenous (photoperiod, temperature) and endogenous factors (neural, hormonal) (Baker and Cardé, 1979; Hollander and Yin, 1982; Webster and Cardé, 1982; Raina et al., 1986, Raina, 1988; Tang et al., 1989). The relationship between calling behavior and pheromone titer had been precisely studied in several moth species such as *Trichoplusia ni* (Hübner) (Sower et al., 1972), *Plodia inter-*

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punctella (Hübner), and *Ephestia cautella* (Walker) (Coffelt et al., 1978); *Platynota stultana* (Walsingham) (Webster and Cardé, 1982); and *Helicoverpa zea* (Boddie) (Raina et al., 1986).

Females of the smaller tea tortrix moth *Adoxophyes* sp. (the species is still unidentified) in Taiwan produce (Z)-11-tetradecenyl acetate (Z11–14 : Ac) and (Z)-9-tetradecenyl acetate (Z9–14 : Ac) as major constituents of its sex pheromone, and the blend ratio is 64 : 36, which is the reverse of the ratio in Japan (Kou et al., 1990). In this study, the level of female calling and the quantity and ratio of the two sex pheromone compounds in the pheromone gland of *A.* sp. are investigated at different times of day. A field-trapping experiment was also carried out to obtain an estimate of the biological significance of the pheromone ratio variation observed at different times during the calling period.

METHODS AND MATERIALS

Insects. Female pupae of the smaller tea tortrix moth used in this study were obtained from the Taiwan Tea Experiment Station, Hsinchu, Taiwan. They were maintained in environmental chambers at 23–26°C under a 14 : 10-hr light–dark photoperiod. Emerged moths were fed with 10% aqueous sucrose solution. Most adults emerged shortly after the onset of a photophase and only these were used in the tests.

Female Calling. Fifty virgin females that had called the first night after emergence were put into a 30 × 30 × 30-cm transparent plastic box. From the beginning of the third photophase, females were observed for 24 hr through the photophase and scotophase at 1-hr intervals. During photophase and scotophase, a 60-W white lamp and a 5-W red lamp were used for observation, respectively. Calling females are easily recognized by their extruded ovipositors and raised vibrating wings.

Pheromone Titer Determination. From the beginning of the third-day photophase, 10 ovipositors of the females were excised at each hour through the photophase and scotophase of that day. The ten ovipositors were then pooled in 10 μl hexane containing 0.5 μg (Z)-11-hexadecenyl acetate (Z11–16 : Ac) (internal standard). The ovipositors were soaked in the solvent for 5 min, and the extract was analyzed for Z11–14 : Ac and Z9–14 : Ac using the internal standard method of quantitative analysis (Kou et al., 1990). One microliter of the extract was injected onto a 35-m fused silica capillary column coated with 0.25-μm film of Carbowax 20 M phase; the column was installed in a Varian 3700 gas chromatograph (GC) equipped with a flame ionization detector. Chromatographic conditions were: nitrogen carrier 7.5 psi, column temperature programmed from 120°C to 200°C, at 4°C/min. The experiment was replicated three times. The ratios of the two pheromonal components were also examined

at 1-hr intervals throughout the third day. Student-Newman-Keuls' test (Steel and Torrie, 1980) was used to analyze the results.

Field Tests. The male response specificity to alternations in sex pheromone component ratios observed in female gland washes was carried out at a tea plantation in Nankang, Taipei, during May 30–August 3, 1990. Synthetic chemicals of five different blend ratios of *Z*11–14 : Ac and *Z*9–14 : Ac (58 : 42, 62 : 38, 64 : 36, 66 : 34, and 68 : 32) were prepared so as to cover most of the observed range of ratios during the calling period. One milligram of each synthetic chemical was dissolved in hexane and loaded in a plastic capsule (capsule for electron microscope, type C050, 8 mm ID × 17.8 mm, TAAB Ltd., Berkshire, England). The plastic capsule was hung on the inside top of a wing-shaped sticky trap. Traps were placed randomly, 10 m apart and 1.5 m above ground. Trap catches were monitored weekly and traps were moved weekly from one location to another during the experiment. Only one set of traps was run in this experiment. The Kruskal-Wallis one-way analysis for multiple comparisons (Siegel and Castellan, 1989) was used to analyze the field test results.

RESULTS

Female Calling. The pattern of calling behavior of the 3-day-old females observed in the present study is shown in Figure 1A. Sixteen percent of the observed females that had called at the end of the second scotophase still continued their calling at 1 hr after the onset of the third photophase, then calling percentage decreased gradually to zero at 4 hr after the onset of the third photophase. At 5 hr after the onset of scotophase, 6% of the observed females started calling, and the calling percentage increased gradually to 87% and 95% at 9 and 10 hr after scotophase, respectively.

Pheromone Titer. The pattern of change in the relative amounts of the two pheromonal compounds throughout the third day was very similar (Figure 1B and C). However, the titer of each compound differed at different times during the 24-hr period. The titers of *Z*11–14 : Ac and *Z*9–14 : Ac decreased gradually from 39.1 and 18.5 ng/female at 1 hr after photophase to 14.8 and 9.0 ng/female at 8 hr into photophase, respectively, and then increased gradually to 45.5 and 27.2 ng/female at 14 hr (the end) of the photophase, respectively. There was no significant difference in the pheromone titer of either compound during the photophase. With the onset of scotophase, *Z*11–14 : Ac and *Z*9–14 : Ac dropped abruptly to 20.4 and 14.6 ng/female at 1 hr after scotophase, respectively, then the titers of both compounds increased progressively. Maximal titers were reached at 8 hr after scotophase, with females containing 94.0 ng of *Z*11–14 : Ac and 43.3 ng of *Z*9–14 : Ac. Both titers were significantly higher than that obtained in any photophase and early scotophase preparations

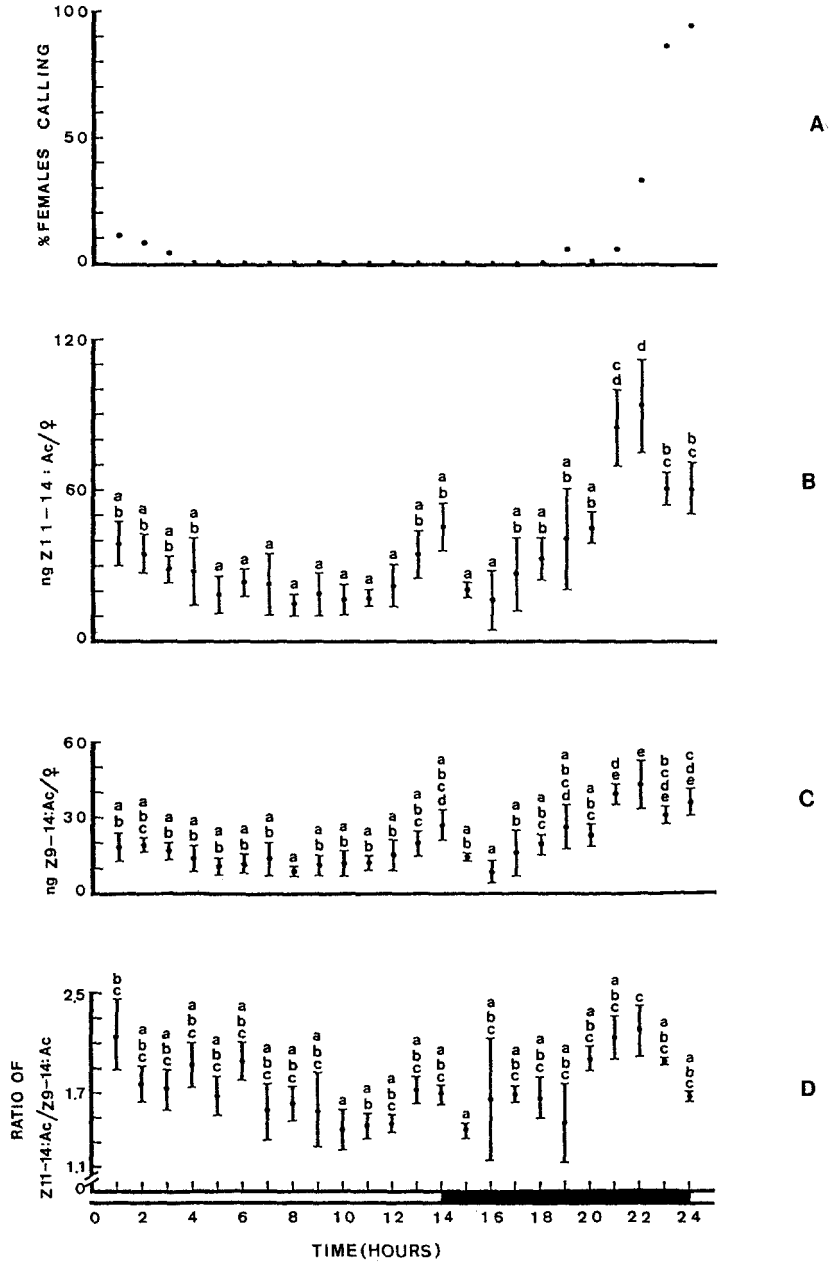

FIG. 1. (A) Periodicity of calling; (B) titer of Z11–14:Ac; (C) titer of Z9–14:Ac; and (D) ratio of Z11–14:Ac to Z9–14:Ac in the female ovipositor, throughout the third day. Shading on the abscissa denotes scotophase. Vertical bars in B, C, and D represent $\pm$SD, and means with different letters are significantly different ($P < 0.05$, Student-Newman-Keuls' test).

($P < 0.05$). The ratio of Z11–14 : Ac to Z9–14 : Ac in the sex pheromone gland was not constant through the third day, with the significantly low ratio (1.40) ($P < 0.05$) at 10 hr after photophase and 1 hr after scotophase (Figure 1D). The highest ratio (2.19) was obtained 8 hr after scotophase.

Field Test. The result of field trapping of wild males with five different blend ratios, which were observed in the gland washes during the calling period, in a tea plantation is shown in Table 1. Among the five different blend ratios, the blends of 64 : 36 (Z11–14 : Ac/Z9–14 : Ac = 1.78) and 66 : 34 (Z11–14 : Ac/Z9–14 : Ac = 1.94) were most attractive (Table 1). The ratio of the males trapped by the most attractive blend (Z11–14 : Ac/Z9–14 : Ac = 64 : 36) to the least attractive one (Z11–14 : Ac/Z9–14 : Ac = 68 : 32) was 2.16. No significant difference of attractiveness existed among the five ratios (Kruskal-Wallis analysis, $P = 0.05$).

DISCUSSION

In the smaller tea tortrix moth, the calling behavior and pheromone titer in the gland appear not to be very synchronous events under the conditions described above. Throughout the third day, the pheromone titer can be easily detected, but calling only occurred during late scotophase with some carry over into photophase. With the onset of scotophase, pheromone titers dropped abruptly, but calling behavior did not commence immediately. Maximal pheromone titer was reached at 8 hr after scotophase, in agreement with a previous observation that the sexual activity was maximized after 7–8 hr into the darkness (Tamaki et al., 1969). On the other hand, maximal calling was reached 2 hr later at 10 hr into scotophase. A same time lag between sex pheromone content and calling behavior also existed in the yellow peach moth, *Conogethes punctiferalis* (Guenée) (Konno, 1986). This time lag between calling behavior and pheromone

TABLE 1. NUMBER (MALES/TRAP/WEEK) OF *Adoxophyes* SP. MALES ATTRACTED TO 5 DIFFERENT SEX PHEROMONE BLEND RATIOS IN A TEA PLANTATION (MAY 30–AUGUST 3, 1990)

Blend ratio of Z11-14 : AC/Z9-14 : AC	Mean catch[a]
58 : 42	3.2 ± 3.4a
62 : 38	3.3 ± 6.2a
64 : 36	4.5 ± 8.8a
66 : 34	4.3 ± 3.2a
68 : 32	2.1 ± 1.9a

[a] Means followed by the same letter are not significantly different (ANOVA, $P > 0.05$).

titer may be explained as follows: In a natural setting, temperature rises and falls during the day and this exogenous factor certainly influences the expression of calling and perhaps the production of pheromone; a decrease in temperature early in the evening might well advance the timing of calling, perhaps thereby bringing these events into closer synchrony than now appears to be the case.

The relationship between calling and pheromone titer found in *A.* sp. was not similar to other moth species. In *Trichoplusia ni* (Hübner) (Sower et al., 1972), pheromone titer of the principal pheromone component remained relatively constant through a 24-hr period, although females called only during the scotophase. In *Plodia interpunctella* (Hübner) and *Ephesia cautella* (Walker) (Coffelt et al., 1978) and in *Helicoverpa zea* (Boddie) (Raina et al., 1986), the rhythm of pheromone titer was synchronous with calling rhythm, titer being highest during calling.

In this study, the ratios of Z11–14:Ac to Z9–14:Ac through the entire photoperiod were not very constant, with some ratios being close to our previously found ratio of 1.77 (Kou et al., 1990). Although the "pooled sample" technique was used in this study, ratio changes were within the range of the most frequently observed ratios from individual analysis (Kou and Chow, 1991). The ratio change through the third day may be attributed to the precise regulation mechanism of biosynthesis and/or release rate of these two compounds.

The result of field tests showed that male response specificity to alternating sex pheromone component ratios observed at different times during the calling period did not differ significantly, but the most attractive ratio (Z11–14:Ac/Z9–14:Ac = 1.78 and 1.94) was very close to our previously identified ratio (Z11–14:Ac/Z9–14:Ac = 1.77). Although in the red-banded leaf-roller, *Argyrotaenia velutinana*, the ratio of Z11–14:Ac/*E*11–14:Ac was found to be precisely regulated to 91:9 (Miller and Roelofs, 1980), males of some moths can respond to a large variation in the ratio of sex pheromone components (Löfstedt et al., 1985; Schlyter and Birgersson, 1989). We shall further investigate the emission rate and the blend ratio of the released sex pheromone of *A.* sp.

Recent studies suggest that in most moth species calling is controlled by input from the brain or other nervous centers (Itagaki and Conner, 1987), whereas the production of pheromone is under neuroendocrine control. A peptide, which in *H. zea* is composed of 33 amino acids, has a molecular weight of 3900 and is localized in the suboesophageal ganglion portion of the brain complex where it regulates pheromone production (Raina et al., 1989). Whether *A.* sp. also uses the same physiological mechanism to control calling behavior and pheromone production needs further study.

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RESPONSE OF FLEA BEETLES, *Phyllotreta* SPP., TO MUSTARD OILS AND NITRILES IN FIELD TRAPPING EXPERIMENTS

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Abstract—Trapping experiments were carried out near Winnipeg, Canada, in the spring of 1987 and 1988 to test attraction of crucifer-feeding flea beetles to volatile glucosinolate (GS) hydrolysis products released from glass vials. Nine isothiocyanates (IC) or mustard oils and three nitriles (CN) were tested. The pattern of attraction was the same for both flea beetle species, *Phyllotreta cruciferae*, and *P. striolata*. Captures in traps baited with allyl IC increased as release rates increased from 0.04 mg/day to 40 mg/day. The lowest rate that attracted large numbers of beetles was 4 mg/day; therefore this rate was used for further experiments. More beetles were captured in traps baited with allyl IC than with any other compound. In 1988 only, four IC in addition to allyl IC were attractive to both species; namely, benzyl IC, ethyl IC, and a mixture of ethyl and methyl 4-isothiocyanatobutyrate (ICB). When captures of the two species were pooled, 3-methylthiopropyl IC, methyl ICB, and *n*-butyl IC were also found to be significantly attractive. Nitriles were the least attractive compounds. The high release rates of mustard oils required to attract flea beetles indicate that fields of *Brassica* crops would release sufficient quantities of IC to attract flea beetles from a distance but individual or small groups of plants normally would not. It is concluded that *Brassica* varietal resistance to flea beetles is unlikely to be affected by manipulating IC release.

Key Words—Coleoptera, Chrysomelidae, Alticinae, *Phyllotreta cruciferae*,

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Phyllotreta striolata, *Phyllotreta pusilla*, host-plant volatile, glucosinolate, host-plant location, insect–plant interaction, *Brassica*.

INTRODUCTION

All plants of the Brassicaceae as well as species in several other plant families are known to contain one or more glucosinolates (GS), a group of naturally occurring anionic compounds whose known number totals almost 100. Cells of these plants also contain the enzyme myrosinase, which facilitates hydrolysis of these compounds to isothiocyanates (IC) or mustard oils, nitriles (CN), and other compounds, depending upon pH and other conditions (Chew, 1988). Some hydrolysis probably takes place during normal catabolysis, particularly during early seedling development, but also occurs rapidly when plant tissue is damaged (Van Etten and Tookey, 1979). As IC have toxic properties to birds, insects, and monogastric mammals (Van Etten and Tookey, 1979; Ahman, 1986), their formation in response to damage is thought to be, at least in part, a plant defense mechanism.

However, a number of insect species feed primarily or exclusively on plants containing GS. Many of these insects are known to suffer no adverse effects from naturally occurring concentrations of GS and IC. It has been well documented that at least some crucifer-specialist insects are stimulated to feed or oviposit in the presence of GS. Some insects are also attracted by one or more of the IC (Chew, 1988).

It has been known for over 30 years that flea beetles are attracted to allyl IC (Görnitz, 1956), a volatile hydrolysis product of allyl GS or sinigrin. It has been assumed that this was the major means by which crucifer-feeding flea beetles located their host plant (Feeny et al., 1970). However, more recent work has demonstrated that this relationship is not nearly so simple. The striped flea beetle, *Phyllotreta striolata* (F.) (Lamb and Palaniswamy, 1990), and the crucifer flea beetle, *P. cruciferae* (Goeze) (Pivnick, unpublished), do not necessarily discriminate between host and nonhost plants until after they are either in very close proximity or have landed on them. These are the two major pest species of flea beetles in western Canada (Lamb and Turnock, 1982), and their host-plant range is confined to the Brassicaceae (Feeny et al., 1970).

Phyllotreta spp. are stimulated to feed in the presence of GS and most GS tested have been similar in their effect (Hicks, 1974; Nielsen, 1978). To date, most feeding specificity of flea beetles within the family Brassicaceae is thought to be due to the presence of various feeding deterrents such as cucurbitacins and cardenolides (Nielsen, 1978). The nonspecificity of the response to GS suggests that attraction to IC would also be nonspecific; indeed, this has often been assumed. However, in a recent study of the northern false chinch bug, *Nysius niger* Baker, a polyphagous insect that has a preference for crucifers, response

to different IC was highly variable (Pivnick et al., 1991). As one step toward understanding the ecological significance of the response to IC by flea beetles and, possibly, toward using this response to manage the pest species, an understanding of the specificity of response to mustard oils is needed. In this paper, we examine the attraction of flea beetles to a number of different GS degradation products, both IC and CN, in field trapping experiments.

METHODS AND MATERIALS

Field trapping was carried out in 1987 on the University of Manitoba campus in Winnipeg, and at Glenlea, Manitoba, and in 1988 at Glenlea only. Trapping sites were next to cultivated fields, along the edge of wooded areas where beetles overwinter, except for one replicate set out on a trail through the woods at Glenlea in 1987. Traps were set out on the ground in a randomized complete block design, with three replicates and a minimum of 10 m between traps within a replicate. Yellow plastic boll weevil traps with a clear plastic funnel and ball trap top were used (Pest Management Specialists, Starkville, Mississippi). Baits were held upright in the center of the bases of the trap with adhesive tape. IC and CN baits, all neat liquids, were released from vials made from glass tubing, 3.5-ml serum vials, and pipets, all sealed at one end, as in Pivnick et al. (1991). The release rate of any given compound was directly proportional to the internal diameter of the container and inversely proportional to the length of the air column above the meniscus of the compound. The dimensions used were designed to give the approximate desired release rates with little change over the period of the tests, which lasted up to a maximum of 25 days. Control of release rates was always approximate, as weather conditions could modify the release rates but would do so uniformly across all baits.

The compounds tested were obtained from: Aldrich Chemical Co. (allyl IC, benzyl IC, 2-phenylethyl IC, ethyl IC, allyl CN, benzyl CN, methyl CN); Eastman-Kodak Co. (*n*-butyl IC, phenyl IC); or synthesized at the Plant Biotechnology Institute (PBI) according to methods described or referenced in Pivnick et al. (1991) [3-methylthiopropyl IC, a 1 : 1 mixture of ethyl and methyl 4-isothiocyanatobutyrate (ethyl/methyl ICB; these are IC), and methyl and ethyl ICB, synthesized individually]. All compounds are known to occur in plants except ethyl ICB and phenyl IC.

Vial dimensions used for release of allyl IC in 1987 were as follows. Air column lengths were 40 mm and internal diameters were 3.3, 0.85, and 0.27 mm for intended release rates of 4, 0.4, and 0.04 mg/day, respectively (based on prior empirical tests). For 40 mg/day, a half-filled 7-dram serum vial was used, capped with a rubber septum with a pipe cleaner inside as a wick. Actual release rates for all compounds were determined by measuring the length of the

air column both before and after the experiment. As vial dimensions used in 1988 for compounds other than allyl IC gave release rates much closer to the desired rates than for the same compounds in the second experiment in 1987, only 1988 dimensions for vials of these compounds are reported here. However, actual release rates are reported for both years. For allyl IC, allyl CN, methyl CN and ethyl IC at 4 mg/day in 1988, dimensions were as for allyl IC at the same rate in 1987. Internal diameters and air column lengths for release at 4 mg/day were 3.3 and 5 mm for methyl ICB, 5 and 5 mm for 2-phenylethyl IC, and 5 and 10 mm for all the other compounds. At 0.04 mg/day, internal diameters and air column lengths were 0.27 and 40 mm for ethyl IC, 0.60 and 10 mm for 2-phenylethyl IC, and 0.60 and 20 mm for the three other compounds.

From April 21 to 28, 1987, a dose–response experiment was carried out with allyl IC released at 0.04, 0.4, 4, and 40 mg/day, to determine the lowest rate at which enough beetles could be captured for comparison of attraction to different compounds. From April 30 to May 25, 1987, a second experiment was carried out to compare the attraction of 11 compounds, plus a mixture of two compounds at an estimated release of 4 mg/day and a blank control. From May 9–16, 1988, the same compounds and blank were tested again, along with one additional IC released at the same rate plus five of the compounds additionally tested at a release rate of 0.04 mg/day. These five compounds had shown weak evidence of attractiveness to flea beetles the previous year. In the two 1987 experiments, traps were emptied five times while in 1988 they were emptied only once at the end of the experiment.

Square-root-transformed data were analyzed by two-way ANOVA followed by unprotected LSD tests. Treatments where no beetles were captured in any of the replicates were not included in the analyses.

RESULTS

In the 1987 allyl IC dose–response experiment, the number of beetles captured increased with release rate in a similar pattern over the entire range tested for all three species caught in significant numbers: *P. cruciferae*, *P. striolata*, and *P. pusilla* Horn (Table 1). A release of 0.4 mg/day appeared to be the lowest rate at which beetles were attracted, although the number caught at this rate was not significantly higher than at 0.04 mg/day. Additional beetles were collected on the tapes that held the baits in place as well as in the bait vials. Beetles in the vials were not abundant enough to significantly affect release rates. This experiment indicated that 4 mg/day was the lowest rate that attracted high numbers of beetles, and thus was the standard rate used in subsequent experiments.

In the second experiment in 1987, only two beetle species were collected:

P. cruciferae and *P. striolata*, and again the trap captures were very similar between the two species (Table 2). Of the baits tested, allyl IC was the only bait that attracted large numbers of beetles, and it was the only bait that attracted significantly more beetles of each species than did the blank. However, there was an indication that other baits might be attractive as several caught more than the blanks. The measurements of change in bait compound levels indicated

TABLE 1. FLEA BEETLE, *Phyllotreta* SPP., CAPTURES IN TRAPS BAITED WITH FOUR RELEASE RATES OF ALLYL ISOTHIOCYANATE AT WINNIPEG, APRIL 21–28, 1987

Approximate release rates (mg/day)	Total number caught in 3 traps[a]				Number of additional beetles caught on tape or in vials (all spp; $N = 3$)
	P. cruciferae	*P. striolata*	*P. pusilla*	All 3 species	
40	131a	344a	44a	519a	48
4	92ab	258ab	23ab	373ab	59
0.4	10b	5ab	2b	17bc	7
0.04	0	1b	0	1c	0

[a]Counts in columns followed by different letters differ significantly at $P < 0.05$.

TABLE 2. FLEA BEETLE, *Phyllotreta* SPP., CAPTURES IN TRAPS BAITED WITH 12 GLUCOSINOLATE DEGRADATION PRODUCTS AT WINNIPEG, APRIL 30–MAY 25, 1987

Bait[a]	Measured release rates (mg/day)[b]	Total number caught in 3 traps[c]	
		P. cruciferae	*P. striolata*
Allyl IC	3.0 ± 0.2(3)	406a	603a
Ethyl/methyl ICB	0.2 ± 0.1(3)	10b	8b
Benzyl IC	0.4 ± 0.1(3)	10b	2b
n-Butyl IC	0.8 ± 0.5(3)	8b	0
Ethyl IC	3.0 ± 0.2(3)	6b	1b
Blank		2b	2b
2-Phenylethyl IC	2.7 ± 0(2)	2b	1b
3-Methylthiopropyl IC	0.3 ± 0(3)	2b	1b
Methyl CN	0.4 ± 0.01(3)	1b	0
Benzyl CN	0.3 ± 0.2(3)	0	1b
Allyl CN	3.4 ± 0.2(3)	0	0
Phenyl CN	0.2 ± 0.1(3)	0	0
Ethyl ICB	0.3 ± 0(3)	0	0

[a]IC = isothiocyanate; ICB = isothiocyanatobutyrate; CN = nitrile.
[b]$\bar{X}$ ± SE; sample sizes in parentheses.
[c]Counts in columns followed by different letters differ significantly at $P < 0.05$.

a lack of uniformity of release rates that may have affected trap captures. Eight of the compounds were released from the baits at approximately one tenth of the desired rates. Partly because of this, and because of the ambiguous results with some of the baits, the experiment was repeated in 1988.

In 1988, trapping took place over a shorter period of time: only one week as compared to 26 days in 1987. Even so, a greater number of beetles were caught in the second year (Table 3). In this experiment, release rates were more uniform, although rates could not be measured in some vials for a variety of reasons. For example, many flea beetles accumulated in the vials of allyl IC making accurate measurement impossible. Relatively more beetles were captured with baits other than allyl IC than in the previous trapping experiment although captures in traps baited with allyl IC were still significantly greater

TABLE 3. FLEA BEETLE, *Phyllotreta* SPP., CAPTURES IN TRAPS BAITED WITH 13 GLUCOSINOLATE DEGRADATION PRODUCTS AT WINNIPEG, MAY 9–16, 1988

	Release rates (mg/day)		Total number of beetles (n = 3) caught[c]			
			In traps		In vials, both	In traps and vials, both
Bait[a]	Estimated	Measured[b]	*P. cruciferea*	*P. striolata*	species	species
Allyl IC	4	NM	296a	158a	450[d]	904a
Benzyl IC	4	4.2 ± 1.4 (2)	138b	49bc	51	238b
Ethyl IC	4	4.1 ± 0.4 (3)	115bc	82b	8	205bc
Ethyl/methyl ICB	4	3.7 ± 0.9 (3)	60bcd	28bcd	22	110cd
3-Methylthiopropyl IC	4	3.7 ± 0.9 (2)	36cde	20cde	47	103cd
Methyl ICB	4	2.5 (1)	23de	8de	57	88de
n-Butyl IC	4	7.7 ± 0.9 (3)	45de	2e	15	62def
2-Phenylethyl IC	4	2.9 ± 0 (3)	21de	18cde	9	48defg
Benzyl IC-low	0.04	NM	6de	5de	17	28efg
Ethyl ICB	4	5.6 ± 0 (3)	1e	1e	21	23efg
Allyl CN	4	7.4 ± 0.7 (2)	1e	1e	15	17fg
n-Butyl IC-low	0.04	0.03 ± 0.02(3)	10de	5de	0	15fg
Ethyl IC-low	0.04	0.02 ± 0 (2)	5de	9de	0	14fg
Benzyl CN	4	2.0 ± 0.9 (3)	1e	5de	6	12fg
Phenyl IC	4	2.9 ± 0 (3)	4e	3e	2	9fg
Ethyl/methyl ICB-low	0.04	NM	2e	5de	0	7g
2-Phenylethyl IC-low	0.04	NM	0	4de	1	5g
Methyl CN	4	11.9 ± 0.4 (3)	0	0	0	0
Blank			0	0	0	0

[a]IC = isothiocyanate; ICB = isothiocyanatobutyrate; CN = nitrile.
[b]$\bar{X}$ ± SE; sample sizes in parentheses; NM = not measured.
[c]Counts in columns followed by different letters differ significantly at $P < 0.05$.
[d]Numbers in vials of allyl IC were estimated to be 150 per vial.

than with any other compound for both species of flea beetle. Three IC—benzyl IC, ethyl IC, and ethyl/methyl ICB—were also significantly attractive for both species. When captures of the two species were pooled, 3-methylthiopropyl IC, methyl ICB, and *n*-butyl IC were also significantly attractive. Because the blank traps caught no beetles, it is likely that captures in traps with some of the other baits also were indicative of attraction. Nitriles were among the least attractive. In all cases, traps baited with compounds at standard release rates (4 mg/day) caught more beetles than did those traps that released at 1% of the standard rates. No important differences were detected in the attractiveness of the baits for the two flea beetles species. In 1988, there was a correlation between the number of beetles caught in the traps and those caught in the vials (for *P. cruciferae*, $r = 0.54$, and for *P. striolata*, $r = 0.61$, with $P < 0.001$ in both cases).

Small numbers of additional flea beetle species, two in 1987 and 13 in 1988, were caught but not included in the tables.

DISCUSSION

These results are the first demonstration that *P. cruciferae* and *P. striolata* discriminate among the hydrolysis products of glucosinolates. Most of the IC tested were attractive to the beetles, whereas the CN tested were only weakly so or not attractive. This difference in the attractiveness of IC and CN is perhaps not surprising; when intact or macerated at room temperature, most crucifers release much more IC than CN, although the relative release rates vary among species (Cole, 1976). The beetles also discriminated among the IC; allyl IC was consistently the most attractive to both flea beetle species. This result contrasts with results for the cabbage stem flea beetle, *Psylliodes chrysocephala* L., which has a strong electroantennogram response to three other IC but not to allyl IC (Blight et al., 1989). The other IC were much less attractive than allyl IC; 2-phenylethyl IC, ethyl ICB, and phenyl IC released at 4 mg/day were not significantly attractive.

In a similar test, the northern false chinch bug also showed strong IC preferences (Pivnick et al., 1991). In this case, ethyl ICB, which does not occur naturally, was the most preferred, although several other IC tested were also attractive. In neither the chinch bug study nor the present one is there an obvious ecological reason for the specificity. Presumably, the most preferred compound or a closely related one is given off by an important present or ancestral host plant.

The pattern of attraction to the baits was similar in both years that the tests were conducted, but there were quantitative differences between years. The relative attraction of allyl IC compared to the other IC was much less in 1988

than in 1987, although allyl IC was the most attractive in both years. Trap saturation did not occur with the numbers caught and could not account for this difference between years. Variation in release rates was also an unlikely explanation for the difference, as can be seen by a comparison of the release rates of ethyl IC, which were similar in the two years, and yet the catches in the traps baited with ethyl IC were relatively much higher in 1988 than in 1987. The release rates of allyl IC in 1988 might have been slightly higher than in 1987 because of the large number of beetles that entered the vials in 1988; a laboratory simulation of this situation showed that the release rate would rise temporarily under these circumstances. However, the dose–response test with allyl IC indicates that an increase in the release rate above 4 mg/day would have increased the attractiveness of the traps baited with allyl IC, not reduced its attractiveness, as required to explain the relatively small difference between allyl IC and ethyl IC in 1988. The relative response of the beetles probably differed in the two years because of differences in the availability of food and their hunger level. In 1987, soil moisture was plentiful and both weedy and crop hosts were abundant, whereas in 1988 there was a severe spring drought and few host plants were available to the beetles. Therefore, the beetles were likely hungrier in 1988 than in 1987. Hungry beetles may have been more likely to respond to the less attractive odors than well-fed beetles (Miller and Strickler, 1984).

Our results do not suggest that both *P. cruciferae* and *P. striolata* are attracted equally to IC, simply that the pattern of attraction is the same. Vincent and Stewart (1984) found that *P. cruciferae* was more strongly attracted to allyl IC than *P. striolata*. We have no way of independently assessing abundance, as our control traps caught very few beetles. This low capture in control traps was surprising, as Vincent and Stewart (1986) found a strong response of the same two species to yellow and white traps in the absence of odors. The low response to our control traps was likely because our traps were old and faded. *P. pusilla*, normally a relatively rare species, was also attracted to allyl IC, but only in the first experiment, which was the only experiment to take place in April.

A release rate of 0.04 mg/day was tested for some compounds in 1988 because our results in 1987 suggested that these compounds may be attractive. Matsumoto (1970) found that some of the less volatile IC were attractive to the vegetable weevil, *Listroderes obliquus* (Klug), at lower rates of release than were some of the other IC. However, his results could also be interpreted to mean that his preparation technique, which involved heating the release substrate to 65°C for 30 min to evaporate solvent, may have eliminated most of the more volatile IC. In any case, we have no evidence of dose–response patterns that differ between IC. Captures in response to all baits at the lower rate were all less than the same baits at 4 mg/day, indicating a pattern similar to the dose–response for allyl IC.

Oriental mustard, *Brassica juncea* (L.) Czern., contains primarily allyl GS and hence releases primarily allyl IC. Measured release rates of allyl IC from *B. juncea* indicate that a trap releasing 4 mg/day of allyl IC is equivalent to the release from 2000 freshly damaged plants or 270,000 intact plants of *B. juncea* at the flower bud stage (Pivnick and Jarvis, 1991). If the plants were younger, even more would be required. Therefore, our results indicate that allyl IC release by individual or small groups of plants normally would not attract many beetles, although release by whole fields certainly would. Canola varieties of *B. napus* L. and *B. rapa* (= *campestris*) L. contain low levels of GS and no allyl GS and hence should release low levels of IC and no allyl IC. The primary mustard oils released by these species, butenyl and pentenyl IC (unpublished data), were not tested but are undoubtedly released in very small quantities. Canola varieties should, therefore, be less attractive than *B. juncea*, although fields of these crops should nonetheless be attractive. Lamb and Palaniswamy (1990) found that intact 2-week-old plants of nine species of crucifers, including *B. juncea*, *B. campestris*, and *B. napus*, were weakly or not at all attractive to *P. striolata*, indicating that long-distance host-plant location of small groups of plants may be largely a random event. Beetles may use IC for host recognition once they are in close proximity to a plant, in which case they would be exposed to greater concentrations of IC than is indicated by release rate measurements from plants. They clearly are not repelled by relatively high concentrations, as evidenced by the flea beetles captured in the vials themselves.

Flea beetles may also use IC to detect sites of damage on a plant, either because of advantages to feeding on weakened plant tissue or to locate conspecifics for group feeding or for mating. *N. niger* and *P. cruciferae* form aggregations that could result from responses to host plant odors released during feeding; they both have very definite IC preferences (Pivnick et al., 1991; this paper).

For purposes of breeding for varietal resistance, it seems unlikely that flea beetle response to IC would be an important consideration in view of the high IC release rates required for attraction. This conclusion is supported by the lack of clear differences in intensity of flea beetle attack between *B. juncea* and the two canola species (Lamb and Palaniswamy, 1990; Palaniswamy and Lamb, 1992), even though *B. juncea* produces much more IC than the other species and is the only one of the three that produces allyl IC, the most attractive IC. However, IC may be important in attraction of flea beetles to fields of *Brassica* crops or in short-range attraction (within centimeters). Of course, glucosinolate (and consequently IC) levels have been and will be manipulated by plant breeders for other reasons; they are important constituents of taste and odor and can affect nutritive qualities (Fenwick et al., 1983). It is also possible that the specific responsiveness to allyl IC may be of use in other ways in the management of flea beetle pests of cruciferous crops.

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EVIDENCE OF AN AGGREGATION PHEROMONE IN THE FLEA BEETLE, *Phyllotreta cruciferae* (GOEZE) (COLEOPTERA: CHRYSOMELIDAE)

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Abstract—Laboratory olfactometer bioassays and field trapping experiments showed that the flea beetle, *Phyllotreta cruciferae* (Goeze), was highly attracted by oilseed rape (*Brassica napus* L.) when flea beetles were on the plant. This attraction was mediated by a flea beetle-produced aggregation pheromone based upon: (1) Oilseed rape damaged mechanically, or by *P. cruciferae*, or by diamondback moth, *Plutella xylostella* (L.), did not attract *P. cruciferae*. (2) Contact with the plants or feeding was required for the production of aggregation pheromone because oilseed rape alone was not attractive when separated from flea beetles by a screen. (3) Equal numbers of males and females were attracted.

Key Words—Aggregation pheromone, olfactometer, field trapping, Coleoptera, Chrysomelidae, *Phyllotreta cruciferae*, *Brassica napus*, crucifer.

INTRODUCTION

The flea beetle, *Phyllotreta cruciferae* (Goeze), feeds primarily on Cruciferae. In the northern Great Plains, it is the most serious pest of spring-planted oilseed rape, *Brassica napus* L. and *B. rapa* L. (Lamb, 1989; Weiss et al., 1991). Damage is caused mainly by the overwintering adult beetles, which feed on the cotyledons and slender stems in the early spring, causing seedling mortality and the crop to mature unevenly (Burgess, 1977; Lamb, 1989).

Our study originated from a field observation that crambe, *Crambe abys-*

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sinica Hochst., sustained significantly less damage by *P. cruciferae* than oilseed rape, *B. napus* L., although these two plant species both belong to the family Cruciferae (Anderson et al., 1992; Peng et al., 1992). The first objective of this study was to test the hypothesis that oilseed rape releases host-finding cues for *P. cruciferae* that are lacking in crambe.

Aggregative feeding behavior has been reported for *P. cruciferae* by Anderson et al. (1992) and Peng et al. (1992). The factors that determine the feeding behavior of *P. cruciferae* have been sought in the chemistry of the host plants themselves (Hicks, 1974; Feeny et al., 1970; Feeny, 1977; Lamb and Palaniswamy, 1990) and virtually nothing is known about the physiology of the beetles that may be relevant to feeding behavior and host-plant location. Therefore, the second objective was to investigate effects of the beetles on host plant location.

Here we present the first account of an aggregation pheromone in this important pest species.

METHODS AND MATERIALS

Laboratory Bioassays

Plants and Insects. Oilseed rape (*B. napus* cv. Westar) and crambe (*C. abyssinica* cv. Meyer) were grown in greenhouse flats using Sunshine Mix #3 as a potting media. Plants were watered as needed and were fertilized the third or fourth day after emergence with 14.5 g/liter 20:19:18 (N/P/K) Peatlite and 0.28 g/liter iron chelate, diluted approximately 1:15. Seedlings were used five to six days after emergence.

Flea beetles were collected from field pennycress, *Thalaspi arvense* L., in the early spring. They were maintained in the greenhouse on horseradish, *Armoracia rusticana* P. Gaertn., B. Mey & Scherb., in vented cages (Burgess and Wiens, 1976) at about 23°C and a photoperiod of 16:8 hr light–dark.

Olfactometer. A two-choice olfactometer (Figure 1) was designed for this study. The olfactometer consisted of a response chamber and two Plexiglas chambers, which are connected with glass tubes. There was an introduction hole on the cover of the response chamber through which beetles were introduced. The response chamber was sealed with sticky foam rubber stripping. Each Plexiglas chamber was divided by a screen into two subchambers: one functioned as a beetle trap and the other as a test chamber in which the test materials were placed. The top and bottom covers of the Plexiglas chambers were removable to facilitate collection of beetles and change of test materials.

Bioassay. All tests were conducted in the laboratory at about 25°C and 30–40% relative humidity between 0900 and 1600 hr (CST) on each date during the summer of 1990 and 1991. Illumination was furnished by two 30-W flu-

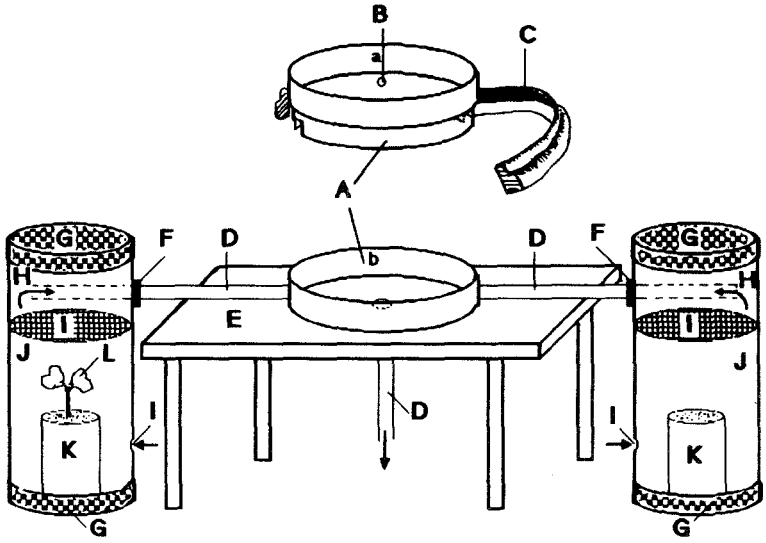

FIG. 1. Two-choice olfactometer; arrows indicate the direction of air flow. A, reaction chamber (10.0 cm inside diameter by 1.6 cm high: a, cover; b, bottom); B, introduction hole (1.0 cm diameter); C, sticky rubber strip; D, glass tube (14.0 cm long × 0.8 cm inside diameter); E, Plexiglas stand; F, rubber; G, plastic cap; H, trap chamber (7.0 cm inside diameter × 4.5 cm high); I, screen (81 mesh/cm^2); J, test chamber (7.0 cm inside diameter × 8.0 cm high); K, glass beaker (40 ml); L, plant.

orescent lamps positioned 1 m above the olfactometer. Air flowed through each glass tube at approximately 300 ml/min.

Timing was begun when about 15 unsexed adult beetles were introduced into the response chamber. After 20 min, the beetles trapped in each chamber and glass tube were counted and removed. The response chamber and two glass tubes were cleaned using 95% ethanol and then dried for about 5 min after each run. Each test treatment was replicated at least eight times. The Plexiglas chamber containing test materials was alternated in consecutive replicates. Blank tests (nothing in the test chambers) were also run.

Three tests were conducted in the summer of 1990. The first test involved a choice between oilseed rape and crambe. Flea beetles were preconditioned for 17 hr in four different ways; beetles were exposed to: (1) oilseed rape; (2) crambe; (3) both plant species; and (4) neither species. Beetles were preconditioned in the growth chamber at 22–24°C, 50–60% relative humidity, and a 16:8-hr light–dark photoperiod (Table 1).

The second test investigated the attractiveness of six odor sources to the flea beetles: intact oilseed rape or crambe, flea beetles, damaged oilseed rape or crambe, and oilseed rape plus beetles (Table 2). A 40-ml glass beaker con-

TABLE 1. ATTRACTION OF PRECONDITIONED FLEA BEETLES TO VOLATILES FROM OILSEED RAPE AND CRAMBE IN A TWO-CHOICE OLFACTOMETER, 1990

Preconditioning	Replicates	*N*	Oilseed rape	Crambe	t^a	*P*
With oilseed rape	12	180	56	64	0.8234	0.4281
With crambe	12	183	54	61	0.6739	0.5143
With both	12	186	61	73	1.1266	0.2839
With neither	8	114	43	39	0.4350	0.6767

[a] Paired *t* tests (SAS Institute, 1987).

TABLE 2. ATTRACTION OF FLEA BEETLES TO VOLATILES OF PLANTS AND BEETLES IN A TWO-CHOICE OLFACTOMETER, 1990

Treatment	Replicates	*N*	Volatiles	Control[a]	t^b	*P*
Intact oilseed rape	8	116	39	31	0.7977	0.4512
Intact crambe	12	145	36	32	0.4690	0.6482
Beetles	12	188	41	50	0.7437	0.4727
Damaged oilseed rape	18	288	61	106	5.6224	0.0001
Damaged crambe	18	165	24	55	2.3112	0.0412
Oilseed rape + beetles	12	166	83	27	5.9696	0.0001

[a] Soil only.
[b] Paired *t* tests (SAS Institute, 1987).

taining soil was used as a control. Eight to 10 seedlings were transferred to a 40-ml glass beaker containing soil, one day before the test. In tests with damaged plants, seedlings were damaged with a pipet tip (2 mm diameter) by crushing the cotyledons in six locations immediately before tests; six new locations were repeated after each three replicates of a bioassay. Tests with flea beetles as an attractant were carried out by releasing about 50 unsexed beetles in the test chamber. Oilseed rape plus beetles were prepared by placing 14 beetles on oilseed rape seedlings in a glass beaker in the test chamber 17 hr before the test. The seedlings and beetles were maintained in the growth chamber described as above. Damage to the cotyledons (i.e., feeding pits) by the beetles was observed before the test.

The third was a choice test conducted with oilseed rape plus beetles versus intact or damaged oilseed rape, respectively. These odor sources were prepared using the method described for the second test.

In 1991, three odor sources were tested in the olfactometer to determine the possible source of attractants: oilseed rape plus beetles, infested oilseed rape

with frass, and infested oilseed rape without frass (Table 3). Test plants were transferred, and a control was prepared in the same way as in the previous tests. Oilseed rape plus beetles was prepared by caging plants and insects in the test chamber immediately before the test, instead of after 17 hr as in the second and third tests of 1990. Infested oilseed rape with frass was prepared by placing a glass beaker containing oilseed rape seedlings and about 50 beetles in a Plexiglas cage (20 × 20 × 30 cm) 17 hr before the test. Beetles were removed from the plants before the test. Frass was observed mostly on the bottom of the cotyledons. Infested oilseed rape without frass was prepared in a similar way. Flea beetles were starved for 24 hr before they were released into the Plexiglas cage and allowed to feed for about 2.5 hr. The feeding that resulted was similar to the damaged area of infested oilseed rape with frass except frass was not observed on the cotyledons.

Field-Trapping Experiment

To measure the attraction of *P. cruciferae* to different combinations of crucifer plants and flea beetles in the field, trapping studies were conducted at Fargo, North Dakota, in the spring of 1991. Flea beetles used in this study were from the same source as in previous tests. Oilseed rape and crambe were grown in 12-cm plastic pots using Sunshine Mix #3 as a potting medium, about 40–50 seedlings per pot, in the greenhouse until they were placed outdoors five to six days after emergence. Plants in the greenhouse were watered and fertilized using the methods described above.

The trap design illustrated in Figure 2 was used to catch *P. cruciferae* adults. Each pot was covered with a fine mesh organdy before test. Therefore volatile chemicals readily passed through the mesh, but field flea beetles were excluded. Each pot was placed on a wooden block in the center of a circular white plastic tray 30 cm diameter and 8 cm deep (Duraco Products, Inc., Streamwood, Illinois). The tray was filled with soapy water to collect flea beetles.

TABLE 3. ATTRACTION OF FLEA BEETLES TO VOLATILES OF PLANTS TREATED IN DIFFERENT WAYS IN A TWO-CHOICE OLFACTOMETER, 1991

Treatment[a]	Replicate	*N*	Volatiles	Control[b]	*t*[c]	*P*
OR + beetles	8	119	51	14	7.0833	0.0002
Infested OR with frass	8	119	55	14	8.0188	0.0001
Infested OR without frass	8	123	19	28	1.6880	0.1353

[a]OR, oilseed rape.
[b]Soil only.
[c]Paired *t* tests (SAS Institute, 1987).

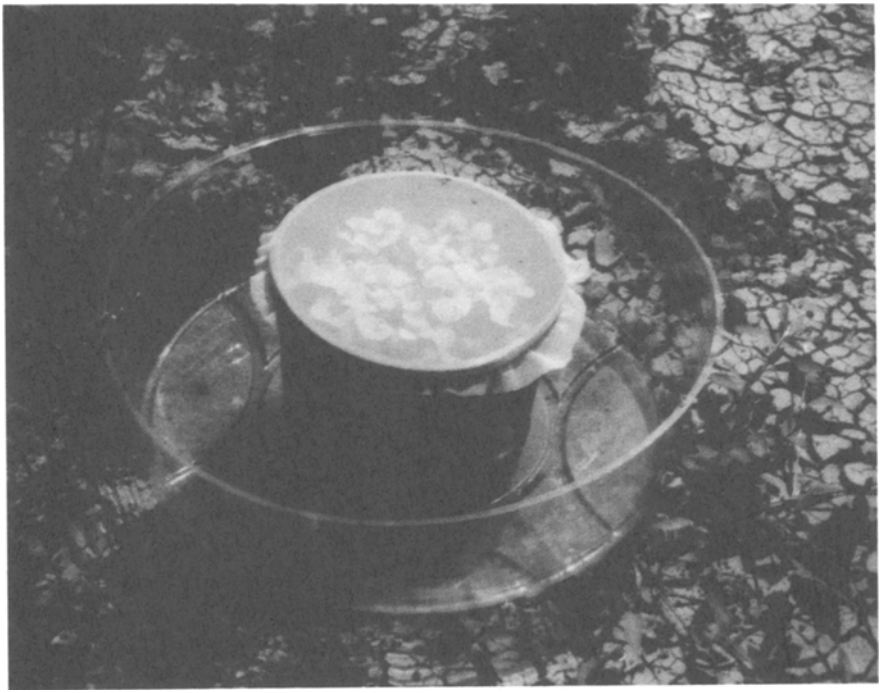

FIG. 2. Trap design for capture of *P. cruciferae*.

Two trapping tests were conducted. Each test consisted of four replications, arranged in a randomized complete block design. The distance between pots in a replicate was about 3 m and about 5 m or more between replicates. Test 1 was conducted May 31 to June 4, 1991, in an area with an array of trees to the east. All traps were positioned about 10 m away from the trees. Six treatments and a control were used: oilseed rape, crambe, beetles, oilseed rape plus beetles with access to the plants, oilseed rape plus beetles without access to the plants, crambe plus beetles with access to the plants, and soil alone (control) (Table 4). Oilseed rape or crambe plus beetles with free access were prepared by releasing approximately 50 beetles into a pot immediately before test. Oilseed rape plus beetles without access was used as a bait by placing a 15-ml vial containing about 50 beetles in a pot. The vial was screened to prevent beetles from feeding on the seedlings present in the pot. In the treatment of beetles, about 50 beetles were released into a pot containing soil only. The second test was conducted June 8–11, 1991, in a field with pennycress, to determine if volatiles released from oilseed rape after damage are attractive to flea beetles. Three treatments and a control were used: oilseed rape plus beetles with access

TABLE 4. MEAN NUMBERS OF ADULT FLEA BEETLES CAUGHT PER TRAP BY TREATMENTS, FROM MAY 31 TO JUNE 4, 1991

Treatment	$\bar{X} \pm$ SD (range)[a]	Sex ratio (M : F)
Oilseed rape (OR)	2.25 ± 1.26(1–4)b	1:0.8
Crambe (CR)	1.25 ± 0.50(1–2)b	1:0.7
Beetles (BE)	1.75 ± 1.26(0–3)b	1:0.2
OR + BE with access to OR	28.25 ± 12.26(12–41)a	1:1
OR + BE without access to OR	3.50 ± 2.38(2–7)b	1:1.3
CR + BE with access to CR	4.75 ± 4.11(0–10)b	1:1.1
Soil only, control	3.25 ± 3.30(1–8)b	1:0.9

[a]Means followed by the same letters are not significantly different [$P = 0.05$, Tukey's studentized range test (SAS Institute, 1987)].

to the plants, damaged oilseed rape, oilseed rape plus diamondback moth larvae with access to the plants, and soil alone (control) (Table 5). Oilseed rape plus beetles with free access and control were prepared in the same way as in the first test. Oilseed rape was damaged with a scissors by making eight 1-cm-long cuts on the cotyledons immediately before test. Eight fresh cuts were added at 1000 hr (CST) daily. In the treatment of oilseed rape plus diamondback moth larvae, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), eight second to third instars were placed in each pot before tests. Diamondback moth larvae were from a colony maintained on crambe in the greenhouse. The beetles caught in the traps were counted and retained for sex determination.

Data Analysis

Results of all olfactometer bioassays were analyzed with a paired t test (SAS Institute, 1987). For the field-trapping experiment, the numbers of *P. cruciferae* caught were analyzed by analysis of variance (ANOVA) and Tukey's studentized range test at the 5% level of significance (SAS Institute, 1987).

RESULTS

Laboratory Bioassays

P. cruciferae did not prefer oilseed rape or crambe, regardless of preconditions (Table 1). Beetles and intact oilseed rape or crambe seedlings themselves were not attractive to *P. cruciferae*. Mechanical damage of the cotyledons on two plant species resulted in significant repellency to *P. cruciferae*. Oilseed rape plus beetles trapped significantly more beetles than the control (Table 2). Attractancy of oilseed rape plants plus beetles was significantly higher than both intact

TABLE 5. MEAN NUMBERS OF ADULT FLEA BEETLES CAUGHT PER TRAP BY TREATMENTS, JUNE 8–11, 1991

Treatment[a]	$\bar{X} \pm$ SD (range)[b]	Sex ratio (M:F)
OR + beetles with access to OR	74.00 ± 58.06(18–154)a	1:1
Damaged OR	1.25 ± 1.89(0–4)b	1:0.7
OR + DBML with access to OR	3.00 ± 0.82(2–4)b	1:1.4
Soil only, control	1.00 ± 0.82(0–2)b	1:3

[a]OR, soilseed rape; DBML, diamondback moth larvae.
[b]Means followed by the same letters are not significantly different [$P = 0.05$, Tukey's studentized range test (SAS Institute, 1987)].

oilseed rape (90 vs. 38, replicate = 12, $N = 174$, $t = 3.7100$, $P = 0.0035$) and damaged oilseed rape (86 vs. 49, replicate = 12, $N = 179$, $t = 3.8442$, $P = 0.0027$).

In the 1991 test (Table 3), oilseed rape and beetles were strongly attractive to *P. cruciferae* when they were put together immediately before tests. Oilseed rape infested by beetles with frass on the cotyledons was significantly attractive to *P. cruciferae*. However, oilseed rape infested by beetles without frass was not attractive.

Field-Trapping Experiment

There were significant differences in numbers of beetles caught by treatments in both tests (test 1: $F = 11.13$, $df = 6, 18$, $P = 0.0001$; test 2: $F = 3.72$, $df = 3, 9$, $P = 0.0385$). Only oilseed rape plus beetles with free access was significantly attractive to *P. cruciferae* among all treatments, and it attracted male and female beetles equally (Tables 4 and 5). Unequal numbers of male and female beetles caught in some of other treatments may be due to minimal catches.

DISCUSSION

Both olfactometer and field-trapping experiments demonstrated that oilseed rape and crambe alone were not attractive to *P. cruciferae*. This result did not support the hypothesis that oilseed rape releases host-finding cues for *P. cruciferae* and crambe lacks the host finding cues. However, oilseed rape plus beetles was strongly attractive to *P. cruciferae*. This attraction was mediated by a flea beetle-produced aggregation pheromone because oilseed rape alone was not attractive to *P. cruciferae*, mechanically damaged oilseed rape or oilseed rape damaged by *P. cruciferae* was not attractive, oilseed rape damaged by

diamondback moth was not attractive, and male and female beetles were caught equally.

The volatiles released from damaged oilseed rape or crambe seedlings were strongly repellent to the flea beetles in olfactometer tests. The cause is yet unknown. Although allyl-isothiocyanate has been shown to be a powerful attractant for *P. cruciferae* (Feeny et al., 1970; Vincent and Stewart 1984), its parent compound, allyl-glucosinolate, was undetectable in the cotyledons of *B. napus* cv. Westar (Bodnaryk and Palaniswamy, 1990). The major glucosinolate in the cotyledons of *B. napus* cv. Westar is glucobrassicin (3-indolylmethyl glucosinolate) (Bodnaryk and Palaniswamy, 1990), which has no volatile hydrolysis products. Allyl-glucosinolate was also not detected in crambe (McGhee et al., 1965).

Feeding or contact with the plants is necessary for the production of aggregation pheromone because use of beetles alone or oilseed rape plus beetles separated by a screen did not attract *P. cruciferae*. Our findings suggest two possible modes of aggregation pheromone production. First, *P. cruciferae* may release pheromone as soon as it has selected a suitable host plant. This is supported by the olfactometer test in which oilseed rape and beetles were attractive when placed together immediately before tests (Table 3). Alternatively, pheromone may be released through frass excreted by beetles on the cotyledons because infested oilseed rape with frass was attractive to *P. cruciferae*, and infested oilseed rape without frass was not attractive (Table 3). These modes of aggregation pheromone production occur in other species of Coleoptera (Birch, 1984; Borden, 1985).

This is the first demonstration of semiochemically induced aggregation in *P. cruciferae*. The aggregation pheromone may play a role in host-plant location because beetles plus crambe (nonhost) were not attractive to *P. cruciferae*. This finding has opened new fields of investigation for a potential improvement of *P. cruciferae* control. Our results have prompted chemical investigations to identify the *P. cruciferae* aggregation pheromone. Synthetic pheromone could be used as a beetle lure to monitor populations and to control them in combination with pesticides.

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FEEDING STIMULATORY AND INHIBITORY CHEMICALS FROM AN ACCEPTABLE NONHOST PLANT FOR *Manduca sexta*: IMPROVED DETECTION BY LARVAE DEPRIVED OF SELECTED CHEMOSENSORY ORGANS

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Abstract—The chemical basis of feeding responses to the acceptable nonhost plant *Vigna sinensis* (cowpea) by larvae of *Manduca sexta* was investigated using chemical isolation techniques directed by a novel chemosensory-based bioassay. The presence of feeding stimulatory and inhibitory compounds in leaves or leaf extracts was determined in a two-choice preference test using leaf disks or glass fiber filter paper disks laced with leaf extract as test substrate and filter paper disks laced with water as control. Larvae strongly prefer the control disks over leaf disks, indicating the presence of feeding inhibitory compounds in the leaf. An ethanol extract of both fresh and dried leaves neither stimulated nor inhibited feeding. The cause of this inactivity was examined by using larvae that respond strongly to either feeding stimulatory or inhibitory compounds due to selective chemosensory deprivation. Larvae having chemosensory organs remaining only on the maxillary palps are stimulated to feed by whole leaf disks and by the ethanol extracts. In contrast, larvae having only the medial and lateral maxillary sensilla styloconica and the epipharyngeal sensilla remaining are strongly inhibited by whole leaf disks and the ethanol extract of fresh leaves. Thus, the ethanol extract contains both feeding stimulatory and inhibitory compounds, which elicit opposite behavioral effects in unoperated larvae, therefore nullifying any stimulatory and inhibitory activity. These compounds can only be demonstrated by using discrimination-enhanced larvae in the choice tests. Further isolation of the feed-

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ing stimulatory principle in *V. sinensis* yielded two separate fractions of neutral compounds, suggesting at least two different chemicals belonging to two different classes: nonpolar and polar lipids. Feeding inhibitory chemicals have apparently polar properties because strong activity was found in the ethyl acetate and methanol extracts of dried leaves. The role of feeding stimulatory and inhibitory compounds in food selection of *M. sexta* larvae is discussed.

Key Words—*Manduca sexta*, Lepidoptera, Sphingidae, food selection, nonhosts, extracts, sensory deprivation, Vigna sinensis.

INTRODUCTION

The oligophagous tobacco hornworm, *Manduca sexta*, confines its food range in nature to selected members of the plant family Solanaceae. Nonhost plant species can be divided in two main groups based on the feeding response by first-instar larvae having no previous contact with food. Unacceptable nonhosts do not stimulate feeding. Acceptable nonhost plant species stimulate feeding and support growth and development (Yamamoto and Flowers, 1982; de Boer and Hanson, 1984). Feeding responses by other instar larvae to these acceptable nonhosts depend on the plant species on which the larvae have been reared. Host-reared larvae are inhibited to feed on acceptable nonhost plant species, while larvae reared on an acceptable nonhost are stimulated to feed on both species (de Boer, 1992). This increased preference found for the rearing plant species relative to the nonrearing plant species is called a diet-induced food preference (de Boer and Hanson, 1984; de Boer, 1992) and also has been demonstrated in several other insect species (cf. Szentesi and Jermy, 1990). Thus, these acceptable nonhost plant species act as nonhosts or to some extent as hosts, depending on the food experience of the larvae. What are the properties of these acceptable nonhost plants that determine their degree of acceptability to *M. sexta* larvae?

Like many phytophagous insects, tobacco hornworm larvae select their food mainly on the basis of its chemical content, particularly feeding stimulatory and inhibitory chemicals. Acceptance or rejection of the food is then based on the balance between sensory inputs that invoke opposite behavioral responses (Dethier, 1982). The chemoreceptor cells involved in food selection by *M. sexta* larvae are located within five different peripheral sensory organs (Schoonhoven, 1987). Each of these chemosensory organs by itself mediates a particular feeding response to a certain plant species (de Boer and Hanson, 1987a, de Boer, 1991). For example, the acceptable nonhost plant cowpea, *Vigna sinensis*, stimulates feeding if sensory input from only the maxillary palps is used for feeding decisions. In contrast, input from the medial and lateral maxillary sensilla styloconica and the epipharyngeal sensilla each mediate inhibition of feeding, and input from the antennae results in neither stimulation nor inhibition. These results

strongly suggest that *V. sinensis* contains both feeding stimulatory and inhibitory chemicals. What are these phytochemicals controlling acceptance of *V. sinensis*?

In this paper we report the isolation of chemicals from *V. sinensis* affecting acceptance of this plant by *M. sexta* larvae. In addition to using chemical fractionation methods, a new technique was developed to distinguish between inactive fractions due to the absence of an active principle and "inactive" fractions due to the presence of both stimulatory and inhibitory chemicals, each counteracting the behavioral effect of the other. During screening for active fractions larvae were used whose detection of either feeding stimulatory or inhibitory chemicals was enhanced by removing selected chemosensory organs. The results show that extracts that are "inactive" for unoperated larvae are active for discrimination-enhanced larvae. Furthermore, some feeding stimulatory compounds in *V. sinensis* belong to the group of nonpolar lipids and others to the group of polar lipids.

METHODS AND MATERIALS

Insects and Plants. Larvae of the tobacco hornworm, *M. sexta* (Lepidoptera: Sphingidae), were reared from eggs obtained from the U.S.D.A. (Beltsville, Maryland). Upon hatching, they were fed foliage of tomato, *Lycopersicon esculentum*. Leaves of the acceptable nonhost plant cowpea, *V. sinensis*, were used as substrates in two-choice food preference tests. All plants were grown in the university glasshouse under long day conditions and kept pest free without using pesticides. Rearing and test conditions of food preference experiments were 16:8 hr light–dark at 28 ± 1°C.

Bioassay. The two-choice test of de Boer and Hanson (1988) was used to assay food preferences of larvae. Three test and control disks of glass fiber filter paper (Whatman GF/A, 14 mm in diameter) were placed in ABAB . . . order around the circumference of transparent containers (10 cm in diameter). The floor of the containers was a layer of paraffin wax into which were inserted pins holding the disks ca. 5 mm above the substrate. Small retainers (3 mm in diameter) punched from acetate sheets (or Teflon when diethyl ether or methylene chloride was used as solvent) kept the disks from sliding down the pin. A wire screen covered a moist piece of filter paper on the floor of the container. A freshly molted fifth-instar larva, deprived of food for 12–24 hr after the molt, was placed in the center of the container. The containers were covered with transparent lids to maintain high humidity. When the larva had eaten approximately 50% of the total area of either test or control disks (time: T_{50}), the larva was removed and the area consumed of each disk was estimated visually. Larvae were tested only once, then discarded. The tests were repeated, usually with 15 or more larvae. The mean area eaten of each substrate (A or B) was expressed

as the percentage of the total area of all disks consumed (e.g., A/A + B) at T_{50}. This value (range 0–100) was taken as a measure of food preference. No effect of the above-mentioned levels of starvation upon food choices was shown in studies using similar choice tests (de Boer and Hanson, 1987a).

The test disks were laced with 0.05 ml of the test solution, which is enough to wet each disk completely. The control disks received 0.05 ml of solvent only. After evaporation of the organic solvent from test and control disks, each disk was wetted with 0.05 ml of distilled water because *Manduca* does not eat dry disks. Every extract and fraction obtained during the isolation procedures was tested in the bioassay at approximately natural concentrations; i.e., the concentration was adjusted by redissolving the dried material in solvent such that 0.05 ml would contain the chemicals from an area of leaf containing 0.05 ml water.

Data were accumulated in a balanced design for experiments within treatment groups as much as possible. The differences in food choices for the test substrates are expressed in a choice index (range -100 to $+100$), which measures the mean percent of total consumption of substrate A minus that of substrate B. Since such consumption scores are not normally distributed, nonparametric statistics were used; a two-tailed Mann-Whitney test was selected for the analysis of the raw scores to determine the significance of difference in food choices.

Sensory Ablation. To screen more effectively for plant chemicals affecting food selection, discrimination-enhanced larvae were tested. Using the microsurgical procedures of de Boer and Hanson (1987a), larvae were deprived of those sensory organs that were shown to differentially mediate stimulation or inhibition of feeding on *V. sinensis* (de Boer, 1991). After immobilizing larvae on melting ice for about 20–45 min, the antennae and the maxillary palps were removed using microdissection scissors to yield larvae having only the medial and lateral maxillary sensilla styloconica, and the epipharyngeal sensilla remaining (MLE larvae). Similarly, the antennae, the medial and lateral maxillary sensilla styloconica, and the epipharyngeal sensilla were removed to produce larvae having only the maxillary palps remaining (MP larvae). Extirpations were performed in the middle of the fourth stadium, except for ablations of the medial and lateral maxillary sensilla styloconica, which were made in fifth stadium within 12 hr of the final larval molt. Operated fourth-instar larvae recovered and fed on leaves until the final larval molt. Ablated larvae appeared to feed normally. The remaining chemosensory organs were examined under the dissecting microscope; animals having unsuccessful operations were withdrawn from further consideration.

Extraction and Isolation. Fresh whole leaves of cowpea (193 g) were soaked in 2 liters of cold ethanol for 24 hr at room temperature. Evaporation of the extract below 40°C yielded 4.1 g of dried material, which was stored at 5°C in the dark. In addition, 20 g of cowpea leaves dried at room temperature was

cut in small pieces and extracted with 300 ml of cold ethanol for 24 hr. This extract was stripped of solvent at room temperature and yielded 0.8 g of dry material. All dried extracts were redissolved in ethanol before testing in the bioassay. The plant residue remaining from the cold ethanol extract of dried cowpea leaves was reextracted with hot ethanol. Further isolation of plant chemicals was done by extraction using another 20 g of air-dried powered cowpea leaves in a glass column using an eluotropic series of solvents (hexane, diethyl ether, methylene chloride, ethyl acetate, and methanol). This sequential extraction leaches from the leaves compounds that dissolve in solvents of increasing polarity. The above extraction methods assume complete extraction within the limits of the technique. All extracts were dried and stored at 5°C in the dark.

Additional chemical separation was accomplished by fractionation of the diethyl ether extract into subclasses of grossly related chemicals using bulk transfer methods (Mitscher et al., 1987). The scheme shown in Figure 1 is widely used to separate natural product mixtures. The diethyl ether extract (called extract 1) is partitioned between 5% hydrochloric acid and chloroform to produce extract 2 (chloroform layer contains neutral substances and acids) and extract 3 (aqueous phase contains alkaloids and water-soluble materials such as sugars, amino acids, and the like). Extract 2 is further fractionated by extraction with 5% sodium hydroxide to produce extract 4 [chloroform layer contains

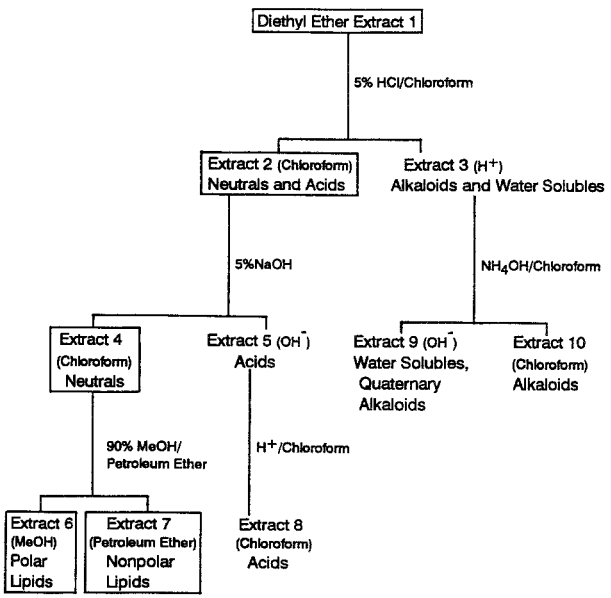

FIG. 1. Bioassay-directed fractionation of *V. sinensis* extracts. Extracts in boxes contain feeding stimulatory activity ($P > 0.05$, $N = 15$ or more).

neutral (neither acidic nor basic) compounds] and extract 5 (basic phase contains acids). Extract 4 is further fractionated by partitioning between 90% methanol and petroleum ether to yield extract 6 (methanol layer contains polar lipids) and extract 7 (petroleum ether phase contains nonpolar lipids). Extract 5 is partitioned between dilute aqueous acid and chloroform to produce extract 8 (chloroform phase contains acids). Extract 3 was partitioned between ammonia and chloroform to yield extract 9 (aqueous phase contains water solubles and quaternary alkaloids) and extract 10 (chloroform layer contains alkaloids).

RESULTS

Larvae significantly preferred the control disks over leaf disks of *V. sinensis* (Table 1: unoperated larvae; choice index −64), suggesting feeding inhibitory chemicals in the leaves. Several extraction methods were used to obtain an active extract from leaves of *V. sinensis*. Larvae consumed equal amounts from disks laced with an ethanol extract of fresh cowpea leaves or no extract, indicating no significant preference for either substrate. (Table 1: unoperated larvae; fresh leaves, choice index −6). Similar results were found with an ethanol extract of dried leaves of *V. sinensis* (Table 1: unoperated larvae; dried leaves, choice index −6). The question arises whether feeding activity remained in the plant residue after cold ethanol extraction. Therefore, this plant residue was reex-

TABLE 1. FEEDING ACTIVITY OF *M. sexta* LARVAE ELICITED BY FRESH LEAVES AND ETHANOL EXTRACTS FROM LEAVES OF ACCEPTABLE NONHOST *V. sinensis*

	Feeding activity[a]		
Vigna sinensis	Unoperated larvae	MP larvae[b]	MLE larvae[c]
Fresh leaves			
Intact	−64***	+56***	−86***
Ethanol extract	−6ns	+34***	−64***
Dried leaves			
Ethanol extract	−6ns	+28**	+6ns
Plant residue	+6ns		

[a] Activity is expressed in a choice index, which is the difference between the mean percent of total consumption of the test substrate (disks with extract) and that of the control substrate (disks with water). Feeding stimulation (+) is indicated by more feeding from the test disks than from the control disks. Feeding inhibition (−) is activity opposite that of feeding stimulation. ***$P < 0.001$; **$P < 0.01$; ns, not significantly different from control (Mann-Whitney test: $P > 0.05$).

[b] Larvae having only their maxillary palps remaining after microsurgery.

[c] Larvae having only the medial and lateral maxillary sensilla styloconica and the epipharyngeal sensilla remaining.

tracted with hot ethanol and tested for feeding activity. However, consumption of the test disks with hot ethanol extract of the plant residue did not differ significantly from consumption of the control disks (Table 1: unoperated larvae; plant residue, choice index +6). Thus, feeding inhibitory chemicals in the ethanol extracts could not be demonstrated.

Perhaps, the above absence of feeding inhibitory activity is due to a loss of a volatile active principle. However, if odors of *V. sinensis* inhibit feeding, then larvae may need to consume very little (bites) or no plant material due to the noncontact mode of olfactory detection. This is not supported by the amount of leaf material consumed by the larvae (18% of total material consumed is from *V. sinensis*). Perhaps the isolates contain both feeding stimulatory and inhibitory compounds. Detection of both groups of chemicals may result in neither feeding stimulation nor inhibition. To distinguish between extracts void of compounds affecting food selection and extracts containing both stimulatory and inhibitory chemicals, we used discrimination-enhanced larvae that were made more selective to either group of chemicals by chemosensory ablations (MP larvae and MLE larvae). Results of a previous study (de Boer, 1991) showed that larvae having only the maxillary palps remaining are stimulated to feed on *V. sinensis* leaves (Table 1: MP larvae, fresh leaves, choice index +56) and larvae having only the medial and lateral maxillary sensilla styloconica and the epipharyngeal sensilla remaining are inhibited from feeding (Table 1: MLE larvae, fresh leaves, choice index −86). Thus, fresh leaves contain both feeding stimulatory and inhibitory chemicals. When MP larvae were used to test the ethanol extract of fresh and dried leaves of *V. sinensis*, a significant stimulation of feeding was found (Table 1: choice indices +34 and +28, respectively, for MP-larvae). In contrast, MLE larvae were strongly inhibited from feeding on the ethanol extract of fresh leaves (choice index −64). However, the ethanol extract of dried leaves did not significantly affect food choice. Thus, an ethanol extract of fresh leaves seems to contain both feeding stimulatory and inhibitory chemicals. On the other hand, the ethanol extract of dried leaves contains mainly or perhaps exclusively feeding stimulatory compounds. Therefore, the dried leaves were used to further isolate the feeding stimulatory principle.

Dried-leaf material was extracted sequentially by solvents of increasing polarity: hexane, diethyl ether, methylene chloride, ethyl acetate, and methanol. Larvae were used that have the same complement of chemosensory organs as those in the above experiments. The results show that unoperated larvae are significantly stimulated to feed by the diethyl ether and the methanol extract (Table 2: unoperated larvae, choice indices; +18). MP larvae were stimulated to feed by all extracts (Table 2: MP larvae). MLE larvae were stimulated to feed by the hexane extract of dried leaves (Table 2: MLE larvae, choice index +10). In contrast, these larvae are significantly inhibited from feeding by the ethyl acetate and methanol extracts (Table 2: MLE larvae, choice indices −24

TABLE 2. FEEDING ACTIVITY OF *M. sexta* LARVAE ELICITED BY DIFFERENT EXTRACTS FROM LEAVES OF ACCEPTABLE NONHOST *V. sinensis*

Extraction solvent	Feeding activity[a]		
	Unoperated larvae	MP larvae[b]	MLE larvae[c]
Hexane	−4ns	+14*	+10*
Diethyl ether	+18**	+26**	−8ns
Methylene chloride	+8ns	+14*	−8ns
Ethyl acetate	−14ns	+54***	−24*
Methanol	+18**	+42***	−70***

[a] Activity is expressed in a choice index which is the difference between the mean percent of total consumption of the test substrate (disks with extract) and that of the control substrate (disks with water). Feeding stimulation (+) is indicated by more feeding from the test disks than from the control disks. Feeding inhibition (−) is activity opposite that of feeding stimulation. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, not significantly different from control (Mann-Whitney test: $P > 0.05$).

[b] Larvae having only their maxillary palps remaining after microsurgery.

[c] Larvae having only the medial and lateral maxillary sensilla styloconica and the epipharyngeal sensilla remaining.

and −70, respectively). The diethyl ether extract stimulates, but does not inhibit, feeding, suggesting a relatively pure extract of feeding stimulatory compounds. This extract was used in the continued isolation of feeding stimulatory chemicals from *V. sinensis*.

Fractionation by bulk transfer starting with the diethyl ether extract (extract 1 in Figure 1) and using MP larvae resulted in stimulatory activity in extracts 2, 4, 6, and 7. Fractions 6 and 7 were repartitioned between 90% methanol and petroleum ether, and again both layers contained feeding stimulatory activity. No activity was found in extracts 8 and 9. Extract 10 did not yield any material. The activity of extracts 6 and 7 must be due to chemicals that belong to the classes of polar and nonpolar lipids, respectively.

DISCUSSION

Although larvae of *M. sexta* are inhibited to feed on leaves of *V. sinensis*, feeding inhibitory chemicals in ethanol extracts or in the plant residue could not be demonstrated (Table 1). This may be due to the absence of a volatile principle lost during the extraction process. However, plant odors seem to play a small, if any, role in the strong inhibition to feed on *V. sinensis*, because most larvae take more than a couple of bites from this plant before they stop feeding. Perhaps, the chemical content of the actual leaf sap sampled by the larvae differs

from the extracts due to mixing all pools of plant chemicals during extraction. Other plant factors also may contribute to the deterrency of this plant species, such as leaf texture. Another possibility is that the isolates contain both feeding inhibitory and stimulatory chemicals, resulting in neither stimulation nor inhibition of feeding. In the latter case, an alternative method to detect feeding activity in "inactive" isolates is needed to understand the chemical basis of feeding responses to *V. sinensis* by *M. sexta* larvae.

In this study a new tool to screen for compounds controlling feeding was developed. Larvae deprived of certain chemosensory organs are used to detect either feeding stimulatory or inhibitory chemicals from *V. sinensis*. This tool became available through detailed studies on the chemosensory basis of feeding responses to *V. sinensis* by *M. sexta* (de Boer, 1991). Extracts inactive on unoperated larvae are now active on larvae having only certain chemosensory organs remaining (Tables 1 and 2). The ability to detect feeding activity using these discrimination-enhanced larvae made it possible to continue the isolation of plant chemicals controlling food choice.

Feeding stimulatory activity was found in extracts that vary in content from mostly nonpolar to mostly polar compounds (Table 2: MP larvae). This suggests that the feeding stimulatory principle in *V. sinensis* may contain compounds with polar and nonpolar properties. The polar extracts may contain the commonly found plant chemicals sucrose and inositol, which have been shown to stimulate feeding in the tobacco hornworm (Städler and Hanson, 1978). The nonpolar extracts and two subfractions from the fraction containing neutral compounds stimulated feeding (Figure 1). These results suggest the presence of at least two different chemicals: one belonging to the class of nonpolar lipids, such as waxes and fats, and the other to the class of polar lipids, such as terpenes and sterols. These findings support and extend the results of de Boer and Hanson (1988), which showed that feeding in tobacco hornworm larvae was stimulated by a leaf surface extract from *V. sinensis* containing neutral compounds. Interestingly, preliminary studies on the isolation of plant chemicals controlling feeding on the host *L. esculentum* also showed that extracts with neutral compounds stimulate feeding (de Boer and Hanson, 1988). Thus, at this level of determination of plant chemicals controlling feeding by *M. sexta*, some similarities are seen between the host *L. esculentum* and the acceptable nonhost *V. sinensis*. Perhaps, the neutral compounds in these two plant species are chemically similar and play the same role in the acceptance of both plant species. Further isolation and identification of these neutral compounds is needed to determine their role in food selection by *M. sexta*.

Feeding inhibitory chemicals in *V. sinensis* must have rather polar properties because inhibitory activity was found in the ethyl acetate and methanol fractions (Table 2). The inhibitory compounds in the methanol fraction deter feeding very strongly (choice index -70 out of a maximum of -100). The

ethyl acetate and methanol fractions also contain feeding stimulatory compounds detected by MP larvae. Therefore, MLE larvae are required in the bioassay for further isolation of feeding inhibitory chemicals from *V. sinensis*.

Larvae detect both feeding stimulatory and inhibitory compounds in extracts of *V. sinensis*. Preliminary isolation studies using *L. esculentum* also yielded fractions containing feeding stimulatory and inhibitory compounds (de Boer and Hanson, 1988). Thus, decisions to feed on an acceptable nonhost as well as a host plant appears to be largely determined by the balance between chemosensory inputs eliciting feeding preference and deterrent responses. This does not support the assumption of Wrubel and Bernays (1990) that maintenance of the restricted host range in *M. sexta* larvae does not rely on avoidance of nonhosts due to feeding deterrence but on attraction and stimulation to feed on hosts. Wrubel and Bernays' conclusion is based on the finding that tobacco hornworm larvae are relatively insensitive to a variety of plant secondary compounds known to occur in some nonhosts compared to other phytophagous insect species tested. Perhaps, nonhosts are less acceptable than hosts because they contain more effective feeding inhibitory and/or less effective stimulatory chemicals than do hosts.

It is important to note that the refinement of the bioassay using MP and MLE larvae is only effective when studying feeding responses to *V. sinensis*. Other plant species may require a different combination of chemosensory organs to mediate feeding responses as was shown by de Boer and Hanson (1987a). In addition, the above two groups of larvae are not exclusively selective for feeding stimulatory or inhibitory compounds. This is shown in Table 2 where feeding of MLE larvae is stimulated by the hexane extract and inhibited by the ethyl acetate and methanol extracts. The chemosensory organs remaining after ablation contain many receptor cells, each with their own selectivity. Indeed, some receptor cells in the maxillary sensilla styloconica have been shown to respond best to feeding stimulatory compounds, while others in the same sensillum respond most strongly to feeding inhibitory compounds (Schoonhoven, 1972). Thus, although the use of discrimination-enhanced larvae can redirect an otherwise halted bioassay-guided isolation procedure, it may not prevent the recurrence of an "inactive" fraction. The latter could be due to chemicals acting on different receptor cells within the same chemosensory organ and eliciting opposite behavioral effects. However, the use of larvae having selected chemosensory organs remaining may be sufficient to evaluate the "inactivity" of a certain fraction, after which unoperated larvae may be used again to complete the isolation process.

The use of discrimination-enhanced larvae in this chemical isolation study underscores the importance of understanding the chemosensory basis of food selection by insects. Moreover, once the particular chemosensory organs and their receptor cells have been identified as the critical mediators for feeding

responses to a certain plant extract, isolation procedures can be directed by measuring receptor cell activity using electrophysiological techniques. Such a screening method promises to be less time consuming and more sensitive than behavioral methods due to the very small amounts of material needed for neural stimulation.

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PHENYLACETIC ACID AS A PHYTOTOXIC COMPOUND OF CORN POLLEN

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Abstract—Phenylacetic acid (PAA), one of the phytotoxic compounds in corn (*Zea mays*) pollen, was identified by GC-MS and by direct comparison with a pure commercial sample of PAA. Bioassays were carried out by testing whole pollen, methylene chloride extract of the pollen, and pure PAA on germination and radical growth of *Amaranthus leucocarpus* and *Echinochloa crusgalli*. The effect of corn pollen was compared with that of *Zea mexicana* (Teosinte), one of the wild relatives of cultivated maize.

Key Words—Pollen, corn, *Zea mays*, teosinte, *Zea mexicana*, allelopathy, phenylacetic acid, phytotoxicity, *Amaranthus leucocarpus*, *Echinochloa crus-galli*.

INTRODUCTION

It has been confirmed that pollen of different species have different growth requirements and contain different amounts of growth hormones. In general, six classes of plant growth hormones have been found in pollen and can potentially

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influence their growth: auxins, gibberellins, kinins, brassins, ethylene, and inhibitors (Stanley and Linskens, 1974).

Some evidence exists on the allelopathic effects of pollen (Kanchan, 1975). Kanchan and Jayachandra (1980) reported that the fruit yield by *Crotalaria pellida* and *Desmodium heterocarpon* was poor when *Parthenium hysterophorus* pollen naturally spread on the leaves and stigmas of the former species. Under culture conditions, germination and pollen tube growth of the test species were inhibited in the presence of *Parthenium* pollen or its extracts. Pollen grains deposited on leaves in large quantities also reduced the chlorophyll content in these target species. Thomson et al. (1982) found that *Diervilla lonicera* (Caprifoliaceae) fecundity was strongly depressed when pollen from *Hieracium floribundum* (Compositae) mixed with *Diervilla* pollen was applied to their stigmas. Murphy and Aarsen (1989) found that the pollen of *Phleum pratense* had allelopathic effects on the pollen germination of 16 sympatric target species.

Jiménez-Osornio and Schultz (1981), in a study of relationships between weeds and cultivated plants in a traditional agroecosystem (chinampa) in the Valley of Mexico, found that weed growth decreased in the middle of the crop cycle when maize was mature and flowering. Jiménez-Osornio et al. (1983) confirmed that a strong inhibitory effect on the radicle growth of *Cassia jalapensis* was observed when corn pollen was sprinkled on its seeds. In relation with this, some peasants of the Valley of Mexico (temperate climate), Tabasco, and Yucatan (tropical climates) assert that the fruiting of squash, bottle gourd, and watermelon is reduced, and also some leaves of beans are "burnt", when corn pollen falls over them (Anaya et al., 1987, 1992). Cruz-Ortega et al. (1988) tested the effects of an ethanolic extract of corn pollen on growth, respiration, and cell division of watermelon and showed the mechanisms of action of the phytotoxic substances of corn pollen.

The chemical analysis of corn pollen has shown various organic compounds to be present, such as: carbohydrates, proteins, lipids, phytosterols, saturate hydrocarbon, phosphatide, and vitamins (Anderson, 1923; Nilsson et al., 1957; Kvanta, 1968; Stanley and Linskens, 1974). Some brassinosteroids (castasterone, typhasterol, and teasterone) found in corn pollen have been associated with stimulating effects (Maugh, 1981; Suzuki et al., 1986). Other compounds reported from corn pollen are the flavonoids quercetin, isorhamnetin, kaempferol, and β-carotene (Katsumata et al., 1974; Ceska and Styles, 1984).

As part of a research program directed towards the isolation of active compounds from Mexican plants, this work describes the identification of one of the bioactive principles from corn pollen (*Zea mays* L., Gramineae) and the results of different bioassays on germination and radicle growth of *Amaranthus leucocarpus* Wats (Amaranthaceae) and *Echinochloa crusgalli* (L.) Beauv (Gramineae).

METHODS AND MATERIALS

General Experimental Methods. GC was carried out on a Hewlett Packard-5890; a methyl silicon 5-m × 0.53-mm column, a temperature program from 150°C to 270°C, and 5°C/min heating speed, using hydrogen as a carrier gas with a 5 ml/min flux. GC-MS was carried out on a Hewlett Packard-5985 B, GC-MS system. The capillary column used in GC was a 30-m × 0.32-mm Bonden FSOT RSL-400. 3UM (OV-210, QF-1, DB-210) of trifluoropropylsiloxane, using helium as a carrier gas and a temperature program of 100°C to 240°C at a rate of 5°/min (EI^+, 70 eV).

One of the preparative TLC fractions (see below), where PAA seems to be present, and a commercial sample of PAA were methylated with CH_2N_2, and a GC-MS analysis of the methylated products was performed. The GC conditions were: 5% HP phenyl methyl silicone 25 m × 0.32 mm × 0.52 μm film capillary column, helium as a carrier gas, and a temperature program of 50°C to 240°C at a rate of 10°/min.

Plant Material. Corn pollen was collected from mature male inflorescences in a field monoculture of corn at Mixquiahuala, Hidalgo, in the months of July and August 1989. The material was dried at room temperature, cleaned, and sieved. A small sample of teosinte pollen [*Zea mexicana* (L.) Schrad] was collected from mature male inflorescences at Ciudad Universitaria, UNAM campus, in August 1989.

Bioassay-Guided Isolation Procedures. Crude extracts of pollen were obtained by macerating at room temperature the dried pollen (450 g) in CH_2Cl_2 (225 ml) for 48 hr. The dried extract was tested for allelopathic activity by the seed germination and radicle growth assay method on *Amaranthus leucocarpus* and *Echinochloa crusgalli* (Anaya et al., 1990).

A preparative TLC (Merck 60F-254) of the CH_2Cl_2 extract was performed using hexane–EtOAc (1:1) as eluent. The bands were visualized under UV light. All the fractions obtained were assayed for allelopathic effects.

General Bioassay Procedure. Whole corn pollen, crude CH_2Cl_2 extract from the pollen, chromatographic fractions of the crude extract, and commercial PAA, all at different quantities and concentrations, were tested on the seeds of *Amaranthus leucocarpus* and *Echinochloa crusgalli*. The bioassays were carried out in Petri dishes containing filter paper or agar (1.5%) as a substrate, as described by Anaya et al. (1990). A complete random block design with four repetitions per treatment was used. The bioassays were carried out using 40 seeds per treatment, at 27°C in the dark during 24 hr for *Amaranthus* and 48 hr for *Echinochloa*. After this time, the percentage of germination and radicle length were measured. All the data were statistically analyzed by ANOVA (Ostle, 1963).

A bioassay comparing the effect of sprinkled whole pollen of corn and teosinte on the radicle growth of *Amaranthus* was carried out in the same manner as described in the case of pollen extracts and their fractions.

RESULTS AND DISCUSSION

Chemical Analysis. The preparative TLC of the crude CH_2Cl_2 extract of the corn pollen showed six bands at R_f 0.05 (fraction 1), 0.11 (fraction 2), 0.22 (fraction 3), 0.28 (fraction 4), 0.51 (fraction 5), and 0.64 (fraction 6), respectively.

The GC analysis of the fraction 3 (Figure 1A) showed a major peak (approximately 10% of the fraction) with a R_T of 1 min. The EI-MS of this peak produced a molecular ion at 136, and a base peak of m/z at 91 (Figure 1B), which confirmed the presence of PAA in this fraction. The identification

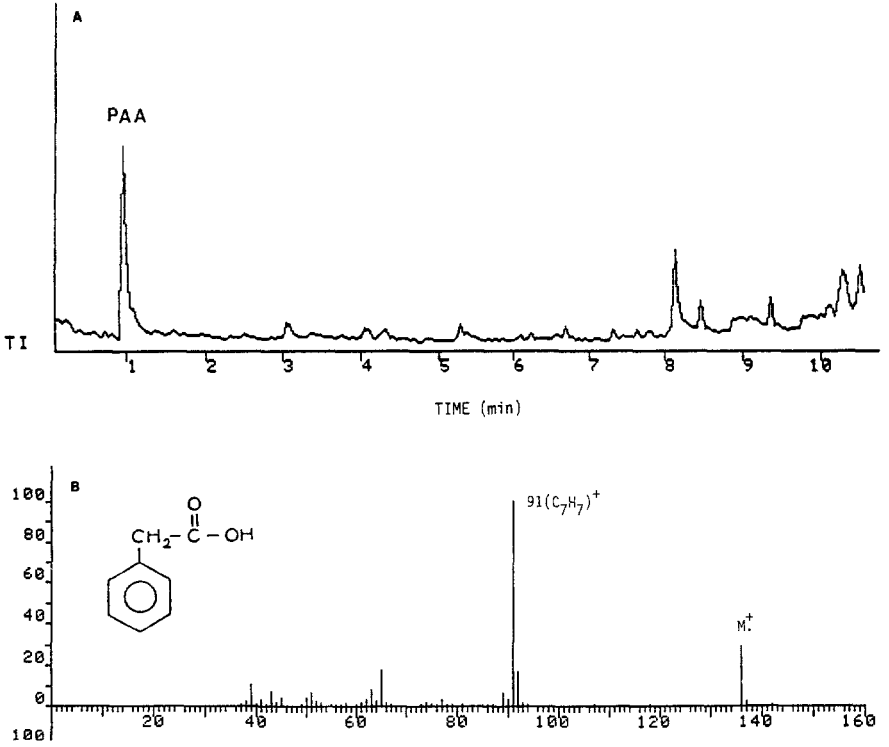

FIG. 1. (A) GC of fraction 3 showing PAA peak. (B) EI-MS spectrum of isolated phenylacetic acid (PAA) from fraction 3.

was further corroborated by comparing it with a commercial sample of PAA. GC-MS analysis of the PAA methyl ester from fraction 3 also was in agreement with the GC-MS data of the methyl ester of commercial PAA.

Biological Analysis. The results obtained from bioassays with the sprinkled corn pollen on germination and radicle growth of *Amaranthus* and *Echinochloa* are shown in Table 1. The radicle growth of both species was significantly affected by the pollen.

The results of the bioassays with the CH_2Cl_2 extract of corn pollen at three concentrations, and the chromatographic fractions of this extract at 100 ppm on the germination and radicle growth of *Amaranthus* are shown in Table 2. The CH_2Cl_2 crude extract caused a significant inhibition at all concentrations tested. On the other hand, almost all the TLC fractions of the CH_2Cl_2 extract (except fraction 6) produced a significant reduction of radicle growth of *Amaranthus*. However, fraction 5 produced a stimulatory effect (28%) on radicle growth. Fraction 3 was the most inhibitory on germination and radicle growth of *Amaranthus*.

Figure 2 shows the comparative effect of fraction 3 and PAA at two concentrations on the radicle growth of *Amaranthus* and *Echinochloa*. This fraction showed the highest inhibition activity on *Amaranthus*. On the other hand, PAA showed the highest inhibition activity on *Echinochloa*. These differences are probably due to the presence of other compounds, in addition to PAA, in fraction 3. The effects of sprinkled pollen of corn and teosinte on the radicle growth of *Amaranthus* are shown in Figure 3. The results confirmed that teosinte pollen has no effect on this test species.

In general, we observed that the percentage of germination was less affected than radicle length in all treatments. This was true of both test species.

Our results indicate that PAA content in corn pollen contributes to the

TABLE 1. EFFECT OF SPRINKLED CORN POLLEN ON GERMINATION AND GROWTH OF *Amaranthus leucocarpus* AND *Echinochloa crusgalli*

	Amaranthus			*Echinochloa*		
Treatments	Germination (%)	Radicle length (mm, $\bar{X}$ of 4 repetitions)	Growth (%)	Germination (%)	Radicle length (mm, $\bar{X}$ of 4 repetitions)	Growth (%)
Sprinkled pollen						
30 mg/10 seeds	72	1.9 ± 0.33	16.4[a]	75	5.1 ± 1.04	27.6[a]
50 mg/10 seeds	89	1.2 ± 0.23	10.4[a]	63	2.0 ± 0.29	10.8[a]
Control	100	11.6 ± 0.83	100	100	18.5 ± 4.46	100

[a] $P < 0.001$.

TABLE 2. EFFECTS OF METHYLENE CHLORIDE EXTRACT AND CHROMATOGRAPHIC FRACTIONS OF CORN POLLEN ON GERMINATION AND RADICLE GROWTH OF *Amaranthus leucocarpus*

Treatment	Germination (%)	Radicle length (mm, $\bar{X}$ of 4 repetitions)	Growth (%)
Methylene chloride extract			
50 ppm	87.5	10.77 ± 2.08	64[a]
100 ppm	80	11.73 ± 2.15	70[a]
200 ppm	90	6.2 ± 0.47	37[a]
Control	100	16.8 ± 0.72	100
TLC fractions of CH_2Cl_2 extract (100 ppm)			
1 R_f 0.05	80	4.2 ± 0.2	44.2[a]
2 R_f 0.11	82	4.1 ± 0.46	43.2[a]
3 R_f 0.22	76.6	2.2 ± 0.56	23.2[a]
4 R_f 0.28	96.6	5.3 ± 0.35	55.8[a]
5 R_f 0.51	90	12.2 ± 1.47	128.4[a]
6 R_f 0.64	90	11.4 ± 1.57	120
Control	100	9.5 ± 0.06	100

[a] $P < 0.01$.

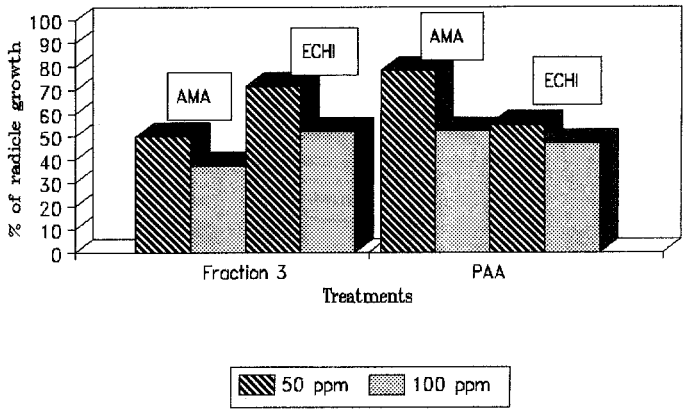

FIG. 2. Effects of fraction 3 from the TLC purification of the CH_2Cl_2 extract of corn pollen and PAA on the radicle growth of *Amaranthus leucocarpus* and *Echinochloa crusgalli*.

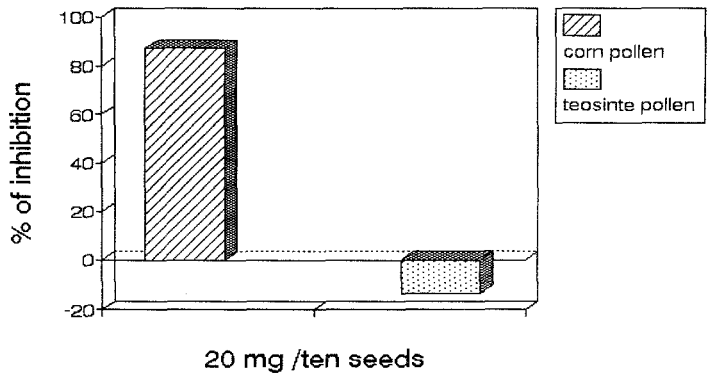

FIG. 3. Effects of sprinkled pollen of corn and teosinte on the radicle growth of *Amaranthus leucocarpus*.

allelopathic effects observed. PAA is a naturally occurring substance in a variety of biological systems. Wightman and Lighty (1982) have demonstrated that PAA fulfills the definition of a nonindolylic auxin and assumed that the amount of PAA present in the shoots of a range of crop plants varies between 1 and 3 $\times$ 10^{-6} M and suggested that the endogenous levels of PAA and IAA are considerably higher in rapidly growing regions. In the case of corn, the PAA content could be higher in pollen.

PAA has been shown to occur in the alga *Undaria pinnatifida*, and it appears to act as an auxin in the red alga *Porphyra tenera* (Abe et al., 1974). Nair and Burke (1988) characterized PAA from *Aspergillus niger* and reported that PAA inhibited the germination of cress and lettuce seeds and also showed antifungal activity against *Cladosporium herbarum*. Chou and Patrick (1976) reported that PAA and other compounds produced during decomposition of corn and rye residues in soil were highly inhibitory to the growth of lettuce.

PAA has been isolated in addition to myrmicacin, IAA, and other acids from metathoracic glands of the leaf-cutting ants *Atta sexdens* from South America. These substances are reported to be used by ants to prevent sprouting of seeds, intrusion of spores, and growth of bacteria in their fungal gardens (Schildknecht and Koob, 1971). Rasmussen et al. (1990) found PAA in temporal gland secretions collected from an Asian bull elephant during a four-month musth episode and examined the role of such secretions as agents for chemical communication between elephants. PAA has also been identified as one of the aromatic acids of sun-cured Greek and Turkish tobacco (Kimland et al., 1973; Chuman and Noguchi, 1977) and was isolated among diverse volatile compounds from swine manure (Yasuhara and Fuwa, 1983). Of special note were the different effects that corn and teosinte pollen had on the radicle growth of

Amaranthus. This is especially interesting considering the taxonomic proximity of both *Zea* species.

The presence of biologically active compounds in corn pollen could be an adaptive mechanism that benefits the species in some way or it could be due to chance. The comparative study of the allelopathic effect of corn pollen and teosinte pollen suggests that the production of allelopathic substances in corn pollen could be an adaptation to management and selection by man or to the new ecological circumstances faced by this plant during its cultivation. The variation of growth regulators in the pollen of both species also confers on them different reproductive and ecological adaptations.

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ATTRACTION OF *Heliothis virescens* (F.) (LEPIDOPTERA: NOCTUIDAE) TO VOLATILES FROM EXTRACTS OF COTTON FLOWERS

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Abstract—Mated and virgin female and virgin male tobacco budworm moths, *Heliothis virescens* (F.), responded via directed upwind flight in wind-tunnel bioassays to volatiles from floral extracts of cotton, *Gossypium hirsutum* L. Significantly more male and female moths landed on cloth dispensers treated with a methylene chloride extract of debracted flowers than on control dispensers treated only with methylene chloride. Only mated females landed in significant numbers on the dispensers treated with extract of cotton squares, flowers (including bracts), or flower petals. After landing, most of the moths examined the dispensers by probing with their antennae, proboscis, and/or ovipositor.

Key Words—Tobacco budworm, *Heliothis virescens*, Lepidoptera, Noctuidae, cotton, *gossypium hirsutum*, oviposition, attractant, floral extracts.

INTRODUCTION

Phytophagous insects often use volatile chemicals emitted from plants to locate suitable oviposition sites and food sources for themselves or their progeny. In studies on the behavioral effects of host and nonhost allelochemicals on *Heliothis* (Lepidoptera: Noctuidae) , we have observed attraction and oviposition stimulation of *H. virescens* (F.) and *H. subflexa* (Guenée) moths by host-plant extracts (Mitchell and Heath, 1987; Mitchell et al., 1990; Tingle et al., 1989, 1990). Volatiles from extracts of cotton leaves and squares (flower buds) and leaves

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from tobacco and *Desmodium tortuosum* (Swartz) de Candolle (a wild host) induced mated *H. virescens* females to exhibit a full array of behaviors (upwind flight, landing, surface examination with antennae, and oviposition on treated substrates) expected of gravid individuals seeking sites to oviposit. Virgin female and male moths were significantly less responsive to the attractant than were mated females. Mitchell et al. (1991) presented data suggesting that cotton squares have a greater concentration of flight- and orientation-stimulating compounds than do the leaves.

Wiesenborn and Baker (1990) found in wind tunnel bioassays that pink bollworm moths, *Pectinophora gossypiella* (Saunders), were attracted to a hexane extract of cotton flowers. They proposed that the flower volatiles act as a food lure, attracting moths to extra floral nectaries. Similarly, Haynes et al. (1991) demonstrated in wind-tunnel assays that olfactory stimuli played an important role in the cabbage looper's, *Trichoplusia ni* (Hübner), ability to locate nectar on honeysuckle flowers, *Abelia grandifora* (Andre). The study reported here describes the behavioral responses exhibited by *H. virescens* females (virgin and mated) and virgin males in wind-tunnel assays when exposed to extract from the flowering parts of cotton, *Gossypium hirsutum* L.

METHODS AND MATERIALS

A Plexiglas wind tunnel (0.6 × 0.6 × 1.95 m) was used to observe flight and other behavioral responses by *H. virescens* to crude extracts of cotton squares (flower buds), whole blooms, blooms with bracts removed, and petals (including pistils and stamens) from blooms. The extracts were prepared from field-grown cotton (variety McNair 220) by washing ca. 400 g of fresh plant material for 30 sec in 1 liter of solvent (methylene chloride), which was filtered and stored in glass containers at 0°C until used (Mitchell and Heath, 1987). Before testing, the extracts were concentrated in a rotary evaporator to 2-g equivalents (2 gE) per 200 μl methylene chloride. Mitchell et al. (1991) determined previously, that 2 gE of cotton square extract attracted over 50% of the mated female *H. virescens* tested in wind-tunnel bioassays.

Test insects were reared in our laboratory on a modified pinto bean diet using the methods described by Guy et al. (1985) and Mitchell et al. (1988). Pupae were held in 5.5-liter plastic cages with screened tops until emergence. Upon eclosion, virgin females and males were held separately or combined for mating (21 females and 14 males/cage). The moths were fed a 10% honey–water solution and held under a reversed 14 : 10-hr light–dark cycle in a holding room maintained at 26 ± 1°C and 65 ± 5% relative humidity for two to four days prior to testing.

On the day of testing, the sex of each moth was verified by gently squeezing

the abdomen to extrude the genitalia. Mated and virgin females and virgin males were confined separately in three 25 × 25 × 25-cm Plexiglas holding cages and placed in the flight tunnel room (3.0 × 2.6 × 2.1 m) ca. 1 hr before scotophase. An intake vent in the wall allowed a continuous flow of fresh unfiltered air from the outdoors into the room, which was maintained at the same environmental conditions as the holding room. An electric timer was used to control overhead fluorescent lights (two banks of two 40-W bulbs). The flight tunnel was the same as described by Tingle et al. (1989). Three incandescent 25-W red light bulbs equally spaced above the tunnel remained on continuously. The light level during scotophase was 2.4 lux inside the tunnel. Air was pulled through the tunnel at ca. 0.4 m/sec, when measured at the center of the tunnel, and exhausted ca. 3 m above the roof covering the flight-tunnel room via a 30-cm-diam. flexible pipe equipped with a fan.

Crude extract (2-gE) from either cotton squares, whole blooms, debracted blooms, or petals from blooms was applied to white cotton muslin cloth that was secured over the end of a glass cylinder dispenser (3.5-cm opening) with a rubber band. On a second cloth-covered dispenser, 200 μl of methylene chloride was applied as a control. After waiting 5 min for the solvent to evaporate, the two dispensers were placed 30-cm apart at the upwind end of the tunnel. Treated and control dispensers were rotated between positions after each replication. Air was blown through each cylinder with an aquarium pump at the rate of 1 liter/min to provide a continuous flow (plume) of the extract odor through the tunnel. The location and form of the plumes were verified by introducing smoke into the dispenser system and observing the smoke trails. The plume from each dispenser remained separate until merging ca. 65 cm from the opposite (downwind) end of the tunnel.

Each moth was removed from the holding cage and placed into a cylindrical 4.0 × 6.5-cm plastic release cage with screened ends. After placement of the release cage into the downwind end of the flight tunnel, the moth was released immediately and observed with the aid of a low-light video camera for 3 min. Behavioral responses, including random and oriented flight, and contacts, landings, and oviposition on extract-treated and control substrates were recorded. Five replicates each of 10 mated females, virgin females, or virgin males were tested individually for responses to each of the four extracts. The data were converted to arcsin $\sqrt{\text{percentage}}$ and analyzed using either the paired or unpaired *t*- test (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Volatiles from extracts of whole (intact) cotton flowers, flowers without bracts, and the extract of petals, pistils, and stamens from flowers attracted significantly more (paired *t*-test) mated and virgin female and male *H. virescens*

to extract-treated substrates than did the corresponding controls (Figure 1, top). The extract from cotton squares attracted significantly more virgin males and mated females (but not virgin females) than did the control. When response to each extract, as demonstrated by upwind flight toward the treated substrate, was compared with the response to each of the other extracts (unpaired *t*-test), the highest significant response was recorded for mated and virgin females to volatiles emitted from the extract of debracted flowers (Table 1). There were no significant differences among the extracts attracting virgin males toward the

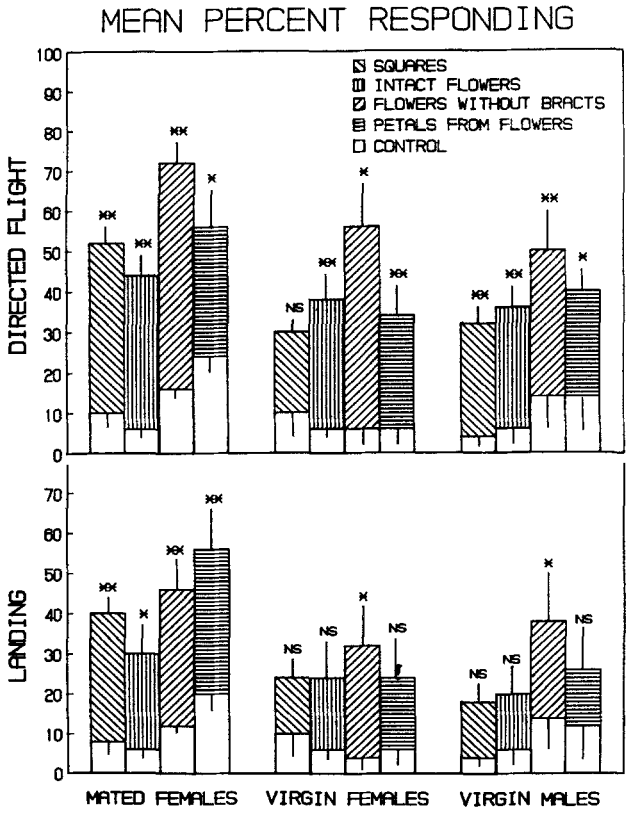

FIG. 1. Response of *Heliothis virescens* moths exposed in a wind tunnel to extracts from cotton. Mean percentages indicate positive anemotactic response by directed flight toward the odor source (top) or landing on extract-treated substrates (bottom). Thin lines above or below the tops of bars indicate standard error of the mean. Significance determined by Student's paired *t*-test from percentage of moths flying to or landing on treated and untreated (control) substrates in choice tests: NS = nonsignificant at $P < 0.05$; * = significant at $P < 0.05$; ** = significant at $P < 0.01$.

TABLE 1. COMPARISON BETWEEN COTTON EXTRACTS FOR ATTRACTING *Heliothis virescens* IN A WIND TUNNEL

Treatment	Flight response (% ± SE)[a]	Treatment[b]			
		Square	Whole flower	Debracted flower	Petals only
Mated females					
Square	53 (3.7)				
Whole flower	44 (5.1)	1.269NS			
Debracted flower	72 (4.9)	3.039*	3.738**		
Petals only	56 (9.3)	0.453NS	1.143NS	1.506NS	
Virgin females					
Square	30 (3.2)				
Whole flower	38 (6.3)	1.054NS			
Debracted flower	56(10.3)	2.409*	1.494NS		
Petals only	34 (7.5)	0.440NS	0.490NS	1.763NS	
Males					
Square	32 (3.7)				
Whole flower	36 (5.1)	0.601NS			
Debracted flower	50 (9.5)	1.756NS	1.054NS		
Petals only	40 (4.5)	1.370NS	0.606NS	0.954NS	

[a] Five replicates per treatment (10 moths tested individually per replicate).
[b] Response to each extract compared with response to each of the other extracts; t values determined by unpaired t test (Steel and Torrie, 1960): NS = nonsignificant, $P < 0.05$; * = significant, $P < 0.05$; ** = significant, $P < 0.01$.

treated substrates. The flight sequence was similar to that previously described by Tingle et al. (1989, 1990) and Mitchell et al. (1991). Most of the moths exited the release cage and began random flight in an upwind direction. Moths that detected the odor plume from the extract flew upwind in a zigzag pattern toward the dispenser. A few moths flew directly to the dispenser after exiting the release cage and landed on the cloth substrate.

Significantly more mated females landed on dispensers treated with each of the extracts (paired t-test) than on control dispensers (Figure 1, bottom). Virgin females and males landed in significant numbers only on the dispensers treated with the extract of debracted flowers. Most of the moths that landed examined the cloth substrates by probing with their antennae, proboscis, and/or ovipositor. Where 20% of the landings by mated females resulted in egg deposition on the treated substrates, only 4% of the landings by virgin females resulted in egg laying. There were no apparent differences in the effect of the various extracts on oviposition. Because it is unclear whether the chemicals that induced the moths to respond by anemotaxis in the flight tunnel are the same

compounds that stimulated oviposition, the oviposition olfactometer developed by Mitchell and Heath (1987) is a better system than the flight tunnel for evaluating oviposition behavior per se.

During previous tests, we had not observed probing of substrates with the proboscis (Tingle et al., 1989, 1990; Mitchell et al., 1991; Tingle and Mitchell, 1991). However, by using a low-light video camera to record behavioral responses to the cotton extracts, we were able to observe extension of the proboscis by males and females (virgin and mated) in this study. Over 50% of the landings on treated substrates by mated females resulted in proboscis extension, which did not vary significantly among the various substrates ($P < 0.05$, unpaired t test). All of the virgin females and males that landed on substrates treated with the extract from whole flowers exhibited proboscis extension, but significantly fewer ($P < 0.05$, unpaired t test) of the moths (47 and 66%, respectively) that landed on the other extract-treated substrates showed this response. Proboscis extension was not surprising because Lepidoptera have contact chemoreceptors located on the proboscis, as well as on the antennae, tarsi, and ovipositor. In a review of contact chemoreception, Stadler (1984) stated that after phytophagous insects have located a potential host plant using olfactory or visual cues, contact with the plant surface appears to be essential before feeding or oviposition is initiated.

Information received via probing with the proboscis may be assessed in conjunction with that from the tarsi, as suggested by Blaney and Simmonds (1988). Their work indicated that feeding behavior by *H. virescens*, *H. armigera* (Hübner), and *Spodoptera littoralis* (Boisdval) adults was correlated with the electrophysiological response of sensilla on the proboscis. They reported that responses of the sensilla to sugar and amino acids were significantly correlated to feeding behavior in each of these species. Additionally, Stadler and Seabrook (1975) determined that sensilla on the proboscis of the eastern spruce budworm, *Choristoneura fumiferana* Chem., were sensitive to sugars, which are involved in feeding behavior, but not to host-specific chemicals that promote oviposition.

We have not yet determined the chemical composition of the cotton extracts that stimulated host-finding, oviposition, and feeding-like responses noted here for *H. virescens*. We also do not know if the same chemical components are present in leaves, squares, and flowers, or if all the components exist in varying ratios throughout the plant. Identification of the chemicals and blends thereof that attract tobacco budworm moths and that stimulate them to feed or oviposit may permit development of systems to regulate populations of this important insect pest.

Plant-produced semiochemicals provide a variety of opportunities to develop manipulative schemes for controlling insect pests on crops. Such schemes may involve: (1) the elimination or reduction of attractants or oviposition stimulants from otherwise desirable cultivars via plant breeding; (2) the development of

baits using attractive substances from plants alone; or (3) the development of baits containing plant-attractive substances in combination with sex pheromones. Such baits could be used to detect or monitor pest insects or for direct control of pest populations using attractive baits containing a toxicant.

The vast majority of attractant chemicals identified have been sex-specific pheromones, primarily produced by females and attractive to males (Mayer and McLaughlin, 1991). Therefore, the use of sex attractant pheromones as predictive tools to forecast pest numbers or potential damage levels in specific crops has been significantly limited because attractants are usually directed at only one sex. Thus, identification of plant constituents that attract *H. virescens* adults, especially females, and that directly affect their feeding or reproductive behavior would greatly expand opportunities for manipulation of the biological and environmental events that control the establishment, development, and dispersal of this important crop pest.

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DO DEFOLIATION AND SUBSEQUENT PHYTOCHEMICAL RESPONSES REDUCE FUTURE HERBIVORY ON OAK TREES?

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Abstract—Perennial plants are thought to respond to partial or complete defoliation by producing new foliage that is less susceptible to herbivores because of induction of allelochemicals. Here, I tested this hypothesis by manually removing primary foliage from branches of *Quercus emoryi* (Fagaceae) at two different times in the season and monitoring changes in protein and tannin levels and the amount of herbivory relative to control branches. New, secondary leaves had 2.5 × greater hydrolyzable tannin content than mature foliage of control branches. Condensed tannins, which constitute a relatively low fraction of leaf mass, were lower, while protein content was temporarily greater, in new secondary leaves relative to mature leaves. Despite large increases in hydrolyzable tannins, herbivory levels were greater on refoliated branches than on control branches. New foliage is susceptible to herbivory regardless of when it is produced in the season, possibly because lower toughness and higher water content override any induced or developmentally related changes in allelochemistry. My results do not support the hypothesis that postherbivore changes in phytochemistry protect perennial plants from future herbivory, at least within a growing season.

Key Words—Oak, *Quercus emoryi*, Fagaceae, defoliation, allelochemical induction, herbivory, tannins, defense.

INTRODUCTION

The foliage of many perennial plants changes in nutrition, allelochemistry, phenology, and morphology following herbivory (for reviews, see Spencer, 1988; Tallamy and Raupp, 1991). These induced responses to herbivory have been viewed as another level of plant defense against herbivore attack, in addition to

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constitutive variation in phytochemistry, phenology, and morphology (cf. Tallamy and Raupp, 1991). Induced responses can be shown to decrease feeding, slow development, or increase mortality of some herbivores in both laboratory and field settings (cf. Tallamy and Raupp, 1991). The critical assumption underlying the notion of induced responses as plant defenses is that responses following herbivory reduce future tissue losses or increase fitness of perennial plants (Schultz, 1988). For example, induced chemical responses may increase mortality of herbivorous insects, but no fitness gain is realized by the plant because compensatory mortality factors maintain constant herbivore population size (Schultz, 1988; Faeth, 1991a; Karban, 1991).

I tested the hypothesis that defoliation reduces future herbivory in *Quercus emoryi* (Fagaceae) by manually removing leaves from branches within trees and monitoring phytochemical changes and amount of arthropod herbivory on new, secondary foliage. Branches were defoliated at two different times in the growing season to assess how timing of defoliation affects regrowth, phytochemistry, and subsequent herbivory.

METHODS AND MATERIALS

Study Trees and Site. Quercus emoryi, Emory oak, is a dominant, semievergreen tree in central Arizona ranging from elevations of 1060 m to 2600 m (Kearney and Peebles, 1960). Emory oak usually grows determinately with new leaves produced in a single flush in mid-April to mid-May as year old leaves abscise. However, secondary, new leaves are produced later in the growing season if primary leaves are damaged physically or consumed by herbivores (Faeth, 1987).

The study site is at the Sierra Ancha Experimental Station (USDA) in a riparian zone surrounding Parker Creek (Gila County, Arizona) at 1554 m elevation, approximately the midrange of Emory oak. Rainfall is high (62.7 cm/year) and soils in the riparian zone are well-developed (Pase and Johnson, 1968) relative to other habitats of Emory oak in Arizona.

Defoliation and Estimation of Herbivory. Ten trees were selected randomly in May 1988 at the study site from a pool of similar-sized trees within a 100-m radius. Within each tree, four branches were chosen randomly from a pool of similar-sized branches at the same canopy height (1–3 m) and same aspect (i.e., cardinal direction) within a tree. One randomly-selected branch was defoliated early in the season, June 2, 1988, and paired with a randomly selected control branch (no defoliation). Paired branches were adjacent secondary branches on the same primary branch. A second randomly selected branch within each tree was defoliated later in the season (June 28, 1988) and also paired with a control branch. Branches within trees were selected as experimental units for

two reasons. First, complete defoliation of branches mimics natural herbivory events for this oak. Arthropod herbivores rarely defoliate entire trees, but early season defoliators such as the western tent caterpillar commonly defoliate whole branches and these branches reflush leaves later in the growing season. Second, the amount of simulated herbivory is $<1\%$ of the total leaf area of the trees. This experimental level of damage is less than that typically removed by endemic herbivores (5–20%) (Faeth, 1987). Previous studies (Faeth, 1986, 1987, 1990) show that induction of allelochemicals is localized within Emory oak trees (i.e., there are no systemic responses in nondamaged branches or leaves within trees) even at much higher levels of simulated and insect damage. Using paired branches within trees permitted better control of biotic and abiotic factors that can influence rates of herbivory among Emory oak trees (Faeth, 1985, 1991b).

Defoliation was accomplished by carefully plucking individual leaves until all leaves were removed from experimental branches. Artificial herbivory may not always produce the same phytochemical responses as does herbivory by arthropods (Baldwin, 1990). However, at least for *Q. emoryi*, artificial herbivory results in similar quantitative changes in tannin and protein content relative to damage by native arthropod herbivores (Faeth, 1986). I artificially defoliated branches to control both extent and timing of damage in the field, which is not feasible with native herbivores.

All defoliated branches were monitored weekly for evidence of refoliation. Budbreak was defined as first appearance of unfurled, secondary leaves from swelled buds. Time of refoliation was measured in days from defoliation and difference between early and late defoliation branches was tested by a Student's t test.

Entire branches were removed at the end of the growing season (April 4, 1989) before leaf abscission began and returned to the laboratory. All leaves were counted and examined for herbivory. Mean ($\pm$SD) number and range of leaves from branches were: control (early and late) $\bar{X} = 662.1 \pm 206.1$, range = 331–957; early defoliation $\bar{X} = 169.4 \pm 117.2$, range = 29–335, and late defoliation $\bar{X} = 262.8 \pm 208.3$, range = 94–735.

The amount of herbivory on new, secondary leaves was compared to leaves on control branches to test the hypothesis that induced responses in secondary leaves of Emory oak reduced further tissue loss. Amount of herbivory was measured in two ways. First, the percent of damaged leaves was calculated by dividing the number of damaged leaves by the total number of leaves for each branch. Second, total leaf area removed was estimated by either randomly selecting a subset of 50 damaged leaves from each branch or, if there were less than 50 damaged leaves within a branch, all of the damaged leaves. The area of each damaged leaf was measured with a leaf area meter, and this area was subtracted from the area of a reconstructed leaf (with opaque tape) to obtain area removed. Each branch was examined thoroughly for petiole scars, which indicate whole

missing leaves. The area of missing leaves was estimated by using the average area of leaves remaining in a terminal cluster. The mean damaged area per leaf was calculated and multiplied by percent of damaged leaves to obtain the total area removed (cm^2). The percent of damaged leaves (arcsine square root transformed), and percent total area removed (arcsine square root transformed) was compared statistically between early and late defoliation and control branches with paired t tests.

I compared size of refoliated leaves to mature leaves by weighing 50 randomly selected, undamaged leaves from each branch and comparing means with a paired *t* test.

Phytochemical Analyses. A small, haphazard sample of leaves (2–20 leaves) was removed from refoliated branches and paired control branches at three 30-day intervals, June 30, July 29, and August 28, 1988, for phytochemical analyses. Leaves were immediately frozen on Dry Ice in the field and returned to the laboratory, where they were lyophilized and then examined for herbivory (see above). Leaves were then ground to a fine powder in a Wiley mill and stored in airtight jars with a desiccant at 5°C until analyses of protein and tannins could be performed.

Details of the phytochemical methods and standardization are in Faeth (1985, 1986). Briefly, relative protein content was determined by the BioRad method (Richmond, California), after washing samples 2× with acetone to remove pigments and tannins. Condensed tannins were determined by a modified acidified vanillin method (Broadhurst and Jones, 1978) after extraction in acetone. Monomeric phenols were first isolated and quantified by removing tannin polymers with a selective adsorbent (Sephadex LH-20). Condensed tannins then were determined by subtracting monomers from total vanillin positive (monomers plus condensed tannin polymers), using a catechin standard prepared for each run. Only results from condensed tannins are presented here since total vanillin positive shows similar trends and because monomeric fractions were very low and varied little among samples. Hydrolyzable tannins were determined by a modified potassium iodate technique (Bate-Smith, 1977) and expressed as percent dry mass tannic acid equivalents. The method is specific for only the galloyl group of hydrolyzable tannins, not ellagitannins, but it is commonly used as an estimate of hydrolyzable tannin content (Schultz and Baldwin, 1982; Baldwin and Schultz, 1983; Faeth, 1990).

All phytochemical analyses were run in triplicate for each leaf branch sample at each date. All phytochemical methods have been standardized and have produced consistent results for Emory oak over an 8-year span (Faeth, 1985, 1986, 1988).

The objective was to sample branches that had just refoliated and then to resample branches 30 days later to determine temporal changes relative to controls. However, since not all branches had refoliated by these dates, especially

the more variable late defoliated branches, and two branches did not refoliate due to insect damage to buds, two consecutive sampling periods were available from six of the early defoliated branches (June 30, July 29) and only three of the late defoliated ones (July 29, August 28). I used ANOVA to determine if phytochemical samples from late defoliated branches could be pooled with early defoliated ones. In other words, I statistically determined if the phytochemistry of new, secondary leaves and 30-day-old leaves on late defoliated branches was the same as that of new and 30-day-old leaves on early defoliated branches, but separated by a 30-day time period. None of the phytochemical parameters varied significantly ($F < 5.10$, $df = 1,7$, $P > 0.10$ for all comparisons) between secondary leaves of the same developmental age on early and late defoliated branches, so samples were pooled in subsequent statistical analyses. I used paired t tests to compare statistically phytochemical measures (arcsine square root transformed) from new, secondary leaves and 30-day-old leaves to their control branch counterparts.

RESULTS

Time to Refoliation. Branches defoliated early in the season produced secondary leaves earlier ($\bar{X} = 21.9 \pm 5.7$ days) than branches defoliated later in the season ($\bar{X} = 36.5 \pm 20.6$ days, $t = 2.05$, $df = 15$, $0.05 < P < 0.10$).

Herbivory Levels. The percentage of leaves damaged by arthropods on branches defoliated early was not significantly different from control branches (Figure 1, $t = 1.13$, $df = 7$, $P > 0.20$). Similarly, the percentage of leaves damaged on branches defoliated late was not different from paired control branches (Figure 1, $t = .37$, $df = 7$, $P > 0.40$).

Percent leaf area removed from branches defoliated early was not significantly different from paired control branches (Figure 2, $t = 1.27$, $df = 7$, $P > 0.20$). However, percent leaf area on branches defoliated late in the season was greater than that removed from control branches (Figure 2), although this difference was only marginally significant ($t = 2.33$, $df = 7$, $0.05 < P < 0.10$). Generally, herbivory tended to be greater on experimentally defoliated branches than control branches (Figures 1 and 2), opposite to predictions that refoliated branches are more resistant to herbivory.

Refoliated leaves on experimental branches were significantly smaller than mature leaves on control branches ($\bar{X} \pm$ SE, refoliated leaves = 19.16 mg $\pm$ 4.92; $\bar{X} \pm$ SE, mature leaves = 47.89 mg $\pm$ 11.97; $t = 9.38$, $df = 15$, $P < 0.0001$). Refoliated leaves were approximately 60% of the size of the mature leaves.

Phytochemistry. Protein content of new, secondary leaves on defoliated branches was significantly greater ($t = 3.32$, $df = 8$, $P < 0.05$) than paired

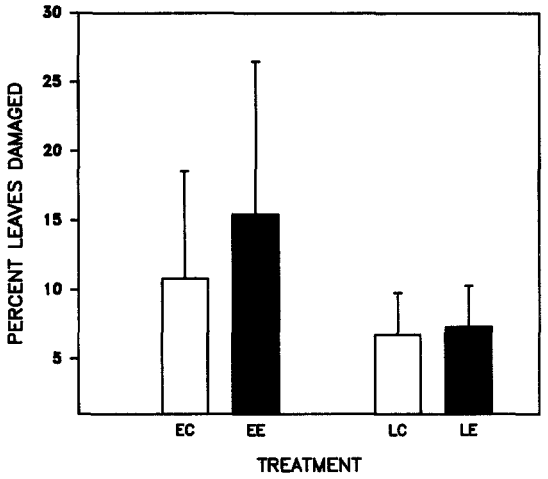

FIG. 1. Mean (±SE) percent of leaves damaged by arthropod herbivores on early-season control (EC) and experimental (EE) and late-season control (LC) and experimental (LE) branches. Data shown are untransformed; percentages were arcsine transformed for statistical tests.

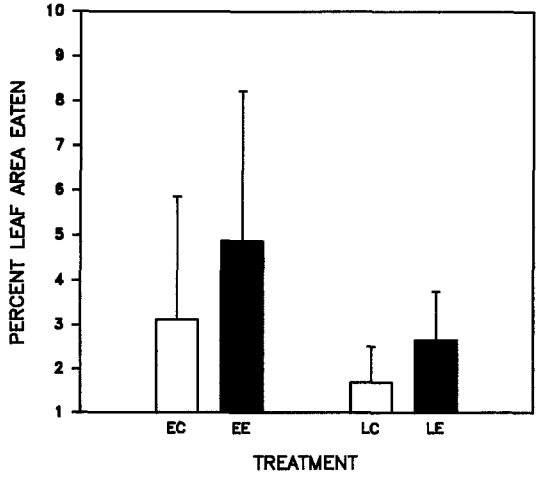

FIG. 2. Mean (±SE) percent of leaf area consumed by arthropod herbivores on early-season control (EC) and experimental (EE) and late-season control (LC) and experimental (LE) branches. Data shown are untransformed; percentages were arcsine transformed for statistical tests.

control branches (Figure 3). Thirty days after reflushing, however, protein content of replacement leaves declined and was not different ($t = 1.03$, $df = 8$, $P > 0.20$) from mature foliage of paired control branches (Figure 3).

Hydrolyzable tannin content of new, secondary leaves on defoliated branches was significantly greater ($t = 5.34$, $df = 8$, $P < 0.001$) on defoliated branches relative to control foliage (Figure 4). Although declining after 30 days, hydrolyzable tannin content on experimental branches was still significantly greater ($t = 6.68$, $df = 8$, $P < 0.001$) than that of control branches.

Condensed tannin content of new, secondary leaves on defoliated branches was significantly less ($t = 3.67$, $df = 8$, $P < 0.01$) than that of control branches (Figure 5). Condensed tannin content of defoliated branches remained significantly lower than control branches ($t = 3.54$, $df = 8$, $P < 0.01$) 30 days after reflushing (Figure 5).

DISCUSSION

New, secondary leaves contain 2.5× more hydrolyzable tannins than mature foliage. Such increases in hydrolyzable tannins in replacement leaves after partial defoliation have been considered induced defenses in *Q. rubrum* against future herbivory (Schultz and Baldwin, 1982). However, it is unclear if changes in secondary foliage of Emory oak are induced responses or simply reflect

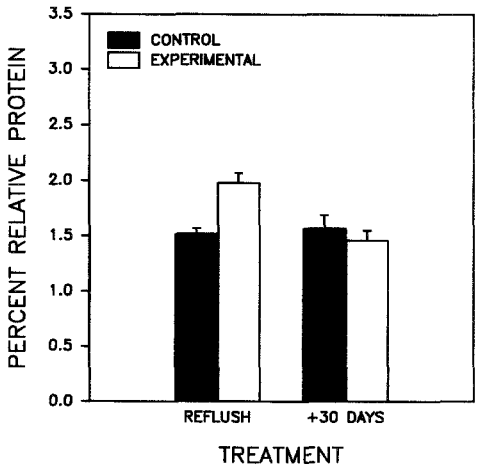

FIG. 3. Mean (±SE) percent relative protein content of control and experimental branches at the time of reflushing of defoliated branches and 30 days later. Data from early and late control and experimental branches were pooled after ANOVA showed no significant differences (see text). Data shown are untransformed; percentages were arcsine transformed for statistical tests.

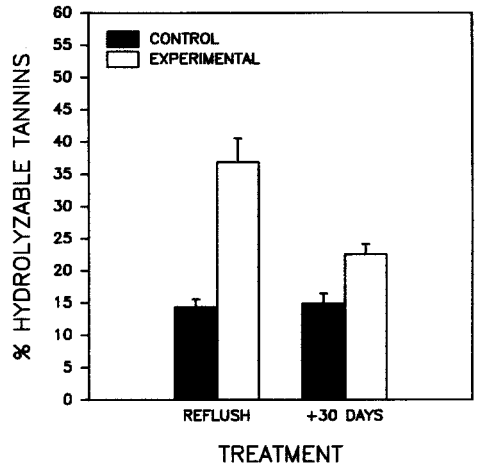

FIG. 4. Mean (±SE) percent hydrolyzable tannin content of control and experimental branches at the time of reflushing of defoliated branches and 30 days later. Data from early and late control and experimental branches were pooled after ANOVA showed no significant differences (see text). Data shown are untransformed; percentages were arcsine transformed for statistical tests.

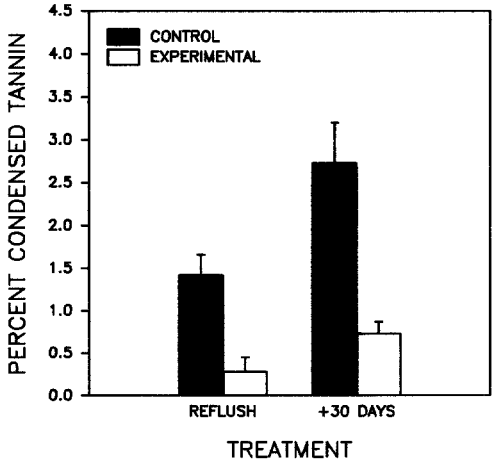

FIG. 5. Mean (±SE) percent condensed tannin content of control and experimental branches at the time of reflushing of defoliated branches and 30 days later. Data from early and late control and experimental branches were pooled after ANOVA showed no significant differences (see text). Data shown are untransformed; percentages were arcsine square transformed for statistical tests.

developmental age of new leaves. The latter is supported by two lines of evidence. First, new foliage on early and late defoliated branches did not differ significantly in protein or condensed or hydrolyzable tannin content (see Phytochemical Analyses in Methods and Materials), indicating secondary leaves are phytochemically similar regardless of when they are produced in the growing season. Secondly, secondary leaves on both types of experimental branches are very similar to new, primary leaves in initial phytochemical composition and subsequent seasonal changes (Faeth, 1985, 1986). Protein and hydrolyzable tannin content of primary leaves is initially high but rapidly declines after 30 days, while condensed tannin content is initially very low but gradually increases throughout the remainder of the growing season (Faeth, 1985).

Whether one considers increased hydrolyzable tannin levels in new, secondary leaves as induced responses or as a consequence of developmental age, these leaves are not less susceptible to future herbivory from arthropods. On the contrary, secondary foliage tended to be more vulnerable to herbivores (Figures 1 and 2). This susceptibility is even more pronounced when one considers that secondary foliage on early and late defoliated branches was exposed to herbivores 54 and 95 days, respectively, (approximate number of days from spring budbreak to refoliation) less, on average, than their paired control counterparts in the year-long growing season. Moreover, two experimental branches were excluded from analysis because their buds were completely destroyed by herbivores before they could unfurl. Clearly, increases in hydrolyzable tannins in secondary foliage do not reduce tissue losses for this perennial plant.

New leaves of defoliated branches were lower in condensed tannin and higher in protein content relative to mature foliage on control branches. These differences may have increased quality of leaves for herbivores and could explain higher rates of folivory on new leaves. However, the increased protein in new leaves is transitory and declines to that of mature leaves within 30 days. Condensed tannins constitute a relatively small fraction of leaf mass compared to hydrolyzable tannins (Figures 4 and 5), and condensed tannins are thought to be less effective deterrents against arthropod herbivores than are hydrolyzable tannins (Zucker, 1983). It is possible that some qualitative change in specific tannin compounds or some other unknown chemical changes explain differences in herbivory levels. However, a more parsimonious explanation may be that higher water content and lower "toughness" of new oak leaves (Feeny, 1970; Faeth, 1985) makes them more susceptible to herbivory no matter when they appear in the growing season and overrides any differences in allelochemistry. Other studies examining defoliation and refoliation in the same growing season indicate that foliage produced after defoliation events is generally more susceptible to herbivory (Rockwood, 1974; Auerbach and Simberloff, 1985; Pullin, 1987; Silva-Bohorquez, 1986; Faeth, 1987; Hunter, 1987) than mature foliage.

There is considerable debate about both the short- and long-term effects of

induced changes on feeding, development, and survival of arthropod herbivores (e.g., Fowler and Lawton, 1985; Schultz, 1988, see reviews in Spencer, 1988; Barbosa and Letourneau, 1988; Karban and Myers, 1989; Tallamy and Raupp, 1991) as well as the mechanisms for plant responses (cf., Bryant et al., 1991). The key, however, to the defensive role of induced responses from the plant's perspective is a reduction in tissue loss or an increase in fitness (Schultz, 1988). Although this study examined only one type and temporal scale of herbivory, defoliation of branches within trees in a growing season, the results do not support a defensive role of postdefoliation chemical changes, regardless of whether the changes are induced in response to herbivory or "incidental" (sensu Neuvonen and Haukioja, 1991) to plant development. Other studies of this oak involving partial damage to individual leaves and long-term effects of past episodes of herbivory on future resistance also fail to support the defensive role of induced changes (Faeth, 1987, 1992).

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Journal of Chemical Ecology, Vol. 18, No. 6, 1992

Book Review

Insect Chemical Ecology. Ivan Hrdý (ed.). Prague, Czechoslovakia: Academia Praha, 1991. 513 pp.

This book consists of a compilation of 75 papers presented at a conference in Tábor, Czechoslovakia, in August 1990. The invited speakers had been asked to present either comprehensive or introductory reports and, as a result, the contributions are of variable length and nature, ranging from relatively extensive reviews to brief research communications.

The book is divided into three sections: (1) chemical communication in insects; (2) chemical insect–plant relationships; and (3) prospects of practical application of pheromones and juvenoids.

Section 1 begins with a review of new methodology for pheromone isolation and identification, with examples of structure–activity studies and some discussion of perception mechanisms. This is followed by a report on the use of photoaffinity labeling to characterize receptors and carrier proteins involved in transport and transduction processes. Subsequent papers describe specific examples of pheromone systems, with emphasis on structure–activity relationships, species specificity, control of pheromone production, and factors affecting insect responses. Included in this group is a well-conceived study of the evolution of moth pheromones. Some additional papers deal with more novel types of pheromones produced by social as well as phytophagous insects. Several contributions are devoted to synthetic methods for pheromones and their intermediates, and others are concerned with factors affecting release rates of pheromones, or perception by and subsequent behavior of the target insect.

Section 2 starts with an introductory review of phytoecdysteroids, followed by several more specific studies of ecdysteroids from various natural and synthetic sources. Other contributions deal with plant compounds that affect feeding, reproduction, or orientation. The methodology for analysis, collection, and identification of active phytochemicals is addressed by several authors.

Section 3 provides many examples of efforts to use pheromones or juvenoids in the control of insect pests. A large number of these reports focus on population monitoring or mass trapping, whereas others describe preliminary attempts to control specific insects using various juvenoids. Also included here are studies of formulation for controlled release of active compounds and metabolic investigations to determine the fate of certain juvenoids. The opening presentation in this section gives several convincing examples of promising

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research areas and new approaches to the practical development of semiochemicals. A review of insect neuropeptides is also included in this section, and another significant paper addresses structure–activity relationships of juvenoids and anti-juvenile hormone compounds.

The quality of papers, although quite variable, is generally good. The involvement of English-speaking coeditors has undoubtedly been useful in the final editing of manuscripts, although some linguistic errors have nevertheless been overlooked. The research appears to be sound in most cases, and some unique ideas have been presented. Since the majority of contributors are from Eastern European countries, this book has particular value as a vehicle for dissemination of ideas and results that were previously suppressed or had limited distribution due to political barriers. The recruitment of participants from a wide variety of other countries has helped to create a truly international flavor and to provide a balance of contributions from both established scientists and developing young investigators.

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Journal of Chemical Ecology, Vol. 18, No. 6, 1992

Book Review

Herbivores: Their Interactions with Secondary Plant Metabolites—Volume 1. The Chemical Participants, 2nd ed., G.A. Rosenthal and M.R. Berenbaum (eds.). San Diego: Academic Press, 1991. 468 pp.

The first edition of *Herbivores: Their Interaction with Secondary Plant Metabolites*, edited by G.A. Rosenthal and D.H. Janzen, played a major role in providing a conceptual framework for studying the chemical ecology of plant–herbivore interactions. In 1979, when the first edition was published, the study of plant–herbivore interactions was in a very rapid stage of growth and change, and it was inevitable that *Herbivores* would be out of date very quickly. The appearance of a revision has been awaited with great impatience and with high expectations. At last the second edition is out, and it meets all of the expectations that have been held out for it.

The second edition is a two-volume set, with the first volume, *The Chemical Participants*, corresponding to Part II of the first edition. Volume I contains 468 pages divided into 12 chapters, which compares with 349 pages divided into 12 chapters in Part II of the original edition. The chapter titles in the second edition are: Nonprotein Amino Acids as Protective Allelochemicals (G.A. Rosenthal), Cyanide and Cyanogenic Glycosides (D.S. Siegler), Alkaloids (T. Hartmann), Glucosinolates (S. Louda and S. Mole), Terpenoids (J. Gershenzon and R. Croteau), Coumarins (M.R. Berenbaum), Cardenolide-Mediated Interactions between Plants and Herbivores (S.B. Malcolm), Iridoid Glycosides (M.D. Bowers), Lectins (I.E. Liener), Tannins and Lignins (A.E. Hagerman and L.G. Butler), Flavonoid Pigments (J.B. Harborne), and Insect Hormones and Antihormones in Plants (W.S. Bowers).

The second edition is a brand new book; it is not a mere update of the first edition. Of the 15 contributors to the second edition, only four had chapters in the first. Of the nine topics covered in both editions, seven are discussed by different authors in the second edition. The second edition includes three chapters devoted to topics (coumarins, cardenolides, and iridoid glycosides) not covered in the first edition. The three topics not revisited in the new edition are toxic seed lipids, saponins, and proteinase inhibitors.

The quality of the individual chapters is uniformly high. Each chapter provides references for isolation and assay procedures, a review of the occurrence and natural distribution of the particular class of chemicals under consideration, and an excellent description of definitive studies of the role of the

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chemicals in plant–herbivore systems. A cursory scan of the bibliographies at the end of each chapter turned up numerous references to articles published in 1989 and 1990, and even a handful from 1991. The structure and style of the second edition, combined with the current state of development of the fields of chemical ecology and plant–herbivore interactions, suggest that obsolescence will not overtake the second edition as rapidly as it did the first.

Clearly, the thrust of this review is that I consider *Herbivores—Volume I. The Chemical Participants* to be an excellent and important book. In trying to construct statements that reflect my judgment that this revision has been a highly successful endeavor, I repeatedly found myself trying to take statements from the Preface and reword them so that I would not be guilty of outright plagiarism. I finally gave up; I simply cannot improve on the review that the editors have written of their own book. I will, therefore, conclude this review by fully endorsing the following comments from the Preface: "This new edition is richer in basic methodology and far more effective in assisting the interested reader who would like to bring the study of plant allelochemicals into their research activities. . . . The second edition focuses sharply on the kinds of important ecological and chemical ecology questions that have been addressed by the study of these compounds and provides insights into current research directions. . . . The second edition is offered because the time has come for an in-depth, updated consideration of how allelochemicals color and shape the many fascinating interactions between plants and herbivores."

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6-METHOXY-2-BENZOXAZOLINONE: A SEMIOCHEMICAL FOR HOST LOCATION BY WESTERN CORN ROOTWORM LARVAE

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Abstract—A bioassay-driven sequential fractionation scheme was used to isolate all portions of a crude dichloromethane corn seedling extract behaviorally active to larvae of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. 6-Methoxy-2-benzoxazolinone (MBOA) was identified as one of the most important components of an attractive crude corn extract. MBOA was found on or in the intact root tissues by injecting an extract of undamaged roots onto an HPLC immediately after extraction. MBOA was demonstrated to be volatile and functions as a semiochemical in conjunction with carbon dioxide in host location by western corn rootworm larvae, which are oligophagous on the roots of maize and several other species of grasses. Because MBOA occurs almost exclusively in maize and other grasses, it offers a simple way for the larvae to distinguish possible hosts from non-hosts. MBOA has previously been reported as a chemical defense against other insect species. This is the first report in grasses of a secondary compound that is toxic or a deterrent to nonadapted insect herbivores but that is used as a semiochemical in host location by a specialist insect species.

Key Words—*Diabrotica virgifera virgifera*, 6-methoxy-2-benzoxazolinone, hydroxamic acids, semiochemical, attractants, western corn rootworm, host location, Coleoptera, Chrysomelidae, *Zea mays*, kairomone.

INTRODUCTION

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is a serious pest of maize (*Zea mays* L.) throughout much of the maize-growing regions of

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North America. Larval damage to maize roots results in reduced plant height, reduced yield, and lodging of maize, which interferes with harvesting (Branson et al., 1980). An estimate of crop losses and treatment costs for corn rootworms is in the range of $1 billion annually (Metcalf, 1986).

Unlike host location in many other herbivorous insect species, western corn rootworm adults do not lay their eggs on a host plant. The eggs are laid in the soil in late fall, and the larvae emerge the following spring (Krysan and Miller, 1986). Host location is then carried out underground by the neonate larvae, which are oligophagous on the roots of a few species of grasses (Branson and Ortman, 1967, 1970). The larvae can distinguish the roots of maize from the roots of broad-leaved plants on the basis of olfactory cues. In choice test bioassays with cut roots, significantly more western corn rootworm larvae were attracted to the roots of maize than to the roots of soybean, squash, or sunflower (Branson, 1982).

We previously found that cryogenic collections of volatiles from germinating maize seeds were attractive to western corn rootworm larvae in laboratory behavioral bioassays (Hibbard and Bjostad, 1988, 1989). One of the attractive compounds is carbon dioxide, which is attractive to western corn rootworm larvae (Strnad et al., 1986; Strnad and Bergman, 1987; Hibbard and Bjostad, 1988) and many other soil organisms (Jones and Coaker, 1977; Doane et al., 1975; Pline and Dusenbery, 1987; and references therein). Carbon dioxide is released into the soil by the roots of maize and many other plant species (Massimino et al., 1980), but because it is a general plant metabolite, it does not appear to provide a basis for selective attraction of western corn rootworm larvae to maize over broad-leaved plants. We also found in choice test bioassays that western corn rootworm larvae rely on chemical cues in addition to carbon dioxide to locate maize plants. In choice test bioassays with equal levels of carbon dioxide on both sides of the choice (verified by infrared gas analysis), larvae chose the maize volatile source significantly more often than the source with carbon dioxide alone (Hibbard and Bjostad, 1988). We used a sequential fractionation scheme modified from Silverstein et al. (1967) to isolate several behaviorally active compounds (Hibbard and Bjostad, 1990). We now report the chemical identification of a volatile secondary maize metabolite that western corn rootworm larvae use in orienting to their host.

METHODS AND MATERIALS

Larvae and Maize Source. A nondiapausing strain of *D. virgifera virgifera* was obtained in June 1986 from the USDA-ARS laboratory in Brookings, South Dakota, and was maintained with the methods of Jackson (1986) as modified by Hibbard and Bjostad (1988). Second-instar larvae (5–7 days old and 5–8 mm

long) were chosen for bioassays because initial bioassays indicated that they behaved similarly to first-instar larvae (the instar that must locate a host), and second-instar larvae were more robust for the large numbers of bioassays required. To prevent possible effects from previous testing, larvae were not reused in the bioassays. Dried whole kernels of maize (3090, Pioneer Hi-Bred International, Inc., Johnston, Iowa), not treated with insecticide or fungicide, were washed and soaked (Hibbard and Bjostad, 1988), and maize seedlings [pre-stage 0 germinated seed (Hanway, 1966)] were removed after 3 to 5 days to obtain maize semiochemicals.

Extraction and Isolation of Behaviorally Active Compound. Approximately 80 g of moist maize seedlings was extracted by placing them in a glass seed-holding tube (30 cm × 30 mm, tapering to 12 mm) for 3 to 6 hr and subsequently dripping dichloromethane through them until 4 ml of solution had been collected. The dichloromethane maize extract was then separated into fractions by column chromatography on 60- to 200-mesh silica gel (J.T. Baker Chemical Co., Phillpsburg, New Jersey), eluted batchwise with mixtures of dichloromethane and diethyl ether (100:0, 97:3, 90:10, 75:25, 50:50, and 0:100). The most active fraction in choice test bioassays with equal concentrations of carbon dioxide in both choices was the 75:25 fraction. The major compound in this fraction was isolated by gas chromatography as described by Hibbard and Bjostad (1990). A 2-mm-ID × 1.83-m 3% OV-101 (100/120 mesh) on Chromosorb Q (Alltech Associates, Inc., Deerfield, Illinois) packed column was temperature programmed at 60°C for 1 min, then 10°C/min to a final temperature of 260°C, in a Hewlett-Packard 5890 gas chromatograph. The 4-ml extract was concentrated to 8 μl under a nitrogen stream and injected onto the gas chromatograph. With the detector and noncolumn gases turned off, the fraction with a retention time of 16–17 min (the retention time on the major component in the 75:25 silica gel fraction) was collected with clean pipettes that were snugly connected to the end of the column with a specially fitted Teflon connector (collection efficiency, 70–85%). The collection was repeated 10 times with fresh extract, and the collection pipette was rinsed with the same 300 μl of solvent each time.

Bioassays with Purified Maize Volatile. Fifteen-microliter aliquots (approximately 28 g equiv of 3-day-old corn seedlings) of the 300-μl sample of purified maize volatile in dichloromethane were placed on 0.1 g of glass wool, and an equal amount of dichloromethane was placed on another piece of glass wool. After the solvent had evaporated, the pieces of glass wool were tested in choice test bioassays with equal levels of carbon dioxide (4 mmol/mol at bioassay onset) on both sides of the choice (verified with infrared gas analysis) as described by Hibbard and Bjostad (1990). Ten larvae were placed in the center dish of the choice test (see Hibbard and Bjostad, 1990), and the number of larvae that had reached either of the end dishes was recorded after 1 hr. Bio-

assays were conducted in the dark at room temperature and were replicated 15 times.

Chemical Analysis. Solid probe electron-impact (EI, 70 eV) and chemical ionization (CI, NH_4^+) mass spectra of the purified maize volatile were obtained with a VG, Inc., mass spectrometer (Model MM-16F). A ^1H NMR spectrum of the purified maize volatile in $CDCl_3$ was obtained with a Bruker AM500 (500 MHz). A diffuse reflectance infrared spectrum was obtained on a Nicolet 60SX Fourier transform spectrometer. A synthetic sample of 6-methoxy-2-benzoxazolinone (MBOA) was obtained (donation from Frank Stermitz, Chemistry Department, Colorado State University, and later from Lancaster Synthesis Ltd., Windham, New Hampshire) and was also analyzed by EI-MS, CI-MS, NMR, and IR as described above. A sample of synthetic MBOA was analyzed by coinjection with the purified maize volatile using an RSL-150, 30-m × 0.33-mm (ID) methyl silicone capillary column programmed at 60°C for 1 min, then 10°C/min to a final temperature of 240°C, in a Hewlett Packard 5890 gas chromatograph. The injection port was set at 240°C and the detector was set at 250°C.

Behavioral Bioassays with Synthetic MBOA. Serial dilutions of synthetic MBOA (Lancaster Synthesis Ltd., Windham, New Hampshire) were prepared and 700 ng of synthetic MBOA was tested in choice test bioassays as described above, with equal amounts of carbon dioxide on both sides of the choice. Seven hundred nanograms was chosen, because the amount of MBOA present in the bioassays of the purified maize volatile was determined to be 700 ng. The number of larvae that were found in either side of the choice test was recorded after 1 hr and the experiment was replicated eight times.

MBOA on Corn Seedlings. The primary naturally occurring precursor to MBOA in corn is DIMBOA-glucoside (Wahlroos and Virtanen, 1959). Hofman and Hofmanová (1971) later corroborated this conclusion, but the limits of detectability of the aglucones in these early papers were not given. Recently, Zúñiga and Massardo (1991) detected a significant amount of the aglucone in the intact tissues of wheat despite inhibiting the β-glucosidase with liquid nitrogen and methanol. They also found only the aglucone in 2-day-old wheat and in undifferentiated wheat cell cultures. Because the limits of detection of the aglucones in corn were not given in the early manuscripts, we wished to test specifically for free MBOA.

Untreated corn kernels were washed and soaked as described above and were placed on moist germination paper for three days. The major root of the germinated seed was then quickly dipped into a 4-ml solution of dichloromethane. A total of 70 germinated seeds was extracted over a time period of 5 min. A portion of the extract (300 μl of 4 ml) was concentrated to 50 μl in a nitrogen stream (which took approximately 1 min) and was immediately injected onto a 25 cm × 4.6-mm, 5-μm Econosphere C-18 HPLC column with a Kratos

Spectroflow UV absorbance detector set at a wavelength of 290 nm. Flow through the system was held constant at 1 ml/min. Operating conditions were as follows: mobile phase, solvents A (20 mmol phosphoric acid, pH 2.3) and B (methanol)—0–5 min 0% B isocratic; 5–20 min 0% B–50% B linear gradient; 20–25 min 50% B–100% B linear gradient; 25–27 min 100% B isocratic, 27–30 min 100% B–0% B linear gradient. In addition to injection onto the HPLC, the same amount of the "quick dip" extract was also injected onto an RSL-150 nonpolar methyl silicone capillary GC column (30 m × 0.25-μm ID × 0.25-μm film thickness). Temperature conditions were as described above. As with the HPLC, the crude extract was also coinjected with synthetic MBOA under the same conditions.

MBOA Volatility. MBOA is a high-melting solid [melting point, 158°C (Chen and Chen, 1976)], but other high-melting solids such as camphor [melting point, 180°C (Weast and Astle, 1981)] have appreciable volatility under ambient conditions. It has been questioned whether MBOA is volatile at room temperature. In order to address this question, we conducted three types of experiments. First, because retention time on a methyl silicone GC column is highly correlated with vapor pressure ($r^2 = 0.724$) (Heath and Tumlinson, 1986), we injected synthetic MBOA and a series of known lepidopteran pheromone components. We used our RSL-150 methyl silicone capillary column with the conditions described above. Second, we extracted MBOA-formulated clay granules after being exposed to room air in a layer of granules one granule thick for periods of 0, 1, 2, 4, 8, and 16 days. Each sample consisted of 0.313 g of clay granules that had previously been formulated with 1600 μg of MBOA/g of clay granules. The samples were extracted with 0.5 ml of dichloromethane and 1 μl was injected onto the RLS-150 capillary column under the conditions described above. The experiment was replicated three times. In a third experiment, volatilized MBOA was collected in a cold trap for analysis. Ten milligrams of synthetic MBOA was placed in a 6.35-mm Teflon tube and held in place by 7.6 cm of tightly packed glass wool. One end of the tube was then connected with Swagelock fittings to a nitrogen tank with a two-stage regulator, and the other end of the tube was connected to a loop of Teflon tubing that was bathed in liquid nitrogen. Glass wool (7.6 cm) was placed in the bottom of the loop in the liquid nitrogen as a condensation site. The nitrogen flow rate through the system was held constant at 10 ml/min. After 26 hr, the tube was removed from the liquid nitrogen and extracted with 4 ml of dichloromethane. One milliliter of the extract was concentrated to 50 μl and injected onto the HPLC system as described above.

Statistical Analysis. The statistical package SAS (SAS Institute, 1990) was used in all data analysis. Proc ANOVA was used alone for tests with only two means, and with Duncan's (1955) multiple-range test for analysis with more than two means.

RESULTS

Behavioral Bioassays with the Purified Maize Volatile. The purified maize volatile was significantly attractive ($P < 0.01$) in choice tests with equal levels of carbon dioxide on both sides of the choice (Figure 1A).

EI and CI Mass Spectra. The EI mass spectrum of the purified maize volatile included peaks at *m/z* 165 (100), 150 (63), 136 (4), 122 (11), 109 (16), 106 (34), and 80 (15). The CI mass spectrum included peaks at 183 (100, M + NH_4), 166 (33, M + 1), 165 (50), 153 (2), 139 (2), 124 (5), 106 (4). The EI and CI mass spectra of the purified maize volatile indicated that the M^+ ion

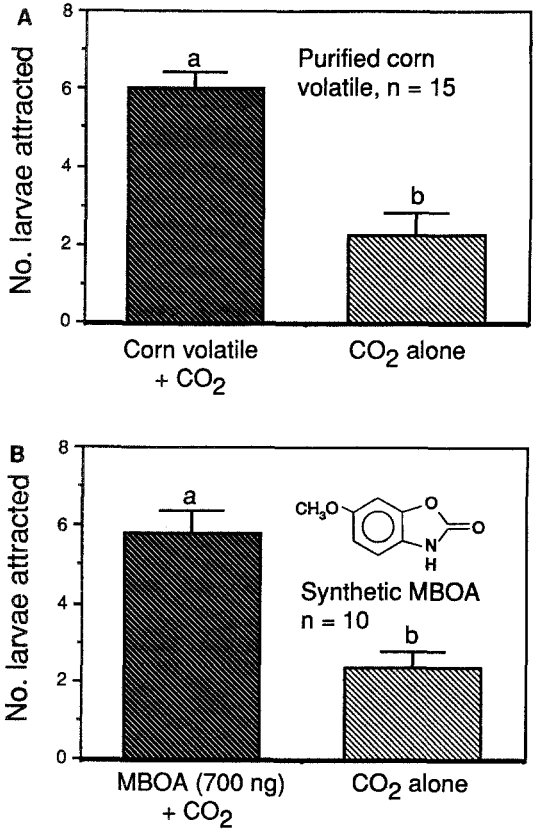

FIG. 1. Choice test bioassays of (A) purified maize volatile and (B) synthetic 6-methoxy-2-benzoxazolinone (MBOA) with equal levels of carbon dioxide on both sides. Treatment means labeled with different lowercase letters were significantly different ($P < 0.01$) according to a one-way analysis of variance (ANOVA). Error bars indicate the standard error of the mean (negative portions not shown).

is m/z 165. The odd molecular weight was consistent with the presence of a nitrogen atom in the molecule.

NMR Spectrum. The ^1H NMR spectrum of the compound is shown in Figure 2. The singlet at δ 3.89 (3H) indicated a methoxy group. The doublets at δ 6.92 (1H) and δ 6.82 (1H) and the double doublet at δ 6.69 (1H) indicated three aromatic hydrogens, with two of the hydrogens adjacent on the aromatic ring. The singlet at δ 8.19 (1H) indicated a proton on nitrogen.

IR Spectrum. The IR spectrum of the purified maize volatile indicated absorptions at 3150, 1770, and 1680 cm^{-1}, which was consistent with N—H, C=O, and C—N, respectively.

Identification. On the basis of the MS, NMR, and IR spectra of the natural and synthetic compound, the structure was determined to be 6-methoxy-2-benzoxazolinone (MBOA). The retention times of synthetic MBOA and the purified maize volatile on an RSL-150 capillary gas chromatograph column were both 14.3 min and this was verified by coinjection. The spectra from EI-MS, CI-MS, NMR, and IR of synthetic 6-methoxy-2-benzoxazolinone were in full agreement with the spectra of the isolated maize volatile.

Behavioral Bioassays with Synthetic MBOA. Synthetic MBOA was significantly attractive ($P < 0.01$) to western corn rootworm larvae at the dose present

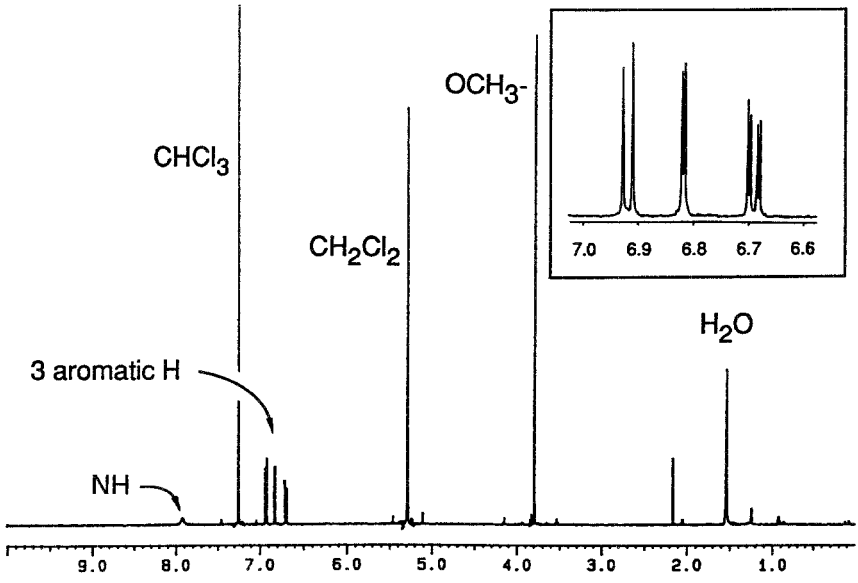

FIG. 2. ^1H NMR spectrum of the purified maize volatile. Inset: Magnification of the three aromatic protons.

in the purified maize volatile (700 ng) on glass wool in choice test bioassays with equal levels of carbon dioxide on both sides of the choice (Figure 1B).

MBOA on Corn Seedlings. Corn root extracts were analyzed using HPLC and GC within 8 min of the extraction of the first corn roots and within 3 min of the extraction of the last corn roots. Using HPLC, 98.7 ng of MBOA/g of 3-day-old corn roots (fresh weight) was found. MBOA was one of the largest peaks in the GC chromatogram, accounting for over 30% of the detected peak area. Over 8 μg of MBOA/g of 3-day-old corn roots (fresh weight) was found by GC with this "quick dip" method. The difference between the two methods can be explained by thermal degradation of MBOA derivable compounds in the injection port of the GC (240°C).

MBOA Volatility. The retention time of MBOA under the described GC conditions was 14.04 min. The retention time of (Z)-7-dodecenyl acetate was 12.49 min; (Z)-11-tetradecenyl acetate, 14.76 min; hexadecyl acetate, 16.74 min; and octadecyl acetate, 18.63 min. Since each of these compounds is known to be a volatile pheromone component for various lepidopteran species, and since retention time on nonpolar methyl silicone columns correlate well with vapor pressure (Heath and Tumlinson, 1986), the retention time of MBOA on our methyl silicone column supports the assertion that it is volatile. In the MBOA volatilization experiment, an average of 298 $\pm$ 35 μg of MBOA was extracted from 0.333 g of clay granules at time 0, 165 $\pm$ 10 μg after 1 day, 135 $\pm$ 20 μg after 2 days, 87 $\pm$ 5 μg after 4 days, and 20 $\pm$ 5 μg after 8 days. After 16 days, no detectable MBOA was found without concentration of the sample. In the cold-trapping experiment of synthetic MBOA crystals, over 40 ng of MBOA was recovered from 26 hr of passing a nitrogen stream over 10 mg of MBOA at a rate of 10 ml/min.

DISCUSSION

We have used a bioassay-driven sequential fractionation scheme to isolate behaviorally active portions of a crude dichloromethane corn seedling extract, and we have identified 6-methoxy-2-benzoxazolinone (MBOA) as one of the most important components of an attractive crude corn extract. Choice test bioassays with equal levels of carbon dioxide of both sides of the choice were used with the crude extract, each fraction of two separate types of separations, the purified natural compound, and the synthetic of our identification. MBOA is the first compound—other than carbon dioxide—identified from corn roots as behaviorally active to western corn rootworm larvae, one of the most important insect pests in the North America.

It was previously believed that MBOA is not an *in vivo* constituent of maize tissue but is produced through the decomposition of a glucoside that occurs in

the maize plant, 2-*O*-glucosyl-2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (Wahlroos and Virtanen, 1959). When the plant is injured, the glucoside is enzymatically hydrolyzed, releasing the aglucone 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), which is subsequently degraded to MBOA (Willard and Penner, 1976). Recently, however, the lack of aglucones in grass tissue has been questioned. Zúñiga and Massardo (1991) concluded that a significant amount of aglucones are present in the intact tissue of wheat. In the present study, we found 98.7 ng of MBOA/g of 3-day-old corn root (fresh weight) by quickly dipping the roots of undamaged corn seedlings in dichloromethane and immediately injecting the sample onto the HPLC. The enzymatic conversion of DIMBOA-glucoside to DIMBOA takes 30 min at room temperature (Lyons et al., 1988). The conversion of DIMBOA to MBOA has a half-life of 5.3 hr at 28°C and pH 6.75 (Woodward et al., 1978a). Since our injection was made within 12 min of the first corn root extracted, our data support the assertion that MBOA is present on or in the tissues of 3-day-old germinated corn.

Many plant species contain specific allelochemicals that provide an effective toxic defense against herbivorous insects (Waller, 1987). Despite this, even a plant species that is chemically well defended may be attacked successfully by a few specialist insect species that not only withstand a particular plant toxin, but use it as a semiochemical to locate and recognize their plant host (Waller, 1987; Harborne, 1988; Rosenthal and Janzen, 1982). No examples of this have been reported for insect herbivores of maize, one of the principal agricultural plant species worldwide. We now report that an important toxin produced by maize and other grasses, 6-methoxy-2-benzoxazolinone (MBOA), is used as a semiochemical for host location by western corn rootworm larvae.

The toxic and antifeedant effects of MBOA have been documented for several insect species, including the European corn borer, *Ostrinia nubilalis* (Hübner) (Klun and Brindley, 1966; Campos et al., 1988; Nicollier et al., 1982), the southwestern corn borer, *Diatraea grandiosella* Dyar (Nicollier et al., 1982), the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Nicollier et al., 1982), the cereal aphid, *Metopolophium dirhodum* (Walker) (Argandoña et al., 1980), the silkworm, *Bombyx mori* (L.) (Kubo and Kamikawa, 1983), the dried fruit beetle, *Carpophilus hemipterus* (L.) (Dowd, 1990), *Spodoptera exempta* (Walker) (Kubo and Kamikawa, 1983), the German cockroach, *Blatella germanica* (L.), and the southern armyworm, *Spodoptera eridana* (Cramer) (Beck and Stauffer, 1957), as well as bacteria (Nicollier et al., 1982; Beck and Stauffer, 1957), and fungi (Beck and Stauffer, 1957; Wahlroos and Virtanen, 1958; Beck and Smissman, 1961). However, MBOA is not toxic to western corn rootworm larvae (Abou-Fakhr et al., 1992).

DIMBOA, the precursor to MBOA, is generally considered a more potent toxin and is toxic to fungi (Long et al., 1975; Guthrie et al., 1985), and bacteria (Woodward et al., 1978b; Corcuera et al., 1978), the European corn borer (Klun

et al., 1967; Reed et al., 1972; Robinson et al., 1982; Campos et al., 1989), the greenbug, *Schizaphis graminum* (Rondani) (Argandoña et al., 1983), other aphids (Beck et al., 1983; Bohidar et al., 1986), and western corn rootworm larvae (Xie et al., 1990). Techniques have been developed that can quantify the amounts of known hydroxamic acids from corn roots (Xie et al., 1991a). The toxicity of DIMBOA to western corn rootworm larvae may be relevant to the isolation of strains of maize that have an antibiosis effect on the insect since quite high amounts of DIMBOA equivalents have been found in the cortex tissues of maize roots (Xie et al., 1991b). DIMBOA acts as a feeding deterrent at high concentrations (Argandoña et al., 1983; Corcuera et al., 1985). DIMBOA is an inducible chemical defense in maize, increasing significantly in concentration after feeding by larvae of *Sesamia nonagrioides* (Lefebvre) (Gutierrez et al., 1988). For more comprehensive reviews of the biology and chemistry of cyclic hydroxamic acids, see Willard and Penner (1976) and Niemeyer (1988).

Hydroxamic acids such as MBOA are well suited as semiochemicals for host location by a soil insect. MBOA has sufficient vapor pressure to diffuse in air, yet it is appreciably water soluble as well. MBOA is volatile under ambient conditions despite its relatively high melting point (158°C) and crystalline structure at room temperature, just as other volatile compounds such as camphor (m.p. 180°C) have similar melting points and crystalline structure at room temperature. In the present paper we demonstrated that MBOA is a volatile compound with vapor pressure comparable to that of many insect pheromones. These characteristics may allow it to move effectively through the soil under a wide range of soil moisture conditions. Hydroxamic acids have been documented in 10 genera of the Gramineae, but there are only two reports outside the Gramineae (Niemeyer, 1988). MBOA provides a simple way for western corn rootworm larvae to limit their orientation to grasses rather than to plants in general. Western corn rootworm larvae are not infallible in their location of appropriate hosts, in that they will orient to many nonhost grass species as well as they do to maize (Branson, 1982), perhaps because MBOA or related compounds are common to both. Larvae are attracted to the roots of sorghum and feed on them, despite the fact that sorghum roots contain cyanogenic glycosides that quickly kill western corn rootworm larvae (Branson et al., 1969; Strnad and Dunn, 1990).

MBOA and carbon dioxide are not the only compounds involved in host selection by western corn rootworm larvae. We have isolated an additional attractive fraction and two repellent fractions by GC separations of dichloromethane extracts of germinating maize (Hibbard and Bjostad, 1990). It is possible that the repellent fractions include compounds that are toxic to western corn rootworm larvae. These behaviorally active fractions are currently under

investigation and may be additional compounds used in discrimination of host plants from nonhosts.

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BEHAVIORAL RESPONSES OF WESTERN CORN ROOTWORM LARVAE TO NATURALLY OCCURRING AND SYNTHETIC HYDROXAMIC ACIDS

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Abstract—Hydroxamic acids have been shown to be toxic to many pest insects and pathogens. In this study, the behavioral responses of western corn rootworm larvae to naturally occurring and synthetic hydroxamic acids were investigated. In a choice test between corn roots treated with hydroxamic acids and roots treated with distilled water (control), western corn rootworm larvae chose to burrow into the control roots significantly more often than compound-treated roots. In addition, when corn roots were treated with different hydroxamic acids in a designed searching-behavior test, neonate larvae of western corn rootworm responded by significantly reducing the number of turns, while the area searched and locomotor rate significantly increased. The responses were dependent on the concentrations of the test compounds. These results suggested that hydroxamic acids were acting as behavior-modifying and possibly feeding-deterrent chemicals.

Key Words—1,4-Benzoxazin-3-ones, hydroxamic acids, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), Coleoptera, Chrysomelidae, *Diabrotica virgifera virgifera*, corn rootworms, nonpreference, search behavior, chemical ecology.

INTRODUCTION

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is a serious insect pest of maize in North America. The eggs are laid in late summer and

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fall and overwinter in the soil. The larval stage is the most destructive, causing a reduction in yield directly through damage to the root system and through lodged plants. Feeding by adults can also cause minor damage by feeding on silks, kernels, and leaves (Branson, 1986). The amount of insecticide used for *Diabrotica* spp. control is more than for any other pest of corn in the United States (Suguiyama and Carlson, 1985).

Behavioral bioassays for western corn rootworm larvae have been developed to determine both the role of carbon dioxide (CO_2) released by roots of corn plant and the effect of volatile semiochemicals from corn seedlings on host location by western corn rootworm larvae (Strnad et al., 1986; Strnad and Bergman, 1987; Hibbard and Bjostad, 1988, 1990). Research has revealed that both a plant primary metabolite (CO_2) and secondary metabolites (volatile semiochemicals) are involved as the attractive agents in the orientation of western corn rootworm larvae. In addition, volatile chemicals from corn silks have been demonstrated to be attractants to adults of western and northern corn rootworm (Prystupa et al., 1988). However, the behavioral response of western corn rootworm larvae to the major group of corn secondary metabolites, the hydroxamic acids, is unknown, although several dichloromethane extracts of germinating corn possibly containing these substances have been found to be attractive or repellent to western corn rootworm larvae (Hibbard and Bjostad, 1990).

The search orientation in animals is recognized to take place in two phases: ranging and local search (Jander, 1975). The former is characterized by relatively straight locomotion in which the animal has limited resource information, and the latter is characterized by increased turning rate and decreased locomotor rate when animals receive information of suitable resources (Bell, 1985). In a host search behavior study with corn rootworms, Strnad and Dunn (1990) demonstrated that search area and locomotor rate decreased and turning rate and path crossings increased after western corn rootworm larva contacted with host roots as compared with nonhost roots.

The hydroxamic acids (1,4-benzoxazin-3-ones) are plant secondary metabolites, that are naturally present as the 2-*O*-β-D-glucosides in intact tissue in several species of Gramineae, such as corn, wheat (Wahlroos and Virtanen, 1959), and rye (Honkanen and Virtanen, 1960). In corn, some of these compounds have been shown to be associated with resistance to insect pests and pathogens (e.g., Klun and Brindley, 1966; Long et al., 1977; Campos et al., 1988, 1989), including corn rootworm (Xie et al., 1990). However, it is not clear what behavioral responses western corn rootworm larvae have after contact with naturally occurring and/or synthetic hydroxamic acids, and it is unknown if the host searching behavior is affected by these secondary metabolites. The objective of this investigation was to characterize the searching behavior of western corn rootworm larvae after contact with maize roots treated with various

hydroxamates and to determine the choice behavior shown by western corn rootworm larvae on maize roots treated with these compounds.

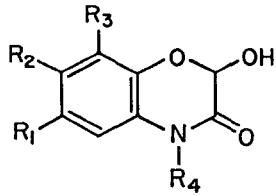
METHODS AND MATERIALS

Source of Compounds. The compounds used in this study include those naturally occurring in maize roots and those synthesized. All compounds were synthesized in our laboratory and represent a good range of known chemical trends, either unimolecular decomposition rates or reduction by thiol (Atkinson, 1989). Complete synthetic details are described by Atkinson (1989). The structures of these chemicals are shown in Figure 1.

Maize Root Materials. Maize kernels of Funks G4017, a currently used Ontario hybrid with a low (<100 μg/g fresh wt) hydroxamic acid content, were washed in detergent solution for 3 min, rinsed thoroughly, and soaked in distilled water for 24 hr. Soaked seeds were rinsed again with distilled water, placed on moistened paper, covered with moistened paper towels, kept in a closed plastic container, and incubated at room temperature (25 °C). Three to four days later, fresh maize roots (2–3 cm long) were removed for bioassays.

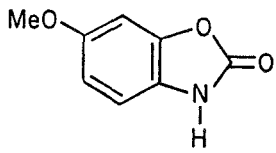
Choice Tests. Fresh maize roots were dipped for 30 sec in an ethanolic solution of various hydroxamates (500 ppm) or distilled water with the same amount of solvent (0.1% EtOH) (solvent control). This concentration was within the range found for 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) in maize root (Xie, 1991). One treated and one control root were placed 1 cm apart on moistened filter paper in a plastic petri dish (9 cm). Then one neonate western corn rootworm was immediately placed between the roots. The dish was covered, sealed with parafilm, and kept in an incubator (25 °C, dark condition). The larval position (in root, on root, or out of root) was examined for each root (treated and control) at 24 hr after infestation. A no-choice control (control roots only) was added in order to determine larval position under untreated condition. Thirty replicates (30 larvae) were prepared for each compound, and the whole experiment was repeated four times.

Host Searching Behavior. The behavioral responses of western corn rootworm neonates to maize roots treated with related compounds were recorded by a modification of the method described by Strnad and Dunn (1990). Fresh maize roots were dipped in a solution of compound (500 ppm) for 30 sec. Distilled water alone served as a true control and distilled water with the same amount of solvent (0.1% EtOH) served as a solvent control. Individual neonates (<24 hr old) of western corn rootworm were placed on the surface of treated roots. After a 5-min period of contact, the larva was transferred to the center of a moistened filter paper (Whatman No. 1, 24 cm) on a square glass plate



1,4-benzoxazin-3-ones

Abbreviation	R_1	R_2	R_3	R_4
DIMBOA	H	MeO	H	OH
DIM_2BOA	H	MeO	MeO	OH
6,7-diMeO	MeO	MeO	H	OH
7-t-Bu	H	t-Bu	H	OH
7-Me	H	Me	H	OH
DIBOA	H	H	H	OH
7-Cl	H	Cl	H	OH
7-F	H	F	H	OH
HMBOA	H	MeO	H	H



6-methoxy-benzoxazolin-2-one (MBOA)

FIG. 1. Chemical structures of naturally occurring and synthetic hydroxamic acids (1,4-benzoxazin-3-ones) and 6-methoxy-benzoxazolin-2-one (MBOA) used in this study.

(30 × 30 cm) with four supports (3 mm high) in each corner and then covered with a second glass plate. An acetate sheet was placed on top of the second glass plate. During a 5-min period, the path of larval travel was traced on the acetate sheet by following the larval travel with a felt pen. Meanwhile, the number of turns (defined as any change of the larval body in axis of travel) was recorded using a hand counter. Thirty larvae were used for each treatment (compounds and controls), and a fresh area of filter paper was used for each larva. Finally, acetate sheets were copied to grid paper (1 square/mm). The area searched (mm^2) was determined by counting the number of squares intersected by the path. Path lengths were measured with a map wheel and converted to locomotor rate (mm/min).

Concentration Dependence of Localized Search. To determine if the effects of hydroxamic acids on the localized search of western corn rootworm neonates depend on the concentrations of the related compounds, different concentrations of the selected compounds were used for testing the search behavior of western corn rootworm larvae. Two compounds, DIMBOA and MBOA, were selected, and three concentrations, 250, 500, and 1000 ppm, for each were used in this study. Number of turns, area searched, and locomotor rate were determined as described above.

Statistical Analysis. The data from choice test were also calculated as percentage control, which was the ratio of the number of larvae responding in the treated group divided by the number of larvae responding in the control group. Since there were no larvae found "on root" or "out of root" in some treatments of the choice test, the number of larvae on root and out of root was transformed [square root $(X + 1)$] (Snedecor and Cochran, 1980) for calculating percentage control. All data were tested by Bartlett's test for homogeneity of variance (Snedecor and Cochran, 1980; Anonymous, 1991), and if the variances were equal, parametric statistics was performed. A t test was applied to compare means of control and treated in the choice test (Table 1).Analysis of variance (ANOVA) and Duncan's multiple-range test were applied for comparisons of data with more than two means. An arcsin transformation was performed for the percentage data before analysis (Steel and Torrie, 1980; SAS Institute, 1982).

RESULTS AND DISCUSSION

Choice Study. All hydroxamates used in this study were shown to be effective deterrents in the choice-test design. The number of larvae found burrowing in the roots of the control side were significantly more than that of the treated side for all test compounds (Table 1). Of the three naturally occurring compounds (1, 2, 9) in maize roots, two (1, 9) strongly affected rootworm choice of the position inside the root, which was only 58.4 and 55.1% of the control and is significantly different from the control (Table 1). Compound 3 showed the least effect, possibly due to its previously observed rapid decomposition, which was the highest of all compounds tested (Atkinson, 1989). There were no significant differences ($P > 0.05$) between the results obtained with the 7-substituted series, except for 7-Cl, which showed the highest behavioral effect (Table 1).

Generally, choice designs are useful in detecting small differences in food acceptability (Schoonhoven, 1982). It is clear that when fresh maize roots were treated with various hydroxamates, the percentage of rootworms found inside the roots significantly decreased and the percentage of rootworms staying on the surface of roots increased (approximately 20–75% more than on the control,

TABLE 1. WESTERN CORN ROOTWORM LARVAL POSITION IN A CHOICE TEST WITH FRESH MAIZE ROOTS TREATED WITH DIFFERENT HYDROXAMATES

Compound		In root[a]			On root[a]			Out of root[a]		
No.	Substituent	Control	Treated	% control[b]	Control	Treated	% control[b]	Control	Treated	% control[b]
	Control	26.8 (1.3)	—	100.0a[c] (0.0)	2.7 (1.1)	—	100.0a (0.0)	0.5 (0.3)	—	100.0bc (0.0)
3	6,7-diMeO	13.5[d] (0.9)	10.5 (1.3)	79.7b (13.2)	0.8 (0.5)	3.8[d] (0.6)	174.8b (19.3)	0.0 (0.0)	1.5 (1.5)	141.4abc (0.0)
2	7,8-diMeO (DIM_2BOA)	14.5[d] (1.3)	11.1 (0.4)	77.8b (7.7)	0.8 (0.3)	2.0 (1.4)	138.4ab (26.5)	0.3 (0.3)	1.0 (0.4)	126.3abc (0.0)
6	7-H (DIBOA)	13.0[d] (1.1)	9.3 (0.5)	73.2bc (8.5)	2.0 (0.4)	3.5[d] (0.3)	125.7ab (12.3)	0.5 (0.5)	1.8 (0.5)	149.1ab (25.5)
4	7-t-Bu	14.3[d] (1.3)	9.8 (1.0)	68.5bc (4.6)	1.8 (1.2)	3.3 (0.6)	147.2ab (23.6)	0.0 (0.0)	1.0 (0.4)	139.0abc (15.0)
8	7-F	17.0[d] (0.6)	11.5 (1.0)	67.9bc (6.9)	0.9 (0.3)	1.3 (1.0)	141.3ab (29.1)	0.3 (0.3)	0.0 (0.0)	92.7c (7.3)

5	7-Me	13.3[d]	8.5	64.3bc	2.8	4.3	121.5ab	0.8	1.3	134.2abc
		(1.0)	(0.7)	(1.6)	(0.3)	(0.8)	(3.9)	(0.5)	(0.5)	(15.5)
10	MBOA	16.8[d]	10.3	62.5bc	0.5	1.0	121.3ab	1.3	0.3	80.2c
		(1.0)	(0.9)	(8.6)	(0.3)	(0.4)	(22.6)	(0.6)	(0.3)	(12.2)
1	7-MeO	15.3[d]	10.0	58.4bc	0.8	2.5[d]	152.8ab	0.5	1.0	115.9abc
	(DIMBOA)	(1.3)	(0.7)	(8.6)	(0.5)	(0.3)	(20.3)	(0.3)	(0.4)	(10.0)
9	HMBOA[e]	17.0[d]	9.3	55.1bc	0.8	1.5	122.2ab	0.0	1.5	153.7a
		(0.9)	(1.1)	(7.2)	(0.5)	(0.7)	(17.4)	(0.0)	(0.7)	(21.5)
7	7-Cl	18.2[d]	8.8	48.1c	0.3	2.3[d]	167.2ab	0.0	0.5	120.7abc
		(0.5)	(0.3)	(2.3)	(0.3)	(0.3)	(16.2)	(0.0)	(0.3)	(11.9)

[a] "In root" indicates that the insects were inside of the roots. "On root" indicates that the insects were in contact with root surface. "Out of root" indicates that the insects were not in contact with the roots.

[b] The resulting ratio of the number of larvae responding in the treated group divided by the number of larvae responding in the control group.

[c] Means in the same column followed by the same letter are not significantly different ($P > 0.05$) in Duncan's multiple-range test (arcsin transformation was performed before analysis). Standard error of mean in parentheses.

[d] Means in the same row within the same position (in root, or on root, or out of root) are significantly different between control and treated ($P < 0.05$; t test).

[e] HIMBOA is an amide.

although not statistically significant) (Table 1). Although these results suggest a change in food acceptability, more direct observation of feeding events is required to confirm this.

Host Searching Behavior. Two controls were designed in order to see if the control with solvent (0.1% EtOH) affected the behavioral responses of western corn rootworm larvae. The results indicated that there were no significant differences ($P > 0.05$) between the solvent-treated control and the true control (distilled water alone) in all behavioral parameters studied (Table 2). Figure 2 illustrates the very distinct path pattern resulting from a 5-min contact with corn roots treated with the hydroxamate 6,7-diMeO as compared to the control. Compared to controls, the searching behavior of neonate western corn rootworm larvae was clearly affected by treating maize roots with hydroxamate solution (Figure 2, Table 2). Neonate western rootworm larvae exposed to maize roots treated with various hydroxamates significantly ($P < 0.05$) decreased the number of turns but increased their search area and locomotor rate significantly ($P < 0.05$) (Table 2). The naturally occurring compounds, DIMBOA, as well as its amide HMBOA and its decomposition product MBOA, and DIM_2BOA showed similar effects on number of turns, area searched (except MBOA), and locomotor rate. In general, the synthesized compounds 3, 4, 5, and 7 of 7-substituted series showed higher activities in this respect (Table 2).

Concentration Dependence of Localized Search. Localized search orientation of insects can be modified by sensory information from internal and external sources (Bell, 1985). For example, the search path of a starved fruit fly, *Drosophila melanogaster*, on and around a 3.5-cm patch of sucrose is dependent on surcose concentration (Bell, 1985). The area searched by *D. melanogaster* on a patch of 0.5 *M* sucrose is significantly larger than that on a patch of 0.125 *M* sucrose (Bell, 1985). The present results show that the effects of hydroxamic acids on the localized search of western corn rootworm larvae are also dependent on the concentrations used (Figure 3). As the concentrations of DIMBOA and MBOA increased, larvae exposed to treated maize roots significantly decreased the number of turns but increased their searched area (except for MBOA) and locomotor rate. At 250 and 500 ppm of DIMBOA and MBOA, the trends were evident but not significant ($P > 0.05$). However, the parameters were significantly different ($P < 0.05$) when insects were exposed to maize roots treated with 1000 ppm of DIMBOA and MBOA (Figure 3). Considering that DIMBOA and MBOA were found to be toxic to western corn rootworm (Xie et al., 1990; Xie, 1991), it is not surprising to find that the number of turns decreased and the area searched and locomotor rate increased when larvae were exposed to maize roots treated with higher concentrations of the compounds.

Generally, an insect's search orientation is controlled by information either stored genetically or learned from sensory systems (Bell, 1985). After neonate

TABLE 2. NUMBER OF TURNS, AREA SEARCHED, AND LOCOMOTOR RATE OF WESTERN CORN ROOTWORM NEONATES DURING A 5-MIN HOST-SEARCHING PERIOD AFTER REMOVAL FROM MAIZE ROOT TREATED WITH HYDROXAMATES SOLUTION

Compound		Number of turns	Area searched (mm^2)	Locomotor rate (mm/min)
No.	Substituent			
	Control (H_2O only)	61.3a[a] (4.9)	91.7f (8.1)	16.8b (1.4)
	Control (with solvent)	58.5a (5.7)	116.9ef (9.2)	20.1b (1.5)
9	HMBOA	36.2bc (2.8)	158.9cde (13.8)	27.5a (2.3)
1	7-MeO (DIMBOA)	30.4bc (3.2)	165.7bcd (11.8)	31.9a (1.9)
2	7,8-diMeO (DIM_2BOA)	26.9cd (3.7)	157.2cd (18.5)	28.1a (2.2)
10	MBOA	25.4cd (2.5)	203.8ab (15.8)	22.5a (2.1)
4	7-t-Bu	24.2cd (1.7)	171.4abc (12.9)	28.8a (1.7)
5	7-Me	22.0cd (2.5)	132.9cdef (17.1)	21.6b (2.3)
8	7-F	20.1d (1.9)	210.5a (15.0)	33.3a (2.0)
7	7-Cl	18.3d (1.7)	176.4abc (14.1)	27.7a (1.9)
6	7-H (DIBOA)	17.6d (2.1)	124.8def (13.7)	20.8b (1.9)
3	6,7-diMeO	16.9d (1.5)	176.8abc (13.3)	28.3a (1.7)

[a] Means in the same column followed by the same letter are not significantly different ($P > 0.05$; Duncan's multiple-range test). Standard error of mean in parentheses.

larvae of western corn rootworm made contact with maize roots, they switched from ranging to a localized search. Our assay involves contact of neonate larvae with maize roots, and hence chemoreception of volatiles is possible. After encountering food resources of the controls in which feeding stimuli are present, neonate rootworm larvae shift their locomotion from a straight to a convoluted patterns. Our results are consistent with the concept of local search (Bell, 1985;

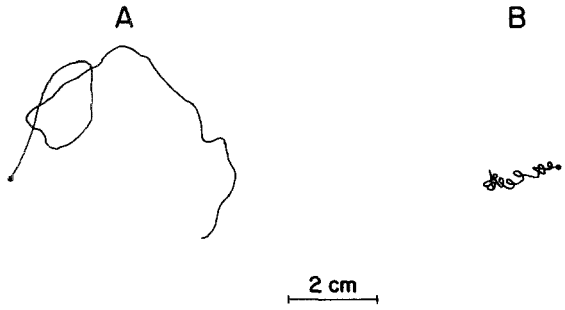


FIG. 2. Pathways of neonate western corn rootworm larvae during a 5-min localized search resulting from 5 min of contact with maize roots treated with hydroxamate (6,7-diMeO) (A) and with distilled water (control) (B). Dot indicates beginning point in each path.

Visser, 1988). When maize roots were treated with different hydroxamates which act as feeding deterrents in other insects (Campos et al., 1988, 1989), neonate larvae of western corn rootworm responded significantly by reducing the number of turns ($P < 0.05$), while the area searched and locomotor rate significantly ($P < 0.05$) increased. The path patterns recorded after larvae contacted maize roots treated with test compounds in our study are similar to those recorded with nonhost plants or possible host plants which contain feeding deterrents (Strnad and Dunn, 1990).

Toxicity of DIMBOA and MBOA against several organisms, including western corn rootworm, has been well documented (Long et al., 1977; Argandoña et al., 1983; Campos et al., 1988, 1989; Xie et al., 1990; Xie, 1991). In this study, when maize roots were treated with hydroxamic acid solutions (500 ppm), western corn rootworm larvae showed significant nonpreference, in three ways: (1) the larvae chose to burrow into untreated roots significantly more than treated roots, (2) the number of larvae avoiding roots was higher in the hydroxamate-treated roots, and (3) host searching behaviors were significantly modified, showing that larvae switched from a localized host-finding behavior to a ranging behavior after contact with treated roots (Table 2, Figure 2). These results strongly indicated that the hydroxamic acids were acting as behavior-modifying and possibly feeding-deterrent chemicals. The deleterious effects of hydroxamic acids on western corn rootworm larvae are similar to those observed with other insects such as greenbug and European corn borer (Robinson et al., 1982; Argandoña et al., 1983; Campos et al., 1988, 1989). Further study of corn rootworm food consumption and utilization is required to clarify the mode of action of hydroxamic acids on this insect.

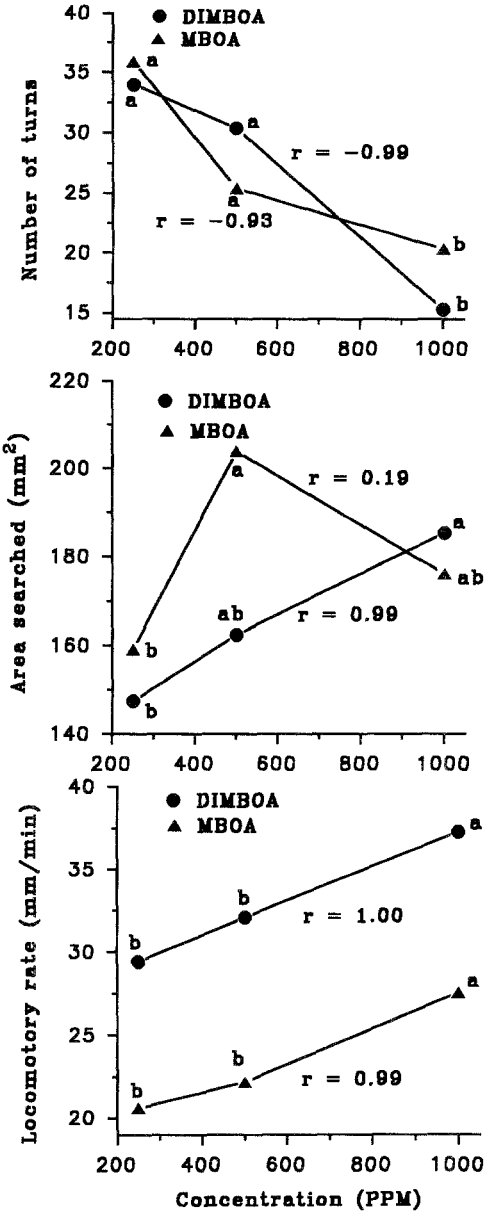


FIG. 3. Number of turns, area searched, and locomotor rate of neonate western corn rootworm larvae during a 5-min host-searching period resulting from 5 min of contact with maize roots treated with various concentrations (250, 500, and 1000 ppm) of DIMBOA and MBOA. Each figure is the mean of 30 larvae tested individually. Means followed by the same letter (within the data for the same compound) are not significantly different ($P > 0.05$; Duncan's multiple-range test).

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CHEMICAL MEDIATION OF BEHAVIOR IN HERMIT CRABS: ALARM AND AGGREGATION CUES

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Abstract—Field studies with three common local species of hermit crabs, *Clibanarius vittatus*, *Pagurus longicarpus*, and *Pagurus pollicaris*, showed that these crabs responded behaviorally to chemicals originating from crushed conspecifics. Hermit crabs are attracted specifically and in a manner similar to previously reported crab responses to odors from dead gastropods. Responses by *C. vittatus* to both kinds of odor are of three types: (1) aggregation/shell investigation responses (previously reported for odors from dead gastropods), characterized by increased locomotor activity, investigation of shells in the vicinity, and switching into empty shells; (2) alarm responses, in which crabs flee the area; and (3) withdrawal responses, in which crabs pull into their shells and do not come out. Studies with *C. vittatus* showed that the stimulatory chemicals originate from hemolymph, are less than 500 D, adsorb to octadecyl silica, and are recovered by elution with 20% methanol. Responses of *C. vittatus* are dependent upon crab size, type of shell occupied, and shell fit. Chemicals originating from dead conspecifics provide a forum for shell acquisition by crabs in relatively small shells and alarm by crabs in relatively large shells.

Key Words—Hermit crabs, shell fit, aggregation cues, alarm cues, anomuran, chemical sensing, *Pagurus*, *Clibanarius*, behavior.

INTRODUCTION

In soft-bottom environments such as the sounds and estuaries of the southeastern Atlantic and Gulf of Mexico coasts of the United States, gastropod shells are

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important as hard substrata (McLean, 1983). The gastropods that produce the shells, predators that empty the shells, and secondary shell users such as epibionts, octopus, fish, and hermit crabs form a community described as the gastropod shell habitat web (McLean, 1975; 1983). Hermit crabs are central in maintaining the shell habitat web. They locate new shells by chemicals given off by the dying gastropod (Rittschof, 1980a). Occupation of the shell ensures that it is not lost to the community by burial in the sediment.

Shells are an important resource for hermit crabs (reviewed by McLean, 1983; Spight, 1985). Shells may control hermit crab population size (Provenzano, 1960; Childress, 1972; Vance, 1972; Bach et al., 1976; Kellogg, 1976; Spight, 1977). With respect to individuals within the population, shells are central to protection and growth (Fotheringham, 1976; Spight, 1985), fecundity (Childress, 1972; Bach et al., 1976; Wilber, 1989), and even copulatory success (Hazlett, 1989; Hazlett and Baron, 1989).

In addition to obtaining shells at gastropod predation events (McLean, 1974, 1975, 1983; Rittschof, 1980a; Hazlett and Herrnkind, 1980; Wilber and Herrnkind, 1982, 1984; Gilchrist and Abele, 1984), crabs find empty shells that resurface after being buried and actively interact inter- and intraspecifically to trade shells (Hazlett, 1966, 1970a,b, 1972, 1983).

Shells could be lost to hermit crab populations through two major pathways: (1) degradation due to erosion, fouling, and shell-crushing predators (see McLean, 1983) and (2) loss of the shell by burial upon the death of the hermit crab occupant. Although degradation of shells is an irreversible process, the ability to recycle occupiable shells that became available through the death of a hermit crab could be a very important means of maintaining the resource. Chemical detection of dead and dying crabs could occur and would be similar to the chemical detection of dying snails (Rittschof, 1980a). Detection of chemicals from dead and dying hermit crabs could enable efficient location of potential shells and would ensure that reusable shells were maintained in the population.

We report here that hermit crabs can chemically locate crushed conspecifics in the field and are stimulated to perform shell acquisition behaviors. The responses of hermit crabs to odors from crushed conspecifics and from fresh conspecific hemolymph are similar to responses to gastropod odors. As is the case for odors from dying gastropods, responses of hermit crabs to dying hermit crabs are highly specific. Although a complex issue (cf. Kuris and Brody, 1976; Liszka and Underwood, 1990; Wilber, 1990), shell fit is important in responses to both odors. Our studies show that small hermit crabs in poor-fitting shells (small shells) are attracted by odors and stimulated to investigate shells in the area. Small crabs in large shells are repelled and flee the area. Crabs in large shells do not respond actively odors. We suggest that hermit crabs actually spend little time in the undirected search for shells. Chemical cues function to stimulate aggregation of crabs "in the market for a new shell" and act as alarm substances

for crabs that are in shells that fit "correctly." As observed by McLean (1974, 1983), Rittschof (1980a), and Chase et al. (1988), the introduction of a single new shell into an aggregation of hermit crabs that is chemically primed (Hazlett, 1979; Rittschof, 1980a) results in a cascade of shell exchanges that may benefit many crabs in the area that are in shells smaller than the introduced shell.

METHODS AND MATERIALS

Reagents and Materials

Seawater used in preparation of odor sources was filtered septically through a 100-kD filter, allowed to stand for a minimum of 3 days, and gravity-filtered through a sterile 0.22-μm filter immediately before use. HPLC-grade methanol and water were from Mallinckrodt, Inc., Paris, KY. Dialysis tubing was 20.4 mm in diameter, 12,000- to 14,000-D cutoff (Spectrum Medical Industries, Inc., Los Angeles, CA). Pressure dialysis membranes were 43-mm-diameter YM2 1000-D cutoff (Amicon Inc., Lexington, MA). Reversed-phase columns were 500 mg octadecyl silica Solid Phase System product 9004 (Burdick and Jackson Inc., Muskegon, MI) .

Test odors were from blood and body tissues. The amount of tissue used to generate odors for bioassays (see below) was based upon weight of both freshly killed and frozen and subsequently thawed tissues.

Gastropod tissues were selected that were known to be attractive to the hermit crabs being tested and included *Busycon carica* (attractive to *C. vittatus*), *Ilyanassa obsoletus* (attractive to *Pagurus longicarpus*), and *Fasciolaria hunteria* (attractive to *P. longicarpus*, *C. vittatus*, and *P. pollicaris*). Body tissue from all three species of hermit crabs and the brachyuran crab *Panopeus herbstii* was also tested. A mass of 3 g of tissue was chosen as a standard sample weight. For large gastropods *Busycon carica* and *Fasciolaria hunteria*, only foot muscle tissue was used. It was collected as three 1-g cubes, approximately 0.5 cm on a side. For the smaller *Ilyanassa obsoleta* we used the flesh of an entire snail without its shell. Approximately 30 *I. obsoleta* provided 3 g of crushed flesh for each experiment. Flesh was not separated from crushed shell. For hermit crabs smaller than 3 g (*P. longicarpus*), several crab bodies totaling the sample weight were crushed and used. Hermit crabs greater than 3 g in body weight (*P. pollicaris* and *C. vittatus*) were sectioned from anterior to posterior to give the correct weight. Hermit crab muscle (*C. vittatus*) was obtained from autoclaved chelae. Exoskeleton was removed, and muscle was blotted and weighed.

Hemolymph was obtained from a major sinus in the chelae. Shells of large *Clibanarius vittatus* were chipped down until the chelae were exposed. The chelae were extended and hemolymph was drawn from the sinus of the major chelae with a 20-gauge needle on a 1-ml tuburculin syringe. The needle was

inserted through the thin cuticle at the second joint from the distal end of the chelae into the hemolymph sinus. Depending on the size of the hermit crab, 0.2 to 1.2 ml of hemolymph was extracted from the sinus.

Hemolymph Fractionation

Nine milliliters of hemolymph was collected and pooled from approximately 25 hermit crabs. Blood cells were removed by centrifugation for 2 min at 1500*g* in a microfuge. One milliliter of hemolymph supernate was saved for assay. Hemocyanin was removed from the remaining 8 ml of hemolymph supernate by centrifugation for 4 hr at 319,000*g* (Beckman TL-100 ultracentrifuge and a TLN-100 Rotor). Five milliliters of supernate was collected and mixed with 5 ml of 100-kd filtered seawater sterilized by gravity filtration through a 0.22-μm membrane filter. The 10 ml of diluted supernate was subjected to pressure dialysis through an Amicon YM 2 (1000-D cutoff) membrane at 60 psi of nitrogen. The first 9 ml through the 1000-D membrane was frozen at $-20°C$ in 1-ml aliquots. Molecules in the >1-kDa fraction were reduced by washing. The 1 ml remaining above the 1000-D membrane was diluted with 5 ml of 0.22-m seawater and reduced to 1 ml three times. The material above the membrane was then diluted to 10 ml with sterile seawater and stored frozen at $-20°C$ in 1-ml aliquots.

Hemolymph factors less than 1000 D were separated on octadecyl silica. Octadecyl silica (500 mg) was prepared for use by washing with 3 ml of 100% methanol and then 3 ml of HPLC-grade water. All of the liquid was extracted with a 3-ml syringe. For each experiment, 1 ml of sample of <1000-D hemolymph was applied to the column. All liquid was pulled through the column by syringe. Next, depending upon the experiment, 1 ml of a test solvent was applied to the column and the procedure repeated. All of the liquid was drawn through with the syringe. Extracted materials were tested in interaction bioassays (see below).

In Situ *Observations of Hermit Crab Behavior in Response to Odors from Crushed Conspecifics*

A preliminary experiment showed that *C. vittatus* were attracted to freshly killed conspecifics. In tests with killed crabs their shells were crushed and placed in a wire-mesh cage so as to prevent removal of the carcass by scavengers. Crushed hermit crabs were placed in approximately 30 cm of water in areas inhabited by conspecific hermit crabs. Crab densities are so low that fewer than one crab would normally pass through the observation area in an hour (Kratt and Rittschof, 1991). The behavior of attracted hermit crabs was identified as feeding or investigatory. Observers stood motionless next to the odor source and recorded behavior of crabs in the immediate vicinity as well as down current

from the source. In a final experiment, a large female hermit crab approximately 40 mm in cephalothorax (carapace length) was taken out of her shell, crushed, and placed in a tide pool being drained by a tidal channel. The female crab was placed in the pool shortly after the tide began to ebb. *C. vittatus* that became active in the tide pool and that moved into the tide pool up the tidal channel were observed. Responding hermit crabs, as determined by shell investigation behavior (see below), were collected and their sex was determined. Observations of crabs in tide pools at low tide showed that most were normally inactive. Crabs did not travel up tidal channels unless chemically stimulated to do so.

Observation of Responses of C. vittatus *in Response to Crushed Conspecifics in the Absence and Presence of an Empty Shell*

Tide pool conditions were simulated by placing 10 freshly collected small- to medium-sized (crabs mainly in *Ilyanassa*, *Littorina*, and oyster drill shells; see last paragraph of Methods and Materials) *C. vittatus* in a bucket containing 3 L of seawater from the environment. After a 15-sec acclimation interval, shell investigation was observed for 3 min. Shell investigation is characterized by the manipulation of other shells with the chelipeds and walking legs and the placement of the legs, chelipeds, and head into the apertures of other shells. Next, a crushed *C. vittatus* was added to the bucket and shell-investigation activity was observed for an additional 3 min. Finally, an empty shell was added and observations of shell investigation and shell switching were made for a period of up to 20 min. When a hermit crab switches shells, it leaves its original shell and enters an empty shell, abdomen first. A total of 50 hermit crabs was tested.

Biological Assays

Three kinds of biological assays described in detail below were used in field trials to test responses of crabs to odors. The first assay, an attraction assay, was a modification of previous hermit crab field assays (Rittschof, 1980a,b; Rittschof et al., 1990; Kratt and Rittschof, 1991) (see below). This assay measured the ability of crabs to find hidden odor sources. The second assay measured chemically stimulated interactions between groups of crabs. The third assay tested chemically stimulated responses of hermit crabs in depressions left by feeding stingrays. This assay was used to separate crabs into specific behavioral categories: (1) no response, (2) investigation behavior, and (3) flight.

Hermit Crab Attraction Assay. Field assays, modified after Kratt and Rittschof (1991), measured responses of hermit crabs to potential sources of attractants. Tests were conducted with *Clibanarius vittatus*, *Pagurus longicarpus*, and *Pagurus pollicaris*. An individual test unit consisted of a 500-ml disposable cup covered with fiberglass window screen, secured with a rubber band. Cups, three

or four for each test substance, were buried along a transect line parallel to the shore 2–3 m apart in approximately 25 cm of seawater, with the screen tops flush with the surface of the sand. Cups contained test substances or were empty (controls). Treatments were alternated in a repeating series. The observer monitored the series of cups by walking the transect slowly. A positive response was recorded when a crab touched the window screen. Responding crabs were captured and held until the end of the 45-min assay. This minimized social interactions and ensured that each crab was counted only once. When a responding crab was captured, other crabs in the area pulled into their shells but returned quickly to normal activity. Frequency distributions of crabs responding to the different treatments were compared using the G statistic [Goodfit (Sokal and Rohlf, 1981)].

Hermit Crab Interaction Bioassay. Observations of behavior of *Clibanarius vittatus* in response to chemicals in tide pools were used to develop a bioassay of hermit crab interaction. Preliminary observations in tide pools and in buckets showed that crabs investigated and switched shells but did not feed. The bioassay used a plastic 12-L bucket as an artificial tide pool. Ten freshly collected *C. vittatus* were placed in a bucket filled with 3 L of seawater. The hermit crabs were then separated manually from each other so that no two were touching. The water was mixed gently, and after 15 sec the hermit crabs were observed for 1 min. Each hermit crab that investigated another hermit crab's shell by touching it with the chelae and first pair of walking legs was scored as an investigator. Each hermit crab was scored only once, and all of the hermit crabs were left in the bucket for the full minute. Next a control value was determined by following the above procedure, with the addition of 100 μl of seawater after the hermit crabs were separated. An experimental trial was then run using the same procedure as the control, with 100 μl of test solution replacing the seawater. Preliminary test odors were presented in all conceivable sequences to determine if order of presentation had an effect. When no effect was observed an ordered sequence of presentation was chosen for simplicity.

Test solutions included 100-μl additions of snail flesh odors, 3 g flesh in 5 ml seawater, crab flesh, hemolymph, and fractionated hemolymph. Three groups of 10 crabs were tested for each test solution. The proportion of crabs responding before addition was compared to the proportion after seawater addition and the proportion responding after seawater addition was compared to the proportion responding after test addition [Z statistic; proportions test (Walpole, 1974)].

Hermit Crab Behavior Assay. To perform a behavior assay, tide pools (0.3–0.5 m in diameter and 2.5 to 10 cm deep) resulting from stingray feeding activity were located. *C. vittatus* were collected haphazardly. Ten to 20 hermit crabs were placed in each tide pool and 0.4 ml of seawater containing *Busycon carica* odor (6 g *B. carica* flesh soaked in 10 ml seawater for 1 hr) was injected into

each pool. Hermit crabs that investigated the shells of other hermit crabs with their walking legs and chelipeds were scored as investigating. Hermit crabs that left the tide pool and walked onto land were scored as fleeing. Hermit crabs that were inactive, that partially buried, or that did not come out of their shells were scored as not responding. In initial experiments, crabs were divided only into categories of "investigating" and "not investigating." In early assays, the "not investigating" group included crabs that either fled or did not respond.

Sex and Shell Fit

Sex and shell fit were determined for hermit crabs attracted to snail flesh in attraction assays. Sex, shell species, carapace length, and shell weight were recorded for each hermit crab. Shells occupied at a low frequency were grouped into categories based upon relative size and shape. The category "oyster drills" was comprised of shells from *Urosalpinx cinerea*, *Eupleura caudata*, and *Pisania tincta*. The "other" category was a mixture of medium- to large-sized shells including shells from *Polinices duplicatus*, *Thais haemostoma floridana*, *Busycon carica*, *Busycon contrarium*, and *Busycon canaliculatum*. The ratio of carapace length to shell weight was used as a measure of shell fit. This ratio has been shown previously to discriminate between crabs attending simulated predation sites and crabs that do not attend (Rittschof, 1980a). Carapace length was measured to the nearest 0.1 mm with vernier calipers. Shells were dried at 60°C for 3 hr and weighed to the nearest 0.1 g.

RESULTS

Preliminary Observations

In preliminary experiments, observations of hermit crab responses to crushed conspecifics were made for *P. pollicaris*, *P. longicarpus*, and *C. vittatus*. All species of hermit crabs responded similarly to crushed conspecifics. The description that follows of responses by *C. vittatus* to a crushed *C. vittatus* is representative of the observations that were made.

A large female *Clibanarius vittatus* approximately 40 mm in cephalothorax length was removed from its shell, killed by crushing, and placed in approximately 2 cm of water on a descending tide in an area containing inactive hermit crabs. Nearby *C. vittatus* became active and were attracted immediately. Directed movements were observed toward the dead hermit crab from as far as 10 m downstream from the source! (These crabs eventually reached the source.) While moving to the source, the hermit crabs crawled upcurrent in and out of the small pools of water. *C. vittatus* reaching the crushed crab did not feed on the flesh. Most did not go directly to the crushed crab source. They investigated shells in

the area including oyster shells and shells occupied by other hermit crabs. The hermit crabs manipulated the shells, turned them over, and tried to crawl into them. Most of the hermit crabs that responded were in small gastropod shells or were in heavily fouled shells. Crabs of both sexes at a 1 : 1 ratio, including ovigerous crabs, were attracted to both sexes of crushed conspecific [$G = 0.15$, $P > 0.05$, 1 df, H_0 1 : 1 ratio (male to female) for crabs responding to a crushed female and $G = 1.0$, $P > 1.05$, 1 df, H_0 1 : 1 (male to female) ratio for crabs responding to a crushed male].

In a final, preliminary experimental series, freshly collected *C. vittatus* were placed in groups of 10 in buckets containing 3 L of seawater and their behavior in response to odor additions was observed. Without chemical additions, approximately one-third of the hermit crabs manipulated and investigated other's shells. The addition of crushed *C. vittatus* immediately resulted in elevated levels of locomotor activity. From 75 to 100% of the hermit crabs displayed shell-seeking activity. Overall, small *C. vittatus* were visibly more active than large *C. vittatus*. After an empty shell was placed in the bucket, one to three hermit crabs smaller than the added shell immediately began to investigate it. The remainder of the crabs continued to investigate each other's shells. When only one hermit crab investigated the shell, it (1) immediately switched shells, (2) displayed repeated shell-search activity on the empty shell and then switched, (3) switched and then switched back to its original shell, or (4) repeatedly investigated the empty shell but did not switch. If there was more than one hermit crab, the crabs fought until one of them managed to switch into the empty shell. The same series of events occurred with each newly vacated shell until the smallest shell in the bucket was empty. Thus, the suggestion from preliminary observations was that hermit crab responses to crushed conspecifics were chemically mediated and involved behaviors associated with shell acquisition.

Hermit Crab Attraction Assays

Three attraction assays with concealed odor sources were used to assess the responses of *C. vittatus*, *Pagurus longicarpus*, and *Pagurus pollicaris* to chemical cues from a series of flesh sources. The test items were crushed hermit crabs of each species, snail flesh known to be attractive to one or more species of hermit crab, and a crushed brachyuran crab *Panopeus herbstii*. Crabs responding by locating and touching the concealed odors were collected and scored as responding (Table 1). Each species of hermit crab responded significantly (P at least < 0.05 compared to blank control) to odors from gastropod flesh whose shell they occupied and to crushed conspecifics. Assay 1 tested responses of *C. vittatus* to four odor sources and blanks. Of the 36 crabs responding, 14 were attracted to crushed conspecific and 22 were attracted to snail odor. No *C.*

TABLE 1. ATTRACTION ASSAY RESULTS[a]

Assay No.	Hermit crab responding	Odor source										
		C.v.	P.h.	P.l.	P.p.	F.h.	I.o.	*B*	H_o	df	*G*	*P*
1	*C. vittatus*	14	0	0	0	22		0				
	C. vittatus	14		0	0	22			1:1:1:1	3	50.05	<0.001
	C. vittatus	14				22			1:1	1	1.77	<0.2
2	*P. longicarpus*	6		48	12			0				
	P. longicarpus	6		48	12				1:1:1	2	44.31	<0.001
	P. pollicaris	6		2	9			0	1:1:1	2	4.66	<0.1
3	*P. longicarpus*	0		53	10		87		1:1:1:1	3	153.81	<0.001
	P. longicarpus			53			87		1:1	1	8.3	<0.01
	P. pollicaris	0		0	4		0		1:1:1:1	3	9.78	<0.05

[a]C.v., *Clibanarius vittatus*; P.h., *Panopeus herbstii*; P.l., *Pagurus longicarpus*; P.p., *Pagurus pollicaris*; F.h., *Fasciolaria hunteria*; I.o., *Ilyanassa obsoleta*; *B*, cup and screen, no added order; H_o, null hypothesis.

vittatus responded to crushed *P. herbstii* or to the blank control. *C. vittatus* was not attracted to either of the crushed pagurid species. Assays 2 and 3 were conducted at night in a region occupied by *P. longicarpus* and *P. pollicaris*. In assay 2, 66 *P. longicarpus* and 17 *P. pollicaris* responded. No crabs responded to the blank controls. Four times as many *P. longicarpus* responded to crushed conspecific odor as to crushed congeneric odors. Twice as many *P. longicarpus* responded to crushed congeneric odor (*Pagurus pollicaris*) as to crushed *C. vittatus* odor. *P. pollicaris* are much less common on the test area, and as a consequence, only one-quarter as many *P. pollicaris* responded. The strongest response of *P. pollicaris* was to crushed conspecific odor.

In assay 3 crushed *Ilyanassa obsoleta* was added to the test series (Table 1). One hundred fifty *P. longicarpus* and four *P. pollicaris* responded. Of the *P. longicarpus*, 58% responded to *Ilyanassa obsoleta* odor and 35% responded to crushed conspecific. The remaining 7% responded to crushed congeneric odor. No crabs in this experiment responded to odor from crushed *C. vittatus*. Regressions of shell weight vs carapace length were similar for the crabs responding to *I. obsoleta* and *P. longicarpus* odors. Carapace-to-shell length ratios were computed for the 87 crabs attracted to *I. obsoleta* and the 53 crabs attracted to *P. longicarpus* odors. Carapace length-to-shell weight ratios were compared by frequency and found to be similar ($G = 4.94$, $P > 0.05$, 4 df; Figure 1). Thus, shell fit of crabs responding to gastropod odors was similar to that of crabs responding to conspecific hemocyanin. Only four *P. pollicaris* responded in assay 3 and they were attracted to the odor of crushed conspecific.

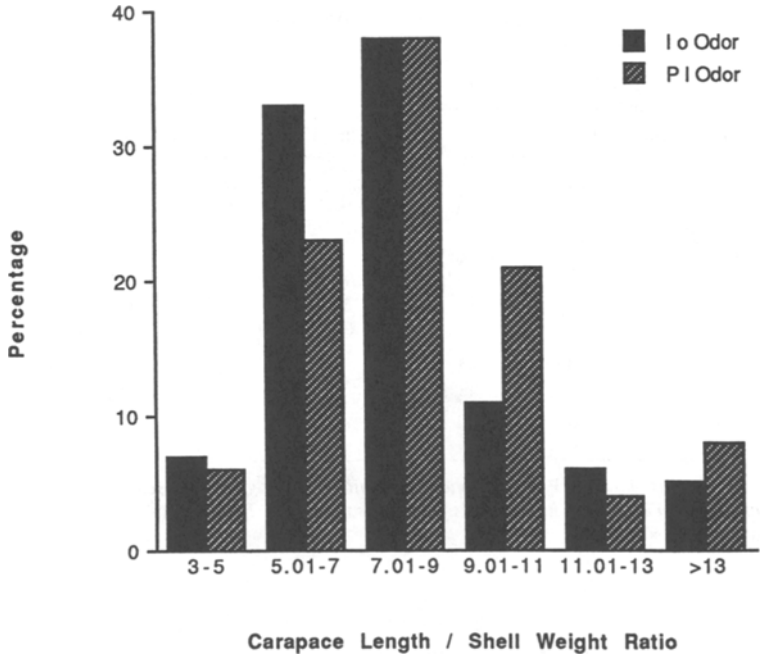

FIG. 1. Frequency distributions of carapace length-to-shell weight ratios for *P. longicarpus* attracted to *Ilyanassa obsoleta* (I o) or to crushed *P. longicarpus* (P l). Virtually all crabs occupied *Ilyanassa obsoleta* shells. Low ratios indicate relatively large shells for the size of the crab.

Shell Occupation by C. vittatus

A fourth assay tested attraction of *C. vittatus* to simultaneous presentation of odors of frozen and thawed *F. hunteria* and *B. carica* flesh, crushed conspecifics, and blanks. In this experiment 96 cups, 24 of each type, were established simultaneously in habitats around the Duke University Marine Laboratory (Beaufort, NC). Sites were monitored for 45 min. At the termination of the experiment every hermit crab within 10 m of the cups was collected and scored as a nonresponder. This search was conducted by 12 people for 20 min. In total, 215 crabs responded by touching a hidden odor source and 73 did not respond. *C. vittatus* were attracted in similar numbers to all three flesh odor sources (H_0, 1:1:1; $G = 1.099$, $P > 0.05$, 2 df).

Frequency distributions of shells occupied by *C. vittatus* attracted to crushed *Clibanarius vittatus*, *Fasciolaria hunteria*, and *Busycon carica* flesh were compared (Figure 2). Shell species distributions for crabs attracted to *C. vittatus* and *F. hunteria* were similar ($G = 1.6$, $P > 0.05$, 4 df). Shell species distri-

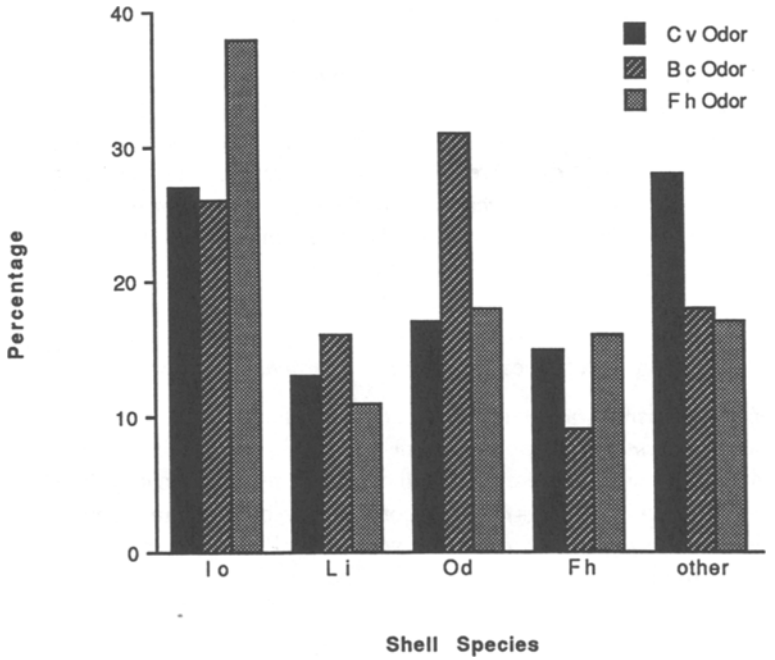

FIG. 2. Shell occupation by *Clibanarius vittatus* attracted to *C. vittatus* (C v) odor, *Busycon carica* (B c) odor, and *Fasciolaria hunteria* (F h) odor. Shell species occupied by the crabs are ordered from smallest, I o (*Ilyanassa obsoleta*), to largest, other (mainly *Busycon* spp. and including *Polinices duplicatus, Murex ponoma, Pleuroploca gigantea*, and *Phalium granulatum*). L i—*Littorina irrorata*; O d—(*Pisania tincta, Urosalpinx cinerea*, and *Eupleura caudata*; F h—*Fasciolaria hunteria*. The "oyster drill" and "other" categories were constructed by combining relatively high frequencies of *P. tincta* and *B. carica*, respectively, with low frequencies of similar-shaped shell species (mainly *U. cinerea* for the oyster drill category and mainly *B. canaliculatum* and *B. contrarium* for the "other" category).

butions for crabs attracted to *C. vittatus* and *B. carica* flesh were not similar ($G = 9.9$, $P < 0.05$, 4 df). Proportionately more crabs in *F. hunteria* shells were attracted to *C. vittatus* odors, and proportionately more crabs in oyster drill shells (mainly *Pisania tincta*) were attracted to *B. carica* odor. Shell species distributions for crabs attracted to *F. hunteria* and *B. carica* were also significantly different from each other ($G = 13.785$, $P < 0.05$, 4 df). Proportionately more crabs in *I. obsoleta* shells were attracted to *F. hunteria* odors and proportionately more crabs in oyster drill shells were attracted to *B. carica* odors (Figure 2). Thus, in more rigorous study of crab shell species in response to odor, some differences in shell occupation behavior by crabs attending *B. carica*

sites as compared to crabs attending crushed *C. vittatus* or *F. hunteria* sites were observed.

Shell occupations of the groups of *C. vittatus* not responding and responding to odors of *C. vittatus* and *B. carica* were compared. Hermit crabs that responded to odors occupied different shell types than nonresponders (G = 107.839, $P < 0.01$, 4 df). Over 80% of the nonresponding crabs were in large shells such as *Fasciolaria hunteria* and *Busycon* spp. Approximately 33% of attracted crabs were in these larger shells. Thus, there were extreme differences in shell occupation frequencies between responsive and unresponsive crabs (Figure 3).

Responses to Busycon carica *Odor in the Interaction Bioassay*

Busycon carica, a potent hermit crab attractant in field tests, was tested in interaction bioassays. Response before the addition of seawater did not change significantly after the addition of 100 μl of seawater [approximately 30% response (Z = 1.04, $P > 0.05$, n = 30)]. In contrast, the hermit crabs responded to the addition of 100 μl of *B. carica* odor with a significant increase in shell investigation activities [approximately 70% response (Z = 12.98, $P < 0.01$, n = 30)].

Responses to Odors from Panopeus herbstii *and* Clibanarius vittatus *Hemolymph and* C. vittatus *Muscle in Interaction Bioassays*

The response of *C. vittatus* was measured with seawater, freshly drawn hemolymph, and odor from hermit crab chelae muscle. Responses to the addition of seawater and *P. herbstii* hemolymph were similar (Z = 0.55, $P > 0.05$, n = 30). Compared to seawater, *C. vittatus* hemolymph significantly increased investigation (Z = 12.97, $P < 0.01$, n = 30). *C. vittatus* claw muscle odor did not increase investigation (Table 2).

Shell Occupation by Investigating and Noninvestigating Crabs in the Interaction Bioassay

An interaction assay using *B. carica* odor and *C. vittatus* hemolymph was performed, with the goal of dividing a large number of haphazardly collected *C. vittatus* into behavior classes so that behavior could be analyzed with respect to shell occupation.

Using the interaction assay hermit crabs were divided into 126 responders and 181 nonresponders. Of the 126 responders, 71 responded to *B. carica* odor and 55 responded to *C. vittatus* odor. Of the nonresponders 96 were to *B. carica* odor and 85 to *C. vittatus* odor. The frequency of responders and nonresponders was similar to both odors (H_0 1 : 1, G = 0.6, 1 df, ns). Shell species distributions

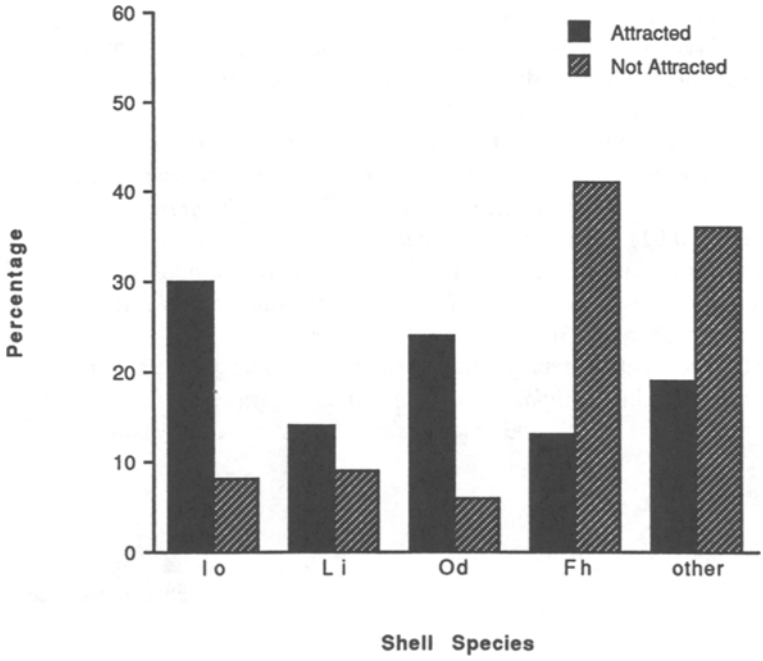

FIG. 3. Shell occupation by *Clibanarius vittatus* attracted to odors in Fig. 2 and in the area but not responding. Shell species are ordered from smallest, I o (*Ilyanassa obsoleta*), to largest, other (mainly *Busycon* spp. and including *Polinices duplicatus, Murex pomona, Pleuroploca gigantea*, and *Phalium granulatum*). L i—*Littorina irrorata*; O d—(*Pisania tincta, Urosalpinx cinerea*, and *Eupleura caudata*; F h—*Fasciolaria hunteria*.

TABLE 2. *C. vittatus* RESPONSE TO HEMOLYMPH FROM *C. vittatus* AND *P. herbstii* AND *C. vittatus* CLAW MUSCLE ODOR

Condition	*n*	Percentage responding	*Z* value	*P*
100 μl seawater	30	40		
100 μ *P. herbstii* hemolymph	30	37	0.55	NS
100 μl seawater	30	33		
100 μl *C. vittatus* hemolymph	30	73	12.97	<0.01
100 μl seawater	30	40	1.13	NS
100 μl *C. vittatus* claw muscle odor	30	47	1.13	NS

of responding and nonresponding crabs were compared and found to differ significantly ($G = 108$, 4 df, $P < 0.01$). Shell species distributions of crabs responding to *B. carica* odor were significantly different from those responding to *C. vittatus* odor ($G = 10.5$, 4 df, $P < 0.05$). This difference was due in large part to responses of crabs in *F. hunteria* shells to *B. carica*. Frequencies of responders within the crabs tested against each odor occupying *F. hunteria* shells were compared. Significantly more crabs in *F. hunteria* shells ($G = 7.4$, 1 df, $P < 0.01$) responded to *B. carica* odor than to *C. vittatus* odor. Shell species distributions of crabs not responding to *B. carica* odor were not different than shell species distributions of crabs not responding to *C. vittatus* odor ($G = 2.8$, 4 df, $P > 0.05$).

Shell occupation data (Figure 4) show that nonresponders were comprised mainly of crabs in *Fasciolaria hunteria* and other large shells, while responding crabs occurred in mainly the smaller shells. Most striking was the response of

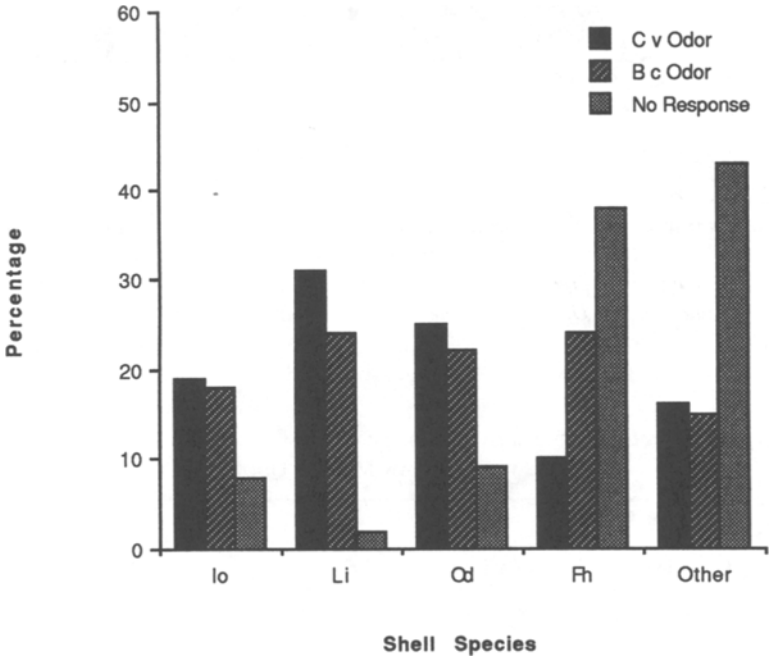

FIG. 4. Shell occupation by *Clibanarius vittatus* attracted to *C. vittatus* (C v) odor or *Busycon carica* (B c) odor and in the area but not responding. Shell species are ordered from smallest, I o (*Ilyanassa obsoleta*), to largest, other (mainly *Busycon* spp. and including *Polinices duplicatus, Murex pomona, Pleuroploca gigantea*, and *Phalium granulatum*). L i—*Littorina irrorata*; O d—*Pisania tincta, Urosalpinx cinerea*, and *Eupleura caudata*; F h—*Fasciolaria hunteria*.

crabs in *Littorina irrorata* shells. Of the 37 crabs in *L. irrorata* shells tested, 34 (92%) responded by investigating. This was in contrast to the response of crabs in the "other" (mainly *Busycon* spp.) shell category. Of the 64 crabs in this category tested, 87% did not respond.

Hermit Crab Behavior Assay

In tide pool behavior assays, *Busycon carica* odor was used to evoke response from *C. vittatus*. *B. carica* odor was added to a tide pool containing 10 to 20 hermit crabs. The crabs responded in one of three ways: (1) they investigated each other's shells and formed aggregations reminiscent of rugby scrums (Rittschof, 1980b), (2) they exited, or (3) they did not respond by either investigating or leaving. In the first series of tide pool assays hermit crabs in category 1 were labeled as "responders," while those in categories 2 and 3 were called "nonresponders." The distribution of shell types between responders and nonresponders was significantly different ($G = 12.401$, $P < 0.05$, 4 df). Significantly more responders were in small shells (*I. obsoleta*, *L. irrorata*), and oyster drill shells: $z = 3.68$, 3.41, and 3.40, respectively; $P < 0.01$ for all categories). Significantly more nonresponders were medium- to large-sized shells, such as *F. hunteria*, and other shell types, which include *Polinices duplicatus*, *Thais haemostoma floridana*, *B. carica*, *B. contratium*, and *B. canaliculatum* ($Z = 6.85$ and 3.76, respectively; $P < 0.01$ for both categories).

A second series of behavior assays with *Busycon carica* odor was used to divide crabs in tide pools into three behavior categories: (1) those that investigated, (2) those that fled the tide pool, and (3) those that did not respond. Carapace, shell species, and shell weight were determined for each behavior class. This series of assays divided 161 *C. vittatus* collected haphazardly into three behavior classes: (1) 37 crabs that did not respond, (2) 65 crabs that left the tide pools , and (3) 59 crabs that investigated. Binary comparisons of frequency distributions of shell species occupied, shell weight, and carapace length were made. Analysis showed highly significant ($P < 0.01$) differences between behavior classes for seven of the nine tests and significant ($P < 0.05$) differences for the other two measures (Table 3).

Inspection of the percentile data for the three behavior classes with respect to shell species occupied, shell weight and carapace length, and carapace length-to-shell weight ratios revealed patterns for each behavior group (Figure 5). Approximately 46% of nonresponding crabs occupied large shells (*Polinices duplicatus*, *Fasciolaria hunteria*, *Busycon* spp.); 21% of the crabs that left the pools occupied these same shell species, while only 7% of the crabs that investigated in response to odor occupied these shells (Figure 5A). Differences observed in shell species occupied were reflected in shell weight and carapace length for the three behavior categories (Figures 5B and C). Finally, comparison

TABLE 3. ANALYSIS OF ANIMAL SIZE AND SHELL WEIGHT FOR *C. vittatus* TIDE POOL ASSAY[a]

Condition	*G* value	df	*P*
Shell species			
No response vs left pool	18.0	3	<0.001
No response vs investigated	48.8	3	<0.001
Left pool vs investigated	11.0	3	<0.02
Shell weight			
No response vs left pool	11.9	3	<0.01
No response vs investigated	39.8	3	<0.001
Left pool vs investigated	9.5	3	<0.02
Carapace length			
No response vs left pool	17.1	3	<0.001
No response vs investigated	41.8	3	<0.001
Left pool vs investigated	6.7	3	<0.05

[a]The null hypotheses were that carapace length, shell weight, and shell species distribution were equal for all groups. Groups were determined by dividing the smallest frequency into four categories with no less than five observations in a category.

of frequency distributions of carapace length-to-shell weight ratios showed significant differences in all three behavior classes (all G values >6.7, all P's $<$ 0.05, all df = 3). Nonresponding crabs had the lowest carapace length-to-shell weight ratio, crabs that left had the next smallest ratio, and crabs that investigated had the highest ratio (smallest-sized shells relative to body size).

Characterization of C. vittatus *Hemolymph Factor*

A series of attraction and interaction assays was used to characterize the biological activities in crab hemolymph with respect to molecular size and adsorption/desorption characteristics on octadecyl silica.

An attraction assay compared hermit crab responses to blanks containing 5 ml seawater in dialysis bags (14-kDa cutoff) with responses to dialysis bags containing 5 ml seawater and 200 μl fresh hemolymph. Twenty-two *C. vittatus* were attracted to the hemolymph sites, as opposed to only one to seawater (G = 23.16, 1 df, $P < 0.001$).

Next, *C. vittatus* hemolymph was fractionated and biological activity determined in the interaction bioassays (Table 4). The blood was separated into three parts: (1) whole hemolymph, (2) hemolymph with molecules less than 1 kDa, and (3) hemolymph molecules greater than 1 kDa. Assays showed that 50- and 100-μl samples of whole blood contained significant biological activity (all Z values >3.52, $P < 0.001$, $n = 30$). The samples of molecules greater than 1 kDa showed low biological activity. One of six tests yielded a significant

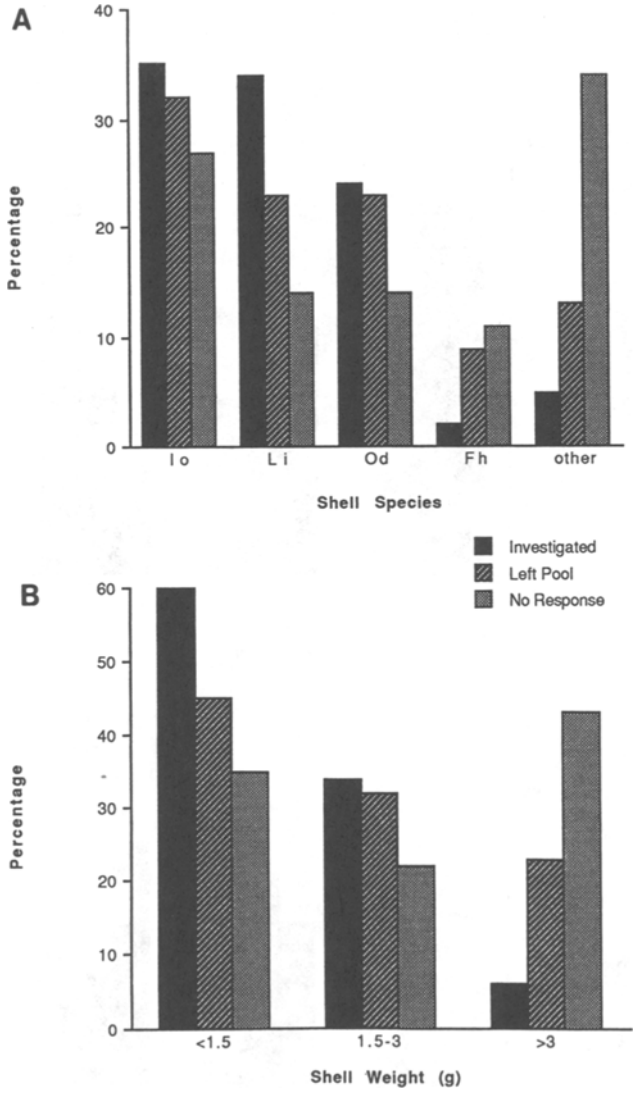

FIG. 5. Shell occupation, shell weight, crab carapace length, and carapace length-to-shell weight ratios of crabs divided into behavior classes in the behavior assay. (A) Shell occupation by *Clibanarius vittatus* that (1) investigated other shells, (2) left the pool, or (3) did not respond when *Busycon carica* odor was added to the pool. Shell species are ordered from smallest, I o (*Ilyanassa obsoleta*), to largest, other (mainly *Busycon* spp. and including *Polinices duplicatus, Murex pomona, Pleuroploca gigantea*, and *Phalium granulatum*). L i—*Littorina irrorata*; O d—*Pisania tincta, Urosalpinx cinerea*, and *Eupleura caudata*; F h—*Fasciolaria hunteria*. (B) Shell weights of the three behavior classes. Categories were determined by dividing the behavior class (no response) into three size groups at obvious breaks in the data. (C) Carapace lengths of the crabs in the three behavior classes. Categories were determined by dividing the behavior class (no response) into three size groups at obvious breaks in the data. (D) Carapace length-to-shell weight ratios.

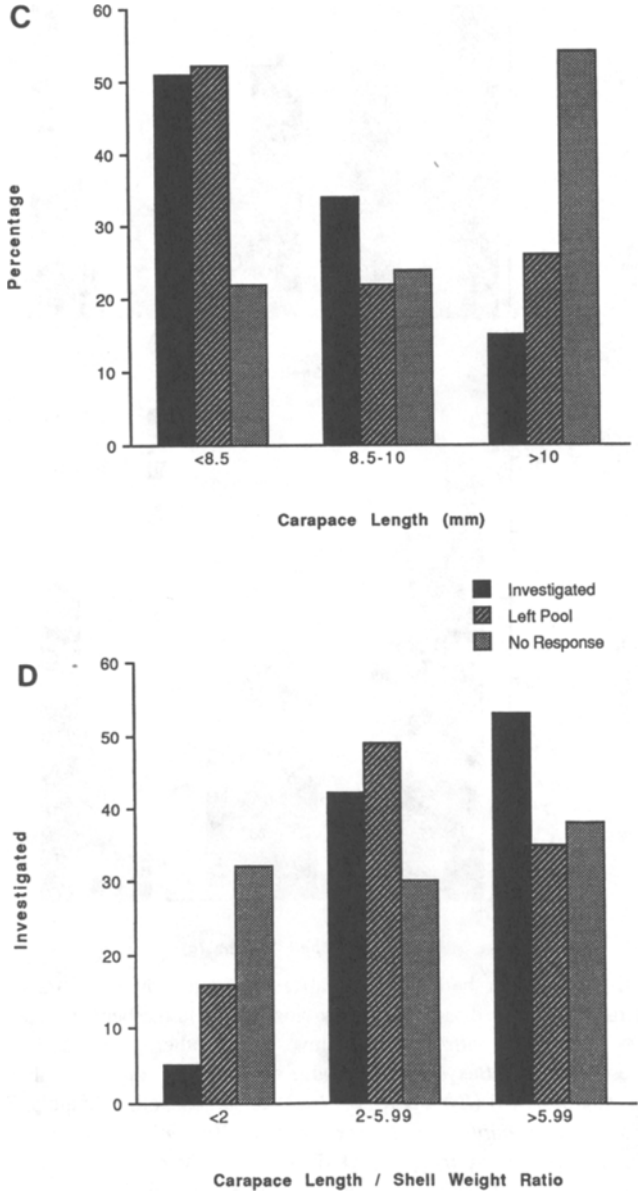

FIG. 5. Continued.

TABLE 4. *C. vittatus* RESPONDING TO *C. vittatus* FRACTIONATED BLOOD

Assay No.[a]	Condition	*n*	Percentage responding	*Z* value	*P*
1	Before addition	30	37		
	100 μl flow through blood	30	33	0.49	>0.05
1	Before addition	30	27		
	100 μl 100% methanol	30	47	3.15	<0.001
2	Before addition	30	33		
	100 μl seawater	30	37	0.49	>0.05
2	Before addition	30	30		
	100 μl flow through	30	43	2.09	<0.05
2	Before addition	30	33		
	100 μl seawater	30	37	0.49	>0.05
2	Before addition	30	37		
	100 μl 20% methanol	30	57	6.12	<0.001
2	Before addition	30	27		
	100 μl seawater	30	37	1.38	>0.05
2	Before addition	30	40		
	100 μl 100% methanol	30	50	2.47	<0.01
2	100 μl 20% methanol	30	57		
	100 μl 100% methanol	30	50	2.01	<0.05
3	Before addition	30	33		
	100 μl seawater	30	37	0.49	>0.05
3	Before addition	30	23		
	100 μl flowthrough	30	40	2.31	<0.05
3	Before addition	30	27		
	100 μl seawater	30	13	1.67	<0.05
3	Before addition	30	27		
	100 μl HPLC-grade water	30	27	0.00	>0.05
3	Before addition	30	20		
	100 μl seawater	30	27	0.84	>0.05
3	Before addition	30	33		
	100 μl 20% methanol	30	50	3.92	<0.001

[a] Every test with the same assay number was performed on the same group of crabs. The entire assay was performed as a continuous series.

response ($Z = 2.53$, $P < 0.01$). All of the samples of hemolymph molecules less than 1 kDa contained significant biological activity (all: $Z > 4.39$, $P < 0.001$, $n = 30$).

The attractant compound in the <1-kDa fraction of hemolymph was fractionated on octadecyl silane using a C18 column. One milliliter of the attractant compound was tested for biological activity in a *C. vittatus* interaction assay. All responses were measured to 100-μl additions of the samples. The first experiment showed that biological activity was retained by the column ($Z = 0.49$, $P > 0.05$, $n = 30$) and could be recovered by 100% methanol elution ($Z = 3.15$, $P < 0.001$, $n = 30$). In the second experiment <1-kDa hemolymph was applied to octadecyl silane. Most activity was retained but a measurable fraction passed through the column ($Z = 2.09$, $P < 0.05$, $n = 30$). Activity was recovered from the column with 20% methanol ($Z = 6.12$, $P < 0.001$, $n = 30$). Some activity was also recovered in the 100% methanol elution ($Z = 2.47$, $P < 0.01$, $n = 30$). More biological activity was present in the 20% methanol elution than in the 100% methanol elution ($Z = 2.01$, $P < 0.05$, $n = 30$). In the third experiment <1-kDa hemolymph was separated into unretained, HPLC-grade water elution and 20 methanol elutions; some material was unretained ($Z = 2.31$, $P < 0.05$, $n = 30$). Elution with HPLC-grade water did recover activity ($Z = 0$, $P > 0.05$, $n = 30$). Elution with 20% methanol recovered most of the biological activity ($Z = 3.92$, $P < 0.001$, $n = 30$). Thus, biological activity in hemolymph adsorbs to octadecyl silane, can be desalted with HPLC-grade water, and can be recovered with 20% methanol.

DISCUSSION

Hermit crabs respond to odors from crushed conspecifics in a manner similar to their previously demonstrated specific response to odors from dead gastropod flesh (McLean, 1975, 1983; Rittschof, 1980a). They have heightened activity, do not attempt to feed upon the carcass, and actively investigate all shells in an area. Hermit crabs attracted by odors from crushed conspecifics aggregate and a cascade of shell switching occurs when an empty shell of the correct size is introduced. All three species of hermit crabs tested are attracted most strongly to odors of specific dead snails (cf. McLean, 1975; Rittschof, 1980a; Herrnkind et al., 1981; Wilber and Herrnkind, 1982; Gilchrist and Abele, 1984; Rittschof et al., 1990) and to the odors of crushed conspecifics. The specificity of the response to crushed conspecifics is reflected in the fact that hermit crabs did not respond to odors from the hemolymph of brachyurans (see also Rittschof, 1980a). Of the hermit crabs tested, the pagurids showed the least specificity, responding at some level to odors from the hemolymph of all three

species of hermit crabs tested. *C. vittatus* was the most specific, responding only to odors from the hemolymph of conspecifics. *C. vittatus* did not respond to odors from *C. vittatus* muscle. There is no evidence for changes in behavior in *C. vittatus* response to food as reported in laboratory studies of *Pagurus pollicaris* (Hazlett, 1979). It is unclear at this time if the specificity of the response is related to similar cues from gastropod flesh and crab hemolymph.

Shell species composition and shell fit are similar for crabs responding to crushed conspecifics and to gastropod flesh. Although similar in size, the *C. vittatus* responding to crushed conspecifics and to *B. carica* occupied shell species in significantly different frequencies. However, differences in shell occupation in response to *B. carica* odor and to *C. vittatus* odor were opposite in the attraction assay and in the behavior assay. Because shells are such an important resource [see Reese (1963) and Spight (1985) for a comprehensive review of the older ecological and behavioral literature on shell fit and Wilber (1990), Hazlett (1990), and Liszka and Underwood (1990) for more recent literature], a variety of factors associated with the shell (Kuris and Brody, 1976; Wilber, 1990) and with the physiological state of the crabs may be involved. These factors warrant further study.

The above observations indicate that odors from crushed conspecifics are potent cues for shell availability. The attraction and resultant aggregation and behavior of crabs are similar to those evoked by shell availability cues from dead and dying gastropods (McLean, 1973, 1974, 1975, 1983; Rittschof, 1980a,b; Hazlett and Herrnkind, 1980; Lepore and Gilchrist, 1988; Gilchrist and Abele, 1984; Rittschof et al., 1990; Kratt and Rittschof, 1991). Thus, specific chemical cues signal the availability of previously used shells from dying hermit crabs just as they signal the availability of new shells from dying gastropods. This suggests that knowledge of hermit crab mortality and its mechanisms is important in assessing the flow of shells through hermit crab populations. Crab mortality could be important in ecological studies of resource use and in the development concepts of shell utilization (cf. Abrams, 1980; Spight, 1985; Chase et al., 1988; Liszka and Underwood, 1990; Wilber, 1990).

In general, *C. vittatus* in large shells were not attracted by odors from either snail flesh or crushed hermit crabs. Large *C. vittatus* did not respond in the interaction bioassay. In the tide pool behavior assay, most larger crabs remained withdrawn and did not respond by investigating or by leaving. This was true even when chemicals were applied directly to the crabs. Finally, shell species occupation by crabs that did not respond and shell fit data suggest that crabs that did not respond were in different species of shells and that they were relatively large crabs in larger shells. These data do not support the hypothesis of Gilchrist and Abele (1984) that chemicals from predation sites somehow signal the size of the prey snail. This suggests that, in spite of the fact that the

only empty shells found during this 5-week study were whelk shells too large and shells too small to be occupied by *C. vittatus*, shells may not be a limiting resource for large *C. vittatus*.

That shells are not limiting for larger crabs, or that most larger crabs do not respond to chemical shell availability cues, explains the observations of Gilchrist and Abele (1984) that classical sampling techniques and chemical attraction techniques resulted in very different population estimates. These observations also explain the discrepancy reported in conclusions on shell fit of responding crabs between Hazlett and Herrnkind (1980) and Rittschof (1980a). Hazlett and Herrnkind (1980) studied shell fit of *C. vittatus* coming within 1 m of simulated gastropod predation sites and reported that responding crabs were in shells similar to the population at large. In contrast, Rittschof (1980a) required that responding hermit crabs touch a 5-cm-diameter target and found differences between shell occupation of crabs responding and that of crabs not responding. The response criteria of Hazlett and Herrnkind (1980) appear to have included large crabs in the area that were actually unresponsive to the cue but that happened to be near the site, while Rittschof (1980a) did not. Thus, minor differences in methods appear to explain this discrepancy in the literature.

In addition to attracting hermit crabs, dead snail and crushed conspecific odors induce increased shell investigation activity. When odors are presented to crabs in tide pools, crabs behave in one of three ways: (1) increasing activity and shell investigation, (2) fleeing onto the land, and (3) remaining inactive. Shell occupation distributions, size of shell patterns, size [carapace length = SHE (Wilber, 1990)], and frequency distributions of shell fit ratio are all significantly different for all three behavior categories. Crabs that are active and investigate shells are smaller crabs in relatively small shells. Crabs that flee the odor onto land are small crabs in relatively large shells. Crabs that are unresponsive are large crabs in large shells. These data support the concept of Herrnkind et al. (1981) that predation sites are gathering areas for shell exchange and the chemical attractants are aggregation cues.

Crabs in low-quality shells responded to aggregation cues. The possibility of trading shells with other attendants gives crabs a potentially large selection of shells. Small crabs in high-quality shells actively leave the area. Leaving is theoretically advantageous because it reduces the likelihood of an encounter with another crab or a predator that could result in the loss of the good shell. Spight (1985) proposes that larger crabs get larger shells through the encounter of larger crabs in smaller shells with smaller crabs in larger shells. The lack of response of large crabs in large shells suggests that the most advantageous response for the larger crabs is to remain withdrawn in their large and relatively roomy shells.

Shell cues for each species of hermit crab are specific, originating from the flesh of different species of gastropods (Rittschof, 1980b), not gastropod he-

molymph (Kratt and Rittschof, 1991), and from the hemolymph of conspecifics. Some of the chemicals responsible for attraction are small peptides that are products of proteolytic activity on protein substrates (Rittschof et al., 1990; Kratt and Rittschof, 1991). Digestion of purified gastropod muscle protein or foreign protein substrates such as porcine chymotrypsinogen or chicken ovalbumin by the correct peptide generating enzymes results in hermit crab attractants. Enzymes can be pure enzymes such as porcine or bovine trypsin (Rittschof, 1980b; Rittschof et al., 1990; Kratt and Rittschof, 1991) or enzymes found in the salivary glands of predatory gastropods (Rittschof et al., 1990).

In contrast to attractant peptides from gastropods, the attractant chemicals in *Clibanarius vittatus* hemolymph are immediately available in the hemolymph and do not originate from hermit crab muscle (Table 2). If these molecules are peptides, they have a very different origin than the peptides from snail muscles (Rittschof et al., 1990).

The behavior altering odors from crushed *C. vittatus* are factors from the hemolymph. The attraction factor is less than 14,000 D. The investigation behavior and fleeing behavior inducers are < 1000 D. We hypothesize that all behaviors are mediated by the same molecules and that behaviors are directly influenced by shell fit. Hemolymph is stimulatory immediately upon removal from hemolymph sinuses. Thus, the factor is probably already present in the blood, and not generated by proteolytic activity, as are hermit crab attractant odors from snail muscle and other proteins (Rittschof, 1980a; Rittschof et al., 1990; Kratt and Rittschof, 1991). If hermit crabs have a complement cascade in the hemolymph (for review see Schiffman, 1982), it is possible that attractants are similar to (but lower in molecular weight than) those that function in leukocyte attraction (Schiffman, 1982) in vertebrates. We look forward to the resolution of this issue.

Factors that stimulate the crabs to leave the area can be considered alarm substances (Hrbacek, 1950; Pfeiffer, 1963; Snyder, 1967; Snyder and Snyder, 1970; Atema and Burd, 1975; Stenzler and Atema, 1976). As is the case for snail alarm substances (Atema and Burd, 1975; Stenzler and Atema, 1976; Atema and Stenzler, 1977), passive release from hemolymph is the source of substances from hermit crabs and response specificity may be related to phylogeny. In contrast to snail alarm substances (Atema and Stenzler, 1977; Stenzler and Atema, 1977), alarm substances from hermit crab hemolymph are relatively small molecules (< 1000 D), while those from snail hemolymph that cause alarm in mudsnails are large, > 100 kDa. The molecular size of the former coupled with the ability to extract and desalt the active material with a reversed-phase column makes it a likely candidate for future purification and structural determination as was accomplished for alarm pheromone from a sea anemone (Howe and Sheikh, 1975).

Thus, depending upon the type of shell and its size relative to the size of

the crab, chemical factors from snail flesh and crab hemolymph act as attractant cues or as alarm substances which may result in inactivity. Because of their chemical sensing capabilities, crabs can detect and locate or avoid potential sources of shells from over 10 m away. Contrary to the reasoning of Spight (1985), hermit crabs that respond to chemical cues by attraction greatly increase the likelihood of obtaining a new shell. Hermit crabs have many more options for obtaining a new shell when in an aggregation than just the one potentially available new shell [which, as Spight (1985) suggests, probably does not fit them!] (Chase et al., 1988). For example, they have the potential of trading with other hermit crabs attracted to the area by the chemicals. In addition, if a new shell becomes available, all crabs in smaller shells benefit. A routine observation at both natural and simulated predation sites is every crab in a shell smaller than the new shell trades up to the next larger size (McLean, 1975; Rittschof, 1980a; Chase et al., 1988). This is because attracted crabs actively determine where the next larger crab is and take a position next to that crab (McLean, 1975; Rittschof, 1980a). Thus, although only one new shell is added, it replaces the smallest shell in the population of crabs attending the site (Rittschof, 1980a; Chase et al., 1988). The empty shell is continually replaced by a smaller shell until the empty shell is buried in the sediment due to lack of manipulation by crabs in the area.

In summary, chemicals from conspecific hemolymph cause dramatic alteration in behavior. The specific behavior response is related to the size of the crab, the size of the shell, and the species of shell occupied. Chemical detection of dead snails and conspecifics increases the ability of crabs to obtain new shells directly from the source of the cue as well as indirectly from other hermit crabs attracted by the odors.

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CHEMICAL VARIATION WITHIN AND BETWEEN INDIVIDUALS OF *Plantago lanceolata* (PLANTAGINACEAE)

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Abstract—Variation in concentrations of leaf nitrogen and iridoid glycosides was examined in replicate plants of five genotypes of *Plantago lanceolata* (Plantaginaceae) grown in an experimental garden. Nitrogen concentration and iridoid glycoside concentration were affected by leaf age. New leaves had nitrogen concentrations 1.7 to 2.7 times higher than mature leaves. Catalpol concentration was highest in new and intermediate-aged leaves. The concentration of aucubin, the biosynthetic precursor to catalpol, was higher in intermediate-aged leaves than in mature leaves, in three of five genotypes. Consequently, the proportion of aucubin relative to total iridoid glycosides increased as leaves aged. Concentration of iridoid glycosides was not correlated with plant size. Plant genotype significantly affected concentration of nitrogen and iridoid glycosides, as well as plant size. Thus, major indicators of hostplant quality for insect herbivores varied considerably both within and among plant genotypes and individuals.

Key Words—*Plantago lanceolata*, iridoid glycosides, aucubin, catalpol, nitrogen, plant size, plant–insect interactions, chemical variation.

INTRODUCTION

A population of plants represents a heterogeneous resource for herbivorous insects. Individual plants may vary in their morphology and architecture (Law-

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ton, 1986); the biomass available to be eaten; and in their location and abundance, which may affect their susceptibility to being found (Rausher, 1981; Mackay and Singer, 1982; Singer et al., 1989; Thomas, 1989, 1990). They may also differ in their nutritional and secondary chemistry (McKey, 1979; Mattson, 1980; Scriber and Slansky, 1981; Louda and Rodman, 1983; Berenbaum et al., 1989; Stamp and Bowers, 1990). At least some of the observed variation in plant secondary chemistry in several plant taxa is genetically based (Gouyon et al., 1983; Berenbaum et al., 1986; Lincoln et al., 1986; Mihaliak et al., 1989), although environmental factors may enhance or override the genetic effects (Lindroth et al., 1987; Mihaliak et al., 1989; Waterman and Mole, 1989).

Within a plant, nutritional quality of leaves may vary, depending on their position and age (Mattson, 1980; Raupp and Denno, 1983; Harper 1989), and phytochemicals may occur in very different amounts in leaves, depending on the location of the leaf, the age of the leaf, and the presence or absence of herbivore damage to the leaf (Lincoln and Langenheim, 1978, 1981; McKey, 1979; Raupp and Denno, 1983; Haukioja and Neuvonen 1987; Baldwin, 1988). For example, new leaves are often higher in nitrogen, water, and secondary metabolites (McKey, 1979; Mattson, 1980; Harper, 1989; Stamp and Bowers, 1990) than mature leaves. Damage to a leaf may induce the production of additional secondary metabolites, which can then alter the suitability of that leaf as food (Haukioja and Neuvonen, 1987; Baldwin, 1988; Baldwin et al., 1990).

Plantago lanceolata (Plantaginaceae), narrow-leaved plantain, is a common introduced weed in North America (Cavers et al., 1980) that serves as a host plant for several species of herbivorous insects (Tietz, 1972; Scott, 1986). *Plantago lanceolata* contains two iridoid glycosides, aucubin and catalpol (Duff et al., 1965). These compounds serve as feeding and oviposition stimulants for some species of specialist insects, including the buckeye butterfly, *Junonia coenia* (Nymphalidae) (Bowers, 1984; Pereyra and Bowers, 1988). Iridoid glycosides are sequestered by buckeye larvae and may help protect them from potential predators (Bowers and Collinge, 1992). Some iridoid glycosides, including aucubin and catalpol, are toxic or deterrent to generalist and nonadapted specialist insects (Bernays and DeLuca, 1981; Bowers and Puttick, 1988, 1989; Puttick and Bowers, 1988).

In this study, we assessed variation in leaf nitrogen content and iridoid glycosides as indicators of food quality for insect herbivores. Plantain can be vegetatively propagated (Wu and Antonovics, 1975), so we were able to analyze replicates of each of five genotypes. In addition, we divided each individual plant into three leaf age classes—new, intermediate, and mature—and measured the nitrogen and iridoid glycoside concentration of the leaves in each age class. Thus we were able to assess the genetic basis for individual variation in these components, as well as the variation within individual plants.

METHODS AND MATERIALS

Five genotypes of *P. lanceolata* were vegetatively propagated in the SUNY Binghamton greenhouse in early May 1989. These genotypes were chosen from an array of genotypes, collected outside Providence, RI, that had been used in other experiments (Fajer et al., 1992; Schmitt et al., 1992). We consider these entities to be separate genotypes for the following reasons: (1) the original parent plants (=genotypes) were collected at least a meter distant from any other parent plant (see Schmitt et al., 1992), (2) *P. lanceolata* is self-incompatible and thus all offspring are the result of out-crossing (Ross, 1973; van Dijk, 1985), and (3) *P. lanceolata* does not reproduce by rhizomes and thus clones of identical individuals do not occur in the field. Three of the genotypes (F8, F9, F14) were originally collected from an old field; the other two genotypes (L11, L15) were from a frequently mowed lawn.

The ramets of each genotype were initially similar in size (three to five leaves) and were grown in pots in the greenhouse for 8 weeks. On June 12, 1989, they were planted in a common garden in Binghamton, NY. The planting design was determined by a concurrent experiment for which these plants served as one type of control. Four pairs of plants of each genotype were planted (N = eight plants of each of five genotypes for a total of 40 plants). Plants within a pair were 0.25 m apart and the pairs were 1.0 m apart. The pairs were oriented alternately, the first pair north–south, the second east–west, and so forth. The planting location in the garden for each pair was determined randomly.

These plants grew in the garden for 4 weeks, after which all of the above-ground parts were harvested. After removing the scapes (=reproductive parts), leaves were separated into new, intermediate, and mature age classes, and the length of each leaf was measured. Leaves of *P. lanceolata* grow in a rosette, allowing leaves to be easily aged. We designated leaf age in the following way: "new" leaves were the five central, incompletely expanded leaves; "mature" leaves were the five most basal leaves; and "intermediate" leaves were the remainder. The number of intermediate leaves therefore varied, depending on the size of the individual plant. Leaf material and scapes were then dried at 50°C to a constant weight and weighed. All leaves of each age class, from each individual, were ground together to a homogeneous powder, then analyzed for iridoid glycosides and nitrogen content.

Iridoid glycoside content of leaf samples was analyzed by gas chromatography (Gardner and Stermitz, 1988; Fajer et al., 1989; Belofsky et al., 1989; Bowers, 1991; Bowers and Collinge, 1992). Twenty-five milligrams of each sample of powdered leaves was extracted overnight in 95% MeOH. GC analyses of iridoid glycosides were performed on the trimethylsilylated derivatives. Phenyl-β-D-glucose was used as an internal standard. The results from two

injections of each plant sample were averaged, and the mean of those was used for statistical analyses.

Total Kjeldahl nitrogen content was determined using a Lachat Flow Injection Ion Analysis system. Samples of 100 mg of dried, powdered plant material were digested in sulfuric acid and a copper sulfate catalyst (1000:30:47, K_2SO_4:anhydrous $CuSO_4$:$CuSO_4 \cdot 5H_2O$) at 360°C for 3 hr. Each run of 40 samples included a set of standards of *P. lanceolata* with known nitrogen concentrations. The *P. lanceolata* standards were from a large batch (several kilograms) of powdered leaves that had had the nitrogen concentration independently analyzed.

Statistical analyses were performed using ANOVA, with both genotype and leaf age as fixed effects. Where appropriate, ANOVAs were followed by Student–Newman–Keuls range tests. Proportions were arcsine-transformed for analysis.

RESULTS

Plants of the five genotypes differed significantly in total biomass (one-way ANOVA, $F = 9.44$, df = 4,35, $P < 0.001$). The plants of genotype L15 were significantly smaller than the others (Table 1). The differences among genotypes in the sum of leaf lengths and plant biomass per unit of leaf length reflected the differences in leaf morphologies among the five genotypes (sum of leaf lengths, $F = 16.90$, df = 4,35, $P < 0.001$; biomass per unit of leaf length, $F = 3.19$, df = 4,35, $P < 0.025$) (Table 1). There were no significant differences among genotypes in number of leaves ($F = 2.01$, $P > 0.10$) or number of scapes ($F = 1.04$, $P > 0.35$) (Table 1).

Nitrogen concentration (Table 2) was significantly affected by both genotype and leaf age (Table 3). New leaves contained 1.7 to 2.7 times as much nitrogen as mature leaves (Table 2). The five genotypes varied in nitrogen concentration by a factor of approximately 1.5 (Table 2).

Percentage dry weight total iridoid glycosides in an entire plant ranged from a mean of 3.36% in genotype L15 to a mean of 5.66% in genotype F8 (Table 1). In addition, iridoid glycoside concentration varied significantly with genotype and leaf age (Tables 2 and 3). Total iridoid glycoside content was highest in new and intermediate leaves and lowest in the mature leaves (Table 2). In three of five genotypes, aucubin was higher in intermediate leaves than in mature leaves, whereas catalpol was highest in new and intermediate leaves (Table 2). The proportion of aucubin relative to total iridoid glycosides (Table 2) increased with leaf age (Table 3) and differed significantly among genotypes (Table 3). For four of five genotypes, new leaves had more catalpol than aucubin, but in intermediate and mature leaves, the amount of aucubin, on average, was as much as three times higher than catalpol (Table 2).

TABLE 1. HARVEST DATA AND TOTAL IRIDOID GLYCOSIDE (IG) CONCENTRATION OF *P. lanceolata* PLANTS OF FIVE GENOTYPES[a]

Genotype	*n*	Biomass (g dry wt)	Sum of lengths of leaves (cm)	Biomass per unit of leaf length (mg/cm)	No. of leaves	No. of scapes	Percentage dry weight, total IGs
F8	8	2.249 ± 0.161[a]	304.8 ± 32.3[a]	7.71 ± 0.64[ab]	20.3 ± 2.0	7.0 ± 1.0	5.66 ± 0.70[a]
F9	8	1.469 ± 0.230[a]	150.8 ± 12.8[c]	9.77 ± 1.37[ab]	20.0 ± 3.0	6.9 ± 1.0	5.41 ± 0.41[a]
F14	8	1.861 ± 0.304[a]	216.9 ± 20.0[b]	8.27 ± 0.71[ab]	21.9 ± 2.3	4.9 ± 1.4	4.10 ± 0.23[ab]
L11	8	2.259 ± 0.224[a]	209.1 ± 7.5[b]	10.80 ± 1.08[b]	26.1 ± 3.6	6.4 ± 0.3	4.75 ± 0.33[ab]
L15	8	0.694 ± 0.064[b]	102.8 ± 6.9[c]	6.72 ± 0.46[a]	16.0 ± 1.0	5.3 ± 0.8	3.36 ± 0.38[b]

[a]Means ± SE are shown. Means within a column followed by different superscript letters were significantly different ($P < 0.05$) by a Student–Newman–Keuls range test. Percentage dry weight total iridoid glycosides (IGs) = (IG concentration of new leaves * biomass of new leaves) + (IG concentration of intermediate leaves * biomass of intermediate leaves) + (IG concentration of mature leaves * biomass of mature leaves).

TABLE 2. NITROGEN AND IRIDOID GLYCOSIDE (IG) CONCENTRATIONS FOR LEAVES OF THE THREE AGE CLASSES AND THE FIVE GENOTYPES

Genotype	Leaf age	Percentage dry wt				Aucubin as percentage of total IGs
		Nitrogen	Aucubin	Catalpol	Total IGs	
F8	New	4.51 ± 0.69[a]	3.07 ± 0.65[a]	5.04 ± 1.26[a]	7.57 ± 1.59[ab]	41.1 ± 4.9[a]
	Intermediate	2.38 ± 0.21[b]	4.11 ± 0.50[b]	3.63 ± 0.72[a]	7.74 ± 1.16[a]	56.0 ± 3.8[b]
	Mature	1.69 ± 0.10[c]	2.17 ± 0.25[a]	1.31 ± 0.43[b]	3.48 ± 0.68[b]	68.2 ± 4.6[b]
F9	New	3.35 ± 0.34[a]	2.90 ± 0.60	3.46 ± 0.30[a]	6.36 ± 0.82[a]	41.9 ± 5.0[a]
	Intermediate	2.13 ± 0.21[b]	3.99 ± 0.27	2.88 ± 0.26[a]	6.86 ± 0.48[a]	58.3 ± 1.7[b]
	Mature	1.46 ± 0.24[b]	2.64 ± 0.31	1.58 ± 0.13[b]	4.23 ± 0.38[b]	61.6 ± 2.7[b]
F14	New	4.27 ± 0.17[a]	2.39 ± 0.27[a]	2.14 ± 0.10[a]	4.53 ± 0.30[a]	51.7 ± 2.9[a]
	Intermediate	2.47 ± 0.12[b]	3.27 ± 0.23[b]	1.46 ± 0.18[b]	4.73 ± 0.38[a]	69.9 ± 1.6[b]
	Mature	2.26 ± 0.29[b]	2.20 ± 0.25[a]	0.70 ± 0.11[c]	2.89 ± 0.36[b]	77.1 ± 1.5[c]
L11	New	4.33 ± 0.18[a]	2.62 ± 0.28	4.21 ± 0.32[a]	6.83 ± 0.54[a]	38.0 ± 1.8[a]
	Intermediate	2.64 ± 0.19[b]	2.89 ± 0.28	2.26 ± 0.27[b]	5.15 ± 0.53[b]	56.4 ± 1.5[b]
	Mature	2.27 ± 0.09[b]	2.18 ± 0.33	1.44 ± 0.20[c]	3.62 ± 0.52[c]	59.9 ± 1.7[b]
L15	New	3.59 ± 0.14[a]	1.72 ± 0.17[ab]	2.39 ± 0.30[a]	4.11 ± 0.42[a]	42.2 ± 3.0[a]
	Intermediate	2.63 ± 0.14[b]	2.36 ± 0.33[b]	1.90 ± 0.30[a]	4.27 ± 0.56[a]	55.6 ± 3.4[b]
	Mature	2.06 ± 0.07[c]	1.47 ± 0.12[a]	0.78 ± 0.13[b]	2.25 ± 0.24[b]	66.9 ± 3.2[c]

[a] Means ± SE are shown. For each genotype, means within a column followed by different superscript letters were significantly different ($P < 0.05$) by a Student-Neuman-Keuls range test.

TABLE 3. RESULTS OF TWO-WAY ANOVAS ON ARC-SINE TRANSFORMED IRIDOID GLYCOSIDE (IG) CONCENTRATION OF FIVE GENOTYPES AND THREE LEAF AGES OF *Plantago lanceolata*

	Genotype (G)			Leaf Age (LA)			G × LA		
	F	df	*P*	*F*	df	*P*	*F*	df	*P*
Aucubin (% dry weight)	5.057	4,100	<0.001	14.889	2,100	≪0.001	0.743	8,100	NS
Catapol (% dry weight)	7.094	4,100	≪0.001	35.671	2,100	≪0.001	0.899	8,100	NS
Total IG's (% dry weight)	6.236	4,100	≪0.001	21.349	2,100	≪0.001	0.903	8,100	NS
Aucubin as % of total IGs	9.284	4,100	≪0.001	71.123	2,100	≪0.001	0.540	8,100	NS
Nitrogen (% dry weight)	8.890	4,90	≪0.001	112.385	2,90	≪0.001	0.873	8,90	NS

To test the hypothesis that plants allocating more resources to growth and reproduction might have less to allocate to chemical defense (i.e., there was a cost of chemical defense) (Gulmon and Mooney, 1986), we tested for a negative correlation between iridoid glycoside concentration and biomass. Concentration of iridoid glycosides was not significantly correlated with plant size, except for the aucubin concentration of genotype L15, which was positively correlated ($r^2 = 0.53, P < 0.05$).

DISCUSSION

Our results showed that these five genotypes of *P. lanceolata* were quite different in terms of biomass, nitrogen content, and iridoid glycoside content. The plant biomass varied substantially among genotypes. The biomass of genotype L15 was about half that of the next smallest genotype, F9, and one-fifth that of the largest genotypes, F8 and L11 (Table 1). Thus, individuals of L15 may be much more quickly decimated by herbivore attack. However, these L15 plants might escape female butterflies searching for suitable oviposition sites, if the females do not notice these small plants or prefer larger ones (Rausher, 1983; Rausher et al., 1981; MacKay, 1985; Thomas, 1989, 1990).

The genotypes also differed significantly in their nitrogen content (Table 2). Because the location of replicate individuals of each genotype in the common garden was randomly determined, the differences in nitrogen content probably reflect differences in photosynthetic capacity (Harper, 1989) or nutrient utilization efficiency, rather than soil nutrient availability. Nitrogen is a limiting factor for insect herbivores (Mattson, 1980; Scriber and Slansky, 1981), and thus a genotype, such as F9, that was relatively low in nitrogen content might be a less adequate nutritional resource than the other genotypes. For example, *Pieris rapae* (Pieridae) caterpillars on low-nitrogen collards grew much more

slowly than caterpillars eating high-nitrogen plants (Loader and Damman, 1991). However, herbivorous insects may consume less biomass from plants that are higher in nitrogen, resulting in relatively more damage to plants lower in nitrogen.

Genotypes also differed significantly in their total iridoid glycoside content, as was found in another experiment with *P. lanceolata* (Fajer et al., 1992). However, in that experiment, plants were grown in pots in environmental chambers, not in the field, and leaves were not separated into different age classes as they were in this experiment. Our data in the present experiment indicate not only that there is a significant genotype effect on iridoid glycoside content but that an individual plant is quite heterogeneous in terms of iridoid glycoside content; new leaves contained an average of twice as much iridoid glycosides as mature leaves.

Our data showed a change in the relative proportion of the two iridoid glycosides, aucubin and catalpol, as the leaves aged. New leaves, in general, were highest in catalpol, except in genotype F14, in which amounts of aucubin and catalpol were about equal. In the biosynthesis of iridoid glycosides in plants, aucubin is the precursor of catalpol (Damtoft et al., 1983). Our data suggest that as leaves age, (1) less catalpol is synthesized, (2) it is more quickly broken down, and/or (3) it is translocated out of the leaf.

We found no indication that higher amounts of iridoid glycosides in these *P. lanceolata* plants resulted in decreased growth or reproduction; that is, there was no cost of a higher investment in chemical defense (Gulmon and Mooney, 1986). These plants were harvested in mid-July and had not completed flowering. Our results suggest that early in the life cycle of a *P. lanceolata* plant, there is no detectable cost (in terms of biomass) to producing higher amounts of iridoid glycosides.

Such chemical variation within as well as among genotypes may affect insects feeding on *P. lanceolata*, and this in turn may determine the impact of those herbivores on the fitness of *P. lanceolata*. For example, laboratory experiments have shown that larvae of the buckeye, *Junonia coenia* (Nymphalidae) grow better on artificial diets with higher amounts of iridoid glycosides (Bowers and Puttick, 1989) and that female *J. coenia* prefer to oviposit on substrates with higher amounts of iridoid glycosides (Pereyra and Bowers, 1988). Herbivory by such specialist insects may selectively remove genotypes high in iridoid glycosides from *P. lanceolata* populations. For insects that feed on *P. lanceolata* and sequester iridoid glycosides, such as larvae of the checkerspot butterfly, *Euphydryas phaeton* (Nymphalidae) (Bowers, 1991), and larvae of the buckeye butterfly, *Junonia coenia* (Nymphalidae) (Bowers and Collinge, 1991), the ability to choose plants and plant parts high in iridoid glycosides may increase their protection from potential predators. Furthermore, the catalpol content of insects appears to be the critical component of unpalatability in those species that

sequester iridoid glycosides (Bowers, 1980; Belofsky et al., 1989). There was a significant effect of genotype on the amount of catalpol in these *P. lanceolata* plants; thus the plant genotype a caterpillar feeds on may determine its relative palatability or unpalatability to potential predators. In addition, new leaves contained more catalpol than intermediate or mature leaves, so preference for new leaves by a caterpillar that sequesters iridoid glycosides may also enhance its unpalatability.

In conclusion, our study shows that nitrogen, an indicator of plant nutritional quality, and iridoid glycosides, major components of plantain defensive chemistry, vary considerably both within and among plant parts. This variation may be important in the relative susceptibility of particular genotypes or plant parts to insect attack and may be important for insects feeding on *P. lanceolata* as well.

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ACCUMULATION OF THE PHYTOALEXIN, GLYCEOLLIN, IN ROOT NODULES OF SOYBEAN FORMED BY EFFECTIVE AND INEFFECTIVE STRAINS OF *BRADYRHIZOBIUM JAPONICUM*

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Abstract—Nitrogen fixation in root nodules formed by strain 2143 of *Bradyrhizobium japonicum* and *Glycine max* (L.) Merr. cv Williams 82 reaches a maximum at 21 to 28 days postinoculation and then begins to decline. The phytoalexin, glyceollin, accumulates in nodules coincident with the decline in nitrogen fixation. Nodules formed by strain 3122, which are unable to fix nitrogen, accumulate even higher levels of glyceollin and do so beginning 21 days postinoculation even though these nodules contain no recoverable bacteria. The typical phytoalexin response occurs within days of infection. The mechanism by which this response in the *Bradyrhizobium japonicum*–soybean combination is delayed 2 to 3 weeks after infection is presently unknown but phytoalexin accumulation could contribute to the inability of the soybean–*Bradyrhizobium japonicum* combination to maintain high levels of nitrogen fixation throughout the growing season.

Key Words—Nitrogen fixation, glyceollin, phytoalexin, *Bradyrhizobium japonicum*, nodulation.

INTRODUCTION

Isoflavonoids and flavonoids are plant-produced, secondary metabolites. Interest in these classes of compounds was renewed in the middle of the 1980s with

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reports that they acted as signal molecules for activation of *nod* genes in *Rhizobium meliloti* (Peters et al., 1986) and *Bradyrhizobium japonicum* (Kosslak et al., 1987). Historically, however, interest in isoflavonoids has centered on their antimicrobial activity and their production by the plant in response to pathogenic insult (Albersheim and Valent, 1978; Ingham, 1982; Hahn et al., 1985; Keen and Kennedy, 1974).

With respect to phytotoxicity and antimicrobial activity, the most widely studied soybean isoflavonoids are the pterocarpans. The pterocarpans include glycinol and the multiple isomers of glyceollin. Their phytotoxicity has been attributed to disruption in membrane functions (Weinstein et al., 1983) and inhibition of electron transport processes (Boydson et al., 1983). Glyceollin has been reported to be bacteriostatic (Fett and Osmond, 1982) and is known to cause at least transient growth inhibition of *B. japonicum* strains (Parniske et al., 1991) of both DNA homology groups, I and II (Hollis et al., 1981).

Only limited information is available about the importance of glyceollin accumulation in the formation and maintenance of functional soybean root nodules. Werner et al. (1985) have investigated the possible role of glyceollin in determining whether combinations of specific strains of *B. japonicum* and soybeans are effective (capable of nitrogen fixation) or ineffective (not capable of nitrogen fixation). They reported that glyceollin I was detected in mature nodules. Since it appeared at much higher levels in ineffective symbiotic combinations, they suggested that it might contribute to the failure of the symbiotic interaction.

Our research is directed toward understanding the maintenance of (rather than the formation of) the effective combination. In our studies on the biochemistry of developing bacteroids in the *B. japonicum*–soybean symbiosis, we see the characteristic rise in nitrogenase activity. This increase is accompanied by a rise in metabolic activities that are presumed to be involved in providing energy for nitrogen fixation (Karr et al., 1984). The rise in nitrogen fixation capacity is transient and is followed by a drop in nitrogen fixation capacity and bacteroid plating efficiency and a cessation or drop in the various indicators of ongoing metabolic activity (Karr et al., 1984; Karr and Emerich, 1988). This decline in nitrogen fixing capacity precedes pod set and should result in a decrease in available biologically fixed nitrogen for pod fill. The purpose of the work described here was to determine whether the failure to maintain maximum nitrogen fixation capacity in effective combinations is accompanied by accumulation of glyceollin in nodules.

METHODS AND MATERIALS

Bacterial Strains and Plant Material. B. japonicum strain 2143, a natural mutant of USDA143 selected for resistance to rifampicin (80 μg/ml) and nalidixic acid (100 μg/ml), forms an effective combination with cv Williams 82

(Karr and Emerich, 1988). *B. japonicum* strain 122 L, a naturally occurring mutant of USDA122 that was selected for large colony size (Emerich et al., 1979), forms an ineffective combination with cv Williams 82. Strain 3122 is a natural mutant of strain 122 L selected for resistance to rifampicin (80 μg/ml). *B. japonicum* strain 122 S, a naturally occurring mutant of USDA122 selected for small colony size (Emerich et al., 1979), forms an effective combination with cv Williams 82. Strain 2122 is a natural mutant of strain 122 S selected for resistance to rifampicin (80 μg/ml). Strains 2143, 3122, and 2122 are members of serogroup 122 and DNA homology group I. Strains USDA143, 122 S, and 122 L were kindly provided by Harold Evans, Laboratory for Nitrogen Fixation Research, Oregon State University. Strain USDA 110 was provided by Dale Blevins, Agronomy Department, University of Missouri.

Soybean seeds [*Glycine max* (L.) Merr. cv Williams 82] were surface-sterilized by washing sequentially for 1 min each in 1.2% (w/v) sodium hypochlorite, sterile, deionized, distilled water, 70% (v/v) ethanol and rinsing thoroughly in sterile, deionized, distilled water. Seeds were germinated on water agar until the roots reached 1 to 2.5 cm in length. Plants were inoculated with the appropriate *Bradyrhizobium japonicum* strain by suspending them for 30 min in a bacterial culture (A_{630} = 0.7) which had been grown for 8 days in yeast extract mannitol medium (YEM) (Vincent, 1970) at 30°C. The inoculated seedlings were planted in perlite–sand–vermiculite potting medium in sterile, modified Leonard jars irrigated with nitrogen-free nutrient solution (Ahmed and Evans, 1960). Growth conditions are detailed elsewhere (Karr and Emerich, 1988). The nodulation pattern of the effective and ineffective strains was different. Nodules formed by *B. japonicum* strains 2143, 2122, and USDA 110 clustered near the crown area of the tap root, while those formed by strain 3122 showed no clustering and were scattered over the entire root system.

Bacteroid Viability and Nitrogen Fixation Capacity. Bacteroid viability was determined from the plating efficiencies of nodule extracts on agar plates in YEM medium amended with rifampicin as described previously (Karr and Emerich, 1988).

Nitrogen-fixation capacity was determined by measuring the capacity of intact root-nodule matrix to reduce acetylene (Schwinghamer et al., 1970). Ethylene formation was measured every 3 min for 18 min. The rate of ethylene formation was calculated from the initial rate and is expressed as micromoles of C_2H_4/hr/g nodule fresh weight (μmol/hr/gfw).

Isolation and Measurement of Glyceollin. Glyceollin was isolated from nodules, the nodulated-root zone, approximately 0.5 cm to either side of the position from which nodules had been detached, and the nonchlorophyllous stem segment just above the root crown. A modification of the procedure described by Keen et al. (1971) was used. The plant material was frozen in liquid nitrogen as it was harvested, and its weight measured. Two to six-gram batches of frozen tissue were macerated in 95% ethanol (2 ml/g tissue) at −20°C with the aid of

a Polytron homogenizer. Debris was removed by 20 min of centrifugation at 20,000*g*. Solvent was removed under vacuum at 40°C on a rotary evaporator. The residue was dissolved in 95% ethanol (2 to 6 g frozen plant material per 2 ml ethanol); 2 ml of the ethanol-soluble fraction was diluted with 4 ml deionized distilled water. This solution was applied to a precleaned C_{18}-RP Seppak (Waters) cartridge and recovered as described previously (Abdullah et al., 1984). Glyceollin recovery was at least 85% as determined by the recovery of authentic glyceollin added to macerated nodules or roots. Glyceollin-containing samples were protected from light and stored at −20°C until analysis.

Samples were analyzed by HPLC; the chromatograph consisted of two Waters 6000A pumps, a U6K injector, a Model 660 gradient programmer, and a Model 440 detector. Glyceollin was separated on a 3-μm C_8-RP column (Supelco, Inc., Bellefonte, PA) using gradient 7 from 60 to 75% (v/v) methanol (1 ml/min). After HPLC, glyceollin was quantitated using a UV detector and an ϵ_{280} of 10,000, a value in agreement with a recent report (Parniske et al., 1991). Glyceollin was identified by UV spectra and by chromatographic identity with authentic glyceollin when subjected to TLC. Analytical TLC was performed on Kodak (Eastman Kodak Company, Rochester, NY) silica gel sheets with fluorescent indicator developed with hexane–ethyl acetate–methanol, 60:40:5 (Keen et al., 1972); benzene–methanol, 95:5; or ethylacetate–hexane, 70:30 (Weinstein et al., 1981). Authentic glyceollin was kindly supplied to us by Dr. N. Keen, University of California—Riverside, and Dr. J. Paxton, University of Illinois. Experiments measuring accumulation of glyceollin in nodules and surrounding root tissues have been repeated five times over a 3-year period and the pattern of glyceollin accumulation was the same in all experiments. The data given in Figures 1 and 2 are from one of the five replications; each data point is the average of at least four samples (three samples for day 13) and three injections of each sample.

Sensitivity to Glyceollin. The sensitivity of *B. japonicum* to glyceollin was measured by the plate bioassay method (Fett and Osman, 1982; Skipp and Bailey, 1977; Wyman and Van Etten, 1978). YEM (Vincent, 1970) agar plates were overlaid with 2.5 ml of 0.8% YEM agar containing 10^7 to 10^8 cells of *B. japonicum* strains that had been cultured for 5 days on YEM liquid culture medium before use. Five-microliter aliquots of glyceollin dissolved in 50% (v/v) methanol or 50% (v/v) ethanol were spotted onto the freshly solidified overlays. Plates were incubated at 28°C, in darkness, and scored after 3 or 4 days of growth. Glyceollin caused the formation of plaques which became hazy with time. The plaque phenotype presumably results from the transient nature of glyceollin-caused growth inhibition of *B. japonicum* (Parniske et al., 1991). The sensitivity of each of the strains was recorded as the lowest glyceollin concentration which caused formation of a visible plaque.

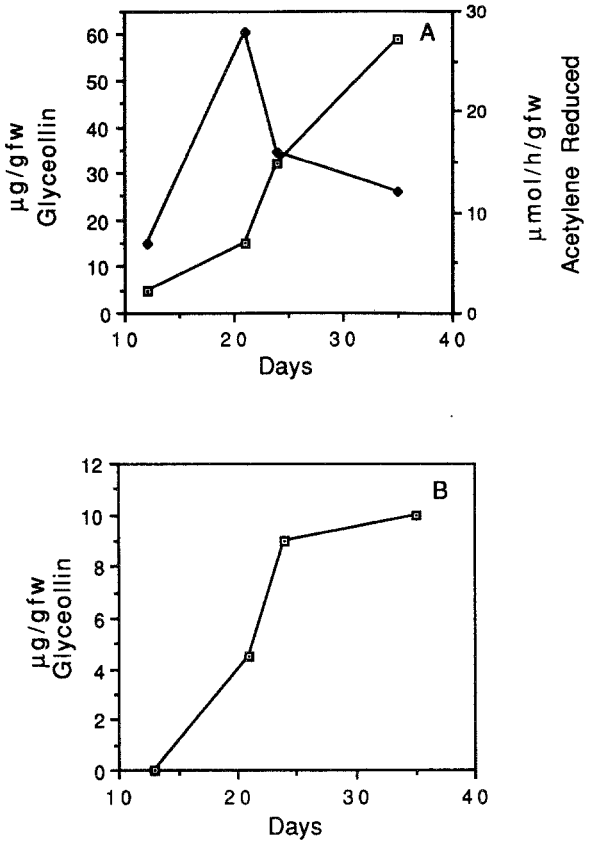

FIG. 1. (A) The relationship between the level of acetylene reduction activity (diamonds) and glyceollin accumulation (open squares) in nodules formed by strain 2143. Glyceollin concentrations in the nodule tissue represent means derived from nodules from four sets of plants (3 for day 13), with each sample being analyzed three times by HPLC. Standard deviations were less than ±10% of the mean for days 21, 24, and 35. The standard deviation for day 13 was ±80%. (B) Accumulation of glyceollin in root tissue adjacent to nodules formed by strain 2143. Standard deviations were less than ±10% of the mean for days 21, 24, and 35.

RESULTS

The pattern of acetylene reduction exhibited by nodules formed by *B. japonicum* strain 2143 on cv. Williams 82 is shown in Figure 1A. Normally, acetylene reduction activity is measurable by the fifteenth day postinoculation

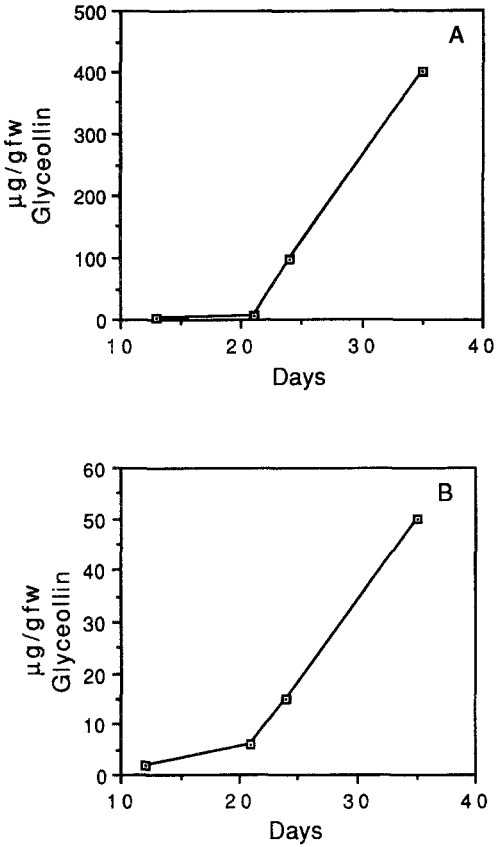

FIG. 2. (A) Glyceollin accumulation in nodules formed by strain 3122. Acetylene reduction was less than 2 µmol/hr/gfw at all time points. Standard deviations were less than ±10% of the mean for days 21, 24, and 35 and ±50% for day 13. (B) Accumulation of glyceollin in root tissue adjacent to nodules formed by strain 3122. Standard deviations were less than ±10% of the mean for days 21, 24, and 35.

(PI), rises to a peak between day 20 and day 28, and then begins to decline. By day 45 acetylene reduction activity is typically no higher than 5 µmol/hr/gfw. This transient pattern of acetylene reduction activity is not specific to nodules formed on cv. Williams 82 by strain 2143. An identical pattern was observed with nodules formed by strains 2122 and USDA 110 on cv. Williams 82 and with nodules formed by strain 2143 on cv. Clark and Chamberlain. The same pattern has been reported previously for nodules formed with a commercial inoculum of *B. japonicum* on cv. Chippewa (Wong and Evans, 1971). Finally, this pattern of acetylene reduction is not specific to plants grown in Leonard

jars and was observed with nodules formed by strain 2143 on cv. Williams 82 grown in commercial potting soil (unpublished data of this laboratory). Acetylene reduction activity was less than 2 μmol/hr/gfw at all sampling times in nodules formed by strain 3122.

This decline in acetylene reduction activity after day 21 was accompanied by a decline in the viability of isolated bacteroids. In the experiment described here (Figure 1A) bacteroids isolated from nodules 21 days PI gave a viability of 105 ± 10%. By day 35 PI bacteroid viability had declined to 39 ± 5%. Viable bacteroids were not recovered from nodules formed by strain 3122.

Glyceollin was readily detectable in nodules formed by strain 2143 by 21 days PI (Figure 1A). Levels of glyceollin continued to rise through day 24 and reached a concentration of 59 μg/gfw by day 35. Glyceollin levels did not increase significantly after day 35 (data not given). A much lower level of glyceollin was also detected in the root zone from which the nodules had been detached (Figure 1B). Glyceollin reached a maximum concentration of only 10 μg/gfw by day 35. The pattern and timing of glyceollin accumulation were the same in roots and nodules. No glyceollin was detected in the nonchlorophyllous stem tissue from plants inoculated with strain 2143.

Glyceollin was also detectable in nodules formed by strain 3122 by 21 days PI (Figure 2A). Levels of glyceollin continued to rise through day 24 and reached a concentration of 400 μg/gfw by day 35. After day 45, nodules formed by strain 3122 became brown inside, presumably due to the accumulation of phenolic, secondary metabolites. The timing and pattern of glyceollin accumulation in nodules formed by strain 3122 were the same as those observed for nodules formed by strain 2143. In plants inoculated with strain 3122, glyceollin levels in root tissue adjacent to the excised nodules (Figure 2B) and stem tissue reached concentrations of 50 and 10 μg/gfw, respectively.

Nodule extracts contained three additional UV-absorbing compounds (designated I, II, and III) which eluted from the HPLC column prior to glyceollin and which appeared in the nodules just previous to the rise in glyceollin levels. Compound I eluted coincident with and had a UV spectrum identical to the isoflavone, genistein. Compounds I and II did not inhibit the growth of *B. japonicum* in the plate assay. Compound III, which is unidentified, caused the formation of a clear plaque in the plate assay.

These experiments were repeated five times over a 3-year period. In separate experiments, the measured peak of nitrogen fixation varied between 21 days (Figure 1A) and 28 days PI. Maximum glyceollin levels obtained from nodules formed by strain 2143 at day 35 PI ranged from 40 to 64 μg/gfw, while levels from nodules formed by strain 3122 at day 35 ranged from 400 to 800 μg/gfw (Table 1). The timing (relative to the period of peak nitrogen fixation) and pattern of glyceollin accumulation were the same in all experiments.

B. japonicum strains 2122, 2143, and 3122 were tested for sensitivity to

glyceollin (Table 2). Sensitivities were reported as end points, because the transient nature of glyceollin inhibition of *B. japonicum* growth makes calculation of ED_{50} values difficult. The three strains differed in their sensitivity to glyceollin. Strain 2122 was the least sensitive, with no plaque formation being observed after application of solutions containing glyceollin at less than 30 μg/ml. The end point for strain 2143 was 10 μg/ml. Strain 3122 was markedly more sensitive to glyceollin. Its growth was inhibited by solutions containing 5 μg/ml, the lowest levels used in this study. The data in Table 2 were obtained using solutions of glyceollin in methanol. Strains 2122 and 2143 were twice as sensitive when glyceollin was applied in 95% ethanol. Glyceollin recovered

TABLE 1. LEVELS OF GLYCEOLLIN DETECTED IN NODULES FORMED BY *B. japonicum* STRAINS 35 DAYS POSTINOCULATION

Experiment No.	Strain	μg/gfw glyceollin in nodule tissue 35 days PI
1	2143	64[a]
	3122	400
2	2143	59
	3122	420
3	2143	40
	3122	450
4	2143	62
	3122	800
5	2143	52
	3122	410

[a] Standard deviations for all values were less than ±10%.

TABLE 2. SENSIVITY OF *B. japonicum* STRAINS TO GLYCEOLLIN

Strain	Lowest glyceollin concentration causing formation of a plaque (μg/ml)[a]
2122	30
2143	10
3122	<5

[a] The values given are for glyceollin applied in methanol.

after HPLC from nodules formed by either strain caused the formation of hazy plaques in the plate assay.

DISCUSSION

The decline of nitrogen fixation in nodules formed by *B. japonicum* strain 2143 clearly coincides with an accumulation of glyceollin in nodule tissue (Figure 1A). A similar pattern of glyceollin accumulation was detectable in roots adjacent to where nodules were detached but not in plant stem tissue. Glyceollin became detectable just preceding the time of maximum measured nitrogen fixation and continued to increase through day 35 postinoculation (PI), after which no further increase was detected. Glyceollin also accumulated at the same time PI in nodules formed by strain 3122. In agreement with the report of Werner et al. (1985), this ineffective combination resulted in the accumulation of much higher levels of glyceollin than the effective combination formed by strain 2143. Root tissue adjacent to nodules formed by strain 3122 and stem tissue also accumulated detectable levels of glyceollin. Indeed, glyceollin levels in such adjacent root tissue were nearly as high as those that accumulated in nodules formed by strain 2143.

It is not surprising that glyceollin accumulation is elicited during infection by *B. japonicum*. Glyceollin belongs to a class of plant-produced, antimicrobial compounds termed phytoalexins whose synthesis is elicited by pathogenic organisms (Paxton, 1980). Phytoalexins have been proposed to play a role in plant disease resistance responses (e.g., Ebel and Grisebach, 1988), and Vance (1983) has noted the similarities between plant diseases and plant nodulation by members of the Rhizobiaceae. What is surprising in the plant nodulation system as reported here is that glyceollin accumulation does not begin until 2 weeks after introduction of the microorganism with maximum accumulation beginning 1 week later. In other plant–microbe combinations where glyceollin is proposed to play a role, glyceollin accumulation is detectable early in the infection process. Parniske et al. (1988) have observed such an early elicitation of accumulation of glyceollin in root hairs soon after infection by *B. japonicum*. That is clearly a different elicitation event than the one described here. It is noteworthy that glyceollin accumulation also begins approximately 3 weeks after infection by strain 3122. Nodules formed by this strain are incapable of nitrogen fixation and do not form detectable leghemoglobin. Plants inoculated with strain 3122 become yellow and stunted if not provided with an exogenous source of nitrogen. While ultrastructural studies have shown that nodules formed by strain 3122 do contain some bacteria in infection thread structures and scattered single bacteroids enclosed in peribacteroid membranes (unpublished results of this laboratory), viable bacteria cannot be isolated from the nodules. These results indicate

that neither the establishment of active nitrogenase nor even the presence of a large number of bacteroids is necessary for triggering the accumulation of glyceollin 2 to 3 weeks PI. While the nature of the elicitation event is unknown, it is possible that the elicitation signal is transmitted early in the infection process and not acted upon by the plant for several weeks or that elicitation of glyceollin accumulation is a natural part of the program of nodule development. While still speculative, the best candidate for elicitation of the defense responses is an oligosaccharide released from the bacterial cell surface. The enzyme responsible for liberation of the oligosaccharide is produced by the bacterial cells at the highest levels in the time period just preceding the decline in nitrogen fixation (unpublished results of this laboratory). Further work will be needed to establish the importance of the oligosaccharide in elicitation of glyceollin accumulation.

While the concentration of glyceollin that accumulates in nodules formed by strains 2143 and 3122 is clearly high enough to inhibit the growth of the respective strain when assayed in culture, it is not certain that glyceollin alone is responsible for the bacteroid viability decline and the observed decline in nitrogen fixation capacity. Parniske et al. (1991) have demonstrated that although the growth of *B. japonicum* in culture is inhibited by glyceollin, this inhibition is transient. Upon continued exposure to glyceollin or treatment with the isoflavonoid genistein, the cells become glyceollin-resistant and resume growth. Yet the situation in soybean nodules 30 days PI is much different than growth in culture. Bacterial division in the determinant nodules formed by *B. japonicum* and soybean has either declined or ceased at 30 days PI. Furthermore, nodule bacteroids exist in an oxygen-limited environment. The effect of glyceollin on *B. japonicum* cells under these conditions is unknown. In addition, other putative plant defense responses are activated in nodules coincident with the accumulation of glyceollin. Such responses include, among others, accumulation of chitinase, β-glucanase, and enzymes of oxygen radical metabolism (unpublished results of this laboratory). These responses may act in concert with glyceollin to cause the decrease in bacteroid viability and decline in nitrogen fixation capacity.

Maximum levels of nitrogen fixation in nodules formed by *B. japonicum* and its economic host, soybean, are clearly transient in nature (Figure 1A) (Karr et al., 1984; Emerich et al., 1979). This observed decline in nitrogen fixation capacity has two important consequences for soybean production. First, biological nitrogen fixation is declining during a period when the plant has a high demand for protein for the production of seeds. Second, since that demand can be met only by removing protein from plant vegetative tissues (Wittenbach, 1983; Craft-Bradner et al., 1984), the value of soybeans as a nitrogen-rich rotation crop is diminished. It is clear that both soybean production efficiency and the utility of soybeans as a rotation crop would benefit if maximum levels of nitrogen fixation activity could be maintained through the period of pod fill.

It is not yet clear that glyceollin-induced growth inhibition alone or glyceollin acting in concert with other putative defense responses is responsible for the decrease in bacteroid viability. It is possible that glyceollin accumulation results from some other, as yet undetected event, which also results in bacteroid death. Whatever the case, it appears likely that an understanding of the unique delayed elicitation response in soybean nodules will permit development of bacterial strains which retain higher levels of nitrogen fixation throughout the lifetime of the plant.

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INFLUENCE OF CABBAGE PROTEINASE INHIBITORS *IN SITU* ON THE GROWTH OF LARVAL *Trichoplusia ni* AND *Pieris rapae*

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Abstract—Trypsin and chymotrypsin inhibitors are proteins that are developmentally regulated in foliage of cabbage plants, appearing at high concentrations in young foliage on mature plants. This temporal and spacial regulation of foliar proteinase inhibitors is synchronized with the appearance and distribution of foliar feeding Lepidoptera. When insects were allowed to select their feeding sites, larval *Pieris rapae* fed on the young foliage of cabbage plants, while larval *Trichoplusia ni* fed on the mature foliage on cabbage plants. Larval *P. rapae* that fed on mature plants were significantly smaller than larvae feeding on young plants, while there was no significant difference between larval *T. ni* feeding on mature plants and those feeding on young plants. Thus, there was a significant inverse correlation between the level of proteinase inhibitory activity in cabbage foliage and larval growth. When *P. rapae* and *T. ni* were provided with an artificial diet containing total protein (including significant levels of proteinase inhibitors) that was extracted from cabbage foliage, there was a significant reduction in growth and development of both species of Lepidoptera.

Key Words—*Pieris rapae, Artogeia rapae, Trichoplusia ni*, Lepidoptera, Noctuidae, Pieridae, trypsin inhibitors, chymotrypsin inhibitors, serine proteinase inhibitors, cabbage, Cruciferae.

INTRODUCTION

Proteinase inhibitors are polypeptides or proteins that commonly occur in plants (Whitaker, 1981). These naturally occurring compounds act *in vitro* as com-

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petitive inhibitors of proteinases by binding tightly to the active site of the enzyme, thus preventing the proteinase from binding to and cleaving the peptide bonds of proteins (Laskowski and Kato, 1980). The role of proteinase inhibitors in plants has not been clearly established, but extensive evidence suggests that they have the potential to act as defensive agents against herbivores (Ryan, 1989). One of the most common and widely studied group of plant proteinase inhibitors inhibits the serine proteinases (e.g., trypsin and chymotrypsin). This group of inhibitors is of particular interest because serine proteinases are commonly found in animals and microorganisms, but they are rarely detected in plants (Ryan and Walker-Simmons, 1981). This suggests that the serine proteinase inhibitors have little or no regulatory function in the plant but may significantly inhibit the primary proteinases in herbivores and pathogens, thus acting as a defensive agent against these pests.

Insect digestive enzymes can be inhibited *in vitro* by plant proteinase inhibitors (Broadway, 1989a; Broadway and Duffey, 1986; Christeller and Shaw, 1989; Gatehouse et al., 1985; Hines et al., 1991). In addition, the growth of herbivorous insects can be significantly reduced by the chronic ingestion of artificial diet supplemented with plant proteinase inhibitors (Birk and Applebaum, 1960; Broadway and Duffey, 1986; Shukle and Murdock, 1983; Steffens et al., 1978). However, proteinase inhibitors can protect plants against herbivores only if the inhibitors are present at the site of attack at effective concentrations.

Very few studies have examined the phenology, *in situ* concentration, and regulation of plant proteinase inhibitors. The most complete study focuses on the serine proteinase inhibitors in solanaceous plants (Pearce et al., 1991; Ryan, 1978). These inhibitors are environmentally regulated in the foliage of cultivated tomato and potato. Mechanical or natural wounding of a single leaf results in the systemic induction of proteinase inhibitors in the foliage of the plant (Broadway et al., 1986; Green and Ryan, 1972). Thus, active proteinase inhibitors are present at maximal levels in the foliage on the plant within hours after herbivory begins. Tomato or tobacco foliage containing high levels of these inhibitors will significantly inhibit the growth of herbivorous Lepidoptera (Broadway et al., 1986; Johnson et al., 1989).

In contrast, some proteinase inhibitors are developmentally regulated, as exemplified by cabbage (Broadway and Missurelli, 1990). Low (background) levels of trypsin inhibitory activity are present in foliage on the young cabbage plant. As the plant matures, trypsin inhibitory activity significantly increases in young and mature foliage but does not significantly increase in senescing foliage. When the plant develops a firm cabbage head, the foliar trypsin inhibitory activity reaches a plateau. At this time the trypsin inhibitory activity is significantly highest in the young foliage (i.e., cabbage head and surrounding cupped leaves), at intermediate levels in the mature foliage (i.e., fully expanded leaves not

touching head), and present at background levels in senescing leaves (Broadway and Missurelli, 1990).

The major lepidopterous pests on cabbage in New York are diamondback moth [*Plutella xylostella* (L.)], imported cabbageworm [*Pieris rapae* (L.)], and cabbage looper [*Trichoplusia ni* (Hübner)] (Andaloro et al., 1982, 1983). Larval *Trichoplusia ni* and *Pieris rapae* primarily secrete trypsin-like and chymotrypsin-like enzymes for the digestion of protein (Broadway, 1989a). In addition, the phenology of trypsin inhibitors in cabbage foliage (Broadway and Missurelli, 1990) is synchronized with the appearance of herbivorous Lepidoptera in cabbage fields (Hoy and Shelton, 1987). This information suggests that the serine proteinase inhibitors in cabbage foliage may function, at least in part, as phytochemical protection against herbivorous Lepidoptera. The following study examines the biological activity of cabbage proteinase inhibitors *in situ* against the growth of larval *Pieris rapae* (L.) (Lepidoptera: Pieridae) and *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae).

METHODS AND MATERIALS

Insects

Eggs from *Trichoplusia ni* were provided by Dr. W.L. Roelofs (NYS Agricultural Experiment Station, Geneva, NY) from a colony that was maintained on a pinto bean-based diet as previously described (Broadway, 1989a). The colony of *Pieris rapae* was established in 1987 with field-collected pupae, and the population was supplemented, annually, by 25–50% with field-collected individuals. Larval *P. rapae* were maintained on a high wheat germ-based meridic diet as previously described (Broadway, 1989a).

Plants

Seeds for the cabbage cultivar Superpack were from Johnny's Selected Seeds (Albion, ME). The seeds were germinated in Cornell Mix (Boodley and Sheldrake, 1977) in 7.5-L plastic pots. The seedlings were thinned to one plant per pot and maintained in a greenhouse under 1-kW metal halide lamps (12L : 12D) at 28°C. The plants were watered daily and fertilized once a week with a water-soluble nutrient mix (16–32–16).

Whole-Plant Bioassays

Two types of whole-plant feeding studies were tested. Type 1 was designed to allow larvae to move about freely on the entire plant, while the Type 2 bioassay confined larvae to a specific leaf on the plant by containing the larvae in a 5-cm-diameter leaf clip cage that was clamped over the leaf and moved

when the foliar tissue inside the cage was consumed. Each type of bioassay used three young (i.e., 10–12 true leaves) and three mature (i.e., 9–11 frame leaves and a 7-cm-diameter cabbage head) plants. In addition, each set of experiments included a control plant (without larvae) that was placed in the same environmental conditions as the treated plants. Foliage on all plants was chemically analyzed at the end of the experiment.

Type 1 experiments employed 40 *T. ni* eggs or 100 *P. rapae* eggs that were placed on each of three young plants per insect species and 70 *T. ni* or 200 *P. rapae* eggs that were placed on each of three mature plants per insect species. (The number of *P. rapae* eggs that was placed on each plant was greater than that for *T. ni.* because there was a greater loss of larval *P. rapae* during experiments.) Each plant was individually caged (to prevent insect migration to other plants) in the greenhouse maintained at 27°C, 12L : 12D. The entire experiment was terminated when a single type of foliage (e.g., the young leaves on a young plant) was almost completely consumed (leaving enough tissue for chemical analyses). All larvae were weighed, and plants were chemically analyzed for trypsin inhibitory activity, chymotrypsin inhibitory activity, total protein, and total glucosinolates. Glucosinolates were measured because they have been implicated as a phytochemical factor that protects crucifers against generalist herbivores and competing plants (Blau et al., 1978; Erickson and Feeny, 1974; Feeny, 1977; Oleszek, 1987).

Type 2 experiments also consisted of three young plants and three mature plants per insect species. Fifty eggs of *P. rapae* or 30 eggs of *T. ni* that were placed inside each small cage (with a recovery of 10–20 first-instar larvae), and as the larvae grew, the group of insects was separated into cages containing three to five larvae. A cage was placed on an expanding leaf (i.e., leaf 3–5, based on the designation of leaf 1 as the youngest expanding leaf and each successive number indicating the next oldest leaf on the plant) on each young plant. For each mature plant, one cage was placed on a young expanding leaf (i.e., leaf 2–4) and one cage was placed on a mature leaf (i.e., leaf 8–10). The entire experiment was terminated when a single type of foliage (e.g., the young leaf tissue on a young plant) was almost completely consumed (leaving enough tissue for chemical analyses). All larvae were weighed, and plants were chemically analyzed for trypsin inhibitory activity, chymotrypsin inhibitory activity, total protein, and total glucosinolates.

Total Plant Protein Bioassay

Total protein was isolated from three types of cabbage foliage: (1) young leaves (i.e., leaf 1–4) from mature plants (i.e., 9–11 frame leaves and a 7-cm-diameter cabbage head), (2) mature leaves (i.e., leaf 8–10) from mature plants, or (3) all leaves from young plants (i.e., total of 8–10 true leaves). Cabbage

foliage (700 g) was homogenized in 500 ml 0.01 *M* sodium citrate, 1 *M* potassium chloride, pH 4.5, buffer (the pH of the leaf fluid). Homogenate was pressed through two layers of cheesecloth, and the liquid was collected, on ice. The leaf tissue was homogenized a second time in 500 ml of buffer, then pressed through the cheesecloth, and the collected liquid was pooled with the first set. The liquid was centrifuged at 4200*g* for 15 min at 4°C. The supernatant was collected, and ammonium sulfate was added to 70% saturation. The mixture was stored overnight at 4°C, then centrifuged at 4200*g* for 15 min at 4°C. The pellet was resuspended in a small volume of distilled water, then dialyzed (12,000–14,000 MWCO) against ice-cold dH_2O for 72 hr. The dialysate was centrifuged at 4200*g* for 15 min at 4°C, then the supernatant was lyophilized. Total recoveries from the young foliage on young plants, young foliage on mature plants, and mature foliage on mature plants were 610, 650, and 710 mg, respectively. The lyophilized powder was chemically analyzed for trypsin inhibitory activity, chymotrypsin inhibitory activity, and total protein. Six hundred milligrams of each protein isolate was used to prepare 600 ml of artificial diet for the determination of the effect of total foliar protein on larval growth and development. The diet used for these tests was the same wheat germ-based diet used for mass rearing of larval *P. rapae*, except that the concentration of casein was reduced from 3.2 to 1.6% (w/v). The bioassays for both species of insect included four treatments, 3 cups/treatment, 30 eggs/cup. Each bioassay was replicated three times. Larvae were provided with diet ad libitum, from neonate until the controls reached the ultimate instar. Then all larvae were weighed.

Chemical Analyses

A standard spectrophotometric assay was used to determine the presence of trypsin inhibitory activity in the cabbage foliage (Walsh, 1970). Leaf juice was expressed from the cabbage foliage, then centrifuged, and the supernatant was diluted to 0.1× with 1 m*M* HCl. The diluted supernatant was mixed 1:1 (v/v) with bovine trypsin (0.1 mg/ml 1 m*M* HCl) and incubated at room temperature for 10 min. Then 100 μl of the mixture was added to 2.9 ml of buffer (0.05 *M* Tris, pH 8.0) containing 1.04 *M* *p*-toluene-sulfonyl-L-arginine methyl ester (TAME) (Sigma). Trypsin activity was monitored at 247 nm for 3 min (UV160U, Shimadzu Scientific). A 50-μl aliquot of trypsin was used to determine uninhibited trypsin activity. Each assay was performed in triplicate.

Chymotrytic inhibitory activity was determined by mixing (1:1, v/v) the diluted leaf juice (prepared as above) with TLCK-treated bovine chymotrypsin (0.1 mg/ml 1 m*M* HCl) for 10 min at room temperature. Then 100 μl of the mixture was added to 2.9 ml substrate (1 m*M* benzoyl-L-tyrosine ethyl ester in 50% MeOH, mixed 1:1 with 0.05 *M* Tris, pH 8.0), and monitored at 256 nm for 3 min (Walsh and Wilcox, 1970). A 50-μl aliquot of chymotrypsin was used

to determine uninhibited chymotrypsin activity. Each assay was performed in triplicate.

To quantify total glucosinolates in cabbage foliage (Heaney and Fenwick, 1981), leaves were ground with a mortar and pestle, and the leaf fluid was collected by centrifugation. A 500-μl aliquot of the supernatant was applied to a 1-ml DEAE-Sephadex A25 column. The column was washed with distilled water, then washed two times with 0.5 ml 0.02 *M* pyridine/acetic acid buffer. Myrosinase, extracted from white mustard meal (Heaney and Fenwick, 1981), was applied, at a concentration of 250 μl of 25 mg/ml pyridine–acetic acid buffer, to the column and allowed to incubate at room temperature for 2 hr. The column was then eluted with distilled water, collecting 1.25 ml of eluant. Three 250-μl aliquots of the eluant were assayed for glucose (Kit 115, Sigma Chemical Co, St. Louis, MO). The standard sinigrin (Nutritional Biochemicals Corp., Cleveland, OH) was also assayed in triplicate.

Total protein concentration was estimated, in triplicate, using bicinchoninic acid (BCA) reagent (Smith et al., 1985) (Pierce Chemical Co.). Purified protein from cabbage foliage was used as the standard.

Statistics

The significant differences ($P < 0.05$) between treatments were determined by analysis of variance (ANOVA) followed by orthogonal comparisons (with P adjusted by the Bonferroni procedure), multiple-range tests, or correlation determinations, using the statistical computer package Systat (Systat Inc., Evanston, IL).

RESULTS

Whole-Plant Bioassay

Larvae that hatched on plants in the large cages were allowed to select their feeding sites. Larval *T. ni* fed on the underside of the oldest leaves of both the young and the mature plants, although they did move up the plant to the fully expanded mature leaves as the oldest leaf tissue was consumed. There was no significant difference in larval weight when comparing *T. ni* feeding on the oldest leaves on young plants with larval *T. ni* feeding on the oldest leaves on mature plants ($P = 0.460$, $n = 220$) (Figure 1). Chemical analyses of the leaf tissue indicated that there was no significant difference in the trypsin inhibitory activity ($P = 0.383$, $n = 35$), chymotrypsin inhibitory activity ($P = 0.389$, $n = 35$), total protein ($P = 0.563$, $n = 35$), or total glucosinolate ($P > 0.05$, $n = 35$) in the oldest foliage from the young and mature plants (Table 1).

Free-roaming larval *P. rapae* fed on the youngest tissue on the plant. They

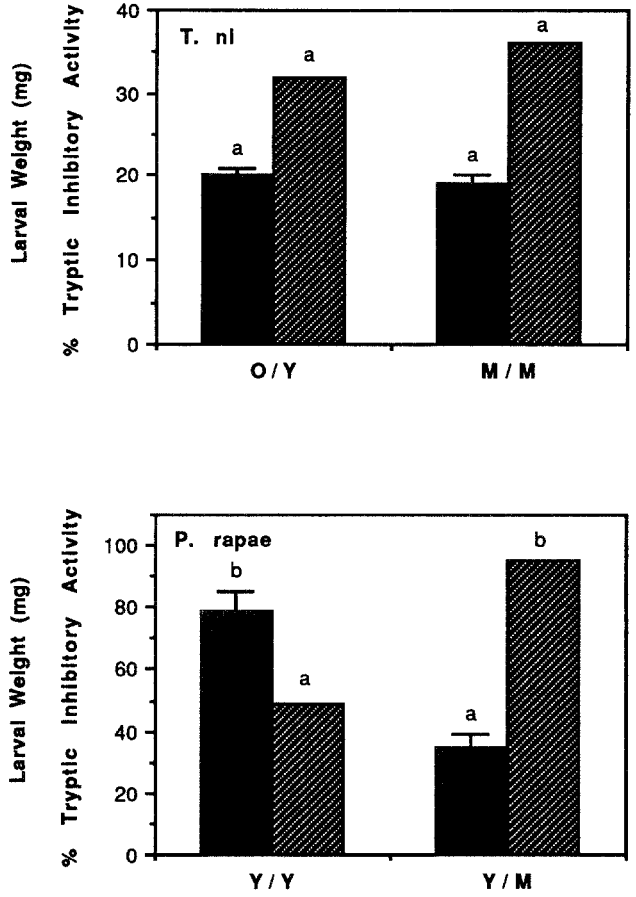

FIG. 1. The relationship between *in situ* trypsin inhibitory activity and growth of larvae allowed to select their feeding site. Inhibitory activity is reported as percentage inhibition of 5 μg trypsin by a 0.05× dilution of leaf fluid. Y/Y indicates young foliage on young plants, O/Y indicates the oldest foliage on young plants, Y/M indicates young foliage on mature plants, and M/M indicates mature foliage on mature plants. Black bars indicate larval weight; hatched bars indicate percentage trypsin inhibitory activity in cabbage foliage consumed by the larvae. Vertical lines indicate standard error of the mean; bars labeled with the same letter are not significantly different at $P < 0.05$.

consumed the apical meristem, then moved down, feeding on the nonvascular tissue at the base of the youngest leaves. They generally did not feed on the fully expanded or oldest leaves of either the young or mature plant. *P. rapae* that fed on the youngest foliage on mature plants were significantly smaller than larvae that fed on the youngest foliage on young plants ($P < 0.001$, $n = 87$)

TABLE 1. LEVELS OF TRYPSIN INHIBITORY ACTIVITY, CHYMOTRYPSIN INHIBITORY ACTIVITY, TOTAL PROTEIN, AND TOTAL GLUCOSINOLATE IN THE FOLIAGE OF CABBAGE USED FOR WHOLE-PLANT FEEDING STUDIES WITH LARVAL *Trichoplusia ni* AND *Pieris rapae*

	%TI[a]	%CI[a]	Total protein[b]	Total glucosinolate[c]
Control				
Y/Y[d]	13 ± 4	13 ± 3	2.9 ± 0.07	6 ± 3
O/Y	7 ± 2	5 ± 1	2.9 ± 0.15	173 ± 62
Y/M	37 ± 15	15 ± 1	3.4 ± 0.25	495 ± 108
M/M	6 ± 2	3 ± 1	2.7 ± 0.04	88 ± 16
T. ni				
Large cage				
Y/Y	63 ± 2	10 ± 1	3.0 ± 0.01	0
O/Y[e]	32 ± 0.6	8 ± 2	2.7 ± 0.02	16 ± 7
Y/M	93 ± 0.3	20 ± 0.2	3.7 ± 0.12	883 ± 28
M/M[e]	36 ± 7	5 ± 1.5	2.8 ± 0.17	37 ± 9
Small cage				
Y/Y[e]	39 ± 1	26 ± 3	2.4 ± 0.04	85 ± 5
O/Y	2 ± 1	5 ± 1	2.5 ± 0.07	170 ± 11
Y/M[e]	96 ± 0.2	41 ± 1	3.9 ± 0.19	130 ± 19
M/M[e]	3 ± 1	11 ± 1	2.9 ± 0.05	82 ± 8
P. rapae				
Large cage				
Y/Y[e]	49 ± 0.6	9 ± 2	3.1 ± 0.03	54 ± 24
O/Y	19 ± 6	7 ± 1	2.6 ± 0.03	32 ± 13
Y/M[e]	94 ± 0.2	24 ± 1.5	4.0 ± 0.12	213 ± 59
M/M	11 ± 2	2 ± 1.3	2.8 ± 0.20	35 ± 16
Small cage				
Y/Y[e]	33 ± 3	13 ± 3	2.6 ± 0.06	185 ± 27
O/Y	4 ± 1	2 ± 1	2.9 ± 0.04	252 ± 14
Y/M[e]	90 ± 2	21 ± 1	3.5 ± 0.13	177 ± 7
M/M[e]	2 ± 1	4 ± 1	2.8 ± 0.05	164 ± 15

[a] The percentage inhibition ± SE of bovine trypsin (%TI) or chymotrypsin (%CI).
[b] Units = mg protein/ml leaf juice ± SE, and include proteinase inhibitors.
[c] Total foliar glucosinolate measured as μg glucose/ml column eluant ± SE (glucose released after digestion of glucosinolate by myrosinase).
[d] Y/Y indicates young foliage on young plant, O/Y indicates oldest foliage on young plant, Y/M indicates young foliage on mature plant, and M/M indicates mature foliage on mature plant.
[e] Site of larval herbivory.

(Figure 1). A comparison of the chemical composition of the young foliage from these two ages of plants indicated that the trypsin and chymotrypsin inhibitory activity in the young foliage on the mature plant was significantly higher than in the young foliage on the young plant ($P < 0.001$, $n = 35$, for both trypsin

and chymotrypsin) (Table 1). Since larval *P. rapae* selectively fed at the base of the young leaves, the trypsin and chymotrypsin inhibitory activities were measured for three sections of the leaf (apex, middle, and base). The results indicate that the trypsin and chymotrypsin inhibitory activities are uniformly distributed within a leaf (Table 2).

Larval *T. ni* that were confined to the oldest leaves on mature plant were significantly larger than larvae feeding on the young leaves on the young plant ($P < 0.001$, $n = 45$) (Figure 2). The larvae restricted to feeding on the young leaves on the mature plant attempted to feed (as indicated by feeding pits on the surface of the leaf), but there was 100% mortality of the larvae in the second instar. Chemical analyses of the plant foliage indicated that there was a significant inverse correlation between larval growth and the level of trypsin or chymotrypsin inhibitory activity (for trypsin inhibitory activity $y = 104.07 - 1.226x$, $r^2 = 0.777$; for chymotrypsin inhibitory activity $y = 153.3 - 4.067$, $r^2 = 0.875$) (Table 1); the trypsin and chymotrypsin inhibitory activities were significantly highest in the young leaves on the mature plant ($P < 0.001$, $n = 35$), at an intermediate level in the young leaves on the young plant ($P < 0.001$, $n = 35$), and at the significantly lowest level in the old leaves on the mature plant ($P < 0.001$, $n = 35$). There was no significant correlation between larval growth and total foliar protein or glucosinolate (for total protein $y = 134.7 - 0.144x$, $r^2 = 0.114$; for total glucosinolate $y = -66.44 + 1.037x$, $r^2 = 0.633$). When larval *P. rapae* were confined to a specific feeding site inside a small cage (Figure 2), their survival was very low on the mature plant (1–6 larvae/treatment). Therefore, although larval growth is not statistically different for larvae feeding on the three types of leaf tissue ($P = 0.348$, $n = 40$), the reliability of the data is questionable.

Total Plant Protein Bioassay

An alternative bioassay, using an artificial diet containing total protein isolated from cabbage foliage, was evaluated, because larval *T. ni* did not feed on foliage with high levels of proteinase inhibitor, and when confined to a single

TABLE 2. THE DISTRIBUTION OF SERINE PROTEINASE INHIBITORY ACTIVITY IN A YOUNG LEAF ON A MATURE CABBAGE PLANT

Leaf section	%TI ± SE[a]	%CI ± SE[a]
Apex	50.4 ± 2.5	39.3 ± 0.7
Middle	49.3 ± 3.4	37.5 ± 0.4
Base	46.3 ± 2.5	38.5 ± 1.4

[a]The percentage inhibition of bovine trypsin (%TI) or chymotrypsin (%CI) ± standard error.

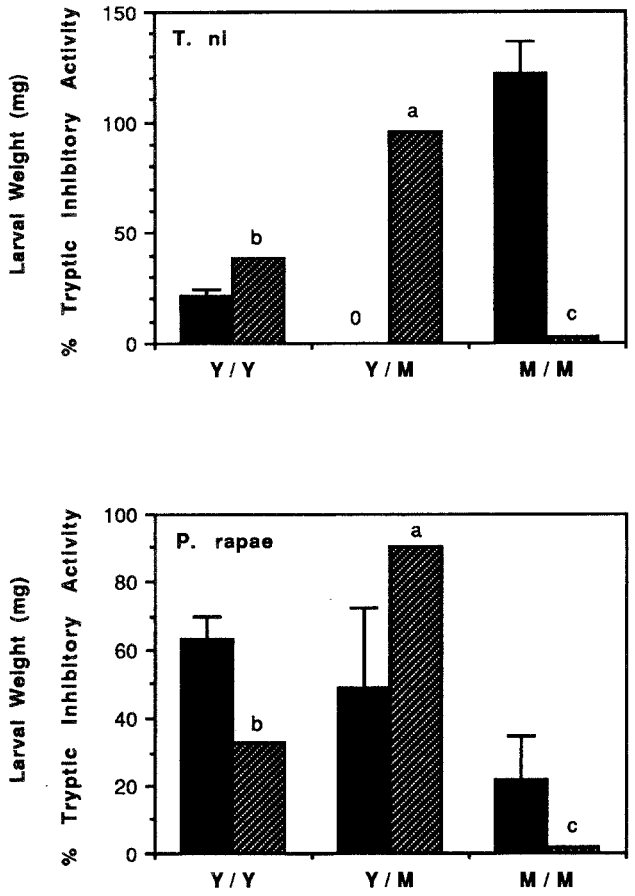

FIG. 2. The relationship between *in situ* trypsin inhibitory activity and growth of larvae confined to a specific feeding site. Inhibitory activity is reported as percentage inhibition of 5 μg trypsin by a 0.05× dilution of leaf fluid. Y/Y indicates young foliage on young plants, Y/M indicates the young foliage on mature plants, and M/M indicates mature foliage on mature plants. Black bars indicate larval weight; hatched bars indicate percentage trypsin inhibitory activity in cabbage foliage consumed by the larvae. Vertical lines indicate standard error of the mean; bars labeled with the same letter are not significantly different at $P < 0.05$.

leaf on a plant, the caged larvae of both species showed poor survival. Chemical analyses of the total protein isolates from the three ages of cabbage leaf indicated that the proteinase inhibitory activity was significantly different in the three isolates (Figure 3). Growth and development of *T. ni* were significantly reduced by dietary amendment with total protein that was extracted from young foliage

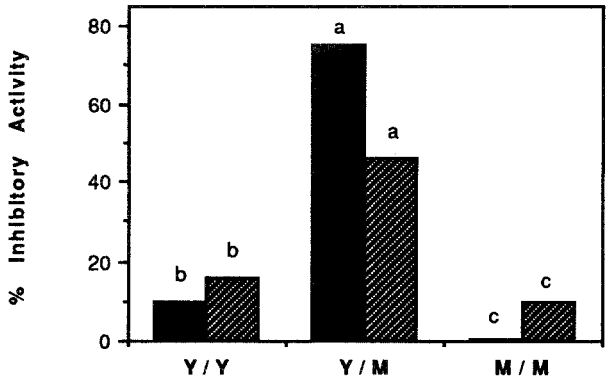

FIG. 3. The level of trypsin and chymotrypsin inhibitory activity in the total protein extracts from cabbage foliage. Inhibitory activity is reported as percentage inhibition of 5 μg trypsin or chymotrypsin by a 0.05× dilution of leaf fluid. Black bars indicate trypsin inhibitory activity; hatched bars indicate chymotrypsin inhibitory activity. Bars labeled with the same letter are not significantly different at $P < 0.05$. Y/Y indicates young foliage on young plants, Y/M indicates young foliage on mature plants, and M/M indicates mature foliage on mature plants.

on mature plants ($P = 0.001$, $n = 915$) (Figure 4). The growth and development of *P. rapae* were significantly reduced by ingestion of protein extract from young foliage on young plants or young foliage on mature plants ($P < 0.001$, $n = 362$) (Fig. 5). In addition, there was a significant inverse correlation between dietary proteinase inhibitory activity and the growth of larval *P. rapae* (for trypsin inhibitory activity $r^2 = 0.919$, $b = -1.02$, $P < 0.001$; for chymotrypsin inhibitory activity $r^2 = 0.939$, $b = -2.16$, $P < 0.001$).

DISCUSSION

Identification of natural phytochemical resistance factors that are effective against specific herbivorous insects requires examination of both the phytochemical producer and the target insects. The present study focused on cabbage and its lepidopterous pests. Cabbage foliage contains proteinaceous inhibitors of trypsin and chymotrypsin (Broadway, 1989b) that have the potential to inhibit the primary proteases in the digestive system of larval *T. ni* and *P. rapae* (Broadway, 1989a), two economically important pests of cabbage (Andaloro et al., 1982). These proteinase inhibitors are not induced in cabbage foliage by mechanical or natural wounding of the tissue (Broadway and Missurelli, 1990). Trypsin inhibitory activity is developmentally regulated in cabbage foliage, appearing at the highest concentration in young foliage on mature plants (Broad-

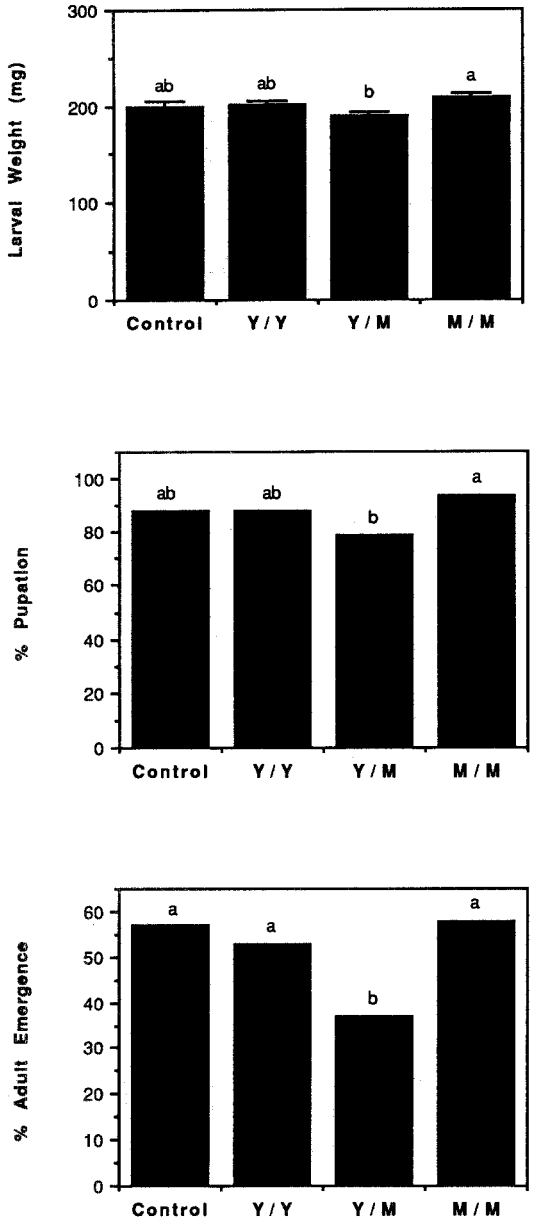

FIG. 4. Growth and development of *T. ni* feeding on artificial diet supplemented with total protein extracted from cabbage foliage. Vertical lines indicate standard error of the mean; bars labeled with the same letter are not significantly different at $P < 0.05$. Y/Y indicates diet containing protein extracted from young foliage on young plants, Y/M indicates protein extracted from young foliage on mature plants, and M/M indicates protein extracted from mature foliage on mature plants.

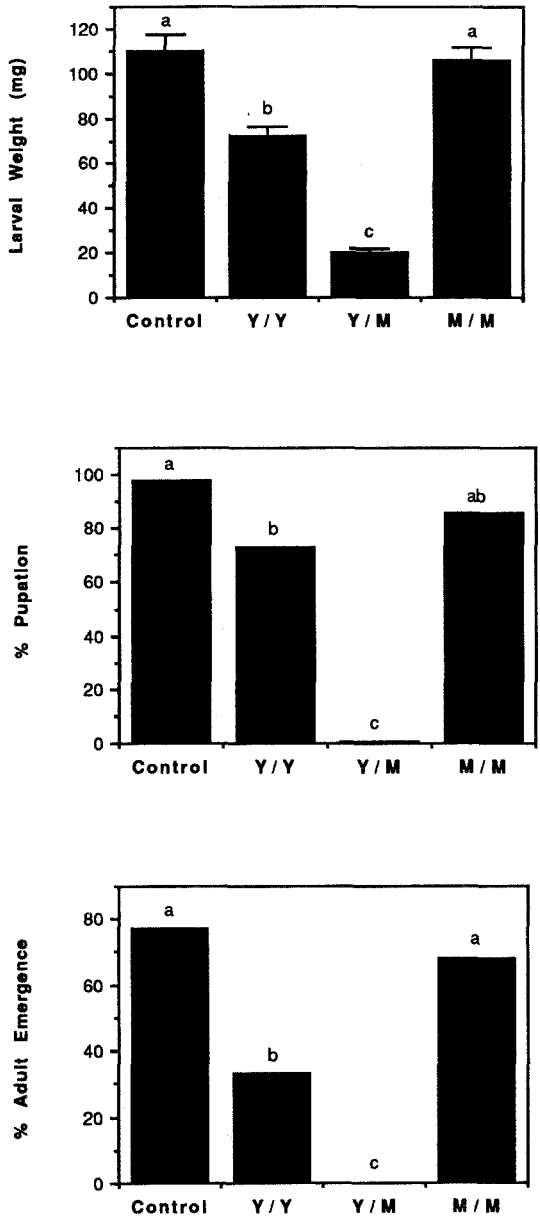

FIG. 5. Growth and development of *P. rapae* feeding on artificial diet supplemented with total protein extracted from cabbage foliage. Vertical lines indicate standard error of the mean; bars labeled with the same letter are not significantly different at $P < 0.05$. Y/Y indicates diet containing protein extracted from young foliage on young plants, Y/M indicates protein extracted from young foliage on mature plants, and M/M indicates protein extracted from mature foliage on mature plants.

way and Missurelli, 1990). The phenology of trypsin inhibitory activity in cabbage foliage is synchronized with the appearance of herbivorous Lepidoptera in cabbage fields. *P. rapae* appears in the field when the cabbage plants reach the six- to eight-leaf stage (Hoy and Shelton, 1987), which is the developmental stage when the level of foliar trypsin inhibitory activity begins to rise. *T. ni* normally appears in the field when the plant reaches the 9- to 12-leaf stage (Andaloro et al., 1982, 1983), which is the developmental stage when the plant is approaching maximum trypsin inhibitory activity. Thus, trypsin inhibitory activity is present in cabbage foliage at the time of invasion by lepidopteran insects and increases as the insect populations increase. Do these proteinase inhibitors function as protective agents *in situ* against lepidopterous pests of cabbage?

The present study indicated that there was a significant inverse correlation between the level of foliar proteinase inhibitory activity and the growth of larval *T. ni* and *P. rapae* and that cabbage proteinase inhibitors may have the potential to inhibit significantly the growth and development of *P. rapae* in the field.

The first criterion for evaluating the potential of a phytochemical to act as a resistance factor is to show that the chemical is present at the site of herbivory. When comparing herbivory of larval *T. ni* and *P. rapae*, the preferred feeding sites were quite different. Larval *T. ni* fed on the underside of the oldest leaves on the cabbage plant, while larval *P. rapae* selected the youngest foliage on the plant. These two different types of foliage contained significantly different levels of trypsin and chymotrypsin inhibitory activity (Table 1). Mature foliage contained little or no proteinase inhibitory activity. Young foliage on young plants contained background levels of proteinase inhibitory activity, while young foliage on mature plants contained significant levels of inhibitory activity. Thus, when larval *T. ni* fed on their preferred cabbage foliage, they did not consume significant levels of serine proteinase inhibitory activity. The results indicate that there was no significant difference in the growth of free-roaming larval *T. ni* feeding on young plants or mature plants (Figure 1). However, when larval *P. rapae* fed on their preferred cabbage foliage, they did consume high levels of proteinase inhibitory activity when feeding on mature plants. Young foliage on mature cabbage plants had two to three times the level of proteinase inhibitory activity as that found in the foliage on young cabbage plants (Table 1). Larval *P. rapae* feeding on young foliage on mature plants were 56% smaller than larvae feeding on the young foliage on young cabbage plants (Figure 1). Thus, under natural conditions, larval *P. rapae* would consume cabbage proteinase inhibitors, while larval *T. ni* would not. Therefore, the proteinase inhibitors *in planta* would not influence the growth of *T. ni*.

However, under artificial conditions, the cabbage proteinase inhibitor mixture did influence the growth of *T. ni*. Growth and development of both *T. ni* and *P. rapae* were significantly reduced when these insects were provided with

an artificial diet that contained a biologically realistic concentration of cabbage proteinase inhibitors (in addition to all other proteins that were extractable from the cabbage foliage at pH 4.5) (Figures 3–5).

These findings are in agreement with the results reported for the wound-inducible proteinase inhibitors in tomato foliage (Broadway et al., 1986; Johnson et al., 1989); there is a significant inverse correlation between the concentration of foliar proteinase inhibitors and the growth of larval Lepidoptera. However, these studies are correlational; there is no indication of causation. Future research will focus on the biological efficacy and mechanism of action of purified cabbage proteinase inhibitors against herbivorous Lepidoptera.

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EFFECTS OF SOME COMPOUNDS ISOLATED FROM *Celaenodendron mexicanum* Standl (EUPHORBIACEAE) ON SEEDS AND PHYTOPATHOGENIC FUNGI

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Abstract—The known compounds friedelin, maytensifolin B, ginkgetin, bilobetin, and amentoflavone as well as the new triterpene 3β-hydroxyfriedelan-16-one were isolated from *Celaenodendron mexicanum* (Euphorbiaceae), an endemic tree of the Pacific Ocean coast of Mexico. The compounds' structures were established on the basis of spectral analysis. The biological effects of aqueous leachates, a $CHCl_3$–MeOH extract and the isolated compounds of leaves and twigs were evaluated on the radicle growth of two plants, *Amaranthus leucocarpus* and *Echinochloa crusgalli*, and on the radial growth of three phytopathogenic fungi, *Fusarium* sp., *Helminthosporium* sp., and *Alternaria* sp. The organic extracts of both leaves and twigs inhibited *Amaranthus* and stimulated *Echinochloa* radicle growth. On the contrary, friedelin and maytensifolin B stimulated *Amaranthus* and inhibited *Echinochloa*. The target fungi showed a different response to each treatment, from inhibition to stimulation.

Key Words—Biological effects, *Celaenodendron mexicanum*, Euphorbiaceae, friedelin, maytensifolin B, ginkgetin, bilobetin, amentoflavone, 3β-hydroxyfriedelan-16-one, *Amaranthus leucocarpus*, *Echinochloa crusgalli*, *Alternaria* sp., *Fusarium* sp., *Helminthosporium* sp., phytopathogenic fungi.

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INTRODUCTION

Euphorbiaceae is a heterogeneous and vast family, particularly in the warmer parts of the world. Many Euphorbiaceae species produce bioactive compounds that have some medicinal uses (*Euphorbia lathytris*, *E. ipecacuanhae*, *Ricinus communis*, *Jatropha* spp., *Manihot utilissima*, *inter alia*).

Furthermore, several species including *Euphorbia corollata*, *E. esula*, *E. supina*, *Croton pyramidalis*, and *C. bonplandianum* have been implicated in allelopathy (Rice, 1984; Anaya et al., 1987).

Celaenodendron mexicanum Standl. is an endemic tree of México which grows in the tropical caducifolia forests in the Pacific Coast (Ferris, 1927). It has a discontinuous distribution and, as distinguished from other trees of this type of forest, *C. mexicanum* keeps its leaves during almost all the dry season and forms dense monospecific stands, with a very sparse understory vegetation, whereas the understory is dense under other species of trees. Furthermore, the leaves of *C. mexicanum* decompose at a very low rate, resulting in a dense mat of dead leaves accumulating on the ground (Filip et al., 1992).

This tree is commonly known as "palo prieto," and it is used as an antiseptic medicinal plant. It is suggested that *C. mexicanum* possess some bioactive compounds that could be ecologically important.

As a part of a project dedicated to search for distinctive natural products in endemic Mexican species, we initiated a study to evaluate the biological activity of secondary metabolites produced by *C. mexicanum* and to identify the more active compounds.

METHODS AND MATERIALS

General Bioassay Procedures

Leaves and twigs from the tree and dead leaves on the soil surface were collected in January 1990. Samples were taken on the route to Barra de Navidad-Puerto Vallarta (km 15), near the Biological Station of UNAM in Chamela, Jalisco State.

Air-dried leaves, twigs, and dead leaves were soaked for 4 hr in distilled water (1 g/100 ml). The leachates were filtered, and the osmotic pressure was measured in a freezing-point osmometer in order to avoid the effects of this factor on germination and growth (Anaya and Rovalo, 1976). The bioassays were performed in petri dishes. Each leachate was mixed (1/1) with 1.5% agar. Distilled water mixed with agar was used as the control. Bioassays were performed with seeds of *Amaranthus leucocarpus* (Amaranthaceae) and *Echinochloa crussgalli* (Gramineae). A completely randomized block design with four repetitions for each target species was used. Ten seeds were sown in each petri

dish. Petri dishes were kept at 27°C in the dark. The radicle length was measured after 24 hr for *Amaranthus* and 48 hr for *Echinochloa*. The data were analyzed by ANOVA ($P < 0.05$).

The effects of a $CHCl_3$–MeOH extract of dry leaves and twigs, and the isolated compounds **(1–6)** also were determinated. In these bioassays, the materials were dissolved at 100 ppm in DMF (dimethylformamide) and then poured on a filter paper disk in petri dishes. After evaporation of the solvent, distilled water was added and the bioassays were carried out under the same general conditions.

Bioassays with Phytopathogenic Fungi

The target species chosen were *Alternaria* sp., *Fusarium* sp., and *Helminthosporium* sp. The bioassays were carried out by incubating a fungal bud in a PDA (potato–dextrose–agar) sterile cultivation medium, to which the aqueous leachates of leaves (1%), the organic extract of leaves and twigs (100 ppm), or pure compounds (100 ppm) were added. Buds 5 mm in diameter were obtained from pure 8-day-old fungal culture. The sown fungi were inoculated in darkness at 27°C. A randomized complete block design with three replications was utilized. The effect of compounds was determined by measuring the radial growth of the mycelium, for which two perpendicular measurements of the colony's diameter were taken and the mean value was obtained. The measurements were made at 3 and 8 days. The mean values of replications were analyzed by ANOVA ($P < 0.05$).

Chemistry

[^{1}H]- and [^{13}C]NMR spectra were performed in $CDCl_3$ or DMSO-d_6 on a Varian VXR-300S spectrometer. Chemical shifts expressed as δ units are reported as parts per million downfield from the internal standard TMS ($\delta = 0$). Mass spectra were determined on a Hewlett-Packard 5985 B spectrometer. IR spectra were obtained in KBr on a Perkin Elmer 599B spectrophotometer. UV spectra were taken on a Beckman DU-7 apparatus. Optical rotations were measured with a Jasco DIP 360 digital polarimeter.

Commercial Merck silica gel 60 (70- to 230-mesh) ASTM was used for column chromatography. Analytical TLC was carried out using precoated silica gel Merck 60F 254 plates. The chromatograms were sprayed with 0.1% $CeSO_4$–2 *N* H_2SO_4 and heated to detect the spots.

Extraction and Preliminary Fractionation

Air-dried and ground leaves of *Celaenodendrum mexicanum* (2.6 kg) were macerated four times at room temperature with 60 L of a mixture of $CHCl_3$–MeOH (1 : 1). The extracts were evaporated at reduced pressure to yield 210 g

of a residue that was column chromatographed on 1.3 kg of silica gel. Elution was accomplished with a solvent gradient of increasing polarity (hexane gradually enriched with $CHCl_3$, and $CHCl_3$ with increasing amounts of MeOH). Fractions of 500 ml were collected.

Isolation of Friedelin (1)

Fractions 119–131 of the original column, eluted with hexane–$CHCl_3$ (8:2), yielded 285 mg of friedelin, mp 245–246°C. The IR, MS, and NMR data were in agreement with those of an authentic sample and its spectral properties were identical to those described previously (Gunatilaka et al., 1983).

Isolation of Maytensifolin B (2) and 3β-Hydroxyfriedelan-16-one (3)

Preparative TLC of fractions 224–286 of the original column eluted with hexane–$CHCl_3$ (7:3), using $CHCl_3$–EtOAc (9:1) as the eluants, allowed the isolation of 103 mg of maytensifolin B **(2)** and 80 mg of 3β-hydroxyfriedelan-16-one **(3)**. The spectral properties of **2** were identical to those described previously (Nosaki et al., 1986). 3β-Hydroxyfriedelan-16-one **(3)**, mp 112–115°C, $[\alpha]_D$ + 10.9° (C 2, MeOH). IR (KBr): 3460, 2920, 2860, 1680, 1440, 1380, 1230, 1190 cm^{-1}. [^{1}H]NMR ($CDCl_3$): 0.84 (s, 3H), 0.88 (s, 3H), 0.92 (s, 3H), 0.96 (d, 3H), 1.04 (s, 3H), 1.17 (s, 3H), 1.25 (s, 3H), 1.28 (s, 3H), 1.3–2.4 (m), 3.74 (dd, J = 3 Hz, 1H). [^{13}C]NMR ($CDCl_3$): 219.3 (C-16), 72.5 (C-3), 61.0 (C-4), 52.3 (C-8), 50.07 (C-15), 49.03 (C-10), 45.2 (C-17), 43.8 (C-18), 41.3 (C-5), 40.3 (C-2), 39.1 (C-6), 37.8 (C-14), 37.2 (C-13), 35.3 (C-9), 35.2 (C-11), 35.08 (C-30), 31.5 (C-19), 31.04 (C-21), 30.7 (C-22), 29.6 (C-12), 29.14 (C-29), 27.5 (C-20), 27.3 (C-28), 20.1 (C-26), 17.89 (C-7), 17.6 (C-1), 16.3 (C-25), 16.1 (C-27), 15.8 (C-24), 11.6 (C-23). EIMS *m/z* (rel intensity): 440 (M^+, 20), 425 (M-15, 31), 355 (17), 275 (6), 219 (43), 124 (29).

Isolation of Ginkgetin (4), Bilobetin (5), and Amentoflavone (6)

Three grams of a yellow powder was precipitated from fractions 551–590 eluted with 95:5 $CHCl_3$–MeOH. Preparative TLC on silica gel (8:2 $CHCl_3$–MeOH) yielded 313 mg of ginkgetin **(4)**, 210 mg of bilobetin **(5)**, and 183 mg of amentoflavone **(6)**.

Ginkgetin **(4)**, mp 198–200°C; $[\alpha]_D$ + 31° (C 2, $CHCl_3$); IR (KBr): 3200, 1680, 1610, 1560, 1480, 1420, 1340, 1280, 1220, 1140, 800 cm^{-1}. [^{1}H]NMR 3.75 (s, 3H, $-OCH_3$), 3.82 (s, 3H, $-OCH_3$), 6.34 (d, J = 3 Hz, H-6, I), 6.42 (s, H-6, II), 6.75 (d, J = 3 Hz, H-8, I), 6.89 (s, H-3, I), 6.91 (s, H-3, II), 6.92 (d, J = 8 Hz, H-3′ and H-5′, II), 7.16 (d, J = 8 Hz, H-5′, I), 7.67 (d, J = 8 Hz, H-2′ and H-6′, II), 8.03–8.06 (m, 2H, H-2′ and H-6′, I), 10.3 (bs,

2H, OH), 12.96 (s, 1H, OH), 13.07 (s, 1H, OH). [^{13}C]NMR ($CDCl_3$): 182.04 (C-4, II), 181.83 (C-4, I), 165.01 (C-2, I), 163.96 (C-7, I), 163.09 (C-2, II), 162.12 (C-7, II), 161.03 (C-5, I and C-4′, II), 160.48 (C-5, II), 159.66 (C-4′, I), 157.21 (C-9, I), 154.46 (C-9, II), 131.34 (C-5′, II), 127.9 (C-2′ and C-6′, II), 122.89 (C-3′, I), 120.78 (C-1′, I), 119.99 (C-1, II), 116.17 (C-5′, I), 114.43 (C-6′, I and C-3′, II), 103.6 (C-8, II), 103.19 (C-10, I), 103.09 (C-3, I and II), 98.67 (C-6, I), 98.01 (C-6, II), 92.61 (C-8, I), 56.01 (OCH_3), 55.49 (OCH_3). UV (MeOH): λ_{max} = 271 nm. *Anal.* Calcd. for $C_{32}H_{22}O_{10}$: C, 67.84%; H, 3.88%. Found: C, 67.77%; H, 3.97%. Bilobetin **(5),** mp 213–215°C; $[\alpha]_D$ −30° (C 2, acetone). IR (KBr): 3150, 1640, 1600, 1490, 1420, 1350, 1270, 1220, 1150, 1020, 800 cm^{-1}. [^{1}H]NMR ($CDCl_3$): 3.76 (s, 3H, OCH_3), 6.2 (d, J = 3 Hz, H-6, I), 6.43 (s, H-6, II), 6.46 (d, J = 3 Hz, H-8, I), 6.84 (s, H-3, I), 6.89 (s, H-3, II), 6.93 (d, J = 8 Hz, H-3′ and H-5′, II), 7.17 (d, J = 8 Hz, H-5′, I), 7.68 (d, J = 8 Hz, H-2′ and H-6′, II), 8.01–8.03 (m, 2H, H-2′ and H-6′, I), 10.35 (bs, 3H, OH), 12.99 (s, 1H, OH), 13.09 (s, 1H, OH). [^{13}C]NMR ($CDCl_3$): 182.04 (C-4, II), 181.64 (C-4, I), 164.0 (C-2, I), 163.69 (C-2, II), 163.1 (C-7, I), 162.08 (C-7, II), 161.93 (C-5, I), 161.35 (C-4′, II), 160.45 (C-5, II), 159.45 (C-4′, I), 157.27 (C-9, I), 154.43 (C-9, II), 131.29 (C-6′, I), 127.87 (C-2′, I and II), 127.74 (C-6′, II), 122.88 (C-3′, I), 120.91 (C-1′, I), 119.89 (C-1, II), 116.09 (C-5′, I and C-3′, II), 114.37 (C-5′, II), 103.91 (C-8, II), 103.62 (C-10, II), 103.6 (C-10, I), 103.13 (C-3, I), 102.92 (C-3, II), 98.73 (C-6, I), 98.61 (C-6, II), 93.93 (C-8, I), 55.4 (OCH_3). UV (MeOH): λ_{max} = 271 nm. *Anal.* Calcd for $C_{31}H_{20}O_{10}$: C, 67.39%; H, 3.62%. Found: C, 67.47%; H, 3.57%.

Amentoflavone **(6),** mp 223–224°C; $[\alpha]_D$ +11.0° (C-2, MeOH). IR (KBr): 3200, 1680, 1610, 1560, 1480, 1420, 1340, 1280, 1220, 1140, 800 cm^{-1}. [^{1}H]NMR (DMSO-d_6): 6.2 (d, J = 3 Hz, H-6, I), 6.39 (s, H-6, II), 6.47 (d, J = 3 Hz, H-8, I), 6.72 (d, J = 8 Hz, 2H, H-3′ and H-5′, II), 6.80 (s, H-3, I), 6.85 (s, H-3, II), 7.14 (d, J = 8 Hz, H-5′, I), 7.6 (d, J = 8 Hz, 2 H, H-2′ and H-6′, II), 8.02 (dd, J = 8, 3 Hz, H-6′, I), 8.05 (d, J = 3 Hz, H-2′, I), 10.6 (bs, 4H, OH), 13.0 (s, 1H, OH), 13.1 (s, 1H, OH). [^{13}C]NMR (DMSO-d_6): 182.01 (C-4, II), 181.6 (C-4, I), 164.05 (C-2, I), 163.8 (C-7, I), 163.5 (C-2, II), 162.6 (C-7, II), 161.3 (C-5, I), 160.9 (C-4′, II), 160.4 (C-5, II), 159.9 (C-4′, I), 157.3 (C-9, I), 154.4 (C-9, II), 131.3 (C-6′, I), 128.1 (C-6′, II), 127.6 (C-2′, I, II), 121.4 (C-3′, I), 120.6 (C-1′, I), 120.2 (C-1′, II), 116.4 (C-3′, II), 115.7 (C-5′, I, II), 104.2 (C-8, II), 103.6 (C-10, I), 103.4 (C-10, II), 102.8 (C-3, II), 102.5 (C-3, I), 98.8 (C-6, II), 98.83 (C-6, I), 94.01 (C-8, I). UV (MeOH): λ_{max} = 271 nm. *Anal.* Calcd. for $C_{30}H_{18}O_{10}$: C, 66.91%; H, 3.44%. Found: C, 66.86%; H, 3.44%. The spectral and physical properties of compounds **4–6** were in good harmony with those described previously for bilobetin (Baker et al., 1963; Joly et al., 1980), ginkgetin (Baker et al., 1963; Garg and Mitra, 1971), and amentoflavone (Garg and Mitra, 1971; Horhammer

et al., 1965; Varshney et al., 1973; Chari et al., 1977). The [^{13}C]NMR data of compounds **4** and **5** have not been previously reported.

Oxidation of 3β-hydroxyfriedelan-16-one **(3).** To a solution of **3** (20 mg) in pyridine (1 ml) was added CrO_3 (24 mg) in pyridine (0.4 ml). The mixture was kept at room temperature for 18 hr, after which up to 7 mg of a pure compound, identical to the natural product **(2),** was obtained.

RESULTS AND DISCUSSION

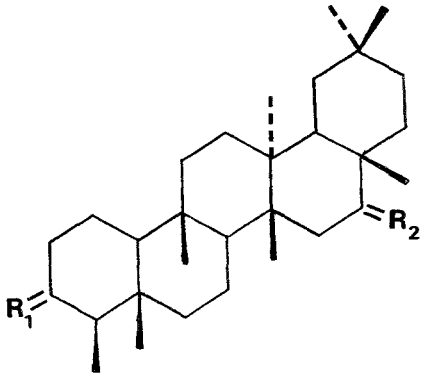
Chemical Analysis

Ground air-dried leaves of *Celaenodendrom mexicanum* were extracted with $CHCl_3$–MeOH (1 : 1). The resulting extract was fractionated via column chromatography on silica gel, and extensive preparative TLC on silica gel of the primary fractions yielded friedelin **(1),** maytensifolin B **(2),** 3β-hydroxyfriedelan-16-one **(3),** ginkgetin **(4),** bilobetin **(5),** and amentoflavone **(6)** (Figure 1).

Compound **(3)** is a new naturally occurring substance. All the isolated compounds were identified by spectral means: in the case of **1,** by comparison of the spectral data with those described previously in the literature. Compound **3,** $C_{30}H_{50}O_2$, was obtained as a white crystalline solid. The NMR (Nozaki et al., 1986; Patra and Kumar, 1987; Kikuchi et al., 1973a,b) and mass spectra (Nozaki et al., 1986; Courtney and Shanon, 1963) data of **1** supported the assignment of a friedelane-type triterpenoid and showed strong similarities to those of maytensifolin B**(2)** (Nosaki et al., 1986). Comparative analysis clearly indicated that the keto group at C-3 in **2** was replaced by a hydroxyl moiety in **3.** Consistent with this proposal were the presence of a signal attributable to a secondary hydroxyl group in the NMR spectra of **3** (δ 72.5 in the [^{13}C]NMR and δ 3.74 in the [^{1}H]NMR) and the chemical conversion of **3** into a diketone identical to maytensifolin B **(2)** by treatment with CrO_3–pyridine. The diagnostically important peaks at *m/z* 355, 219, and 124 in the mass spectra of **3** (Nosaki et al., 1986) as well as the chemical shift value of C-4 (δ 61.0) and C-23 (δ 11.6) were in agreement with the placement of the ketone and hydroxyl groups at C-16 and C-3, respectively. The stereochemistry at C-3 was unequivocally ascertained from the chemical shift and the splitting pattern observed for the signal at 3.74 (dd, J = 3 Hz) in the [^{1}H]NMR of **3** (Kikuchi et al., 1973a,b).

Bioassays with Seeds

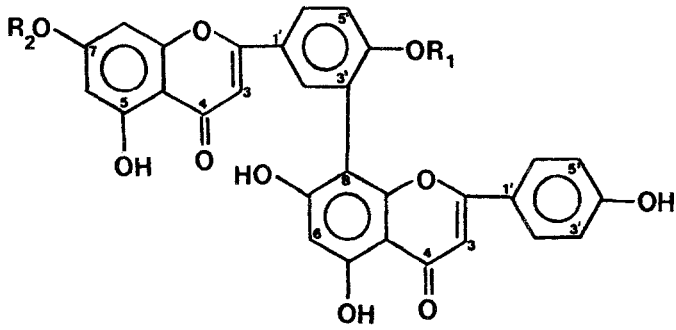
Aqueous leachates of dry leaves produced a significant stimulation of radicle growth of *Amaranthus* and *Echinochloa* (Table 1). In the case of the organic extract of leaves and twigs, a selective inhibitory effect on the growth of *Ama-*



1 $R_1 = O$ $R_2 = H$

2 $R_1 = R_2 = O$

3 $R_1 = OH\ \beta$ $R_2 = O$



4 $R_1 = R_2 = CH_3$

5 $R_1 = CH_3$ $R_2 = H$

6 $R_1 = R_2 = H$

FIG. 1. Structures of compounds **1–6.**

ranthus was observed. On the other hand, these extracts induced a significant stimulation of radical growth in *Echinochloa*.

In general, the growth responses of *Amaranthus* and *Echinochloa* (with the isolated compounds **1–6**) were opposites (Figure 2). Two of the compounds,

TABLE 1. EFFECTS OF AQUEOUS LEACHATES AND CHLOROFORM–METHANOL EXTRACT OF THE LEAVES AND TWIGS OF *Celaenodendron* ON THE RADICLE GROWTH OF *Amaranthus* AND *Echinochloa*

Treatment	Amaranthus		Echinochloa	
	Radicle growth (mm)	% of inhibition (stimulation)	Radicle growth (mm)	% of inhibition (stimulation)
Aqueous leachates				
Control	9.25 + 0.65	0	12.45 + 3.45	0
Dry leaves	11.47 + 2.24	(24)*	15.42 + 0.38	(24)*
Dead leaves	9.07 + 0.04	2.2	13.92 + 1.35	(8)
Chloroform-methanol extract				
Control	16.0 + 1.46	0	18.55 + 2.77	0
Dry leaves	8.82 + 0.1	45*	20.05 + 3.43	(12.4)*
Twigs	11.27 + 2.9	30*	23.15 + 2.95	(24.3)*

*$P < 0.05$.

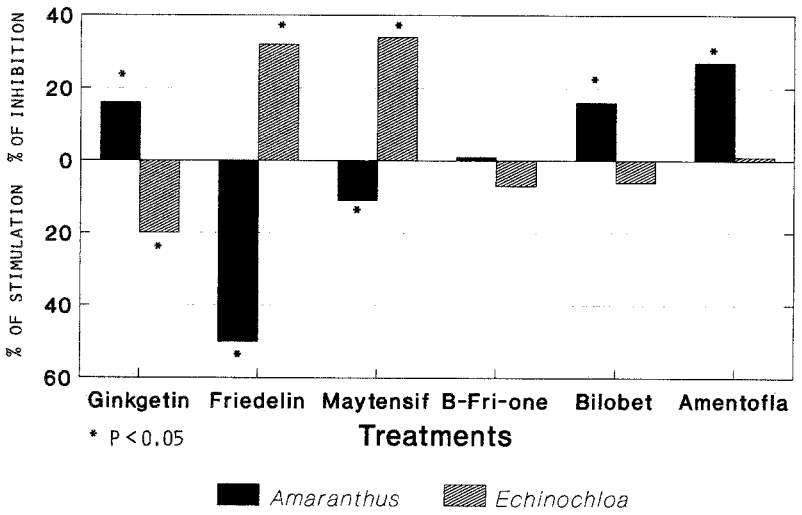


FIG. 2. Growth responses of *Amaranthus* and *Echinochloa*.

friedelin **(1)** and maytensifolin B **(2)**, produced a stimulatory effect on the radical growth of *Amaranthus*. However, they were the only compounds that significantly inhibited *Echinochloa*. The biflavonoids tested **(4–6)** inhibited the radical growth of *Amaranthus*; however, ginkgetin **(4)** significantly stimulated that of *Echinochloa*.

Bioassays with Phytopathogenic Fungi

Aqueous leachates produced only two significant effects on the target fungi (Table 2). Dry and dead leaves stimulated *Alternaria* and *Fusarium*, respectively, at 3 days of growth. Organic extracts of dead leaves stimulated *Alternaria* at 3 days of growth, but that of dry leaves significantly inhibited this species at 8 days. Organic extract of dry leaves significantly stimulated *Helminthosporium* at 3 and 8 days. On the other hand, a significant inhibitory effect of dead leaves on this species can be observed at three and eight days of growth.

The effects of compounds **3–6** on the fungi are shown in Figures 3 and 4. Ginkgetin had no effect on the three fungal species tested. 3β-Hydroxyfriedelan-16-one **(3)** produced a significant inhibition on *Alternaria* at 3 days, whereas *Fusarium* at 3 days and *Helminthosporium* at 8 days were significantly stimulated by this compound. Bilobetin **(5)** produced only a significant stimulation on *Alternaria* at 3 days. Finally, amentoflavone **(6)** significantly inhibited *Helminthosporium* at 3 and 8 days and stimulated *Alternaria* at 3 days. These results show that the target fungi had a clear difference in sensitivity and in response to each compound.

TABLE 2. EFFECTS OF AQUEOUS LEACHATES AND CHLOROFORM–METHANOL EXTRACT OF LEAVES AND TWIGS OF *Celaenodendron* ON THE RADIAL GROWTH OF *Alternaria*, *Fusarium*, AND *Helminthosporium*

	% of inhibition/(stimulation)					
	Alternaria		*Fusarium*		*Helminthosporium*	
Treatment	3 days	8 days	3 days	8 days	3 days	8 days
Aqueous leachates						
Control	0	0	0	0	0	0
Dry leaves	(22.4)*	(2.6)	(2)	3.4	(9.7)	(1.5)
Dead leaves	(8.5)	(8.1)	(11)*	(2)	(9.4)	(3)
Chloroform–methanol extract						
Control	0	0	0	0	0	0
Dry leaves	(7.2)	11.5*	(6.3)	(1.8)	(20.3)*	(11.8)*
Dead leaves	(18.6)*	(3.4)	(2.1)	0.3	13.6*	17.6*

*$P < 0.05$.

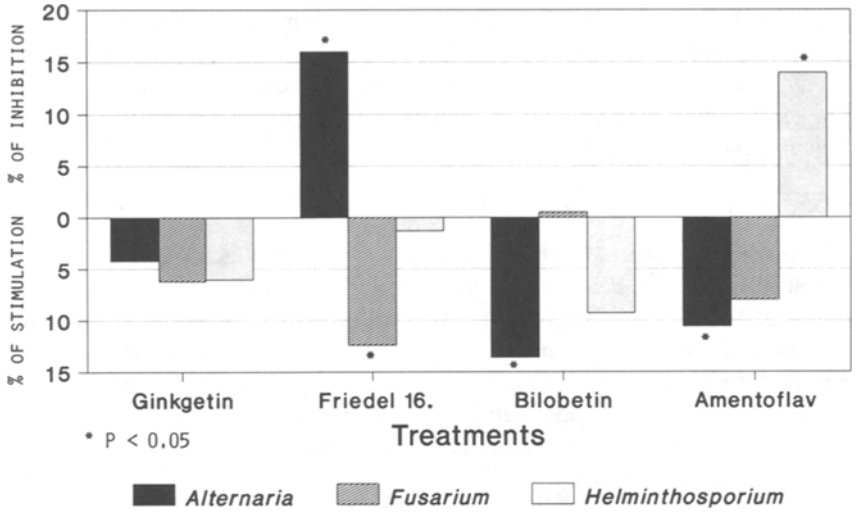


FIG. 3. Effects of compounds **3–6** on the fungi (3 days).

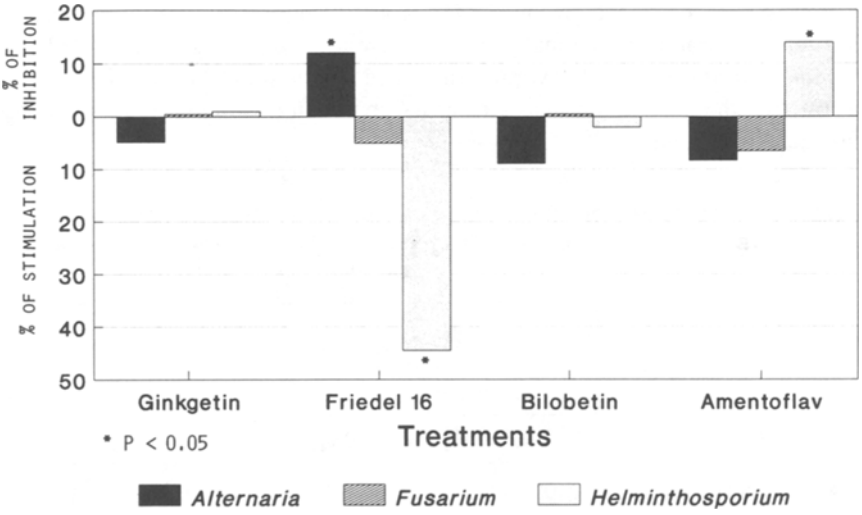


FIG. 4. Effects of compounds **3–6** on the fungi (8 days).

In relation to the two types of compounds identified in *Celaenodendron*, monoterpenes and sesquiterpenes are the terpenes more commonly involved in allelopathic effects on plants and microorganisms. (Rice and Pancholy, 1973; Del Amo and Anaya, 1978; González de la Parra et al., 1981; Robinson, 1983; Rice, 1984). Triterpenes have not been studied as allelopathic agents.

Flavonoids are compounds that have a wide range of biological activity. Some of these are implicated in allelopathy and antifeedant activities (Borner, 1959; Williams, 1960; Fottrell, 1964; Rice and Pancholy, 1974; Lodhi and Killingbeck, 1980; Corcuera et al., 1987; Moreland and Novitzky, 1987). As a result of the present study, it is possible to observe in general, that under *in vitro* conditions, biflavones inhibited *Amaranthus* and triterpenes inhibited *Echinochloa*.

In natural conditions, *Celaenodendron* cause a significant ecological effect on different organisms in the community. For example, the bearing of foliage almost year-round creates a constant light reduction by the tree that necessarily affects the growth of other species. In addition to the physical qualities of the microenvironment created by *Celaenodendron*, we hypothesize that the richness of this species in secondary compounds could contribute to this influence.

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AN INVESTIGATION OF HUMAN APOCRINE GLAND SECRETION FOR AXILLARY ODOR PRECURSORS

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Abstract—Recently completed studies from our laboratories have demonstrated that the characteristic human male axillary odors consist of C_6 to C_{11} normal, branched, and unsaturated aliphatic acids, with (*E*)-3-methyl-2-hexenoic acid being the most abundant. To investigate the mechanism by which the odor is formed, it is necessary to determine the nature of the odorless precursor(s) found in the apocrine secretion which is converted by the cutaneous microorganisms to the characteristic axillary odor. Pooled apocrine secretion was obtained from several male volunteers by intracutaneous injection of epinephrine. Partitioning this secretion into aqueous and organic soluble fractions was followed by hydrolysis of each fraction with NaOH or incubation with axillary microorganisms (cutaneous lipophilic corynebacterium). Analysis by gas chromatography/mass spectrometry (GC/MS) revealed the presence of (*E*)- and (*Z*)-3-methyl-2-hexenoic acid in the aqueous phase hydrolysate and aqueous phase incubated with bacteria; however, only a trace amount was seen in the resultant organic phase mixtures. These results

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suggest that a water-soluble precursor(s) is converted by the axillary flora to the characteristic axillary odors.

Key Words—Human axillary odors, human apocrine gland secretion, (*E*)-3-methyl-2-hexenoic acid, axillary odor precursors, androstenol.

INTRODUCTION

Recent reports from our laboratory have shown that the compounds imparting the characteristic odor to axillary secretions consist of C_6–C_{11}, normal, branched, and unsaturated acids, with the most abundant being (*E*)-3-methyl-2-hexenoic acid (3M2H) (Zeng et al., 1991; Leyden et al., 1990). Although organoleptic descriptions (Labows, 1988) and instrumental analysis (Preti et al., 1987; Labows et al., 1981) had previously suggested the presence of C_2–C_{18} acids in axillary secretions and extracts of this fluid, the exact structure of the acids and their relationship to the characteristic axillary odor was only recently determined (Zeng et al., 1991). Prior to this, the compounds suggested as responsible for axillary odor were thought to be volatile androst-16-enes, such as androstenone (5α-androst-16-en-3-one), androstadienone (androst-4,16-en-3-one), and androstenol (5α-androst-16-en-3α-ol) as well as isovaleric acid (Froebe et al., 1990; Labows, 1988, Gower, 1988).

In an attempt to better understand the mechanism by which the characteristic axillary odor is formed, the precursors to the axillary odors have been sought in the apocrine secretion. Our study is the first to look for the precursor(s) to 3M2H since it was not previously reported in axillary sweat or thought to be related to the characteristic axillary odor.

The research of Hurley and Shelly (1960) as well as Shehedah and Kligman (1963) demonstrated that freshly produced secretion is odorless and that the action of the cutaneous microorganisms upon the secretion creates the odor. Most of the published studies in this area have examined the apocrine secretion for steroidal-type molecules, namely, C_{19}-androgen sulfates and/or glucoronides (Labows 1988; Gower, 1988; Froebe et al., 1990). However, several lines of evidence suggested that the precursors to the odor might be lipid in nature. First, the axillary diptheroids, which are generally regarded to produce the more characteristic malodors (Leyden et al., 1981; Labows, 1988), have a lipid requirement for growth (McGinley et al., 1985); second, the structures of the compounds constituting the characteristic odor suggest that they are not formed by catabolism of amino acid substrates but may be secreted bound to glycerol and hydrolyzed to the free acids; third, the odor is formed fairly rapidly when axillary diptheroids are mixed with apocrine secretion (personal observations), suggesting that a rapid process (hydrolysis?) forms the odor; and fourth, other researchers have also alluded to possible nonvolatile, lipid-type molecules such as

triglycerides being present in the apocrine secretion, which could be degraded to give aliphatic acids such as 4-ethyloctanoic acid (Labows et al., 1981; Labows, 1988).

In this paper we report fractionation of apocrine secretion in aqueous and organic phases with subsequent saponification via NaOH and incubation with axillary diphtheroid bacteria. These experiments show that the precursor(s) to the characteristic axillary odors does not appear to be a lipid but is found in the water-soluble portion of the apocrine gland secretion.

METHODS AND MATERIALS

Apocrine Secretion Donors

Several collections of apocrine secretion were made to supply the material necessary for the experiments performed. Healthy male volunteers between 20 and 40 years of age served as paid voluntary donors. Informed consent was obtained prior to each collection. In the first collection, eight donors supplied a total of 8 μl of materia; seven donors supplied 6 μl in the second collection, and seven donors supplied 120 μl for the third collection, and six donors supplied 30 μl for the fourth collection.

The axillae was prepared for sampling by shaving the underarm area on the morning of the collection day (Labows et al., 1979). The donors rested in a supine position, the axillary region was washed with a nonionic detergent (0.1% Triton X-100), rinsed with water, dried, and finally, washed with hexane.

By this procedure, desquamated horny cells, most of the cutaneous flora, and skin surface lipids were removed, yielding apocrine secretion with minimal contamination by bacteria, cell debris, and sebum. To stimulate the apocrine glands, 0.1 ml of 1:2000 adrenalin (in physiologic saline) was intradermally injected. Droplets of apocrine secretion, which emerge from the ductal orifices, were collected with 10 μl glass micropipettes.

The secretions collected in the pipettes were pooled in 0.2-ml conical-shaped vials precooled to 0°C (React-a-Vial, Pierce, Rockford, IL). The four collections yielded the amounts detailed above. The third collection was unusual due to the large amount obtained. We think that it was due to several new donors being used. Since they were not familiar with the procedure, it was stressful enough to cause a larger volume of secretion to be expressed. The secretions were stored at −60°C until analysis.

Separation of Apocrine Secretions

The apocrine secretion microdroplets were diluted first with 300 μl distilled water, followed by 400 μl chloroform. The chloroform was mixed well with the apocrine secretions, and the mixture centrifuged for 5 min to form two layers.

The bottom chloroform layer was removed via pipette. The remaining aqueous layer was extracted two more times with chloroform (2 × 400 μl) and subsequently separated by the same procedure as above. This procedure gave a total volume of 1.2 ml of chloroform solution and 300 μl of aqueous apocrine solution.

In the experiment where the large amount (120 μl) of apocrine secretion was employed, aliquots of the aqueous (10 μl) and chloroform layers (10 μl) were set aside for analysis using sodium dodecyl sulfate (SDS)/polyacrylamide gels, as described later.

Aqueous Phase Hydrolysis of Apocrine Secretion

The aqueous apocrine solution was refluxed for 20 min with 1 ml of 5% sodium hydroxide aqueous solution under a stream of nitrogen. The mixture was allowed to cool to room temperature and acidified with 2*M* HCl to pH 2. A slight axillary-like odor developed in the reaction mixture upon acidification. The acidified mixture was extracted with 3 × 150 μl chloroform.

In the first two experiments, the combined chloroform extracts were concentrated to approximately 5 μl for organoleptic evaluation and GC/MS analysis. However, in the experiments employing the larger volumes of apocrine secretion, we concentrated to approximately 12 μl.

Organic Phase Hydrolysis of Apocrine Secretion

In each experiment, the chloroform extract obtained from apocrine secretion was concentrated to approximately 100 μl and refluxed for 20 min with 5% NaOH in 2 ml of a 1 : 1 solution of methanol : water under a stream of nitrogen: the same conditions used for the aqueous phase hydrolysis. After removal of the solvent, the reaction mixture was acidified with 2 *M* HCl to a pH of 2; no odor was detected. This acidic solution was extracted with chloroform (3 × 0.5 ml) and concentrated to approximately 5 μl.

Incubation of Apocrine Secretion Aqueous and Organic Phases with Diphtheroid Bacteria

A combined male apocrine secretion sample (~30 μl) from six donors was separated into aqueous and organic soluble phases as described above. Each phase was transferred to 50-ml, round-bottomed flasks, and the $CHCl_3$ removed from each by rotary evaporation. To each of these flasks was added 200 μl of McFarland Barium Sulfate Standard No. 2 (Balows, 1991) containing ~600,000,000 colony forming units/ml of cutaneous lipophilic corynebacterium (CLC). These are the most abundant species of diphtheroid bacteria found in the human axillae. An equal amount of McFarland Standard No. 2 with CLC

was added to an empty, sterile, 50ml round-bottom flask to use as a blank. All three flasks were sealed with parafilm and incubated at 37°C for 24 hr.

Following this time the contents of each flask were transferred to three centrifuge tubes. Each flask was rinsed with 300 μl doubly distilled water and each rinse was combined with its respective solution. Sodium chloride (20 mg) was added to each centrifuge tube, and the three solutions were extracted separately with chloroform (3 × 500 μl). The extraction procedure was identical to that described above following the hydrolysis with NaOH. Each chloroform extract was concentrated to 10 μl by the vacuum concentration and analyzed by GC/MS.

Sodium Salt of 3-Methyl-2-Hexenoic Acid: Extraction and Hydrolysis

To form the sodium salt, 500 ng of a 10:1 synthetic mixture of *E*- and *Z*-3-methyl-2-hexenoic acid was added to a solution (0.5 ml) of 5% NaOH in a (1:1) water:ethanol mixture. This solution was adjusted to pH 2 by dropwise addition of 6 *N* HCl. The acidified solution was extracted with chloroform (2 × 1 ml). The chloroform was divided into two equal portions. One of these portions as well as the remaining aqueous layer was treated with the 5% NaOH solution and refluxed under N_2 for 20 min. The solutions were cooled to room temperature, acidified to pH 2 with 6 *N* HCl, and extracted with chloroform (2 × 0.5 ml). These two extracts and the unreacted half of the chloroform extract (see above) were each concentrated to approximately 5 μl by vacuum concentration for GC/MS analysis.

Examination of Apocrine Secretion for the Sodium Salt of 3-Methyl-2-Hexenoic Acid

To test if the 3-methyl-2-hexenoic acids are present in apocrine secretion as their sodium salts and therefore remain in the aqueous phase during the partitioning with $CHCl_3$, a sample of combined apocrine secretion from 12 donors (~40 μl) was adjusted to pH 2 by the addition of approximately 50 μl 6*N* HCl. The acidified sample was diluted to 300 μl with doubly distilled water and extracted with 3 × 400 μl of $CHCl_3$. One microgram of D_{19}-decanoic acid was added to each phase as an internal standard to gauge efficiency of recovery. One-half the $CHCl_3$ extract and the remaining aqueous phase were subjected to hydrolysis and analyzed as described above. The portion of extract not subjected to hydrolysis was concentrated to ~10 μl by vacuum concentration and analyzed by GC/MS.

Synthesis of Both 3-Methyl-2-Pentenoic and 3-Methyl-2-Octenoic Acids

The synthesis of these compounds was similar to that employed for (*E*)- and (*Z*)-3-methyl-2-hexenoic acid (Zeng et al., 1991).

To a mixture of NaH (1.33 g, 55.3 mmol) and toluene (80 ml), triethyl

phosphonoacetate (12.4 g, 55.3 mmol) was added dropwise at room temperature for 30 min. The temperature was raised to 40°C. After 1 hr of stirring, 2-butanone (3.98 g, 55.3 mmol) or 2-heptanone (6.31 g, 55.5 mmol) was added dropwise, and stirring was continued at 40°C for 2 hr. The reaction mixture was poured into ice water (70 ml) and extracted with diethyl ether (3 × 40 ml). The extract was dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*, and the residue was added to a solution of KOH (5.0 g) in methanol (30 ml) and water (30 ml). The reaction mixture was refluxed for 2 hr, cooled to room temperature, then poured into ice water (100 ml) and extracted with diethyl ether (2 × 60 ml). The aqueous layer was acidified with dilute sulfuric acid and extracted with *n*-hexane (3 × 100 ml). The extract was dried over anhydrous sodium sulfate and concentrated to give an oil (5.04 g, 80% yield for 3-methylpentenoic acids; and 7.14 g, 83% yield for 3-methyl-2-octenoic acids).

Analysis by GC/MS showed that both products contain the (*E*)- and (*Z*)-isomers at a ratio of approximately 3.6:1. The H^1 and C^{13}-NMR data for these isomers have been previously reported (Ogura et al., 1970; Gahiez et al., 1976; Hagen et al., 1980). The mass spectra and relative chromatographic retention times [ethyl ester unit; EEU (van den Dool and Kratz, 1963)] are given here.

(Z)-*3-Methyl-2-Pentenoic Acid.* Mass spectrum: *m/z* (rel. int.) *m/z* (rel. int.) M+ 114 (100), 99 (42), 98 (16), 97 (18), 95 (20), 85 (13), 81 (19), 70 (31), 69 (25), 67 (32), 59 (10), 53 (23), 45 (9), 43 (50), 42 (13), 41 (45). EEU = 12.27.

(E)-*3-Methyl-2-Pentenoic Acid.* Mass spectrum: M^+ 114 (100), 99 (52), 98 (15), 97 (20), 95 (20), 85 (15), 81 (19), 72 (14), 71 (10), 70 (30), 69 (30), 67 (32), 59 (8), 55 (20), 53 (22), 45 (5), 43 (35), 41 (50). EEU = 12.63.

(Z)-*3-Methyl-2-Octenoic Acid.* M^+ 156 (23), 141 (5), 113 (100), 100 (82), 97 (28), 95 (78), 85 (24), 82 (28), 72 (75), 67 (29), 55 (30), 43 (75), 41 (33). EEU = 14.93.

(E)-*3-Methyl-2-Octenoic Acid.* M^+ 156 (3), 138 (10), 127 (1), 113 (37), 100 (100), 95 (42), 85 (22), 81 (19), 72 (24), 71 (17), 70 (13), 67 (17), 57 (15), 56 (15), 55 (22), 43 (10), 42 (19), 41 (18). EEU = 15.45.

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

A Finnigan 4510 GC/MS data system equipped with a split/splitless injector, a fused silica capillary column, and capabilities for operation in both electron impact and chemical ionization modes, was used for analysis. The column employed was a 30 m × 0.32-mm (I.D.) fused silica column with a 1.00-μm coating of Stabilwax (cross-bonded polyethylene glycol; Restek, Port Matilda, PA). The mass spectrometer is interfaced to a Nova 4X computer, which utilizes the Super Incos software for data acquisition, analysis, and quantitation. The

mass range employed during these analyses was m/z 40–400. This mass range was scanned once each second and a typical run included 4000 scans. The data system also included the NBS library of 42,000 compounds.

Identifications were based on comparison of unknown spectra with both the NBS library and interpretation of the resulting spectrum with mass spectra generated from synthetic standard compounds. In addition, the relative chromatographic retention times of unknown and known standards were compared. A mixture of fatty acid ethyl esters was used to determine the relative retention times (van den Dool and Kratz, 1963).

Organoleptic Evaluation of Hydrolyzed Apocrine Secretion Samples

This procedure was performed using a panel of three or four judges in a manner similar to that used for evaluation of chromatographic eluants (Zeng et al., 1991). Judges were presented in a blind manner with the acidified, hydrolyzed aqueous or organic portions of apocrine secretion and asked which had an odor that most resembled axillary odor. A small (2- to 5-μl) aliquot of concentrated axillary extract on a filter-paper strip (in a sealed vial) was on hand for reference if requested by a judge.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Apocrine Secretion and Extracts

SDS-PAGE was done according to Laemmli (1970). The stacking gel was 5% acrylamide and the separating gel was 15%. Samples of interest were dried down to approximately 5 μl, then mixed with 5 μl sample buffer containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, and 50 m*M* Tris–HCl at pH 6.8. After electrophoresis, the gels were stained with Coomassie blue.

RESULTS

The amount of apocrine secretion collected in each of the collections was highly variable and appeared to be dependent on the nature of the subject rather than their number. Certain individuals yielded as much as 20–30 μl, possibly due to their being tense about the collection procedure.

The first two experiments, performed with small amounts of apocrine secretion (7 and 6 μl, respectively), yielded identical results: the concentrated chloroform extract made from the acidified aqueous layer hydrolyzate had a weak but distinct axillary odor when $\sim$2 μl was allowed to evaporate to dryness on clean, odorless filter-paper strips. The identical extract obtained from the organic layer hydrolyzate had no axillary odor or any other odor quality distinguishable from solvent.

Gas chromatography/mass spectrometry (GC/MS) analysis of the axillary smelling extract did not yield any distinct mass spectra which could point to the presence of odorous components such as (*E*)-3-methyl-2-hexenoic acid (*E*-3M2H) or any of the other acidic components known to be present in the axillary odor bouquet (Zeng et al., 1991). This result is consistent with the olfactory threshold of these compounds being below the instrumental detection level.

The third experiment performed with a large amount of apocrine secretion (~ 120 μl) yielded a chloroform extract from the acidified aqueous layer hydrolysate, which had a strong axillary odor upon concentration (~ 12 μl). One microliter (1 μl) was employed for GC/MS analysis, as was 1 μl of the concentrated extract of the organic layer hydrolyzate.

Figure 1 shows the area in the computer reconstructed chromatogram where previous studies (Zeng et al., 1991) have shown the characteristic axillary odors, (*E*)- and (*Z*)-3M2H elute. Figure 1A is the organic phase hydrolysate, while Figure 1B is the aqueous phase hydrolysate. Above each of the chromatograms is a plot of *m/z* 128, the ion representing the intact, ionized, 3-methyl-2-hexenoic acids, eluting in this area. Only a trace amount of the (*E*)-3M2H, is seen in the organic phase hydrolysate (Figure 1A). From comparison of the area of the ions for *m/z* 128 with injection of known amounts of 3M2H, we estimate that the aqueous phase hydrolysate contained approximately 50.0 ng/μl of the (*E*)-isomer and 4.2 ng/μl of the (*Z*)-isomer. The organic phase hydrolysate contained approximately 0.5 ng/μl of the (*E*)-isomer. We recently reported (Zeng et al., 1991) that the (*E*)-isomer-to-(*Z*)-isomer ratio in a combined male extract was 10:1; the same approximate ratio is also seen here (Figure 1B).

Similar results were obtained from the experiment involving incubation of the diphtheroid bacteria with separate samples of apocrine secretion aqueous and organic phases. Analysis of these samples showed that bacteriolysis of the aqueous phase produced 5.13 ng/μl of the *E*-isomer and 0.12 ng/μl of the *Z*-isomer, while the organic phase yielded only a trace of the *E*-isomer (0.02 ng/μl). Analysis of the blank, which contained only the McFarland medium, did not show any *E*- or *Z*-3M2H. The results of both the NaOH hydrolysis and the bacteriolysis are summarized in Table 1.

Figure 2 shows the portion of the computer-reconstructed chromatogram where previous studies have shown the C_6 to C_{11} acids constituting the axillary "bouquet" elute (Zeng et al., 1991). Each of the compounds is labeled and its identity is given in Table 2. These data show that the aqueous and organic phase hydrolysates contained many of the straight-chained acids which we have reported in the axillary extract (Preti et al., 1987; Zeng et al., 1991), albeit at relatively low levels. In addition, the aqueous phase extract also contained small amounts of three other unsaturated acids. We have identified two of these as 7-octenoic acid (Peak I), (*E*)-3-methyl-2-pentenoic acid (Peak B, a lower homologue of

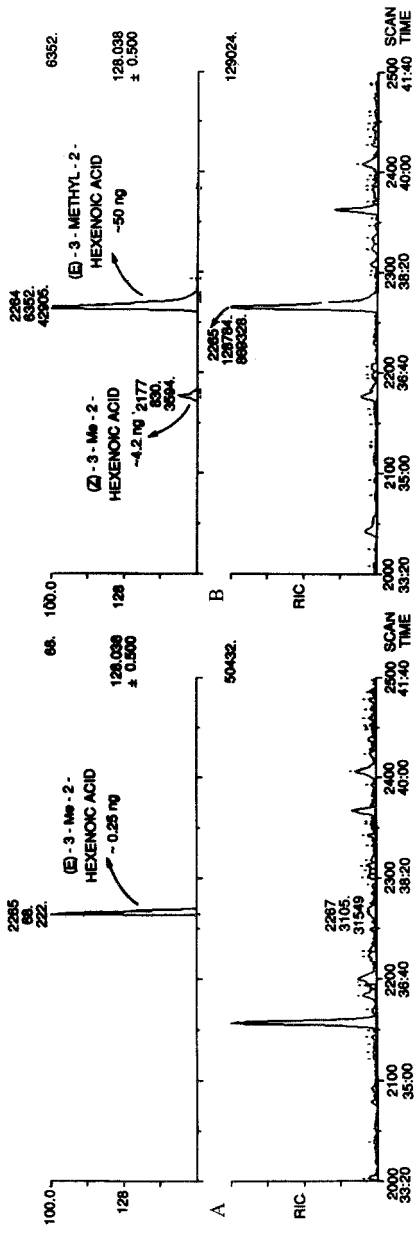

FIG. 1. Reconstructed ion chromatogram showing the area where (Z)- and (E)-3-methyl-2-hexenoic elute. (A) The organic phase hydrolysate; (B) the aqueous phase hydrolysate. The top half of A and B shows a mass chromatogram of m/z 128, the intact, ionized molecular ion of (E)- and (Z)-3-methyl-2-hexenoic acid. The aqueous phase hydrolysate contains approximately 200 times more 3-methyl-2-hexenoic acid than the organic phase hydrolysate.

TABLE 1. AMOUNTS OF 3-METHYL-2-HEXENOIC ACID LIBERATED FROM APOCRINE SECRETION BY NaOH HYDROLYSIS AND BACTERIOLYSIS

	Nanograms per microliter			
	5% NaOH hydrolysis		Bacteriolysis	
	E-Isomer	*Z*-Isomer	*E*-Isomer	*Z*-Isomer
Aqueous phase	50.0	4.20	5.13	0.12 ng/μl
Organic phase	0.25	0	0.02	0

3M2H), and a C_9-unsaturated acid (Peak L). The structure of 3-methyl-2-pentenoic acid was confirmed by comparison (MS and relative retention time) to a synthesized sample. The C_9-unsaturated acid had a mass spectrum and relative retention time identical to that of an unknown C_9-unsaturated acid we reported in our study of male axillary odor components (Zeng et al., 1991). We also synthesized 3-methyl-2-octenoic acid to see if it corresponded to this unknown C_9-unsaturated acid; it did not (see Table 2). No evidence was seen for these three unsaturated acids in the organic phase hydrolysate.

We tested the possibility that 3M2H and other acids might be present in apocrine secretions as their salts and therefore left in the aqueous phase after extraction with $CHCl_3$. To do this we first determined what would result from the $CHCl_3$ extraction and subsequent hydrolysis of a basic solution containing a known amount (500 ng) of *E*- and *Z*-3M2H. Acidification of the basic solution produced the characteristic axillary-like odor of the 3M2H. After extraction with $CHCl_3$ half of this extract (E_1) and the remaining aqueous phase (A_R) were refluxed with 5% NaOH as described above. The other half of the extract (E_2) was concentrated and analyzed by GC/MS. The results of the GC/MS analysis of each sample are given in Table 3. These data show that all the 3M2H were extracted out of the aqueous phase and approximately 58% of the acids extracted into the $CHCl_3$ were recovered.

This experiment was repeated using a 40-μl sample of apocrine secretion. Upon adjusting to pH 2, we noted a weak but distinct odor. The smell was not that of 3-methyl-2-hexenoic acid but a burnt/onion, sulfurous odor. After dilution and extraction of the acidified sample, half the $CHCl_3$ extract (AE_1) and the remaining aqueous phase (AAR) were refluxed with 5% NaOH and analyzed as described above. The other half of the $CHCl_3$ extract (AE_2) was concentrated and analyzed. The results of each analysis are given in Table 4. The recovery of the D_{19}-decanoic acid internal standard for each of the three samples was 44, 52, and 71% for AE, AAR, and AE_2, respectively.

The data in Table 4 shows that the hydrolysis procedure liberated 3-methyl-

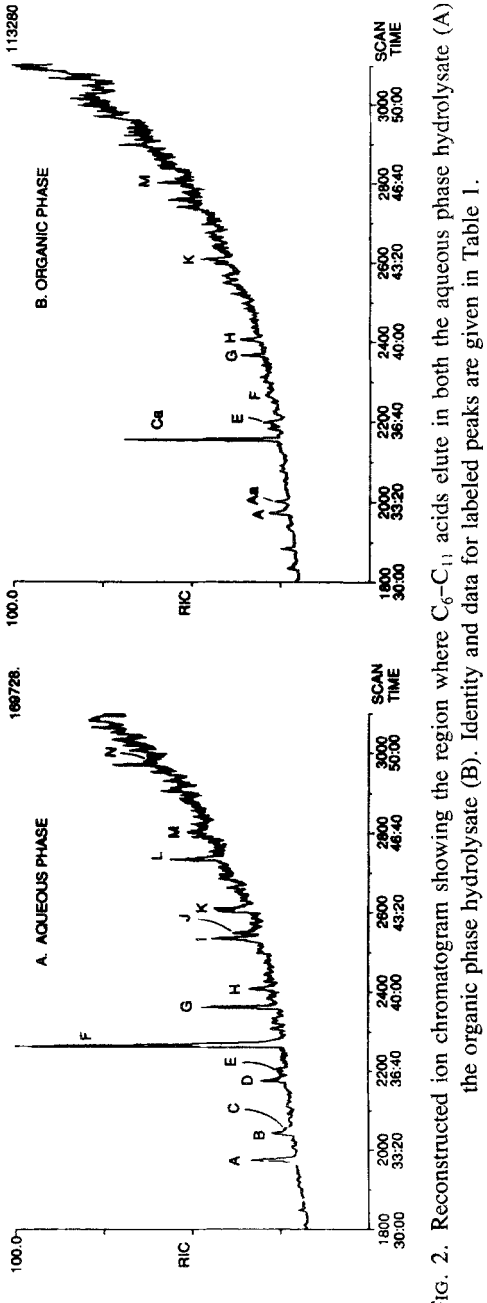

FIG. 2. Reconstructed ion chromatogram showing the region where C_6–C_{11} acids elute in both the aqueous phase hydrolysate (A) and the organic phase hydrolysate (B). Identity and data for labeled peaks are given in Table 1.

TABLE 2. IDENTIFICATION OF COMPONENTS IN FIGURE 2[a]

	Peak	MW	Relative retention time (EEU)[b]
A	Hexanoic acid (C)	116	12.20
Aa	Unknown[c]	—	12.14
B	(*E*)-3-Methyl-2-pentenoic acid (C)	114	12.55
C	3-Methylhexanoic acid (C)	130	12.60
Ca	Unknown[d]	—	12.80
D	(*Z*)-3-Methyl-2-hexenoic acid (C)	128	13.05
E	Heptanoic acid (C)	130	13.23
F	(*E*)-3-Methyl-2-hexenoic acid (C)	128	13.50
G	Unknown[e]	—	14.00
H	Octanoic acid (C)	144	14.30
I	7-Octenoic acid (C)	142	14.91
J	Unknown[f]		15.00
K	Nonanoic acid (C)	158	15.31
L	C_9-Unsaturated acid[g]	156	15.96
M	Decanoic acid (C)	172	16.30
N	Undecanoic acid (C)	186	17.30

[a] C—correspondence of mass spectrum and relative chromatographic retention times with commercially available or synthetic sample; T—tentatively assigned from mass spectral and relative retention time data.

[b] Ethyl ester units.

[c] Unknown at 12.14 EEU found only in organic phase hydrolyzate: *m/z* (relative intensity) 43 (95), 56 (20),70 (27), 71 (100), 96 (8), 100 (5), 111 (8).

[d] Unknown at 12.80 EEU found only in organic phase hydrolyzate: *m/z* (relative intensity) 45 (20), 59 (15), 74 (100), 87 (8), 107 (7), 139 (12), 141 (6). Although this contains some salient mass spectral characteristics of a methyl ester, it was not one.

[e] Unknown at 14.00 EEU found only in aqueous phase hydrolyzate: *m/z* (relative intensity) 41 (10), 43 (45), 55 (7), 56 (11), 57 (21), 69 (8), 71 (100).

[f] Unknown at 15.00 EEU found only in aqueous phase hydrolysate: *m/z* (relative intensity) 45 (100), 55 (100), 56 (12), 60 (8), 67 (11), 68 (15), 70 (8), 71 (9), 72 (15), 81 (18), 82 (21), 87 (9), 95 (5), 110 (3), 142 (5).

[g] Mass spectrum of unknown, C_9-unsaturated acid: *m/z* (relative intensity) M^+ 41 (70), 43 (85), 45 (92), 55 (100), 56 (55), 58 (35), 67 (55), 68 (70), 69 (72), 70 (22), 71 (22), 72 (25), 81 (45), 83 (35), 85 (25), 88 (25), 96 (57), 97 (38), 114 (35), 138 (3), 156 (10). EEU = 16.04. Does not compare with mass spectrum of 3-methyl-2-octenoic acid in Methods and Materials or 8-nonenoic acid reported previously (Zeng et al., 1991).

2-hexenoic acid from the aqueous phase; this is in contrast to A_R in Table 3, where no 3-methyl-2-hexenoic acids were found.

The apocrine secretion aqueous and organic phase hydrolysates were also investigated for the presence of volatile steroids and/or steroid sulfates by GC/MS using previously established procedures employing bonded methyl silicone columns (Preti et al., 1987; Zeng et al., 1991). No evidence of these compounds was found in the organic phase hydrolysate. In contrast, the aqueous

TABLE 3. RECOVERY OF SODIUM 3-METHYL-2-HEXENOATE AFTER HYDROLYSIS

Sample	Nanograms of 3-methyl-2-hexenoic acid
E_1	160.7
E_2	130.6
A_R	0

TABLE 4. RECOVERY OF 3-METHYL-2-HEXENOIC ACID FROM ACIDIFIED APOCRINE SECRETION AND SUBSEQUENT HYDROLYSIS

Sample	Nanograms of 3-methyl-2-hexenoic acid
AE_1	46.1
AE_2	106.2
AA_R	136.5

phase hydrolysate contained detectable amounts of the steroid sulfates; 17-oxo-5-androsten-3α-yl sulfate (dehydroepiandrosterone sulfate) and 17-oxo-5α-androstan-3α-yl sulfate (androsterone sulfate) were seen as their pyrolysis products (Labows et al., 1979; Preti et al., 1987). The free steroids, androsterone and dehydroepiandrosterone, were also present. No evidence of the volatile steroids (e.g., androstenone and androstenol) was seen in these samples.

Similar analyses were carried out on the aqueous and organic phase following bacteriolysis. No evidence was found for the presence of C_{19}-steroids in the organic phase. However, the aqueous phase contained both dehydroepiandrosterone sulfate and androsterone sulfate. In addition, androsternol was found in this sample along with dehydroepiandrosterone and androsterone. Although quantitation was not done, the level of androstenol was comparable to the levels of dehydroepiandrosterone sulfate.

To determine how much of the steroid sulfates would be converted to the free steroids by the hydrolysis procedure, we performed an experiment employing 100 μg of dehydroepiandrosterone sulfate and 50 μg of androsterone sulfate. These are the approximate amounts expected in 120 μl of apocrine secretion as per previous studies (Labows et al., 1979). One milliliter of 5% aqueous NaOH was added to 0.12 ml of doubly distilled water containing the above sulfate levels and the hydrolysis performed as described under Methods and Materials. Workup and analysis of the resultant hydrolysate showed that only 17% of the sulfates were cleaved by the procedure. Previous studies have shown that these

sulfates do not appear to be precursors for androstenol (Labows, 1988; Froebe et al., 1990).

Further investigation of the nonhydrolyzed organic and aqueous layers was performed using SDS/polyacrylamide electrophoresis. Figure 3 shows the Coomassie blue-stained gel on which were loaded comparable aliquots of organic soluble compounds (lanes 1 and 2) and aqueous soluble compounds (lane 3) prior to hydrolysis. These were saved from the experiment which utilized the large amount (~ 120 ml) of apocrine secretion (as noted above).

The aqueous portion of the apocrine secretion appears to be extremely rich in proteins, while little or no protemaceous material is seen in the organic phase. Several protein bands can be seen in lane 3 including major protein components found at apparent molecular weights of 66,000, 40,000, and 26,000.

DISCUSSION

The finding that the water-soluble components of apocrine secretion contained nonodorous molecules that could be hydrolyzed to yield (*E*)- and (*Z*)-3M2H by both hydrolysis with NaOH and incubation with axillary diphtheroids was unexpected. It suggests that an important, characteristic human axillary odor is not being synthesized on the skin surface. Possibly, this is made elsewhere in the body, stored in the apocrine gland, and secreted in the apocrine secretion with the water soluble components. Consequently, this axillary odor appears on the axillary skin surface bound to a precursor and is liberated by the action of the cutaneous microflora.

The experiments in which the sodium salts of *E*- and *Z*-3M2H and apocrine secretion were acidified, extracted, and hydrolyzed further support our conclusion of an aqueous soluble precursor. Acidification of the basic solution containing the sodium 3M2H yielded the distinct characteristic odor of the 3M2H; subsequent extraction of the aqueous phase with $CHCl_3$, hydrolysis with NaOH, and analysis by GC/MS showed no 3M2H in the resulting hydrolysate (Table 3, Ar). This suggests that the 3M2H was removed from the acidified aqueous phase. However, such a result was not seen when the similar procedure was performed with apocrine secretion. The odor produced by the acidified apocrine secretion was not that of the 3M2H but had a distinct burnt, sulfur/onion odor, which suggested that a reaction was occurring which liberated 3M2H as shown in Table 4. However, a larger amount of 3M2H remained in the aqueous phase of the acidified apocrine secretion than in its $CHCl_3$ extract.

These data are consistent with the 3M2H being associated, in some fashion, with a water-soluble precursor molecule. Addition of HCl to the apocrine secretion either cleaves a precursor–3M2H covalent bond or disrupts the intermolecular forces holding the precursor–3M2H together. If the precursor molecule is

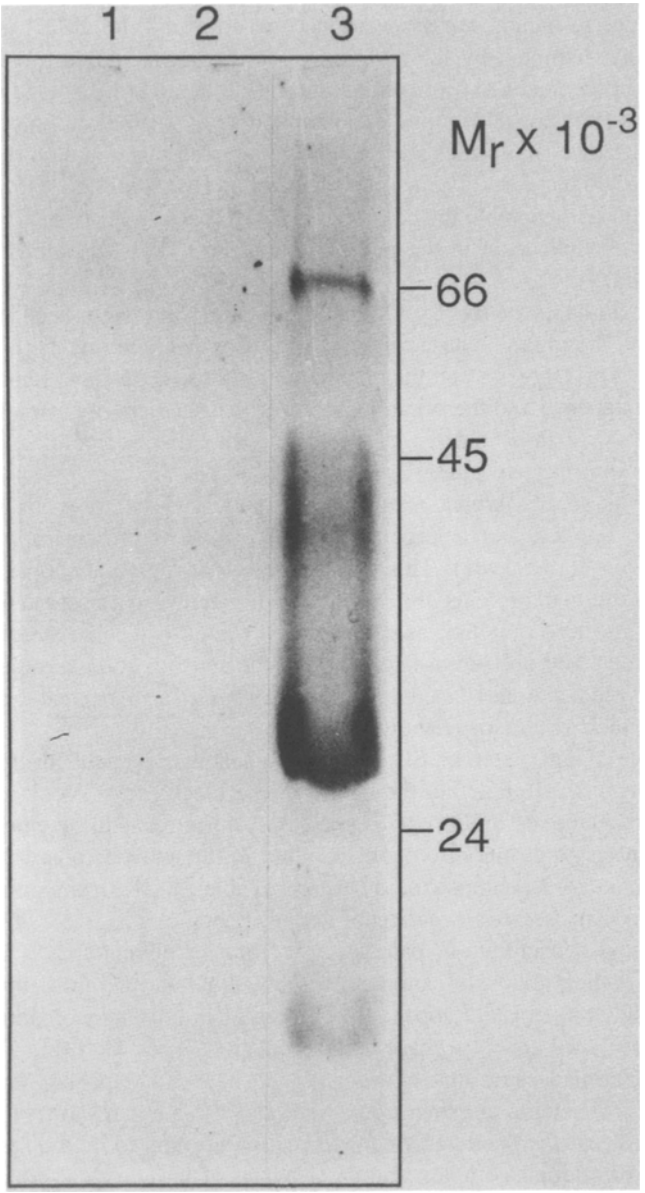

FIG. 3. The SDS–polyacrylamide gel electrophoretic profile of the unhydrolyzed organic phase (lanes 1 and 2) and aqueous phase (lane 3). The aqueous soluble molecules contain a number of proteins as shown by Coomassie blue staining. No proteinaceous material is seen in lanes 1 and 2. Molecular weight markers (not shown) were used to generate the scale at the right.

proteinaceous in nature, the association between it and the 3M2H may be one that is easily disrupted by the addition of acid or base. Some proteins, under acidic conditions, even at room temperature, will begin to hydrolyze and/or lose their three-dimensional structure (Hunkapillar et al., 1983; Kopple, 1966). In the latter instance, molecules such as 3M2H intercathelated within the protein's structure would be released. The odor noted when HCl was added to the apocrine secretion did not resemble the acid but may have been indicative of bond cleavage (thioester linkages?) in the putative precursor. These are areas for further investigation.

Small amounts of the C_6–C_{11} straight-chain acids were seen in both the aqueous and the organic phase mixtures following bacterial incubation and base hydrolysis. This suggests that the precursors for these acids have some solubility in both the aqueous and the organic phases or that they are present at low levels (below olfactory threshold) in the collected apocrine secretion and partition themselves into the two phases, prior to hydrolysis.

Previous studies which examined the apocrine secretion for its various components suggest that a large amount of protein is present: approximately 10% (Labows et al., 1981). This percentage seems high and should be reevaluated. In addition to proteins, the steroid sulfates were also present in the aqueous portion of apocrine secretion as expected. However, a volatile steroid (androstenol) was detected only upon interaction of the aqueous soluble molecules with the diphtheroid bacteria; consequently, axillary bacteria are needed to metabolize them from their precursor (Labows, 1988).

Our initial data based on SDS-PAGE suggest that the aqueous phase is rich in proteinaceous material, but the nature of the precursor molecules associated with the characteristic axillary odor (i.e., 3M2H) remains to be elucidated.

One interesting minor component found in the aqueous phase hydrolysate was (*E*)-3-methyl-2-pentenoic acid (Peak B, Table 2). This compound does not appear in axillary secretions collected in pads (Zeng et al., 1991). This may be due to its low concentration, precluding it from instrumental detection and its coelution, in the previously examined combined extract with 3-methylhexanoic acid. The latter compound appears to be present in only trace quantities in the freshly hydrolyzed aqueous phase component (i.e., Peak C, Table 2).

Our data also show that hydrolysis with base accomplishes much of the same effect as bacterial enzymes do *in vivo*, albeit in a much shorter time frame. The main role of the bacteria in odor formation appears to be the disruption of the precursor/odorant complex and/or cleavage of the covalent bonds holding the acid molecules to the precursor.

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SEX PHEROMONE COMPONENTS OF THE GEOMETRID MOTHS *Lobophora nivigerata* AND *Epirrhoe sperryi*[1]

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Abstract—3Z,6Z,9Z-Nonadecatriene (3Z,6Z,9Z-19:H; other abbreviations follow the same pattern) has been identified as a female sex pheromone component of the geometrid moth *Epirrhoe sperryi* (H.). 3Z,6Z,9Z-18:H and 6Z,9Z-19:H were also identified in pheromone gland extracts but had no apparent biological activity. 3Z,6Z,9Z-21:H was tentatively identified as a female sex pheromone component of a second geometrid species, *Lobophora nivigerata* (Wlk.). Attraction of male moths to this compound was strongly synergized by the addition of small amounts of 6Z,9Z-21:H to lures.

Key Words—Sex attractant, pheromone, 3Z,6Z,9Z-octadecatriene, 3Z,6Z,9Z-nonadecatriene, 3Z,6Z,9Z-heneicosatriene, 6Z,9Z-nonadecadiene.

INTRODUCTION

Unsaturated hydrocarbons with one to four *cis* double bonds separated by single methylene units have been identified as sex attractants and sex pheromone components for a variety of moths from several insect families. Compounds of this type are usually produced by the female but can also be produced by males (Heath et al., 1988; Descoins et al., 1990). These types of compounds have been found as single component pheromones in the winter moth and Bruce spanworm (Roelofs et al., 1982; Underhill et al., 1987), which both use

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1,3Z,6Z,9Z-nonadecatetraene as a single-component pheromone. More commonly, unsaturated hydrocarbons occur as blends, either with each other or with similar components such as unsaturated epoxides, aldehydes, or ketones (Millar et al., 1990a, and references therein). Hydrocarbon blends may occur as mixtures of homologues with the same number of double bonds [*Anticarsia gemmetalis* (Heath et al., 1983); *Mocis disseverans* (Landolt et al., 1986); *Caenurgina erechtea* (Underhill et al., 1983)] or as mixtures of compounds with the same or different carbon-chain lengths, with different numbers of double bonds [*Arctia villica* (Einhorn et al., 1984); *Utetheisa ornatrix* (Jain et al., 1983); *Alsophila pometaria* (Wong et al., 1984); *Paleacrita vernata* (Millar et al., 1990a)].

Wong et al. (1985) reported 3Z,6Z,9Z-nonadecatriene (3Z,6Z,9Z-19:H; other abbreviations follow the same pattern) as a sex attractant for male *Epirrhoe sperryi* (H.) moths. We report here (1) the identification of 3Z,6Z,9Z-18:H, 3Z,6Z,9Z-19:H, and 6Z,9Z-19:H in *E. sperryi* female pheromone glands, (2) the tentative identification of 3Z,6Z,9Z-21:H in *L. nivigerata* female pheromone glands, and (3) the results of field tests with blends of the identified components.

METHODS AND MATERIALS

Insects and Electroantennography. Male moths were captured in light traps, in sweep nets, or in traps baited with synthetic sex attractants. Female moths of unknown mating status were taken in light traps or in sweep nets. Male antennal responses to synthetic compounds were measured by EAG (Chisholm et al., 1975), using 1-μg amounts of compounds applied to filter-paper disks and by the GC-EAD method of Arn et al. (1975). Synthetic compounds tested in EAG and/or GC-EAD studies included C_{17-22} chain lengths of the following types of compounds: 6Z,9Z-, 3Z,6Z,9Z-, 1,3Z,6Z,9Z-, 3Z,6Z,9Z,11Z-, and 3Z,6Z,9Z,11*E*-unsaturated hydrocarbons; combined mixtures of the monoepoxide regioisomers from nonselective oxidation of the diene or triene hydrocarbons above (combined monoepoxides, CME); racemic and chiral forms of 3Z,6Z-*cis*-9,10-, 3Z,9Z-*cis*-6,7-, and 6Z,9Z-*cis*-3,4-epoxydienes; and racemic and highly enantiomerically enriched forms of 6Z-*cis*-9,10- and 9Z-*cis*-6,7-epoxymonoenes.

Extracts of sex pheromone glands from field-collected female moths were prepared by a 20-min pentane extraction of the terminal three or four abdominal segments. The extracts were concentrated under a stream of nitrogen, internal standards (*n*-heptadecane and *n*-tetracosane) were added, and the extract was submitted to coupled gas chromatography–electroantennogram detection (GC-EAD) as described previously (Arn et al., 1975). The signals from the flame ionization detector and the insect antenna were recorded simultaneously with a

matched pair of Hewlett-Packard 3392A integrating chart recorders (Hewlett-Packard, Avondale, PA).

Coupled gas chromatography–mass spectrometry (GC-MS) analyses were carried out in the selected ion monitoring (SIM) mode, using chemical ionization (methane), with a Finnigan 4000E instrument interfaced to an Incos 2300 data system. A DB-5 column (50 m × 0.32-mm ID; J&W Scientific, Folsom, CA) or an Ultra-2 column (50 m × 0.32-mm ID; Hewlett-Packard) were used for analyses.

GC-MS analysis of an *E. sperryi* female pheromone gland extract was conducted as follows. Full-scan CI mass spectra of synthetic standards were obtained to select characteristic ions. A pentane solution of the standards with octadecane and tetracosane internal standards (100 or 400 pg of each compound injected) was then run in the SIM mode, followed by a blank run. Finally, the concentrated pentane extract of the pheromone glands from field-collected female moths of unknown mating status was analyzed in SIM mode (pentane solution of octadecane and tetracosane internal standards added prior to injection).

Insect Trapping. Field experiments were carried out in a mixed forest area approximately 100 km northeast of Saskatoon. Phero Tech wing traps (Phero Tech Inc., Delta, B.C., Canada), similar in design to Pherocon 1 CP traps (Scentry Inc., Buckeye, AZ) were used throughout and were baited with red rubber septa containing the synthetic lure blends. These lure blends (usually 500 μg of test compound total weight) were loaded onto septa as hexane solutions. Baits were stabilized by the addition of several drops of a dilute acetone solution of butylated hydroxytoluene.

Traps were hung from branches 10–15 m apart, at a height of ca 1.5 m, and were set out in a randomized block design. Moth captures were recorded twice weekly throughout the flight season. Because some treatments had no captures, the raw data were subjected to a nonparametric Kruskal–Wallis test, followed by a Waller–Duncan *K*-ratio comparison of means test (SAS Institute, 1988).

Field survey traps in 1984 were baited with the following C_{18-22} compounds, alone or as binary mixtures at ratios of 10:1 and 1:10: 3*Z*,6*Z*,9*Z*-trienes, 6*Z*,9*Z*-dienes, mixtures of the combined monoepoxides derived from nonspecific monoepoxidation of those dienes and trienes (CME-X:H), racemic *cis*-3,4-, *cis*-6,7-, and *cis*-9,10-epoxydienes, and 3*Z*,6*Z*,9*Z*,11*E*-tetraenes.

Field survey traps in 1985 were baited with the following compounds, generally in chain lengths of C_{16-22}, alone or as binary mixtures at ratios of 10:1 and 1:10: 3*Z*,6*Z*,9*Z*,12*Z*-, 3*Z*,6*Z*,9*Z*,13*E*-, 3*Z*,6*Z*,9*Z*,13*Z*-, 3*Z*,6*Z*,9*Z*,14*Z*-, or 3*Z*,6*Z*,9*Z*,14*E*-tetraenes with 3*Z*,6*Z*,9*Z*-trienes; 3*Z*,6*Z*,9*Z*-trienes with enantiomers of *cis*-6,7- and *cis*-9,10-epoxydienes; and epoxide enantiomers with each other or with regioisomers. Blends were made up with compounds of the same chain length or differing by one carbon in chain length.

Field survey traps in 1986 were baited with the following compounds, generally in chain lengths of C_{16-21}, alone or as binary mixtures at ratios of 10:1 and 1:10: 3Z,6Z,9Z-trienes; 1,3Z,6Z,9Z-tetraenes; enantiomers of 6Z,9Z-*cis*-3,4-epoxydienes; 6Z,9Z-dienes; enantiomers of 6Z-*cis*-9,10- and 9Z-*cis*-6,7-epoxymonoenes; and 6Z,9Z-dien-3-ols and 6Z,9Z-dien-3-ones.

Synthetic Chemicals. The syntheses of the compounds used in the field surveys and EAG or GC-EAD studies described in this paper have been published elsewhere, as follows: 6Z,9Z-and 3Z,6Z,9Z-unsaturated hydrocarbons and the corresponding racemic *cis*-3,4-, *cis*-6,7-, and *cis*-9,10-monoepoxides (Underhill et al., 1983; Wong et al., 1985; Millar et al., 1987); enantiomers of *cis*-9,10-epoxydienes (Wong et al., 1985); enantiomers of *cis*-6,7-epoxydienes (Millar and Underhill, 1986); and enantiomers of *cis*-3,4-epoxydienes (Millar et al., 1990d).

With regard to the specific experiments described in this report, the trienes and dienes were >99% chemically and isomerically pure by capillary gas chromatography (DB-5 and DB-1701 columns).

RESULTS

Lobophora nivigerata *Wlk.* Male moths of this species were caught in low numbers for several years in traps baited with lures containing 3Z,6Z,9Z-21:H as a single or major component. For example, in 1984, 35 specimens were attracted to 17 different lures, 13 of which contained 3Z,6Z,9Z-21:H as the major component.

In EAG studies, strong antennal responses (approx 2 mV) were obtained by antennal stimulation with 3Z,6Z,9Z-21:H, 3Z,6Z,9Z-20:H, and 3Z,9Z-*cis*-6,7-epoxy-20:H and -21:H. Smaller responses (approx 1.5 mV) were seen to 6Z,9Z-21:H, 6Z,9Z-20:H, and homologues of the above compounds. In GC-EAD studies, a male antenna gave strong responses to C_{20-21} trienes and 3Z,9Z-*cis*-6,7-epoxy-21:H and lesser responses to 6Z,9Z-21:H and to homologues.

GC-EAD analysis of a female gland extract revealed a single strong response at the retention time of 3Z,6Z,9Z-21:H (Figure 1, bottom traces). In the same run, a flame ionization detector (FID) peak was obtained at the retention time of 3Z,6Z,9Z-21:H but not at that of 6Z,9Z-21:H.

A pheromone gland extract from a second female moth was analyzed by coupled gas chromatography–mass spectrometry, using chemical ionization (CI; methane) in the selected ion monitoring (SIM) mode, with a DB-5 column. An exact retention time match and a fair ion ratios match were obtained for 3Z,6Z,9Z-21:H versus a 100-pg standard [standard, *m/z* 291 (74, M + 1), 289 (100, M − 1), 275 (23); insect extract *m/z* 291 (83), 289 (100), 275 (11)]. 6Z,9Z-21:H was not detected in the extract.

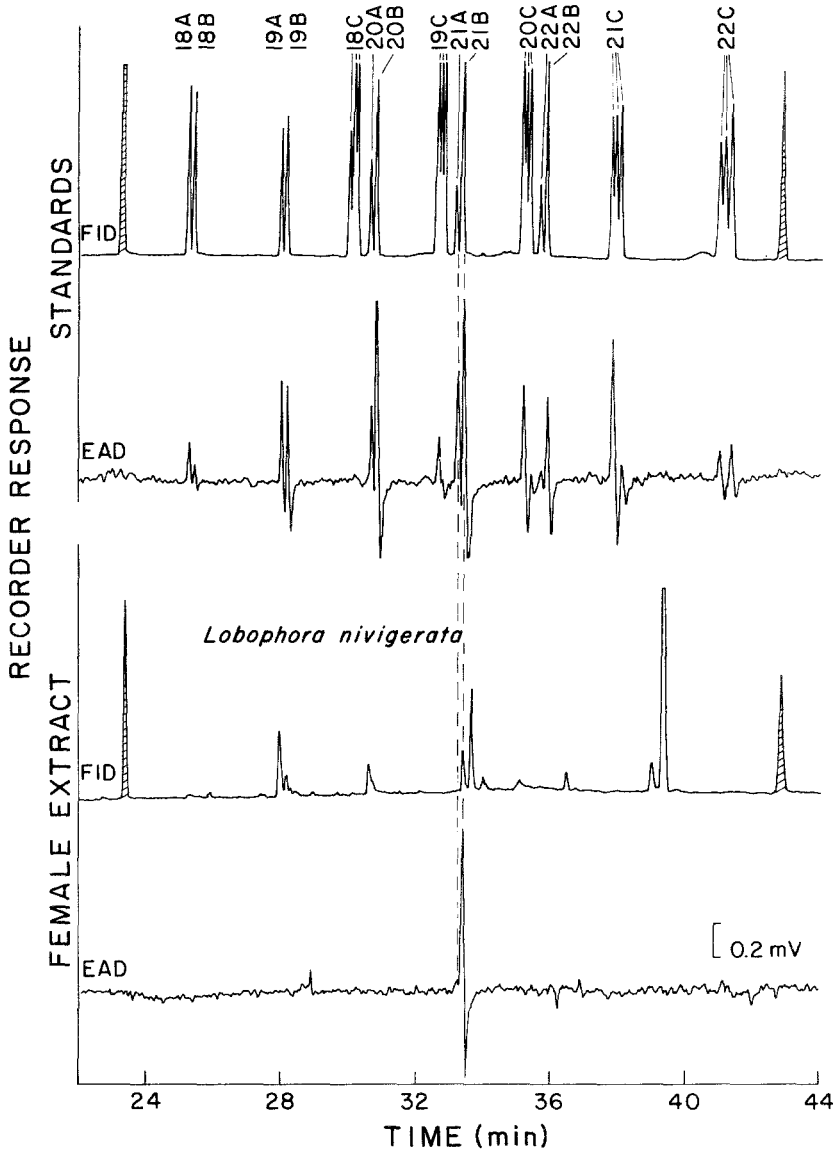

FIG. 1. Simultaneously recorded flame ionization detector (FID) and electroantennographic detector (EAD) traces using antennae of male *Lobophora nivigerata*. Upper pair of traces is in response to a mixture of C_{18-22} 6Z,9Z-diene hydrocarbons (designated by the letter A and preceded by the carbon-chain length), 3Z,6Z,9Z-triene hydrocarbons (designated by the letter B), and their monoepoxydiene analogues (designated by the letter C); the latter eluted from the DB-5 column in the order 6,7-, 3,4-, and 9,10-monoepoxydiene. Internal standards: 17:H and 24:H (shaded). Lower pair of traces is in response to a pheromone gland extract from a conspecific female. GC conditions: splitless, 40°C/0.5 min, 20°/min to 90°C, 4°/min to 230°C and hold.

In 1985 field tests, 3Z,6Z,9Z-21:H and 6Z,9Z-21:H as single components and a 1:1 blend were inactive, whereas mixtures of 19:1 and 4:1 (triene:diene) were attractive (Table 1). There was strong synergism between the two components, and the blend ratio was obviously important.

Epirrhoe sperryi *(H).* GC-EAD analysis of a female gland extract revealed EAD-active compounds at the retention times of 3Z,6Z,9Z-18:H, 3Z,6Z,9Z-19:H, and 6Z,9Z-19:H (Figure 2). The compounds were present at a ratio of 1:98:1, respectively.

A second female extract was submitted to GC-MS analysis in the SIM mode on an Ultra-2 column with methane CI. Exact retention time matches were obtained for all three compounds, and excellent ion ratio matches versus synthetic standards were obtained for 3Z,6Z,9Z-18:H [400-pg standard, *m/z* 249 (45, M + 1), 247 (47, M − 1), 109 (100); insect extract, *m/z* 249 (45), 247 (46), 109 (100)] and 6Z,9Z-19:H [400-pg standard, *m/z* 263 (100, M − 1), 249 (12), 109 (50); insect extract 263 (100), 249 (19), 109 (52)]. The SIM match between the 3Z,6Z,9Z-19:H standard [400 pg, *m/z* 263 (45, M + 1), 261 (48, *M* − 1), 247 (6), 206 (18), 109 (100)] and the compound in the insect extract [*m/z* 263 (100), 261 (100), 247 (18), 206 (46), 109 (100)] was poor because the amount of material in the extract was so large that the resulting signal saturated the detector.

In 1985, 530 male moths were attracted to 56 lure blends, the majority of which contained 3Z,6Z,9Z-19:H as the major component. There was no clear indication of synergistic or antagonistic effects due to the minor components, which included dienes, tetraenes, and monoepoxydienes. In 1986, traps containing 37 different lure blends caught 619 specimens, of which 610 were caught in traps baited with 3Z,6Z,9Z-19:H as a significant (> 10%) or major component.

In a second 1986 field test (Table 2), 3Z,6Z,9Z-19:H alone was signifi-

TABLE 1. ATTRACTION OF *Lobophora nivigerata* MOTHS TO LURES CONTAINING 3Z,6Z,9Z-21:H AND 6Z,9Z-21:H AS SINGLE COMPONENTS OR BLENDS

Lure (μg)	Male moths captured[a]
3Z,6Z,9Z-21:H (500)	4 b
6Z,9Z-21:H (500)	1 b
3Z,6Z,9Z-21:H (475) + 6Z,9Z-21:H (25)	54 a
3Z,6Z,9Z-21:H (400) + 6Z,9Z-21:H (100)	47 a
3Z,6Z,9Z-21:H (250) + 6Z,9Z-21:H (250)	0 b

[a]Traps were replicated three times and set out June 18–July 10, 1985. Trap captures followed by the same letter are not significantly different ($P < 0.05$, Waller–Duncan *K*-ratio multiple-comparisons test).

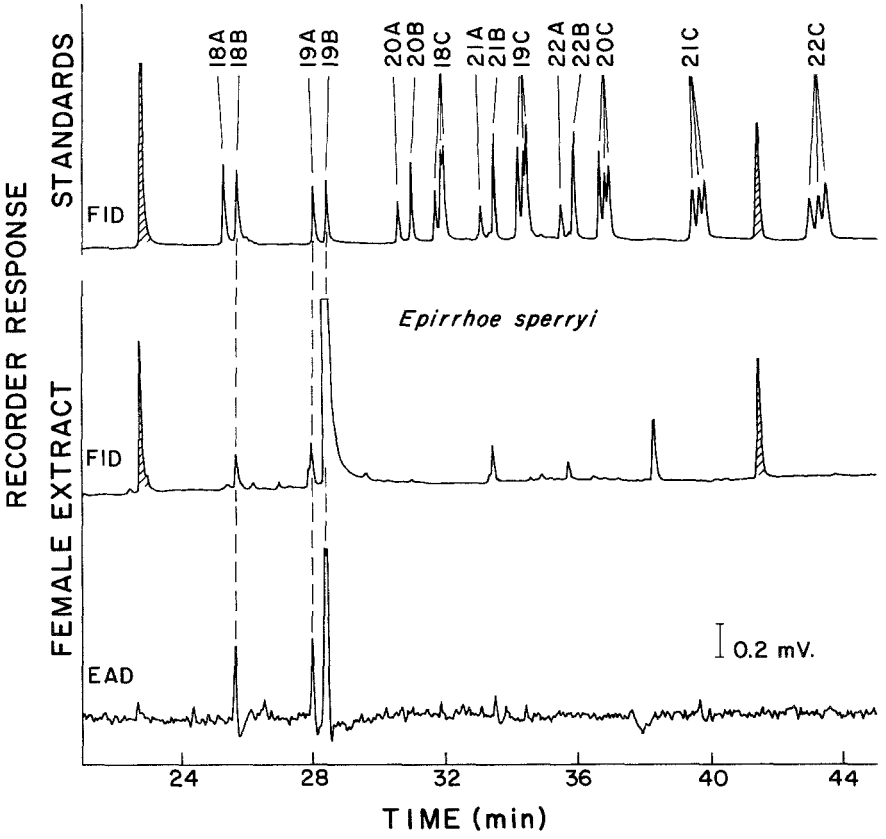

FIG. 2. FID and EAD traces (antennae of male *Epirrhoe sperryi*); upper trace is an FID trace from standards blend. Lower pair of traces is in response to a pheromone gland extract of a conspecific female. Number/letter designations and GC conditions the same as in the legend to Fig. 1.

cantly attractive, whereas 3Z,6Z,9Z-18:H and 6Z,9Z-19:H were not. When added in amounts equivalent to 1 or 10% of the total lure blend, 3Z,6Z,9Z-18:H had no significant effect on trap captures. 6Z,9Z-19:H had no significant effect at the 1% level but completely suppressed trap captures when present as 10% of the blend.

The three-component blend of 3Z,6Z,9Z-19:H, 3Z,6Z,9Z-18:H, and 6Z,9Z-19:H (100:1:1, the approximate ratio found in the pheromone gland extract) was not significantly more attractive than 3Z,6Z,9Z-19:H alone. However, the same three components at a ratio of 9:1:1 were significantly less attractive, supporting our finding that increased amounts of 3Z,6Z,9Z-18:H decreased trap captures.

TABLE 2. ATTRACTION OF *Epirrhoe sperryi* MOTHS TO 3Z,6Z,9Z-19:H, 3Z,6Z,9Z-18:H, AND 6Z,9Z-19:H AS SINGLE COMPONENTS OR BLENDS

Lure (μg)	Male moths captured[a]
3Z,6Z,9Z-18:H (500)	1 e
6Z,9Z-19:H (500)	0 e
3Z,6Z,9Z-19:H (500)	38 bc
3Z,6Z,9Z-19:H (500) + 3Z,6Z,9Z-18:H (5)	15 de
3Z,6Z,9Z-19:H (450) + 3Z,6Z,9Z-18:H (50)	51 ab
3Z,6Z,9Z-19:H (500) + 6Z,9Z-19:H (5)	26 cd
3Z,6Z,9Z-19:H (450) + 6Z,9Z-18:H (50)	0 e
3Z,6Z,9Z-19:H (500) + 6Z,9Z-19:H (5) + 3Z,6Z,9Z-18:H (5)	56 a
3Z,6Z,9Z-19:H (450) + 6Z,9Z-19:H (50)+ 3Z,6Z,9Z-18:H (50)	14 de
Blank	0 e

[a]Traps were replicated three times and set out May 28–June 26, 1986. Values followed by the same letter are not significantly different ($P < 0.05$, Waller–Duncan K-ratio multiple-comparisons test).

DISCUSSION

Male *L. nivigerata* moths were attracted to the synergistic blend of 3Z,6Z,9Z-21:H and 6Z,9Z-21:H, with the presence and ratio of both compounds being important. No other potential pheromone components were detected, either by GC-EAD or by GC-MS examinations of pheromone gland extracts. Blends of dienes and trienes are not uncommon as lepidopteran pheromones. For example, the sex pheromone of the noctuid species *Mocis latipes* consists of a blend of the same two components (Descoins et al., 1986), and the arctiid species *Creatonotos transiens, C. gangis*, and *Utetheisa ornatrix* have both these compounds admixed with several other components in their pheromones (Jain et al., 1983; Bell and Meinwald, 1986; Wünderer et al., 1986). There have also been several reports of homologous diene and triene blends as lepidopteran sex pheromones [*Paleacrita vernata* (Millar et al., 1990a)] or sex attractants [*Alsophila quadripunctata* (Szöcs et al., 1984); *Boarmia repandata* (Bogenschütz et al., 1985)].

Preliminary data on attraction of male moths of *E. sperryi* to 3Z,6Z,9Z-19:H have been published (Wong et al., 1985), along with EAG data showing strong responses of male moth antennae when stimulated by C_{18-20} 3Z,6Z,9Z-trienes and the combined monoepoxides derived therefrom. *E. sperryi* antennae are highly receptive to three components from the female pheromone gland, but only one of these, 3Z,6Z,9Z-19:H, is significantly attractive alone to males. The other two components have variable biological activity, depending on the

blend ratio at which they are presented. At low doses, the combination of the two minor components appears to synergize the response to the major component, while at higher doses, at least one of the minor components (6Z,9Z-19:H) appears to be inhibitory. It is possible that a wider ratio of 3Z,6Z,9Z-19:H to the other two components (e.g., 1000:1:1) may have enhanced trap catches.

E. sperryi is also unusual in that its behavioral responses to the attractive 3Z,6Z,9Z-19:H were not affected by a wide variety of homologues and other similar compounds, including 6Z,9Z-dienes, 3Z,6Z,9Z,X-tetraenes (*X* = 12*E* or *Z*, 13*E* or *Z*, 14*E* or *Z*), chiral or racemic *cis*-3,4-, 6,7-, or 9,10-epoxydienes, and 6Z,9Z-dien-3-ones or 6Z,9Z-dien-3-ols. Of all the compounds tested in combinations with 3Z,6Z,9Z-19:H, the only compound which significantly altered trap captures, either positively or negatively, was 3Z,6Z,9Z-20:H, which strongly suppressed trap captures, as had been previously reported (Wong et al., 1985). None of the epoxides, either of the same chain length or homologues, had any apparent effect, despite the strong responses to these compounds by male antennae in EAG studies (Wong et al., 1985).

In our region of Saskatchewan, *E. sperryi* fly from mid-May to early July (e.g., 1984, first moth caught May 21; last moth caught July 10). During this period, there is a wide variety of geometrid and noctuid moths flying, and 3Z,6Z,9Z-19:H is a common component of sex attractants and sex pheromones for some of these species (Wong et al., 1985; Millar et al., 1990b,c,d, 1991a). *E. sperryi* males were attracted to a wide variety of lures containing 3Z,6Z,9Z-19:H as a component (e.g., in 1986, 619 moths caught with 37 different lure blends), suggesting that other physiological or behavioral mechanisms (e.g., closely synchronized female calling and male response) are operative in minimizing male attraction to pheromone blends containing 3Z,6Z,9Z-19:H produced by female moths of sympatric species.

Obtaining hard analytical data to support conclusive identifications of the pheromone components has been difficult, as we have obtained only a few hand-collected female moths of unknown mating status for analysis of pheromone gland chemistry. The evidence that has been presented in support of the identification of 3Z,6Z,9Z-21:H as a pheromone component for *L. nivigerata* consists of retention time matches on two capillary GC columns and a reasonable CI SIM ion ratios match versus a synthetic standard, plus the strong electroantennogram activity and biological activity of the synthetic compound as an attractant. Furthermore, previous studies in our laboratory have shown that under the GC-EAD conditions used, only one (an *EEZ* isomer) of the eight possible geometric isomers of 3Z,6Z,9Z-21:H is chromatographically indistinguishable from 3Z,6Z,9Z-21:H (Millar et al., 1991b). However, until hard analytical evidence can be obtained to determine unambiguously the double-bond geometries and positions, our identification remains tentative.

The evidence for the identification of 6Z,9Z-21:H as a pheromone com-

ponent is tenuous, consisting of antennal responses to standards by male moth antennae in GC-EAD studies and antennal responses in EAG studies. This identification is strongly supported by the obvious synergism between this compound and 3*Z*,6*Z*,9*Z*-21:H in field trials (Table 1).

Much stronger evidence was obtained for the identifications of the components isolated from *E. sperryi* pheromone glands. The major biologically active compound, 3*Z*,6*Z*,9*Z*-19:H, had retention times identical to those of a synthetic standard on two capillary GC columns. The SIM mass spectral match was poor, due to the saturation of the detector by the large amount of 3*Z*,6*Z*,9*Z*-19:H in the gland extract. However, the major ions at *m/z* 263 ($M + 1$) and 261 ($M - 1$) provided support for the molecular weight and formula, and the major ion at *m/z* 109 $(C_8H_{12} + H)^+$ was diagnostic for the 3,6,9-triene structure (Heath et al., 1983). Furthermore, we have previously demonstrated that under the GC-EAD conditions used, only the *EEE* isomer and one of the *EEZ* isomers had retention times which were potentially indistinguishable from that of 3*Z*,6*Z*,9*Z*-19:H. Given the strong field attraction (Table 2) and EAD responses (Fig. 2) to this compound, the chance of the pheromone component being one of the other two isomers seems remote.

Evidence to support the identity of 3*Z*,6*Z*,9*Z*-18:H in pheromone gland extracts consisted of exact retention time and CI SIM matches versus a synthetic standard, including the presence of a strong *m/z* 109 ion confirming the 3,6,9 placement of the double bonds. The same types of data confirmed the gross structure of 6*Z*,9*Z*-19:H in the extract, and in addition, all four geometric isomers of this compound are readily distinguishable by capillary GC (Millar et al., 1990a).

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ROLE OF THE ISOFLAVONOID COUMESTROL IN THE CONSTITUTIVE ANTIXENOSIC PROPERTIES OF "DAVIS" SOYBEANS AGAINST AN OLIGOPHAGOUS INSECT, THE MEXICAN BEAN BEETLE

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Abstract—The antixenosic properties of the isoflavonoid, coumestrol, were tested in dual-choice leaf disk bioassays with the Mexican bean beetle (*Epilachna varivestis* Mulsant). *E. varivestis* preferred the methanol-treated (solvent-control) disk when the coumestrol concentration was 1.8 or 0.9 μg/leaf disk. No preference was observed between the coumestrol-treated and the solvent-control disks when the coumestrol concentration was higher, at 3.6, or lower, at 0.45 μg/leaf disk. Coumestrol alone clearly is not responsible for the significant constitutive antixenosic properties of "Davis" soybeans, *Glycine max* (L.) Merrill, because the amount of coumestrol in these plants is significantly less than the minimum concentration which was antixenosic in this study. However, it might contribute to a constitutive antixenosis in "Davis" involving a profile of allelochemicals. A computer-aided densitometer, adapted to measure the leaf disk area, increased the resolution of the leaf area 250 (×)-fold as compared to the standard LI-COR leaf area meter.

Key Words—Allelochemicals, isoflavonoids, coumestrol, antixenosis, *Epilachna varivestis*, Mexican bean beetle, Coleoptera; Coccinellidae, *Glycine max*, *Phaseolus lunatus*, feeding bioassay.

INTRODUCTION

A holistic interpretation of plant resistance to herbivores entails multiple interacting parameters involving anatomical, morphological, and biochemical entities

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(Norris and Kogan, 1980). In *Glycine* the anatomical traits associated with certain resistance include stem diameter, shorter stem internodes, and secondary xylem (Chiang and Norris, 1983a). Morphological characteristics include trichome density and length (Norris and Kogan, 1980; Chiang and Norris, 1983b; Khan et al., 1986). Regarding interactions between phytochemistry and insect behavior, growth, development, or reproduction as components of plant resistance, at least two levels of resistance have been identified in *G. max*. One involves both volatile straight-chain hydrocarbons (Liu et al., 1988, 1989) and relatively nonvolatile metabolites, e.g., phenylpropanoids (Chiang et al., 1987; Khan et al., 1986; Norris et al., 1988; Norris, 1990). This at least dual-based chemical resistance is effective against polyphagous insects such as the cabbage looper, *Trichoplusia ni* (Hubner). A second, and lesser, level of *G. max* resistance involving phenylpropanoid metabolites is effective against oligophagous species such as the Mexican bean beetle (*Epilachna varivestis* Mulsant), which feeds exclusively on Leguminosae (Turnipseed and Shepard, 1980). The latter resistance, which is effective against some oligophages, needs further investigation regarding whether the causal chemistry is one or a few compounds or is a more complex mixture (profile) of chemicals. To address this question experimentally, the profiles of phenylpropanoids of two legumes, "Henderson" lima bean (*Phaseolus lunatus* L.) and "Davis" soybean (*G. max*), were analyzed in the present study. *E. varivestis* completes its life cycle on the preferred host "Henderson" lima beans, but larvae do not complete development and female adults experience reproductive failure (Burden and Norris, 1993) on the "Davis" cultivar, which has phenylpropanoid resistance.

Phenylpropanoid-based resistance in *G. max* especially involves flavonoids (Hedin and Waage, 1986). Some isoflavonoids, a subgroup of flavonoids, are important as phytoalexins and antiherbivory compounds in legumes (Fischer et al., 1990; Neupane and Norris, 1990, 1991; Sharma and Norris, 1991). Isoflavonoid-based constitutive and inducible resistance in *G. max* to *E. varivestis* has been correlated with phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL) activities, two key enzymes in phenylpropanoid (e.g., isoflavonoid) biosynthesis (Chiang et al., 1986, 1987). Phytoalexin-rich, and thus presumably isoflavonoid-rich, soybean hypocotyls deterred feeding by adult and fourth-instar *E. varivestis* (Hart et al., 1983). Glyceollins, a major group of isoflavonoids, were identified as feeding deterrents to *E. varivestis* (Fischer et al., 1990). Coumestrol, an isoflavonoid in the same general biosynthetic pathway with glyceollins (Ebel, 1986), is a feeding deterrent to the scarab beetle, *Heteronychus arator* (F.) (Sutherland et al., 1980).

Coumestrol is present in several of the host legumes of the beetle, *E. varivestis*, and was reported in the extractables from *G. max*: leaf tissue (Vaughn and Hymowitz, 1984; Smith, 1985; Porter et al., 1986), hypocotyls (Keen et al., 1972), and roots (Morandi and Le Quere, 1991; Cho and Harper, 1991).

Coumestrol is also present in *Phaseolus vulgaris* (L.) leaf tissue (Beggs et al., 1985) and in the seedlings of *P. lunatus* L. (O'Neill et al., 1986); these are the two primary host plants of *E. varivestis*. Thus, in the present study the role of coumestrol as an antixenosic agent in "Davis" soybean against *E. varivestis* was emphasized.

Accurate analysis of any effects of isoflavonoids such as coumestrol on insect feeding requires highly sensitive bioassays. Analyzing such effects on *E. varivestis* feeding has been especially difficult because both the adults and the larvae eat by a unique piercing-chewing action which does not create cleanly cut holes through the leaves. Most previous studies of *E. varivestis* feeding have used leaf area measurements lacking the resolution required to detect a significant correlation between feeding preference and some test compound. Jones et al. (1981) only visually estimated the percentage of leaf disk marked by *E. varivestis* feeding ridges. The majority of other previous studies used a LI-COR leaf area meter (Lambda Instrument Corporation, Lincoln, NE) to estimate the unconsumed leaf tissue (Chiang et al., 1987; Fischer et al., 1990; Lin et al., 1990). The sensitivity of the LI-COR is only 1 mm^2 (LI-COR Instrument Manual); thus, accurate measurement of the area removed (consumed) from the leaf by *E. varivestis* is not possible. In this paper we report bioassay results on the effects of coumestrol concentration on *E. varivestis* adult feeding as quantified by a high-resolution computerized densitometer system. These findings are also discussed within the perspective of the differential constitutive concentrations of coumestrol in the leaves of the *G. max* nonhost, "Davis," and the preferred host, *P. lunatus*.

METHODS AND MATERIALS

Plants

"Henderson" lima bean (*Phaseolus lunatus*) and "Davis" soybean (*G. max*) were used for high-performance liquid chromatography (HPLC). They were grown in the University of Wisconsin Biotron under the controlled conditions: 70% relative humidity, a 14-hr photoperiod, and day and night temperatures of 28 and 21°C, respectively. Plants were harvested every 24 hr until 120 hr. The 0-hr plants were at the V7 stage (Fehr et al., 1971). The "apical" leaves involved node four and above for the *G. max* leaves and nodes three and above for the lima beans. The leaf tissue was quick-frozen, lyophilized, and then sealed at room temperature in a darkened glass jar in a desiccator.

"Henderson" lima beans used for the feeding bioassays were grown in a greenhouse (14:10 hr, L:D) in a potting mixture of field soil, sand, and vermiculite (2:1:1). The plants were uniformly watered once a day and were maintained at 27 ± 5°C and 60 ± 10% relative humidity.

HPLC Analyses

The lyophilized leaf tissue was ground with a mortar and pestle, divided into 0.5-g aliquots, and homogenized with a TekMar Tissuemizer (Tekmar Company, Cincinnati, OH) for 60 sec at 55% maximum speed in 40 ml methanol. The homogenized tissue–methanol mixture was filtered by vacuum filtration using a Buchner funnel and Whatman No. 1 filter paper. The filtrate was collected and stored at 5°C, and the tissue residue was added to 100 ml of methanol in an Erlenmeyer flask and placed on a shaker for 16 hr. Two 16-hr extractions of each residue with methanol were conducted. The two extractions were pooled, reduced to 2 ml by rotoevaporation, and stored in a sealed vial at −5°C. Each concentrated extract was filtered through a Gelman Nyaflo 0.45-μm filter, and the volume was adjusted to 7.5 ml either by adding methanol or by reducing the volume with a stream of nitrogen (N_2) gas. At least three 20-μl injections of each extract were analyzed by HPLC using a Beckman Ultrasphere ODS column (4.6 × 250 mm). The elution gradient was 90:10 2% acetic acid:acetonitrile for 5 min, then a linear gradient to 100% acetonitrile in 15 min. The total chromatographic time was 30 min. The elution was monitored with a UV detector at 254 nm and the peak area was integrated with a Spectra Physics 4400 integrator. Each sample's analyses are an average of at least three replicate injections. Each harvest time represents at least three plants. The coumestrol peak in each extract was quantified based on a standard curve constructed from the HPLC-resolved mean peak areas for known concentrations of injected authentic coumestrol.

Insects

The *E. varivestis* were raised on "Henderson" lima beans in a greenhouse under the conditions described previously for growing lima beans. To obtain precisely aged *E. varivestis* adults for use in assays, pupae were collected and enclosed individually in 13 × 100-mm test tubes. Emergence of adults was monitored daily, and each was sexed and aged; males and females were placed in separate cages. Sex determination was based on the method of Burden and Norris (1993).

Bioassays

The feeding arena was a 6-cm plastic petri dish with a base of paraffin (2–3 mm) in its bottom, which was covered with filter paper (5.5-cm-diameter, Whatman No. 2). A similar wetted paper lined the inside of the top of the petri dish. The moistened filter paper in the lid helped to maintain the turgidity of the leaf disks. The first-trifoliolate (T1) leaf from 10-day-old, V2 "Henderson" lima beans was used as the feeding substrate for bioassays. In two-choice feeding

tests, one leaf disk was treated with a known concentration of coumestrol (ICN, Cleveland, OH) dissolved in methanol and the other disk was treated with methanol. The solutions (20 μl) were applied to the abaxial surface of the leaf. The two disks per assay were centered, abaxial side up, in an opposed manner in each arena and were secured with insect pins (7 mm long). One 5- to 6-day-old virgin adult female *E. varivestis*, starved but water satiated for 24 hr, was randomly placed in each feeding arena. An individual beetle was used only once in the assays. Coumestrol was tested at 3.6, 1.8, 0.9, and 0.45 μg/leaf disk. The highest treatment value (3.6 μg) represents the saturation concentration of coumestrol in methanol. The average weight and abaxial surface area of a leaf disk was 1.39 mg and 56.6 mm^2, respectively. A series of the feeding arenas containing methanol-treated leaf disks was exposed to *E. varivestis*; this control treatment was termed 0.0 μg/leaf disk. This solvent–solvent control thus tested for potential biases in feeding preference due to the nontreatment conditions of the bioassay (i.e., positioning of the leaf disks, side effects due to lighting, etc.).

Each assay was conducted in darkness, or light, as indicated in an incubator at 25°C. The amount of feeding was monitored every 2 hr. Each assay was terminated when at least 50% of one leaf disk was consumed and a clear preference for one of the disks was established. If a preference was not observed, that individual assay was excluded from the analyses. The beetle was then removed from the arena and the disks were stored at 5°C until they were measured.

Quantitation of Feeding

The two leaf disks from each assay were mounted between two microscope slides and the areas were measured. Leaf disk images were scanned into files using a computer system which consisted of a Microtek MSF-300G image scanner (Microtek Lab, Inc., Torrance, CA), a Microtek MS-SCSI computer adapter, a MacIntosh II computer (Apple Computer, Inc., Cupertino, CA), and a GrayScan image scanning software program (Microtek Lab, Inc.). Images were analyzed for remaining disk area using the information extraction and reduction program, Collage (Image Dynamics, Madison, WI).

Statistics

The coumestrol concentrations in "Henderson" lima beans and "Davis" soybeans for all harvest times were evaluated by analysis of variance, and the differences between the individual means within a cultivar were separated with a least-significant difference test. The total means of coumestrol content for all harvest times were compared between "Henderson" lima bean and "Davis" soybean by a two-sample t test. Chi-square tests were used to generate the probabilities of insect feeding preference between coumestrol-treated and meth-

anol (solvent)-treated leaf disks. The chi-square tests were followed by Z tests to check for differences in feeding preference between and within the different coumestrol concentrations (SAS Institute, 1985).

RESULTS

Coumestrol in Plant Tissue

HPLC chromatographs of the resolved methanol extractables from apical "Henderson" lima bean and "Davis" soybean leaves are shown in Figure 1; the small resolved coumestrol peak is identified in each chromatograph. Cou-

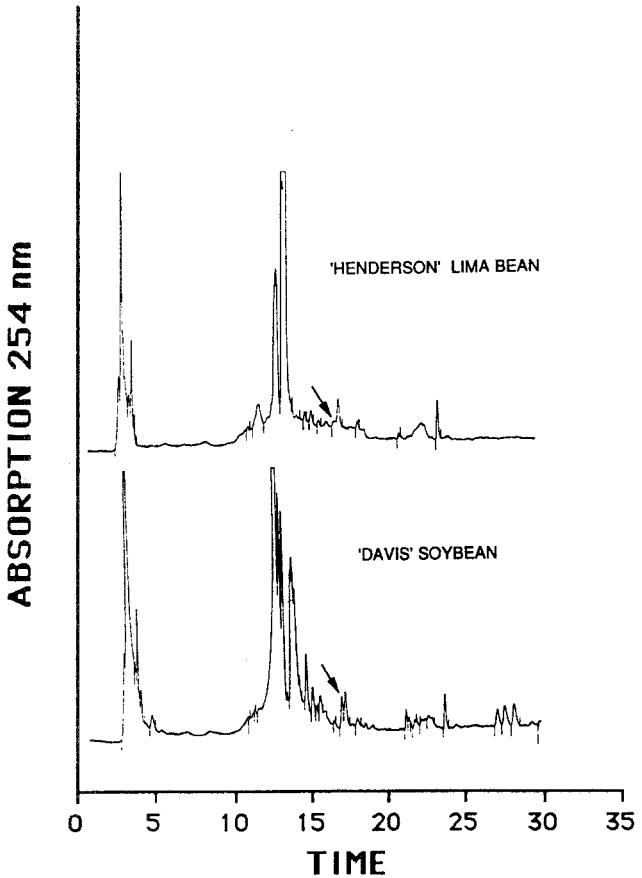

FIG. 1. HPLC chromatographs of methanol extractables from the leaves of "Henderson" lima bean and "Davis" *G. max*. The arrow marks the coumestrol peak in each chromatograph.

mestrol concentration was significantly lower ($P < 0.05$) in lima bean than in soybean. The standard line, $y = -435.36 + 511.4x$, used to quantify the coumestrol concentration in leaf tissue has $r^2 = 0.996$. Based on this curve, "Davis" soybean and "Henderson" lima bean leaves contained 0.069 and 0.018 μg coumestrol/mg dry leaf wt, respectively (Table 1). These data are averages of all samples from all harvest times (0–120 hr). There were 56 analyses for both "Davis" and "Henderson." Due to limitations in the peak recognition threshold of the HPLC integrator, 30 smaller "Henderson" coumestrol peaks were not integrated. Thus, there are fewer quantified peak area values for "Henderson" than for "Davis," thus resulting in a larger variance for "Henderson." The coumestrol peak was integrated in all of the 56 "Davis" analyses. The coumestrol concentration in "Henderson" and "Davis" at 24-hr intervals is also given in Table 1. The amount of coumestrol in "Davis" peaked to 0.092 μg/mg dry leaf wt at 72 hr. The relatively low coumestrol content of "Henderson" gradually increased with time but remained significantly lower than "Davis" except for 48 and 72 hr.

Bioassay Conditions

Bioassays were initially conducted in darkness. In these cases the cumulative percentage of assays completed in 12 hr was below 40% (Figure 2). A much higher percentage (>95%) of the bioassays was completed in 12 hr when they were conducted under light. The accelerated feeding rate of *E. varivestis* in light versus darkness is well illustrated by the difference in the percentage of

TABLE 1. MEAN ± SE COUMESTROL CONCENTRATIONS[a] IN 'DAVIS' SOYBEAN AND 'HENDERSON' LIMA BEAN APICAL LEAVES

Time[b]	'Davis'	N[c]	'Henderson'	N
0	0.074 ± 0.027 ab[d,e]	3	0.014 ± 0.014 a	3
24	0.067 ± 0.015 ab[e]	5	0.017 ± 0.015 b	3
48	0.067 ± 0.028 ab	3	0.016 ± 0.015 ac	3
72	0.092 ± 0.046 a	3	0.019 ± 0.015 a	3
96	0.047 ± 0.015 b[e]	3	0.020 ± 0.014 bc	3
120	0.066 ± 0.021 ab[e]	3	0.021 ± 0.013 ac	3
Mean	0.069 ± 0.019[e]	20	0.018 ± 0.013	18

[a] μg coumestrol/mg dry leaf wt.
[b] Plants were harvested every 24 hr starting at the V7 stage.
[c] Number of plants used in the analyses.
[d] Means followed by the same letter within a column are not significantly different ($P < 0.05$, LSD).
[e] Mean between 'Davis' and 'Henderson,' within a row, is significantly different ($P < 0.05$, t test).

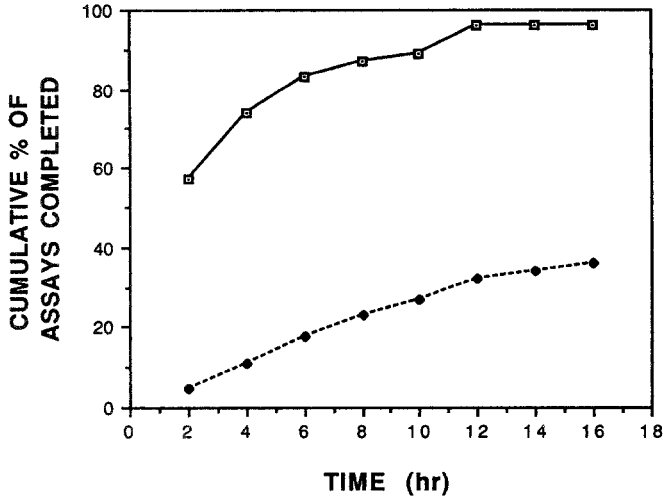

FIG. 2. Cumulative percentage of *Epilachna varivestis* assays completed in light (——) versus darkness (– – –) over time as measured in the two-choice bioassay.

completed assays under the two conditions at 2 hr after the beginning of the assays (Figure 2).

Effects of Assayed Coumestrol on Insect Feeding

Dosages of 1.8 and 0.9 μg coumestrol/leaf disk caused a significant ($P < 0.05$) *E. varivestis* feeding preference for the methanol (solvent)-treated versus the coumestrol-treated lima bean disk (Figure 3). At 0.45 μg/leaf disk *E. varivestis* favored numerically the coumestrol-treated disk, but this difference was not statistically significant at $P < 0.05$. A neutral response was also obtained at the higher dosage, 3.6 μg/leaf disk. There was a significant ($P < 0.05$) difference in feeding preference between 0.9 μg/leaf disk and 0.3 and the 0.0 μg/leaf disk.

DISCUSSION

Coumestrol, an isoflavonoid in legumes, has received considerable study as an allelochemical (Hart et al., 1983; Smith, 1985; Rose et al., 1988; Sharma and Norris, 1991). The present study demonstrates that coumestrol can be a feeding deterrent to *E. varivestis* but that such antixenosis depends on concentration. There appears to be a small window between 0.9 and 1.8 μg coumestrol/leaf disk where coumestrol is antixenosic to *E. varivestis*.

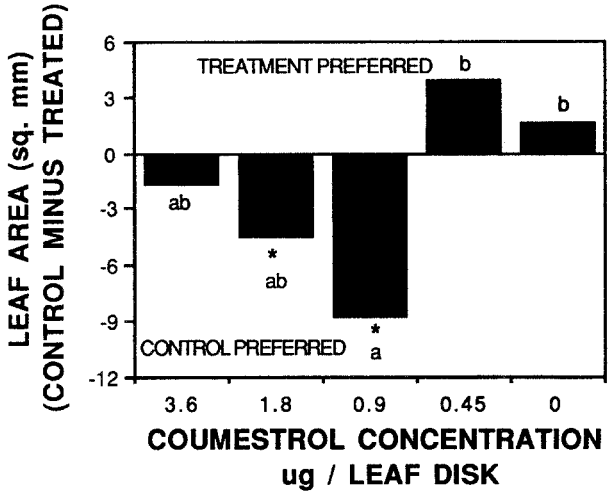

FIG. 3. Each bar represents the average nonconsumed leaf disk area for the coumestrol-treated disk subtracted from that area for the methanol-treated (solvent-control) disks. There were 84, 100, 66, 47, and 80 replicate bioassays for the concentrations of 3.6, 1.8, 0.9, 0.45, and 0.0 μg coumestrol/leaf disk, respectively. One asterisk (*) indicates a significant ($P < 0.05$) preference for the control versus the coumestrol-treated disk within a concentration. Bars labeled by the same letter are not significantly ($P < 0.05$) different.

Our bioassay results indicate that the constitutive antixenosic properties of "Davis" soybean apical leaves to *E. varivestis* apparently are not dependent on coumestrol. This conclusion is based on the fact that the "Davis" leaves contain about 10 (×)-fold less coumestrol than our smallest tested amount (0.7 μg coumestrol/mg dry leaf wt) which had a significant ($P < 0.05$) effect on the insect's feeding. The endogenous amount of coumestrol in the "Henderson" leaf disk used in the bioassays apparently would not have influenced *E. varivestis* feeding preference, because the amount, 0.018 μg coumestrol/mg dry leaf wt, is much below the minimum deterrent concentration of 0.7 μg/mg (0.9 μg/leaf disk).

The effect of coumestrol on feeding by other insects appears to depend also on concentration and on the species of insect. Lane et al. (1985) found that coumestrol at 0.2 to 2 × 10^{-3} μg/mg cellulose powder was not a significant feeding deterrent to the polyphagous root-feeding grass grub, *Costelytra zealandica* (White). Coumestrol at 200 μg/ml of cellulose powder (formed into a disk) deterred feeding in *Heteronychus arator* (F.) (Sutherland et al., 1980). Conversely, coumestrol at up to 1000 ppm was not a feeding deterrent to the pea aphid, *Acyrthosiphon pisum* (Harris) (Dreyer et al., 1987).

Coumestrol quantities do significantly change temporally. During a 5-day period the coumestrol concentration fluctuated in the nonhost "Davis" but just gradually increased in the host "Henderson." The plants were harvested at the same time each day, so it is doubtful that the variation is due to diurnal effects. These temporal changes probably reflect generic differences between *Glycine* and *Phaseolus*. Intraspecific variation in flavonoid concentrations is also known to occur, and this variation is often temporal (Bohm, 1987).

Developing a bioassay which adequately mimics the natural situation can be difficult. Our bioassay using leaf disks has the limitation that the distribution of the test chemical(s) in the total disk tissue is unknown. Because the coumestrol was topically applied to just the abaxial leaf surface (56.6 mm^2), it may be assumed that it was not uniformly distributed within the 1.4-mg dry weight disk. The resultant coumestrol per milligram of dry disk would vary with the percentage of the disk which actually received the chemical (Figure 4).

The timing of, and the environmental conditions for, antixenosic bioassays with *E. varivestis* were refined. The cumulative percentage of completed *E. varivestis* bioassays plateaued at 12 hr regardless of whether the tests were conducted in light or darkness. Similar results were reported by Jones et al. (1981). These combined results indicate that the more efficient bioassays with

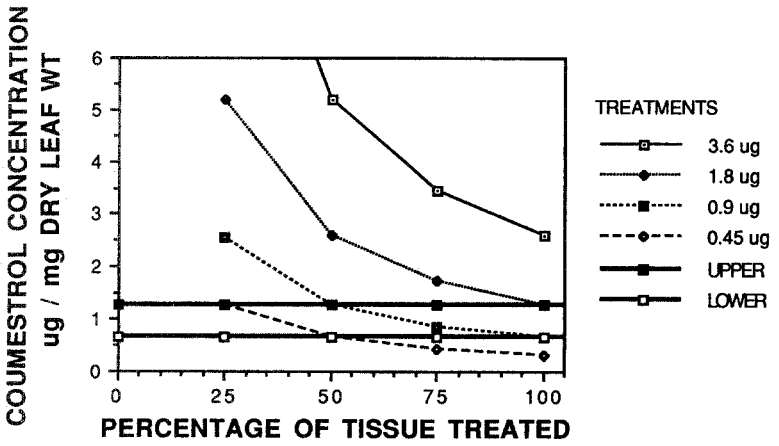

FIG. 4. Resultant coumestrol concentration per milligram leaf disk from each of the four quantities applied to the abaxial surface of the leaf disk assuming the indicated four percentages (*X* axis) of leaf disk tissue involvement. A significant *E. varivestis* preference for the methanol-treated (solvent-control) disk occurred in the two-choice assay with the two coumestrol treatments illustrated as upper (1.6) or lower (0.65) horizontal lines. The area between these two horizontal lines and the plot of each of the four applied amounts (0.45–3.6 μg coumestrol) per disk indicate the μg/mg concentrations which would exist assuming the indicated (*X*-axis) percentage of disk involvement.

E. varivestis should be conducted in light and do not need to extend beyond 12 hr.

The Collage measurement program used in this study increased the resolution of leaf area by 250 (×)-fold as compared to a LI-COR machine. The major basis for this improvement is that the Collage program resolves 1 pixel, which is 1/250 mm^2, whereas the resolution limit for the LI-COR machine is 1 mm^2. A second factor in the improved measurement is that the remaining *E. varivestis* "skeletonized" portion of the leaf tissue is not counted as "eaten area" by the densitometric-based Collage system, but is by the LI-COR machine.

Our results clearly indicate that statements regarding the constitutive antixenosic and antibiotic properties of given isoflavonoids to a given herbivore should include the involved plant cultivar(s), the specific compound, its detected concentration, and the specific involved plant tissue. Our overall findings indicate that the feeding response of a herbivore to a plant tissue is likely determined by the "net input" provided by the complex profile of negative and positive plant stimuli. In this regard, we are currently studying the temporal phenylpropanoid profiles of other host-versus-nonhost legumes of *E. varivestis*.

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EFFECTS OF SOME NATURAL VOLATILE COMPOUNDS ON THE PATHOGENIC FUNGI *Alternaria alternata* AND *Botrytis cinerea*

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Abstract—A bioassay system was developed to test the effects of volatile compounds on the growth of hyphae from germinating fungal spores. Volatiles from crushed tomato leaves inhibited hyphal growth of two fungal pathogens, *Alternaria alternata* and *Botrytis cinerea*. Aldehydes, including C_6 and C_9 compounds formed by the lipoxygenase enzyme pathway upon wounding leaves, inhibited growth of both fungal species. Terpene hydrocarbons, 2-carene and limonene, had no significant effect on hyphal growth. The quantities of volatile compounds in the vapor phase of the bioassay system were measured by direct headspace sampling and GC analysis.

Key Words—Headspace, aldehydes, *E*-2-hexenal, monoterpenes, lipoxygenase, spores, hyphae, wounding, *Lycopersicon esculentum*, *Alternaria alternata*, *Botrytis cinerea*.

INTRODUCTION

Previously, we screened foliage from several plant species to evaluate the effects of volatile compounds released from the tissues on pollen germination and growth *in vitro* (Hamilton-Kemp et al., 1991). Crushed tomato leaves emitted volatiles that were inhibitory, and among these the most active substances detected were unsaturated aldehydes derived from the lipoxygenase pathway [reviews by Galliard and Chan (1980) and Hatanaka et al. (1987)]. Since spores from pathogenic fungi may come in contact with volatiles from wounded or damaged leaves, we

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decided to evaluate the effects of vapors from tomato leaves and the major classes of their volatile components, namely, terpenes and aldehydes, on spores of two widely occurring pathogens, *Alternaria alternata* (Fr.) Keissl. (*A. tennis* Nees) and *Botrytis cinerea* Pers. ex Fr. (Nyvall, 1979). A bioassay for these fungi was developed and the concentrations of individual compounds in the vapor phase to which the organisms were exposed were estimated by GC analysis.

METHODS AND MATERIALS

Fungal cultures were established by isolating *A. alternata* from lesions of tobacco leaves onto V-8 juice agar saturated with $CaCO_3$ at pH 6.2 (Spurr, 1973) and maintained on this medium. *B. cinerea* was isolated from infected strawberry fruit, established on potato dextrose agar, and maintained on V-8 juice agar at pH 7.2. Both cultures were kept at laboratory temperature (24 ± 2°C) and continuous fluorescent lighting was used to stimulate sporulation of *A. alternata*.

The system developed to test effects of volatiles on conidiospores from *A. alternata* and *B. cinerea* is shown in Figure 1. Spores were placed on a 1-cm^3 block of 2% water agar by adding a drop of spore suspension or brushing the spores over the surface of the block with a sterile cotton swab followed by the addition of a drop of water. The agar block had been placed in the bottom portion of a 5-cm-diameter glass petri dish. This dish was in a 9-cm-diameter glass petri dish which contained either crushed leaf tissue or a 1-cm-diameter glass sample dish. The sample dish contained 1 ml of water, to which 10 μl of a solution of the test compound (10 μl of solvent in controls) was added. The

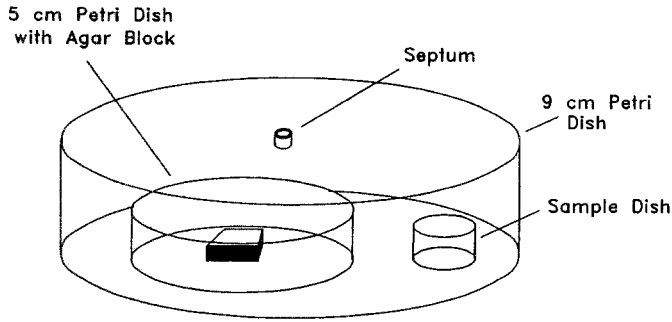

FIG. 1. Bioassay system for testing the effects of volatile compounds on *A. alternata* and *B. cinerea*. Spores were spread on the surface of the 2% water agar block and 10 μl of a solution of the volatile test compound was placed in the sample dish. Headspace samples from the system were withdrawn through the septum with a syringe and analyzed by GC. At the conclusion of an experiment hyphal lengths were measured.

top cover of the large petri dish was placed over the system (volume 120 ml), which was wrapped with Parafilm. All glassware was sterilized prior to setup of an experiment, and the spores were handled under aseptic conditions. The bioassays with *A. alternata* were carried out for 5–6 hr, and those with *B. cinerea* were for 12–14 hr.

At the conclusion of an experiment, the hyphal lengths were measured using a microscope with a net micrometer ocular at 100× magnification. (A drop of neat *E*-2-hexenal could be added to the 9-cm petri dish to stop a bioassay and permit subsequent measurements at a convenient time.) At least two petri dishes were set up for each concentration of compound evaluated. The lengths of five hyphae from each agar block were measured, means calculated, and the data analyzed statistically by analysis of variance.

For bioassays of plant tissue, 5-g samples of immature succulent leaves from " Mountain Pride" tomato plants grown in a greenhouse were harvested and crushed with a mortar and pestle.

Synthetic samples of volatile aldehydes tested in bioassays, *E*-2-hexenal, hexanal, and *E*-2-nonenal, were dissolved in 1,2-propanediol (Sigma). Nonanal, 2-carene, and (*D*)-limonene were dissolved in paraffin oil 335/350 (Fisher) due to insolubility in 1,2-propanediol. The solutions were prepared as for the pollen experiments; that is, a 10% solution (w/w) of the test compound was made and diluted serially (w/w) to obtain 1 and 0.1% solutions.

A rubber septum was fitted into a hole within the top cover of the bioassay system (Figure 1). The concentrations of *E*-2-nonenal and nonanal in the vapor phase within the bioassay dishes were determined at 24 ± 2°C after 1 hr in a manner similar to that described for pollen bioassays (Hamilton-Kemp et al., 1991). (The vapor phase concentrations of the remaining compounds had been determined earlier.) A 250-μl sample of headspace vapor from the bioassay system was withdrawn through the septum with a gas-tight syringe and injected into a Varian 3700 GC equipped with a 0.53 mm × 1-m fused silica precolumn and a 0.53 mm × 30-m DB-wax (polyethylene glycol) or DB-5 (methylsilicone) column for *E*-2-nonenal and nonanal, respectively. The operating conditions were as follows: inlet, 220°C; column oven, 50°C for 3 min, then programmed at 2°/min to 200°C; FID, 240°C; He flow rate, 6 ml/min. The response factors for the volatiles on the GC were determined using a 2-liter static dilution bottle following an EPA method (Winberry et al., 1988) using argon for dilution.

RESULTS AND DISCUSSION

The bioassay system used in these studies provided a convenient means for testing the effects of volatile compounds on spore germination and hyphal growth. The spores could be spread over the top surface of the agar block and kept moist during the bioassay. Hyphal lengths in controls and treatments were readily

measured on the agar surface. The vapor phase levels of compounds within the bioassay system could be measured by withdrawing gas samples through the rubber septum using a gas-tight syringe and analyzing them by capillary GC.

The results obtained from exposure of *A. alternata* and *B. cinerea* spores to compounds emitted by crushed tomato leaves are shown in Figures 2 and 3. In the case of both organisms the length of hyphae which emerged after germination was significantly inhibited by foliage vapors. Hyphal length was subsequently used to evaluate the effects of individual compounds at various concentrations. Tube growth from germinated pollen in earlier bioassays (Hamilton-Kemp et al., 1991) was not as markedly affected as hyphal growth by vapor phase concentrations of volatiles.

Tomato leaf tissue is known to produce lipoxygenase pathway-derived volatile compounds such as *E*-2-hexenal and hexanal, which increase markedly

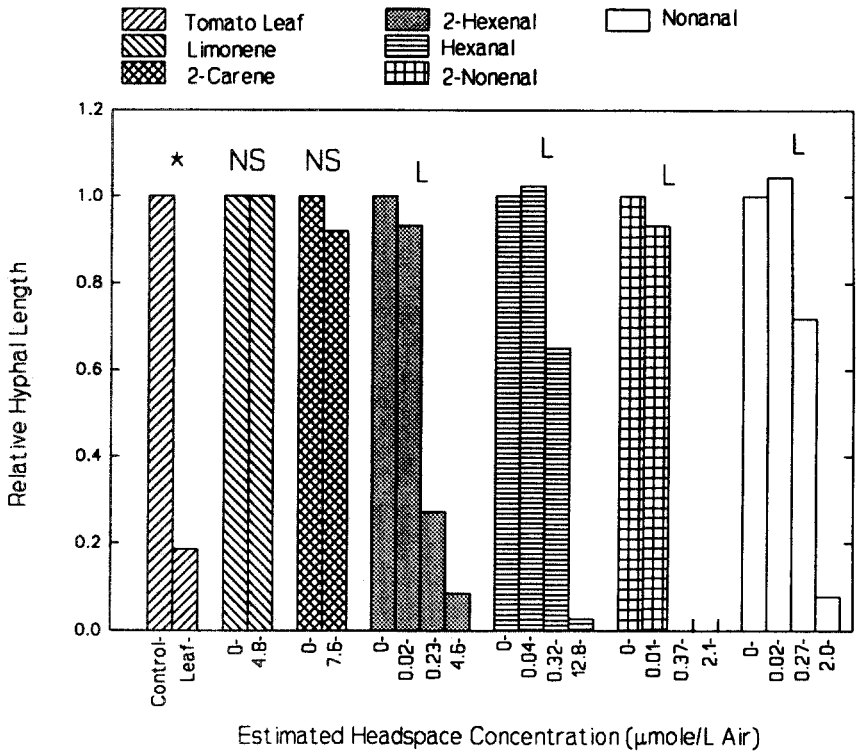

FIG. 2. Lengths of hyphae, relative to controls, after exposure of *A. alternata* spores to crushed leaves and various vapor phase concentrations of aldehydes and monoterpene hydrocarbons. An asterisk denotes a significant difference, and NS no significant difference, between means at $P < 0.05$ using Student's t test. L denotes a significant linear correlation ($R^2 > 0.90$) between hyphal length and volatile concentrations.

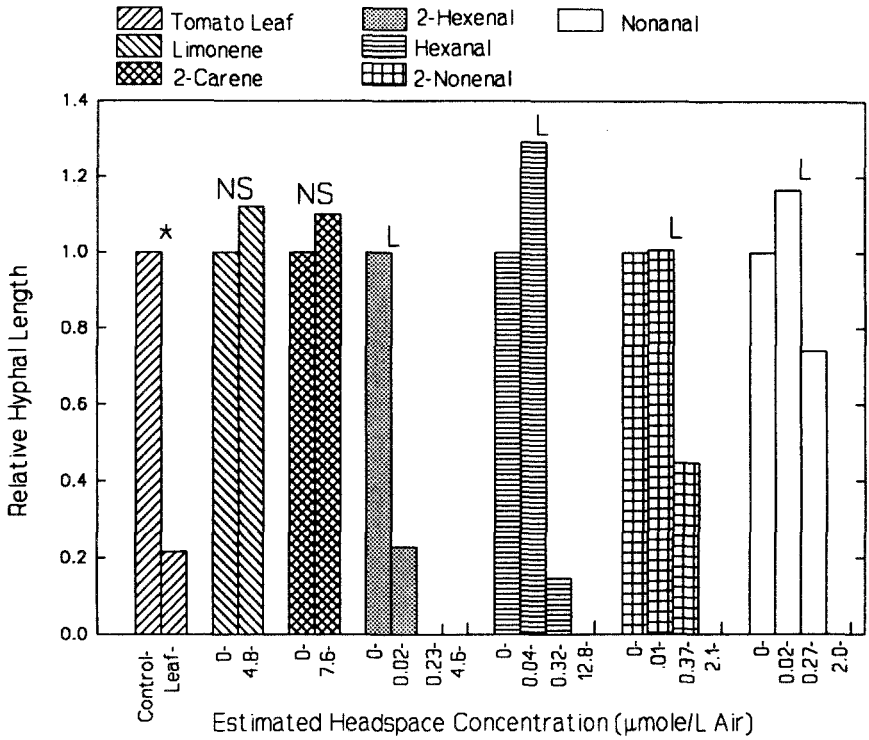

FIG. 3. Lengths of hyphae, relative to controls, after exposure of *B. cinerea* spores to crushed leaves and various vapor phase concentrations of aldehydes and monoterpene hydrocarbons. An asterisk denotes a significant, and NS no significant, difference between means at $P < 0.05$ using Student's t test. L denotes a significant linear correlation ($R^2 > 0.90$) between hyphal length and volatile concentrations.

upon wounding (Buttery et al., 1987; Hamilton-Kemp et al., 1991). Preliminary tests indicated that the amounts of these compounds released depended on the stage of development of the leaves, and young expanding leaves emitted the largest quantities of lipoxygenase pathway products. In addition, tomato leaves also released monoterpene hydrocarbons as the other major fraction of their volatile component emission.

Bioassays with synthetic samples of the tomato leaf components *E*-2-hexenal and hexanal showed that these compounds inhibited *A. alternata* hyphal growth (Figure 2). These compounds were tested by adding the three levels of solutions as described under Methods and Materials to the bioassay system and the resultant mean concentrations of compounds in the headspace vapor phase as estimated by GC are shown in Figure 2. Tests with vapors from neat solvents indicated that these materials alone had no significant effects on the growth of

fungal hyphae. *B. cinerea* spores responded similarly to those of *A. alternata* when exposed to the six-carbon aldehydes (Figure 3). Bioassays of the homologous nine-carbon aldehydes, *E*-2-nonenal and nonanal, showed that these volatile compounds also inhibited hyphal growth in both fungal species (Figures 2 and 3). The results obtained after treatment of *A. alternata* spores with the highest concentration of *E*-2-nonenal compared to the results with a control are shown in Figure 4. The nine-carbon homologues are found in certain crushed tissues such as wheat leaves (Hamilton-Kemp et al., 1987).

In previous studies we found that the concentration of *E*-2-hexenal produced by 5 g of crushed tomato leaves in the bioassay system was approximately 0.2 μmol/L air (Hamilton-Kemp et al., 1991). As noted above, there was inhibition of hyphal growth for both fungi observed at this concentration of *E*-2-hexenal. Thus, it appears that the level of this compound in the vapor phase was high enough to contribute significantly to the inhibition observed in crushed tomato leaf tests.

Among the aldehydes studied, *E*-2-hexenal has often been reported previously as an inhibitory compound in processes such as seed germination (Bradow and Connick, 1990; Gardner et al., 1990), fungal spore germination (Major et al., 1960), mycelial growth (Urbasch, 1984; Zeringue and McCormick, 1989), and protozoan movement (Schildknecht and Rauch, 1961).

Tests with other major types of volatile components from crushed tomato

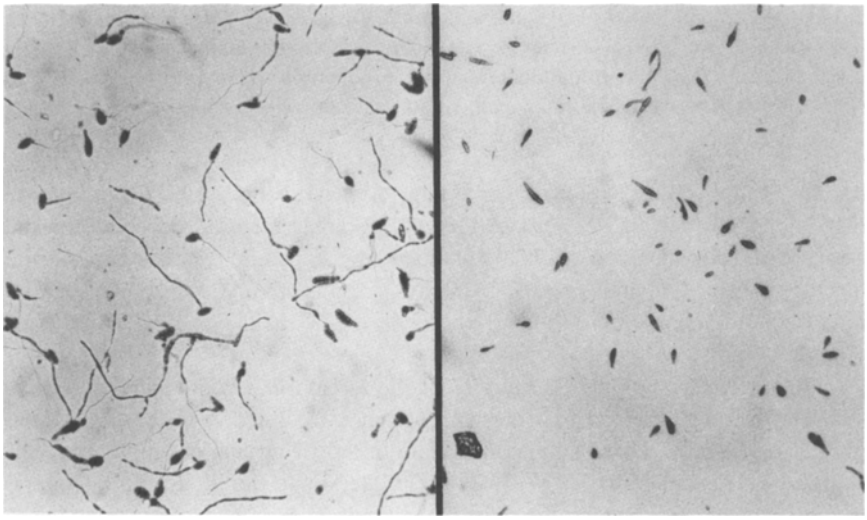

FIG. 4. Photomicrographs of bioassay of *Alternaria alternata* spores after 6 hr: (l) control and (r) *E*-2-nonenal (2.1 μmol/L air).

leaves, terpene hydrocarbons, were also carried out. The commercially available terpenes, 2-carene and limonene, at concentrations comparable to the highest concentrations of the aldehydes tested had no significant effect on either *A. alternata* or *B. cinerea* (Figures 2 and 3). The bioassay vapor phase concentrations of 2-carene and limonene were 7.6 and 4.8 μmol/L and greatly exceeded the levels produced by tomato leaves, 0.2 and 0.1 μmol/L air (Hamilton-Kemp et al., 1991). Hence these terpenes did not contribute to the inhibition produced by crushed tomato leaves. However, the possibility cannot yet be excluded that there are certain synergistic or additivity effects on the bioactivity of volatiles that result from the combination of the various compounds released from the crushed leaf tissue.

The observation that crushed tomato leaves inhibited growth of both *A. alternata* and *B. cinerea* hyphae was consistent with the inhibition exhibited by crushed leaves against pollen in earlier studies. In addition, the observation that the aldehyde components but not the terpene hydrocarbon components inhibited the fungi also agrees with the results for pollen. In pollen bioassays, the α,β-unsaturated aldehyde, *E*-2-hexenal, was considerably more active than the saturated aldehyde, hexanal. The unsaturated aldehydes, *E*-2-hexenal and *E*-2-nonenal, appeared to be more active than their saturated counterparts in bioassays with both fungal species, although the differences were not as pronounced as in the pollen bioassays.

Two compounds that were identified in tomato earlier, the monoterpene β-phellandrene and *Z*-3-hexenal, were not available commercially and were not tested. Synthetic *Z*-3-hexenal was inhibitory to pollen; however, this compound can isomerize enzymatically (Phillips et al., 1979) or chemically rearrange to *E*-2-hexenal and a similar reaction may have occurred with pollen.

Previous studies showed that α,β-unsaturated aldehydes can react with thiol groups in proteins or form Schiff's bases with amino groups as described by Schauenstein et al. (1977). Also, certain members of this class of compounds oxidize and form adducts with DNA (Sodum and Chung, 1989). Saturated aliphatic aldehydes were found to react with tryptophan residues to cause protein cross-linking as described by Kaneko et al. (1989). However, the mode of action of the aldehydes in inhibiting the growth of fungal hyphae remains to be elucidated. One area which merits investigation in the future is the interaction of volatile compounds with fungal membranes.

The concentrations of volatile aldehydes produced at the boundary layer of plant tissues after wounding and during infection processes involving fungal pathogens are not known. Since plants are capable of rapidly producing lipoxygenase pathway-derived aldehydes such as 2-hexenal, which are inhibitory to fungal growth, future studies are needed to clarify the possible role of natural volatile compounds in plant–fungal interactions.

Urbasch (1984) studied the effects of *E*-2-hexenal and hexanal, referred to

as wound gases from tomato leaves, on the radial growth of a culture of *B. cinerea*. She found that *E*-2-hexenal was more inhibitory than hexanal when aliquots of the neat liquids were compared. Levels of the vapors in the bioassay dishes to which the cultures were exposed during the experiments were not reported. Urbasch (1987) subsequently showed that *B. cinerea* cultures converted *E*-2-hexenal to the corresponding unsaturated alcohol, *E*-2-hexen-1-ol, or to 1-hexanol. Wilson et al. (1987) recently tested the effects of fruit volatiles on spore germination and fungal growth and found that aromatic compounds such as benzaldehyde inhibited germination and growth of *B. cinerea*.

French and co-workers, in numerous studies on fungal pathogens that cause rusts and other diseases, showed that several volatile compounds stimulate germination (French and Gallimore, 1971; French et al., 1977; French, 1990). In the present work, the saturated aldehydes, hexanal and nonanal, appeared to stimulate hyphal growth at their lowest concentrations. However, further studies involving additional dilutions would be needed to establish such an effect.

Interpreting these data, it seems useful to note results in which both organisms (which represent different genera) gave a similar response, such as inhibition of hyphal growth caused by the aldehydic function and the absence of effects resulting from exposure to terpenes. The specific response observed for one organism to a single compound, for example, should probably be interpreted cautiously since several variables such as the strain of the organism and culture conditions could affect the results of an experiment. Accordingly, only general trends observed for both organisms are noted here.

In conclusion, a rapid, simple bioassay system for the evaluation of the effects of volatile compounds on fungal spore germination and hyphal growth has been developed. Volatile compounds produced by crushed tomato leaves inhibited growth of both *A. alternata* and *B. cinerea* hyphae. Aldehydes which constitute a major group of volatiles from the leaves were inhibitory, whereas the terpene hydrocarbons 2-carene and limonene yielded no significant effect on hyphal growth. The results with aldehydes and terpenes were similar to those observed earlier in pollen bioassays.

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SEX ATTRACTANTS FOR THE WHITE-BARRED GROUNDLING MOTH (*Recurvaria leucatella* CLERCK) AND THE LESSER BUD MOTH (*R. nanella* HÜBNER) (LEPIDOPTERA: GELECHIIDAE)

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Abstract—In field tests synthetic (3*E*,5*Z*)-3,5-tetradecadienyl acetate was found to attract large numbers of males of *Recurvaria leucatella* Clerck into sticky traps. The other geometrical isomers did not influence catches. Of the corresponding monoenic compounds, (3*E*)-3-tetradecenyl acetate was highly attractive to a close relative, *Recurvaria nanella* Hübner. The catches of the latter species were suppressed by higher amounts of (5*Z*)-5-tetradecenyl acetate added to the bait, and the addition of (3*E*)-3-tetradecenol or (3*Z*)-3-tetradecenyl acetate was without effect. The attractive compounds can be used in traps for monitoring the two pests.

Key Words—Sex attractant, (3*E*,5*Z*)-3,5-tetradecadienyl acetate, (3*E*)-3-tetradecenyl acetate, (5*Z*)-5-tetradecenyl acetate, *Recurvaria leucatella*, *Recurvaria nanella*, Lepidoptera, Gelechiidae

INTRODUCTION

Presently, there are several hundred moth species for which sex pheromone components have been identified (Arn et al., 1986; Mayer and McLaughlin, 1991). Information can be obtained on the types of chemical structures involved

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in reproductive communication of Lepidoptera from conventional, complete, identification projects or by screening compounds structurally similar to known pheromones. Such sex attractants, once established, can be applied in practical, plant-protection programs by similar methods as natural pheromonal products, despite the fact that their natural occurrence in the female emitted pheromone has not been firmly established. In the present paper, we report the discovery of two sex attractants for orchard pests; the white-barred groundling moth (*Recurvaria leucatella* Clerck) and the lesser bud moth (*R. nanella* Hübner) (Lepidoptera: Gelechiidae).

METHODS AND MATERIALS

Instrumentation

Instrumentation used in the purifications and final analyses included a Hewlett-Packard Model 5890A gas chromatograph equipped with a split injection system. The column was a 50 m × 0.32-mm-ID, 0.25-μm film thickness, 007 series methyl silicone (Quadrex Corporation, New Haven, CT). The oven temperature was held at 60°C for 1 min, then programmed to 160°C at 40°C/min and held at that temperature for 47 min. High-performance liquid chromatography was performed with a Kratos Spectroflow 400 solvent delivery system with the mobile phase (ethyl acetate–hexane, 0.35:99.65) delivered at a flow rate of 5.0 ml/min. A Kratos Spectroflow 757 variable-wavelength ultraviolet absorbance detector set at 230 nm was used for sample detection. The column used was 250 × 12.5-mm-ID stainless steel, packed with Lichrosorb 60 (5 μm) (E. Merck).

Chemicals

The four isomers (E,Z; E,E; Z,E; Z,Z) of 3,5-tetradecadienyl acetate were prepared as described by Doolittle and Solomon (1986). The monoene acetates and alcohols were either purchased from commercial sources or synthesized by standard, well-documented procedures (Sonnet, 1984; Mori, 1979, 1981; Henrick, 1978; Rossi, 1977) and were purified by high-performance liquid chromatography (HPLC) on silver-nitrate-impregnated silica gel as described by Heath et al. (1977). Gas chromatographic analysis by capillary gas chromatography (GLC) as described by Doolittle and Solomon (1986) gave isomeric purities >99%; overall purities were >99.8%.

Purification of Diene Acetates

(3Z,5E)-3,5-Tetradecadienyl Acetate. The material used for this purification contained small amounts (≤2%) of each of the other three isomers. The E,E isomer was removed by treatment with an excess [relative to the estimated

(GLC) amount of E,E present] of *N*-phenylmaleimide in benzene. This produced a Diels-Alder adduct that could be removed from the diene acetates by simple gravity flow chromatography on a silica-gel column with hexane as the eluting solvent. The remaining two isomers were removed by HPLC on the column described above. The final GC analysis on the product used in the 1990 field test had traces of E,E, Z,Z, and E,Z lower than 0.1% (which was our detection limit).

The diene alcohols were purified by low-temperature recrystallization from dilute (10%) solutions in hexane using a modification (Figure 1) of equipment described by Schlenk (1961). The temperature in the apparatus was reduced slowly and uniformly, then held at that temperature where crystals of each alcohol began to form, by circulating methyl alcohol through the coil in the apparatus from a thermostatically controlled Multi-Cool Model MC-8-80A-2 low-temperature circulating system (FTS Systems, Inc., Stone Ridge, NY). The purified alcohols were subsequently acylated with acetic anhydride by standard techniques.

The analyses (GLC) of the diene acetates used in the 1990 field tests were as follows:

E,E had 0.26% Z,Z, 0.4% E,Z, and 0.4% Z,E.
E,Z had 0.5% E,E and $\leq$0.1% Z,E and Z,Z.
Z,Z had 1% Z,E, 1% E,Z, and 1.7% E,E.

The purities (GLC) of the diene acetates tested in 1987 and 1989 were as follows:

E,Z had 2.5% E,E, 1.2% Z,Z, and 1.2% Z,E.
E,E had 0.4% Z,Z, 4.0% E,Z, and 3.6% Z,E.
Z,Z had 2.5% Z,E, 2.3% E,Z, and 0.66% E,E.
Z,E had 5.5% E,Z, 4.8% Z,Z, 5.8% E,E.

Dispensers

The diene acetates were impregnated into 11-mm silicone rubber stoppers (Wheaton Co., Millville, NJ; Catalog No. 224091). The stoppers were extracted with methylene chloride before use. The test samples were placed on the stoppers as dilute solutions in hexane (50–200 μl) and the solvent was allowed to evaporate in a fume hood in a stream of air for a minimum of 24 hr, then sealed in screw-cap jars. The jars were stored in a freezer (-30°C) except while in transit to Hungary. Sustane 6 (Universal Oil Products Co., East Rutherford, NJ) was added to all samples at a ratio of 10 parts Sustane 6 to 1 part test chemical. Sustane 6 is a food-grade antioxidant containing tertiary-butyl-4-hydroxyanisole (BHA) and 2,6-ditertiary-butyl-4-methylphenol (BHT) in corn, cottonseed, or

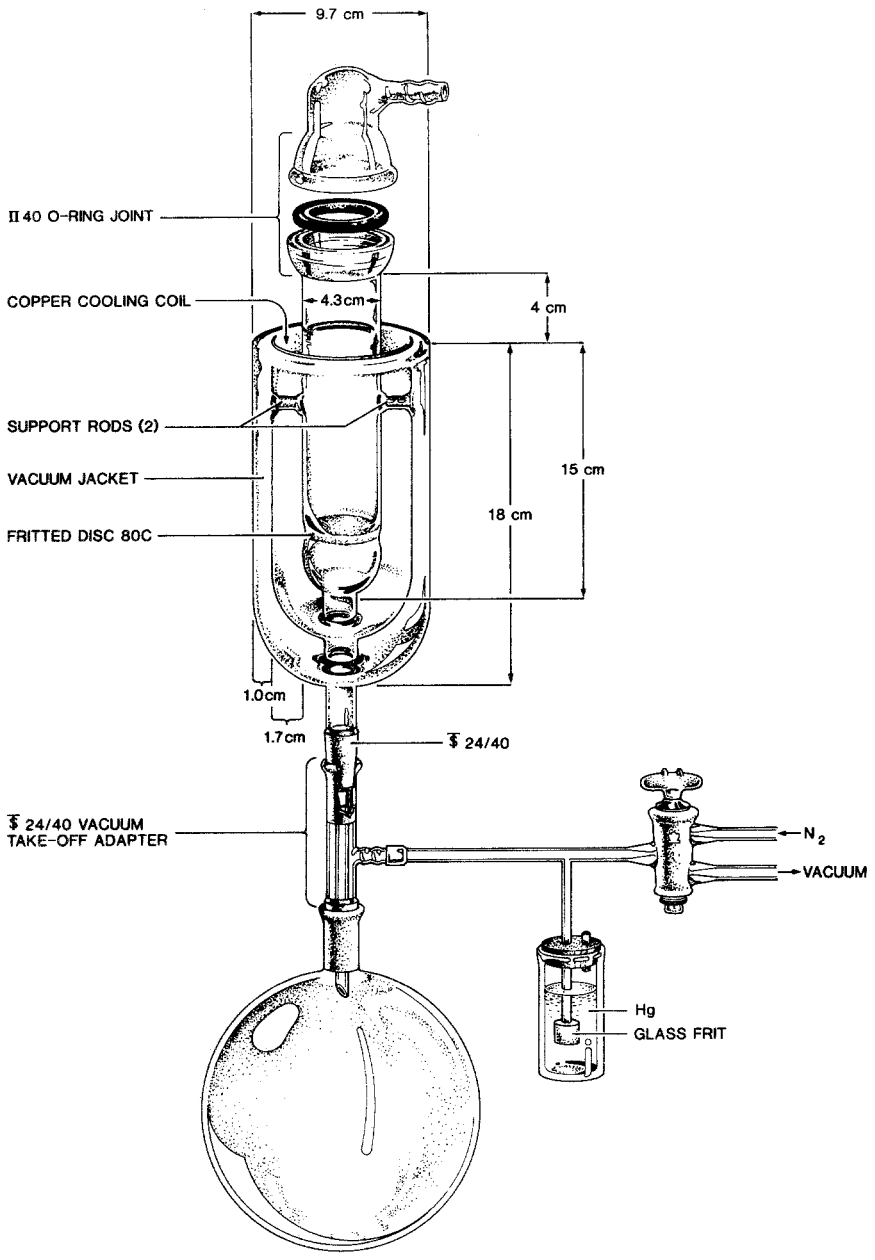

FIG. 1. Low-temperature crystallization apparatus used in purification of synthetic tetradecadienols.

soybean oil and has been used routinely in field tests with these diene acetates (Doolittle et al., 1976a, 1980).

Dispensers for tests with monoenes were prepared in Budapest, using pieces of rubber tubing (Taurus, Budapest, Hungary; No. MSZ 9691/6; extracted three times in boiling ethanol for 10 min, then three times in methylene chloride overnight, prior to usage). To make each lure, the required amounts of compounds were administered to the surface of the dispensers in hexane solutions. Prepared lures were stored at −65°C until use.

Trapping

Field tests were conducted at the Julianna major Experimental Station of the Plant Protection Institute, Hungarian Academy of Sciences, Budapest (Hungary), in an apple orchard (*R. nanella*), or at the edge of an apple orchard and a mixed oak forest (*R. leucatella*). In the tests triangular traps with removable sticky inserts made from polyethylene sheets, similar in shape and size to those described by Arn et al. (1979), were used. Traps were suspended 1.5 m above the ground from the branches of orchard trees. Traps containing different lures were set up in rectangular blocks. The distance of traps within a block was 4–5 m. The distance between blocks ranged from 100 to 1000 m. Traps were moved one position forward within a block at each occasion when the traps were inspected. At the same time, the number of captured males was recorded and sticky inserts changed to new ones. In statistical analyses, capture data were transformed to $\log(x + 1)$ and differences between means were tested for significance by Duncan's new multiple-range test (DNMRT). Catch of a trap at an inspection was taken as a replicate.

RESULTS

R. leucatella

When the four geometrical isomers of 3,5-tetradecadienyl acetate and the corresponding alcohols were screened preliminarily in 1987 in Hungary, large catches of male *R. leucatella* were observed at two sites in traps baited with 100 μg of (3*E*,5*Z*)-3,5-tetradecadienyl acetate [E3Z5-14Ac; names of compounds are abbreviated according to Arn et al. (1986)]. Few males were caught in traps baited with the other acetates. The alcohols alone or added to the active acetates did not increase catches.

In more detailed tests conducted in 1989, aimed at the comparison of the activity of the different geometrical isomers of the acetate, the E,Z isomer was the most effective (Table 1). Again, fewer moths were caught in some traps baited with the other three isomers. We hypothesized that the small catches by the other isomers can be attributed either to the low attractive activity of the

TABLE 1. CATCHES OF MALES OF *R. leucatella* IN TRAPS BAITED WITH 3,5-TETRADECADIENYL ACETATES OR THE CORRESPONDING MONOENIC ACETATES (JULY 17–AUGUST 1, 1989)[a]

	Chemical applied lure (μg)								Mean No. of males/ trap/inspection (SE)
	Z3-14Ac	E3-14Ac	Z5-14Ac	E5-14Ac	Z3E5-14Ac	Z3Z5-14Ac	E3Z5-14Ac	E3E5-14Ac	
Caps replaced by	100	—	—	—	—	—	—	—	0.0 c (0.0)
new ones at	—	100	—	—	—	—	—	—	0.0 c (0.0)
2- to 3-day intervals	—	—	100	—	—	—	—	—	0.0 c (0.0)
	—	—	—	100	—	—	—	—	0.0 c (0.0)
	—	100	100	—	—	—	—	—	0.0 c (0.0)
	—	—	—	—	100	—	—	—	0.5 bc (0.2)
	—	—	—	—	—	100	—	—	1.0 b (0.5)
	—	—	—	—	—	—	100	—	35.5 a (5.4)
	—	—	—	—	—	—	—	100	0.9 b (0.3)
Same caps left in	100	—	—	—	—	—	—	—	0.0 c (0.0)
traps throughout	—	100	—	—	—	—	—	—	0.0 c (0.0)
all the test	—	—	100	—	—	—	—	—	0.0 c (0.0)
	—	—	—	100	—	—	—	—	0.0 c (0.0)
	—	100	100	—	—	—	—	—	0.0 c (0.0)
	—	—	—	—	100	—	—	—	1.4 b (0.4)
	—	—	—	—	—	100	—	—	1.3 b (0.4)
	—	—	—	—	—	—	100	—	26.7 a (4.5)
	—	—	—	—	—	—	—	100	1.0 b (0.3)

[a] For each treatment four traps were set up. Traps were inspected at 2- to 3-day intervals. Means followed by the same letter are not significantly different at $P \leq 0.05$ (DNMRT).

compound itself or to the small amounts of the E,Z isomer known to be in the synthetic samples. Since the numbers attracted to the respective baits were similar when bait dispensers were renewed at 2- to 3-day intervals or when the same dispenser was used throughout the test period (Table 1; $t = 1.43$, $F = 23$; not significantly different in comparison to baits containing E3Z5-14Ac by Student's t test), the ratio of the E,Z isomer in the samples did not seem to increase through isomerization under the field conditions of this test.

No moths were caught in traps baited with the corresponding monoenic acetates or in traps baited with a 1 : 1 mixture of (3*E*)-3-tetradecenyl and (5*Z*)-5-tetradecenyl acetates (E3-14Ac; Z5-14Ac) (Table 1), showing that attractive activity was restricted to the dienic compound.

The comparison of the activity of the isomers was evaluated again in 1990 using newly purified samples (Table 2). The purities of the samples used in the 1990 tests are listed under Methods and Materials. In these tests, only the purified E,Z isomer showed attractive activity; catches of the other acetates did not differ significantly from the controls.

In a test designed to determine the possible effect of the addition of small amounts of the other isomers to E3Z5-14Ac, no significant effect was observed by addition of the other three isomers to the E,Z isomer (Figure 2). When pure E3Z5-14Ac was compared with a mixture containing this isomer and 3% of each of the other three isomers, no difference was observed between catches of the two treatments at any of the dose levels tested (Figure 3). Finally, in a dosage test of pure E3Z5-14Ac, traps baited with 3000, 1000, 300, 100, 30, 10, 3, and 1 μg captured an average of 35.80 (a), 22.10 (b), 6.60 (c), 4.35 (c), 2.75 (d), 1.10 (d), 0.25 (e), and 0.20 (e) males (per trap per inspection; means followed by the same letter are not significantly different at $P \leq 0.05$ by DNMRT), respectively.

TABLE 2. CATCHES OF MALES OF *R. leucatella* IN TRAPS BAITED WITH 3,5-TETRADECADIENYL ACETATES (JUNE 28–JULY 2, 1990)[a]

Chemical applied to lure (μg)				Mean No. of males/ trap/inspection (SE)
Z3E5-14Ac	Z3Z5-14Ac	E3Z5-14Ac	E3E5-14Ac	
100	—	—	—	0.00 b (0.00)
—	100	—	—	0.30 b (0.20)
—	—	100	—	6.87 a (1.42)
—	—	—	100	0.00 b (0.00)

[a]From each treatment 10 traps were set up. Traps were inspected daily. Means followed by the same letter are not significantly different at $P \leq 0.05$ (DNMRT).

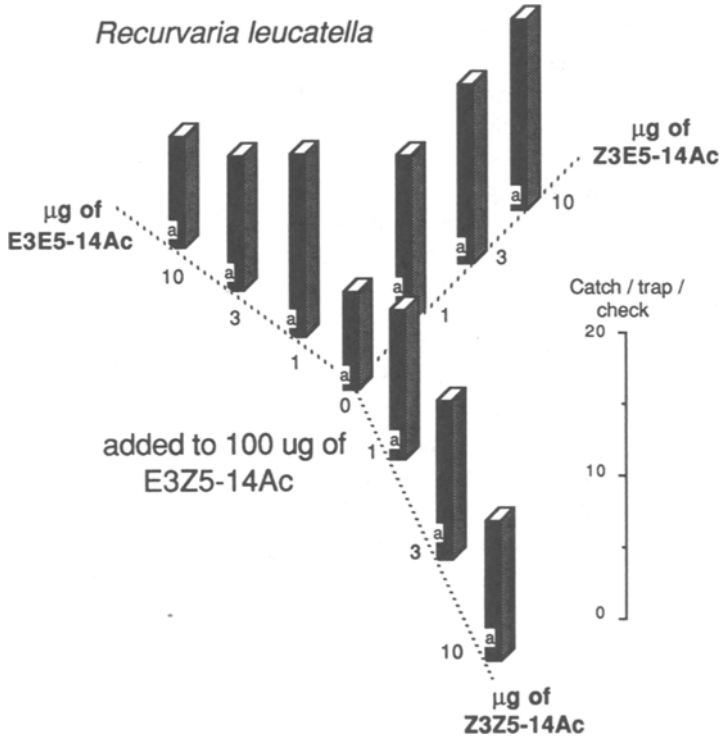

FIG. 2. Catches of male *R. leucatella* in traps baited with E3Z5-14Ac and its binary mixtures with the geometrical isomers (June 28–July 2, 1990). Ten traps of each treatment were used. Traps were inspected daily. Columns labeled with the same letter are not significantly different at $P \leq 0.05$ by DNMRT.

R. nanella

In the 1989 tests, which were originally aimed at studying *R. leucatella* (results shown in Table 1), traps baited with 100 µg of E3-14Ac caught a total of 578 specimens of *R. nanella* males, a close relative to *R. leucatella*. No other compounds caught any males of *R. nanella* in this test, and the mixture of E3-14Ac with Z5-14Ac was not attractive.

In the 1990 tests, the effect of the addition of the corresponding geometrical isomer (3*Z*)-3-tetradecenyl acetate (Z3-14Ac), and of the corresponding alcohol (3*E*)-3-tetradecenol (E3-14OH), to E3-14Ac was determined (Figure 4). No significant change in catches was observed when these compounds were added at 3, 10 or 30% to E3-14Ac (Fig. 4A). However, since catches with the lower amounts of the alcohol or with the highest amount of Z3-14Ac added were numerically higher than in traps with E3-14Ac alone, further binary and ternary

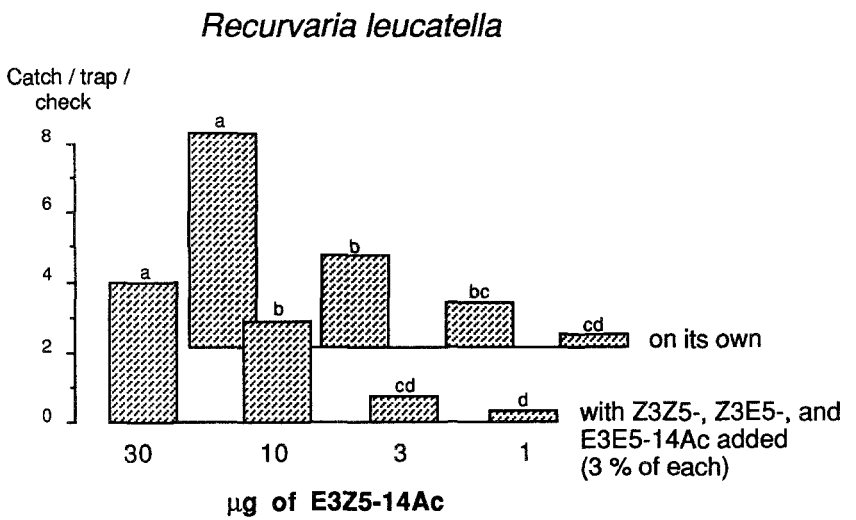

FIG. 3. Catches of male *R. leucatella* in traps baited with different dosages of E3Z5-14Ac or a quaternary mixture containing all four geometrical isomers (July 10–20, 1990). Five traps of each treatment were used. Traps were inspected each second day. Columns labeled with the same letter are not significantly different at $P \leq 0.05$ by DNMRT.

combinations of the three compounds were compared. In this test, none of the combinations caught significantly higher numbers of moths compared to the catch of pure E3-14Ac (Figure 4B).

When Z5-14Ac was tested in binary mixtures with E3-14Ac, the addition of 3 or 10% was without significant effect, while the addition of 30% decreased catches (Table 3). This agreed with the results of the 1989 test, where no insects were caught with a 1 : 1 mixture of the two compounds (Table 1).

In a dosage test of E3-14Ac, equally high numbers of males were caught by 3000, 1000, and 300 µg (Table 4). Significantly less males were caught at lower doses.

DISCUSSION

In the present study, E3Z5-14Ac and E3-14Ac were established as male sex attractants for the orchard pests *R. leucatella* and *R. nanella*, respectively. The described compounds can be used in amounts of 1 mg as baits in traps for monitoring the respective pest species.

E3-14Ac has already been found to attract one other gelechiid, *Chionodes perpetuella* H. Sch. (Priesner, 1987). The corresponding Z isomer has also been reported as sex attractant in a number of other gelechiids (Priesner, 1987). The

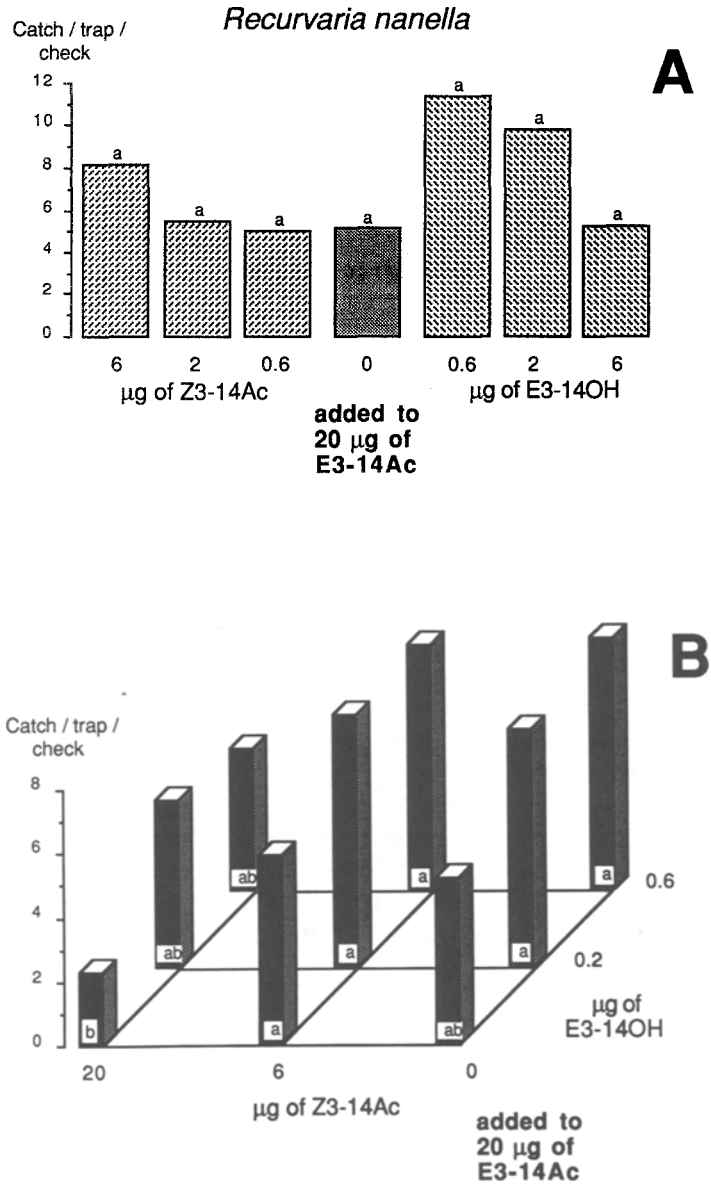

FIG. 4. Catches of male *R. nanella* in traps baited with E3-14Ac and its mixtures with Z4-14Ac and E3-14OH. (A) June 28–July 4, 1990; 10 traps of each treatment were used. (B) July 23–27, 1990; six traps of each treatment were used. Traps were inspected daily. Columns labeled with the same letter are not significantly different at $P \leq 0.05$ by DNMRT within each diagram.

TABLE 3. CATCHES OF MALES OF *R. nanella* IN TRAPS BAITED WITH E3-14Ac AND ITS MIXTURES WITH Z5-14Ac (JULY 4–11, 1990)[a]

Chemical applied to lure (μg)		Mean No. of males/ trap/inspection (SE)
E3-14Ac	Z5-14Ac	
20	—	4.05 a (0.54)
20	0.6	3.23 a (0.39)
20	2	2.70 a (0.38)
20	6	0.52 b (0.12)

[a]From each treatment 10 traps were set up. Traps were inspected daily. Means followed by the same letter are not significantly different at $P \leq 0.05$ (DNMRT).

TABLE 4. CATCHES OF MALE *R. nanella* IN TRAPS BAITED WITH DIFFERENT DOSAGES OF E3-14Ac (JULY 10–20, 1990)[a]

Chemical applied to lure (μg), E3-14Ac	Mean No. of males/ trap/inspection (SE)
3000	9.85 a (1.98)
1000	5.76 ab (1.59)
300	7.20 a (1.17)
100	3.96 b (0.80)
30	1.84 c (0.43)
10	1.16 c (0.27)
3	0.24 cd (0.10)
1	0.12 d (0.07)

[a]Five traps of each treatment were used. Traps were inspected each second day. Means followed by the same letter are not significantly different at $P \leq 0.05$ (DNMRT).

shorter-chain length homologue, (3*E*)-3-dodecenyl acetate, besides being attractive to some gelechiids in the field (Priesner, 1987; Renou et al., 1980), has been found in the pheromone of *Scrobipalpa ocellatella* Boyd. (Renou et al., 1980) and *Scrobipalpopsis solanivora* Povolny (Nesbitt et al., 1985). (3*E*)-3-Tridecenyl acetate, a similar compound having an odd number of carbons in its chain, has been identified from the pest *Scrobipalpa heliopa* Lower (Baker et al., 1985). So far, tetra-, tri-, or dodecenyl compounds with an unsaturation at the δ3 position have been described almost exclusively from Gelechiidae. The only exception is the cossid *Prionoxystus robiniae* Peck (Doolittle and Solomon, 1986), where E3-14Ac was found to synergize the attraction of (3*Z*,5*E*)-3,5-tetradecadienyl acetate.

The compound attracting *R. leucatella* in this investigation, E3Z5-14Ac, has not been described as a pheromonal component for any other species. Its geometrical isomers have been known to attract the cossids *Acossus centerensis* Litner (Doolittle et al., 1976b), *Prionoxystus piger* Grote (Landolt et al., 1985), and *P. robiniae* (Doolittle et al., 1976a; Doolittle and Solomon, 1986). The occurrence of this structure in gelechiids is not unexpected, as isomers of the shorter-chain length homologue, (3*E*,5*Z*)-3,5-dodecadienyl acetate, and (3*Z*,5*E*)-3,5-dodecadienyl acetate attract males of *Chionodes lugubrella* Fabr. (Reed and Chisholm, 1985).

At present, it is not known whether the attractant chemicals described in the present investigation are components of the sex pheromones of female *R. leucatella* and *R. nanella*. The compounds share chain length (14 carbons), functional group (acetate), and 3*E*-unsaturation. If indeed they represent the major components of the natural pheromone, the possible evolutionary splitting of these two *Recurvaria* species may have happened via pheromone biosynthesis mutation, i.e., the introduction of a second double bond into E3-14Ac at the fifth carbon atom, yielding E3Z5-14Ac. More effective reproductive isolation of the *leucatella* form from the *nanella* form may have resulted from the apparent inhibitory activity of the 5*Z* double bond toward males of the latter form. The inhibitory activity of Z5-14Ac toward *R. nanella* in this study may represent indirect evidence supporting the above hypothesis.

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PARASITIC *Meloidogyne* AND MUTUALISTIC *Acremonium* INCREASE CHITINASE IN TALL FESCUE[1]

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Abstract—Tall fescue (*Festuca arundinacea* Schreb.) is a C-3 perennial grass noted for its persistence in harsh environments. Tall fescue persistence is enhanced by *Acremonium coenophialum*, a mutualistic fungal endophyte that increases resistance to drought, pathogens, and insects. This research was conducted to identify and elicit biochemical mechanism(s) that could account for tall fescue persistence. In initial studies, two cultivars known to differ in persistence were analyzed for chitinase, an antifungal hydrolase associated with disease resistance in other plants. *Acremonium*-infected Kentucky 31 (KY31), a persistent cultivar, and Johnstone, a nonpersistent cultivar, were inoculated with the parasitic nematode, *Meloidogyne marylandi*, grown for 50 days, and analyzed at 10-day intervals. Chitinase fluctuated throughout the 50-day period of seedling development, and activity was highest in the persistent *Acremonium*-infected KY31. In addition, chitinase was elicited by parasitic *M. marylandi* and expressed systemically. Subsequent studies were conducted to determine whether or not mutualistic *Acremonium* could increase chitinase activity. Genetically identical KY31, with and without *Acremonium*, were grown for 25 days and analyzed for chitinase at 5-day intervals. After 20 days, *Acremonium*-infected KY31 expressed more chitinase than *Acremonium*-free KY31. We concluded that chitinase is related to tall fescue persistence; it was highest in the most persistent cultivar, increased under pathogen attack, and increased in the presence of *Acremonium*, a symbiont known to enhance disease resistance.

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Key Words—*Festuca arundinacea*, *Acremonium coenophialum*, *Epichloë typhina*, *Meloidogyne marylandi*, pathogenesis-related proteins, elicitor, plant defense, fescue toxicosis, nematodes.

INTRODUCTION

Tall fescue (*Festuca arundinacea* Schreb.) is a pasture grass widely used throughout the world. It is often preferred over other grasses because it persists under adverse environmental conditions (Burns and Chamblee, 1979). The use of tall fescue has extended pastureland into environmental zones which would not normally support livestock production; it currently represents approximately 35 million acres of pastureland in the United States [National Research Council (NRC), 1989]. Unfortunately, tall fescue is infected with *Acremonium coenophialum* Morgan-Jones and Gams (Bacon et al., 1977; Morgan-Jones and Gams, 1982), an endophytic fungus that causes fescue toxicosis and costs U.S. beef producers in excess of $200 million annually (Siegel et al., 1985).

In an effort to remove toxicity, *Acremonium*-free cultivars have been developed. Although the new cultivars are nontoxic, they are less persistent (Bacon and Siegel, 1988). According to recent studies, *Acremonium*-free cultivars are more susceptible to drought (West et al., 1988), insects (Johnson et al., 1985; Clay et al., 1985), and pathogens (West et al., 1990; Kimmons et al., 1990). While mechanisms that account for drought and insect resistance are being postulated (Johnson et al., 1985; West et al., 1988), those responsible for disease resistance have not been described. When these mechanisms are identified, they can be incorporated into *Acremonium*-free germplasm and permit development of a persistent, non-toxic fescue.

In other plants, disease resistance reactions are associated with a complex of plant responses, often including the accumulation of pathogenesis-related (PR) proteins (Van Loon, 1985). Synthesis of such proteins may be elicited by either biological or chemical stresses (Van Loon, 1983). The mechanism by which PR proteins contribute to disease defense responses is unknown, but certain of the proteins, such as β-1,3-glucanases, are capable of releasing elicitors of phytoalexin synthesis (Keen and Yoshikawa, 1983; Takeuchi et al., 1990). Other PR proteins, such as chitinases, possess antifungal activity (Schlumbaum et al., 1986; Boller, 1985). PR proteins are present in annual grasses (Fink et al., 1988; Sock et al., 1990; White et al., 1987) and may be present in perennial grasses, such as tall fescue.

This research included several objectives. The first was to identify chitinase activity in tall fescue. If activity was detected, an additional objective was to determine if chitinase activity was higher in a persistent cultivar and elicited with the pathogenic nematode, *M. marylandi*. The final objective was to deter-

mine if chitinase increased in the presence of the mutualistic endophyte, *Acremonium coenophialum*. If these objectives resulted in positive data, that is, that chitinase activity was highest in the persistent cultivar, was elicited by pathogenic stress, and increased in the presence of a mutualistic organism known to increase persistence, then chitinase could be postulated as a possible mechanism for disease defense in tall fescue.

METHODS AND MATERIALS

Chitinase in Persistent Cultivars and as Affected by Nematodes

Two cultivars of tall fescue were treated with the parasitic nematode, *Meloidogyne marylandi* (Jepson and Golden) (Jepson, 1987), a species reported to infect tall fescue (Kimmons et al., 1990). Cultivars were selected with persistence as the primary criterion; they included *Acremonium*-infected Kentucky 31 (KY31), a highly persistent cultivar (Stuedemann and Hoveland, 1988), and Johnstone, a less persistent, low-alkaloid, *Acremonium*-free, *Festuca* × *Lolium* hybrid (Buckner et al., 1983). Seed were surface-sterilized with 30% Clorox, then rinsed with sterile distilled water, and 10-g lots were planted in 500 g of autoclaved, sandy soil. Soil was inoculated with 0 or 16,000 eggs and second-stage juveniles (J2) of *M. marylandi*, while controls were not inoculated. Plants were allowed to grow in a greenhouse for 60 days. Each pot received 25 ml of a modified Hoagland's solution every 5 days (Epstein, 1972). Nematode root infection was verified under magnification (Byrd et al., 1983). Plant samples were collected at 10-day intervals throughout the first 50 days of seedling development. The main effects of nematode, cultivar, and time were arranged in a factorial design, and each combination was replicated three times.

As plants were collected, root and foliar tissues were separated and stored at −20°C. Tissues were allowed to thaw and duplicate 3-g samples were analyzed for chitinase activity according to the procedure of Boller et al. (1983). Briefly, this procedure involved homogenation in a 0.1 *M* sodium acetate buffer (pH 5.1), incubation with washed, powdered chitin for 20 hr at 30°C, colorimetric assay with a blank control as reported by Reissig et al. (1955), and protein quantification as reported by Bradford (1976). Main effects and interactions were considered significant at the 0.05 probability level in analysis of variance (Snedecor and Cochran, 1989).

Chitinase as Affected by Acremonium

In order to evaluate the isolated effect of endophytic *Acremonium* on chitinase activity, the confounding effect between plant genotype and *Acremonium* level was eliminated by evaluating genetically identical populations of tall fes-

cue. Two lots of KY31 seed were secured (International Seeds, Inc., Halsey, OR); one lot was *Acremonium*-free, while the other was *Acremonium*-infected. Ten grams of seed was surface-sterilized with 30% Clorox, then planted into 500 g of autoclaved vermiculite and allowed to grow for 25 days. Seedlings were collected at 5-day intervals, beginning at germination. Tissues were stored and analyzed for chitinase activity according to the procedure described previously (Boller et al., 1983). Statistical analyses were performed according to procedures cited previously (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

Chitinase in Tall Fescue

Results indicated that tall fescue contained chitinase in both roots and foliage (Figures 1 and 2). To our knowledge, these data report the first instance of chitinase production in a perennial grass. Results also indicated that activity fluctuated throughout seedling development. Chitinase activity in the roots

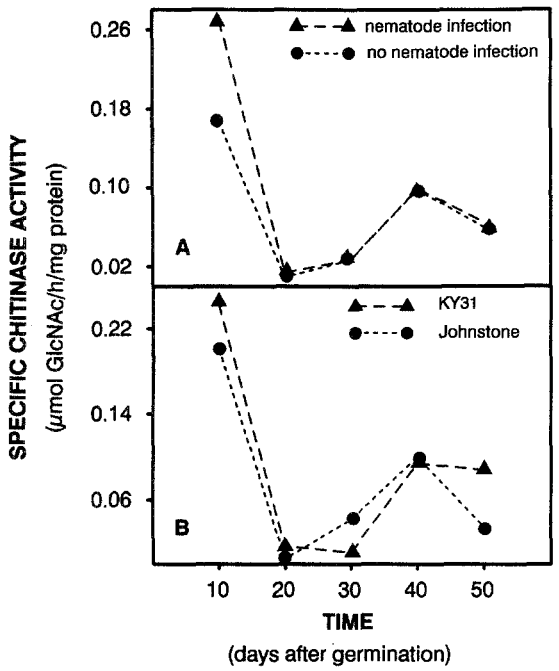

FIG. 1. Chitinase activity in tall fescue roots (A) inoculated with the parasitic nematode, *Meloidogyne marylandi*, and (B) in two fescue cultivars.

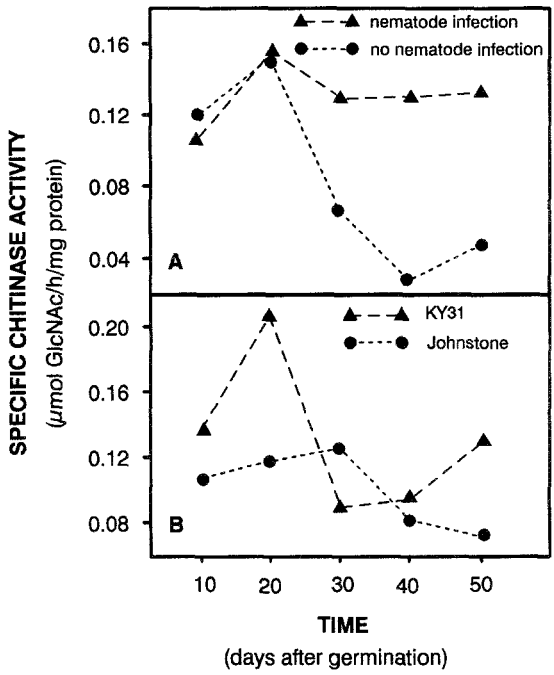

FIG. 2. Chitinase activity in tall fescue foliage (A) inoculated with the parasitic nematode, *Meloidogyne marylandi*, and (B) in two cultivars.

decreased sharply upon germination and remained low throughout the 50-day period. Activity in the foliage decreased also, yet the decrease was delayed by a slight increase 10 days after germination.

Chitinase as Elicited by Nematodes

In general, chitinase in the roots was not affected by nematode infection (Figure 1A). Activity in the roots of inoculated and control plants was almost identical except for 10 days after germination, when a significantly higher response was measured in the inoculated plants. Chitinase in the foliage, however, was greatly affected by nematode infection (Figure 2A). When tall fescue was inoculated with nematodes, chitinase activity remained high throughout the 50-day period of seedling development. At 40 days after germination, activity in inoculated plants remained at 0.13 μmol GlcNAc/hr/mg protein, while in control plants, activity had decreased to 0.02 μmol GlcNAc/hr/mg protein.

These data do not prove that chitinase is involved in a disease resistance mechanism. They do, however, support the hypothesis. If chitinase is involved, activity would be expected to increase over constitutive levels under pathogen

attack. In this study, plants responded systemically (Métraux and Boller, 1986; Roby et al., 1988) when infected with nematodes, producing a hydrolase specific for chitin, a product not found in higher plants, yet ubiquitous in insect exoskeletons and fungal cell walls. It is probable, then, that chitinase is involved in a defense response, and is not merely an expression of evolutionary speciation. When elicited with nematodes in other crops, protein accumulation was associated with disease resistance (Hammond-Kosack et al., 1989).

Chitinase Levels Among Cultivars

Chitinase activity in the roots did not differ greatly between cultivars (Figure 1B). Root chitinase was similar until 50 days after germination and then increased only slightly in *Acremonium*-infected KY31 while it remained low in Johnstone. In the foliage, however, the difference between cultivars was more pronounced (Figure 2B); activity in *Acremonium*-infected KY31 was high and variable, while in Johnstone it was lower and more constant.

These data support a hypothesis that involves PR proteins in fescue persistence. If chitinases are involved in a resistance mechanism, the highest activity would be expected in the persistent cultivar, *Acremonium*-infected KY31. As expected, *Acremonium*-infected KY31 exhibited high chitinase activity, and its chitinase level was related to visual observations of persistence (Figure 3A). *Acremonium*-infected KY31 persisted throughout the 50-day growing period, while Johnstone did not. By the end of the growing period, Johnstone, the cultivar with low chitinase activity, was easily recognized by its thin, senescent stand (Figure 3B).

These data do not establish a relationship between endophytic *Acremonium* and chitinase activity; although KY31 is *Acremonium*-infected and Johnstone is not, the cultivars differ genetically. With this in mind, the study was continued in order to investigate the possible relationship between endophytic *Acremonium* and chitinase activity.

Chitinase as Increased by Acremonium

Chitinase levels were higher in *Acremonium*-infected KY31 fescue (Figure 4). Since the populations were genetically identical, it is obvious that elevated levels of chitinase resulted from the presence of the endophyte. It was not possible to determine whether the excess chitinase activity originated from the endophytic fungus or the plant. It is likely, however, that the increased activity originated from the plant; *Acremonium* is not highly concentrated in seedling development.

Like data from the initial studies, these data do not prove that chitinase is involved in a disease defense mechanism. Nevertheless, they do establish a relationship between chitinase and *Acremonium* (Figure 4) and, thereby, support

FIG. 3. Two cultivars of tall fescue treated with the parasitic nematode, *Meloidogyne marylandi*, and grown for 50 days. *Acremonium*-infected Kentucky 31 (A), a cultivar noted for its persistence in adverse environments, contained high chitinase activity (Fig. 2) and persisted well throughout the entire study. In contrast, Johnstone (B), a *Festuca* × *Lolium* hybrid, exhibited lack of persistence, which corresponded with low chitinase activity (Fig. 2).

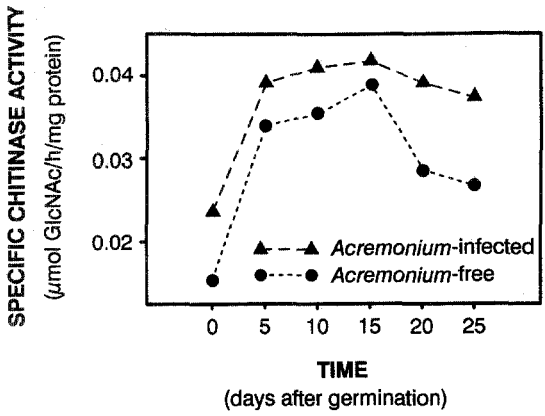

FIG. 4. Chitinase activity in genetically identical populations of Kentucky 31 tall fescue seedlings as influenced by the mutualistic endophyte, *Acremonium coenophialum*.

the hypothesis that involves chitinase in disease defense. The highest chitinase level was in *Acremonium*-infected KY31, a symbiosis known for tolerance and resistance to pathogens and insects.

CONCLUSIONS

Since there is presently no information on the nature of disease defense responses in tall fescue, we chose to measure chitinase, a PR protein associated in plant defense reactions in a wide variety of host–pathogen combinations. As expected for a PR protein, chitinase activities in fescue increased in response to infection by a nematode. Potentially important to tall fescue ecology, chitinase increased also in response to the mutualistic fungus, *Acremonium*. If these and other fescue PR proteins can be individually characterized, they can provide markers for plant geneticists attempting to develop persistent, *Acremonium*-free germplasm. Chitinase and other PR proteins are already being incorporated into other plants using recombinant DNA technology (Dixon, 1991).

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ATTRACTION OF MALE BEETLES TO GRUBS: EVIDENCE FOR EVOLUTION OF A SEX PHEROMONE FROM LARVAL ODOR

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Abstract—Females of the scarabaeid beetle *Cyclocephala lurida* produce a volatile sex pheromone which attracts conspecific males. Field experiments demonstrated that larvae of both sexes also emit volatile chemicals that stimulate similar responses in adult males, including attempts by the attracted males to mate with the nonreproductive immature stage. Significantly more adult males were caught in traps baited with conspecific male or female larvae or adult females than in blank control traps. Hexane extracts of both male and female grubs were at least as effective as live larvae in trapping male adults, demonstrating that the behavioral responses are mediated by volatile chemicals. Sensory and behavioral responses of males to sex pheromones emitted by adult females are part of the functional communication system. However, their response to grubs is not functional, because grubs are normally temporally and spatially inaccessible to mate-seeking males. In theory, the evolution of a communication system is problematic because it requires the development of a signal in one sex and the sensory and behavioral attributes to respond to that signal in the other sex. The ontogeny of sex pheromone communication in *C. lurida* suggests a partial solution to this evolutionary problem. We propose that this sex pheromone communication system is probably derived from noncommunicative volatile chemicals that are lost in adult males and retained by adult females.

Key Words—*Cyclocephala lurida*, Coleoptera, Scarabaeidae, sex pheromone, chemical communication, evolution of signals.

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INTRODUCTION

Sex pheromone communication involves release of a chemical signal by individuals of one sex and specialized sensory receptors for, and behavioral responses to, that signal in members of the other sex (Payne et al., 1986). The evolution of chemical communication and systems relying on other sensory modalities is problematic because of the disparate requirements for effective communication in the signaler and receiver (Krebs and Dawkins, 1984; Otte, 1974; Ryan and Wilczynski, 1988; Smith, 1977). The initial evolution of chemical communication may be facilitated by modification of existing cues and/or biosynthetic pathways for signal production. For example, in the crab *Pachygrapsus crassipes*, the female's excretion of crustecdysone, a hormone that regulates molting, stimulates search and display mating behaviors in males and thus takes on a pheromonal function in this context (Kittredge and Takahashi, 1972). In scolytid bark beetles, biosynthetic modification of host-tree compounds is often involved in the production of species-specific aggregation pheromones (see references cited by Vanderwel and Oehlschlager, 1987). The ontogeny of the sex pheromone signal in the southern masked chafer, *Cyclocephala lurida* (Coleoptera: Scarabaeidae), suggests another, previously undescribed evolutionary route for the development of chemical communication, in which sex pheromones are derived from larval odors.

As is typical of other scarabaeid beetles and many other insects (Tamaki, 1985), adult females of *C. lurida* produce a sex pheromone which attracts conspecific males (Potter, 1980). Release of this pheromone is behaviorally controlled by the female beetle, which emerges from the soil beneath pasture or turfgrass shortly after sunset, in close daily synchrony with the emergence and flight of conspecific males. Unmated females remain on the ground or climb grass blades. Males fly close to the ground, zigzagging upwind toward females. Mating follows, often with several males attempting to copulate with a single female (Figure 1A). Male beetles will show a similar response to hexane or ether extracts of adult females, demonstrating the chemical nature of this communication system (Potter, 1980). Mated females quickly burrow into the soil, often before the male has disengaged. After the mating period, which lasts from dusk until about 11 PM (EST), the remaining beetles burrow into the soil. If unmated females are held aboveground in traps after this mating period, they are attractive to a closely related sympatric scarabaeid species, *C. borealis*, which normally mates after midnight (Potter, 1980). This provides evidence that effective pheromone emission is behaviorally controlled by emergence of the female beetle from the ground. There is one generation per year, mating flights generally occurring during late June and early July (Potter, 1981).

Initially we observed male beetles attempting to mate with conspecific grubs, which if developing normally would not be present when males are active.

FIG. 1. (A) Normal mating behavior of *Cyclocephala lurida*. Male beetles are attracted to a sex pheromone emitted by the female. (B) Males are attracted to and attempt to mate with the nonreproductive grub. Males will mount grubs and probe the grub with their aedeagus.

These grubs were naturally infected with milky disease [*Bacillus popilliae* (*Cyclocephala* strain)], a bacterial disease that delays their development. For an unknown reason a few infected individuals were found on the ground surface. In this report, we provide evidence that both healthy male and female larvae produce a volatile chemical cue that attracts adult male beetles and stimulates them to attempt to copulate with the nonreproductive immature stage. This response of males to the immature stage is clearly not an adaptive feature of sexual communication, because the root-feeding grubs are not ordinarily present during late June and July when males are active, nor are they normally above-

FIG. 1. Continued.

ground. However, the response suggests that sex communication in this species may have evolved by loss of the signal in males and specialization of the males' sensory system to detect the same signal from the adult female.

METHODS AND MATERIALS

Last-instar grubs were collected in May by digging up grass sod and examining the root systems for the feeding larvae. Grubs were stored in sod at 18°C to delay development. Grubs were separated by sex, based on well-defined sexual characteristics (Tashiro, 1987). Adult male and female beetles displaying characteristic precopulatory behavior were collected on a golf course fairway the night before an experiment. Adult females can be distinguished from males by their behavior, the structure of their tarsal claws, and other morphological differences (Potter, 1980; Tashiro, 1987). When an individual was collected, its sex was determined and it was placed with other members of the same sex in a paper carton with moistened paper towels. The beetles were stored until the next day at 8°C.

The following evening, traps were baited with male or female adults or male or female grubs and placed on golf course fairways or adjacent roughs. Experiments were conducted at the Lexington Country Club Golf Course, Lexington, KY, in late June 1991. We used standard metal Ellisco Japanese beetle traps placed on the ground and supported by wire stands. Traps were at least 5 m apart and were arranged in a randomized complete block with five replications. Three individuals were placed in the bait well of each insect-baited trap. One trap per replicate was left unbaited to serve as a control. The position of traps was rerandomized within each block at 15-min intervals. After the nightly flight period, traps were retrieved and beetles were counted. Data were analyzed using a two-way (treatments and experimental blocks) analysis of variance on log-transformed data [log $(x + 1)$]. Means of insect-baited traps were compared to blank traps using the LSD procedure for preplanned comparisons.

In the second experiment, grubs were collected at field sites in May and brought back to the laboratory to be extracted. The extraction procedure followed a protocol that has proved to be successful in extracting pheromone from adult females (Potter, 1980; Haynes and Potter, unpublished data). Seventeen male and 19 female grubs were rinsed with 5 ml of hexane in separate 50-ml flasks. Approximately 4 ml of the hexane was recovered, and the extract was then diluted to 3 grub equivalents/ml. At sunset, traps were baited with hexane extracts of male or female grubs by dispensing 1 ml of these solutions into a 3.5-cm-diameter aluminum dish. Other traps were baited with aluminum dishes containing three male or three female grubs. Blank traps contained an aluminum dish treated with 1 ml of hexane. Other procedures were identical to the first experiment.

RESULTS AND DISCUSSION

Both male and female grubs attracted large numbers of adult male beetles (Figure 1B). Traps baited with larvae of either sex caught significantly more male *C. lurida* than blank traps (Figure 2A). Adult female *C. lurida* also attracted conspecific male beetles as expected. In contrast, traps baited with adult males and blank control traps caught very few beetles. Thus, males become unattractive to other males as adults, while females retain their attractiveness to males.

Attraction of males to grubs is mediated by a hexane-extractable chemical or chemicals. Hexane extracts of male or female grubs attracted significantly more male *C. lurida* than hexane alone (blank traps) (Figure 2B). Male or female grub extracts were as attractive as live grubs.

Attraction of other adult arthropods to signals from conspecific immature stages is not without precedent (Thornhill and Alcock, 1983). However, in each of the previously documented examples this attraction represents an adaptive

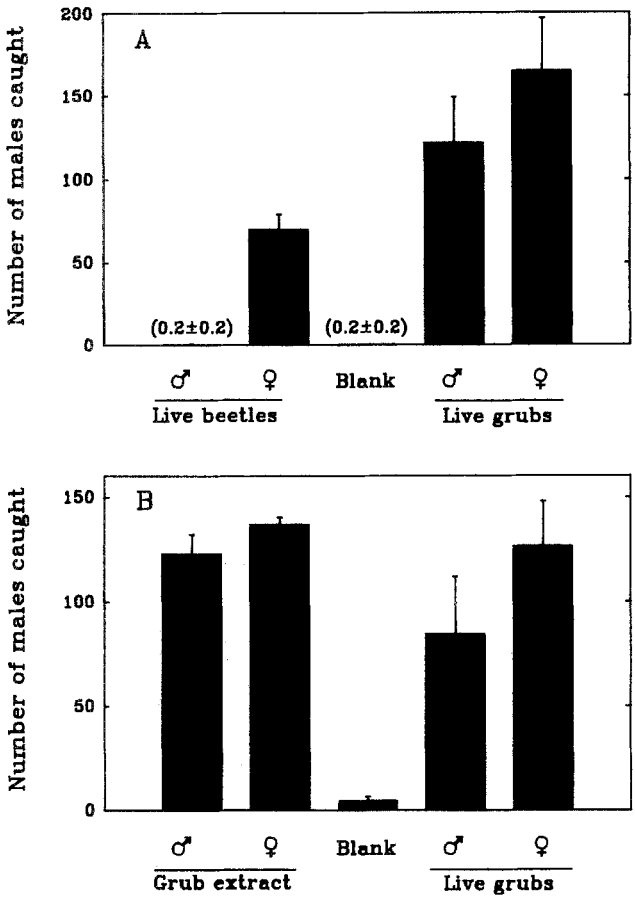

FIG. 2. (A) Attraction of adult male *Cyclocephala lurida* to live adults and grubs. Traps baited with adult females, male grubs, or female grubs caught significantly more males than blank control traps (LSD test after two-way analysis of variance; $P < 0.001$, df = 4,16, $F = 183.5$). Error bars show 1 SE. (B) Extracts of both male and female grubs caught significantly more adult male beetles than blank control traps (LSD test after two-way analysis of variance; $P < 0.001$, df = 4,16, $F = 31.5$).

feature of reproductive behavior with the immature individual close to maturation. For example, male spider mites are attracted to quiescent female deutonymphs, which they guard until emergence and mating (Potter et al., 1976). The pupal stage of some heliconiid butterflies is attractive to males, with mating usually occurring on the pupal case as the female emerges (Gilbert, 1976).

In normal circumstances grubs and reproductively active adult male *C.*

lurida do not co-occur. Activity of grubs is confined to underground feeding on grass roots, while mating activity occurs aboveground. The combined prepupal and pupal period lasts 15–21 days. This species has a single generation per year, with adult activity confined to a few weeks (Potter, 1981). Thus, there would normally be little or no temporal overlap between adult males and grubs. Because chemically mediated sex attraction of adult males to larvae of holometabolous insects is unexpected, it may not have been investigated before. This phenomenon may be common to scarabaeid beetles (a family which includes many species of economic importance) or may even be phylogenetically widespread in species in which adults and larvae do not co-occur in the same habitat.

The evolution of communication systems is complicated by the fact that signaler and receiver must coevolve. De novo and simultaneous evolution of both signal and receptors is unlikely. The attraction of male *C. lurida* beetles to the immature grub stage suggests that it may be possible for the evolution of sex pheromone communication to begin with odors common to all developmental stages. Apparently, males lose the attractive components during metamorphosis.

An alternative explanation of the lack of attraction to adult males could be that they produce a repellent. This possibility is not supported by observations or experimentation. It is common to find clusters of males surrounding a single pheromone-releasing female (Potter, 1980). Traps baited with females captured large numbers of males. When extracts of males and females are combined, there is no decrease in the number of males captured (unpublished data).

In summary, these experiments have demonstrated that adult females and both male and female larvae produce a volatile sex attractant. This observation appears to be unprecedented for *C. lurida* or for any other insect species. We hypothesize that the evolution of the communication system in this species involved sex-specific loss of this chemical cue in the male, combined with specialization of the adult male's sensory receptors to detect that cue.

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FURANOCOUMARIN CONTENT AND PHOTOTOXICITY OF ROUGH LEMON (*Citrus jambhiri*) FOLIAGE EXPOSED TO ENHANCED ULTRAVIOLET-B (UVB) IRRADIATION

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Abstract—Rooted cuttings of *C. jambhiri* were grown under enhanced levels of UVB radiation for 95 days. Bacterial phototoxicity and furanocoumarin content were determined in extracts made from various tissues from the aboveground biomass. Young, newly expanded leaves contained significantly higher concentrations of furanocoumarins than older leaves and stems. Additionally, the proportional concentration of psoralen was higher in young leaves than in old leaves. While treatment with UVB did not result in a change in the overall level of furanocoumarins, it did cause an increase in the ratio of psoralen to bergapten. Bacterial phototoxicity paralleled the distribution of furanocoumarin content among tissue types; analysis of covariance suggested that the phototoxic properties of the extracts could be accounted for on the basis of furanocoumarin content alone.

Key Words—*Citrus jambhiri*, furanocoumarins, phototoxins, ultraviolet-B radiation, plant-herbivore interactions, psoralen, bergapten.

INTRODUCTION

Gradual reductions in levels of stratospheric ozone, attributable at least in part to anthropogenic inputs of chlorofluorocarbon and nitrogen oxide pollutants, are

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associated with an increase in the incidence of shortwave solar ultraviolet-B radiation (UVB; 280–320 nm), particularly in the region between 290 and 315 nm (Caldwell et al., 1989). Enhanced UVB radiation disrupts a number of physiological processes in plants; exposure to high levels of UVB radiation is associated with decreases in rates of photosynthesis and nitrogen fixation, reduced enzyme activity, destruction of chlorophyll, growth anomalies, and lowered levels of soluble protein (Teramura, 1983; Caldwell et al., 1989).

There is also great potential for enhanced UVB radiation to influence ecological interactions between plants and their pathogens and herbivores. Enhanced UVB radiation alters patterns of secondary, as well as primary, metabolism and can induce biosynthesis of a number of shikimic acid derivatives, including flavonoids and coumarins (Caldwell et al., 1989). In that these compounds frequently serve as defensive agents against pathogens and herbivores, alterations in levels of secondary metabolism may affect the relative susceptibility of plants to damage. For instance, treatment with UVB can increase disease severity in cucumbers (Orth et al., 1990), although the mechanism by which susceptibility increases is not known. Ecological alterations may be particularly striking for those plants with secondary metabolites that are photoactive—that is, compounds that can absorb ultraviolet light to form an excited state. These excited molecules can react directly with biological macromolecules such as DNA or proteins to disrupt biological function or can react with ground-state oxygen to form a variety of toxic oxygen species, which can then proceed to damage cells and tissues (Downum et al., 1991). The efficacy of UVB radiation in activating plant photosensitizers is largely unknown, but the possibility exists that UVB may be as effective as UVA (ultraviolet-A; 320–400 nm) at enhancing the toxicity of photosensitizers.

Species in the genus *Citrus* are grown worldwide in tropical, subtropical, and semitropical areas where levels of UVB are naturally high (Caldwell, 1981); herbivores and pathogens on exposed foliar surfaces will be exposed to these high irradiance fluxes. Foliage of *Citrus* and other rutaceous species is phototoxic to a variety of organisms, including humans (Knowlton and Ockert, 1950; White, 1986; Israel, 1985). Like many other rutaceous species (Waterman and Grundon, 1983; Fisher and Trama, 1979), *C. jambhiri* produces furanocoumarins. These UV-absorbing compounds are phototoxic to a wide variety of organisms (Berenbaum, 1991; Murray et al., 1982) and are responsible, at least in part, for the resistance of some *Citrus* species to pathogenic fungi (Martin et al., 1966; Pirone et al., 1960). *Citrus jambhiri* is frequently used as a rootstock in commercial citriculture, where it is valued for its disease resistance (Whiteside et al., 1988). Although not reported from *C. jambhiri*, rutaceous plants produce a number of other photosensitizers that are biosynthetically and structurally distinct from the furanocoumarins; these include benzylisoquinoline alkaloids, β-carboline alkaloids, simple coumarins, and pyranocoumarins (Towers, 1984).

In this study, we examined the effects of UVB radiation exposure on furanocoumarin production in *C. jambhiri* foliage. In addition, we used an ultrasensitive bacterial bioassay to estimate changes in phototoxicity resulting from UVB exposure (Tuveson et al., 1986; Ashwood Smith et al., 1986; Downum et al., 1991). Such an assay is particularly suitable for our purposes for several reasons. It allows for rapid and sensitive screening of multiple extracts, and it provides data that correlate well with phototoxicity against a broad spectrum of organisms, including insects (Downum et al., 1991; Larson et al., 1988). Further, it allows us to estimate total phototoxicity of foliar extracts, including not only that attributable to furanocoumarins but also to other, as yet uncharacterized, phototoxins in the plant.

METHODS AND MATERIALS

Plants. Apical cuttings from three randomly selected clones of *C. jambhiri* were rooted in the greenhouse with a commercially available rooting compound. Clones had been taken from a group of about 80 parental plants donated by a commercial supplier. After 6 weeks, the cuttings had begun to produce roots and were transplanted into a light soil mixture (Perlite:Peat:Soil, 2:1:1) in 10.2-cm clay pots. After 3 weeks in these pots, the cuttings were established and growing vigorously. We then selected 10 plants of similar size and degree of apical dominance from each clone. Half of the plants within each clone were randomly assigned to the control group. Thus, there were 15 plants, 5 in each clone, in the control and treatment groups. The plants were then repotted to 15.2-cm clay pots and placed on a greenhouse bench for UVB exposure. UV light was supplied by banks of Westinghouse FS-72 fluorescent bulbs which were filtered on the control side of the bench with Type S Mylar film (Gar-Ron Plastics, Baltimore, MD). This film does not transmit UVB wavelengths (280–320 nm); bulbs on the treatment side of the bench were filtered with cellulose acetate film (3 mil, Folex, Inc., Palmyra, NJ), which allows UVB transmission but filters wavelengths below 280-nm (Mirecki and Teramura, 1984). A sheet of Mylar, suspended between the two light banks, shielded the control side of the bench from the treatment side. The UVB bulbs were turned on for 8 hr daily, centered in the 14-hr photoperiod of the experiment. Plants were irradiated with a UVB dose of 19.1 kJ day^{-1} BE_{300} (biologically effective radiation, normalized at 300 nm). This dose measurement expression gives the integrated fluence over the UVB waveband weighted according to Caldwell's generalized plant damage spectrum for UVB normalized to 300 nm (Caldwell et al., 1983). Constancy of this dose was maintained by adjusting the height of the bulbs over the plants or by occasionally lengthening the period of exposure as needed. Supplemental visible light was supplied by 8 HID metal halide lamps situated

over the UVB light banks. Maximum levels of photosynthetically active radiation at midday ranged from 600 to 1000 μmol m^{-2} sec^{-1}, depending on the cloudiness of the day.

We grew the rough lemon plants under UVB for 95 days through the spring and summer of 1990. Plants were watered as needed and fertilized ca. every 2 weeks with 20–20–20 NPK supplemented with $MgSO_4 \cdot 7H_2O$. UVB fluence levels were checked and adjusted frequently (five times per week) using a UVB radiometer (SED-240, International Light Inc., Newburyport, MA) that was periodically calibrated with a spectroradiometer (Optronics OL 752, Optronics Labs. Inc., Orlando, FL). Periodically, the bench sides were switched and the plants were randomized within a bench side. Cellulose acetate and Mylar were changed as needed.

Following the period of growth under enhanced UVB, the plants were harvested and the aboveground biomass was divided into four tissue classes. Young leaves were defined as the soft, bright green leaves that either were not fully expanded or had been fully expanded for a short period. Intermediate leaves were fully expanded leaves that had begun to harden but were still a light green shade. Old leaves were easily distinguished by their dark green color and extreme hardness in comparison to intermediate leaves. Finally, aboveground tissue remaining after all leaves had been removed was harvested as stems. Harvested tissues were immediately frozen and stored at −25°C. The frozen material was then freeze-dried and ground in a Wiley Mill to pass through a 40-mesh sieve.

Furanocoumarin Assay. Three 50-mg (±1.5-mg) subsamples were taken from each plant/tissue combination. The subsamples were homogenized in 5 ml cold Tris buffer (50 m*M*, pH 8) with a polytron. Furanocoumarins were then extracted with 3 × 2 ml EtOAc with vortexing. Clean phase separation was achieved by first bringing the aqueous homogenate to 0.0125% cetyltriammonium bromide (CTAB), followed by mild heat denaturation of solubilized proteins (60°C, 20 min). Particles were removed and phase separation was effected by centrifugation at 2000*g* for 10 min. The combined extracts from each subsample were evaporated and redissolved in 400 μl EtOAc. Furanocoumarins present in the resulting solution were assayed by normal-phase HPLC (Berenbaum et al., 1984). The presence of psoralen and bergapten had been previously confirmed by GC-MS of the nonphenolic lactone fraction (Berenbaum, 1981; McCloud, unpublished data) and by retention time matching in normal- and reversed-phase HPLC systems. After HPLC, the remaining liquids from each subsample were combined, evaporated, and redissolved in MeOH at an approximately constant level of tissue representation, ca. 0.75 mg dry weight of tissue/ml. After resolvation in MeOH, phototoxicity of the tissue extracts to *Escherichia coli* was measured.

Bacterial Culture and Bioassay. The complete genotype of the *E. coli* strain, RT7h(tet^r), used in these investigations is described by Leonardo et al.

(1984) and Sammartano et al. (1986). The bacteria were grown on modified Luria-Bertani (LB) medium (Miller, 1972). The minimal medium used to assess survival consisted of minimal A medium (Miller, 1972) supplemented with arginine, lysine, and histidine (40 μg ml^{-1}).

Cells were grown at 37°C in a side-arm flask (Bellco) containing 50 ml of LB, and growth was monitored with a Klett-Summerson colorimeter. When the culture had reached stationary phase, it was diluted with an additional 450 ml of LB containing 25% glycerol. Ten-milliliter samples of the cells were dispensed into 15 × 150-mm screw-top sterile test tubes and quickly frozen in a dry ice–ethanol bath. The frozen cells were stored at −55°C. Prior to bioassay, a 10-ml sample of the frozen cells was removed from the freezer and rapidly unfrozen by running cold tap water over the tube. The cells were then transferred to a sterile 15-ml Corex tube and pelleted in a Sorvall refrigerated centrifuge at 10,000 rpm for 10 min. The supernatant was poured off and the cells were resuspended in cold (ice bath-temperature), 0.067 *M*, pH 7.0, phosphate buffer (K-K). The cells were pelleted two additional times at 4k, with resuspension being in the same buffer. Final cell density was about 5.0 × 10^8 cells ml^{-1}. One-milliliter aliquots of the cell suspension were distributed into 13 × 100-mm test tubes. To each tube either 10 μl of extract in methanol (experimental) or 10 μl of methanol (control) was added, following which the experimental tubes were exposed to UVA (4.8 kJ m^{-1}). The approximate tissue representation in each tube was 7.4 μg dry weight/ml of cells. Following exposure to UVA, the cells were diluted appropriately in 0.067 *M*, pH 7.0, phosphate buffer and three 100-μl samples were spread on three plates of supplemented minimal A medium to obtain colony counts. The plates were incubated at 37°C for 2 days. The mean colony counts from the experimental tubes were divided by the mean colony counts from the control tubes, giving the surviving fraction. The reciprocal of the surviving fraction was taken as a measure of the relative toxicity of the particular extract.

The broad-spectrum UVA source used for the bacterial experiments (Tuveson and Jonas, 1979) consisted of a bank of four lamps (GE40BLB, integral filter) with a range of emission from 300 to 425 nm with a maximum emission at 350 nm as in the study by Tuveson and Jonas (1979). The source was maintained in a cold room at 10°C to reduce heating effects. Sham UVA irradiation of foil-wrapped cell suspensions showed no inactivation (Tuveson and Jonas, 1979). The fluence rate was between 40 and 43 J sec^{-1} m^{-2}.

Statistical Analyses. Data on furanocoumarin concentrations and bacterial toxicity were analyzed as a three-way ANOVA in a completely crossed design with the SAS statistical package [Statistical Analysis System (SAS) Institute, 1982]. Bacterial toxicity was scored as the log-transformed reciprocal of the surviving fraction, and since all extracts could not be assayed simultaneously, we tested the extracts in five batches that were completely balanced with respect

to the independent variables. Thus, we used the assay batch as an additional blocking factor. Furanocoumarin ratios were compared by the distribution-free Kruskal–Wallis nonparametric one-way analysis of variance (Sokal and Rohlf, 1982). We estimated variability around mean furanocoumarin proportions as the back-transformed 95% confidence intervals in order to provide the most meaningful estimate of dispersion (Sokal and Rohlf, 1982). For simplicity, interaction terms were dropped from the model when the P value was much greater than 0.25. Because we anticipated a relationship between furanocoumarin levels in extracts and bacterial toxicity (Tuveson et al., 1986), we examined this relationship using linear regression. In addition, the effect of the independent variables, corrected for furanocoumarin concentration and tissue representation in the assay, was investigated using ANCOVA. Differences between individual means were examined with Bonferroni's test as needed [Statistical Analysis System (SAS) Institute, 1982].

RESULTS

Psoralen and bergapten levels varied with tissue type (Table 1); higher levels of furanocoumarins were present in young leaves, and leaves had higher amounts than stems. Also, we found that clonal origin accounted for a significant portion of the variation in bergapten content $F_{2,22} = 7.03$, $P = 0.0043$). Psoralen and bergapten concentrations were highly correlated ($r^2 = 0.83$, $P = 0.0001$) over all plant parts and treatments; UVB exposure resulted in a slight decrease in the degree of correlation between them ($r^2 = 0.89$, $P = 0.0001$, UV−; $r^2 = 0.82$, $P = 0.0001$, UV+).

While exposure to UVB had no significant effect on mean levels of bergapten or psoralen in aerial parts of *C. jambhiri*, UVB treatment increased the

TABLE 1. MEAN FURANOCOUMARIN LEVELS (SE) IN ABOVEGROUND TISSUES OF *C. jambhiri*[a]

Plant part	Furanocoumarin (μg/g dry weight)	
	Psoralen	Bergapten
Young leaves	541.6 (41.1) a	379.6 (20.5) a
Intermediate leaves	492.2 (32.2) a	362.1 (16.6) a
Old leaves	73.5 (10.9) b	158.6 (9.3) b
Stems	35.6 (5.4) b	44.5 (5.3) c

[a] Means within a column followed by the same letter are not significantly different at $P = 0.05$, Bonferroni's multiple-comparison test.

ratio of psoralen to bergapten (Table 2). This increase in relative psoralen concentration was apparent in all tissues. Also, the ratio showed significant variation among plant parts (Figure 1). In a pattern similar to that for the combined levels of psoralen and bergapten, the proportional representation of psoralen was higher in younger leaves and, overall, leaves had a higher relative concentration than stems.

Bacterial toxicity was highly correlated with the furanocoumarin content of extracts (Figure 2); samples with higher levels of bergapten and psoralen

TABLE 2. FURANOCOUMARIN CONTENT AND RATIO IN ABOVEGROUND BIOMASS OF *C. jambhiri*[a]

	UV+	UV−
Psoralen	313.7 (37.7)	271.4 (34.4)
Bergapten	232.5 (19.5)	246.4 (22.2)
Psoralen:bergapten	1.18	0.91[b]

[a] UV+ foliage had been exposed to 19.1 kJ day^{-1} UVB (BE_{300}; see text). Means (SE) are μg/g dry weight.
[b] $P = 0.04$, Kruskal–Wallis test.

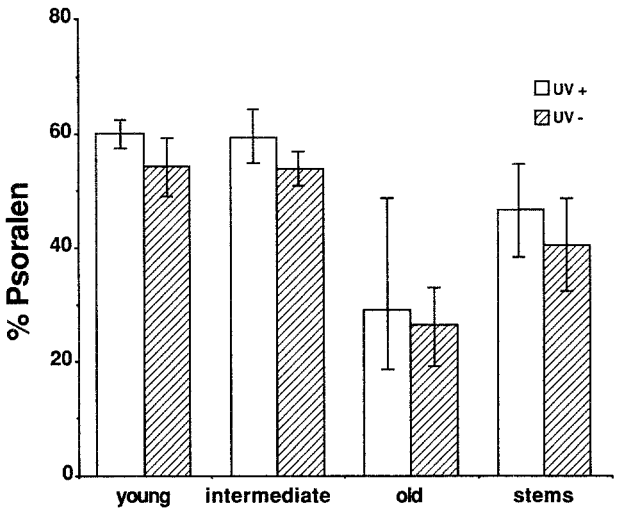

FIG. 1. Proportion of psoralen in different parts of the aboveground biomass of *C. jambhiri* exposed (UV+) and not exposed (UV−) to 19.1 kJ day^{-1} UVB (BE_{300}; see text). Error bars are back-transformed 95% confidence intervals from arcsin-transformed data.

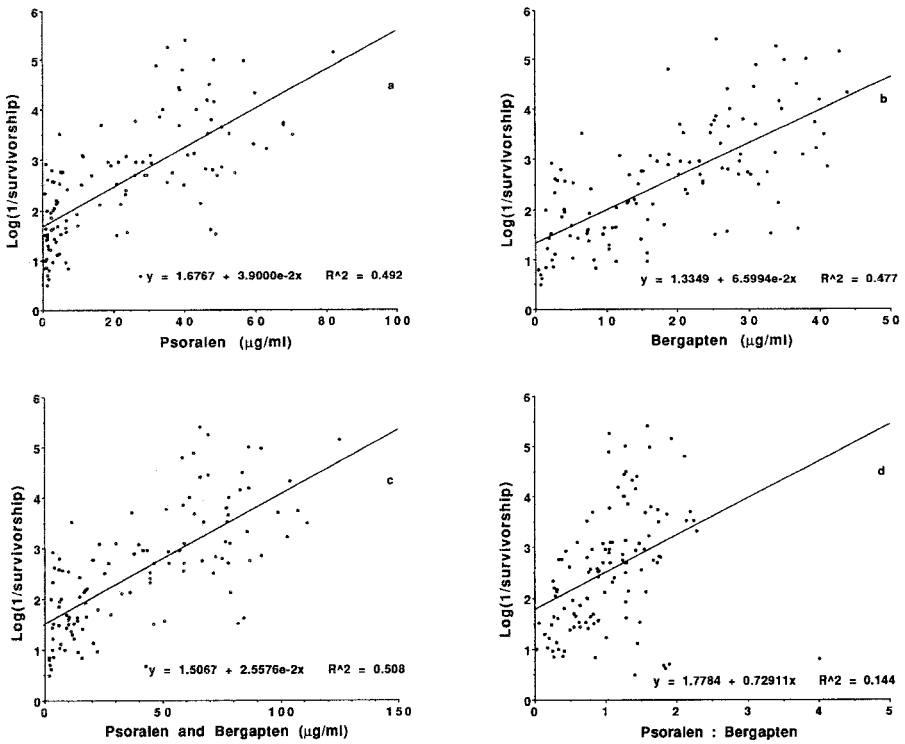

FIG. 2. Regressions of bacterial toxicity to RT7 cells on furanocoumarin contents and psoralen-to-bergapten ratios of extracts from *C. jambhiri*. All regressions are significant at $P < 0.01$.

were more toxic in our assays. Also, psoralen-to-bergapten ratios showed a highly significant correlation with toxicity; however, the amount of variation that could be accounted for by the correlation was low (Figure 2). UVB treatment had no significant effect on the toxicity of extracts, although the effect of plant part was highly significant (Table 3). Clonal variation in bergapten levels was not sufficient to elicit a clonal effect on bacterial toxicity.

We used ANCOVA to examine the effect of plant part corrected for furanocoumarin content. In this way, we sought evidence for other effects of plant part extracts on bacterial toxicity which could not already be explained by furanocoumarin variation among the extracts. Neither tissue, clonal origin, nor UVB irradiation could account for any additional variation in bacterial toxicity when corrected for furanocoumarin content (Table 4). Similar results were obtained when psoralen or bergapten concentrations alone were used as covariates. Further, a heterogeneity of slopes analysis showed that there was no

TABLE 3. ANALYSIS OF VARIANCE[a]

Source	df	SS	*F*	*P*
UV	1	0.0208	0.03	0.8536
Clone	2	0.6109	0.5	0.6059
Tissue	3	69.7164	38.33	0.0001
Batch	4	26.1462	10.78	0.0001
UV * Clone	2	1.6852	1.39	0.2544
Clone * Tissue	6	5.7584	1.58	0.1612
Error	90	161.4552	—	—

[a] Effects of UVB treatment (19.1 kJ day^{-1}, BE_{300}; see text), clonal origin, and plant tissue on toxicity of *C. jambhiri* extracts to RT7 cells. Assays were blocked by batches.

TABLE 4. ANALYSIS OF COVARIANCE[a]

Source	df	SS	*F*	*P*
Furanocoumarins	1	6.304	11.64	0.001
Tissue	3	2.538	1.56	0.202
Clone	2	0.127	0.12	0.889
UVB	1	0.069	0.13	0.721
Error	88	47.61	—	—

[a] Effects of plant tissue, clone, and UVB treatment on toxicity of *C. jambhiri* extracts to RT7 cells. Furanocoumarin content (psoralen and bergapten) is the covariate.

difference in the relationship between furanocoumarin content and bacterial toxicity in different tissues.

DISCUSSION

Our analysis of furanocoumarin variation in aerial parts of *C. jambhiri* revealed several patterns consistent with those reported in other species. Furanocoumarin concentrations are highest in young foliage and lower in older leaves and stems. Similar patterns have been reported in several umbellifers and rutaceaous species (Berenbaum, 1981; Zobel and Brown, 1989, 1991). Variation in mean furanocoumarin levels of leaves amounted to a nearly fourfold difference in total foliar furanocoumarins. The difference between leaves and stems was even greater (up to 11.5 fold). Moreover, the relative concentration of psoralen was higher in younger leaves. Zobel and Brown (1991) termed bergapten a "senescence compound" based on its higher relative concentrations in older

leaves and callus tissue from *Ruta graveolens*. Here, our results show a parallel trend; the proportion of psoralen is highest in young leaves and bergapten proportions are highest in old leaves. The age-dependent change in relative representation of psoralen is due to a more rapid decline in psoralen concentration than bergapten (Table 2) (see Zobel and Brown, 1991). Psoralen precedes bergapten in furanocoumarin biosynthesis (Murray et al., 1982). Our results suggest that, as leaves age, furanocoumarins are degraded at a greater rate and that psoralen is more rapidly converted to bergapten. This scenario remains speculative and depends on the extent to which turnover occurs. Alternatively, Mihaliak et al. (1991) have recently demonstrated lack of monoterpene turnover when assays were performed on whole plants. So if furanocoumarin turnover in *C. jambhiri* is low or constant, then furanocoumarins accumulated after an initial burst of synthesis early in leaf ontogeny might then be continuously degraded at a constant rate. Psoralen would be converted to bergapten, and bergapten to nonfuranocoumarin degradation products.

Regardless of the mechanism, there are several possible implications of furanocoumarin distribution among tissues for herbivores and pathogens of *Citrus* spp. Young foliage of *Citrus* spp. is less tough and has a higher water content than older leaves (McCloud, unpublished data). Because preference for younger foliage is a hallmark of many leaf-chewing herbivores of woody perennials (Scriber and Slansky, 1981; Slansky and Rodriguez, 1987), our results suggest that *Citrus*-feeding herbivores may have to consume larger quantities of furanocoumarins in order to feed on young, nutrient-rich foliage. Similarly, leaf pathogens on citrus may face a tradeoff between penetration of more sclerophyllous older leaves and increased phototoxicity in tender young leaves. Consumers of young citrus foliage, then, not only will ingest higher levels of phototoxins, but also will experience a higher light regime on these young, terminal leaves.

Foliar bergapten levels vary significantly between *C. jambhiri* clones. That we were able to detect clonal variability in furanocoumarin accumulation using only three different clones suggests that such variability may be quite high in this species.

Analysis of covariance suggests that phototoxicity in leaves of *C. jambhiri* can be accounted for on the basis of furanocoumarin content alone. While phototoxicity in our bacterial assay varied nearly 44-fold with the type of tissue extract, tissue type had no significant influence on phototoxicity over and above furanocoumarin content.

Levels of UVB reaching the earth's surface are expected to increase in the coming decades as a result of continuing stratospheric ozone depletion (Bowman, 1988; Blumthaler and Ambach, 1990). Our results suggest that, given this predicted change in incident UVB, phototoxin-mediated effects on *Citrus* enemies will depend on the genetic identity of the variety in question, the species,

and the tissue-specific furanocoumarin content as well as the amount of UVB enhancement. In our study, we failed to document effects of UVB enhancement on the phototoxicity of leaf and stem extracts, although we found changes in furanocoumarin concentration. *Citrus* species are native to subtropical habitats and several authors have presented evidence that plant populations native to low latitudes are more resistant to UVB (Robberecht et al., 1980; Caldwell et al., 1982; Barnes et al., 1987). Inasmuch as increased accumulation of phenylpropanoids constitutes a stress response, lack of increase in mean furanocoumarin concentrations suggests that our dose (equivalent to 39% O_3 reduction at 28°N, latitude) did not stress the plants. DeLucia et al. (1991) have shown that penetration of UVB into the leaves of woody perennials is limited; thus, damaging wavelengths may not reach their targets in *Citrus*. However, the change in the ratio of psoralen to bergapten observed indicates that UVB radiation did affect furanocoumarin metabolism in this plant despite its possibly limited penetration. While we designed our *E. coli* assay system to be a sensitive model of possible effects on herbivore and pathogen interactions with *C. jambhiri*, we sought only phototoxin-mediated effects. Other changes induced by exposure to UVB remain untested, as do effects of proportionally altered furanocoumarin accumulation. Our results, then, warrant future work, in which the consequences of increased UVB exposure for herbivores and pathogens are directly addressed.

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POSSIBLE FUNCTION OF ECDYSTEROID-22-*O*-ACYLTRANSFERASE IN THE LARVAL GUT OF TOBACCO BUDWORM, *Heliothis virescens*

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Abstract—In the larval gut of the tobacco budworm, *Heliothis virescens*, most ingested ecdysteroids are transformed to ecdysteroid-22-acyl esters by a novel enzyme, ecdysteroid-22-*O*-acyltransferase. This conversion is different from the normal metabolic pathway in which the compounds are usually transformed to more water-soluble products. Most ecdysteroid-22-*O*-acyltransferase activity was found in the midgut epithelial-cell membrane and was active only during feeding stages. The activity decreased as the larvae became committed to pupation, and it was not enhanced by feeding ecdysteroids to the fifth-instar larvae. Our preliminary results showed that the esterification was very specific to 22-OH in ecdysteroids. We suggest that detoxification is the major function of ecdysteroid-22-*O*-acyltransferase.

Key Words—Detoxification, ecdysteroid, metabolism, brush border membrane, epithelial cell.

INTRODUCTION

Ecdysteroid metabolic studies, most of which were made through the injection of ^{3}H-ecdysteroids, have shown that hydroxylation and conjugation were the major metabolic pathways by which ecdysteroids are converted to more polar products (Koolman, 1982; Lafont and Koolman, 1984), although apolar metabolites, such as ecdysteroid-22-acylesters, which might serve as storage forms, are found in ovaries and eggs of some insects (Connat, and Diehl, 1986; Hof-

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mann, 1986; Slinger et al., 1986; Slinger and Isaac, 1988a,b; Whiting and Dinan, 1988a,b, 1989). Ecdysteroids fed to insects such as *Heliothis virescens* (Kubo et al., 1987) and *H. armigera* (Robinson et al., 1987), as well as to other arthropods (Connat et al., 1988a,b), however, are metabolized in a different way. They are converted to apolar products, ecdysteroid-22-acyl esters, which were excreted with the feces. Unlike the production of ecdysteroid-22-acyl ester in the insect ovaries, the conversion of the ecdysteroids to their 22-acyl esters might be a pathway of detoxification of ingested noxious compounds (Kubo et al., 1987; Connat et al., 1988a,b; Robinson et al., 1987).

One of the enzymes involved in this ecdysteroid esterification is a novel enzyme, ecdysteroid-22-*O*-acyltransferase, which is being characterized in our lab. Because most of the ingested ecdysteroid was transferred to its ester and excreted into the feces, we suggest that the major function of the ecdysteroid-22-*O*-acyltransferase is to detoxify the foreign compounds ingested with the food.

There are some common characteristics of the detoxifying enzymes. First, they are located in the tissues where toxins are first encountered or most abundant, e.g., in the fat body and gut tissues. Second, most of the detoxifying enzyme activity can be observed during the feeding stage because most of the toxins are obtained through feeding (Ahmad et al., 1986). Third, the detoxifying enzyme can be induced by the introduction of the toxins (Yu, 1986). In this paper we demonstrate that ecdysteroid-22-*O*-acyltransferase shares most of these characteristics.

METHODS AND MATERIALS

Insects. H. virescens larvae were reared on artificial diet (Chan et al., 1978) under the conditions described by Kubo et al., 1987. Fifth-instar larvae were used for the experiment.

*Location of Ecdysteroid-22-*O*-Acyltransferase.* Fifth-instar larvae at the digging phase (Webb and Dahlman, 1985) were dissected at 4°C in Tris buffer containing 50 m*M* Tris–HCl, pH 7.4, 50 m*M* 2-mercaptoethanol, and 1 m*M* EDTA. Different tissues were collected and homogenized in Tris buffer with a Duall Tissue Grinder. Protein content in the homogenate was measured using the method described by Bradford (1976).

In ecdysteroid-22-*O*-acyltransferase assay, 0.5 ml of mixture containing homogenate (containing 0.1 mg of protein), 10 m*M* phosphate buffer, pH 7.0, 0.06 m*M* oleoyl-CoA, and 4.2×10^{-7} m*M* ^{3}H-ecdysone (47.3 Ci/mmol; NEN Wilmington, DE) was incubated at 20°C for 20 min. The reaction was stopped by adding 200 μl of 1 *N* HCl. Two hundred microliters of 1 *N* KOH was then

added to neutralize the acid condition. Two milliliters of *n*-hexane–ethyl acetate (3 : 1) was added to the mixture and mixed on a vortexer 10 times (2 sec each time) to extract ecdysone-22-oleate. Using authentic ecdysone-22-oleate synthesized by the method of Dinan (1988), we have found that the efficiency of hexane–ethyl acetate extraction was over 99%. An aliquot of extract was added to 4 ml of Ready Safe liquid scintillation cocktail (Beckman, Fullerton, CA), and the radioactivity was measured in LS 5000 TA liquid scintillation counter (Beckman), One unit of enzyme activity (IU/mg) is defined as the formation of 1 μmol of ecdysone-*O*-oleate/min/mg of protein at 20°C. Controls were treated identically but lacked oleoyl-CoA in the assay mixture to determine the radioactivity background in the product extract.

In the gut tissues alkaline phosphatase has been reported to localize in the epithelial-cell membrane and concentrate in the brush border region, which will remain in the supernatant when centrifuged at 3000*g* and will precipitate when further centrifuged at 30,000*g* (Wolfersberger et al., 1986). We used the alkaline phosphatase as a marker for the region in the epithelial-cell centrifugal fractions, which were obtained as follow: the gut homogenates were centrifuged at 3000*g* for 15 min to give precipitate (3000*g* pp) and supernatant (3000*g* sp) which were further centrifuged at 30,000*g* for 30 min to give precipitate (30,000*g* pp) and supernatant (30,000*g* sp). Ecdysteroid-22-*O*-acyltransferase activities in these fractions were compared with the alkaline phosphatase activities, which were measured as described by Ferreira and Terra (1982).

*Ecdysteroid-22-*O*-Acyltransferase Activity in the Fifth-Instar Larval Stadium.* The developmental phases of fifth-instar larvae of *H. virescens* were identified as described by Webb and Dahlman (1985). The gut tissues from these larvae were dissected and the ecdysteroid-22-*O*-acyltransferase activities were measured as described above.

*Induction of 20-Hydroxyecdysone to Ecdysteroid-22-*O*-Acyltransferase.* First-day fifth-instar larvae were fed diet containing 0, 50, 100, 200, 400, or 800 ppm of 20-hydroxyecdysone for 2 days and the effect of the ingested 20-hydroxyecdysone on the change of ecdysteroid-22-*O*-acyltransferase activity was determined by measuring the enzyme activity (IU/mg) in the 30,000*g* centrifuged precipitate of the epithelial homogenate which was obtained from the treated larvae.

*Competition of Different Ecdysteroids as Substrates for the Ecdysteroid-22-*O*-Acyltransferase.* Each 0.5 ml of enzyme assay mixture contained 10 m*M* phosphate buffer, pH 7.0, 0.06 m*M* oleoyl-CoA, 100 μg epithelial-cell homogenate, and 10–100 μ*M* ecdysone (2×10^{-4}–2×10^{-2} μCi/ml), plus 0.5 m*M* 20-hydroxyecdysone or 0.5 m*M* 20-hydroxyecdysone-22-acetate as the competitors.

In another assay, the normal assay mixture (see assay for location of the

enzyme) was added with 0.02–0.5 μM 20-hydroxyecdysone, 20-hydroxyecdysone-2-acetate, and 20-hydroxyecdysone-22-acetate, respectively, to compete with ecdysone as the substrate.

Ecdysone-22-oleate formed after 20 min of incubation at 20°C in the presence or absence of competitors was extracted with *n*-hexane : ethyl acetate (3 : 1) and quantified by measuring the radioactivity.

HPLC Analysis. To confirm the major product formed in the enzyme assay, we analyzed the extract in HPLC. An aliquot of hexane–ethyl acetate extract was pooled, evaporated to dryness, and redissolved with methanol. HPLC (Eyela PLC-5 with a UV spectrometer set at 254 nm; Tokyo Rikakikai Co., Ltd., Tokyo) analyses were performed in a Zorbax ODS column (4.6 mm × 25 cm; Dupont) with 93% methanol eluted at 1 ml/min. Fractions (1 ml each) were added to 4 ml of Ready Safe liquid scintillation cocktail and the radioactivity was measured. Authentic ecdysone-22-oleate used for cochromatography with enzyme product extracts in HPLC analysis was synthesized by the method of Dinan (1988).

RESULTS

*Identification of Ecdysteroid-22-*O*-Acyltransferase Product.* The major product formed in the enzyme assay was found to cochromatograph with the synthetic ecdysone-22-oleate in the HPLC analysis (Figure 1). The major background of radioactivity in the product extract was due to the partition of some ^{3}H-ecdysone into the organic phase.

*Location of the Ecdysteroid-22-*O*-Acyltransferase.* Most of the ecdysteroid-22-*O*-acyltransferase activity was observed in the gut tissue. However, we could not detected this enzyme activity in hemolymph, fat body, or Malpighian tubules. When the gut tissues were further analyzed, the ecdysteroid-22-*O*-acyltransferase activity was found to concentrate in the anterior part of the midgut (Figure 2).

When we compared the ecdysteroid-22-*O*-acyltransferase activity and alkaline phosphatase activity in different epithelial fractions, we found that the distribution of these two enzymes was very similar (Figure 3), suggesting that ecdysteroid-22-*O*-acyltransferase localized in the epithelial cell membrane and possibly concentrated in the brush border membrane.

*Age Distribution of the Ecdysteroid-22-*O*-Acyltransferase.* Ecdysteroid-22-*O*-acyltransferase activity was found to be very high in the fifth-instar larvae feeding stage (Figure 4). The activity decreased as the larvae stopped feeding and entered the stage committed to pupation.

*Induction of the Ecdysteroid-22-*O*-Acyltransferase.* No induction of ecdysteroid-22-*O*-acyltransferase activity was observed in fifth-instar larvae of *H. virescens* fed diets containing 20-hydroxyecdysone of different concentrations

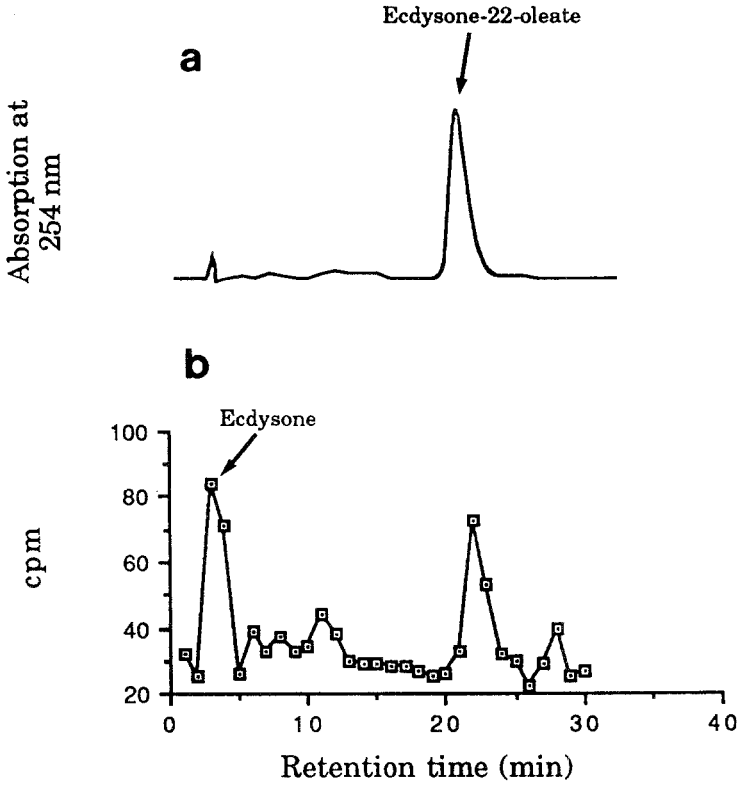

FIG. 1. HPLC analysis of ecdysteroid-22-*O*-acyltransferase product. (a) Synthetic ecdysone-22-oleate; (b) extract of the enzyme product.

(Figure 5). The enzyme activity was already high even without the introduction of 20-hydroxyecdysone.

Substrate Competition. When the ecdysteroid-22-*O*-acyltransferase assay mixture contained 20-hydroxyecdysone, ecdysone-22-oleate formation was inhibited (Figure 6), and the amount of ecdysone-22-oleate formed decreased as the concentration of 20-hydroxyecdysone or 20-hydroxyecdysone-2-acetate increased (Figure 7). But 20-hydroxyecdysone-22-acetate had no effect on ecdysone-22-oleate formation (Figures 6 and 7).

DISCUSSION

Our studies have shown that the majority of the ecdysteroid-22-*O*-acyltransferase activity was in the anterior part of the midgut. The ecdysteroid-22-*O*-acyltransferase activity detected in the foregut was probably due to the presence of some midgut tissue in the foregut homogenate.

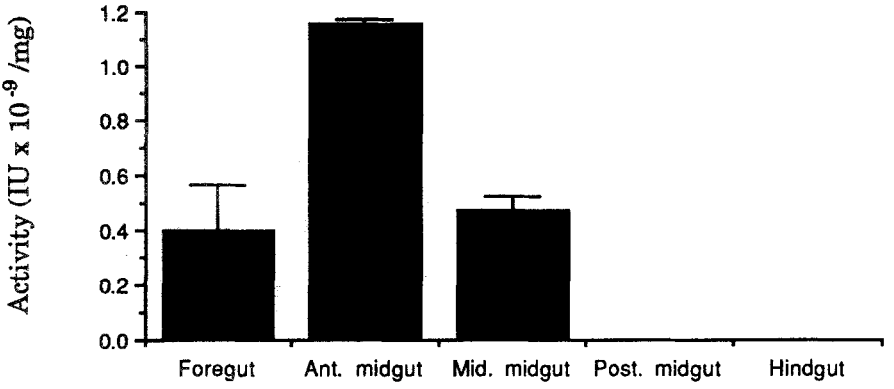

FIG. 2. Ecdysteroid-22-*O*-acyltransferase activities in different parts of gut tissues: anterior (ant.) midgut, middle (mid.) midgut, and posterior (post.) midgut. Each result is the mean of three replicates ± SE.

The codistribution of the ecdysteroid-22-*O*-acyltransferase and alkaline phosphatase activity in different epithelial centrifuge fractions suggested that ecdysteroid-22-*O*-acyltransferase localizes in the epithelial plasma membrane and possibly concentrates in the brush border membrane, which serves as a first barrier to the ingested noxious compounds.

Many noxious chemicals are commonly found in natural diets, and high detoxifying enzyme activities in the insect feeding stage are required in many cases. This may also be true for the ecdysteroid-22-*O*-acyltransferase activity, which is also very high during the feeding stage, to metabolize the noxious compounds.

However, the naturally occurring substrate for this enzyme has not been determined because it is still questionable whether ecdysteroids occur in the host of *H. virescens*. Besides, ecdysteroid-22-*O*-acyltransferase activity was very high even without the introduction of 20-hydroxyecdysone, and the activity did not increase after the introduction of 20-hydroxyecdysone (Figure 5), indicating that there may be other compounds that had already existed in the diet and that could induce this enzyme activity.

Substrate competition studies can give us some structural characteristics of the possible natural substrates of the ecdysteroid-22-*O*-acyltransferase. Our studies have shown that 20-hydroxyecdysone and its acetyl esters which contain unesterified 22-OH can compete with ecdysone as the substrate for ecdysteroid-22-*O*-acyltransferase and that, when 22-OH was blocked by forming an acetyl

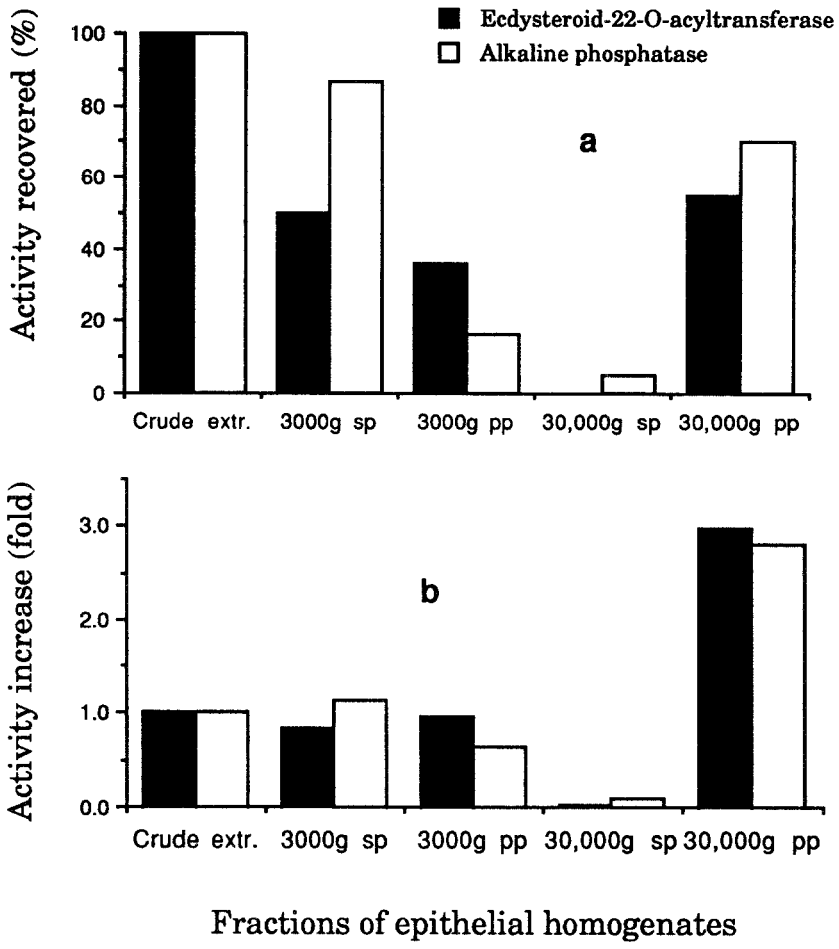

FIG. 3. Ecdysteroid-22-*O*-acyltransferase activity recovered (a) and activity increase (b) in different *H. virescens* larval midgut epithelial homogenate centrifugal fractions. Crude extr., crude gut homogenate; 3000*g* sp, 3000*g* supernatant; 3000*g* pp, 3000*g* precipitate; 30,000*g* sp, 30,000*g* supernatant; 30,000*g* pp, 30,000*g* precipitate. Each result is the mean of three replicates.

ester, leaving the other hydroxyl groups intact, the ecdysteroids cannot compete with ecdysone in the enzyme assay, suggesting that this enzymatic esterification was restricted to the 22-OH in ecdysteroids and that the potential substrates occurring in nature should have similar structural characters.

Based on the fact that ecdysteroid-22-*O*-acyltransferase shares some common characteristics with other detoxifying enzymes with regard to location and

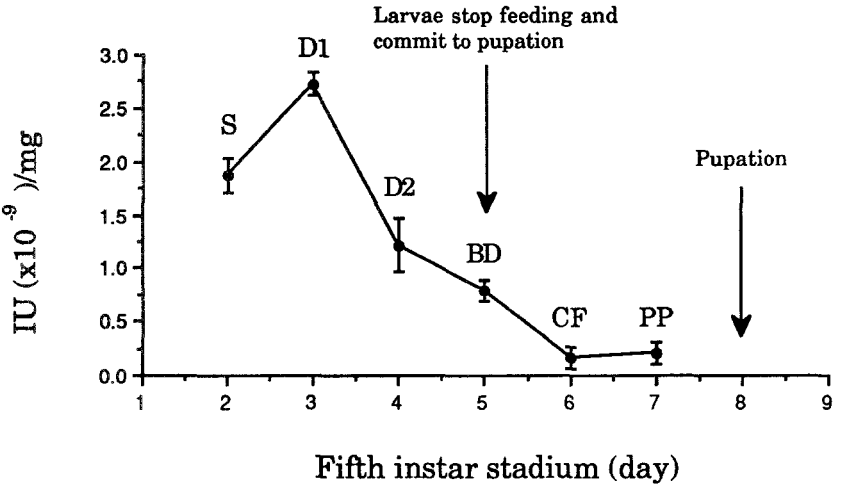

FIG. 4. Ecdysteroid-22-*O*-acyltransferase activity in different phases of fifth-instar larvae of *H. virescens*. S, slender phase; D1 and D2, first and second days of the digging phase; BD, burrow-digging phase; CF, cell formation phase; PP, pharate pupa. Each point is the mean of three replicates ± SE.

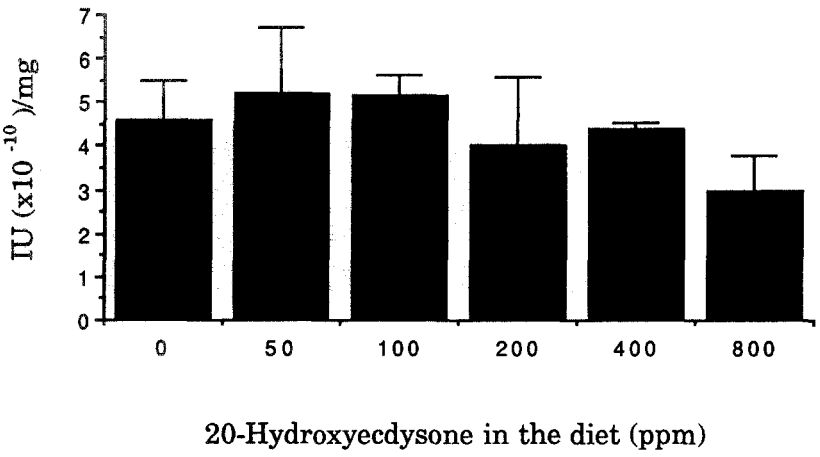

FIG. 5. Induction of ingested 20-hydroxyecdysone on ecdysteroid-22-*O*-acyltransferase activity. Each result is the mean of three replicates ± SE.

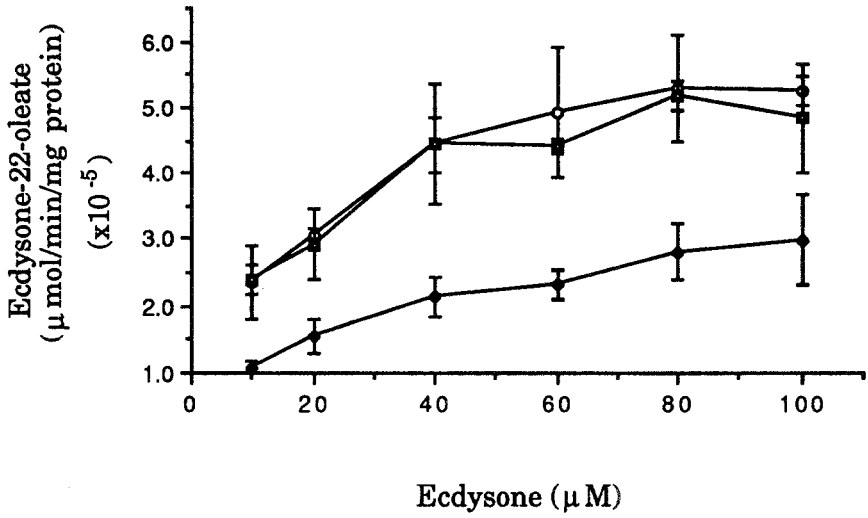

FIG. 6. Rate of ecdysone-22-oleate formation in the ecdysteroid-22-*O*-acyltransferase assay with the absence of competitor (open circles) and with the presence of 0.5 m*M* 20-hydroxyecdysone (filled circles) and 0.5 m*M* 20-hydroxyecdysone-22-acetate (filled squares) as the competitors. Each point is the mean of three replicates ± SE.

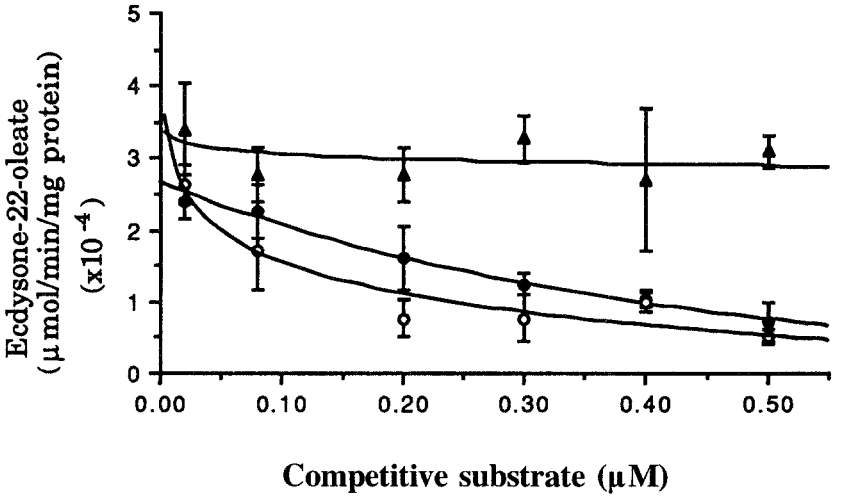

FIG. 7. Effects of the competitors on ecdysone-22-oleate formation in the ecdysteroid-22-*O*-acyltransferase assay. Filled triangles, 20-hydroxyecdysone-22-acetate; filled circles, 20-hydroxyecdysone; open circles, 20-hydroxyecdysone-2-acetate. Each point is the mean of three replicates ± SE.

age distribution, we suggest that the major function of ecdysteroid-22-*O*-acyltransferase is to detoxify the noxious compounds ingested with the food. As the result of this enzyme reaction, the noxious compounds would be deactivated and excreted with feces. We have shown that ecdysteroid-22-acyl esters were over 100 times less active than 20-hydroxyecdysone in inducing the evagination of *Drosophila* imaginal disks (unpublished). Some of the deactivated products might be reabsorbed in the gut, but because of their high lipophilicity they would be restricted to the fat bodies and kept from reaching the target tissues. More studies are required to determine the spectra of naturally occurring substrates for this enzyme.

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INFLUENCE OF THE PLANT ANTIFEEDANT, PINOSYLVIN, ON SUPPRESSION OF FEEDING BY SNOWSHOE HARES

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Abstract—This study investigated the influence of the plant antifeedants, pinosylvin and pinosylvin methyl ether (PME), on suppression of feeding by snowshoe hares (*Lepus americanus*). Screening bioassays showed clearly that pinosylvin suppressed feeding by hares when sprayed directly on coniferous tree seedlings. Pinosylvin odor, when associated with food (but not mixed with it), also significantly reduced consumption of rabbit chow by hares. Large pen bioassays indicated that both pinosylvin and PME significantly reduced feeding on tree seedlings by hares when the antifeedants were sprayed directly on trees. In field bioassays near Prince George, British Columbia, Canada, pinosylvin sprayed on trees and encapsulated in controlled-release devices of PVC plastic, with an internal wire as a twist-tie for attachment to tree, significantly reduced feeding by hares. Thus, pinosylvin will generate an "avoidance response" in terms of feeding by snowshoe hares. This response is likely triggered by an olfactory pathway based on positive results with controlled-release devices attached to seedlings. Our study reports the first practical utilization of plant antifeedants for forest crop protection and wildlife management.

Key Words—Plant antifeedants, pinosylvin, pinosylvin methyl ether,

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snowshoe hare, *Lepus americanus*, herbivore, lodgepole pine, forest plantation, release devices, feeding suppression, crop protection.

INTRODUCTION

The snowshoe hare (*Lepus americanus*) is widely distributed across the boreal and subboreal forests of North America. This rabbit population fluctuates dramatically in abundance, with peaks occurring every 9–10 years (Keith, 1963). Hares prefer habitats dominated by early and midsuccessional deciduous and coniferous trees and shrubs which provide both food and cover (Conroy et al., 1979; Wolff, 1980; Pietz and Tester, 1983; Sullivan and Sullivan, 1983).

During peak years of the cycle, intense browsing may severely damage this natural food supply (Fox, 1978; Pease et al., 1979; Wolff, 1980). A consequence of this damage is the production of adventitious shoots which are very unpalatable to snowshoe hares (Bryant and Kuropat, 1980). These adventitious shoots of several common boreal forest trees contain significantly higher concentrations of terpene and phenolic resins than the mature growth form twigs of the same species (Bryant, 1981). In general, these resins are repellent to snowshoe hares and likely explain the avoidance of adventitious shoots by hares (Bryant, 1981).

One such boreal tree species associated with snowshoe hares is Alaskan green alder (*Alnus crispa*). Hares will feed on internodes but avoid juvenile growth forms of this species. This feeding preference is governed by concentrations of two deterrent secondary metabolites, pinosylvin and pinosylvin methyl ether; laboratory bioassays have confirmed the repellency of these two compounds to hares (Bryant et al., 1983; Clausen et al., 1986).

Feeding damage to coniferous plantations by the snowshoe hare (*Lepus americanus*) is a serious problem in many areas of forest regeneration in boreal and subboreal forests of North America (Radvanyi, 1987; Bergeron and Tardiff, 1988). Hares damage seedlings by clipping terminal and lateral shoots and by girdling stems. The most severe damage occurs in the 2–3 years during each peak in the 10-year cycle (Sullivan and Sullivan, 1982, 1988).

Repellents have been advocated as a potential means to reduce this damage (Sullivan and Crump, 1984; Radvanyi 1987). Some success has been achieved with predator odor repellents at the field level (Sullivan and Crump, 1984, 1986), but there have been no investigations of plant antifeedants as repellents for tree seedlings and snowshoe hares.

This paper reports on the influence of two plant antifeedants, pinosylvin and pinosylvin methyl ether, in suppressing snowshoe hare feeding on coniferous tree seedlings. The study was designed to test the hypothesis that these phenolic resins, when associated with tree seedlings, will elicit an "avoidance response" in terms of feeding by snowshoe hares.

METHODS AND MATERIALS

Synthesis of Compounds. Pinosylvin (3,4-dihydroxystilbene) **(5)** (Figure 1) was prepared in the following manner: the bistetrahydropyranyl ether of ethyl 3,5-dihydroxy benzoate **(1)** was reduced with lithium aluminium hydride in diethyl ether to the benyl alcohol **(2),** which was subsequently oxidized with pyridinium chlorochromate in dichloromethane to the benzaldehyde derivative **(3).** ^{1}H NMR ($CDCl_3$, 60 MHz): 3.2–3.9 (2H, bm, H_1'', and H^1), 7.0 (H, bs, H_4), 7.3 (2H, bs, H_2, H_6), 9.9 (1H, S, CHO). Benzyl bromide (11 ml) and triethylphosphite (29 ml) were heated under reflux for 2 hr and then excess reagents were distilled out (70°C water pump vacuum). The crude diethylbenzyl phosphonate was added to sodium hydride (3.4 g) in dimethyl formamide at 0°C, followed by the benzaldehyde derivative **(3)** (21.8 g). The mixture was stirred for 1 hr at 15°C, heated at 100°C for 1 hr, and then stirred at 15°C overnight. Workup and purification by silica gel chromatography gave the protected stilbene **(4),** 17.6 g. Deprotection was effected by heating the crude product in aqueous methanoic oxalic acid on a steam bath for 2 hr. Neutralization with $NaHCO_3$ followed by ether extraction gave pinosylvin, 9.4 g; mp 55–57°C (lit. 55–56°C). ^{1}H NMR ($CDCl_3$ 60 MHz): 6.45 (1, m, H_4), 6.90 (2, m, H_2, H_6), 7.10 (2, s, alkene), 7.10–7.50 (5, m, phenyl).

Pinosylvin methyl ether (3-hydroxy-5-methoxystilbene) **(6)** was similarly prepared from ethyl 3-hydroxy-5-methoxybenzoate. ^{1}H NMR ($CDCl_3$, 90 MHz): 3.78 (3, s, OCH_3), 6.35 (1, m, H_4), 6.40 (2, m, H_2, H_6), 6.67 (2, s, alkene), 7.24–7.44 (5, m, phenyl); M^+ 226. Purities of pinosylvin and PME were greater than 95%.

The vanillin PME analogue, 4-hydroxy-3-methoxystilbene **(7),** was similarly prepared from vanillin (4-hydroxy-3-methoxybenzaldehyde). ^{1}H NMR ($CDCl_3$, 60 MHz): 3.8 (3, s, OCH_3), 7.0 (2, bs, alkene), 6.8–7.6 (8, bm, aromatic); M^+ 226.

Compounds and Preparations. With respect to compounds and preparations, pinosylvin was tested as a 20% solution in methanol at 50 mg/tree. Seedlings were dipped in the solution and allowed to dry prior to the start of a given trial. As a prelude to the testing of controlled-release devices, a 5-cm-wide cloth band was impregnated with pinosylvin and attached around a 500-ml plastic bowl containing commercial rabbit chow. This compound was also tested in a clay pellet (activated alumina, Catalog No. H026-00-050, Edwards High Vacuum, Oakville, Ontario, Canada) which acted as a controlled-release device, in Wheaton vials, and on paper sohxlet extraction thimbles. Four or five pellets (or an extraction thimble) were attached to each seedling in a collar of Scotch tape. Untreated pellets (or thimbles) were attached to control trees. A final screening bioassay assessed the influence of a pinosylvin methyl ether **(7)** analogue on feeding. Feeding by hares on seedlings or chow (amount eaten)

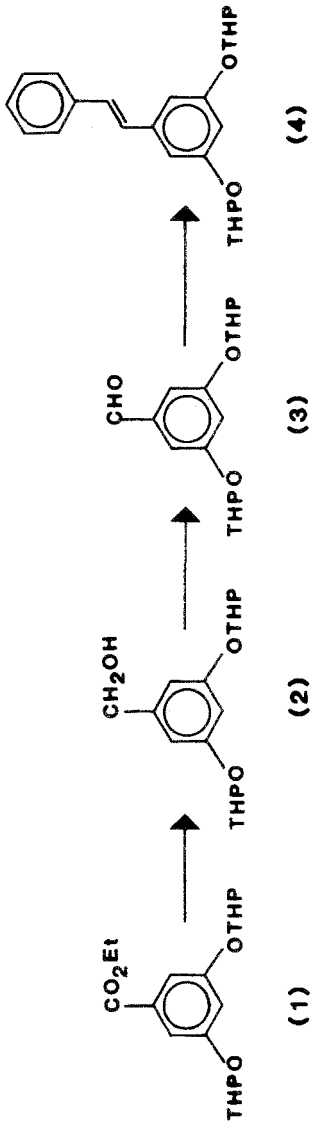

FIG. 1. Structural formulas of compounds.

was recorded daily in a given trial. Three bioassays were conducted with pinosylvin directly on trees, and two bioassays were done for each of the clay pellets, Wheaton vials, and thimbles.

Screening Bioassays. Bioassays were conducted at the Applied Mammal Research Institute, Langley, B.C., Canada. The bioassay enclosure (9.15 × 18.3 m) was composed of three sections or pens set in a natural environment (see Sullivan and Crump, 1984). The entire enclosure was covered by a fiberglass roof to eliminate effects of adverse weather. The control and experimental pens (A and B) were enclosed by polyethylene on the walls to keep odors within the respective treatment section. These pens had all natural vegetation cleared prior to start of trials. Douglas fir (*Pseudotsuga menziesii*) bark mulch covered the ground of each pen to a depth of 20 cm. Each pen had three experimental units, each composed of 10 Styrofoam blocks to anchor coniferous seedlings. One-year-old (nursery stock) lodgepole pine seedlings were used in all trials with one seedling per block. Thus, there was a maximum of 30 seedlings in each of the control and treatment pens (A or B) at the start of a given trial.

The natural habitat (pen C) was not cleared of native vegetation and provided cover and some grasses and forbs for hares. Thus, this pen served as a potential refuge area for hares. Access of hares to pens A or B from pen C was through 30 × 30-cm openings in each adjoining wall. Hares moved freely among all three pens and new animals were always allowed at least 5 to 7 days to acclimate to this experimental configuration. To exert adequate feeding pressure on the experimental material and keep social interactions at a minimum, one or two hares were used in each trial. All hares used were from our northern study areas at Prince George, B.C. Hares were replaced at regular intervals to overcome potential variability and habituation among animals. Rabbit pellets, grass hay, and water were available ad libitum throughout these trials.

Large Pen Bioassays. Two trials were conducted in a 4.5-ha enclosure at the Pineridge Forest Nursery, 130 km northeast of Edmonton, Alberta. The pen was composed of 2-m-high steel mesh and was buried in the ground to a depth of 30 cm. This study area was located in the boreal mixed wood zone (Strong and Leggat, 1981), with jack pine (*Pinus banksiana*) and aspen (*Populus tremuloides*) being the main tree species. Wild rose (*Rosa* spp.) and saskatoon berry (*Amelanchier alnifolia*) were the dominant shrub species. Within this area, four blocks (30 m × 30 m) were established and separated by at least 100 m. In July 1984, each block was cleared of trees and scarified in a manner similar to a typical logging and site preparation prescription. After planting, these blocks acted as plantations of lodgepole pine (*P. contorta*) seedlings in trials. Lodgepole pine is preferred by snowshoe hares over all other coniferous species (see Sullivan et al., 1985). Each block had 100 (10 × 10) seedlings for a given trial. The enclosure was stocked with snowshoe hares captured locally and from Prince George. This stocking allowed us to approach a peak population [4–5

hares/ha (Keith and Windberg, 1978)] of hares, thereby providing a rigorous test of various predator odor compounds in a simulated "field" situation. Both spring and fall represent periods when coniferous seedlings are particularly vulnerable to snowshoe hare feeding attacks. Snow cover is not adequate to protect seedlings and alternative summer herbaceous foods are not available.

In the first trial, pinosylvin and pinosylvin methyl ether (PME) were each made into 20% solutions with methyl alcohol. Fifty seedlings were dipped in the pinosylvin solution and another 50 seedlings were dipped in the PME solution. The 100 seedlings were then planted in a random manner in the experimental block. Methanol was applied to 50 trees in a control block which also had a total of 100 seedlings. The trial commenced on October 9, 1985, with browsing of seedlings checked on October 18 and 29 and November 7 (snow had essentially covered the seedlings by this time). Feeding (clipping) on the terminal was considered as mortality to a given seedling. All trees were completely covered with snow by mid-November and remained so until the spring assessment on May 2, 1986.

In the second trial, a solution of pinosylvin (5 g/37.7 ml methanol) was sprayed/dripped by syringe onto 50 (5 × 10) planted trees. A second set of 50 trees was treated with a similar solution of PME (5 g/37.7 ml methanol). The pinosylvin-treated trees were planted in one-half of the experimental block and the PME-treated trees in the other half. A second block acted as a control and had methanol only applied to 50 (5 × 10) of 100 trees. The trial began on May 2, 1986, and hare browsing of seedlings was checked on May 9, 22, and 28 and June 18.

A common observation in the pen trials at Pineridge Nursery was the apparent preference by hares to feed on seedlings adjacent to the forest edge within each block. Therefore, separate analyses have been conducted which exclude one row of perimeter trees from each block in the overwinter and spring trials. These adjusted results are compared with the standard results for each trial.

Field Bioassays. Two trials were conducted in central British Columbia during a snowshoe hare peak in population in 1988–1990. In the first trial, a plantation was located both at Shelley, 30 km northeast of Prince George, and near the Salmon River, approximately 60 km northwest of Prince George. Both sites were planted in the spring of 1988, interior spruce (*Picea glauca* × *Picea engelmannii*) at Shelley and lodgepole pine at Salmon River. In each plantation, all control and treatment blocks were located adjacent to areas with heavy vegetation cover within or on the perimeter of the plantations. Feeding damage by snowshoe hares tends to be most severe near such cover. Three blocks of 200 trees each were chosen at each plantation. A 20% solution of pinosylvin in methanol was sprayed directly on each of the 200 trees in one treatment block at each plantation. The same concentration of pinosylvin encapsulated in a

6-cm-long PVC plastic rope with internal copper wire as a twist-tie (PheroTech Inc., Delta, B.C., Canada) was attached to each tree in the second treatment block at each plantation. The third block acted as a control. Prior to application, 10% of the trees on each respective treatment and control block were flagged as sample trees. The applications were installed on October 30–31, 1988, and efficacy of treatments was evaluated on May 15–16, 1989. As for the large pen trials, feeding on the terminal was considered as mortality to a given seedling.

In the second trial, three plantations were located near the Salmon River, and one large plantation was located 80 km east of Quesnel. Again, control and treatment blocks were established near heavy vegetation cover or on the perimeter of the plantations. At each study site, pinosylvin was dispensed via a spray application (50 mg/tree) directly onto trees (1-year-old lodgepole pine and interior spruce seedlings planted in spring 1989), in controlled-release devices (25 mg/tree) of PVC plastic with the internal wire, plastic bubble caps, and C-flex rubber tubing. Each treatment was applied to a total of 400 trees with three replicate blocks at the Salmon River site and four replicates at the Quesnel site. Replicate control blocks completed the experimental design at each site. This trial was installed during October 15–30, 1989, and feeding damage to seedlings was assessed during May 21–31, 1990.

Statistical Analysis. Comparison of the number of seedlings surviving in control and treatment blocks was analyzed by chi-square, with significance levels of $P < 0.05$ and $P < 0.01$.

RESULTS

Screening Bioassays. In the initial two bioassays, pinosylvin sprayed directly on trees reduced feeding by hares over a 2-day period with respect to percentage of seedlings surviving (trial 1—control, 0.0 and 0.0, treatment, 66.7 and 33.3; trial 2—control, 13.3 and 0.0, treatment, 90.0 and 90.0). A more intensive assessment was conducted in trial 3 (Figure 2). Pinosylvin clearly suppressed feeding by hares compared with the control block and declined in efficacy only after control trees stopped being replaced at day 8.

The excellent results of trial 3 set the stage for testing controlled-release devices for pinosylvin. A contact repellent will not protect new tree growth and is not durable to prevailing weather conditions. Some type of encapsulation is necessary since an effective repellent should be present as an odor in the plantation environment but not necessarily attached to tree foliage. Results of four replicate trials assessing the influence of pinosylvin odor (soaked in cloth) on feeding by hares indicated a significant reduction in percentage of chow consumed after each 24-hr trial: (1) control, 39.0%, and treatment, 0.0%; (2) control, 54.3%, and treatment, 8.4%; (3) control, 68.2%, and treatment 7.2%;

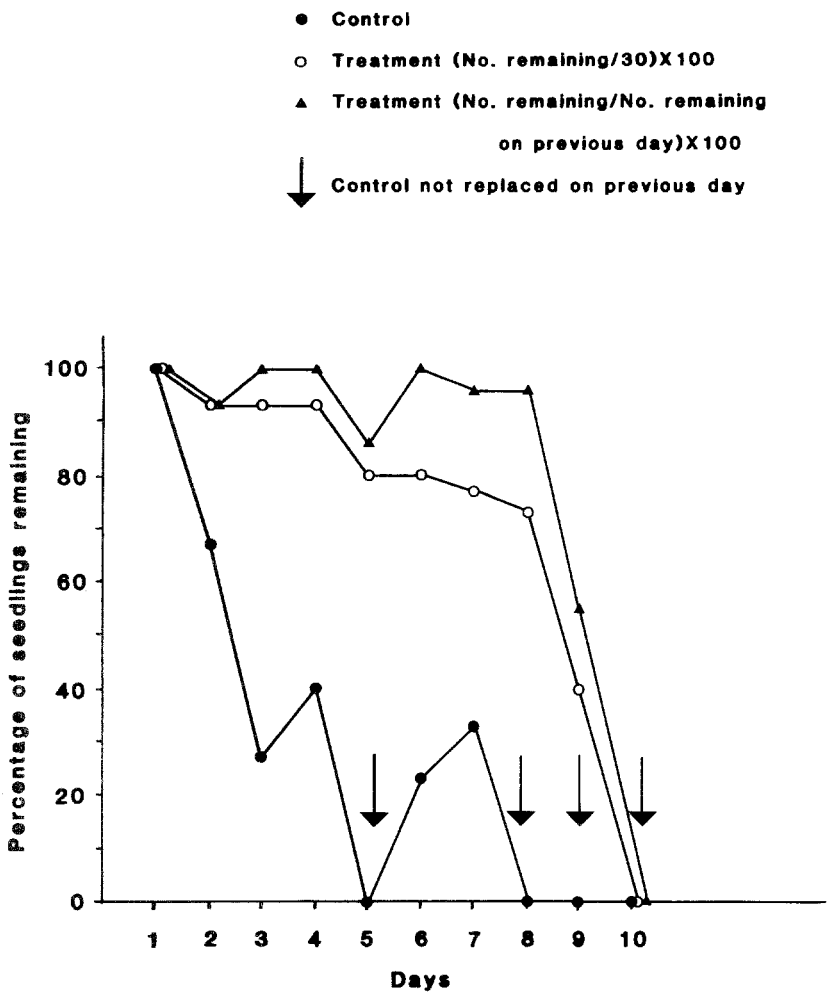

FIG. 2. Screening bioassay of the efficacy of pinosylvin in suppressing feeding on lodgepole pine seedlings by snowshoe hares.

(4) control, 46.0%, and treatment, 16.9%. The Wheaton vials and extraction thimbles attached to trees were not effective as controlled-release devices for pinosylvin. However, the clay pellets, when soaked in the pinosylvin for 24 hr prior to a trial, did significantly reduce feeding over a 2-day period with respect to the percentage of seedlings surviving (control, 10.0 and 0.0; treatment, 87.0 and 53.0). A lower concentration of pinosylvin in the pellets or fewer pellets per tree did not reduce feeding.

A final screening bioassay on the pinosylvin methyl ether analogue (7) showed that this compound had no effect on feeding by hares with respect to the percentage of seedings surviving over a 2-day period: control, 50.0 and 3.3; treatment, 43.3 and 10.3.

Large Pen Bioassays. Pinosylvin and PME significantly reduced hare feeding on pine seedlings throughout the fall trial of 1985 and, again, in the spring of 1986 (Figure 3A). There was no difference between pinosylvin and PME in reduction of feeding, and so these data were combined. In addition, survival of nonperimeter trees was significantly higher than that recorded when perimeter trees were included in the analysis. This trial was terminated on May 2. The spring 1986 trial with pinosylvin and PME, in separate halves of the block, also indicated that these compounds significantly reduced hare feeding (Figure 3B). These results provide temporal replication and, when combined with the screening bioassays, clearly show the impressive potential of pinosylvin as an antifeedant for snowshoe hares.

Field Bioassays. In the first trial, pinosylvin sprayed directly on trees and as an antifeedant odor from a plastic controlled-release device significantly reduced feeding damage to lodgepole pine seedlings (Table 1). There was insufficient browsing on spruce seedlings but the tendency was for less feeding in the pinosylvin-treated blocks.

In the second trial, one of three plantation replicates had sufficient feeding pressure to make comparisons among control and treatment blocks at the Salmon River site. The pinosylvin spray and PVC + wire and plastic bubble cap significantly reduced feeding compared with the control and C-flex tubing treatment blocks (Table 2). There was insufficient browsing on seedlings throughout all control and treatment blocks to make any statistical comparisons at the Quesnel site.

DISCUSSION

This study has clearly demonstrated that the plant antifeedant, pinosylvin (and PME), will suppress feeding by snowshoe hares. This feeding suppression corroborates those results obtained by Bryant et al. (1983) and Clausen et al. (1986) for bioassays with pinosylvin and laboratory foods. Our study reports the first test and use of pinosylvin associated with tree seedlings as a plant antifeedant repellent. Therefore, we accept the hypothesis that pinosylvin will generate an "avoidance response" in terms of feeding by snowshoe hares. This response is likely triggered by an olfactory pathway based on our results with the controlled-release devices attached to seedlings. The odor of pinosylvin was clearly an effective means of discouraging hares from feeding.

There are several studies of other plant antifeedants deterring snowshoe

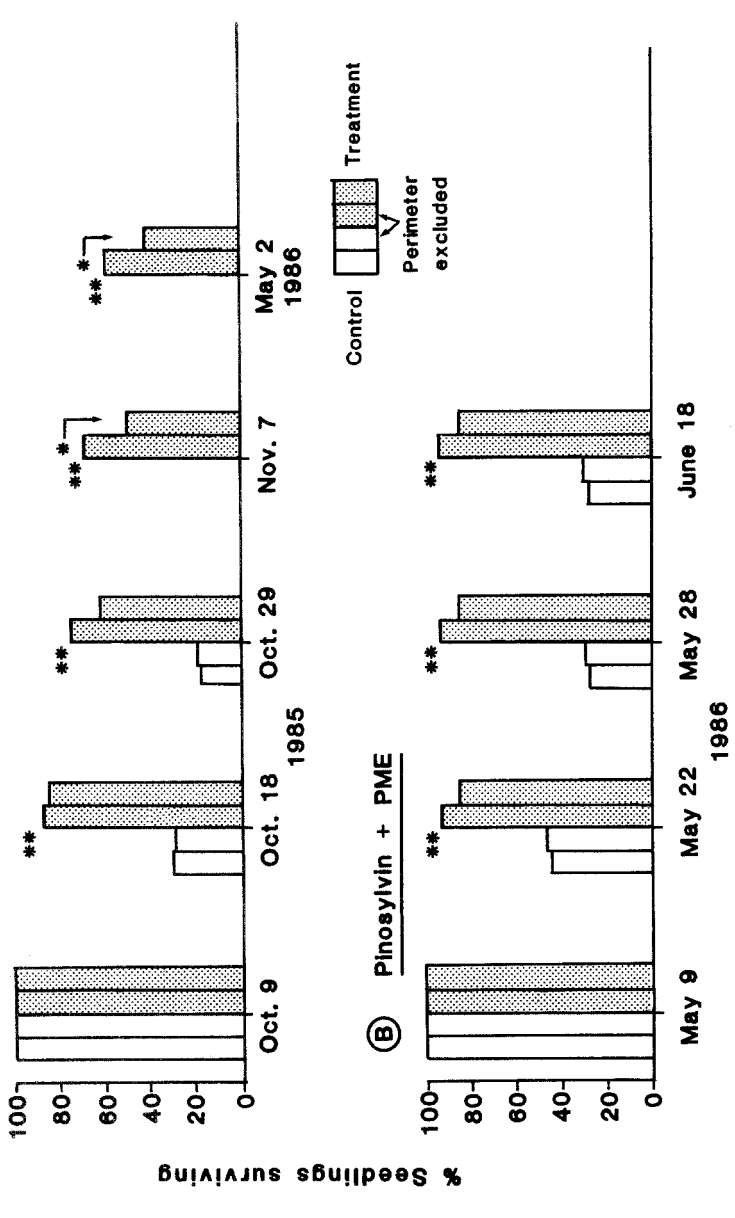

FIG. 3. Efficacy of pinosylvin + pinosylvin methyl ether (PME) in suppressing feeding on lodgepole pine seedlings by snowshoe hares during: (A) fall (October–early November) and overwinter to the spring (early May); (B) spring (May–June). Analyses were conducted with and without one row of perimeter trees in each block. (*) $P < 0.05$; (**) $P < 0.01$; significant difference by chi-square.

TABLE 1. PERCENTAGE SURVIVAL OF TREE SEEDLINGS FROM FEEDING DAMAGE BY SNOWSHOE HARES AT PRINCE GEORGE STUDY AREAS, OVERWINTER 1988–1989[a]

	Lodgepole pine		Spruce	
	Pinosylvin	Control	Pinosylvin	Control
Release device	100.0[b] (20)	25.0 (20)	90.0 (20)	75.0 (20)
Spray application	100.0[b] (20)	—	100.0 (20)	—

[a] Sample size in parentheses.
[b] $P < 0.01$; significant difference by chi-square.

TABLE 2. EFFICACY OF PINOSYLVIN (PLANT ANTIFEEDANT) IN TERMS OF PERCENTAGE SURVIVAL OF TREE SEEDLINGS FROM FEEDING DAMAGE BY SNOWSHOE HARES AT THE PRINCE GEORGE (SALMON RIVER) STUDY AREA, OVERWINTER 1989–1990[a]

Plantation	Control	Spray	PVC + wire	Plastic bubble	C-flex tubing
1	91.1 (79)	91.5 (130)	92.1 (38)	91.9 (62)	NB
2	NB	NB	NB	NB	NB
3	60.6 (94)[abc]	86.5 (111)[ag]	84.9 (86)[bh]	97.6 (85)[ci]	68.7 (99)[ghi]
Total	74.6 (173)[def]	89.2 (241)[dj]	87.1 (124)[ek]	95.2 (147)[fl]	68.7 (99)[jkl]

[a] Sample size in parentheses. Superscripts a–a, b–b, c–c, d–d, e–e, and f–f, $P < 0.01$; g–g, h–h, i–i, j–j, k–k, and l–l, $P < 0.05$; significant difference by chi-square. NB, no browsing by hares.

hares from feeding. These include papyriferic acid from Alaska paper birch (*Betula resinifera*) (Reichardt et al., 1984), phenolglycoside and catechin fractions of willow (*Salix* spp.) bark (Tahvanainen et al., 1985), camphor from white spruce (Sinclair et al., 1988), germacrone from labrador tea (*Ledum groenlandicum*) (Reichardt et al., 1990a), and several compounds from balsam poplar (*Populus balsamifera*) (Jogia et al., 1989; Reichardt et al., 1990b). An intriguing follow-up to these studies would be testing of the various antifeedants on tree seedlings to determine their repellency to snowshoe hares. In particular, camphor from juvenile white spruce suppresses feeding on rabbit chow by hares in a laboratory situation (Sinclair et al., 1988). Plantations of nursery-raised white and interior spruce seedlings on cutover forest land are fed on by snowshoe hares during peak populations. Therefore, a critical test for camphor as an antifeedant is to apply the compound to tree seedlings in a field situation as we have done in our study. Camphor is presumably at a low concentration in nursery-raised spruce, or other metabolic constituents may mask the antifeedant effect of this compound.

Similar feeding damage to tree seedlings is often caused by high populations of voles of the genus *Microtus* (Green, 1978; Roy and Bergeron, 1990a; Sullivan and Martin, 1991). Preference for certain coniferous species and rejection of others was related to concentration of phenolic compounds (Hansson et al., 1986; Roy and Bergeron, 1990a). In addition, voles will manipulate their food resource by cutting branches of young seedlings, and after 3 days, the cut branches have significantly less phenolics and condensed tannins (Roy and Bergeron, 1990b). By this manipulation, a low-quality resource is transformed into one compatible with vole feeding patterns and detoxification mechanisms. The senior author has also observed this behavior in snowshoe hares, which may cut and leave seedlings in plantations. Thus, mammals are able to evaluate the palatability of potential foods on the basis of secondary metabolites. Pinosylvin clearly plays a role in this selection for snowshoe hares and, along with other plant antifeedant compounds, may regulate the 10-year cycle of abundance (Bryant and Kuropat, 1980; Bryant et al., 1983; Coley et al., 1985).

The specificity of chemical structure of pinosylvin appears to be of critical importance to the snowshoe hare. Presumably pinosylvin and other repellent antifeedants have specific receptor sites within the olfactory system of the snowshoe hare and perhaps other small mammal herbivores. The ineffectiveness of the PME analogue (7) as an analogous compound to pinosylvin supports this specificity. This result is similar to that recorded for mustelid scent gland compounds and their analogues (Sullivan and Crump, 1984).

The use of avoidance-inducing compounds as area repellents to reduce feeding damage to tree seedlings is clearly important to forest protection and management. A plant-derived repellent, pine oil, which is a mixture of terpene alcohols and monoterpenes has reduced feeding by snowshoe hares and voles (*M. townsendii*) when mixed with laboratory rations (Bell and Harestad, 1987). Similarly, red deer calves (*Cervus elaphus*) consumed less laboratory rations when this food was mixed with odors of crushed lodgepole pine and various monoterpenes (Elliot and Loudon, 1987). Again, as discussed previously, the critical test for plant antifeedants, as repellents to reduce feeding by herbivores, is application of the synthetic compounds to tree seedlings in field situations. Our study reports the first practical utilization of plant antifeedants for crop protection and wildlife management.

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EVIDENCE FOR THE ACCESSORY GLANDS AS THE SITE OF PRODUCTION OF THE OVIPOSITION ATTRACTANT AND/OR STIMULANT OF *Lutzomyia longipalpis* (DIPTERA: PSYCHODIDAE)

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Abstract—The phlebotomine sandfly *Lutzomyia longipalpis* Lutz and Neiva, the vector of visceral leishmaniasis in South America, has recently been shown to produce an oviposition semiochemical. In the present study it was found that a nonpolar extract of eggs was attractive and/or stimulatory to ovipositing females. A chromatographic investigation indicated the presence of similar compounds in accessory glands and egg extracts. Extract of accessory gland was also found to elicit a positive oviposition response. It is concluded that the pheromone is produced in the accessory glands and is secreted onto the eggs during oviposition.

Key Words—*Lutzomyia longipalpis*, Diptera, Psychodidae, oviposition, pheromone, accessory gland, bioassay.

INTRODUCTION

Pheromones from the eggs of a number of heamatophagus Diptera have been shown to attract ovipositing females; these include *Culex quinquefasciatus* Say Wiedmann (Bruno and Laurence, 1979; Sakaibara et al., 1984; Laurence et al., 1985; Laurence and Pickett, 1985; Hwang et al., 1987). A review of the various *Culex* species oviposition site selection cues, including orientation toward egg pheromone sources, is given by Bentley and Day (1989). Ovipositing *Aedes atropalpus* Coquillett are also attracted to an oviposition attractant produced by immature stages (Kalpage and Brust, 1973). Furthermore, Soman and Reuben

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(1970), showed that gravid *Aedes aegypti* (L.) females were attracted to water containing immature stages of the same species.

Recently, Elnaiem and Ward (1990, 1991a) showed that eggs of the sandfly *Lutzomyia longipalpis* Lutz and Neiva have an attractant and/or stimulant effect, related to the presence of a semiochemical, perceived by conspecific, ovipositing females. When eggs were washed in a series of polar and nonpolar solvents, the oviposition response was no longer elicited. This demonstrated that the oviposition attractant and/or stimulant effect was chemically mediated, and not due solely to a visual or tactile effect of the eggs.

The field behavior of *L. longipalpis* during oviposition is little studied. The females are known to be gonotrophically concordant and pass through more than one gonotrophic cycle (Guilivard et al., 1980, Dye et al., 1987). A major problem in the study of the transmission of leishmaniasis is the very high mortality rate found at oviposition. This is attributed to the difficulty in matching laboratory and field conditions. Females are known to lay up to 100 eggs in a single oviposition but the normal number is 40. In the wild, the eggs are thought to be laid in microhabitats, such as cracks and crevices (Young et al., 1926; Lewis and Kirk, 1954; Ward, 1974), rich in organic nutrients and are difficult to find in normal circumstances. The eggs are laid in humid conditions and it is known that control of temperature and humidity is an essential requirement of colony maintenance (Killick-Kendrick, 1977).

The photoperiod does not affect the success of oviposition of *L. longipalpis* but does markedly alter the oviposition periodicity (Ready, 1976). Based on laboratory observations it appears that some species are capable of long periods of dormancy (Johnson and Hertig, 1961). While capable of withstanding desiccation, the eggs are resistant to prolonged immersion (Chaniotis, 1967). This is a useful adaptation in a terrestrial insect that may often suffer temporary inundation due to rainfall.

Accessory glands of many female insects produce secretions which are present in or on oviposited eggs (Adiyodi and Adiyodi, 1989). In the present study, therefore, we investigated if organic or aqueous extracts of *L. longipalpis* eggs retained biological activity similar to that induced previously by the use of whole eggs. In addition, a chromatographic investigation was carried out to determine if the female accessory glands had a role in the production of the semiochemical.

METHODS AND MATERIALS

Colonization

The sandflies used in the experiments were *L. longipalpis* established from flies collected at Jacobina, Bahia, Brazil, and maintained in the laboratory for

6 years using methods described by Modi and Tesh (1983), at 95% RH, a room temperature of 28°C, and a 12h L:D photoperiod.

Female *L. longipalpis* were fed on Syrian hamsters anesthetized with 12 mg/ml of sodium pentabarbitone and were kept for 4 days to allow for complete defecation and oogenesis before use in bioassays.

Sample Preparation

All eggs used for extraction purposes were taken from colony rearing pots 1 to 2 days after oviposition. To prepare the nonpolar egg extract, 1000 eggs were placed in a 2-ml microvial (Alltech) and left for 24-hr at −70°C in 100 μl of pesticide-grade hexane (BDH Chemicals). Accessory gland extracts were prepared from 50 gravid females that had been blood-fed 4 days previously. The glands were excised under a stereoscopic microscope and the tissue was transferred to a 2-ml microvial using a micropipette and extracted as described above. The remaining female bodies were also similarly extracted, to provide material for a negative control. To prepare the polar extract 1000 eggs were placed in a 2-ml microvial and extracted in 100 μl of double-distilled, deionized H_2O at 3°C for 24 hr; 100 accessory glands and the remainder of the female bodies were also similarly extracted. The preparation of material for HPTLC and GC analysis used the same protocol as described above. Five hundred *L. longipalpis* eggs and 50 excised female accessory glands were extracted in 20 μl of hexane. Further, for GC analysis, five whole male, whole female, and female flies without accessory glands were extracted in 40 μl of hexane. Samples were stored at −70°C.

Bioassay

Only eggs laid in the test and control sites were counted. The bioassay was an adaptation of that used by Elnaiem and Ward (1991). The bioassay chambers were modified polymethylpentene (BDH apparatus) rearing pots. Modifications included the use of two oviposition areas, 2 cm^2, termed test and control, with lightly demarcated perimeter grooves on the plaster of paris base.

Six bioassays were conducted, each with 10 replicates, using 20 females per replicate. In each oviposition chamber a 10 μl aliquot of sample extract was pipetted onto the plaster of paris base at the test site. The control was 10 μl of pure extraction solvent. This test volume consistently gave an equivalent of 100 eggs, accessory glands from five females, or five female bodies without accessory glands. Bioassays performed were (a) nonpolar egg extract versus hexane control, (b) nonpolar accessory gland extract versus hexane, (c) polar egg extract versus water control, (d) polar accessory gland extract versus water, (e) nonpolar extract of females without accessory glands versus hexane, and (f) polar extract of females without accessory glands versus water.

After loading the oviposition areas in the bioassay chambers with the test

and control samples, 20 gravid females were introduced and left for 3 days under normal colonization conditions. The females were provided with a small wad of cotton wool, soaked with a saturated solution of sucrose, placed on top of the oviposition chamber. Provision of a sugar meal is known to increase the number of eggs laid per sandfly (Ready, 1979).

Statistical Analysis

The bioassay design gives a matched pair of results, which was found to be nonparametric. As such, Wilcoxon's rank paired test was employed to a 95% confidence level.

Chemical Analysis

High-Performance Thin-Layer Chromatography (HPTLC). High-performance TLC plates were 10 × 20 cm, coated with a 200-μm-thick layer of silica (Whatman Lab Supplies). The developing solvent was a 1 : 1 chloroform/hexane solution (BDH Chemicals), described by Kates (1986), for nonpolar lipids. HPTLC plates were prepared by baking at 200°C for 24 hr and then cleaned by running twice in the solvent system. Twenty-microliter aliquots of egg and accessory gland extract (25 female equivalents) were applied to the plates with 2-μl capillary tubes. Twenty micrograms of each of five standards (cholesterol, cholesterol linolenate, cholesterol stearate, squalene and caryophyllene oxide; Sigma Chemicals) was applied to the plate. Plates were visualised by ultraviolet light at 302 nm, followed by charring with concentrated H_2SO_4/EtOH solution (1 : 1, v/v) and baking at 110°C for 10 min. Comparisons of samples and standards were carried out using R_f values.

Gas Chromatography. Gas chromatography was performed on a Shimadzu GC-15a (Dyson Instruments), fitted with a Grob splitless injection system with a 0.6-min sampling time. The column used was a 30-m, fused silica DB1 capillary column, with a 0.25-μm film thickness (J. and W. Scientific). The temperature program was as follows: 45°C initial, increased at 15°C min^{-1} to 250°C, held for 4 min, increased at 10°C min^{-1}, and then held at 350°C for 4 min. The carrier gas was helium at a pressure of 4.5 kg cm^3 and the injection block and detector temperatures were 250 and 350°C, respectively. Integration was carried out with the Shimadzu CR5a Chromatopac (Dyson Instruments).

Only nonpolar extracts were analyzed by GC. Samples included extracts of eggs, female accessory glands, whole males, whole females, and females without accessory glands prepared as described above. Before being analyzed by GC, extracts, cooled on ice, were reduced under N_2 to 1 μl.

RESULTS

Bioassays

The bioassay results are summarized in Table 1. The response of *L. longipalpis* during oviposition to hexane extracts of egg and accessory gland material showed a highly significant difference between the test and the control sites. Neither the hexane nor the aqueous extracts of female bodies without accessory glands produced a statistically different number of eggs at the test and control sites. Aqueous extracts of both egg and accessory glands failed to induce an oviposition response. Thus nonpolar extracts of both egg and accessory gland material were the only samples to elicit the biological response which has previously been associated with whole eggs.

Thin-Layer Chromatography

The HPTLC analysis demonstrates chemicals present in both egg and accessory gland samples that are of a very similar polarity (Table 2). A compound which has a R_f value similar to that of cholesterol, lane 1 ($R_f = 0.12$), is present in both, as is a compound that is more polar than cholesterol. Two other pairs of matching spots are observed on the HPTLC plate. The spots at $R_f = 0.51$ (accessory, lane 6) and $R_f = 0.50$ (egg, lane 7) are only slightly less polar than caryophyllene oxide, lane 4 ($R_f = 0.48$). At $R_f = 0.62$ (accessory) and $R_f = 0.66$ (egg) two spots were exhibited of a polarity similar to that of cholesterol stearate, lane 5, and cholesterol linolenate, lane 1.

TABLE 1. OVIPOSITION BIOASSAY WITH POLAR AND NONPOLAR EXTRACTS OF EGGS, ACCESSORY GLANDS, AND WHOLE FEMALE *L. longipalpis* WITHOUT ACCESSORY GLANDS[a]

Extract	Solvent	Oviposition response (mean ± SD)		*P*
		Test	Control	
Egg	Hexane	95.40 ± 13.79	20.80 ± 8.68	0.006
Egg	Water	22.00 ± 8.16	26.20 ± 4.76	0.186[b]
Accessory gland	Hexane	98.80 ± 12.31	26.40 ± 11.54	0.006
Accessory gland	Water	23.60 ± 6.26	24.3 ± 4.90	0.610[b]
Whole ♀ without gland	Hexane	25.90 ± 6.49	26.50 ± 6.98	0.799[b]
Whole ♀ without gland	Water	25.70 ± 5.60	23.50 ± 4.20	0.262[b]

[a]Each bioassay was composed of 10 replicates employing 20 females in each. *P*, probability. Consistently 100 egg equivalents, 5 female equivalents of accessory gland material, and 5 whole-female equivalents without glands were used in each respective bioassay.
[b]Nonsignificant by Wilcoxon ranked pair test.

Gas Chromatography

The retention times of major peaks found during the GC investigation are shown in Table 3. Chromatograms of the following extracts are shown in Figures 1A–C; females without accessory glands, eggs, and accessory glands. There is a similar pattern to all the traces, showing a series of compounds common to

TABLE 2. R_f VALUES OF STANDARDS AND EXTRACTS OF ACCESSORY GLAND AND EGG SAMPLES BY HPTLC[a]

Sample	1	2	3	4	5	6	7	8
Cholesterol linolenate						0.69		
Cholesterol	0.12							
Squalene							0.72	
Caryophyllene				0.48				
Cholesterol sterate						0.67		
Accessory gland	0.12				0.51	0.62		
	0.03							
Egg	0.12				0.5	0.66		
	0.06							

[a] Plates were loaded at 20 μl standards, 500 equiv. eggs, 25 female equiv. accessory glands. Solvent phase, 1 : 1 chloroform/hexane, with a silica gel plate.

TABLE 3. CHROMATOGRAM RETENTION TIMES FOR EXTRACTS OF EGG, ACCESSORY GLAND, WHOLE-FEMALE, AND WHOLE-MALE SAMPLES BY GAS CHROMATOGRAPHY[a]

	Retention time (min)					
	11	12	13	14	15	16
Egg	11.86		13.52	14.82		16.21
				14.99		
Accessory gland	11.89		13.50	14.84		16.20
				14.99		
Whole ♀	11.88		13.34	14.86	15.00	16.2
			13.50			
Whole ♂	11.05			14.85		16.19
	11.51			14.99		
Corresponding number on chromatogram[a]	1		2	3		4

[a] Numbers correspond to peaks on chromatograms in Fig. 1.

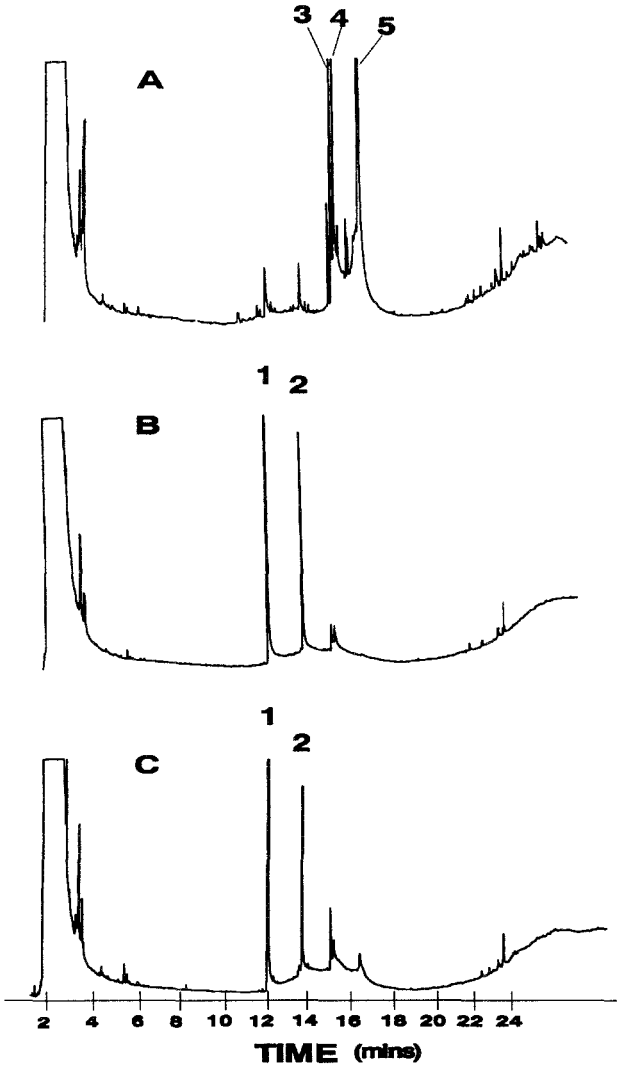

FIG. 1. Gas chromatograms of hexane extracts of (A) females without accessory glands, (B) eggs, and (C) accessory glands. Peak numbers correspond to those given in Table 3. The experimental conditions are given in the text.

all the samples, with peaks 1 and 2 being specific to eggs and accessory glands. Male *L. longipalpis* has two major peaks, at 11.052 and 11.511. These are known to originate from the tergal glands (Hamilton, personal communication). Whole-female extract has major peaks at 11.887 and 13.509, which are not

present in the whole male samples. These two peaks (peaks 1 and 2 on the chromatogram) do occur in female accessory gland extract (11.896 and 13.502) and in egg material (11.862 and 13.521) and are greatly reduced in samples of females with excised accessory glands.

DISCUSSION

This is the first demonstration that the oviposition semiochemical present on the eggs of *L. longipalpis* may be extracted in hexane, indicating that the molecule is a relatively nonpolar organic compound. Furthermore, the extract does not lose its oviposition attractant and/or stimulant properties when bioassayed. The hexane extract of the accessory gland material also elicited similar biological activity. In contrast, hexane extracts of female bodies from which accessory glands had been excised did not elicit an oviposition response, nor did any of the aqueous extracts.

The gas chromatographic analysis revealed that two compounds which occur in extracts of whole female flies and accessory glands are also present in extracts of eggs. These compounds are absent in females without accessory glands and from whole males. The HPTLC results indicate that two compounds of comparable polarity are present in egg and accessory gland material. These compounds are of a polarity similar to that of cholesterol linolenate or cholesterol stearate and caryophyllene oxide, all of which are oxygenated compounds.

Chromatographic and bioassay evidence suggests that the semiochemical(s) present on *L. longipalpis* eggs is produced by the accessory glands of the female. It appears that as eggs are laid, the semiochemical is coated onto them from this gland. It is known that secretions from the accessory gland include a sticky substance, which is used to adhere the egg to oviposition substrates (Davis, 1967; Wu and Tesh, 1989). Adiyodi and Adiyodi (1989) reviewed the role of the accessory glands of female insects, noting them to have a secretory role in both egg maturation for ootheca production and during oviposition.

In contrast, the presence of oviposition-deterring pheromones in sticky secretions used to adhere eggs to oviposition substrates has been shown in the sorghum shoot fly, *Atherigona soccata* (Raina, 1981). Here, a water-soluble glue is used to stick eggs to the leaves of sorghum.

Little is known about the attractant and/or stimulant role of the *L. longipalpis* oviposition pheromone. It is possible that the pheromone acts over a limited range, with the female being directed to oviposition sites by long-range volatile chemicals from organic substrates and physical characteristics of the oviposition substrate. In this situation the pheromone would be the final phase in the oviposition site selection mechanism. Elnaiem and Ward (1991b) produced some circumstantial evidence for the above hypothesis, showing that

oviposition *L. longipalpis* females were attracted to an aqueous extract of rabbit feces, a component of the laboratory larval rearing medium. Schlein et al. (1990) also found that *Phlebotomous papatasi* chose oviposition sites by physical and chemical stimuli in the oviposition substrate. Furthermore, it is known that *Culex* females select oviposition sites by environmental, physical, chemical, and pheromonal cues.

Use of an oviposition pheromone by *L. longipalpis* may lead to larval aggregation, which may be advantageous in overcoming factors such as predation and parasitism (Manning, 1979). However, attempts to locate sandfly larval breeding sites in soil samples have only shown sparsely scattered immature stages (Hanson, 1961; Thatcher, 1968; Rutledge and Mosser, 1972). However, Bettini et al. (1986), using emergence traps, were successful in collecting 27,405 emergent flies from a 25-m^2 breeding site in 1 year. Further investigation is therefore needed to clarify the apparent disparity between the results from these two sampling techniques and to investigate the role of the oviposition pheromone in the field. It is recognized that some oviposition pheromone-like activity has been associated with the presence of volatiles from bacterial activity, as with *Lucilia cuprina* Wiedmann (Emmens and Murray, 1983). As accessory gland extract of female *L. longipalpis* elicited a response and samples of female bodies without these glands did not, it is thought that this phenomenon is not occurring here. Further work is currently being carried out to clarify this point and to determine the structure of the components of egg and accessory gland material that are responsible for the biological activity.

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SPECIES-SPECIFIC, TWO-COMPONENT, VOLATILE SIGNALS IN TWO SYMPATRIC ANT-LION SPECIES: *Synclysis baetica* AND *Acanthaclisis occitanica* (NEUROPTERA, MYRMELEONTIDAE)

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Abstract—The thoracic glands of males in two ant-lion species *Synclysis baetica* and *Acanthaclisis occitanica*, which occur sympatrically in Israel, were found to contain a volatile secretion with two-component blends of nerol oxide and (*R*,*Z*)-6-tridecen-2-ol (approx 1:5) and nerol oxide and 10-homonerol oxide (approx. 1:2), respectively. Chemical analyses were performed using gas chromatography–mass spectrometry, chiral gas chromatography, and ozonolysis, and the proposed structures were confirmed by synthesis. The species-specific, few-component volatile signals are thought to function as a

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reproductive isolation mechanism between the two sympatric species. Biochemical relationships between the nerol derivatives and between the unsaturated secondary alcohols are discussed.

Key Words—Ant lions, Neuroptera, Myrmeleontidae, (*R*,*Z*)-6-tridecen-2-ol, nerol oxide, 10-homonerol oxide, biosynthesis, enantiomers, gas chromatography–mass spectrometry, sympatric species isolation.

INTRODUCTION

Synclysis baetica and *Acanthaclisis occitanica* belong to Acanthaclisini, a tribe well defined by larval and adult characteristics that, in contrast to most other tribes, exhibits limited diversity (Stange and Miller, 1985). Both species have a holo-Mediterranean distribution and are widespread sympatrically along the coastal sand dunes of Israel. Aspects of their biology and coexistence in Israel were described by Simon (1988).

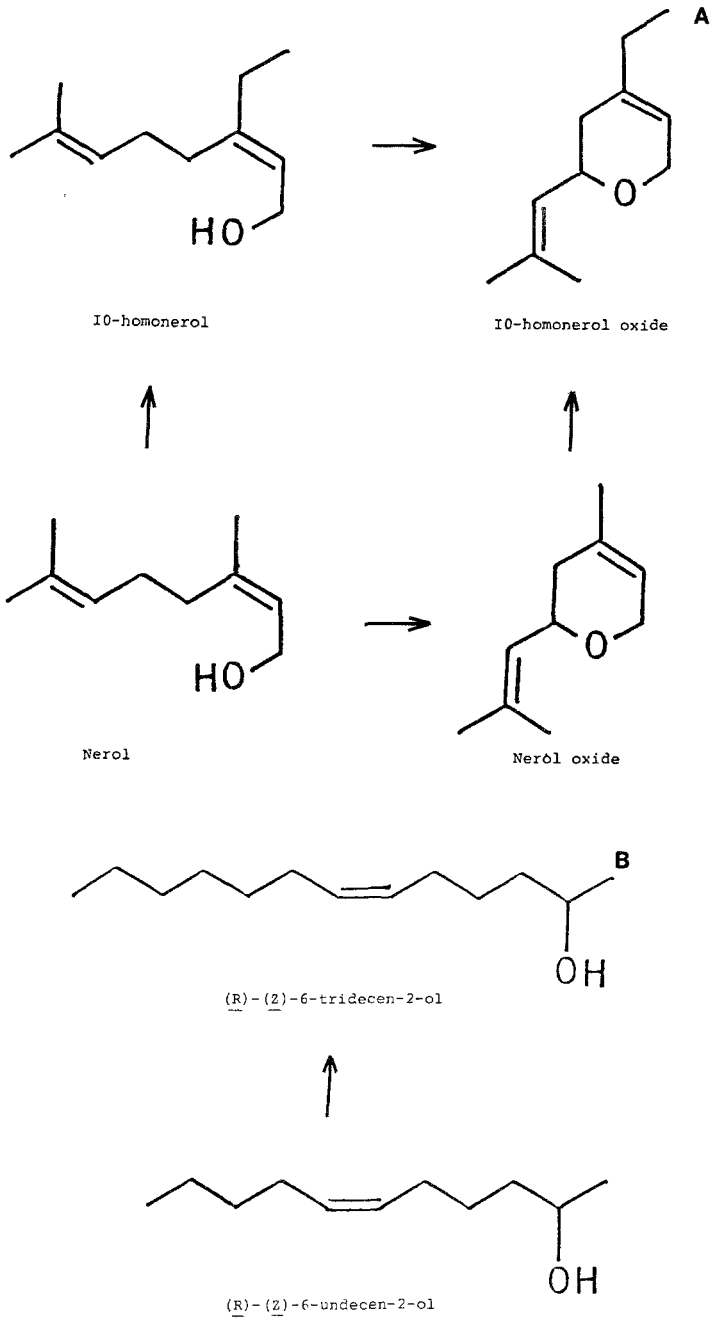
Male ant lions produce two-component secretions in their thoracic glands, which are species specific and thought to function as a reproductive isolation mechanism (Elofson and Löfqvist, 1974; Löfqvist and Bergström, 1980; Baeckström et al., 1989). Components of the secretions in three species have been identified as derivatives of the monoterpene alcohol nerol and a straight-chain, unsaturated C_{11}-alcohol, "nostrenol" (Baeckström et al., 1989). Females of the same species contain nerol oxide in small amounts.

In the present report we have analyzed the thoracic gland secretion of two additional species from Israel, *Synclysis baetica* Rambur 1842 and *Acanthaclicis occitanica* Villers 1789, as a part of an evolutionary study of volatile signals in ant lions. The biochemical pattern of volatile compounds reported earlier in *Euroleon nostras*, *Grocus bore*, and *Myrmeleon formicarius* (Baeckström et al., 1989) is now reinforced by the finding that, also in the two species now studied, each produces a different two-component combination on the same theme (Scheme I). It is thus shown that even in these species, specificity is achieved by a blend of just two compounds.

METHODS AND MATERIALS

Collection and Preparation of Insect Material

Third-instar larvae of both species were collected from the coastal sand dunes of Israel. They were reared individually in plastic vials and fed house fly maggots. Emerging adults were kept alive for 2–4 days, after which they were



SCHEME I. Possible biochemical relationship between the chemical compounds identified from five species of ant lions. (A) Nerol-related compounds; (B) monounsaturated, secondary alcohols.

frozen and immediately dissected under water. The thoracic glands were removed and placed in methylene chloride for extraction.

Identification of Volatiles

Methylene chloride extracts of thoracic glands were analyzed by GC-MS (Finnigan 4021 equipped with an Incos data system) on a fused silica capillary column coated with Superox FA (25 m × 0.15-mm I.D., df = 0.3 μm). Nerol oxide and 10-homonerol oxide were identified by comparing gas chromatographic retention values and mass spectra of the natural components with those of synthetic reference compounds (Baeckström et al., 1989).

Gas Chromatography of Enantiomers

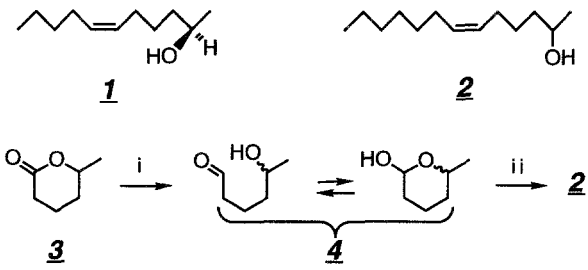
Separation of the nerol oxide and 10-homonerol oxide enantiomers was carried out on Ni(II)-bis[3-heptafluorobutyryl-1(*R*)-camphorate] (CC&CC, Postfach 14, D-7402 Kirchentellinsfurt, Germany) and Mn(II)-bis[3-heptafluorobutyryl-1(*R*)-camphorate] columns. Enantiomers of (*Z*)-6-tridecen-2-ol were separated as isopropyl carbamate derivatives on a XE-60-(*S*)-valine-(*S*)-2-phenylethylamide column, according to König and Francke (1982). For further GC data, see Fig. 3.

Ozonolysis

The unknown compound was isolated by preparative gas chromatography (Carlo Erba 4160), according to Wassgren and Bergström (1984), on a capillary column coated with OV-351 (20 m × 0.32-mm I.D., df = 0.64 μm). The collected fraction was then dissolved in hexane and ozonized according to Beroza and Bierl (1967). Separation and identification of the ozonolysis products were obtained by GC-MS.

Synthesis

5-Hydroxyhexanal or Cyclic Hemiketal (Compound 4; Scheme II). δ-Hexanolide (**3**) was prepared from 5-oxohexanoate (Merck) according to the literature (Ansell et al., 1968). δ-Hexanolide (**3**; 0.5 g, 4.2 mmol) was stirred in dry tetrahydrofuran (THF; 5 ml) in an argon atmosphere at −50°C. Diisobutylaluminium-hydride (1.5 *M* in toluene, 20 mmol, 13.3 ml, dissolved in THF, 5 ml) was added dropwise during 20 min and stirred at −50°C for an additional 40 min. GC analysis indicated no remaining starting material. At the same temperature $Na_2SO_4 \cdot 10H_2O$ (6 g) mixed with the same volume of Celite was then added. The reaction mixture was slowly warmed to room temperature, then filtered, and the solids were washed with diethyl ether. The solution was dried (with $MgSO_4$), filtered, and concentrated carefully to give a toluene solution.



SCHEME II. (i) Diisobutylaluminiumhydride, tetrahydrofuran, −50°C. (ii) $CH_3(CH_2)_5CH{=}P(Ph)_3$, dimethyl sulfoxide, 25°C.

Most of the toluene was removed by distillation through a column at 100 mm Hg, leaving a mixture of toluene and the desired product, **4**) (shown in Scheme II) in its hemiketal form, which was used directly in the next step. IR (film): 3650–3100 s broad, no peak in the carbonyl region. NMR (60 MHz, $CDCl_3$): δ1.1 and 1.2 (3H, two d, J = 5 Hz), 1.3–2.0 (6H, m), 3.2–4.1 (1H, m, on irradiating at δ1.15 ppm, two broad s appeared), 4.2–4.7, (1H, disappeared on shaking with D_2O), 4.6–5.0, and 5.1–5.3 (1H, two broad m) ppm and signals due to toluene.

(Z)-6-Tridecen-2-ol (Compound 2; Scheme II). Sodium hydride (50% in oil, 0.405 g, 8.26 mmol) was washed twice with dry THF and the residual THF was evaporated in an argon atmosphere. Dimethyl sulfoxide (DMSO; 30 ml freshly distilled from CaH_2, stored over 4-Å mol sieves) was added under argon at room temperature. The mixture was stirred and warmed at 60°C until hydrogen evolution stopped and a clear grayish solution formed (2 hr). After cooling to 20°C, heptyltriphenylphosphonium bromide (3.65 g, 8.26 mmol) was added under a blanket of argon. The deep red heptylidenephosphorane was formed instantaneously. After 0.5 hr the toluene solution (from the step above) containing product **4** (approximately 4.2 mmol) was diluted with DMSO (10 ml) and added dropwise during 0.3 hr under cooling. After stirring for an additional 1.5 hr, the brownish-black solution was poured into water and extracted with hexane several times. The combined organic extracts were dried (with $MgSO_4$) and concentrated to 10 ml. Storage in the freezer overnight caused the triphenylphosphine oxide to separate, and it was then removed by filtration. After evaporation of the solvent the residue was distilled bulb to bulb (110°C/0.1 mm), furnishing a colorless oil (0.450 g, 54% calculated on the hexanolide) that tested pure by GC. Attempts to analyze the *Z/E* ratio by GC on several phases were not successful. However, the *Z/E* ratio was estimated to be 91/9 on the basis of analysis of the *N*-isopropyl carbamate derivative (see Figure 2). *Anal.* Calc. for $C_{13}H_{26}O$: C, 78.7%; H, 13.2%. Found: C, 78.6%; H, 13.4%. n_D 1.4563. IR (film): 3500–3100 s broad, 3025, no medium or strong peak at

950–1000 cm^{-1}. NMR (60 MHz, $CDCl_3$): δ0.93 (3H, t), 1.32 (3H, d, J = 5 Hz), 1.12–1.45 (12H, m), 1.7 (1H, b s, disappeared on shaking with D_2O), 1.80–2.30 (4H, m), 3.62–4.0 (1H, m, gave a narrower m when the δ1.32 peak was irradiated), 5.40 (2H, distorted t, gave a broad s on irradiation at δ2.05) ppm. MS (70 eV, m/e; rel intensity): see Figure 1.

(R)-(−)-(Z)-6-Tridecen-2-ol (Compound ***R-2****; Scheme III) of Low Enan-*

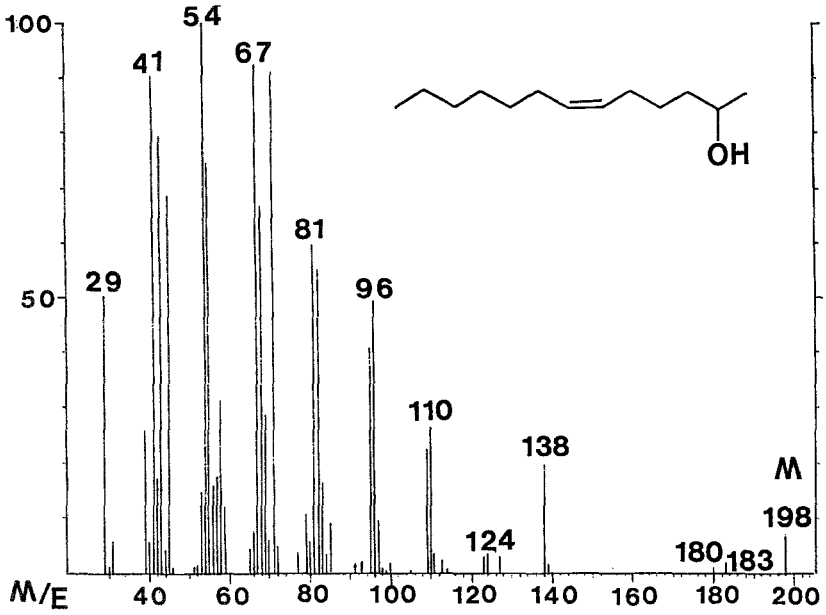
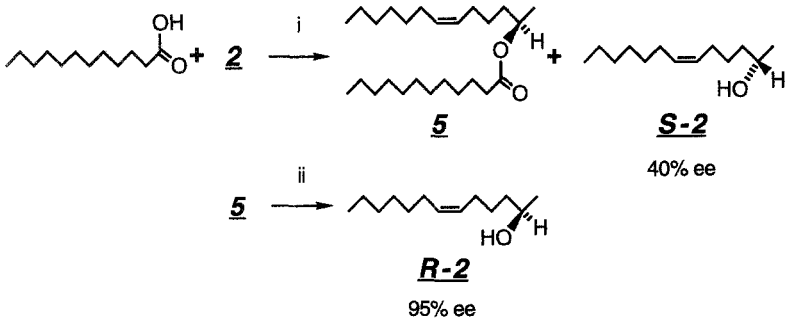


FIG. 1. Mass spectrum of natural (*R*,*Z*)-6-tridecen-2-ol from *S. baetica*.



SCHEME III. (i) Porcine pancreas lipase (PPL), heptane, 70°C, 16 hr. (ii) KOH, MeOH, 25°C, 16 hr.

tiomeric Purity. δ-Hexanolide was chromatographed on triacetylated cellulose using the methodology described earlier (Högberg et al., 1987). The combined fractions with positive rotations containing [*R*]-(+)-**3** (Pirkle and Adams, 1979) were subjected to the reaction sequence described above, yielding the title compound with [*R*] configuration $[\alpha]_D^{20}$ −0.4° (neat), which corresponds to approximately 5% ee (enantiomeric excess).

Enzymatic Resolution of rac-(Z)-6-Tridecen-2-ol. Dodecanoic acid (200 mg, 1 mmol) and rac-(*Z*)-6-tridecen-2-ol **(2)** (198 mg, 1 mmol) and PPL (porcine pancreas lipase) (Sigma L-3126; 4 g) were stirred in heptane (40 ml) at 70°C for 16 hr. The reaction was stopped by cooling and filtering off the enzyme. Chromatography of the concentrated filtrate on silica gel (60, 230–400 mesh, eluted with 0–10% ethyl acetate in hexane) furnished the dodecanoate ester (120 mg, 32%) and (*S*)-(+)-(*Z*)-6-tridecen-2-ol **S-2,** 110 mg (55%; $[\alpha]_D^{20}$ +3.0°; c 5.4, hexane), after bulb-to-bulb distillation (120°C/0.3 mm Hg), which was then analyzed as an *N*-isopropyl carbamate derivative and found to be of 40% ee, i.e., 70% **S-2** and 30% **R-2.**

(R)-*(−)-(Z)-6-Tridecen-2-ol* ***(R-2).*** The dodecanoate ester from above was saponified by stirring under argon in methanol (15 ml) containing dissolved potassium hydroxide (0.5 g). After 16 hr the solution was poured into water (50 ml) and extracted several times with hexane. After drying ($MgSO_4$) the hexane solution was concentrated and the residue chromatographed as described above for **S-2** to give a colorless oil which was distilled bulb to bulb (120°/0.3 mm Hg), 40 mg (20%). $[\alpha]_D^{20}$ −8.0° (c 1.8, hexane). GC analysis as an *N*-isopropyl carbamate derivative established that this compound was of >95% ee (i.e., >97.5% *R*) and that it was identical to the natural unknown compound from *S. baetica.*

Manganese-Chelate Stationary Phase

Manganese(II)–bis[3-heptafluorobutyryl-(*1R*)-camphorate] was prepared from 3-heptafluorobutyryl-(*1R*)-camphor and manganese(II) chloride, according to Shurig et al. (1981). The product was obtained as a green solid in a total yield of 48%.

Preparation of Mn-Chelate Capillary Column

The fused silica capillary column was leached with 6 *N* hydrochloric acid, sealed at both ends, heated for 3 hr at 100°C, rinsed with 0.01 *N* hydrochloric acid, and dried.

Deactivation. A capillary was deactivated with Superox-4 0.2% in methylene chloride twice with the static method. Then the capillary was deactivated for 1 hr at 336°C.

Coating. A column was coated with a 0.8% solution of OV-101 in pentane

by the static method, then filled with a mixture of 30 mg OV-101 and 5.7 mg Mn-(II)–bis-[3-heptafluorobutyryl-1 (*R*)-camphorate] dissolved in 5 ml pentane, using the static procedure.

RESULTS AND DISCUSSION

Chemical Analysis of S. baetica *Males*

Analysis by GC-MS (one sample from 1985 and five samples from 1987) showed that the thoracic gland secretion was composed of nerol oxide (MW = 152) and (*Z*)-(*R*)-6-tridecen-2-ol (MW = 198). The proportion between the two components was 1:5. The former was found to be racemic. A mass spectrum of natural (*R*,*Z*)-6-tridecen-2-ol is shown in Figure 1. The double-bond position in the alcohol was determined by ozonolysis, producing the two aldehydes with molecular weights ($C_7H_{14}O$) = 114 and ($C_6H_{12}O_2$) = 116. The (*Z*,*R*) configuration was proved by comparison with the synthetic product; see Figure 2.

Chemical Analysis of A. occitanica *Males*

This species was found to contain nerol oxide and 10-homonerol oxide in the proportion 1:2. Gas chromatography on chiral phases, (a) Ni- and (b) Mn-chelates, respectively, showed that both compounds occurred as racemates; see Figure 3. The enantiomer separation of 10-homonerol oxide was unsatisfactory on the Ni-chelate column; however, the Mn-chelate column gave better results.

Confirmation of Chemical Structures

The structure of the unknown compound (Figure 2) isolated from *S. baetica* was established in the following way. Its mass spectrum indicated that it was a chain-elongated homologue of nostrenol, **1** (Scheme II), containing two methylene groups in the hydrocarbon end relative to the double bond. The proposed structure, **2** (Scheme II), was then confirmed by synthesis.

The synthetic procedure (Scheme II) was similar to that used earlier by us for the preparation of racemic nostrenol, **1** (Baeckström et al., 1989). Thus racemic δ-hexanolide, **3** (Ansell et al., 1968), was reduced to a mixture of the two cyclic ketals of the corresponding hydroxyaldehyde, **4**, or its ring-closed hemiketal forms, and this mixture was reacted with heptylidenetriphenylphosphorane in dimethyl sulfoxide to give racemic (*Z*)-6-tridecen-2-ol **2** (*Z*/*E* ratio, 91/9). Reactions in this solvent give predominantly the *Z*-isomer (Baeckström et al., 1989), which was also confirmed by the IR spectrum (no large peak at 950–1000 cm^{-1}). This compound gave both a mass spectrum and GC retention

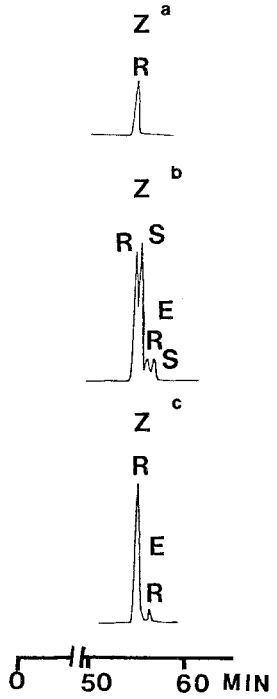


FIG. 2. Enantiomer separation of (*Z*)-6-tridecen-2-ol as isopropyl carbamate derivatives on a 30 m × 0.23-mm-I.D fused silica capillary column, coated with XE-60-(*S*)-valine-(*S*)-2-phenylethylamide, df = 0.24 μm. Temperature program: isothermal at 130°C for 5 min, then programmed temperature 2°C/min to 170°C. Carrier gas, nitrogen (13 psi). (a) Natural (*Z*)-6-tridecen-2-ol, obtained from *S. baetica*; (b) synthetic (*R,S*)-(*Z*)-6-tridecen-2-ol, isomer composition ×91% (*Z*) and 9% (*E*); (c) (*R*)-(−)-(*Z*)-2-tridecen-2-ol ×95% (*Z*) and 5% (*E*).

times on several phases which were identical to those of the natural compound, thus establishing its structure apart from its stereochemistry.

Tridecenol *2* has one chiral center and the natural compound could be either one of its two enantiomers or a mixture of them. When analyzed as the *N*-isopropyl carbamate derivatives on a chiral capillary GC column [XE-60-(*S*)-valine-(*S*)-2-phenylethylamide], the natural compound gave only one peak with the same retention time as the first of the two major peaks that were obtained from the racemic synthetic material (two minor peaks due to the pair of *E*-enantiomers were also observed) (see Figure 2.). When natural or synthetic nostrenol is subjected to the same GC conditions, the natural *R*-form gives rise to the first peak (see Figure 2). If the tridecenol, **2** (Scheme II), behaves as nostrenol, the configuration of the natural material should also be *R* (Scheme

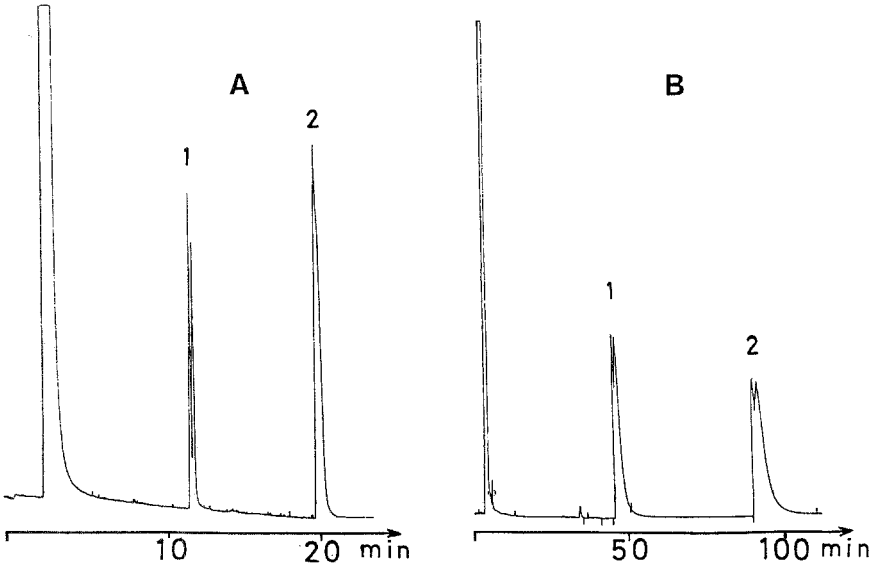


FIG. 3. Separation of extract from *A. occitanica* containing the two enantiomeric pairs (1) nerol oxide and (2) 10-homonerol oxide. Chromatographic conditions: (A) column, Ni-(II)-bis[3-heptafluorobutyryl-1(*R*)-camphorate], 25 m × 0.25-mm i.d; 93°C isothermal; N_2 (8 psi). (B) column, Mn-(II)-bis[3-heptafluorobutyryl-1(*R*)-camphorate], 25 m × 0.25-mm i.d.; 63°C isothermal; N_2 (8 psi).

III). To confirm this we synthesized the two enantiomers of *Z*-6-tridecen-2-ol (**2**) using an enzyme-catalyzed resolution. PPL is known to resolve 2-octanol through selective partial esterification with dodecanoic acid at 70°C in heptane (Gerlach et al., 1987). Thus, (*S*)-(+)-2-octanol and the dodecanoic acid ester of (*R*)-(−)-2-octanol are formed. In analogy with this, we found that when treated with PPL in heptane at 70°C for 16 hr, (*Z*)-6-tridecen-2-ol **2** (Scheme II) and dodecanoic acid gave a 30% yield of the ester and an approximately 60% yield of the (+)-alcohol of approximately 40% ee (compare below). Alkaline hydrolysis of the dodecanoate furnished (*Z*)-(−)-6-tridecen-2-ol **(R-2)** of 95% ee, which was found to be identical to the natural material when analyzed as described above. Secondary alcohols (Beilstein, 1973) such as (*R*)-2-butanol, (*R*)-2-hexanol, (*R*)-2-octanol, and nostrenol **(1)** (Baeckström et al., 1989) all show negative rotation, and by analogy, the natural tridecenol should again be **R-2**.

Independent proof for this assignment was gained from the following experiment. We found that the starting δ-hexanolide, **3** (Scheme II), was partially resolved into its enantiomers by preparative liquid chromatography on triacetylated cellulose using the methodology described earlier (Högberg et al., 1987).

The (+)-form is known to have the (*R*)-configuration (Pirkle, 1979), which was obtained in approximately 5% ee and was converted to (−)-(*R*,*Z*)-6-tridecen-2-ol, **R-2** ($[\alpha]_D^{20}$ −0.4°) using the synthetic protocol described above, thus proving the assigned configuration.

Biochemical Relationships Between Ant-Lion Volatiles

The two species fall into the biochemical pattern found in the three species analyzed earlier. Both species, like *E. nostras*, have nerol oxide (racemic) as one of their two components. *A. occitanica* is characterized by possessing 10-homonerol oxide (racemic), similarly found in *G. bore*, whereas *S. baetica* is characterized by the newly identified tridecenol.

Scheme I shows possible biogenetic relationships (a) between the nerol-derived compounds and (b) between the monounsaturated, secondary alcohols. Mechanisms that could lead to structure variation of the nerol basic skeleton are cyclization (to nerol oxide) and side-chain elongation (to 10-homonerol). The two mechanisms could combine to produce 10-homonerol oxide. Compare, though, studies on the biosynthesis of homoterpenes by Boland and Gäbler (1989) and Gäbler et al. (1991). Nerol oxide occurs in three of the five species studied so far. Furthermore, it is the sole compound, in small amounts, in the females of the first three species studied. Chain elongation of (*Z*)-6-undecen-2-ol adds two carbon atoms.

The composition of the two-compound blends in the five species studied is summarized in Table 1. The six compounds give 15 different theoretical two-compound combinations. Of these, five are seen in the five species; see Scheme I. It is interesting to note that *M. formicarius*, which possesses the biochemically most "simple" (original) composition, is also considered to be a "primitive" species. It is our intention to study other Myrmeleontidae species from other regions, thereby comparing sympatric and nonsympatric species. Behavioral and electrophysiological experiments are planned.

TABLE 1. COMPOUNDS AND THEIR ENANTIOMERIC COMPOSITION IN ANT-LION SPECIES[a]

	E.n.	G.b.	S.b.	A.o.	M.f.
Nerol					—[b]
10-Homonerol					—
Nerol oxide	R,S		R,S	R,S	
10-Homonerol oxide		R,S		R,S	
(*Z*)-6-Undecen-2-ol	R	R			
(*Z*)-6-Tridecen-2-ol			R		

[a]E.n., *E. nostras*; G.b., *G. bore*; S.b., *S. baetica*; A.o., *A. occitanica*; M.f., *M. formicarius*.
[b]Nonchiral.

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RESISTANCE OF CEREALS TO APHIDS: THE INTERACTION BETWEEN HYDROXAMIC ACIDS AND UDP-GLUCOSE TRANSFERASES IN THE APHID *Sitobion avenae* (HOMOPTERA: APHIDIDAE)

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Abstract—UPD-glucose transferases are found in the cytosolic and microsomal fractions of the grain aphid *Sitobion avenae* F. Gel filtration and SDS-PAGE revealed that the microsomal fraction contained several forms of the enzyme. The molecular weights of the three most active fractions might be 68,000, 66,000, and 36,500. There was a negative correlation between the enzymes' activity in extracts of aphids and the concentration of DIMBOA-aglucone in the winter wheat variety fed on by the aphid. A strong inhibition of the activity of the UPD-glucose transferases was observed *in vitro* at a concentration of DIMBOA as low as 0.01 m*M*. There was a greater activity of the enzymes in aphids fed on seedlings of susceptible than on moderately resistant wheat cultivars. Prolonged feeding on resistant cultivars resulted in a further reduction in the activity of the aphid's enzymes. The significance for cereal aphids of the role of their UDP-glucose tranferases in the detoxification of plant allelochemicals and adaptation to resistant varieties of cereals is discussed.

Key Words—Grain aphid, *Sitobion avenae*, Homoptera, Aphididae, detoxi-

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fying enzymes, UDP-glucose transferases, resistance, cereal allelochemicals, DIMBOA.

INTRODUCTION

Three groups of allelochemicals—phenols, hydroxamic acids, and indole alkaloids—are involved in the resistance of cereals to aphids (Todd et al., 1971; Argandoña et al., 1980; Leszczynski et al., 1985; Zuñiga et al., 1985; Dixon, 1987). At least two of them, phenols and hydroxamic acids, are present in undamaged host-plant tissues as glucosides (Wahlross and Virtanen, 1959; Pridham, 1960; Harborne, 1989). When herbivorous insects disrupt plant cells, the glucosides are enzymatically converted into aglucones (Katagiri, 1979; Chipoulet and Chararas, 1985; Lindroth, 1988). As the aglucones are more toxic to insects than glucosides, this results in an increase in the antibiotic effect of the allelochemicals (Ahmad et al., 1986).

Previously it was found that aphid feeding causes an increase in the level of the free aglucone of the hydroxamic acid 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4)-one (DIMBOA) in wheat (Niemeyer et al., 1989a; Leszczynski and Dixon, 1990). Hydroxamic acids are ingested by cereal aphids (Niemeyer et al., 1989b), and almost all of them are subsequently excreted in the glucoside form (Leszczynski and Dixon, 1990). Thus there is a strong indication that cereal aphids, at least to some extent, can metabolize the toxic aglucones.

One mechanism involves the use of detoxifying enzymes, e.g., polysubstrate monooxygenases, which can actively catalyze NADPH-linked cytochrome P-450 reactions and enzymes that conjugate toxic aglucones with either glutathione or a sugar (Morello et al., 1971; Ahmad et al., 1986; Lindroth, 1989). In the aglucone–glucoside interaction the most important detoxifying enzymes are UDP-glucuronyl transferases (EC 2.4.1.17) and UDP-glucose transferases (EC unclassified) (Morello and Repetto, 1979). Detoxification by means of β-glucoside formation occurs widely in the animal kingdom, especially in insects (Smith, 1968). The UDP-glucose transferases (UDPGT) are relatively unknown biochemically and occur in some species in the soluble portion of the cytoplasm (cytosolic fraction) and in others in the microsomes and/or mitochondria (microsomal fraction) (Mehendale and Dorough, 1972; Yang, 1976). A little is known about their role in the metabolism of insecticides (Dorough, 1979; Dauterman, 1985), but nothing about their role in the detoxification of plant allelochemicals.

The activity of UDP-glucose transferases in grain aphid tissue was measured and the molecular weights of some of the UDPGTs present in the microsomal fraction were determined. In addition, the effect of DIMBOA on the activity of these enzymes *in vivo* and *in vitro* was studied.

METHODS AND MATERIALS

Plants. Four varieties of winter wheat were investigated: two moderately resistant, Grana and Saga; and two susceptible, Emika and Liwilla (Leszczynski, 1987). Seeds of the wheats were germinated in a glasshouse kept at 20–25°C during the 16-hr day and 10–15°C during the 8-hr night. The seedlings were grown in a nutrient medium, consisting of fine compost plus sand (F3, Fisons plc), in 10 × 10-cm plastic pots. Twenty-five plants were grown per pot. The plants were regularly watered and no extra fertilizer was added.

Aphids. A green-color form of the grain aphid, *Sitobion avenae* (F), from a stock culture kept at the University of East Anglia, was used. The aphids were reared on 3-week-old seedlings of winter wheat. Aphid performance was measured by placing two fourth-instar aphids on each of 40 seedlings, of each variety, and recording the number of aphids present 7 days later.

Chemicals. DIMBOA was isolated from 500 g of etiolated 6-day-old seedlings of maize var. Gracia using the method of Queirolo et al. (1983). Chemicals used in the protein assay were purchased from BioRad Laboratories (Richmond, CA). Gel filtration was done on a Superose 12 column obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Chemicals required for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) came from Serva (Heidelberg, Germany). All reagents used for estimating the activity of UDPGT were obtained from Sigma Chemical Co. (St. Louis, MO).

DIMBOA Analysis. The concentration of DIMBOA was determined in 5-day-old seedlings using HPLC as described earlier by Leszczynski and Dixon (1990).

Effect of Feeding on Susceptible and Resistant Cultivars of Wheat on the Activity of the Aphid's UPD–Glucose Transferases. Four-day-old seedlings of the four wheat varieties were infested with grain aphids, which were allowed to feed for either 1, 2, 4, or 8 days. Then a mixture of 100 third- and fourth-instar aphids was collected from each cultivar and immediately bioassayed to determine the activity of their UDPGTs.

Enzyme Preparation. The batches of 100 aphids were each placed in 6 ml of cold 0.1 *M* Na-phosphate buffer, pH 7.5, and ground in a glass homogenizer. The homogenate was centrifuged on a L8-70M Beckman Ultracentrifuge at 3000*g* for 15 min and the residue discarded. The supernatant remaining after centrifugation was further centrifuged at 105,000*g* for 1 hr to separate the cytosolic and microsomal fractions. The supernatant, which contained the cytosolic fraction was collected and stored at 0°C until required for analysis, and the precipitate was dissolved and homogenized in another 6 ml of 0.1 *M* Na-phosphate buffer, pH 7.5. All extractions were done at 0–4°C. The cytosolic and microsomal fractions were used to determine the UDP–glucose transferases activity of aphid tissue.

Enzyme Assay. The general activity of UDP-glucose transferases was determined using a slightly modified version of Morello and Repetto's (1979) method. The activity was measured, after several trial runs, in a mixture that appeared to be optimum for such measurements, especially considering the results of Morello and Repetto (1979). It consisted of 1 ml of aphid extract in 0.1 *M* Na-phosphate buffer, pH 7.5, and 0.833 m*M* UDP-glucose, 0.167 m*M* p-nitrophenol, 16.7 m*M* $MgCl_2$, and 0.8% Triton X-100 buffered to pH 7.5. The reaction was started by the addition of UDP-glucose and then the mixture was incubated at 30°C for 30 min, after which the reaction was stopped by the addition of 4 ml of 0.1 *M* trichloroacetic acid. The mixture was then centrifuged at 4000*g* for 15 min, after which 0.1 ml of 12 *M* KOH was added to the supernatant and its absorbance at 400 nm, compared to the blank (without UDP-glucose), was measured in a UV/VIS spectrophotometer. The protein content of the cytosolic and microsomal fractions was determined using the method of Bradford (1976). The activity of the UDP-glucose transferases is expressed as micromoles of conjugated *p*-nitrophenol per 30 min per milligram of protein.

Effect of DIMBOA on the Activity of UDP-Glucose Transferases. The grain aphids used in this study were fed for 24 hr on 4-day-old seedlings of Emika. The activity of the enzymes present in aphid extracts was measured using the method described above. The control contained all the chemicals except DIMBOA and the test mixtures contained either 0.005, 0.01, 0.05, or 0.1 m*M* DIMBOA. All samples were measured against a blank lacking UDPG and DIMBOA.

Gel Filtration. The microsomal fraction extracted from 2000 aphids fed on seedlings of Emika was freeze-dried and suspended in a small volume of 0.1 *M* Na-phosphate, pH 7.5. After centrifugation at 14,000*g* for 30 min, 200 μl of the supernatant was loaded onto a Superose 12 gel filtration column (Pharmacia Fine Chemicals) previously equilibrated with 0.1 *M* Na-phosphate buffer, pH 7.5, contained in 0.15 *M* NaCl. The column was calibrated with reference proteins of known molecular weights. The fplc separation was done on a Pharmacia Fine Chemicals (Uppsala, Sweden) apparatus, the column of which had been eluted with 20 ml of 0.1 *M* Na-phosphate buffer, pH 7.5, contained in 0.15 *M* NaCl (flow rate, 0.5 ml/min). Fractions of 0.25 ml were collected and their enzyme activity was determined.

Gel Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was done on pooled fractions following the method of Laemmli (1970). The gel (70 × 100 × 1 mm) contained 12.5% acrylamide. Bromophenol blue was the tracking dye, and electrophoresis was carried out at 10 mA. After separation the protein bands were stained with Coomassie brilliant blue R-250 and destained in 10% acetic acid. The presence of subunits of UDPGT was revealed using SDS-PAGE as described by Webber and Osborn (1969). The relative mobility of the subunits of UDPGT and the standard proteins—lysozyme (MW 14,300), β-lactoglobulin

(MW 18,400), trypsinogen (MW 24,000), ovalbumin (MW 45,000), and albumin (MW 66,000) was used to estimate the molecular weights of the subunits of UDPGT.

Statistical Analyses. The results given in all the tables and figures are averages of three replicates. The grain aphid performance, the quantity of hydroxamic acids in the wheat seedlings, and the activity of UDP-glucose transferases in the aphid's tissues were subjected to an analysis of variance followed by Duncan's test.

RESULTS

Intracellular Location of the UDP-Glucose Transferases. The UDPGTs were found in extracts of whole aphids. The enzymes were present in both the microsomal and the soluble fractions (Table 1). The general level of activity of the enzymes was greater in the microsomal than in the cytosolic fraction. Gel filtration of the microsomal fraction revealed several forms of the UDPGT. There were three major peaks in the enzymes' activity. The greatest activity occurred in fractions 36–39, with a lesser activity in fractions 52–59 and fractions 67–69 (Figure 1). Apart from the third peak of the UDP-glucose transferase activity, there was no association with absorbance at 280 nm. SDS-PAGE of the active fractions 36–39 revealed two protein bands, which had molecular weights of 68,000 and 66,000, and of fractions 52–59, a protein of molecular weight 36,500, which might be the molecular weights of the enzymes concerned. However, no clear protein band staining with Coomassie brilliant blue R-250 could be detected in fractions 67–69 (Figure 2).

Effect of Feeding on Moderately Resistant and Susceptible Wheats on the Activity of the Aphid Enzymes. The activity of the UDP-glucose transferases in the aphids fed on moderately resistant wheat varieties was much lower than in the aphids fed on susceptible wheats. For the resistant wheats, lower activities

TABLE 1. THE ACTIVITY (μMOL *p*-NITROPHENOL CONJUGATED/30 MIN/MG PROTEIN ± SE) OF THE UDP-GLUCOSE TRANSFERASES PRESENT IN THE CYTOSOLIC AND MICROSOMAL FRACTIONS OF GRAIN APHIDS FED ON THE WHEAT VARIETIES EMIKA AND GRANA

	Intracellular fraction	
Wheat variety	Cytosolic	Microsomal
Emika	0.246 ± 0.048	0.824 ± 0.245
Grana	0.146 ± 0.021	0.550 ± 0.209

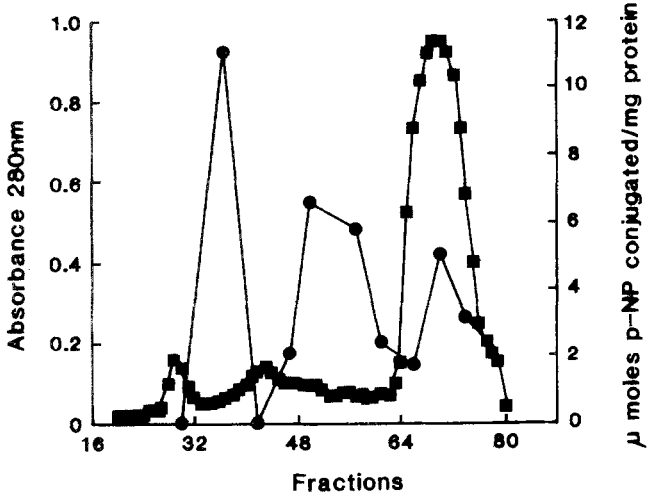

FIG. 1. The protein content, indicated by absorbance at 280 nm (■——■), and the UDP-glucose transferase activity as μmol p-NP conjugated/mg protein (•——•), of fractions of the microsomal extract of the grain aphid.

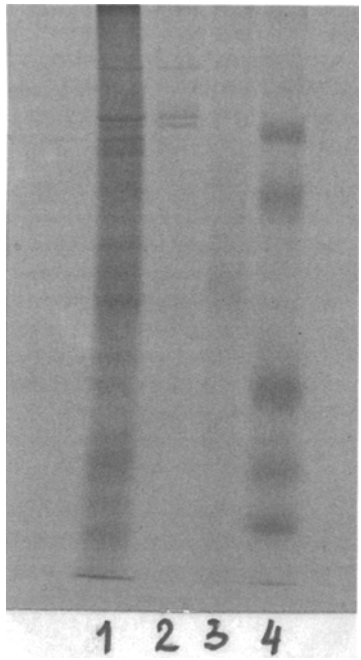

FIG. 2. SDS-polyacrylamide gel electrophoresis of the grain aphid's UDP-glucose transferases: (1) microsomal fraction; (2, 3) fplc of fractions 36–39 and 52–59, respectively; (4) standards lysozyme, β-lactoglobulin, trypsinogen, ovalbumin, and albumin.

were recorded in the aphids fed on seedlings of Saga than in those fed on Grana (Table 2). There was a positive correlation between aphid performance and the activity of UDPGTs in their tissues. However, there was a negative correlation between the activity of the aphid's enzymes and the concentration of hydroxamic acids in the seedlings, especially DIMBOA-aglucone (Table 2).

The highest activity of UDP-glucose transferases was found in aphids that had fed for 24 hr on the wheat seedlings. The activity slowly decreased with time spent feeding on all the varieties (Figure 3). There was a much higher activity in the aphids fed on Emika than on the moderately resistant Saga and Grana varieties. During the aphids' adaptation to feeding on the different varieties of wheat, a bigger reduction in enzyme activity was observed in the aphids fed on the susceptible variety (Figure 3).

Effect of DIMBOA on UDPGT Activity in Vitro. In comparison to the control without DIMBOA (1.308 μM p-NP conjugated/mg protein = 100%), all the concentrations of this allelochemical, which ranged from 0.005 to 0.1 mM, decreased the relative activity of the UDP-glucose transferases (Figure 4). A concentration as low as 0.01 mM reduced the activity of the enzymes by about 65%. Further increases in the amount of hydroxamic acid caused a further reduction in the activity of the UDPGT, but not to the same dramatic extent.

DISCUSSION

Conjugation of a foreign compound with glucose appears to be one of the major means of detoxification in insects. They then excrete and/or store allelochemicals in the less toxic glucoside form (Kent and Brunet, 1959; Smith and Turbert, 1961; Teas, 1967). Although UDP-glucose transferases have been reported from many insects (Smith, 1968), they are reported here for the first time from cereal aphids. There is some UDPGT activity in the cytosolic fraction, but most of it occurred in the microsomal fraction. Because of their microsomal location, these enzymes can easily interact with the cytochrome P-450-linked monooxygenases.

There was a clear negative correlation between the activity of the UDP-glucose transferases in aphids and the concentration of DIMBOA in the wheat variety on which they had been reared. This suggests that high concentrations of DIMBOA, the main hydroxamic acid in wheat (Niemeyer, 1988), reduces the activity of the grain aphid's UDPGT. This was particularly clear when the aphids fed on Saga, which contains the highest concentration of the highly toxic DIMBOA-aglucone. The inhibitory effect of DIMBOA on the activity of UDPGT was confirmed *in vitro*, where a marked reduction in enzyme activity (about 65%) was observed even at a concentration as low as 0.01 mM. However, from our results it is not clear whether it is acting as a competitive or a non-

TABLE 2. THE ACTIVITY (μMOL *p*-NITROPHENOL CONJUGATED/30 MIN/MG PROTEIN ± SE) OF THE UDP-GLUCOSE TRANSFERASES IN EXTRACTS OF GRAIN APHIDS FED FOR 48 HR ON SEEDLINGS OF MODERATELY RESISTANT AND SUSCEPTIBLE VARIETIES OF WHEAT AND THE CONCENTRATION OF DIMBOA DERIVATIVES (μMOL/G FRESH WEIGHT ± SE) AND APHID PERFORMANCE (NUMBER OF APHIDS/SEEDLING ± SE AFTER 7 DAYS) ON THESE VARIETIES[a]

Variety of wheat	Enzyme activity	DIMBOA[b]			Aphid performance[b]
		Glucoside	Aglucone	Total	
Resistant					
Saga	0.14 ± 0.037 c	1.24 ± 0.07 b	1.44 ± 0.07 a	2.68 ± 0.14 b	6.75 ± 2.05 a
Grana	0.495 ± 0.059 b	2.19 ± 0.14 a	1.16 ± 0.07 b	3.35 ± 0.21 a	8.93 ± 4.03 a
Susceptible					
Liwilla	0.606 ± 0.094 a	1.44 ± 0.11 b	0.68 ± 0.05 d	2.12 ± 0.16 c	12.80 ± 6.60 a
Emika	0.666 ± 0.081 a	1.04 ± 0.12 c	0.85 ± 0.04 c	1.89 ± 0.15 c	11.78 ± 2.68 a

[a]The values in a column not followed by the same letter are significantly different ($P < 0.01$, Duncan's test).
[b]After Leszczynski and Dixon (1990).

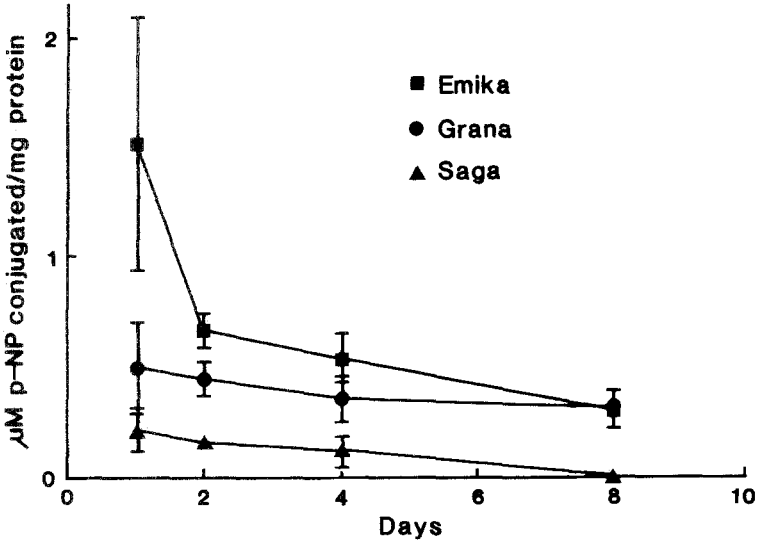

FIG. 3. The activity of the UDP-glucose transferases (±SE) in extracts of grain aphids fed on moderately resistant and susceptible winter wheat cultivars for 1, 2, 4, or 8 days.

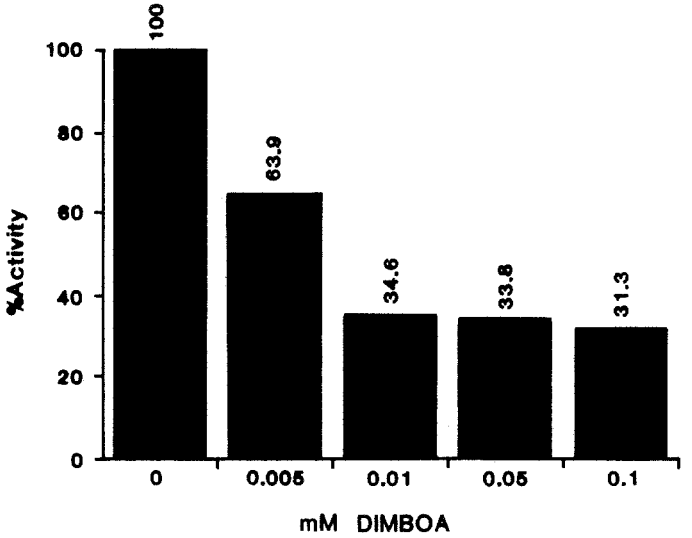

FIG. 4. Effect of a range of concentrations of DIMBOA on the activity of the grain aphid's UDP-glucose transferases.

competitive inhibitor, but hydroxamic acids are well-known as inhibitors of other enzymes such as ribonucleotide reductase (Kjoller et al., 1982), urease (Fishbein, 1982), ATPase, and enzymes involved in ATP synthesis (Queriolo et al., 1983) as well as α-chymotrypsin (Cuevas et al., 1990) and papain (Perez and Niemeyer, 1989).

Therefore it is possible that some of the DIMBOA-glucoside ingested passes through the aphid's alimentary canal and is excreted in the honeydew. However, because there may be several forms of UDP-glucose transferases, which are able to conjugate many substances differing in molecular structure (Trivelloni, 1964), it is possible that not all of the izoenzymes are inhibited by DIMBOA. In addition, it is also possible that the biosynthesis of the DIMBOA-glucoside is another mechanism by which DIMBOA is detoxified.

However, our results indicate that the hydroxamic acids in moderately resistant wheat cultivars not only exert a direct antibiotic influence on cereal aphids (Argandoña et al., 1983) but also seriously reduce the effectiveness of one of the most important biochemical means of detoxifying other groups of xenobiotics. This is especially so for the phenolic compounds, which have a very adverse effect on cereal aphid performance (Todd et al., 1971; Dreyer and Jones, 1981; Leszczynski et al., 1985), because UDP-glucose transferases play a significant role in the detoxification of phenolic aglucones (Smith, 1968; Ahmad et al., 1986). Thus the allelochemicals in moderately resistant wheat cultivars

might act synergistically (Berenbaum, 1985) and protect the wheat's resistance mechanisms against the grain aphid's detoxifying enzymes.

The results presented here and in an earlier study (Leszczynski and Dixon, 1992) indicate that, of the two groups of transfer enzymes in the grain aphid, the glutathione *S*-transferases are less sensitive to the concentration of hydroxamic acids naturally occurring in wheat tissues than are the UDP-glucose transferases. Thus the cytosolic glutathione *S*-transferases seem to be more effective in detoxifying cereal allelochemicals and might play a more important role in the aphid's adaptation to their host plants. To prove this, a further study of the aphid detoxification system, in particular the role of the cytochrome P-450-dependent polysubstrate monooxygenases in detoxifying cereal allelochemicals, is needed.

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Dendroctonus pseudotsugae HOPKINS (COLEOPTERA: SCOLYTIDAE): PRODUCTION OF AND RESPONSE TO ENANTIOMERS OF 1-METHYLCYCLOHEX-2-EN-1-OL

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Abstract—Female Douglas-fir beetles, *Dendroctonus pseudotsugae*, produced an average 45:55 mixture of the (*S*)-(−)- and (*R*)-(+)-enantiomers of 1-methylcyclohex-2-en-1-ol (MCOL). The (*S*)-(−):(*R*)-(+) ratio of 3-methylcyclohex-2-en-1-ol (seudenol) produced by this population of females was 34:66. Lindgren funnel traps baited with (*R*)-(+)-MCOL attracted significantly more males and females than (*S*)-(−)-MCOL-baited traps, which captured significantly more beetles than unbaited controls. The combined effect of the enantiomers was additive, rather than synergistic. Either enantiomer of MCOL increased catches by frontalin-baited traps. Racemic MCOL can be used for trapping Douglas-fir beetles in south-central British Columbia.

Key Words—*Dendroctonus pseudotsugae*, Douglas-fir beetle, Coleoptera, Scolytidae, aggregation pheromone, production, response, methylcyclohexenol, seudenol, enantiomers, trapping.

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INTRODUCTION

The chemical ecology of the Douglas-fir beetle, *Dendroctonus pseudotsugae* Hopkins, is perhaps better known than for any other species of North American bark beetles (Borden, 1982; and references therein). The main aggregation pheromones, frontalin [1,5-dimethyl-6,8-dioxabicyclo(3.2.1)octane] and seudenol (3-methylcyclohex-2-en-1-ol), and the antiaggregation pheromone MCH (3-methylcyclohex-2-en-1-one) have been known for this insect for many years (Pitman and Vité, 1970; Kinzer et al., 1971; Vité et al., 1972). Libbey et al. (1983) identified MCOL (1-methylcyclohex-2-en-1-ol), an isomer of seudenol, as an additional aggregation pheromone for this species.

In recent years chiral-specific activity of pheromones and the intra- and interspecific roles of their enantiomers have been studied in scolytids (Birch et al., 1980; Borden et al., 1980, 1986; Miller et al., 1989). Data of Mori et al. (1987) suggested that racemic MCOL acted synergistically with frontalin for *D. pseudotsugae*, but additional tests were proposed to determine the effect of the two enantiomers.

The objective of this study was to determine the mean ratio of MCOL enantiomers produced in a Douglas-fir beetle population and the response by *D. pseudotsugae* to the enantiomers alone and in combination when used as attractants by themselves and in combination with frontalin.

METHODS AND MATERIALS

Experimental Beetles and Volatile Collection. Douglas-fir bolts, *Pseudotsuga menziesii* (Mirb.) Franco, naturally infested with Douglas-fir beetles, *D. pseudotsugae*, were obtained near Kamloops , British Columbia. Beetles emerging from caged logs were collected daily and sexed. Females were confined individually with gelatin capsules (Borden, 1967) on uninfested, overwintered Douglas-fir logs. Female-produced frass was removed from the capsules every 24 hr and accumulated in a cylindrical Pyrex glass aeration chamber over the 5-day aeration. In this manner a total of 50 cm^3 of frass from 150 females was used in order to obtain sufficient material for the analysis. Charcoal-filtered air was drawn through the aeration chamber by a water aspirator at 0.5 ml/min for 5 days, and volatiles continuously trapped on Porapak Q. Volatiles were eluted from the Porapak Q with 30 ml of redistilled pentane. The solution was cooled in ice water and concentrated under a fine stream of nitrogen to 0.2 ml.

Volatile Analyses. GC and GC-MS analyses of volatiles were carried out with a Hewlett Packard 5830A gas chromatograph equipped with a glass column (30 m × 0.5-mm i.d.) coated with SP-1000 (Supelco Inc., Bellefonte, PA 16823) and a Hewlett Packard 5985B GC/MS/DS fitted with a fused silica column (30 m × 0.32-mm i.d.) coated with SP-1000 (J&W Scientific Inc.,

Folsom, CA 95630). Micropreparative GC was conducted with a Varian 1200 instrument fitted with a 3.05 m × 3.18-mm o.d. stainless-steel column packed with 10% SP-1000 on Chromosorb W AW (100/120) (Supelco Inc. Bellefonte, PA 16823), a 10:1 effluent splitter, and a thermal gradient collector (Brownlee and Silverstein, 1968). Helium was used as carrier gas at a flow rate of 40 cm/sec. Optical isomers of MCOL and seudenol were analyzed with a Hewlett Packard 5985B GC-MS fitted with a Chirasil-Dex (8) column (25 m × 0.25-mm i.d.) (V. Schurig, University of Tübingen, Germany).

Field Experiments. Two hundred milligrams each of (*R*)-(+)-MCOL and (*S*)-(−)-MCOL were produced as described by Mori et al. (1987). The enantiomers were loaded (20 μl) into glass capillaries (45 × 1-mm i.d.) sealed at one end. The release rate from each capillary was estimated at 0.1 mg/24 hr at 24°C by measuring the drop of the meniscus. Actual release rates in the field are temperature dependent. The enantiomeric purity of the enantiomers was estimated by Mori et al. (1987) as about 90% enantiomeric excess (95:5 ratio) for both enantiomers.

On May 3, 1989, Lindgren 12-funnel traps were set up in a 4 × 4 Latin square design experiment at the Malcolm Knapp Research Forest, Maple Ridge, in the coastal Douglas-fir zone of British Columbia. Treatments consisted of (1) unbaited control, (2) one capillary of (*R*)-(+)-MCOL, (3) one capillary of (*S*)-(−)-MCOL, and (4) one capillary of each enantiomer. In this manner the release rate of each enantiomer was held constant among treatments 2 to 4. Beetles were collected on May 10 and May 16 and stored at −18°C until the beetles could be counted and their sex determined.

A second 4 × 4 Latin square was established on May 24 at Scottie Creek Road, near Cache Creek, in the interior Douglas-fir zone. The traps were set up in a stand of mature Douglas-fir with patches of immatures. The treatments were as above except that all traps were also baited with frontalin released from Flex lures (Phero Tech Inc., Delta, B.C., Canada) at 0.6 mg/24 hr at 24°C as measured by GC analysis of Porapak Q-collected volatiles in the laboratory. Beetles were collected on June 5 and stored as above until counting and sex determinations could be done.

At the end of the second experiment all frontalin release devices were removed, and the MCOL treatments were reallocated by rerandomization. Beetles from this experiment were collected on June 15 and stored as above.

Statistical Analysis. Pooled data from each of the experiments were subjected to analysis of variance ($\alpha = 0.05$), and when significant, means were separated by Duncan's multiple-range test. There was no treatment by experiment interaction in experiments 1 and 3, so these were analyzed as a replicated Latin square (Steel and Torrie, 1960). Furthermore, there were no other significant interactions, so these effects were pooled into the error term. Prior to analysis of numbers of beetles captured, the data were transformed as $x' =$

$\log(x + 1)$ to correct for heterogeneity of variances. Percentage data were transformed as $p' = \arcsin\sqrt{p}$, where p is a proportion.

RESULTS AND DISCUSSION

Volatile Analyses. Frass volatiles of female *D. pseudotsugae* contained all four known pheromones: frontalin, MCOL, seudenol, and MCH (Figure 1). MCOL produced by this population of female *D. pseudotsugae* was comprised of 45 and 55% of the (*S*)-(−)- and (*R*)-(+)-enantiomer, respectively (Figure 2). Seudenol produced by these females contained 34 and 66% of the (*S*)-(−)- and (*R*)-(+)-enantiomer, respectively. Intrapopulation variation in the production of MCOL enantiomers could not be determined since the amount produced by individual beetles is too low to be analyzed. Therefore, it is possible that considerable variation within and among populations occurs, as was found for *I. pini* (Miller et al., 1989).

Field Experiments. Traps baited with (*R*)-(+)-MCOL caught significantly more *D. pseudotsugae* of both sexes than traps baited with (*S*)-(−)-MCOL

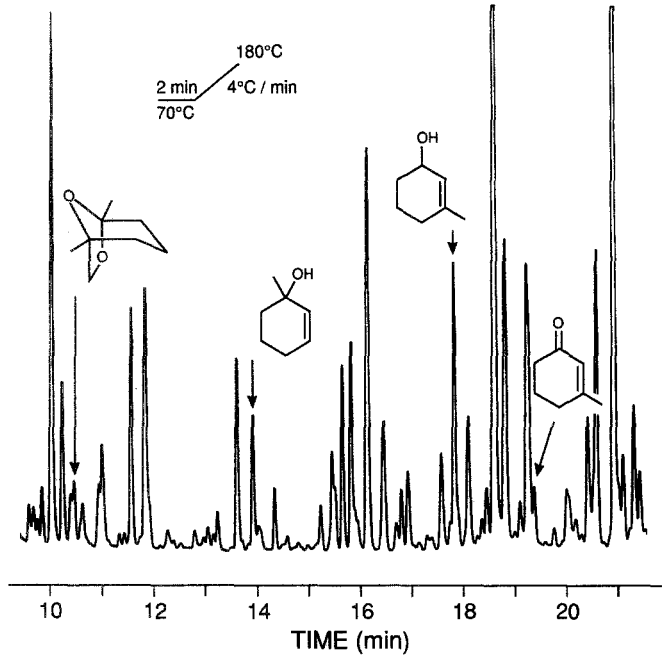

FIG. 1. Gas chromatogram of volatiles from frass of female *D. pseudotsugae*. Unidentified peaks are mainly terpenoid host compounds.

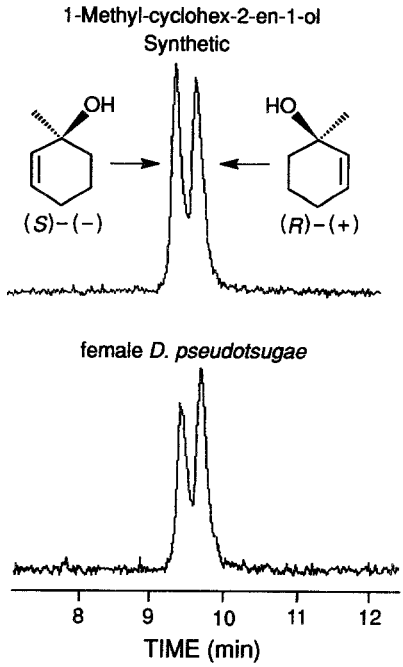

FIG. 2. Selected ion chromatogram of the base ion (m/z 97) of synthetic (top) and female-produced MCOL (bottom). Column, Chirasil-Dex (8); temperature program, 50°C (0.5 min)-20°C/min-85°C (10 min).

(Table 1). (*S*)-(−)-MCOL-baited traps caught significantly more beetles than unbaited control traps when both sexes were analyzed together (Table 1). Traps baited with racemic MCOL did not capture significantly more beetles than (*R*)-(+)-MCOL (Table 1). However, the analysis of variance of enantiomer interaction indicates that the combined effect of (*R*)-(+)- and (*S*)-(−)-MCOL is additive ($P = 0.841$ for males, $P = 0.998$ for females, and $P = 0.631$ for males and females), rather than synergistic, and both enantiomers are required for maximum response. Due to the limited availability of the enantiomers, their optimal ratio could not be determined in this study.

In the second experiment MCOL significantly increased catches to frontalin-baited traps (Table 2). Racemic MCOL significantly increased the proportion of females over (*S*)-(−)-MCOL and frontalin alone. (*R*)-(+)-MCOL significantly increased the proportion of females over (*S*)-(−)-MCOL but was not different from racemic MCOL or frontalin. (*S*)-(−)-MCOL did not change the proportion of females captured significantly compared to frontalin alone.

As (*R*)-(+)-MCOL caught significantly more beetles than (*S*)-(−)-MCOL

TABLE 1. CATCHES OF DOUGLAS-FIR BEETLES IN LINDGREN FUNNEL TRAPS BAITED WITH ENANTIOMERS OF 1-METHYLCYCLOHEX-2-EN-1OL (MCOL)[a]

Treatment	Mean number (±SD) of beetles caught[b]		
	Males	Females	Total
Control	0.13 (±0.35) a	0.00 (±0.00) a	0.13 (±0.35) a
(*S*)-(−)-MCOL	2.38 (±5.15) a	2.13 (±4.85) a	4.50 (±9.96) b
(*R*)-(+)-MCOL	5.88 (±4.26) b	4.38 (±3.34) b	10.25 (±7.11) c
Racemic MCOL	10.63 (±7.42) b	9.00 (±7.21) b	19.63 (±13.98) c

[a] Malcolm Knapp Research Forest, Maple Ridge, B.C., May 3–16, and Scottie Creek Road, Cache Creek, B.C., June 5–15, 1989 (N = 8).
[b] Means within columns followed by the same letter are not significantly different, Duncan's multiple-range test (α = 0.05).

TABLE 2. EFFECT OF ENANTIOMERS OF 1-METHYLCYCLOHEX-2-EN-1-OL (MCOL) ON CATCHES OF DOUGLAS-FIR BEETLES IN LINDGREN FUNNEL TRAPS BAITED WITH FRONTALIN[a]

Treatment	Mean number (±SD) of beetles caught[b]			% (±SD) females[b]
	Males	Females	Total	
Frontalin (F)	167.0 a (±131.5)	116.5 a (±104.9)	283.5 a (±236.2)	29.1 bc (±5.5)
F + (*S*)-(−)-MCOL	290.3 b (±160.3)	176.3 b (±151.2)	498.5 b (±309.6)	31.9 c (±4.0)
F + (*R*)-(+)-MCOL	352.3 b (±162.2)	184.0 b (±142.2)	536.3 b (±304.3)	37.9 ab (±5.7)
F + racemic MCOL	360.5 b (±198.4)	208.3 b (±155.1)	536.8 b (±346.3)	40.3 a (±8.8)

[a] Scottie Creek Road, Cache Creek, B.C., May 25–June 5, 1989 (N = 4).
[b] Means within columns followed by the same letter are not significantly different, Duncan's multiple-range test (α = 0.05).

(Table 1) and, when combined with frontalin, significantly increased the proportion of females captured (Table 2), there is evidence of enantiospecific activity of MCOL. Enantiospecific production, reception, and behavioral activity of scolytid pheromones such as bicyclic acetals and terpene alcohols have been documented previously (Vanderwel and Oehlschlager, 1987). Our data show that *D. pseudotsugae* successfully discriminates between enantiomers of a

cyclohexenol. Since the spruce beetle, *D. rufipennis* (Kirby), produces and responds to the same pheromone components as *D. pseudotsugae*, it is possible that enantiospecific responses to MCOL prevent cross attraction in areas where the two species are sympatric. For example, in south-central British Columbia *D. rufipennis* responds preferentially to (*R*)-(+)-MCOL, while the (*S*)-(−)-enantiomer is inhibitory (J.H. Borden, Simon Fraser University, Burnaby, B.C., Canada, personal communication).

From a management perspective, racemic MCOL can be used for monitoring or mass trapping of Douglas-fir beetle populations, at least in the areas where these experiments were conducted. However, there may be merit in further investigating geographic variation in production and response by Douglas-fir beetles to MCOL and determining the optimal enantiomeric ratio, MCOL dose, and optimal ratios of MCOL and frontalin.

Considering the 34:66 ratio of the (*S*)-(−)- and (*R*)-(+)-enantiomers of seudenol produced by female Douglas-fir beetles, and sex-specific effects of seudenol at higher doses (Lindgren and Miller, unpublished), the role of this pheromone should be further investigated. Furthermore, there is a potential of interaction between MCOL and seudenol which may be important for optimum aggregation. Further refinement of a lure for Douglas-fir beetle trapping may provide a useful tool for population management.

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DEVELOPMENT AND EVALUATION OF SYSTEMS TO COLLECT VOLATILE SEMIOCHEMICALS FROM INSECTS AND PLANTS USING A CHARCOAL-INFUSED MEDIUM FOR AIR PURIFICATION

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Abstract—A system is described for the collection of volatiles produced by plants and insects that minimizes stresses on the plant or insect in an environment that is free from chemical impurities. Air entering a volatile collection chamber containing insects and/or plants was purified using a nonwoven fabric medium infused with charcoal. When three layers of this material were used, the total amount of impurities detected by gas chromatography was less than 40 ng/hr at a collection rate of 1 L/min. The air filtration system can maintain this level of air purification for 96 hr at an air flow of 0.43 m/sec, or a total volume of approximately 750,000 L of air. The air filtration system did not alter the relative humidity of the purified air compared to the relative humidity of ambient air. A multiport collector system was developed for use with the insect volatile collection system and enabled up to three samples to be collected without disturbing the system.

Key Words—Collection system, volatile collections, air purification, charcoal cloth.

INTRODUCTION

To understand insect chemical communication systems fully and to maximize the potential development and practical use of volatile semiochemicals produced by insects and plants for insect control, the chemicals emitted must be collected

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and analyzed both qualitatively and quantitatively. Although it is possible to gain much information about insect semiochemicals from solvent extracts of insects or parts of insects (see, e.g., Sower et al., 1973; Klun et al., 1980; Teal et al., 1981), the blend of volatiles actually emitted by the insect is often uncertain. Many compounds found in extracts of insect ovipositor glands are not volatilized by the insect, and often those compounds that are volatilized are not released at the same ratio as found in the gland (Teal et al., 1986; Heath et al., 1991). Similarly, identification of "volatile" components from plants using methods such as extraction with solvent or steam distillation most often results in the identification of a large number of organic compounds that are not representative of the chemicals released by the plant (Tollsten and Bergstrom, 1988). The results of these studies prompted us to develop systems that can be used to collect volatiles from various biological sources in such a manner that we minimized the stress that is placed on the organism being studied, as well as minimizing the collection of unwanted background organic compounds from the surrounding environment.

Several systems for the collection of volatiles from insects have been published (e.g., Baker et al., 1981; Tumlinson et al., 1982; Golub et al., 1983; Shani and Lacey, 1984; Golub and Weatherston, 1984). However, the efficiency of these collection systems has not been well defined and the stress placed on the plants and/or insects contained in these systems is often considerable. Development of systems for collection of volatiles from unaltered plants in their natural environment has been limited. Advances in this area of research have been restricted due to the inability to purify the large volumes of air at high flow rates that are needed to surround completely either the whole plant or the parts of the plant under investigation. These large volumes of pure air are required to prevent the intrusion of ambient air from back-flowing into the system, resulting in contamination of samples collected from a plant.

We report in this paper a method that permits purification of large volumes of air and the development of systems for use in the collection of volatiles from insects and plants.

METHODS AND MATERIALS

Volatile Collection System (VCS)

The collection system consisted of four main components (Figure 1). An air delivery system (ADS) is attached to a passive air filtration system (AFS) which purifies the incoming air. Attached to the AFS is a volatile collection chamber (VCC), a cylindrical glass sleeve in which purified air surrounds the subject from which volatiles are being collected, and finally, the volatile col-

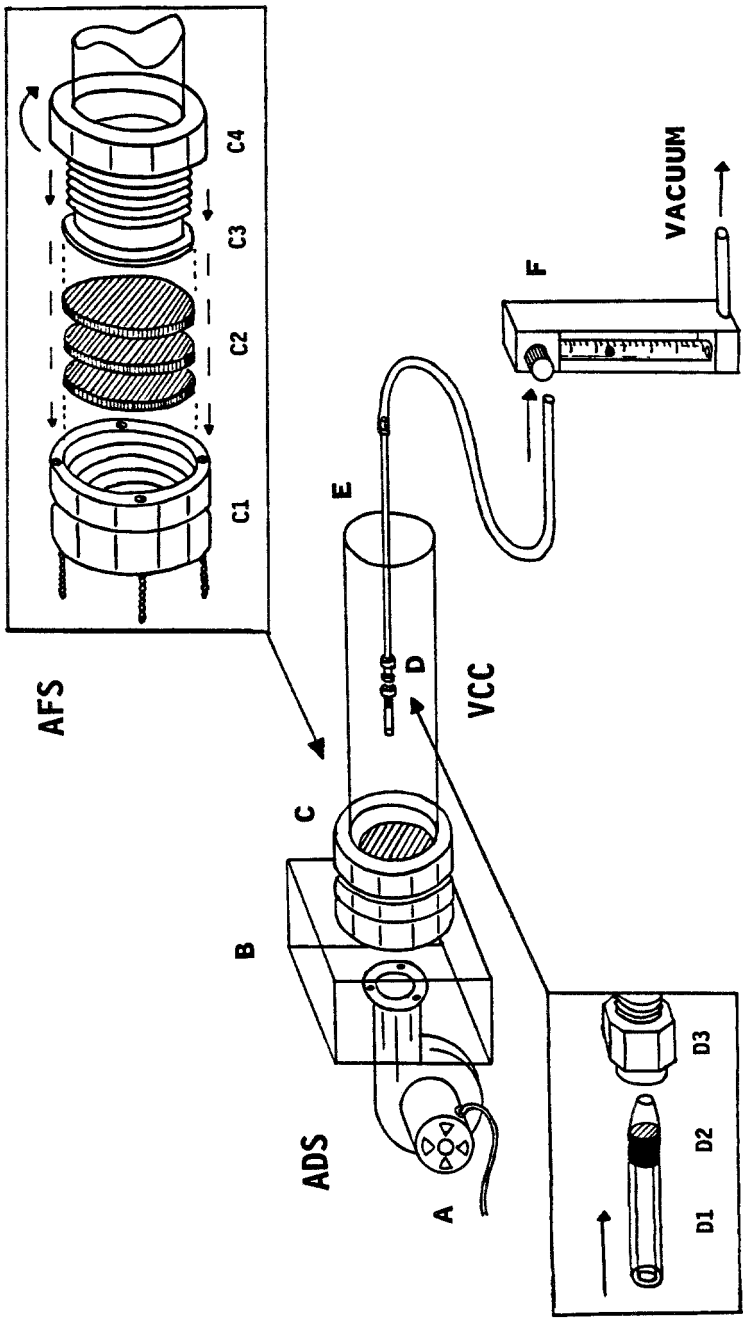


FIG. 1. Drawing of the collection system designed for the collection of volatiles released from plants. System components are as follows: air delivery system (ADS), air filtration system (AFS), volatile collection chamber (VCC), and volatile collector trap (VCT).

lector trap (VCT), which contains a polymeric adsorbent used to entrap volatile organics within the chamber for further chemical analyses.

Air Filtration System (AFS)

The AFS consists of two components, the filtering medium and the holder (Figure 1, C-AFS). The filter holder assembly (Figure 1, C1–C4) is an 8.9-cm phenolic coupler (Model S-6750-010, Southern Scientific Inc., Micanopy, FL). Filter material used in this assembly (Figure 1, C2) was a nonwoven polyester fiber infused with activated carbon. The filter media tested in this investigation included 9.0 oz (fabric weight) material containing 150% activated carbon (13.5 oz carbon/sq yd, P/N ACF-NWPE-9-150P) and 12.0 oz material also containing 150% activated carbon (18 oz carbon/sq yd, P/N ACF-NWPE-12-150P). This material was obtained from Lewcott Corp. (Millbury, MS). The activated carbon material (1.27 cm in thickness) was cut into 9.5-cm-diameter circles and placed between the first half of the coupler (C1) and the glass flange (C3) as shown in Figure 1. The other half of this coupler (C4) is placed over the open end of the collection chamber glass tube (E) and slid down till it reaches a glass flange (vide infra) on the opposite end. This piece (C4), along with the glass flange (C3), is then used to compress one to three sections of filter material on an internal flange on the mounted coupler (C1). The entire assembly is screwed together by hand force.

Air Delivery System (ADS)

Ambient air was pushed through the filters using a "squirrel-cage" type shaded pole blower (Figure 1, A) capable of delivering 15 cfm in free air at 3160 rpm at 115 AC and 0.21 A, with an air-flow cutoff at 0.22 in. of water static pressure (Dayton Electric Model 2C782, W.W. Grainger, Jacksonville, FL). The blower was connected to an AC variable autotransformer (Powerstat Type 116B, Superior Electric Co., Bristol, CT) and adjustment of the line voltage to the blower motor varied the air velocity through the volatile collection system. The output of the blower was directed through a 2.54-cm-diameter hole contained in a 13.5 cm square × 6.7 cm wide Plexiglas box. This box (Figure 1, B) was used for connecting the blower motor to the filter section as well as serving as an air diffuser to ensure an evenly distributed air flow across the filter medium. The AFS (C) was mounted to the air diffuser box by four bolts centered on an 8.9-cm-diameter hole.

For comparing this system with previously developed air purification systems, air provided by a laboratory air compressor was pushed through two in-line charcoal filters. These filters were made of 1.6-cm-OD (0.05-mm wall thickness) × 12.7-cm stainless-steel tubing and contained 28-mesh activated charcoal (Alfa Products, Danvers, MA) packed between two 325-mesh stainless-

steel screens held in place by 1.6- to 0.64-cm reducing unions (Swagelok Co., Solan, OH). Prior to use the two in-line filters were cleaned by baking them at 200°C for 24 hr with a flow of ultrapure nitrogen at 2 ml/min. These filters were placed in a vertical position to prevent the formation of channels or voids that might allow air to pass without being filtered by the charcoal.

Volatile Collection Chamber (VCC)

Two chamber configurations for the VCS were constructed of 0.32-cm-thick Pyrex glass by Southern Scientific Inc. Both chambers were 25.7 cm long and 7.6 cm in ID and contained a 9.21-cm-OD × 0.064-cm-thick flange (Figure 1, C3) attached to one end of the Pyrex glass cylinder. The system used for providing purified air over plants (P-VCS) used a chamber that was open at one end (Figure 1, E). The insect volatile collection system (I-VCS), shown in Figure 2, used a VCC that contains a 50/55-mm male ground-glass joint. A multiport collector base (Figure 2, MCB) constructed of Pyrex contained (in addition to the 50/55-mm female ground-glass joint for connecting to the insect chamber), two 10/18-mm joints and one 14/20-mm female ground-glass joint. A vacuum tube thermometer adapter was connected to the 14/20-mm joint and provided a port (Figure 2, MCB-A) that could be used to purge the system. A 0.64-cm-OD × 0.5-cm-ID stainless-steel tube is inserted through each of the three joints and connected using Teflon thermometer adapters (Ace Glass, Vineland, NJ). The collector traps were connected to the stainless-steel tubing using 0.64-cm unions and 0.64-cm-ID Teflon ferrules. The outlet of the stainless-steel tubes and the purge outlet were connected to a vacuum manifold using Tygon tubing. The vacuum manifold (Figure 2, MPV) consisted of a four-way, multiport valve (Whitey B-43ZF2, Swagelok Co., Solan, OH), a flow meter set at 1 L/min, and a vacuum pump. The manifold directed the vacuum flow for the collection of volatiles to one of the three collector traps or enabled the purging of the system. Initially, all glass was acid-washed and heated to 400°C for 48 hr. Before each volatile collection, the I-VCC or P-VCC was washed with deionized water and acetone and heated at 400°C for a minimum of 6 hr.

Volatile Collector Trap (VCT)

The collector traps (Figure 1, D, and Figure 2, VCT) used to trap organic volatiles were made from a 4.0-cm-long × 4.0-mm-ID piece of glass tubing (Figure 1, D1) and contained 20 mg of Super-Q (Altech Assoc. Inc., Deerfield, IL) as the adsorbent. Two stainless-steel frits were used to contain the adsorbent (Figure 1, D2). The collector traps were connected to stainless-steel tubing using 0.64-cm unions and 0.64-cm-ID Teflon ferrules (Figure 1, D3). These traps were cleaned by soxhlet extraction using ultra-high-purity methylene chloride (Burdick & Jackson GC^2 GC-MS grade solvent, Catalog No. GC60616-4,

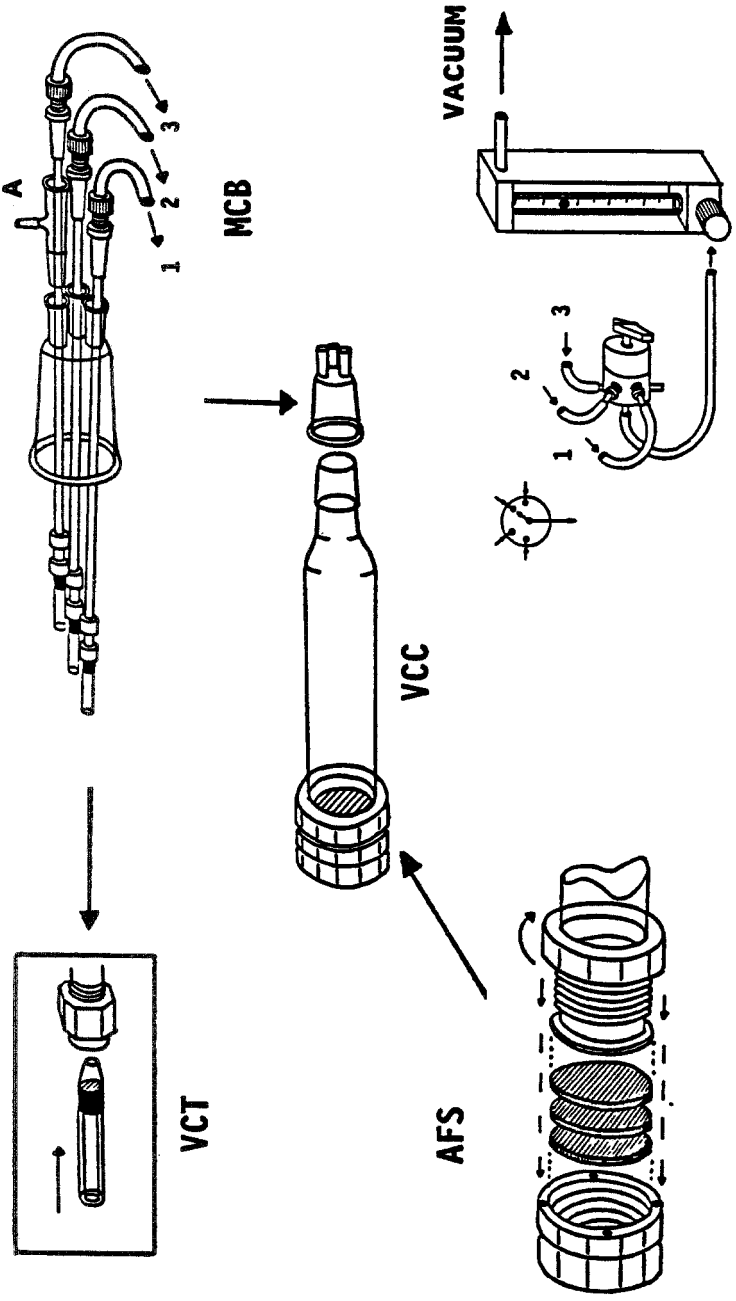


FIG. 2. Drawing of the collection system designed for the collection of volatiles released from insects. System components are as follows: air filtration system (AFS), volatile collection chamber (VCC), volatile collector trap (VCT), and multiport collector base (MCB).

Muskegon, MI) for 24 hr and dried in a fume hood prior to use. Volatiles collected on the traps were eluted with 100 μl of the high-purity methylene chloride and then an appropriate amount of tridecan-1-ol acetate was added as internal standard for subsequent analyses.

Analyses of Volatiles

Gas chromatographic analyses were conducted using a Hewlett-Packard Model 5890A gas chromatograph, equipped with a cool on-column capillary injector (septum injector) and flame ionization detector (FID). Helium was used as the carrier gas at a linear flow of 18 cm/sec and the temperature program was initially isothermal at 60°C for 2 min, then temperature programmed at 20°/min to 180°C. The chromatographic data were collected and processed using the Perkin-Elmer Nelson Turbochrom II chromatographic software package running on an IBM-type PC/AT/386 system. Capillary gas chromatography (CGC) was done using a retention gap column prior to the capillary column. This system permitted the injection of samples without concentration in 5–100 μl of solvent (Grob, 1982; Murphy, 1989). A combination of three fused silica columns connected in series using GlasSeal connectors (Supelco Inc., Bellefonte, PA) was used. The primary deactivated fused silica column, 8 cm × 0.5-mm ID, was connected between the injector and the retention gap column. This primary column permitted the use of 0.4-mm-OD stainless-steel needles with a septum injector. The retention gap columns used were 10 m × 0.25-mm-ID deactivated fused silica (Quadrex, New Haven, CT). The analytical columns used for analyses were a 30 m × 0.25-mm-ID Supelcowax 10 (bonded Carbowax) and a 50 m × 0.25-mm-ID BP-1 purchased from Quadrex.

Experiment 1. Initial tests were conducted to determine the volume of air that could be supplied through the AFS containing one to three layers of the 12-oz, 150-carbon infused fabric. Various air flows were obtained via control of the fan motor voltage. Air velocities were measured using a Teledyne Hastings-Raydist hot-wire anemometer (Model AB-27, Hampton, VI). System back-pressures developed at these various air velocities using one to three filters were recorded using an MKS Instruments baratron tube electromechanical pressure transducer (Model 223BD-00010AA, Burlington, MS) attached to a Hewlett Packard 4.5-digit system voltmeter (Model HP3437A, Loveland, CO) capable of sampling voltages at rates up to 5700 samples/sec. The pressure transducer measured the system back-pressures between the blower and the filter medium, covering a range of 0 to 5 in. of water static pressure ("H_2O" SP) and producing a linearized DC voltage output between 0 and 5 V DC.

Experiment 2. Additional tests were conducted to determine the ability of the AFS to pass water vapor through its filtering medium in order to provide a humid environment for either plants or insects. Tests were performed by increas-

ing the relative humidity (RH) content of the incoming ambient lab air by using a household centrifugal-type humidifier (Kaz Model 564B, New York). Equilibrium times for changes in percentage relative humidity (% RH) were determined by initially measuring the ambient humidity inside the volatile collection chamber and then comparing the humidity over time in the VCC when 100% RH saturated air was introduced and then removed from the air intake of the blower. RH measurements were made using an Omega digital thermohygrometer (Model RH411, Stamford, CT) with simultaneous digital and linearized DC voltage output of both temperature and RH connected to a strip-chart recorder.

Experiment 3. The ability of the AFS to purify air at an air flow of 0.43 m/sec was determined using one, two, and three filters of the 12-oz, 150-carbon material over a 144-hr period using the P-VCS. Air samples were collected for 60 min at 1 L/min at 24-hr time intervals during a 144-hr period and analyzed by CGC. This experiment was replicated five times. Similarly, 60-min collections ($n = 3$) were made from an AFS containing three layers of 12-oz, 150-carbon material at 0.43, 0.23, and 0.12 m/sec after the filter material had been on line for 24 hr at an air velocity of 0.44 m/sec. Air samples were also collected and analyzed ($n = 5$) from the AFS using three layers of the 9-oz, 150-carbon material. Similarly, experiments were performed on the I-VCS using the same method described for the P-VCS except air was pulled through the system at 1 L/min. Impurities that were not removed by the AFS and collected on the VCT were determined by analyses of CGC peak areas obtained from analyses using the Supelcowax 10 column. The volatile collector traps were extracted with a total of 100 μl of methylene chloride in three aliquots and a total of 20 ng of internal standard was added to this extract. Areas of detected peaks were then converted to amounts per hour based on the FID response obtained with the 20 ng of tridecan-1-ol acetate internal standard for all analyses of purified air. For analyses of ambient air, 2 μg of tridecan-1-ol acetate was added as the internal standard. The impurities not removed by the AFS were compared with air purified with two in-line charcoal filters and also to compounds detected in the ambient laboratory air.

Experiment 4. We demonstrated the use of the system in collecting pheromone from insects by using sexually mature male Caribbean fruit flies, *Anastrepha suspensa* (Loew). Flies used in this study were obtained as pupae from the Florida Department of Agriculture, Division of Plant Industries, Gainesville. Adult flies were sorted by sex 2 days posteclosion and placed in screen cages (30 × 30 × 30 cm). The flies were given water and a 3:1 mixture of refined cane sugar:protein hydrolysate. Males were maintained in a greenhouse located in Gainesville at ambient temperature and humidity during September 1991. Five males 9–11 days old were placed in the I-VCS and collection of pheromone was made from 1800 to 2000 in the greenhouse. Volatiles collected from the flies extracted from the VCT, 500 ng of tridecan-1-ol acetate was added as the

internal standard, and the samples were analyzed on the BP-1 column. Pheromone collections were compared to collections made with the I-VCS which did not contain flies.

Experiment 5. We demonstrated the use of the system in collecting volatiles from plants by using mature cotton plants *Gossypium hirsutum* that were in their flowering stage. Selected branches containing a flower were enclosed in the P-VCS system and collections were made from 1300 to 1400 in the greenhouse during September 1991. Volatiles collected from the flower, leaves, and branch were extracted from the VCT, 20 ng of tridecan-1-ol acetate was added as the internal standard, and the samples were analyzed on the Supelcowax 10 column. The collection from the cotton plant was compared to background collection of the P-VCS.

RESULTS AND DISCUSSION

Static back-pressures of one, two, and three filters containing 12-oz, 150-carbon infused fabric at various air velocities are shown in Table 1. It is interesting to note that the volume of air required to provide an air velocity of 0.43 m/sec for a 24-hr period through a cylinder having an ID of only 8.9 cm is approximately 170,000 L/day. Thus, sampling of volatiles from plants for a 5-day period would require in excess of 850,000 L of purified air. Static back-pressure through three filters approaches the capacity of the blowers used in these experiments when an air velocity of 0.43 m/sec was used.

Experiments to determine the equilibrium time for changes in relative humidity indicated that three layers of the 12-oz, 150-carbon material provided sufficient flow-through of water vapor to track changes in ambient relative humidity. Tests done at a constant air velocity of 0.25 m/sec (approximately 0.5 mph wind speed), moved 69.5 L of air/min (4171 L/hr) through the system. This slower speed (instead of the 0.43 m/sec used in other experiments) was used because it was the maximum capacity of the humidifier to supply 100% humidified air to the intake of the blower. Results presented in Figure 3 show that humidity inside the VCC increased immediately once the humidified air was introduced into the blower intake. The humidity increased from 45% RH (ambient) to 62% RH in under 1 min and continued to increase until it equilibrated at 98% RH in 20 min with the incoming air supply (time to saturate the material completely). This demonstrated that the filter material does not block a significant amount of water vapor from passing through and will allow for changes in the humidity of the air in a very short time within a VCC. When the humidity source was removed, the humidity within the chamber began to drop within 1 min and shortly returned to the initial ambient conditions. Equilibrium time for the P-VCS when RH was decreased from 98 to 49% (ambient

TABLE 1. AIR VELOCITY AND STATIC PRESSURE OF THE AIR FILTRATION SYSTEM USING ONE, TWO, AND THREE FILTERS OF 12-OZ CARBON CLOTH FILTERS

Rheostat setting (%)	Material				Static pressure (H_2O)
	mph	m/sec	L/min	L/day	
	1 layer 12-oz, 18% carbon load				
100	1.59	0.71	194.65	280,293	0.089
50	1.53	0.69	187.70	270,282	0.073
40	0.91	0.41	111.23	160,167	0.020
39	0.85	0.38	104.28	150,157	0.016
38	0.74	0.33	90.37	130,136	0.012
35	0.68	0.30	83.42	120,125	0.008
32	0.45	0.20	55.61	80,084	0.004
	2 layers 12-oz, 18% carbon load				
100	1.25	0.56	152.94	220,230	0.155
50	1.14	0.51	139.03	200,209	0.128
40	0.97	0.43	118.18	170,178	0.076
39	0.51	0.23	62.57	90,094	0.028
38	0.45	0.20	55.61	80,084	0.020
35	0.40	0.18	48.66	70,073	0.011
32	0.19	0.09	23.64	34,036	0.005
	3 layers 12-oz, 18% carbon load				
100	0.97	0.43	118.18	170,178	0.206
50	0.91	0.41	111.23	160,167	0.170
40	0.80	0.36	97.32	140,146	0.135
38	0.68	0.30	83.42	120,125	0.096
35	0.28	0.13	34.76	50,052	0.019
32	0.08	0.04	9.73	14,015	0.009

RH) was 27 min at this air flow, however, the largest changes occurred in 10 min while taking the remaining time to return to ambient humidity (see Figure 3). The ability for the P-VCS to track changes in RH suggests that, under normal environmental conditions, plants in the P-VCS and surrounded by purified air will not be subjected to large differentials in RH compared to plants in the environment.

The results of analyses of air purification provided by one, two, and three layers of the 12-oz, 150-carbon charcoal-infused medium after 48 hr in the P-VCS at an air velocity of 0.43 m/sec demonstrated that the use of additional layers of filtering material resulted in increased air purification (Figure 4). With one layer of the 12-oz material, $860.6x \pm 468$ ng/hr ($n = 5$) of impurities was

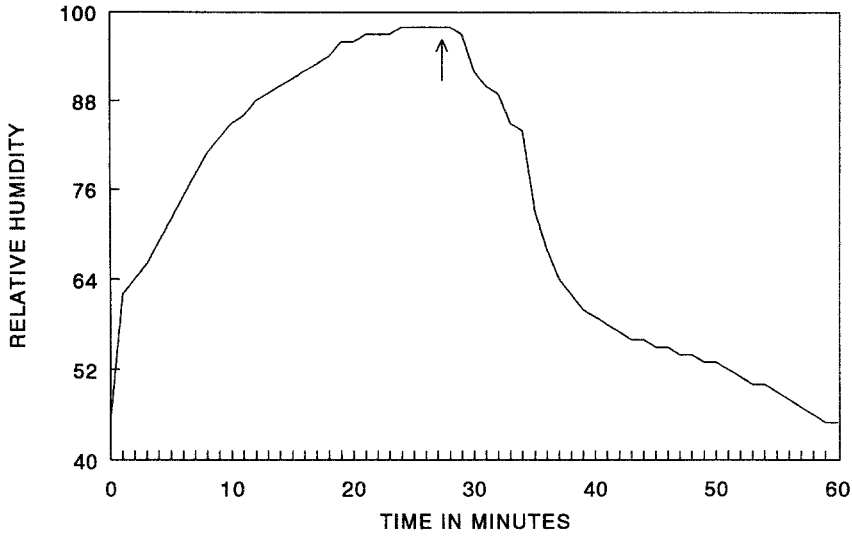


FIG. 3. Graph of the time required for air purified through the three layers of 12-oz, 150-carbon-impregnated cloth to change relative humidity. Air velocity was 0.25 cm/sec and 0 time indicates inlet RH changed from ambient (46%) to 100%. Inlet RH was returned to ambient (47%) after 28 min (indicated by arrow).

detected in 60 L of air collected in 1 hr based on the summation of the 70 major peaks detected by CGC from 6 to 30 min. The impurities detected when two layers of filter material were used decreased to 440.8 $\pm$ 165.8 ng/hr (n = 5). When three layers of filtering material were used, total impurities detected were 36.0 $\pm$ 6.4 ng/hr. Three sections of filter material were the maximum number of layers that could be compressed within the coupler/flange assembly (Figure 1, C1–C4) of this size AFS. Additionally, three layers of filter material produced a system back-pressure that approached the blower cutoff static pressure, and thus the desired air velocity of 0.43 m/sec was reduced. Any additional layers would prevent air flow at the required velocities using this size system and blower motor size. The use of three layers of the 9-oz, 150-carbon material resulted in the detection of 583.2 $\pm$ 42.2 ng/hr of impurities. Because of its reduced performance relative to the 12-oz material, additional evaluation of this material was not pursued. Analyses of impurities detected after 24, 48, 72, and 96 hr of continued air purification using three layers of 12-oz, 150-carbon material indicated that for the first 96 hr, the average amount of impurities detected at the various time intervals was similar to those found at 48 hr. At 120 hr the average amount of impurities detected was 77.68 $\pm$ 5.8 ng/hr (n = 3), and at 144 hr the average amount of impurities detected increased to 87.8 $\pm$ 8.8 ng/hr (n = 3). Studies conducted with decreased air velocities of 0.23 and 0.12

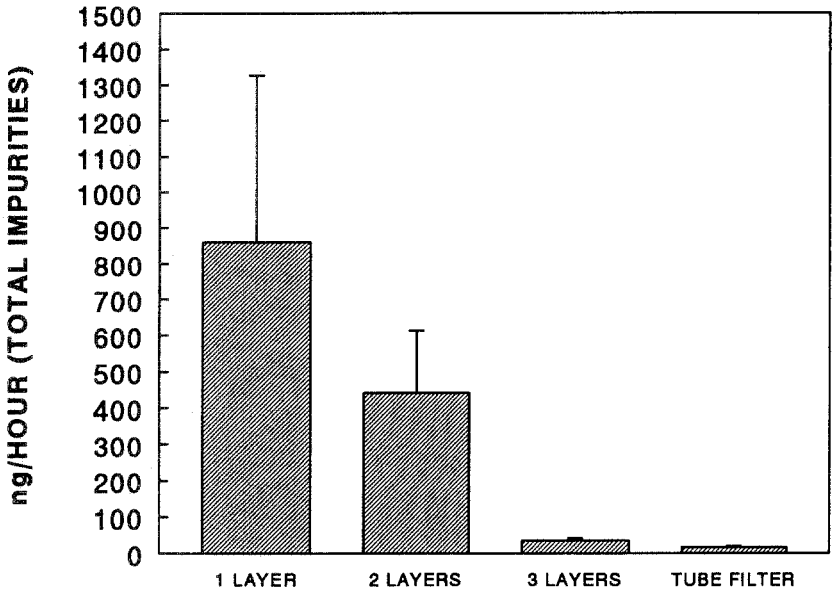


FIG. 4. Comparison of the amount of impurities found in air that was purified by one, two, and three layers of 12-oz, 150-carbon charcoal fabric material using the plant volatile collection system and impurities detected in air purified by two charcoal line filters. Error bars indicate standard deviation (n = 5).

m/sec indicated that air purification was related to the total air flow through the AFS. At 0.23 m/sec the average total amount of impurities present was determined to be 28.8 $\pm$ 9.2 ng/hr (n = 3), and at 0.12 m/sec the amount of impurities decreased to 22.3 $\pm$ 7.1 ng/hr (n = 3).

A comparison of impurities detected using CGC was made among ambient laboratory air, air purified by three layers of 12-oz, 150-carbon material in the P-VCS, and air purified by two charcoal in-line filters (Figure 5). Two charcoal in-line filters provided the best air purification (Figures 4 and 5), with only 8.2 $\pm$ 1.2 ng/hr (n = 3) of impurities detected in the 60 L of air collected. Impurities detected in volatiles collected with the P-VCS consisted of 16 peaks using a peak detection threshold of greater than 0.5 ng/hr. Major impurities occurred with retention times less than 9 min and were typically less than 5 ng/hr. Impurities detected in air collected using the I-VCS were identical in retention time to impurities seen in the P-VCS system. The average amounts of impurities seen in the I-VCS was approximately 3% of that detected in the P-VCS due to the decreased amount of air being purified. The volume of air purified by the I-VCS is 1 L/min, compared with 118.2 L/min of air purified by the P-VCS. Figure 6 shows the retention times and the average amount of the impurities

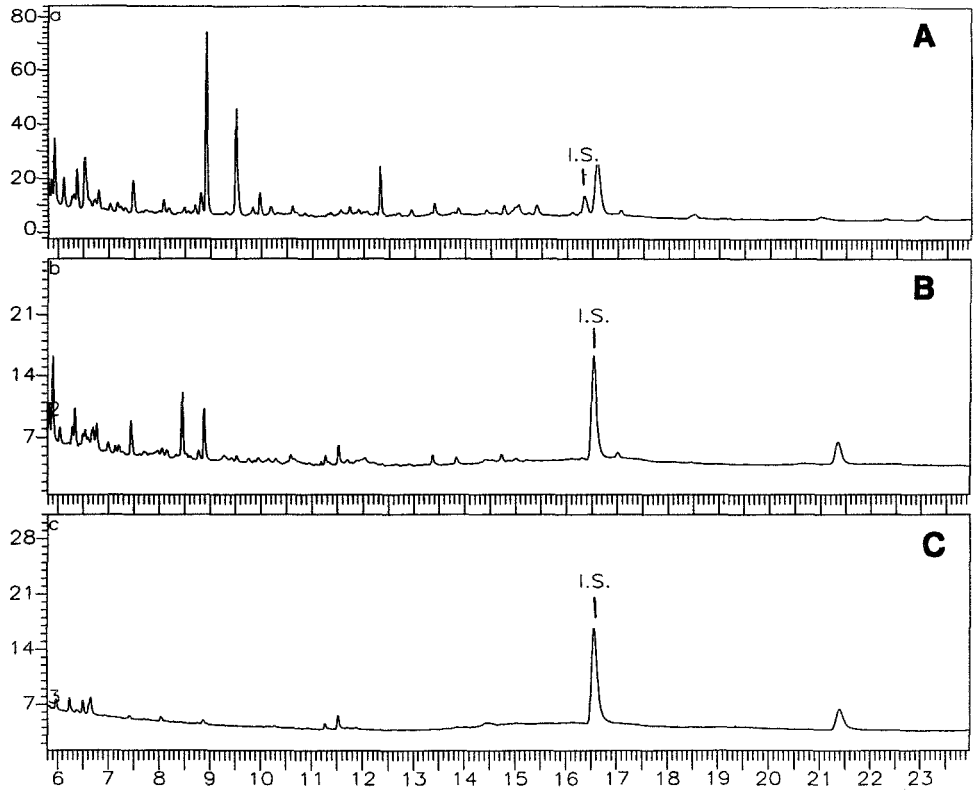
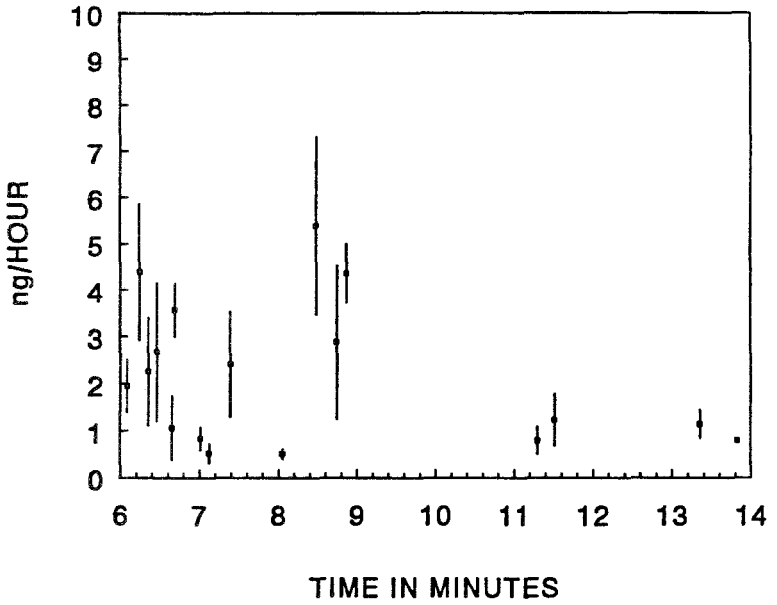


FIG. 5. Typical chromatogram obtained from a 15-min collection of laboratory air (A), a 1-hr collection of air purified by three layers of 12-oz, 150-carbon material in the plant volatile collection system (B), and a 1-hr collection of air purified by two charcoal line filters (C). I.S., internal standard—2 μg of tridecan-1-ol acetate for A and 20 ng for B and C. Column used was a 30 m $\times$ 0.25-mm-ID Supelcowax 10 (see text for operating conditions).

detected from five systems assembled using three layers of 12-oz, 150-carbon-infused fabric as filters. Analyses of air samples from 15-min collections of laboratory air (n = 2) indicated that approximately 55012.8 $\pm$ 856 ng/hr of volatile material was detected by CGC (Figure 5A).

Gas chromatography analysis shown in Figure 7A of volatiles collected from male Caribbean fruit flies indicated that the previously identified compounds, i.e., ocimene [retention time (RT) of 8.4 min], (Z)-3-nonenol and (Z,Z)-3,6-nonadienol (RT, 9.3 min), suspensolide (RT, 14.2 min), E,E-α-farnesene (RT, 14.5 min), β-bisabolene (RT, 14.7 min), anastrephin (RT, 15.3 min), and epi-anastrephin (RT, 15.6 min), were collected in amounts and at



RETENTION TIME	AMOUNT	± STD	RETENTION TIME	AMOUNT	± STD
6.09	1.95	1.14	7.40	2.41	2.25
6.25	4.39	2.96	8.06	0.51	0.23
6.36	2.26	2.32	8.49	5.39	3.86
6.47	2.67	2.97	8.75	2.88	3.28
6.66	1.07	1.36	8.87	4.36	1.29
6.69	3.57	1.17	11.52	1.23	1.11
7.02	0.84	0.50	13.36	1.15	0.61
7.13	0.53	0.43	13.83	0.81	0.11

FIG. 6. Retention times of impurities detected by gas chromatography and average amount of the impurities ($n = 5$) detected in air purified with three layers of 12-oz, 150-carbon-infused fabric as filters. Column used was a 30 m × 0.25-mm ID Supelcowax 10 (see text for operating conditions).

ratios similar to those previously reported (Nation, 1990). Impurities not removed by the charcoal filters in the I-VCS are minimal and, as shown in Figure 7B, would not compromise detection of pheromonal components volatilized by male flies.

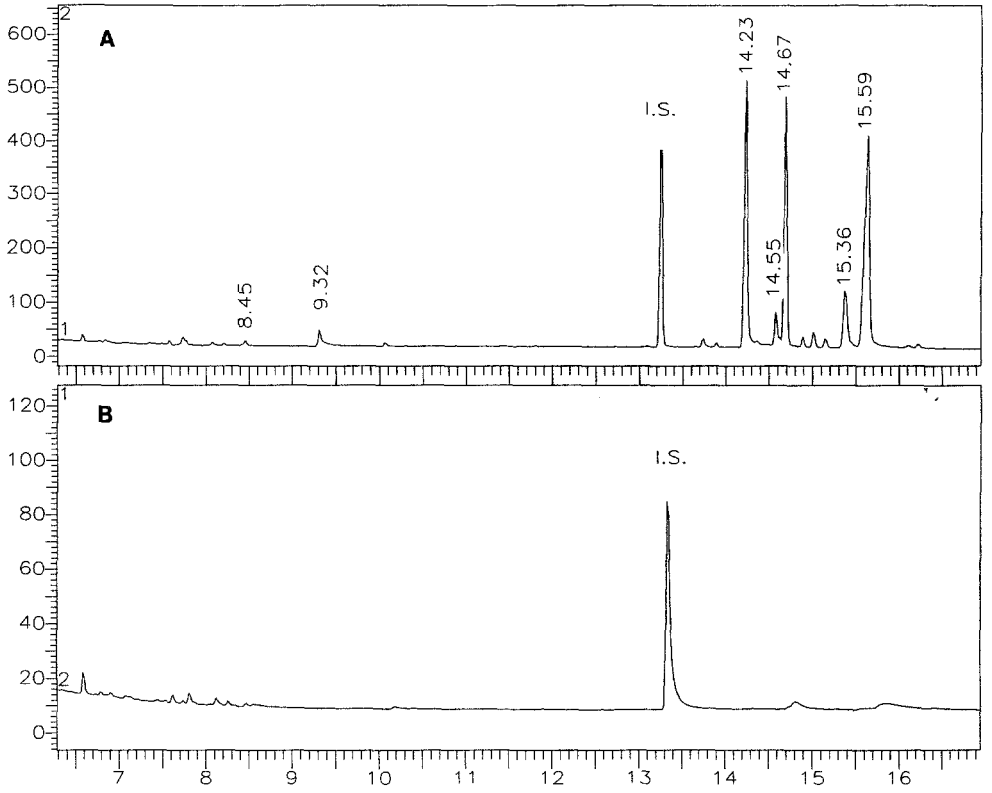


FIG. 7. Comparison of gas chromatograms using the insect volatile collection system for collection of pheromone from sexually mature male Caribbean fruit flies (A) and volatiles collected using the system without flies (B). Column used was a 50 m × 0.25-mm-ID BP-1 (see text for operating conditions and pheromonal peak identification). I.S., internal standard—500 ng tridecan-1-ol acetate.

Analysis of volatiles from a cotton flower, surrounding leaves, and branch resulted in the detection of numerous peaks in relatively small amounts (Figure 8A). Amounts of impurities not removed with the P-VCS are shown in Figure 8B. Although the identification of the compounds is unknown at this time, the chromatograms shown in Figure 8 illustrate the ability to use the P-VCS for collection of plant volatiles. It is apparent that the amount of volatiles collected from the cotton plant investigated was minimal. It is important to note that the amount of air that was subjected to collection on the VCT is only 60 L of the total air (calculated to be approximately 6600 L) that was passed over the plant during the 1-hr collection period. Reduced total air flow and increased air flow through the VCT would increase the amount of material collected.

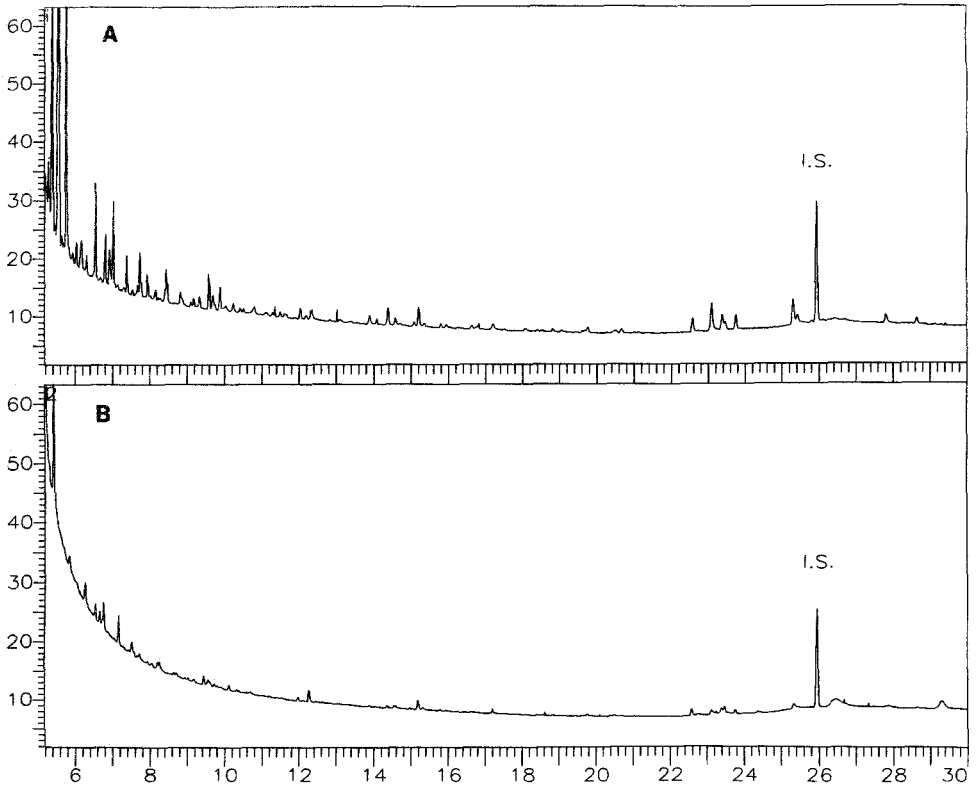


FIG. 8. Comparison of gas chromatograms obtained using the plant volatile collection system for collection of volatiles from a flower, leaves, and branch of a cotton plant (A) and volatiles collected using the system without a plant (B). I.S., internal standard—20 ng of tridecan-1-ol acetate. Column used was a 30 m × 0.25-mm-ID Supelcowax 10 (see text for operating conditions).

CONCLUSION

The air filtration system (AFS) using three layers of 1.27-cm-thick, 12-oz, 150-carbon fabric containing a 150% activated carbon loading provides a purification of laboratory air sources by a factor of approximately 1500 over ambient air, while air purified using two charcoal traps in series provides a purification factor of approximately 3100. The performance of the current volatile collection system (VCS) design demonstrates that a considerable air filtration factor can be obtained using the charcoal-infused fabric to purify very large volumes of air at high flow rates. Evaluation of this material suggests that it can perform well for at least 96 hr or the equivalent of the purification of approximately

760,000 L of air through a 70.88-cm^2 (7.07-$in.^2$) cross section of filter. Currently, systems used for the collection of volatiles from insects generally are done at system air flows of 0.1 to 1 L/min (Shani and Lacey, 1984; Teal et al., 1986; Heath et al., 1991). This suggests that a filter system containing three layers of the 12-oz, 150-carbon material at the diameter tested will perform at these reported levels for approximately 1.5 years before they need to be replaced (760,000 L at 1 L/min flow). The ability of the AFS to adjust quickly to changes in RH and not block water vapor from passing through is important because large volumes of dry air passed over the plants (or insects) would cause them to desiccate.

The design of air filtration systems required for the collection of plant volatiles is more difficult than those needed for collections from insects. This is due largely to the fact that studies of whole plants or plant segments often require a much larger system. The volumes of air that must be purified are also large, and the corresponding flow rates associated with these volumes cannot be pushed through ordinary in-line charcoal trap filters. Another factor which adds to the large volumes of air required for plant collection systems is the increased flow rates through the system used for positive purging to provide a barrier to the intrusion of unpurified air from entering the system from the open end. High air velocities are needed to minimize any momentary disruptions in flow or turbulence caused by movements of objects passing by the volatile collection system. In addition to the problems with size and flow rates, the level of purity of incoming air must be at least the same as, or greater than, that often used to collect insect pheromones. Therefore, relatively low background impurities in air sources must be obtained before any released organic volatiles can be discerned.

The need for still larger systems like the one evaluated, for delivering purified air to plants so that trace semiochemical emissions can be detected, is apparent when one wants to study whole plant and insect interactions in an undisturbed, natural, or artificially duplicated environment. This requires that the system fully enclose the plant or plant and insects without damaging the plant by cutting it to a smaller size to fit a vessel or remove it from its roots or soil. In either case, it is difficult to discern whether the chemical release is caused by the plant–insect interaction or by the plant itself, since it has been severely stressed due to physiological damages or drastic changes to its environment. The nonwoven-fiber carbon medium has the potential for use in such larger-scale systems requiring larger volumes and flow rates that cannot be achieved using standard in-line filtration methods for purifying air.

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ODORS INFLUENCE CHOICE OF OVIPOSITION SITES BY *Diabrotica virgifera virgifera* (COLEOPTERA: CHRYSOMELIDAE)[1]

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Abstract—Female *Diabrotica virgifera virgifera* LeConte were allowed to choose between oviposition substrates that were and those that were not associated with potential sources of semiochemicals. Females deposited over five times more eggs on moist towelettes that were treated with homogenates of female abdomens than on towelettes treated with distilled water. Similar results were obtained when screening separated the homogenates from the towelettes, indicating that odors alone could elicit the response. In contrast, females did not choose towelettes that had previously been used for oviposition or towelettes containing eggs over unused towelettes. Further tests with homogenates of abdomens and a bacteriostatic agent (sorbate) indicated that the females were probably responding to bacterial odors rather than an oviposition-enhancing pheromone. Four strains of bacteria were isolated from a homogenate of female abdomens; females deposited 4 to 16 times more eggs on substrates with odors of the bacteria than on substrates with odors of uninoculated nutrient agar. In no-choice tests, bacterial odors did not increase the number of eggs deposited per female beetle; however, in choice tests with dishes that tended to retain any beetles that entered, there were more eggs per female (but not more beetles) after 24 hr in dishes with bacterial odors than in those without the odors. Females also chose dishes with odors of excised maize (*Zea mays* L.) roots or elevated levels of carbon dioxide over "control" dishes.

Key Words—Western corn rootworm, *Diabrotica virgifera virgifera*, Coleoptera, Chrysomelidae, bacteria, carbon dioxide, pheromone, semiochemicals, *Zea mays*.

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[1] Mention of a commercial or proprietary product does not constitute an endorsement for its use by USDA.

INTRODUCTION

Semiochemicals mediate a variety of important life-history events for western corn rootworms, *Diabrotica virgifera virgifera* LeConte. Mating is facilitated by a female-produced sex attractant, 8*R*-methyl-2*R*-decyl propanoate (Guss et al., 1984). Volatiles from the beetles' primary host, maize (*Zea mays* L.), attract adult beetles (Prystupa et al., 1988), and first-instar larvae are attracted by carbon dioxide and other volatiles from maize seedlings (Strnad et al., 1986; Hibbard and Bjostad, 1990). Feeding stimulants (cucurbitacins) and volatile attractants have also been identified from Cucurbitaceae, which are ancestral hosts of *Diabrotica* beetles (Metcalf et al., 1980; Andersen and Metcalf, 1986; Lampman and Metcalf, 1987; Lampman et al., 1987; Metcalf and Lampman, 1989).

Semiochemicals have not been reported to play a role in selection of oviposition sites by *D. v. virgifera*. Females deposit several batches of eggs beneath the soil surface in late summer. The eggs hatch late in the following spring, and the larvae can develop on roots of several species in the family Graminae (Branson and Ortman, 1970). Due to the restricted motility and limited host range of the larvae, the choice of oviposition site is an important determinant of subsequent survival (Branson and Krysan, 1981). In the rearing facility of the USDA–ARS Northern Grain Insects Research Laboratory, female *D. v. virgifera* appear to concentrate their eggs arbitrarily in one of the two or more egg dishes (petri dishes filled with moist soil) in any given cage (T.F. Branson, personal communication). This indicates that semiochemicals might be involved in the selection of oviposition sites. This study was designed to identify possible sources of semiochemicals that influence choice of oviposition sites by female *D. v. virgifera*.

METHODS AND MATERIALS

General Protocol. Adult *D. v. virgifera* were taken from F_1–F_7 laboratory cultures that were maintained at the Northern Grain Insects Research Laboratory. Larvae were reared on seedling maize (Branson et al., 1988). Adult beetles were held in screen cages (30 × 30 × 30 cm) at 25°C, 40–50% relative humidity, and a 14-hr photophase. Each cage that was used for assays initially contained 100 females (newly eclosed) and 50 males (5–7 days old). Beetles were provided with artificial diet (Branson and Jackson, 1988) and a moisture source (0.6% agar).

An oviposition substrate was continually available to all females that were 2 weeks or more past adult eclosion. Female *D. v. virgifera* readily oviposit on moist, loosely crumpled kitchen towelettes (Handi Wipes, Colgate Non-woven Co., New York; approximately 13 × 27 cm). Each towelette was placed into

a 100 × 20-mm plastic petri dish with five 17-mm holes in the lid. Branson et al. (1975) noted that *D. v. virgifera* "prefer to oviposit beneath an object" (p. 6), so a fluted sheet of aluminum (9-cm diameter) was placed on top of each dish (Figure 1A). New towelettes were boiled through several changes of distilled water to remove manufacturer's cleaning agents; previously used towelettes were also boiled for 10–15 min before being placed in cages.

Oviposition choice tests were initiated when females were 2 to 3 weeks old. Two or three oviposition dishes, each representing an individual treatment, were placed simultaneously into a single cage for 1–3 days, depending on the test. Eggs were then rinsed from towelettes onto filter paper and counted under

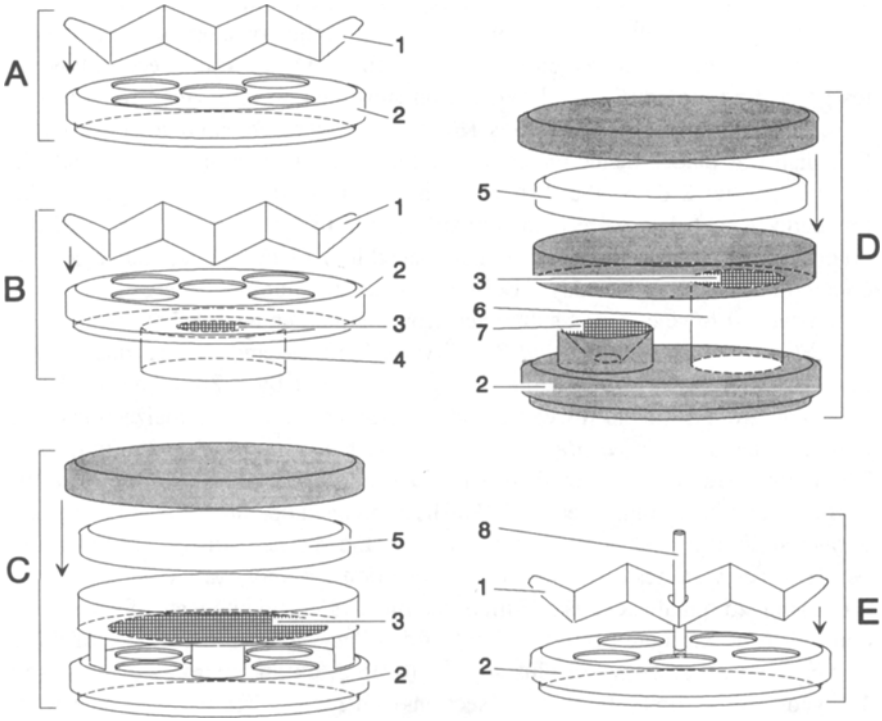

FIG. 1. (A–E) Dishes used in oviposition choice tests (shaded portions were painted black). (1) Circular fluted sheet of aluminum. (2) Plastic petri dish that contains oviposition substrate (moist towelette). (3) Screen (1.2 mm) to keep beetles from touching the sources of odor. (4) Reservoir dish; holds sources of odors that were tested for effects on oviposition. (5) Bottom of petri dish (inverted) containing nutrient agar. (6) Clear acrylic tube allowing odors to pass from culture plate to oviposition substrate. (7) Inverted cone of screening to hinder movement of beetles out of oviposition dish. (8) Tube for introducing gasses into oviposition dish.

a dissecting scope. Results of a test were discarded if a total of < 100 eggs was deposited in the cage during the period. Individual tests were replicated 8 to 20 times, although greater numbers of replicates were run in some cases if results of initial tests were inconclusive or accurate quantitative estimates were desired. Wilcoxon's rank-sign test was used to analyze data from two-way choice tests; data from three-way tests were analyzed with Friedman's test (Wilkinson, 1990).

Tests for an Oviposition Pheromone. Two types of tests were conducted to detect possible pheromonal influences on choice of oviposition sites by *D. v. virgifera*. In the first type, homogenates of tissues from *D. v. virgifera* were assayed for activity. Initially, entire reproductive tracts were removed from 25 females, homogenized at room temperature in approximately 7 ml of distilled water, and applied directly to a towelette in an oviposition dish (Figure 1A). Females were offered the choice of ovipositing on this towelette or on a second towelette that had been treated only with distilled water. In the second type of test, eggs and previously used oviposition substrates were assayed for activity. Three dishes containing towelettes (Figure 1A) were prepared for each cage. One dish was placed inside the cage, and the other two were placed just outside the cage. After 2 days, the dish was removed from the cage; all eggs on the towelette in that dish were manually transferred to the towelette in one of the other dishes. All three dishes were then placed into a single cage, and eggs were counted after another 2–3 days. Before analysis, the number of eggs transferred to the second towelette was subtracted from the egg count for that towelette.

Effects of Homogenates and Root Tissue. For these and all subsequent tests, potential sources of semiochemicals were separated by 1.2-mm mesh plastic screening from both the towelette and the beetles. Effects of maize roots and homogenates of *D. v. virgifera* tissues were tested using the dish in Figure 1B. The bottom side of the petri dish was glued concentrically to the top of a 50-mm-diameter × 20-mm-"reservoir" dish; a screened opening (25-mm diameter) allowed odors to pass between the reservoir and the petri dish. The following odor sources were tested in various combinations: homogenate of reproductive tracts from 25 female beetles in 7 ml of distilled water, homogenate of abdominal contents from 25 male beetles in 7 ml of distilled water, homogenates of these tissues from *D. v. virgifera* plus sorbate (0.01 g, an antimicrobial agent), and 1.5 g of maize roots (1- to 4-cm sections) on moist filter paper. Roots were obtained from postflowering maize plants (various varieties) that were grown in pots in a greenhouse.

Odors from Bacterial Cultures. Bacteria were isolated from homogenates of female *D. v. virgifera* abdomens that had been held at approximately 23°C for 24 hr. Loops of the homogenate were streaked on plates of Bacto Nutrient Agar (Difco Laboratories, Detroit, MI) so as to produce isolated colonies. After 48 hr, four types of colonies (based on visual characteristics) appeared to be prevalent on the plates. Four strains were isolated by streaking bacteria from

four colonies (one of each type) onto individual plates of sterile nutrient agar. The four newly inoculated plates were incubated at room temperature (22–27°C) for 2 days; bacteria were then removed from one isolated colony on each plate and streaked onto a separate plate of sterile nutrient agar. The latter process was repeated several times to ensure pure cultures. The four strains were tentatively identified using the following criteria: cell morphology, characteristics of colonies, gram reaction, indophenol oxidase activity (oxidase reagent droppers, Marion Scientific, Kansas City, MO), and growth on MacConkey agar (Difco Laboratories, Detroit, MI). In addition, 20 metabolic tests were run using Minitek identification system (Marion Scientific, Kansas City, MO).

To determine the effects of bacterial odors on oviposition, sterile petri dishes of nutrient agar were heavily inoculated with one of the four strains. After 24 hr, petri dishes were placed (culture side down) over oviposition dishes (Figure 1C). For the next 2 days, female *D. v. virgifera* were offered the choice of ovipositing in dishes with odors from bacterial cultures or in dishes with odors from nutrient agar alone. Data were not used if >5% of one or both culture plates was covered with contaminating organisms at the completion of the test.

Odors from bacterial cultures were also tested for adult attraction and oviposition using the dishes shown in Figure 1D. With these dishes, beetles entered through a 0.5-cm-diameter hole in the bottom of a screen cone (Figure 1D, No. 7). The screen cones were to retain beetles that entered the dish. To test how readily beetles escaped from the dishes, dishes containing 25 females were placed in empty cages for 24 hr. Tests were then run by placing dishes (without beetles) for 24 hr in cages containing 100 females and 50 males. At the end of a test, females in each dish, as well as eggs, were counted.

Effects of Carbon Dioxide. In a final series of tests, female *D. v. virgifera* were allowed to choose between dishes that did and dishes that did not contain elevated levels of carbon dioxide. Air or 5% carbon dioxide in air was pumped (peristaltic pump) from large Mylar balloons into dishes (Figure 1E) at 17, 40, 70, or 140 ml/hr for 48 hr.

RESULTS

Tests for an Oviposition Pheromone. Mixed results were obtained in initial tests to determine if oviposition of *D. v. virgifera* was influenced by a pheromone. Oviposition was greatly enhanced by homogenates of female reproductive tracts. Females deposited a mean of 405 ± 41 (SE) eggs on towelettes that had been treated with homogenate but only 79 ± 14 ($N = 47$; $P < 0.001$) on towelettes treated with distilled water. In contrast, a female's choice of towelette did not appear to be influenced by the presence of eggs or by the previous use

of a towelette as an oviposition substrate. In those tests, females deposited 328 ± 67 eggs on the single towelette that was initially placed in each cage. When those eggs were transferred onto one of two unused towelettes and all three towelettes were placed in the cage, females deposited 312 ± 62 eggs on the towelette that had previously been used as an oviposition substrate, 811 ± 349 additional eggs on the towelette containing eggs (but only 485 ± 133 without one replicate in which 5383 eggs were deposited in the dish for this treatment), and 484 ± 229 eggs on the towelette containing only distilled water ($N = 15$; $P = 0.76$).

Effects of Homogenates and Root Tissue. Odors of homogenated *D. v. virgifera* tissues or excised roots of maize were sufficient to enhance oviposition. In tests with the reservoir dishes (Figure 1B), homogenates of female reproductive tracts clearly enhanced oviposition, and odors from homogenates of male abdomens had a similar effect (Table 1). In several of the tests, the odor of the homogenate (i.e., to human senses) at the end of the test strongly suggested the presence of putrifying bacteria. In an attempt to reduce bacterial growth, sorbate

TABLE 1. NUMBERS OF EGGS DEPOSITED BY FEMALE *Diabrotica virgifera virgifera* IN THE PRESENCE OF ODORS FROM VARIOUS SOURCES IN TWO- AND THREE-WAY CHOICE TESTS

Choice	Source of odor at oviposition substrate	N	Eggs/dish ($\bar{X}$ ± SE)	P^a
A vs	Homogenate of 25 *D. v. v.* ovaries	11	247 ± 67	0.005
B	Distilled water		31 ± 12	
A vs	Homogenate of 25 *D. v. v.* ovaries	25	262 ± 86	0.016
B vs	Homogenate of 25 male abdomens		190 ± 52	
C	Distilled water		87 ± 29	
A vs	Distilled water	17	164 ± 67	0.407
B	Distilled water + 0.01 g sorbate[b]		160 ± 34	
A vs	Homogenate of 25 *D. v. v.* ovaries	19	486 ± 87	0.002
B	Homogenate of 25 ovaries + 0.01 g sorbate		103 ± 26	
A vs	Homogenate of 25 ovaries + 0.01 g sorbate	30	91 ± 18	0.005
B vs	Homogenate of 25 male abdomens + sorbate		97 ± 18	
C	Distilled water + 0.01 g sorbate		52 + 19	
A vs	Excised maize root (1.5 g)	25	263 ± 52	0.005
B	Moist filter paper (blank)		94 ± 20	

[a] Data analyzed using Wilcoxon's rank-sign test for two-way choices and Friedman's test for three-way choices.
[b] Bacteriostatic agent.

(0.01 g) was added to the 7 ml of homogenate in each dish. The sorbate eliminated overt putrification of homogenates during the tests and did not, by itself, affect the beetles' choice of oviposition substrate (Table 1). Despite this, sorbate reduced the effect of the homogenate on oviposition (Table 1), suggesting that the females' responses to the homogenates were actually, at least in part, responses to microbial metabolites. Regardless, females deposited somewhat greater numbers of eggs in dishes with homogenate plus sorbate than in dishes with sorbate in distilled water (Table 1). Females in the latter test also may have been responding to microbial metabolites rather than to volatiles from the beetles because sorbate often does not completely inhibit microbial growth.

Odors from Bacterial Cultures. The bacterial strains from homogenates of female *D. v. virgifera* abdomens were tentatively identified as *Bacillus* sp., *Flavobacterium* sp., an unpigmented biotype of *Serratia marcescens*, and a second *Serratia* species that was likely either *S. liquefaciens* or a second strain of *S. marcescens*.

Dishes with odors of bacterial cultures received an average of 4 to 16 times more eggs than did dishes with odors from only nutrient agar (Table 2); the differences were statistically significant for three of the four strains. Data obtained with dishes that tend to retain beetles (Figure 1D) were pooled across bacterial strains before analysis (Table 3) because the beetles appeared to respond to odors from all of the bacterial cultures. When 25 females were placed directly in dishes that were designed to retain beetles, odors from the bacteria did not affect the number of eggs deposited on the towelettes or the percentage of females (approximately 60% of the original 25) that were present in the dishes after 24 hr (Table 3). In contrast, when the same dishes were placed, without beetles, into cages of beetles, females deposited significantly more total eggs and more eggs per female in dishes with bacterial odors than in those without the odors (Table 3). The number of females trapped per dish, however, was

TABLE 2. NUMBERS OF EGGS DEPOSITED BY FEMALE *Diabrotica virgifera virgifera* IN THE PRESENCE OR ABSENCE OF ODORS FROM BACTERIAL CULTURES

Strain	*N*	Mean eggs per dish (±SE)		*P*[a]
		Dishes with bacteria	Dishes without bacteria	
1. *Flavobacterium* sp.	13	503 ± 103	31 ± 9	0.001
2. *Serratia* sp.	15	529 ± 120	66 ± 22	0.006
3. *S. marcescens*	8	276 ± 103	67 ± 33	0.093
4. *Bacillus* sp.	15	559 ± 137	65 ± 21	0.001

[a] Wilcoxon rank-sign test.

only slightly (and not significantly) higher for the bacteria-odor treatment than for the blank agar treatment.

Effects of Carbon Dioxide. Female *D. v. virgifera* chose oviposition dishes with elevated levels of carbon dioxide over those from the air treatment (Table 4). The effect was evident at all flow rates of 40 ml/hr of 5% carbon dioxide (2 ml/hr carbon dioxide) or higher but not at 17 ml/hr (0.85 ml/hr carbon dioxide).

TABLE 3. EFFECTS OF ODORS FROM BACTERIAL CULTURES ON NUMBERS ADULT FEMALES AND EGGS OF *Diabrotica virgifera virgifera* ON OVIPOSITION SUBSTRATES IN DISHES DESIGNED TO RETAIN BEETLES

Choice	Odor treatment	*N*	$\bar{X} \pm$ SE		
			Eggs per dish	Females per dish	Eggs per female
	Tests with 25 females placed directly in oviposition dishes				
A vs	Bacteria[a]	15	256 ± 50	15.2 ± 1.2	15.9 ± 2.7
B	Nutrient agar		293 ± 48	16.3 ± 1.3	17.9 ± 3.0
(*P*)			(0.609)	(0.589)	(0.532)
	Tests with oviposition dishes placed in cages containing beetles				
A vs	Bacteria[a]	38	486 ± 69	12.7 ± 1.0	41.8 ± 5.7
B	Nutrient agar		217 ± 45	10.9 ± 0.9	17.6 ± 2.7
(*P*)			(0.002)	(0.084)	(0.001)

[a]Combined data from tests with *Bacillus* sp., *Flavobacterium* sp., and *Serratia* sp.
[b]Wilcoxon rank-sign test.

TABLE 4. NUMBERS OF EGGS DEPOSITED BY FEMALE *Diabrotica virgifera virgifera* IN DISHES WITH AIR OR 5% CO_2 PUMPED IN AT VARIOUS RATES

Flow rate (ml/hr)	*N*	Mean eggs per dish (± SE)		*P*[a]
		5% CO_2	Air	
17	10	85 ± 27	142 ± 47	0.663
40	15	209 ± 38	57 ± 13	0.009
70	20	373 ± 86	48 ± 10	0.002
140	14	383 ± 86	52 ± 19	0.001

[a]Wilcoxon rank-sign test.

DISCUSSION

Although odors clearly influenced the selection of oviposition sites by *D. v. virgifera*, data from these studies do not support the hypothesis that a pheromone is involved. Initial tests with tissue homogenates suggested a possible pheromonal effect, but this effect was greatly reduced by adding a bacteriostatic agent. A bacterial influence on choice of oviposition sites is also consistent with the females' lack of selectivity among unused towelettes, towelettes that had previously been used for oviposition, and towelettes containing eggs. Benzon and Apperson (1988) likewise demonstrated that responses to bacterial odors had initially been mistaken as the influence of an oviposition-enhancing pheromone in *Aedes aegypti* (L.).

Odors from bacteria are known to have direct or indirect effects on oviposition-site selection in a number of insect species, primarily in the Diptera (e.g., Eddy et al., 1975; Hough et al., 1982; Drew and Lloyd, 1987; Benzon and Apperson, 1988; Hough-Goldstein and Bassler, 1988). In some instances, the bacteria are presumably indicative of suitable larval habitats (e.g., Benzon and Apperson, 1988) or are a source of food for adults (e.g., Drew and Lloyd, 1987). Bacteria appear to be important in the nutritional ecology of the soil-dwelling larvae of onion maggots, *Delia antiqua* (Meigen), and seedcorn maggots, *D. platura* (Meigen). Accordingly, adults of these species are attracted by odors of specific bacteria in association with host tissues (Hough et al., 1982; Hough-Goldstein and Bassler, 1988).

With *D. v. virgifera*, an oviposition-enhancing effect specific to bacterial odors would not serve any obvious ecological function. At this point, very little is known of possible ecological associations of *Diabrotica* with bacteria. Species of *Serratia* and *Flavobacterium* have been isolated from other *Diabrotica* spp. (Schalk et al., 1987; Tran and Marrone, 1988), although members of these genera (as well as *Bacillus*) are common in the environment and may have been contaminants in the tissue homogenates. Regardless, because female *D. v. virgifera* appeared to respond to odors from all four strains, the effect is probably a generalized response to carbon dioxide and perhaps other compounds rather than a response to specific bacteria. Such odors could perhaps indicate suitable soil conditions, the presence of host roots, or host root tissue that is decaying following attacks by rootworm larvae. First-instar *D. v. virgifera* locate roots, in part, by being attracted to sources of carbon dioxide (Strnad et al., 1986). Other compounds emitted by maize also influence responses of the larvae and presumably increase the specificity of their host-seeking efforts (Hibbard and Bjostad, 1990). In laboratory assays, odors from excised maize roots enhanced oviposition by female *D. v. virgifera*, but these odors may have differed substantially from those produced by intact roots in the soil. In any case, possible

ecological functions of oviposition-enhancing odors are made more obscure by the fact that eggs of *D. v. virgifera* overwinter before hatching.

The behavioral or physiological mechanisms by which odors influence oviposition-site selection are also obscure. Specifically, contrasting results were obtained in the tests with oviposition dishes that tend to retain beetles (Figure 1D). In the no-choice tests (females placed directly into the dishes), bacterial odors affected neither the number of eggs deposited nor the proportion of females remaining in the dishes after 24 hr. In those tests, the dishes retained approximately 60% of females for 24 hr; for the choice tests, those dishes may provide an index of relative visitation rates to dishes with and without bacteria. In the choice tests, however, numbers of eggs per female (but not females per dish) were greater for dishes with bacteria than for those without. Thus, results of the no-choice tests indicate that bacterial odors enhance oviposition primarily by acting as attractants or arrestants, whereas results of the choice tests indicate that the odors were primarily stimulants or primers of oviposition behavior. One possible explanation for this discrepancy is that bacterial odors did, in fact, attract females that were seeking oviposition sites but that the bulk of the eggs was deposited by very few beetles. In that case, most females in the dishes of the choice tests could have crawled in by chance, obscuring any differences in numbers of females that had been attracted by the odors.

Overall, the findings presented here clearly demonstrate that odors can influence the choice of oviposition sites by *D. v. virgifera*. Understanding the ecological relevance and behavioral mechanisms involved in this phenomenon will require additional study.

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A FOUR-COMPONENT ATTRACTANT FOR THE MEXICAN FRUIT FLY, *Anastrepha ludens* (DIPTERA: TEPHRITIDAE), FROM HOST FRUIT

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Abstract—Sixteen chemicals found in fermented chapote fruit odor were evaluated as attractants for hungry adult Mexican fruit flies. Ethyl octanoate, ethyl benzoate, terpinyl acetate, ethyl salicylate, and (−)-α-copaene proved slightly attractive. Several of the chemicals also were tested for their ability to increase the attractiveness of the previously developed chapote-derived attractant (CEH) consisting of 1,8-cineole, ethyl hexanoate, and hexanol. Combinations containing CEH with ethyl octanoate, ethyl benzoate, 4-terpineol, (−)-α-cubebene, or α-terpineol were significantly more attractive than CEH alone. The two most attractive four-component combinations were ethyl octanoate with CEH (CEHO) and ethyl benzoate with CEH. No combinations containing greater numbers of chemicals were significantly more attractive than CEHO. Therefore, CEHO was selected for further study in this paper. Of CEHO component ratios that were tested, the most attractive was 10:1:1:100 for the chemicals 1,8-cineole, ethyl hexanoate, hexanol, and ethyl octanoate, respectively. Formulations of CEHO into rubber septa and polyvinyl chloride (PVC) were aged 0–15 days and tested against *Torula* yeast in competing McPhail traps in a flight chamber. Summed over all lure ages, rubber septa and PVC dispensers, respectively, were 1.2 and 1.5 times more attractive than *Torula* yeast. PVC dispensers aged 10–15 days were approximately 2.1 times more attractive than *Torula* yeast.

Key Words—Attractants, Mexican fruit fly, Diptera, Tephritidae, *Anastrepha ludens*, host fruit, chapote fruit, 1,8-cineole, ethyl hexanoate, hexanol, ethyl octanoate.

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INTRODUCTION

Robacker et al. (1990a) demonstrated that odor from fermenting, immature fruit of yellow chapote, a native host of the Mexican fruit fly (*Anastrepha ludens* Loew), was attractive to hungry male and female adult *A. ludens* of almost any age and at most times of the day. Robacker (1991) determined that hunger for sugar rather than hunger for protein was the drive primarily responsible for attraction to the odor. Robacker et al. (1990b) developed a three-component mixture of chemicals found in the fruit odor that was at least as attractive as natural volatiles collected from the fermented fruit. Also, the mixture was 1.8 times as attractive as *Torula* yeast, a commonly used bait for *A. ludens*, in greenhouse flight chamber experiments. The mixture was composed of 1,8-cineole, ethyl hexanoate, and hexanol (CEH) at a 10:1:1 ratio.

This paper reports further research toward developing an attractant for *A. ludens* from odor of fermented chapote fruit. Sixteen chemicals identified by Robacker et al. (1990b) but not tested previously were evaluated for individual attractiveness and for their ability to increase the attractiveness of CEH when combined with it. The final four-component mixture developed in this work was formulated into rubber septa and polyvinyl chloride (PVC) and the attractiveness to *A. ludens* of these formulations was evaluated in greenhouse flight chamber experiments.

METHODS AND MATERIALS

Insects and Test Conditions. Flies were from a culture that originated from yellow chapote fruit from Nuevo Leon, Mexico, in 1987 and had been maintained on laboratory diet for about 24 generations. Mixed-sex groups of 180–200 flies were kept in 473-ml cardboard cartons until used in tests. Flies were tested when 2–3 days old and had never been fed as adults. Flies were not deprived of water. All tests were conducted between 0830 and 1400. Responses to chapote odor are relatively constant during this period of the day (Robacker et al., 1990a). Laboratory tests were conducted under a combination of fluorescent and natural light. Laboratory conditions were 22 ± 2°C, 50 ± 20% relative humidity, and photophase from 0630 to 1930.

Identification of Chemicals from Chapote Fruit. The fermented, immature chapote fruit, collection of volatiles, and identification of chemical components were described previously (Robacker et al., 1990a,b).

Gas Chromatography–Mass Spectrometry. A Finnigan MAT 4500 GC-MS data system (Finnigan MAT, San Jose, CA) was operated in the electron-impact ionization mode (emission current, −0.30 mA; electron energy, 70 eV; multiplier voltage, −1300 V) to identify impurities in commercial-grade ethyl octanoate. Mass spectral data were acquired over a mass range of 33–350 amu, using

a 1-sec scan rate. A DB-Wax fused silica column was used for sample component separation (J & W Scientific, Folsom, CA; 60 m × 0.32-mm ID, 0.25-μm film thickness; 12-psi constant head pressure; temperature programmed from 50 to 230°C at 4°/min.).

Chemicals Evaluated in Bioassays. Sixteen chemicals previously identified in volatiles from fermented chapote fruit by GC-MS but not evaluated in earlier work were obtained for behavioral assays. These chemicals were chosen because their retention times coincided with attraction behavior of Mexican fruit flies in a tandem GC/behavior bioassay conducted previously (Robacker et al., 1990b). All 16 chemicals had retention times greater than those tested in the earlier work. Names, sources, and purities of the chemicals are listed in Table 1. Purities stated on product labels were verified by GC. Note that Table 1 lists

TABLE 1. CHEMICAL NAMES, SOURCES, PURITIES, AND RELATIVE CONCENTRATIONS IN CHAPOTE VOLATILES OF 19 CHEMICALS EVALUATED BEHAVIORALLY IN VARIOUS EXPERIMENTS

Chemical	Source[a]	Purity (%)[b]	Amounts in chapote volatiles (%)[c]
Tetradecane	Sigma	>99	0.042
Ethyl octanoate	Aldrich	>99	<0.01
(−)-α-Cubebene	Fluka	98	0.18
(−)-α-Copaene	Fluka	96	1.18
Linalyl acetate	Roth	94	<0.01
(−)-α-Gurjunene	Fluka	97	0.17
Ethyl benzoate	Aldrich	>99	0.82
4-Terpineol	Roth	96	0.38
α-Terpineol	Aldrich	98	0.028
Naphthalene	Sigma	>99	0.042
(−)-*trans*-Caryophellene	Sigma	98	9.24
(−)-Alloaromadendrene	Fluka	98	1.72
Terpinyl acetate	P&B	88	0.028
α-Humulene	Sigma	98	1.77
Methyl salicylate	Roth	99	<0.01
Ethyl salicylate	Aldrich	99	<0.01
Methyl octanoate	Sigma	99	Not found
Ethyl decanoate	Aldrich	>99	Not found
Undecane-2-one	Aldrich	>99	Not found

[a] Sigma Chemical Company, St. Louis, Missouri; Aldrich Chemical Company, Inc., Milwaukee, Wisconsin; Fluka Chemical Corp., Ronkonkoma, New York; Carl Roth GmbH & Co., Karlsruhe, Germany; Pfaltz & Bauer, Inc., Waterbury, Connecticut.

[b] Determined from label information and GC analysis.

[c] Optical rotations of the isomer(s) in chapote volatiles are unknown; percentages refer to each chemical without regard to optical rotation.

specific enantiomers for several chemicals even though GC-MS does not distinguish optical isomers. Since we did not know which were the natural enantiomers of yellow chapote, we tested those that were readily available.

Cage-Top Bioassay Procedure. Cage-top bioassays were used to evaluate attractiveness of chemicals and mixtures of chemicals in most experiments. Although this bioassay does not rigorously separate attraction from locomotor arrest of flies, observations from this and previous work indicated that responses of flies are best described as attraction.

Bioassays were conducted by placing four filter papers (3 cm/side) containing test chemicals or controls near the corners on the top of an insect cage (30 cm/side, aluminum-screened) and counting the numbers of flies beneath each filter paper once each min for 5 min, then rotating the positions of the papers by 90° on the cage top and counting flies for another 5 min. Generally, two papers contained test chemicals and were positioned diagonally from each other on the cage top, and likewise for two papers containing controls. The filter papers were raised 5 mm above the cage top using plastic rings as in Robacker et al. (1991) to ensure that olfaction and not contact chemoreception was solely responsible for the response of the flies. One carton containing 180–200 flies was used in each bioassay cage. Certain other modifications were made for each experiment.

Experiments 1–3. Cage-Top Bioassays of Individual Chemicals and Combinations of the Chemicals with CEH. Solutions of the 16 chemicals from chapote volatiles were prepared in hexane and tested using cage-top bioassays in three experiments. The bioassay procedure was modified in that one filter paper was loaded with hexane, one with one of the chemicals (in hexane), one with CEH (1,8-cineole, ethyl hexanoate, hexanol in hexane), and one with the chemical and CEH. Five counts of flies were made, at 1-min intervals, at each of the four treatments in their initial positions on the four corners of the cage-top. Then new papers with new loadings of the same four treatments were placed on the corners but rotated by 90° into new positions for five more counts, and so on, until all four treatments occupied each of the four positions for five counts. The CEH mixture contained 1,8-cineole, ethyl hexanoate, and hexanol at 40:4:4 ng/μl. Each of the 16 test chemicals from chapote volatiles was prepared at 40 ng/μl. Two amounts of each chemical were tested in separate experiments: 400 ng in 10 μl (Expt 1) and 4 μg in 100 μl (Expt 2). Amounts of CEH solution were varied accordingly: 10 μl of CEH was used in bioassays of 400 ng of the test chemicals, and 100 μl of CEH was used with the 4-μg quantities. In Experiment 3, the same bioassay procedure was used to test 100 μl of hexane, 40-μg quantities of the test chemicals ethyl octanoate or ethyl benzoate in 100 μl of hexane, 100 μl of CEH, and the combination of 40 μg of test chemical with 100 μl of CEH.

The two objectives of these experiments were (1) to determine which of

the 16 chemicals were more attractive than hexane and (2) to determine which of them in combination with CEH were more attractive than CEH alone. The two objectives were accomplished simultaneously in each of the three experiments using the bioassay procedure described above. The numbers of replications that were conducted were set initially at five for Experiment 1 and four for Experiment 2, pending the outcomes of those replications. Chemicals that had not increased the attractiveness of CEH by at least 30% after the preset number of replications were not tested further. The others were tested two or three more replications and reevaluated. Those that were borderline were tested further, up to 12 replications. Six replications of Experiment 3 were conducted.

Experiments 4–8. Cage-Top Bioassays of Complex Mixtures. The purpose of these experiments was to determine the most attractive combination of chemicals identified from fermented chapote fruit that contained the fewest components. Because the complete procedure would have required testing hundreds of thousands of combinations of the various chemicals, we chose an arbitrary set of rules to select and eliminate components so that the task could be simplified to a manageable level, hopefully without sacrificing too much accuracy.

Complex mixtures were those containing five or more chemicals, usually CEH plus two or more chemicals from Table 1. Selection rules for incorporation of chemicals into complex mixtures were (1) 1,8-cineole, ethyl hexanoate, and hexanol were included because they had proven the most attractive combination in earlier work (Robacker et al., 1990b); (2) each chemical must have been identified from an attractive fraction of the gas chromatogram (Robacker et al., 1990b); (3) the chemical must have significantly increased the attractiveness of CEH at either the 400-ng or the 4-μg test quantity in Experiments 1 and 2; and(4) chemicals were incorporated into mixtures at 4 ng/μl if the 400-ng test quantity used in Experiment 1 was more attractive than the 4-μg test quantity used in Experiment 2 or at 40 ng/μl if the 4-μg test quantity used in Experiment 2 was more attractive than the 400-ng test quantity used in Experiment 1. Application of these rules led to Mix 8 (Table 2).

A set of elimination rules was used to develop Mixes 7A, 7B, 6A, 6B, and 6C (Table 2): (1) (−)-α-cubebene was deleted from Mix 7A because it was relatively unattractive alone (Expts 1–2), it increased the attractiveness of CEH only at the 4-μg test quantity (Expt 2), and it is relatively expensive (for development of an inexpensive commercial lure); (2) 4-terpineol was deleted from Mixes 7B–6C because it was relatively unattractive alone (Expts 1–2), it increased the attractiveness of CEH only at the 400-ng test quantity (Expt 1), it is relatively expensive, and it is similar in structure to the less expensive α-terpineol, which was not deleted from any of these mixes; and (3) ethyl benzoate, ethyl hexanoate, and ethyl octanoate were deleted sequentially from Mixes 6A–6C to determine the relative importance of each in the blend.

Low quantities (10 μl) of Mixes 8–6C were tested as Experiment 4 (n =

TABLE 2. CHEMICAL CONCENTRATIONS (NG/μL) IN COMPLEX MIXTURES TESTED FOR ATTRACTIVENESS TO *A. ludens* IN CAGE-TOP BIOASSAYS (EXPERIMENTS 4–7)

Chemical	Mix									
	8	7A	7B	6A	6B	6C	6D	5A	5B	5C
Hexanol	4	4	4	4	4	4	4	4	4	4
Ethyl hexanoate	4	4	4	4	—	4	4	4	4	4
1,8-Cineole	40	40	40	40	40	40	40	40	40	40
Ethyl octanoate	40	40	40	40	40	—	40	40	—	40
(−)-α-Cubebene	40	—	40	40	40	40	—	—	—	—
Ethyl benzoate	40	40	40	—	40	40	40	—	40	40
4-Terpineol	4	4	—	—	—	—	—	—	—	—
α-Terpineol	40	40	40	40	40	40	40	40	40	—

15 replications) and high quantities (100 μl) as Experiment 5 (n = 15 replications). Both experiments were randomized complete blocks with test days as blocks containing each of the six treatments. Cage-top bioassays as described under Cage-Top Bioassay Procedure were conducted. Controls were 10 and 100 μl of CEH (as described in Experiments 1–3), respectively, for the low and high test quantities of Mixes 8-6C. The results of these experiments were used to develop Mixes 6D, 5A, 5B, and 5C.

Mix 6D contained all chemicals of Mix 8 except (−)-α-cubebene and 4-terpineol, which added no attractiveness to the blend in the presence of the other components (Expts 4–5). Ethyl benzoate, ethyl octanoate, and α-terpineol were deleted sequentially from Mixes 5A–5C to determine the relative importance of each in the remaining blend. Cage-top bioassays like those used to test Mixes 8–6C were conducted. Low quantities (10 μl) of Mixes 6D–5C were tested as Experiment 6 (n = 18 replications) and high quantities (100 μl) as Experiment 7 (n = 12 replications), both as randomized complete block designs.

Experiment 8 evaluated Mix 5C vs a four-component mixture (Mix 4) equal to Mix 5C minus ethyl benzoate. The two mixtures were tested using a cage-top bioassay in which Mix 5C was loaded onto two filter papers and Mix 4 onto the other two papers, serving as the control. Forty-one replications were conducted.

Experiment 9. Cage-Top Bioassays of Impurities of Commercial Ethyl Octanoate. The purpose of this experiment was to determine if the attractiveness of commercial-grade ethyl octanoate was due to impurities. Each of three major impurities of commercial-grade ethyl octanoate was evaluated for attractiveness in mixtures containing CEH (40, 4, and 4 ng/μl) and one or all three components at 20 ng/μl each, relative to the attractiveness of CEH alone. Cage-top bioassays

like those used to test complex mixtures were used. Only 10-μl quantities were tested. Nine replications were conducted. Sources and purities of the three chemicals (methyl octanoate, ethyl decanoate, and undecane-2-one) are shown in Table 1.

Experiment 10. Cage-Top Bioassays to Evaluate Ratios of Ethyl Octanoate to CEH. The purpose of this experiment was to determine the most attractive ratio of ethyl octanoate to the three components of CEH. Test solutions (in hexane) contained CEH (40, 4, and 4 ng/μl) with either 4, 40, 400, or 4000 ng/μl of ethyl octanoate. Each mixture (10 μl) was tested against 10 μl of CEH as the control in a randomized complete block design. Cage-top bioassays like those used to test complex mixtures were used. Twenty-two replications were conducted.

Experiment 11. Comparison of TY and CEHO in McPhail Traps. The purpose of this experiment was to compare the attractiveness of CEHO to an aqueous solution of *Torula* dried yeast and borax (TY), a common bait used for detection of *A. ludens* and other *Anastrepha*.

TY traps were prepared using six *Torula* yeast/borax "bait pellets" (Sit-Khem Corp., Michigan City, IN) in 300 ml of water in a McPhail trap (Baker et al., 1944). The TY was allowed to ferment for 4–5 days before tests.

CEHO was also tested in McPhail traps. For use in these traps, CEHO was formulated into rubber septa and polyvinyl chloride (PVC) by Scentry, Inc. (Buckeye, AZ). Rubber septa and PVC dispensers were loaded with 110 and 120 mg, respectively, of a neat mixture of 1,8-cineole, ethyl hexanoate, hexanol, and ethyl octanoate (CEHO) at relative concentrations of 10:1:1:50. CEHO dispensers were suspended in the entrance holes on the undersides of the traps. Traps also contained 300 ml of amber-colored water to mimic the color of TY. Amber-colored water was prepared from red, yellow, and green McCormick food colors (McCormick & Co., Inc., Baltimore, MD). The amber-colored water also contained 0.01% Triton X-100 (Rohm and Haas Co., Philadelphia, PA) as a wetting agent. Dispensers were aged 0–15 days in the greenhouse before testing.

The two McPhail traps containing TY and CEHO were compared in an aluminum-screened flight chamber (2.0 m long $\times$ 0.7 m wide $\times$ 1.3 m high) with an airflow of 1–2 m/sec in the greenhouse. Approximately 800–1000 2-day-old flies were released into the downwind end of the cage at the beginning of each replication. Pupae were irradiated with 7000–9200 rads (cobalt 60 source) 1–3 days before adult eclosion in this experiment because it was conducted in an unsecure greenhouse and *A. ludens* is a quarantined insect in Texas. The traps were removed from the cage after 1 hr. A total of 22 replications was conducted using rubber septa of various ages and 28 replications using PVC.

Emissions from CEHO Dispensers. To measure emission rates of the four chemicals from rubber septa and PVC, dispensers were suspended in the entrance

holes on the undersides of McPhail traps in a greenhouse with an airflow of 1–2 m/sec to age for 1–21 days. Periodically, dispensers were taken into the laboratory and put into an emission-collection apparatus for 1 hr, then returned to the greenhouse. The collection apparatus was designed and built by Heath and Manukian (1992). Briefly, air was pulled through three 7-cm-diameter, 0.5-cm-thick charcoal discs (Activefilter, ACF-NWPE-9-150 Lewcott Corp., Millbury, MA), then passed over the dispenser and through a trap containing 20 mg of Super-Q (Alltech Associates, Inc., Deerfield, IL). Chemicals were extracted from the traps with 200 μl of methylene chloride. To analyze emissions, we used a Shimadzu GC-9A instrument (Shimadzu Scientific Instruments, Inc., Columbia, MD) with a flame ionization detector. Analyses were made on a SPB-1 capillary column (Supelco, Inc., Bellefonte, PA) (60 m, fused silica, 0.32-mm ID, 1-μm film).

Statistical Analyses. Experiments 1–3 and 8–9 were analyzed by paired t tests to compare total counts beneath the control papers to total counts beneath treatment papers. Experiments 4–7 and 10 were randomized complete block designs and were analyzed by analyses of variance to compare treatments with each other. This was done by subtracting total counts at control papers from total counts at treatment papers to obtain differences that were then subjected to analyses of variance. Means were compared by Fisher's protected least significant difference method. For Experiment 11, captures in McPhail traps containing CEH were compared with captures in McPhail traps containing TY by paired t tests.

RESULTS AND DISCUSSION

Experiments 1–3. Objective 1. Attractiveness of Chemicals vs Hexane. Five of the 16 chemicals were more attractive than hexane controls. At the 400-ng test quantity, ethyl octanoate, (−)-α-copaene, terpinyl acetate, and ethyl salicylate were significantly attractive at the 5% level by paired t tests (smallest $t = 2.8$, df = 4) (Table 3). At the 4-μg test quantity, only ethyl octanoate and ethyl benzoate were significantly attractive at the 5% level (smaller $t = 5.0$, df = 11) (Table 4). At the 40-μg test quantity, ethyl octanoate (mean = 26.5 flies/rep) was significantly more attractive than hexane (mean = 14.3) ($P < 0.01$, $t = 6.8$, df = 5), but ethyl benzoate (mean = 27.3) was not significantly more attractive than hexane (mean = 18.7) ($P = 0.1$, $t = 2.1$, df = 5).

Experiments 1–3. Objective 2. Attractiveness of Chemicals + CEH vs CEH Alone. Only two of the five chemicals that were more attractive than hexane also significantly increased the attractiveness of CEH when added to it. They were ethyl octanoate at the 4- and 40-μg test quantities and ethyl benzoate at the 4-μg test quantity. Data for the 4-μg quantities are shown in Table 4. For

TABLE 3. MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING HEXANE, 400 NG OF 1 OF 16 CHEMICALS, CEH, OR COMBINATIONS OF THE CHEMICALS WITH CEH IN CAGE-TOP BIOASSAYS (EXPERIMENT 1)

Chemical	*n*	Hexane	Chemical	CEH[a]	Chemical + CEH[a]
Tetradecane	5	13.8	16.8	38.2	36.6
Ethyl octanoate	5	10.8	25.0[b]	40.8	52.0
(−)-α-Cubebene	5	19.8	22.2	40.6	44.8
(−)-α-Copaene	5	11.6	17.2[b]	49.6	41.8
Linalyl acetate	5	13.4	16.8	36.2	34.8
(−)-α-Gurjunene	5	13.2	17.0	42.6	46.8
Ethyl benzoate	5	12.6	25.4	44.2	52.8
4-Terpineol	8	19.5	19.0	59.2	74.2[c]
α-Terpineol	5	14.2	18.0	51.2	62.8
Naphthalene	5	14.8	16.4	51.2	48.4
(−)-*trans*-Caryophellene	5	18.4	19.0	53.0	51.8
(−)-Alloaromedendrene	5	13.2	16.6	47.2	51.6
Terpinyl acetate	5	10.4	20.8[b]	35.4	35.0
α-Humulene	5	21.0	18.8	54.8	57.4
Methyl salicylate	8	16.8	18.4	61.1	66.6
Ethyl salicylate	5	16.8	26.0[b]	45.4	49.4

[a] CEH—400 ng 1,8-cineole, 40 ng ethyl hexanoate, 40 ng hexanol.
[b] The mean response to an individual chemical is significantly greater than the response to hexane by paired *t* tests ($P < 0.05$).
[c] The mean response to a combination is significantly greater than response to CEH by paired *t* tests. ($P < 0.05$).

the 40-μg quantity of ethyl octanoate, the mean response to the ethyl octanoate + CEH combination was 57.0 flies/rep, compared to 39.8 for CEH alone ($P < 0.05$, $t = 2.7$, df = 5). In contrast, the mean response to the 40-μg quantity of ethyl benzoate in combination with CEH was 43.8 flies/rep compared to 41.2 for CEH alone ($P > 0.5$, $t = 0.6$, df = 5).

In addition, 4-terpineol significantly increased the attractiveness of CEH at the 400-ng test quantity (Table 3) and (−)-α-cubebene and α-terpineol significantly increased the attractiveness of CEH at the 4-μg test quantity (Table 4). Ethyl octanoate, ethyl benzoate, 4-terpineol, (−)-α-cubebene, and α-terpineol were chosen for further testing in more complex mixtures because they increased the attractiveness of CEH in Experiments 1–3.

Experiments 4–8. Attractiveness of Complex Mixtures. Mixtures containing five to eight components were all significantly more attractive than CEH by paired *t* tests (smallest $t = 2.6$, df = 29, $P < 0.05$). These paired *t* tests were conducted on the combined data from low and high test quantities. Within low

TABLE 4. MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING HEXANE, 4 μG OF 1 OF 16 CHEMICALS, CEH, OR COMBINATIONS OF THE CHEMICALS WITH CEH IN CAGE-TOP BIOASSAYS (EXPERIMENT 2)

Chemical	*n*	Hexane	Chemical	CEH[a]	Chemical + CEH[a]
Tetradecane	4	8.2	13.8	42.2	45.0
Ethyl octanoate	12	15.3	29.3[b]	42.2	57.3[c]
(−)-α-Cubebene	12	17.3	18.5	50.1	66.2[d]
(−)-α-Copaene	4	13.0	16.0	34.8	37.8
Linalyl acetate	4	14.2	11.5	36.8	41.8
(−)-α-Gurjunene	4	15.0	14.0	32.5	33.5
Ethyl benzoate	12	15.4	28.2[b]	49.8	65.6[c]
4-Terpineol	6	12.8	15.2	47.3	42.3
α-Terpineol	12	13.7	15.2	51.2	63.2[d]
Naphthalene	4	13.5	16.5	44.5	45.8
(−)-*trans*-Caryophellene	4	11.5	11.2	32.0	38.2
(−)-Alloaromedendrene	4	10.0	11.5	39.0	46.5
Terpinyl acetate	4	10.5	14.0	27.0	30.2
α-Humulene	4	9.0	16.0	39.5	40.0
Methyl salicylate	6	13.3	17.2	40.7	42.7
Ethyl salicylate	4	10.2	11.0	32.2	32.8

[a]CEH—4 μg 1,8-cineole, 400 ng ethyl hexanoate, 400 ng hexanol.
[b]The mean response to an individual chemical is significantly greater than the response to hexane by paired *t* tests ($P < 0.01$).
[c]The mean response to a combination is significantly greater than the response to CEH by paired *t* tests. ($P < 0.01$).
[d]The mean response to a combination is significantly greater than the response to CEH by paired *t* tests. ($P < 0.05$).

and high test quantities, Mixes 8, 7A, 7B, 6A, 6B, and 6C were not significantly different from each other in attractiveness (Table 5). Likewise, Mixes 6D, 5A, 5B, and 5C were not significantly different from each other. Note that experimental design did not allow direct comparisons of Mixes 8–6C with Mixes 6D–5C or of low with high test quantities.

Experiments 4–7 demonstrated that all test mixtures containing any five or more components (within the limits of those tested) were more attractive than CEH. However, the results also suggested that some chemicals were more attractive than others despite the lack of significant differences among the eight mixtures in Table 5. Ethyl hexanoate is one such attractive chemical. Evidence includes its attractiveness in previous work (Robacker et al., 1990b) and the relatively low attractiveness of Mix 6B (Table 5), the only mixture that did not contain ethyl hexanoate (Table 2). The conservative decision was made to retain ethyl hexanoate along with the other components of CEH in the blend.

TABLE 5. MEAN COUNTS OF *A. ludens* AT FILTER PAPERS CONTAINING CEH OR COMPLEX MIXTURES CONTAINING FIVE TO EIGHT CHEMICALS IN CAGE-TOP BIOASSAYS (EXPERIMENTS 4–7)[a]

	Low test quantity[b]			High test quantity[c]			Overall (L + H)		
Mix	*n*	CEH	Mix	*n*	CEH	Mix	CEH	Mix	Mix/CEH
8	15	37.5	49.0	15	32.9	38.5	35.2	43.7	1.24
7A	15	43.5	59.6	15	28.9	34.7	36.2	47.1	1.30
7B	15	35.0	52.7	15	28.6	38.5	31.8	45.2	1.42
6A	15	44.1	58.1	15	33.6	41.5	38.8	49.8	1.28
6B	15	38.9	49.5	15	32.5	33.6	35.7	41.5	1.16
6C	15	39.8	54.2	15	29.9	39.0	34.8	46.6	1.34
6D	18	50.6	70.3	12	31.5	43.6	42.9	62.0	1.44
5A	18	49.6	66.1	12	34.1	48.8	43.4	59.1	1.36
5B	18	49.8	68.4	12	37.5	48.4	44.9	60.4	1.35
5C	18	43.4	65.1	12	35.4	46.3	40.2	57.6	1.43

[a] All mixes were significantly more attractive than CEH, but no mixes were significantly different from each other in attractiveness by Fisher's protected LSD.
[b] CEH—400 ng 1,8-cineole, 40 ng ethyl hexanoate, 40 ng hexanol in 10 μl of hexane; mix—10 μl of mix solution (Table 2).
[c] CEH—4 μg 1,8-cineole, 400 ng ethyl hexanoate, 400 ng hexanol in 100 μl of hexane; mix—100 μl of mix solution (Table 2).

Other decisions regarding retention or deletion of chemicals were based on combinations of factors. The five-component mixtures (Mixes 5A, 5B, 5C), which contained the least expensive and generally most attractive components in Experiment 1–3 (Tables 3 and 4), were at least as attractive as six- to eight-component mixtures (Table 5), which contained the additional components (−)-α-cubebene and 4-terpineol. Thus, the conservative decision was made not to include the more expensive and generally less attractive components (−)-α-cubebene and 4-terpineol. This decision left only six components under consideration: hexanol, ethyl hexanoate, 1,8-cineole, ethyl octanoate, ethyl benzoate, and α-terpineol. Because Mix 5C was numerically the most attractive of the five-component mixtures and contained the most attractive components in Experiments 1–3 (Tables 3 and 4), the conservative decision was made to consider further only the five chemicals in Mix 5C and to delete α-terpineol from the blend.

Ethyl octanoate and ethyl benzoate were about equally attractive in Experiments 1–2 (Tables 3 and 4) and Experiments 6–7 (Table 5: Mixes 5A vs 5B). However, in Experiment 3, the 40-μg quantity of ethyl octanoate significantly increased the attractiveness of CEH, while the 40-μg quantity of ethyl benzoate did not. The decision was made to keep ethyl benzoate under consideration only

if it further increased the attractiveness of the mixture containing CEH and ethyl octanoate. In Experiment 8, Mix 5C was 8% more attractive than Mix 4, which contained the same components as Mix 5, except ethyl benzoate ($P < 0.05$, $t = 2.2$, df = 40). Because of the added cost of ethyl benzoate and the limited gain in attractiveness, we eliminated ethyl benzoate from further consideration in this study. The attractant blend that we chose for comparison with *Torula* yeast thus contained hexanol, ethyl hexanoate, 1,8-cineole, and ethyl octanoate. Further testing of ethyl benzoate with CEH or with CEH + ethyl octanoate would be a logical extension of the present study.

Impurities of Commercial Ethyl Octanoate. The commercial ethyl octanoate used in this study was found to be >99% pure by GC-MS. The three major contaminants were as follows: ethyl decanoate, 0.062%; undecane-2-one, 0.055%; and methyl octanoate, 0.050%. Thus, at the most attractive test quantities of ethyl octanoate (4–40 μg), 2.5–25 ng of ethyl decanoate also was present on the filter papers. The other contaminants were present in lower quantities.

Experiment 9. Cage-Top Bioassay of Impurities of Commercial Ethyl Octanoate. Mixtures containing combinations of CEH with 200 ng of ethyl decanoate, undecane-2-one, methyl octanoate, or all three chemicals were not significantly more attractive than CEH alone. The most attractive of the four mixtures, CEH plus ethyl decanoate, was only 1.05 times more attractive than CEH. It seems unlikely that these chemicals would be maximally attractive at 2.5–25 ng and not at all attractive at 200 ng, especially since similar chemicals such as ethyl hexanoate and hexanol were maximally attractive at quantities of 40–400 ng and were only slightly attractive at 4 ng (Robacker et al., 1990b). We conclude that the attractiveness of commercial ethyl octanoate was due to ethyl octanoate.

Experiment 10. Cage-Top Bioassays to Evaluate Ratios of Ethyl Octanoate to CEH. Figure 1 shows the results of Experiment 10. The most attractive mixture contained 400 ng of 1,8-cineole, 40 ng of ethyl hexanoate, 40 ng of hexanol, and 4000 ng of ethyl octanoate. Although the apparent optimum ratio is 10:1:1:100, the actual optimum could lie anywhere between 10:1:1:10 and 10:1:1:1000, at least for the test quantity range that was tested. The optimum ratios and test quantities of the components of CEH were determined previously (Robacker et al., 1990b). It is possible that addition of ethyl octanoate to CEH could alter optimum ratios and concentrations of 1,8-cineole, ethyl hexanoate, and hexanol to each other, but this was not tested.

Receptor-Site Physiology. Robacker et al. (1990b) argued that 1,8-cineole, ethyl hexanoate, and hexanol bind to separate receptor sites owing to the additivity of their attractivenesses and the finding that each could not be replaced in the mixture with greater concentrations of the others. We now suggest that ethyl octanoate binds to yet another receptor site. There are several lines of evidence. First, its effect on attractiveness is additive to the three components of CEH.

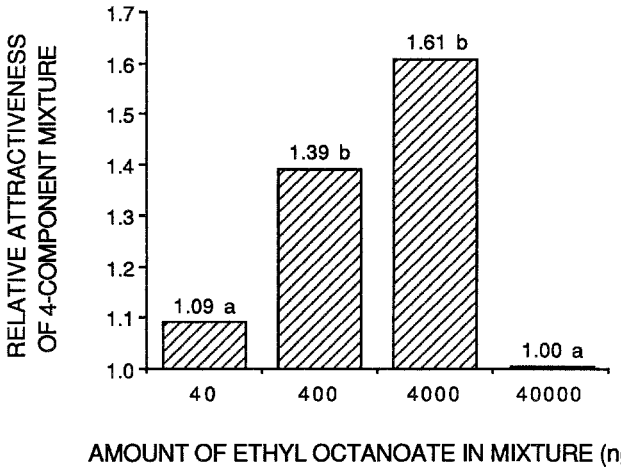

FIG. 1. Means of counts of *A. ludens* at filter papers containing various amounts of ethyl octanoate combined with CEH (400 ng of 1,8-cineole, 40 ng of ethyl hexanoate, and 40 ng of hexanol), divided by counts at filter papers containing only CEH, in cage-top bioassays (Experiment 10). Means followed by the same letter are not significantly different from each other at the 5% level by LSD.

Second, ethyl hexanoate, the component of CEH most similar to ethyl octanoate in structure, remains an important part of the mixture even in the presence of ethyl octanoate (Table 5: Mix 6B). Third, increasing the concentration of ethyl hexanoate decreased the attractiveness of CEH (Robacker et al., 1990b), while ethyl octanoate was most attractive at concentrations 100 times higher than ethyl hexanoate.

Relationship to Chapote Odor. Although the four chemicals of CEHO are found in chapote odor, all four are very minor components. Therefore it is not correct to refer to CEHO as odor of chapote fruit. Further, experiments showing how attractiveness of chapote odor extract to *A. ludens* adults is affected by physiological state of the fly and by environmental factors (Robacker et al., 1990a) do not necessarily hold true for CEHO.

Emissions from Rubber Septa and PVC Dispensers. PVC dispensers emitted CEHO more uniformly over the course of the 21 days than did rubber septa, in both the amounts emitted and the ratios of the components to each other (Figure 2). Neither dispenser type emitted the chemicals at the apparent optima of 10:1:1:100 determined in Experiment 10. Average emission ratios from rubber septa were closer to the optima than those from PVC, but as discussed above, they were not consistent.

Experiment 11. Comparison of TY and CEHO in McPhail Traps. McPhail traps baited with either rubber septa or PVC dispensers of CEHO captured more

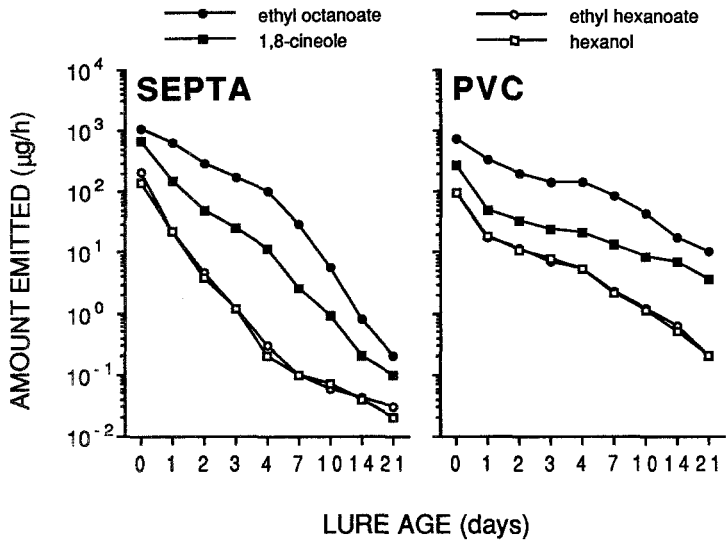

FIG. 2. Emissions of four chemicals from rubber septa and PVC dispensers of various ages.

male and female flies than McPhail traps with TY, at all lure ages between 0 and 15 days. Summed over lure ages of 0–15 days, these results were significant for both rubber septa and PVC traps (Table 6). Captures in traps with PVC dispensers generally increased with lure age by 1.1% of the flies in the cage per day of lure age ($F = 8.1$; df = 1,16; $P < 0.05$). Captures in traps with rubber septa did not change significantly with lure ages between 0 and 15 days.

Traps with PVC dispensers captured more flies than traps with rubber septa, although the difference was not significant by a t test of capture ratios (PVC/TY vs rubber septa/TY). It is noteworthy that McPhail traps baited with polyethylene microcentrifuge tubes containing 25 or 100 µl of CEH captured 1.8 times as many flies as TY in tests identical to those conducted here except that the flies were from a different laboratory culture and microcentrifuge tubes were not aged before tests (Robacker et al., 1990b). This is a surprising result considering that CEHO was 1.3–1.6 times more attractive than CEH in direct comparisons in this work (Tables 3 and 4; Figure 1). Perhaps the explanation is that the rubber septa and PVC dispensers emitted CEHO at suboptimal levels most of the time. If only data from PVC ages 10–15 days are considered, then traps with PVC dispensers captured 2.1 (±0.2 SE) times as many flies as those with TY. The attractiveness of CEH and CEHO relative to TY in the field remains to be tested.

TABLE 6. CAPTURES OF ADULT *A. ludens* BY COMPETING PAIRS OF MCPHAIL TRAPS BAITED WITH RUBBER SEPTA OR PVC DISPENSERS LOADED WITH A 10:1:1:50 MIXTURE OF 1,8-CINEOLE, ETHYL HEXANOATE, HEXANOL, AND ETHYL OCTANOATE (CEHO) VS *Torula* YEAST (TY) IN A FLIGHT CHAMBER IN A GREENHOUSE (EXPERIMENT 11)[a]

Bait	Number of replications[b]	Males[c]	Females[c]	Males + females[c]	CEHO/TY (M + F)
Rubber septa	22	30.3b	24.6b	27.4b	1.22
TY		24.5a	20.5a	22.5a	
PVC	28	35.1b	32.3b	33.7b	1.52
TY		23.9a	20.6a	22.2a	

[a]TY traps contained 300 ml of water and six *Torula* yeast bait pellets; rubber septa and PVC dispensers were loaded with 110 and 120 mg of CEHO, respectively; CEHO traps contained 300 ml of amber-colored water to mimic TY color.
[b]Summed over dispenser ages of 0–15 days following removal from −20°C.
[c]Data are mean percentages of the total flies in the flight chamber captured by each trap. Means of competing traps followed by different letters are significantly different at the 1% level by paired *t* tests.

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QUALITATIVE AND QUANTITATIVE ANALYSES OF VOLATILES AND PHEROMONE GLAND EXTRACTS COLLECTED FROM *Galleria mellonella* (L.) (LEPIDOPTERA: PYRALIDAE)

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Abstract—Chemical analyses of pheromone-gland extracts and volatiles released by male *Galleria mellonella* (L.), the greater wax moth, resulted in the identification and quantification of the aldehydes, primary alcohols, and fatty acids of nonane and undecane. Adult females released none of these six compounds. Mean percentages of the aldehydes and alcohols in the gland extracts were 19.0% undecanal, 3.9% nonanal, 48.3% 1-undecanol, and 28.8% 1-nonanol. Mean percentages of the aldehydes and alcohols in volatiles collected during the first hour of scotophase from moths 1 to 9 days old were 77.4% undecanal, 20.8% nonanal, 1.46% 1-undecanol, and 0.36% 1-nonanol. Age did not significantly affect the amount of each compound collected. The collection of volatiles from moths 3 to 5 days old during a 12-hr scotophase indicated that production was greatest during the first and third quarters of the period. Methylation of group volatile samples allowed the quantification of the fatty acids.

Key Words—Lepidoptera, Pyralidae, Gallerinae, *Galleria mellonella*, greater wax moth, sex pheromone gland, nonanoic acid, undecanoic acid, 1-nonanol, 1-undecanol.

INTRODUCTION

The greater wax moth (GWM), *Galleria mellonella* (L.), is an important pest of the honey bee, *Apis mellifera* L. The larval stage of the GWM feeds on the

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wax, pollen, and honey produced by honey bees. Wax moth infestations in strong active colonies are effectively controlled by worker bees which remove GWM larvae and adults. However, extensive losses occur when the members of active colonies have been weakened by exposure to pesticides or bee diseases and are unable to defend the colony. The heaviest economic losses occur when beekeeping equipment containing only comb and debris has been placed in storage.

Some studies have been conducted on the use of pheromone traps for capturing the GWM (Finn and Payne, 1977; Flint and Merkle, 1983). Male adults of the GWM produce a sex pheromone in glands located on their forewings (Roller et al., 1968). The pheromone attracts females during mating (Finn and Payne, 1977). Female adults are not known to produce any pheromone. In a study by Flint and Merkle (1983), traps were baited with nonanal and undecanal, the reported male sex pheromone components for the GWM (Roller et al., 1968; Leyrer and Monroe, 1973). The response of females to the synthetic bait was not as high as their response to live males, suggesting that the pheromone blend tested by Roller et al. (1968) and Leyrer and Monroe (1973) may have been incomplete. Minor but biologically active components of the pheromone may have escaped detection in the original chemical analyses.

Since the success of pheromone traps is dependent on the identification of all pheromone components and an accurate estimate of the relative ratios and amounts of the components, studies were initiated to verify the components of the pheromone of the GWM and establish their release rates. Two sources of male pheromone were analyzed: volatiles of the GWM collected by a glass-adsorption technique and glands extracted with solvent.

METHODS AND MATERIALS

Insect Rearing

Adults were obtained from the first, second, or third generation of laboratory cultures initiated from wild stock collected at the Townsend House—Apiculture Field Station, University of Guelph. Insects were reared on an artificial diet (Dutky et al., 1962) in jars kept in the dark at 27 ± 3°C and 70% RH. Last-instar larvae were transferred to containers with fan-folded pieces of stiff paper and were maintained at 25°C in a growth chamber with a reverse photoperiod of 12L:12D. Individuals were separated by sex during the pupal stage. Adult males and females were collected daily 2 hr prior to the initiation of the scotophase and were kept individually in 14-ml plastic vials in growth chambers maintained in separate buildings. Adults were not provided with food because this stage does not feed (Oertel 1962).

Gland Extracts

Forewings were dissected from live unanesthetized male moths. Scales on the dorsal and ventral surfaces of the wings were removed by gentle scraping with the edge of a metal spatula. The wing tissue was cut so that a rectangular piece of the wing containing the pheromone gland was excised. Six glands from three moths of the same age were dipped for 0.5 min in 30.0 μl of isooctane (Caledon, distilled in glass) which also contained 30.0 ng of an internal standard, tridecanyl acetate (C13 : AC). Glands from moths 0 to 5 days old were extracted. The time of extraction was recorded with all extractions occurring between the first and the sixth hours after the initiation of scotophase. Five controls were prepared by extracting pieces of female forewings in the same manner as described for the males. Gland extracts were allowed to evaporate at room temperature to a volume of <5.0 μl. Two microliters of the extract was injected into a Hewlett Packard 5790A gas chromatograph. A total of 32 samples of pheromone gland extracts was analyzed.

Volatiles

Study I: Release by Male GWM of Different Ages. Volatiles were obtained with a glass-adsorption technique similar to Baker et al. (1980). Individual moths were placed in stoppered 100-ml volumetric flasks. Prior to the introduction of the moths, 1000 ng of the internal standard, C13 : AC, dissolved in 10 μl of isooctane, was added to each flask and the solvent was allowed to evaporate. Male moths aged 1, 3, 5, 7, and 9 days were placed in flasks 2 hr prior to the initiation of scotophase to allow the insects to become accustomed to the containers. Total production for each moth was measured 0, 1, 2, or 3 hr after scotophase initiation. Measurements were taken at the beginning of scotophase because male GWM have been observed to be sexually active during this period (Nielson and Brister, 1977). Only one sample per moth was taken. Four replications of each combination of age and collection time were obtained ($n = 80$). To collect the extract, flasks were placed in the freezer at $-15°C$ for 10 min, after which the moths were removed. Flasks were rinsed three times, once with 1.0 ml isooctane and twice with 0.5 ml isooctane. For each rinse, the isooctane was swirled continuously for 30 sec and then filtered through glass wool. The glass wool was rinsed once with 0.2 ml isooctane.

Study II: Release by Male GWM During a 12-hr Scotophase. Results from Study I indicated that moths 3 to 5 days old produced the greatest quantities of pheromone. Therefore, in Study II, production by moths 3 to 5 days old was monitored for the entire scotophase. Individual moths were placed in stoppered 500-ml boiling flasks 2 hr prior to the initiation of scotophase. Each flask contained 1500 ng of the internal standard, C13 : AC. Total production was measured 3, 6, 9, or 12 hr after the initiation of scotophase. Only one sample per

moth was taken. A total of five replications for each combination of age and collection period was obtained ($n = 60$). Flasks were rinsed twice with 2.0 ml isooctane, and the rinses were filtered through glass wool. The glass wool was rinsed once with 0.4 ml isooctane.

Study III: Release by Groups of Male GWM. Group effects on production were studied by placing five male moths of the same age in a 500-ml boiling flask. Each flask contained 20.0 μg of C13:AC. Male moths aged 3, 4, or 5 days old were placed in flasks 2 hr before the start of scotophase. Total production was measured 3 hr after the initiation of scotophase so that comparisons could be made between the results of the 3-hr collection periods in each of Studies I, II, and III. Flasks were rinsed as described in Study II. Four replications of each age were prepared for a total of 12 samples. Three controls were prepared by placing five female moths of the same age (3, 4, or 5 days old) in each of three flasks and collecting the volatiles in the same manner as described for the males.

Methylation of Fatty Acids

Quantification of fatty acids from six arbitrarily selected samples in Study III was achieved by converting them to their methyl esters (Morrison and Smith, 1964). A 100-μl aliquot was transferred from a volatile sample to a glass vial. The isooctane was allowed to evaporate and 100 μl of 14% boron trifluoride in methanol (Sigma Chemical Co., St. Louis, MO) was added. The vial was capped and the contents were heated for 45 min at 60°C. One hundred microliters of distilled water was added, and the sample was extracted three times with isooctane (total volume, 1.0 ml). Crystals of sodium sulfate were added to the combined extract to absorb any water remaining in the sample. The sample was concentrated to a volume of <100.0 μl using low heat. Two microliters of the concentrate was injected into the gas chromatograph.

Chemical Analyses

All pheromone samples and synthetic standards were analyzed on a Hewlett Packard 5790A gas chromatograph equipped with a splitless injector and a flame ionization detector. The gas chromatograph was interfaced to a Hewlett Packard 3390A integrator. The fused silica capillary column used was a Supelcowax10, 30 m × 0.25-mm i.d. (Supelco, Oakville, Ontario). Hydrogen was used as a carrier gas at a linear flow velocity of 38 cm/sec. Injector and detector temperatures were set at 225 and 250°C, respectively. Samples were injected splitless at an initial oven temperature of 75°C. For the gland extracts, the oven temperature remained at 75°C for the first 2.00 min and then increased at 25°/min to a final temperature of 160°C. For the volatile extracts, the GC temperature rose at 15°/min to a final temperature of 200°C. Chromatograms were run for

at least 30 min. Retention times of major peaks were compared to the retention time of the internal standard, C13 : AC, tridecanyl acetate. The relative retention times of authentic standards were determined. Compounds identified from the natural product samples were quantified by comparing the peak heights to those produced by authentic standards of C9 : Al, C11 : Al, C9 : OH, and C11 : OH and the synthetically produced methyl esters of C9 : acid and C11 : acid. All chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO) with the exception of nonanal, which was obtained from the Aldrich Chemical Company Inc. (Milwaukee, WI).

Peak assignments determined from the analyses on the Supelcowax10 column were confirmed by the analysis of selected samples and synthetic standards on a 15 m × 25-mm-i.d. SPB1 nonpolar column (Supelco, Oakville, Ontario). GC conditions were as described previously with the exception of the temperature program, which had an initial temperature of 75°C, a final temperature of 160°C, and a temperature change of 15°/min.

Mass spectral analyses were performed on a Kratos MS890 double-focusing mass spectrometer interfaced to a Carloerba/Kratos gas chromatograph fitted with a 30 m × 0.32-mm DB-5 column (J and W Scientific, Folsom, CA). Helium was used as a carrier gas at a flow rate of 1 ml/min. The injector temperature was 230°C. Samples were injected at an oven temperature of 75°C. The temperature was held at 75°C for 2 min, then increased at 15°/min to a final temperature of 250°C. Both electron impact (EI) and chemical ionization (isobutane) (CI) spectra were obtained. All spectra were generated at 70 eV. Spectra of compounds from the pheromone extracts were compared to those of authentic compounds.

Statistical Analyses

Data were transformed by natural log prior to an analysis of variance. The ages of the moths, the date of sampling, and the time of collection were considered in the model. Data were analyzed twice: (1) to determine the mean amounts of the compounds in the samples and (2) to determine the relative percentages of the compounds in the samples. A Protected LSD test was performed on the transformed data to compare mean values at the 5% level (Fisher, 1935).

RESULTS

Isolation and Identification of Chemical Compounds

GC–mass spectral analyses of male GWM volatiles demonstrated the presence of six compounds. Two of the peaks had fragmentation patterns and retention times identical to those of nonanal (C9 : Al) and undecanal (C11 : Al), the

previously reported sex pheromone components of the GWM (Roller et al., 1968; Leyrer and Monroe, 1973). Two other peaks had EI and CI spectra which suggested that the compounds were 1-nonanol (C9:OH) and 1-undecanol (C11:OH). The two remaining peaks gave EI and CI spectra which corresponded to those of nonanoic acid (C9:acid) and undecanoic acid (C11:acid).

The parent ion peak for the compound identified as C9:OH (MW 144) did not appear in the EI spectrum, while the M + 1 peak *m/e* = 145 (addition of H^+) did not appear in the CI spectrum. This is quite common for alcohols (Pavia et al., 1979). In the EI spectrum, a small peak at *m/e* = 126 corresponded to the loss of water from the parent ion (M − 18). Another peak at *m/e* = 98 corresponded to the simultaneous loss of water and ethylene (M − 46). In the CI spectrum, a peak at *m/e* = 127 corresponded to the loss of water from the M + 1 peak. In the CI spectrum, a small peak at *m/e* = 143 could have been a M − 1 peak, although this is not common for alcohols (G. King, personal communication).

The parent ion peak for the compound identified as C9:acid (MW 158) appeared in the EI spectrum, while the M + 1 peak *m/e* = 159 was the most intense peak in the CI spectrum. An intense even-numbered peak at *m/e* = 60 in the EI spectrum resulted from a McLafferty rearrangement, which is common for carboxylic acids (Pavia et al., 1979).

The interpretation of the EI and CI spectra of the compound identified as C11:OH (MW 172) was similar to that given for C9:OH. The EI spectrum had fragments corresponding to M − 18 (154) and M − 46 (126). The CI spectrum had a fragment corresponding to M + 1 − 18 (155). A peak at *m/e* = 171 could have been a M − 1 peak.

The parent ion peak for the compound identified as C11:acid (MW 186) appeared in the EI spectrum, while the M + 1 peak *m/e* = 187 appeared as the most intense peak in the CI spectrum. An intense even-numbered peak appeared at *m/e* = 60 in the EI spectrum as the result of a McLafferty rearrangement.

All six peak assignments from the GC–mass spectral analyses of male GWM volatiles were confirmed by cochromatography of the natural product samples with authentic standards on both the slightly polar Supelcowax10 column and the nonpolar SPB1 column. The aldehydes, C9:Al and C11:Al, and the alcohols, C9:OH and C11:OH, were consistently found in the gland extracts and the volatiles of the males. The tentative assignments of the alcohols from the GC–mass spectral analyses were confirmed by cochromatography of authentic C9:OH and C11:OH with the natural product samples on the Supelcowax10 column. The two acids, C9:acid and C11:acid, produced broad tailing peaks on the Supelcowax10 column with retention times which varied according to the concentrations of the compounds. Peaks for the acids on the SPB1 column were more distinct but C9:acid could not be resolved from the large peak for

C11 : Al. The presence of peaks for C9 : acid and C11 : acid in the gland extracts and volatiles of the GWM males was variable due to the fact that acids chromatograph so poorly without conversion to their methyl esters. Methylation of selected volatile samples indicated that the acid assignments were correct because the methyl esters of the acids, methyl nonanoate and methyl undecanoate, were detected by cochromatography with synthetic standards.

Gland Extracts. The relative percentages of the aldehydes and alcohols in the gland extracts of the males were 3.9% C9 : Al (95% CI = 3.39–4.34), 19.0% C11 : Al (95% CI = 17.09–20.51), 28.8% C9 : OH (95% CI = 27.11–29.66), and 48.3% C11 : OH (95% CI = 45.36–50.02). C9 : acid and C11 : acid are not included because peaks for these compounds were not always detectable. Wing extracts from the females did not contain any of the aldehydes, alcohols, or acids or nonane and undecane that were found in the male extracts.

Volatile Samples. The aldehydes, C9 : Al and C11 : Al, and the alcohols, C9 : OH and C11 : OH, were found in all volatile samples. However, chromatograph peaks for C9 : acid and C11 : acid were not always detectable. Volatiles collected from female GWM did not contain the aldehydes, alcohols, and acids of nonane and undecane that were found in the male volatiles.

Study I: Release by Male GWM of Different Ages. Although the largest quantities of the aldehydes and alcohols were collected from moths which were 3 to 5 days old, age did not significantly affect the amount of each component collected ($P > 0.05$, ANOVA). Variations between individuals were thus greater than any variations which may have been due to age. In addition, the date of sampling also did not significantly affect the amounts of the aldehydes and alcohols collected ($P > 0.05$, ANOVA). However, the time of collection was a highly significant factor ($P < 0.0001$, ANOVA). Data from moths of all ages were pooled and mean values for the amounts of four components collected at the four sampling times (i.e., 0, 1, 2, and 3 hr into scotophase) were calculated (Table 1).

From Table 1, it can be seen that the release rates for C11 : Al during the first, second, and third hours of the scotophase were 25.5, 8.6, and 6.7 μg, respectively. Similarly, the release rates for C9 : Al were 6.8, 2.7, and 2.4 μg. The release rates for the alcohols for the first, second, and third hours of scotophase were 0.38, 0.66, and 0.61 μg for C11 : OH and 0.11, 0.23, and 0.17 μg for C9 : OH.

Mean percentages of the aldehydes did not change significantly during the first 3 hr of scotophase ($P > 0.05$, ANOVA) although the mean percentages for the alcohols did change ($P < 0.0001$, ANOVA) (Table 2).

Study II: Release by Male GWM During a 12-hr Scotophase. The mean cumulative amounts of C11 : Al and C9 : Al for the collections made at 3 hr into scotophase and 6 hr into scotophase were not significantly different ($P > 0.05$, protected LSD), indicating that the aldehydes collected in the second quarter

TABLE 1. MEAN AMOUNT (μg) OF COMPOUNDS FOUND IN VOLATILES EMITTED BY INDIVIDUAL ADULT MALES OF *G. mellonella*, DURING THE FIRST 3 HR OF SCOTOPHASE[a]

Hours into scoto.	C11:Al mean (μg)	95% CI	C9:Al mean (μg)	95% CI	C11:OH mean (μg)	95% CI	C9:OH mean (μg)	95% CI
0	0.23 a	0.17–0.33	0.08 a	0.06–0.10	0.11 a	0.08–0.14	0.01 a	0.01–0.02
1	25.77 b	20.15–32.95	6.90 b	5.52–8.64	0.49 b	0.40–0.59	0.12 b	0.08–0.17
2	34.42 c	29.40–40.31	9.56 c	7.70–11.87	1.15 c	0.96–1.39	0.35 c	0.30–0.40
4	41.10 c	32.71–51.63	11.94 c	8.93–15.68	1.76 d	1.42–2.19	0.52 d	0.41–0.64

[a]Amounts are cumulative, from 2 hr prior to scotophase to 0, 1, 2, or 3 hr after the initiation of scotophase (n = 20 for each time indicated). C11:Al, undecanal; C9:Al, nonanal; C11:OH, 1-undecanol; C9:OH, 1-nonanol. Raw data were transformed by natural log prior to statistical analyses. Within each column, means followed by the same letter are not significantly different at $P < 0.05$ (protected LSD).

TABLE 2. MEAN PERCENTAGES OF FOUR COMPOUNDS FOUND IN VOLATILES EMITTED BY INDIVIDUAL ADULT MALES OF *G. mellonella*, DURING THE FIRST 3 HR OF SCOTOPHASE[a]

Hours into scoto.	C11:Al (%)	95% CI	C9:Al (%)	95% CI	C11:OH (%)	95% CI	C9:OH (%)	95% CI
1	77.43 a	74.63–78.80	20.75 a	18.46–22.94	1.46 a	1.20–1.76	0.36 a	0.26–0.48
2	75.68 a	73.43–76.92	21.01 a	19.04–22.88	2.54 b	2.11–3.01	0.77 b	0.65–0.88
3	74.44 a	71.29–75.73	21.44 a	18.93–23.68	3.19 b	2.50–3.97	0.93 b	0.75–1.13

[a]Amounts collected for each hour were cumulative, from 2 hr prior to the initiation of scotophase (n = 20 for each hour). C11:Al, undecanal; C9:Al, nonanal; C11:OH, 1-undecanol; C9:OH, 1-nonanol. Raw data were transformed by natural log prior to statistical analyses. Within each column, means followed by the same letter are not significantly different at $P < 0.05$ (protected LSD).

were not appreciable (Table 3). The amounts of the aldehydes in the samples collected at 9 hr into scotophase were almost double the amounts collected in the 3- and 6-hr samples, suggesting that the insects also released aldehydes during the third quarter of the 12-hr scotophase. The amounts of the two aldehydes in the samples collected at 12 hr into scotophase were not significantly different ($P > 0.05$, protected LSD) from the amounts collected in the 9-hr samples, suggesting that the aldehydes were not released during the last quarter of the 12-hr scotophase.

The quantities of C11:OH collected in the samples at 3 and 6 hr into scotophase were significantly different ($P < 0.05$, protected LSD) (Table 3), suggesting that C11:OH was being released during the second quarter of the 12-hr scotophase. Amounts of C9:OH collected in the samples at 3 and 6 hr

into scotophase were not significantly different ($P < 0.05$, protected LSD) (Table 3). The amounts of the alcohols collected at 9 hr into scotophase were significantly greater ($P < 0.05$, protected LSD) than the amounts collected at 6 hr into scotophase. However, the amounts of C11:OH and C9:OH collected in the 9- and 12-hr into scotophase samples were not significantly different ($P > 0.05$, protected LSD), suggesting that the alcohols also were not released during the last quarter of the 12-hr scotophase.

Mean percentages of the aldehydes for the 3- and 6-hr samples were significantly different ($P < 0.05$, protected LSD) from the mean percentages of the aldehydes for the 9- and 12-hr samples (Table 4). The mean percentage of C11:Al decreased over time, while the mean percentage of C9:Al increased.

TABLE 3. MEAN AMOUNT (μg) OF COMPOUNDS FOUND IN VOLATILES EMITTED BY INDIVIDUAL ADULT MALES OF *G. mellonella*, DURING A 12-HR SCOTOPHASE[a]

Hours into scoto.	C11:Al mean (μg)	95% CI	C9:Al mean (μg)	95% CI	C11:OH mean (μg)	95% CI	C9:OH mean (μg)	95% CI
3	79.72 a	67.64–93.95	23.18 a	19.99–26.88	0.82 a	0.66–1.01	0.32 a	0.27–0.38
6	88.08 a	71.46–108.55	28.53 a	21.36–38.11	1.24 b	0.96–1.61	0.45 a	0.34–0.61
9	143.09 b	118.77–172.39	60.73 b	49.49–74.52	3.12 c	2.51–3.89	1.20 b	0.88–1.65
12	150.04 b	123.37–182.47	74.10 b	53.43–102.78	3.60 c	2.89–4.47	1.27 b	0.93–1.75

[a] Amounts are cumulative, from 2 hr prior to scotophase to 3, 6, 9, or 12 hr after the initiation of scotophase ($n = 15$ for each time indicated). C11:Al, undecanal; C9:Al, nonanal; C11:OH, 1-undecanol; C9:OH, 1-nonanol. Raw data were transformed by natural log prior to statistical analyses. Within each column, means followed by the same letter are not significantly different at $P < 0.05$ (protected LSD).

TABLE 4. MEAN PERCENTAGES OF FOUR COMPOUNDS FOUND IN VOLATILES EMITTED BY INDIVIDUAL ADULT MALES OF *G. mellonella*, DURING A 12-HR SCOTOPHASE[a]

Hours into scoto.	C11:Al (%)	95% CI	C9:Al (%)	95% CI	C11:OH (%)	95% CI	C9:OH (%)	95% CI
3	76.62 a	73.39–78.80	22.28 a	19.46–25.12	0.79 a	0.64–0.94	0.31 a	0.24–0.39
6	74.45 a	69.19–77.85	24.11 a	20.20–27.98	1.05 b	0.90–1.19	0.39 a	0.33–0.44
9	68.75 b	64.47–71.63	29.18 b	25.38–32.78	1.50 c	1.23–1.78	0.57 b	0.43–0.75
12	65.50 b	59.27–69.41	32.36 b	26.72–37.56	1.57 c	1.26–1.87	0.56 b	0.43–0.69

[a] Amounts collected for each hour were cumulative, from 2 hr prior to the initiation of scotophase ($n = 15$ for each hour). C11:Al, undecanal; C9:Al, nonanal; C11:OH, 1-undecanol; C9:OH, 1-nonanol. Raw data were transformed by natural log prior to statistical analyses. Within each column, means followed by the same letter are not significantly different at $P < 0.05$ (protected LSD).

The mean percentages of the alcohols increased over time. However, the mean percentages of C9:OH and C11:OH for the 9- and 12-hr samples were not significantly different ($P > 0.05$, protected LSD).

Study III: Release by Groups of Male GWM. Mean percentages of the aldehydes and alcohols in the samples collected from groups of five male GWM in glass flasks were 69.5% C11:Al (95% CI = 65.44–72.50), 27.5% C9:Al (95% CI = 23.50–31.68), 1.96% C11:OH (95% CI = 1.63–2.31), and 0.97% C9:OH (95% CI = 0.81–1.15).

Methylation of six of the group samples resulted in the conversion of the fatty acids to their methyl esters and the aldehydes to dimethyl acetals (Figure 1). The mean percentages of the derived dimethyl acetals, fatty acid methyl esters, and alcohols in the six group samples were 57.76% C11:DMA (95% CI = 50.99–62.96), 27.06% C9:DMA (95% CI = 23.14–30.45), 1.52% C11:OH (95% CI = 1.18–1.87), 0.78% C9:OH (95% CI = 0.63–0.95), 10.35% C11:Me (95% CI = 5.61–18.35), and 2.53% C9:Me (95% CI = 1.68–3.66). The concentrations of the two fatty acids were thus intermediate to the concentrations of the aldehydes and the alcohols. Eleven-carbon compounds were always at higher concentrations than their corresponding nine-carbon compounds.

DISCUSSION

In our study, chemical analyses of volatiles and pheromone gland extracts of male GWM resulted in the isolation and identification of six related compounds, four of which had not previously been identified. The compounds were the aldehydes, alcohols, and fatty acids of nonane and undecane. The aldehydes have already been demonstrated to play a role in the attraction of female GWM (Finn and Payne, 1977; Flint and Merkle, 1983). Even though the acids and alcohols represent a small percentage of the volatiles in comparison to the aldehydes, they also may be components of the pheromone blend. Studies of other species have shown that the minor components of a pheromone may not be attractive by themselves but when they are combined with the major components, they induce a greater response than would be achieved with the major components alone (Sweeney et al., 1990). The roles of the fatty acids and alcohols produced by the GWM should be established. If these compounds are proven to be pheromone components, the addition of these compounds to synthetic lures could result in greater attraction of females than was achieved previously (Finn and Payne, 1977; Flint and Merkle, 1983).

Behavioral bioassays should probably be initiated with a blend similar to that collected at the beginning of scotophase since this appears to be the period during which male GWM usually mate (Nielson and Brister, 1977). In Study

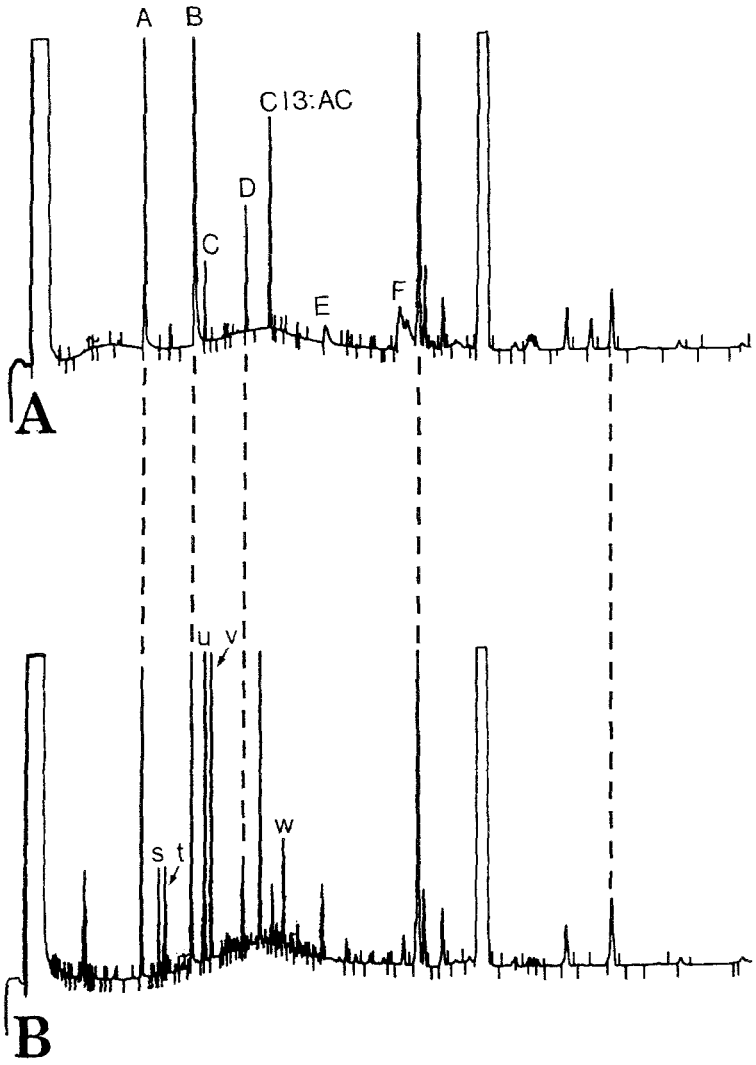

FIG. 1. A comparison of GC chromatograms from (A) a volatile sample collected from a group of five adult males of *G. mellonella* kept for 3 hr in a glass flask and (B) the same sample after methylation with 14% boron trifluoride in methanol. Dashed lines connect corresponding peaks. C13:AC, tridecanyl acetate = internal standard; A, nonanal (C9:Al); B, undecanal (C11:Al); C, 1-nonanol (C9:OH); D, 1-undecanol (C11:OH); E, nonanoic acid (C9:acid); F, undecanoic acid (C11:acid); s, 1,1-dimethoxy nonane; t, methyl nonanoate; u, 1,1-dimethoxy undecane; v, methyl undecanoate; w, tridecanol (product of methylation of C13:AC).

II, GWM males appeared to release pheromone during the third quarter of the 12-hr scotophase. However, behavioral studies have shown that GWM males do not call extensively during this time period (Romel and Scott-Dupree, unpublished). Pheromone release, which is usually initiated in response to the onset of scotophase, may have a rhythm independent of the length of scotophase.

Leyrer and Monroe (1973) conducted quantitative studies on the release of pheromones by male GWM and obtained a 3:7 ratio of C11:Al to C9:Al in the volatiles. In our study, C11:Al was the major component of the GWM pheromone, with the ratio of C11:Al to C9:Al at approximately 3:1. Leyrer and Monroe (1973) collected male GWM volatiles for 24 consecutive hr from newly emerged adults which may not have been fully mature. Also, different strains of the same species sometimes have different pheromone blends (Foster et al., 1989). Studies of different strains of the GWM should be conducted prior to any testing of synthetic pheromone in the field.

A comparison between the mean percentages of the compounds in the gland extracts and the volatiles illustrates that the alcohols were predominant in the gland extracts, while the aldehydes were predominant in the volatiles. Teal et al. (1986) determined that the alcohols found in pheromone gland extracts of *Heliothis virescens* (F.) and *H. zea* (Boddie) were the immediate precursors of their analogous aldehydes found in the volatiles. The two alcohols, C9:OH and C11:OH, in the pheromone glands of the GWM may be the precursors to C9:Al and C11:Al in the volatiles. The two acids, C9:acid and C11:acid, are also structurally similar to the main components of the GWM volatiles and the biosynthesis of these compounds may be interrelated.

The compounds found in the volatiles of male GWM could also have roles in interspecific communication. The major component, C11:Al, in the volatiles of the GWM is a pheromone component of the lesser wax moth (LWM), *Achroia grisella* F., although the function of C11:Al in the communication system of the LWM has not yet been determined (Dalm et al., 1971). The GWM also shares two components of its volatiles with *Achroia innotata obscurevittella* Ragonot, a pest of both *Apis cerana japonica* F., the Japanese honey bee, and *Apis mellifera* L. *A. innotata obscurevittella* releases a pheromone composed of 96.8% C9:OH and 3.2% C9:Al (Nemoto et al., 1990). C9:Al has no known function in the communication system of *A. innotata obscurevittella*. The major pheromone component of *A. innotata obscurevittella*, C9:OH, is a minor component in the volatiles of the GWM. Reproductive isolation of these related wax moth species is probably maintained by differences in the release rates and/or ratios of the common components and by the release of compounds unique to each species.

Behavioral bioassays will be necessary in order to determine the roles of the various components of the volatiles of male GWM in wax moth commu-

nication. A pheromone blend which is as attractive to female GWM as that emitted by live males could then be developed. At the very least, this pheromone blend could be used in traps in apiaries and storage facilities for beekeeping equipment for the monitoring and detection of GWM populations.

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ATTRACTION OF FEMALE FATHEAD MINNOWS, *Pimephales promelas*, TO CHEMICAL STIMULI FROM BREEDING MALES

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Abstract—Female fathead minnows, *Pimephales promelas*, were attracted to water that had contained conspecific males in breeding condition. The attraction was particularly strong in the morning and occurred in both females with mature gonads and gonadally regressed females. Females were also attracted to water that had contained other females but this attraction was weaker than the attraction to breeding males and tended to occur in afternoon trials. When offered a choice, females preferred breeding male water over regressed male water or female water. Swarming behavior, in which females formed a very active group near the water inlet, occurred primarily in test locations receiving water from breeding males. Our results indicate that breeding male *P. promelas* produce water-borne chemical stimuli that attract females and that females distinguish breeding male stimuli from female or regressed-male water-borne stimuli.

Key Words—Pheromone, fathead minnow, *Pimephales promelas*, Cyprinidae, mate attraction, breeding behavior, fish behavior.

INTRODUCTION

Pimephales promelas, the fathead minnow, inhabits freshwater systems of North America (Andrews, 1970). In southern Saskatchewan, large aggregations of males and females form in the spring (e.g., Smith and Lemly, 1986). As day

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length and water temperature increase, males exhibiting breeding tubercles, a dorsal mucus pad, and nuptial coloration associated with elevated androgen levels (Smith, 1974) move into the shallows. There they establish breeding territories associated with either rock cobble, floating, or submerged objects (Lord, 1927; Marcus, 1934; Isaak, 1961; Andrews and Flickinger, 1973). Spawning occurs when females leave the aggregation, move inshore, and oviposit on a suitable spawning substrate within a male's territory, after which the male tends the embryos until hatching (Marcus, 1934; McMillan and Smith, 1974; Sargent, 1988, 1989; Unger, 1983; Unger and Sargent, 1988).

During reproduction, male *P. promelas* use visual displays to solicit female attention and to indicate their readiness to spawn (Cole and Smith, 1987a) and females release chemical stimuli that increase male courtship behavior (Cole and Smith, 1987b). This study tested the hypothesis that mature male *P. promelas* release chemical stimuli that are attractive to females.

METHODS AND MATERIALS

Experimental Fish

Major collections of *P. promelas* were made at Bradwell Reservoir, 45 km southeast of Saskatoon, Saskatchewan (latitude 52°, longitude 106°) from late May to mid-July, which includes the reproductive season and the period directly preceding it. Fish of both sexes were collected by dip net from offshore aggregations, while sexually mature males, with breeding tubercles, dorsal pad, and nuptial coloration, were collected by dip net from spawning sites near shore. Although no direct examination was made of the testicular state, the presence of tubercles, dorsal pad, and nuptial coloring is associated with mature testis (Smith 1978). Some of these fish were brought directly into the laboratory for experimentation, while others were placed in large, outdoor holding pools under natural photoperiod and seasonally appropriate temperatures for use the following winter.

Eight weekly collections of 20 or more fish each were made throughout this period in order to provide baseline information on seasonal gonad development. All individuals from the weekly collections were killed with the fish anesthetic, 2-phenoxyethanol, and body weight and gonad weight were determined for each to provide gonosomatic index values (GSI = gonad weight as percentage of total body weight).

Experimental Apparatus

The experimental apparatus was designed to simulate the choices faced by female fathead minnows in nature. Females may remain offshore away from

male spawning sites or they may approach suitable sites and choose among those sites, some unoccupied and others occupied by males of various status. Our apparatus allowed females to remain in the open area of the tank or to enter spawning sites containing chemical stimuli from conspecifics.

This was done by introducing donor water (obtained from donor tanks) at separate point sources located in suitable spawning sites at either end of a 216-L test tank. Any subsequent attraction to particular donor waters on the part of recipient fish could then be measured by recording the time spent in close proximity to either donor water source.

Donor tanks (28.4 L) receiving a continuous flow from a central water supply (20 ml/min) contained with a single male (male donor tanks; $n = 4$), a single female (female donor tanks; $n = 4$), or no fish (control donor tanks; $n = 4$) (Figure 1). Donor water taken from donor tanks was then transferred to

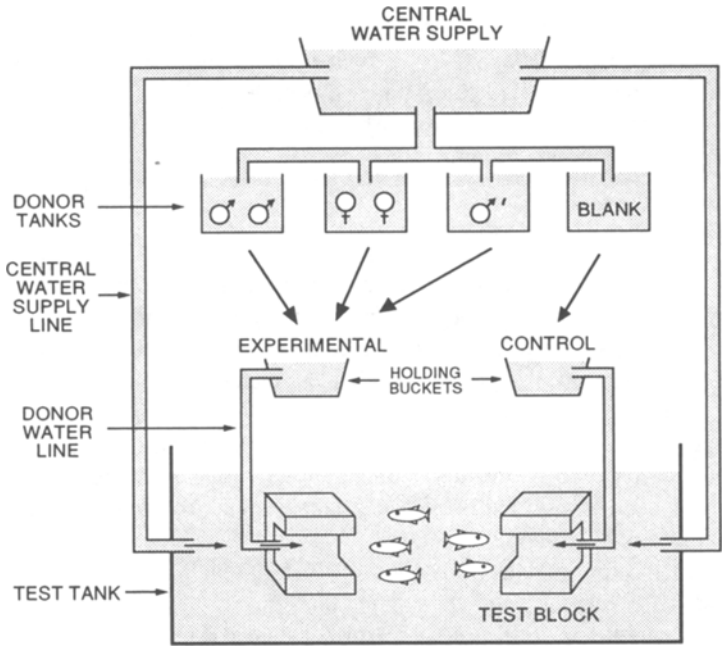

FIG. 1. The experimental apparatus. Water could flow into the test tank directly from the central water supply or stimulus water from donor tanks containing fish in various combinations of sex, maturity, and number could be introduced into the cavities of test blocks. Stimulus waters could be switched from one test block to another. The test blocks were inherently attractive to minnows but test stimuli could alter the relative attractiveness of the two blocks.

1-L holding buckets. From there, donor water could be introduced into test tanks holding recipient females.

Four 216-L test tanks were used in all experimental trials. Each test tank contained a pair of vertically stacked c-shaped concrete blocks in each corner. These blocks (190 × 190 × 190 mm) attract *P. promelas* because of the visual presence of overhanging ceilings that are preferred as spawning surfaces (McMillan and Smith, 1974). Water, which was taken from donor tanks during experimental trials and the central water supply at all other times, entered the test tanks via the top block (test block) in each pair (Figure 1). This block was placed so that the open portion was directed forward, while clear plastic plates on each side formed a partially enclosed space that received the donor water. Water was introduced into the cavity through a rigid, J-shaped tube that passed through a hole in one of the plastic plates and which could be connected with plastic tubing to various elevated holding buckets of donor water or to the central water supply. Donor water then flowed by gravity into each of the two concrete test blocks, with a constant flow rate (30 ml/min) controlled by tubing clamps.

Both donor and recipient fish in the laboratory were maintained in flow-through water conditions at 20–22°C both prior to and during portions of the experimental trials and at all times under a photoperiod matched to natural day length. Prior to experimental trials, fish were fed twice daily to satiation with commercial fish food (Tetramin).

Experimental Protocol

Each experimental trial consisted of recording the responses of a group of five female *P. promelas* before and after the introduction of various donor waters. Each five-fish test provided one comparison, or replicate. Each experimental trial consisted of eight such replicates, using new females for each test ($n = 8$ groups).

Experimental donor water was obtained by taking (1) an equal mixture of water taken from two of the four donor tanks (chosen at random) containing same-sex fish or (2) a mixture of water taken from two control donor tanks, containing no fish, for control water.

For each experimental trial, each of 20 test females was marked with a unique set of notches on the caudal fin, then placed in a small aquarium for several hours until any release of alarm substance (Smith, 1973) was likely complete. After this, marked females were divided into four groups of five each and placed in one of four test tanks. In addition, several white cloud minnows (*Tanichthys albonubes*) were added to each of the test tanks to provide a dither effect (Barlow, 1967). Trials began 24 hr after females had been marked and placed in test tanks.

A trial consisted of recording female behavior during three intervals: (1) a

prestimulus 15-min interval, with water from the central supply flowing into both blocks; (2) an initial 15-min stimulus interval, with control water entering one block and experimental donor water entering the other; and (3) a second 15-min stimulus interval, with the water lines interchanged so that control water flowed into the former experimental block and experimental water into the former control block. In each of the intervals, female response was measured by recording the amount of time each of the five females spent in each of the two test blocks, then summing their responses for a group total.

In addition to block occupancy, test females in summer trials frequently exhibited an unusual and conspicuous behavior that we termed "swarming." Swarming was defined as occurring when three or more females aggregated in one of the test blocks and exhibited rapid and jerky swimming motions inside the block, rapid circling of the test block ceiling, and nibbling motions directed toward the ceiling or the inlet tube. Occasionally one or more of the females appeared to rub the ceiling with the head or dorsum. Aggressive interactions were markedly absent during swarming behavior. The number and duration of swarming bouts were recorded and compared among the various experimental conditions.

Following testing, females were killed with 2-phenoxyethanol and GSI values calculated. Although GSIs provide only a rough index of gonadal state, they do distinguish between fish that are near sexual maturity and those that are not in breeding condition. In addition, these values could be compared with those obtained from females collected from the field in weekly collections made from May to July.

Experimental Trials

Summer Trials. Following collection from the field, fish were brought back to the laboratory and placed in holding tanks for 24 hr to adjust to the laboratory environment. Subsequently, test females (summer females) were marked with caudal fin notches as described above, placed in groups of five in test tanks with whitecloud minnows, then tested 24 hr later.

In the morning (A.M. trials), each group was tested with control water versus water from either male or female donors. Then 5–6 hr later, during the afternoon (P.M. trials), the same females were tested with control water versus water from donor tanks of the other sex. Eight groups of summer females were tested with female donor water first and eight with male donor water first.

Winter Trials. A second series of trials used females that had been held in outdoor pools for 6 months following the end of the reproductive season (winter females). Winter temperatures in the pools were maintained near 10°C. Following a 36-hr acclimation period to adjust to the 18–20°C temperature in the

laboratory, groups of five females were individually marked with caudal notches, placed in test tanks with white cloud minnows, then tested 24 hr later.

Donor water was obtained from tanks containing one of three types of fish: (1) adult males showing full breeding morphology, tubercle development, dorsal pad, and nuptial coloration (i.e., developed males); (2) gonadally regressed males (i.e., regressed males); or (3) winter-condition (i.e.,gonadally regressed) females. All donor fish were held at a density of one per tank. As in summer trials, water from two of four tanks of similar donor type were chosen at random and mixed together prior to introduction into test tanks.

Unlike summer trials, recipient females in winter trials were given a choice between two simultaneously presented, conspecific stimuli, following a 15-min prestimulus interval. In 12 recipient female groups, donor water from developed males was introduced at the same time as donor water from regressed males during the first 15-min stimulus interval. The donor lines were then changed for the second 15-min stimulus interval so that the stimulus conditions in the two test blocks were reversed. In nine additional recipient winter-condition female groups, developed male donor water was paired with water from regressed females.

All winter trials were carried out in the morning. At the end of each trial the test females were killed and GSI values were calculated, as described above, for each individual. At the end of the experiment the donor fish were also killed and their gonadal status was checked by determining GSI values.

Data Analysis

In all trials, the total number of fish-seconds spent by the five recipient females in each of the two test blocks was recorded for each interval of the test. Since each test interval was 15 min long, each group of five test females could accumulate a maximum of 4500 fish-sec. Comparisons were then made between prestimulus (before introduction of donor water) and successive stimulus intervals. First, the total number of fish-seconds spent in each of the two test blocks during the prestimulus interval was compared with that of the first 15-min stimulus interval (the introduction of either experimental or control donor water into the test blocks in summer trials, the simultaneous introduction of two types of experimental donor water into test blocks in winter trials). Second, the total number of fish seconds spent in each of the two test blocks during the first stimulus interval was then compared with that of the second 15-min stimulus interval which followed the exchange of donor types between test blocks. The Wilcoxon matched-pairs, signed-rank test (Siegel, 1956) was used to test for significance. Since trials were conducted on eight sets of five females, there were eight comparison pairs ($n = 8$) for the Wilcoxon tests. In some tests, ties reduced the effective sample size to seven.

RESULTS

Summer Trials

Summer females with mature ovaries showed a strong attraction to test blocks receiving water from reproductive males during the first stimulus interval, compared to the prestimulus interval, in both morning and afternoon trials (Figure 2). When donor sources were interchanged in the second stimulus interval,

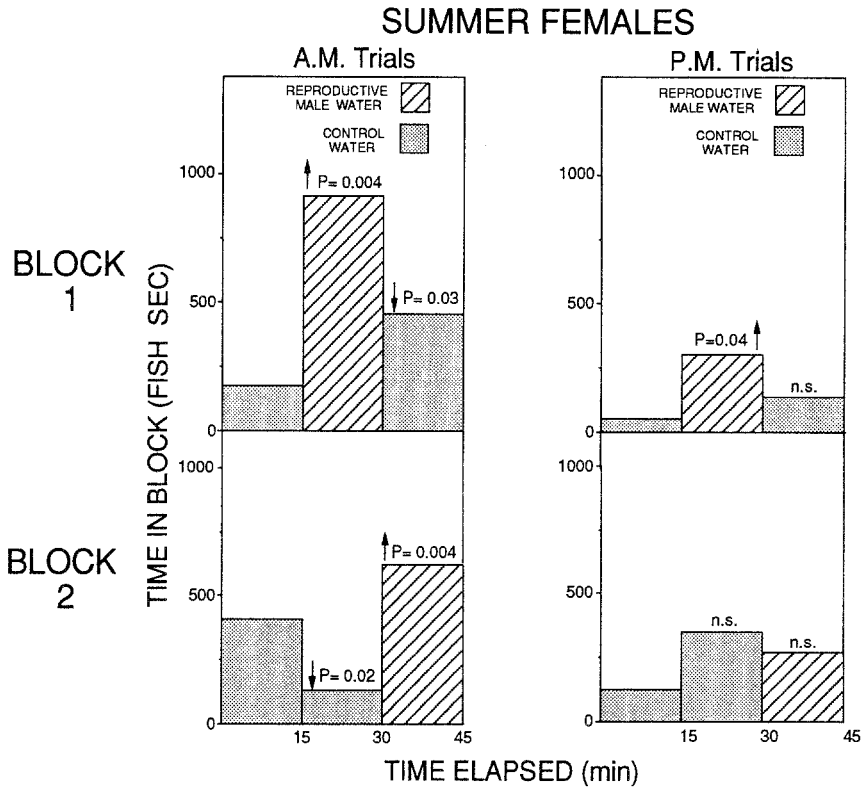

FIG. 2. Median times (fish-seconds) spent by summer test females in two test blocks during the introduction of control water to both blocks for 15 min, followed by the introduction of reproductive-male donor water into the least favored block for 15 min, then interchange of the two stimuli for a final 15 min. Eight groups of five females were tested in morning trials (A.M. trials) and eight groups of five in afternoon trials (P.M. trials). Probabilities are based on comparing occupancy rates in the same test block for successive observation periods in eight separate trials [Wilcoxon matched-pairs, signed-rank test (Siegel, 1956)].

the attraction to reproductive male donor water persisted in A.M. trials but not in P.M. trials (Figure 2).

A difference was also evident in females' response to test blocks in the morning compared to the afternoon. There was a significantly greater degree of test block occupancy (both control and experimental test blocks) in morning trials than in afternoon trials in the first stimulus interval [$U = 8$, $N = 8$, $P = 0.005$, Mann–Whitney U (Siegel, 1956)]. Also, response to male donor water was less pronounced in afternoon trials compared to morning trials (Figure 2) and block occupancy did not change significantly after the exchange of experimental and control stimuli.

Summer females were unresponsive to female donor water during morning trials (Figure 2). A significant attraction was evident during afternoon trials, but only at its initial introduction; when experimental and control lines were interchanged, the attraction response disappeared (Figure 3).

In male donor trials, the occurrence and duration of swarming behavior was significantly greater during stimulus intervals (9 of 16 trials; durations per trial, 13, 63, 15, 37, 399, 420, 17, 169, and 22 sec) than during prestimulus trials [one instance, 120 sec; $N = 9$, $P = 0.002$, sign test (Siegel, 1956)] and occurred more frequently in the male-donor blocks (seven trials) than in the control-donor blocks (two trials) [$P = 0.005$, Fisher exact test (Siegel, 1956)]. Swarming frequency declined in the second stimulus interval, occurring in only 2 of 16 trials, both in the male-donor block.

There was also a temporal pattern to swarming behavior. Male donor water induced swarming behavior in morning trials for longer periods than in afternoon trials [$N = 8$, $P = 0.014$, Mann–Whitney U (Siegel, 1956)].

The introduction of male donor water also induced more swarming behavior than did the introduction of female donor water, regardless of the time of the trial [$N = 9$, $P = 0.01$, Wilcoxon matched-pairs, signed-ranks test (Siegel, 1956)]. Within female donor water trials, swarming did occur but did not significantly increase from prestimulus levels after the introduction of female donor water [prestimulus interval—one 10-sec swarming event; first stimulus interval—four events of 15, 16, 6, and 6 sec, respectively, all in experimental donor blocks; $N = 5$, $P = 0.188$, sign test (Siegel, 1956)]. No swarming was seen during the second stimulus interval of female donor water trials.

There was a wide range of gonadal development in field-caught females in the eight collections made over 53 days covering the natural breeding season (Table 1). Test females used in the summer trials also exhibited a wide range of GSIs but reflected elevated values typical of midbreeding season (mean GSI $= 11.2 \pm 3.9$ SD; range, 3.1–20.9; $n = 73$).

Among test groups of summer females that were responsive to male stimuli, there was no correlation between responses of individual females, as measured

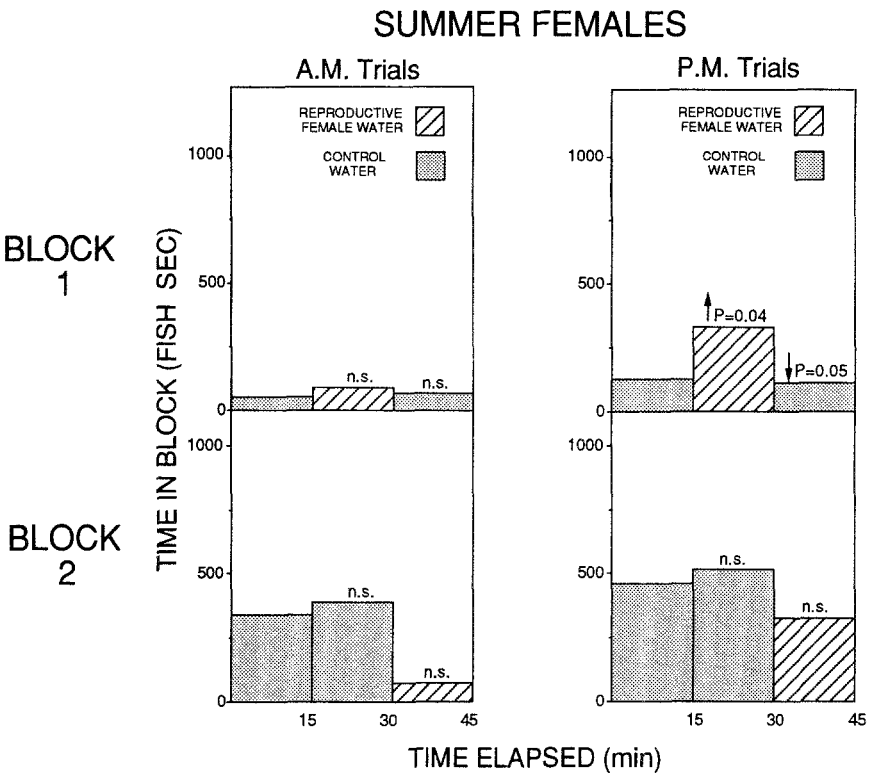

FIG. 3. Median times (fish-seconds) spent by summer test females in two test blocks during the introduction of control water to both blocks for 15 min, followed by the introduction of reproductive-female donor water into the least favored block for 15 min, then interchange of the two stimuli for a final 15 min. Eight groups of five females were tested in morning trials (A.M. trials) and eight groups of five in afternoon trials (P.M. trials). Probabilities are based on comparing occupancy rates in the same test block for successive observation periods in eight separate trials [Wilcoxon matched-pairs, signed-rank test (Siegel, 1956)].

by occupancy time in experimental test blocks, and their GSI value (slope = 0.0149; $r = 0.2980$; $n = 50$).

Winter Trials

In winter trials, females showed an increase in occupancy of the test block receiving reproductive-male donor water. However, these same recipient females made no distinction between blocks receiving donor water from either nonre-

TABLE 1. MEAN GSI VALUES AND STANDARD LENGTHS (SL; MM) OF FIELD-CAUGHT FEMALES OBTAINED IN EIGHT COLLECTIONS[a]

	Collection date							
	May 20	May 26	June 2	June 9	June 15	June 22	June 29	July 12
GSI	8.3 (1.2)	7.5 (1.2)	8.5 (2.6)	13.6 (4.1)	16.6 (3.7)	14.2 (2.5)	13.1 (3.1)	8.2 (2.7)
SL	48.8 (3.4)	46.5 (2.7)	47.3 (3.7)	51.2 (3.5)	48.5 (3.1)	49.9 (4.9)	48.9 (3.2)	48.8 (2.7)
n	20	15	24	14	9	14	27	34

[a] Standard deviations for each mean value are given in parentheses. *n* is sample size.

productive males or females (Figure 4). The attraction to reproductive male donor water was short-lived, however, compared to summer trials since switching of the donor sources in the second stimulus interval did not lead to a change in block occupancy patterns by test females.

Winter females also exhibited swarming behavior. In nine trials using donor water from sexually mature males tested against water from regressed males, no swarming was seen during the prestimulus interval but swarming did occur during the first stimulus interval in four of the nine trials (durations, 111, 37, 240, and 82 sec). In all cases, the swarming occurred in the block receiving developed-male water. In the second stimulus interval, after donor lines were interchanged, swarming was seen in three trials, twice in the developed-male donor tank (69 and 3 sec) and once in the regressed-male donor block (41 sec).

In developed-male versus undeveloped-female donor trials, swarming increased significantly following the first introduction of the two donor waters [prestimulus interval—one 68-sec swarming event; first-stimulus interval—12 swarming events, of 55, 388, 122, and 92 sec in the developed-male test block and 280, 341, 9, 49, and 21 sec in the undeveloped-female block; $X = 0$, $N = 10$, $P = 0.001$, sign test (Siegel, 1956)]. However, no preference was seen for swarming in a block receiving a particular donor water [$P = 0.30$, Fisher exact test (Siegel, 1956)].

The calculation of GSIs for 103 winter test females (gonads of two fish were damaged during histological preparation) confirmed that females were in a gonadally regressed state (mean female GSI of test females in male versus female donor trials = 5.0 ± 1.1, range = 2.6–7.4, $N = 60$; mean female GSI in developed male versus undeveloped male trials = 3.6 ± 2.5, range = 1.7–14.4, $N = 43$).

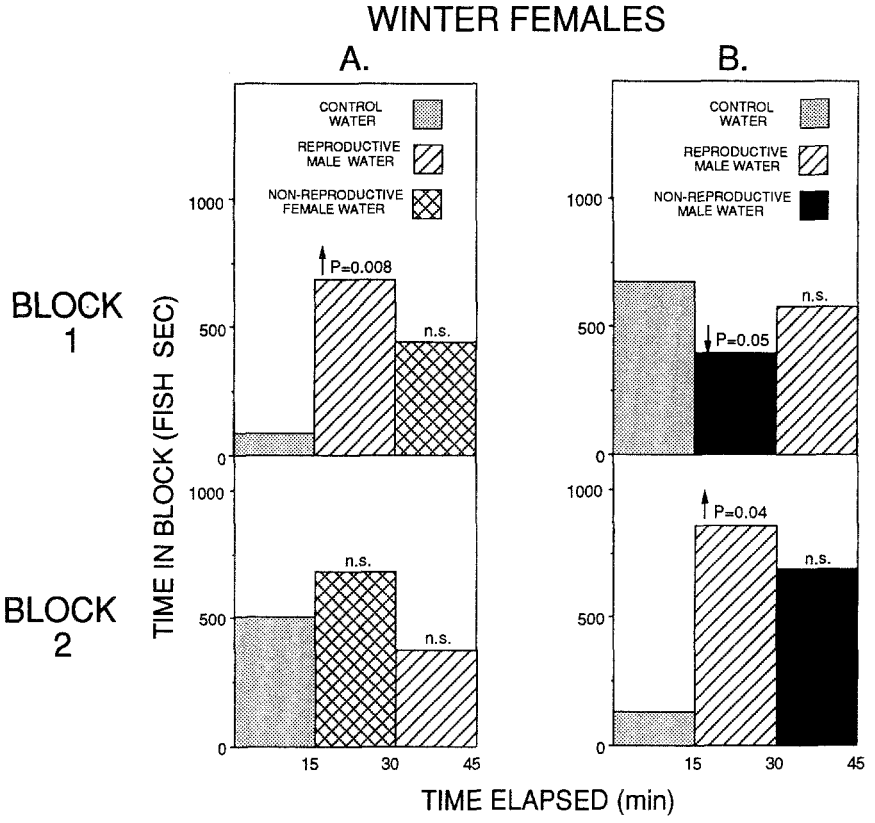

FIG. 4. (A) Median times (fish-seconds) spent by eight groups of five winter test females in two test blocks during the introduction of control water to both blocks for 15 min, followed by the introduction of reproductive-male donor water into the least favored block and nonreproductive female water into the other block for 15 min, then interchange of the two stimuli for a final 15 min. Probabilities are based on comparing occupancy rates in the same test block for successive observation periods in eight separate trials [Wilcoxon matched-pairs, signed-rank test (Siegel, 1956)]. (B) Median times (fish-seconds) spent by eight groups of five winter test females in two test blocks during the introduction of control water to both blocks for 15 min, followed by the introduction of reproductive-male donor water into the least favored block and nonreproductive male water into the other block for 15 min, then interchange of the two stimuli for a final 15 min.

Visual examination of the gonads of undeveloped males and females that were used as donor sources confirmed their sex and their regressed gonadal state.

DISCUSSION

Female *P. promelas* tested during the reproductive season were strongly attracted to water taken from tanks holding male conspecifics with mature gonads and secondary sexual characters during both morning and afternoon trials. Water from female *P. promelas* was also attractive to summer females, but only during afternoon trials, and responses to both male and female donor water in afternoon trials were consistently lower than the response to male water in the morning. Gonadally regressed females tested in the winter were also attracted to water from tanks holding developed males but not to water, introduced at the same time, from tanks holding either undeveloped males or undeveloped females.

The increased occupancy by test females at sites receiving water that had contained reproductive male *P. promelas* suggests that males produce a waterborne chemical substance(s) that is(are) attractive to females. These cues may function to direct females to males that are ready to spawn. The stronger response of summer females in morning trials indicates either diel variation in female responsiveness to male and female cues or diel variation in the release of chemical stimuli by the donor fish. Spawning in *P. promelas* has been reported to occur primarily in the morning. Gale and Buynak (1982) found that captive *P. promelas* spawned 73.8% of their eggs between midnight and 1000. Our findings are consistent with this observation, inasmuch as females were significantly more responsive to male chemical stimuli in the morning than in the afternoon.

Substances that are produced by males and attract females to suitable spawning sites have been described in various fish species, including rainbow trout, *Oncorhynchus mykiss* (Newcomb and Hartman, 1973), *Blennius pavo* (Laumen et al., 1974), zebrafish, *Brachydanio rerio* (Bloom and Perlmutter, 1977), and the black goby, *Gobius jozo* (Colombo et al., 1980). Additional examples are reviewed by Stacey et al. (1986). Colombo et al. (1980) suggest that male *G. jozo* that defend contiguous territories may contribute to a group signal that attracts females at some distance from the spawning site. The active component is apparently a steroid glucuronide. This is consistent with the finding that male African catfish, *Clarias gariepinus*, release steroid glucuronides from the seminal vesicles that attract females and stimulate ovulation and vitellogenesis, acting through the olfactory receptors and the medial olfactory tract (Resink et al., 1989a–c,; 1990; Van Weerd et al., 1990). In *P. promelas*, breeding males often defend closely associated territories and other members of the population tend to form aggregations near the spawning areas (Cole and Smith,

personal observation). Hence, attractive substances produced by male *P. promelas* may identify nest sites of defending males.

Substantial research has been conducted on steroid and prostaglandin pheromones released by female fish (e.g. Sorensen et al., 1988; Sorensen et al., 1990). However, less is known about the sources and chemistry of the water-borne cue(s) released by male fish. The results presented here indicate that *P. promelas* may provide a potential assay system for the analysis of the female-attracting chemical cue(s) produced by males. Potential anatomical sources of such cues include the gonads and the secretory dorsal pad (Smith and Murphy, 1974).

The occurrence of a strong attraction to water-borne reproductive male cues in both summer females in a range of gonadal states and winter females with regressed ovaries indicates that female responsiveness to male stimuli is not tightly linked to ovulation or to a developed ovarian state. This is in contrast to female *G. jozo*, which respond to attractant pheromones from males only between the time of ovulation and egg deposition (Colombo et al., 1980). In a related study we have found that female *P. promelas* can express sexual behavior, including elements of ovipositing behavior (without egg release), while in a gonadally regressed state (Cole and Smith, 1987a).

While gonadally regressed female *P. promelas* were attracted to blocks receiving water exposed to reproductive males, occupancy rates in blocks receiving water exposed to either nonreproductive females or nonreproductive males did not show a similar increase. However, due to the experimental protocol used, undeveloped female and undeveloped male donor water were both introduced into test blocks previously having high occupancy rates. Any minimal attraction response that would have been obvious in low-occupancy blocks may have been obscured. Thus, we cannot rule out the possible occurrence of a weak attraction of winter females to a conspecific chemical(s) from females or nonreproductive males.

Swarming behavior by test females in reproductive condition was exhibited primarily in response to reproductive-male donor water. However, it is unclear whether swarming was triggered solely by chemical cues or if successive females were attracted by the behavior of the first females entering the test block; some combination of chemical and visual cues from introduced donor water, other females, or potential spawning surfaces may release swarming responses. The function of swarming behavior is unknown but is resembles behavior seen in the field and laboratory when several females intrude into a male's territory together, some attempting to spawn and others eating eggs (R.J.F.S., personal observation).

In winter trials, gonadally regressed females also exhibited swarming behavior but they swarmed equally in blocks receiving water from developed males and regressed females. Chemical substances released by males artificially

maintained in a reproductive state may differ in form or potency from those of naturally reproductive males, resulting in more ambiguity in female response. Alternatively, conspecific odors from gonadally regressed females may also stimulate swarming responses in other gonadally regressed females. If so, the virtual absence of swarming in response to female donor water in summer female trials suggests that the production of this stimulus may be absent in summer females, or its stimulus value minimized in females with mature ovaries.

The attraction of female *P. promelas* to water exposed to reproductively developed male conspecifics suggests that male *P. promelas* release a waterborne chemical substance(s) that attracts females to male territories and spawning sites. Such attractants may be particularly important in the turbid habitat often occupied by *P. promelas* and during the predawn hours when spawning often occurs in nature. Our findings, however, leave several phenomena that deserve further study. These include the apparent diel variation in female responsiveness, the source and nature of the attractant male chemical cues, and the role of the endocrine system in the control of the male and female components of the system.

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Book Review

Ecological Chemistry and Biochemistry of Plant Terpenoids, Proc. Phytochem. Soc. Europe No. 31. J.B. Harborne, and F.A. Tomas-Barbenan. Oxford, UK: Clarendon Press, 1991. $115.00, 459 pp.

The title of a book should convey the essence of its contents. It is therefore unfortunate that the 31st publication from the Proceedings of the Phytochemical Society of Europe pretends to cover ecological chemistry and biochemistry of plant terpenoids when it gives ecological chemistry a scant one-eighth of the contents: two chapters, to be more precise—one of which comes at the very end, when it would have made a nice introduction, particularly in the absence of an overall survey of plant terpenoids.

The editors, in their introduction, claim that the book is divided "somewhat arbitrarily into four sections: chemistry and chemotaxonomy; biochemistry, biosynthesis and physiology; pharmacology and toxicology; and ecological biochemistry." All these topics are indeed covered, but there is so much overlap that the desirable sections are unfortunately far from evident. The book is divided into 16 chapters, with contributions coming mainly from the United Kingdom and Spain (the venue for the September 1989 meeting on which the proceedings are based). It therefore took 2 years to put all this information together, a rather slow process. Papers, however, do include references which appeared in 1990 (e.g., Perspectives on Plant Competition, edited by T.A. Grace and D. Telman). There is a table of contents, subject index, and index of organisms, the latter being very useful indeed. The illustrations are of excellent quality.

The introduction is in fact an overview of the book contents, and it is difficult to review this publication without repeating what the introduction states. The first four chapters, which, according to the editors, constitute the first section (Chemistry and Taxonomy), discuss the recent chemistry of conifer terpenoids, the terpenoids of marine plants, the chemistry, biogenesis, and chemotaxanomy of the diterpenoids of *Salvia* (of the Labiatae family—a genus quite restricted in its geographic distribution), and environmental control of essential oil production in Mediterranean plants.

Chapters 5 to 8 are devoted to several aspects of the biochemistry and physiology of terpenoids. This section starts with an interesting review of terpenoids production in plant cell cultures, followed by the biosynthesis and distribution of plant iridoids in angiosperms. Chapter 7 deals with various aspects of biosynthesis, catabolism, and regulation of terpenoid phytoalexins, the latter

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term following the definition of Paxton (1981): low molecular weight, antimicrobial compounds synthetized and accumulated in plant cells after exposure to microorganisms. This chapter contains a useful table of terpenoid phytoalexins produced in nine plant families; the last chapter in the section deals with inhibition of phytosterol biosynthesis and the consequences for plant growth.

Section three is essentially devoted to pharmacology and toxicology. Chapter 9 concentrates on the newer (diterpenoids) and the more interesting findings from a pharmacological point of view, while Chapter 10 is devoted to the chemistry and molluscicidal action of saponins. As expected, the molluscicidal saponins of *Phytolacca dodecandra* berries are discussed, considering the importance of this plant for an eventual natural control of Schistosomiasis.

The chemical ecology announced in the title of the book finally gets its meaning from Chapter 11 (Chemical Ecology of Terpenoid and Other Fragrances of Angiosperm Flowers). Chapter 16 (Recent Advances in the Ecological Chemistry of Plant Terpenoids) would have been a better introduction to this fourth section. Other contributions deal mainly with various aspects of plant–insect relationships where terpenoids play a role: lower terpenoids as natural insect control agents; sequestration of iridoids by insects. A chapter is also devoted to plant terpenoids as allelopathic agents.

Altogether, this book, which covers mostly the literature appearing in the second part of the 1980s, will provide researchers with ample material for further investigations in what remains an exciting area of natural products chemistry and potential promising applications.

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INHIBITION OF GROWTH OF *Phytophthora parasitica* VAR. *nicotianae* BY AROMATIC ACIDS AND COUMARINS IN A LABORATORY BIOASSAY

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Abstract—Hydroxy-, dihydroxy-, trihydroxy-, methoxy-, dimethoxy-, hydroxy- + methoxy-, amino-, chloro-, and nitro-substituted benzoic, phenylacetic, phenylpropanoic, and phenylpropenoic (cinnamic) acids were evaluated for activity against the growth of *Phytophthora parasitica* var. *nicotianae*, Races 0 and 1, in a laboratory bioassay. Several substituted coumarins were also tested. In general, for Race 0, the phenylpropenoic acids were more active (on a millimolar basis), than the corresponding benzoic, phenylacetic, or phenylpropionic acids (9 of 14 series). Among the most active acids were *o*-hydroxycinnamic and the chloro- and methoxycinnamic acids. The activities of unsubstituted benzoic and phenylpropionic acids were comparable to the most active compounds tested. Monohydroxyaromatic acids were more active than most dihydroxy acids of the same chain length. Dihydro-3,4-dihydroxycinnamic acid was slightly more active than the corresponding cinnamic acid, while the reverse was true for the mono-*p*-hydroxycinnamic acid versus *p*-hydroxyphenylpropionic acid. Coumarin was more active than its hydroxy, methyl, hydroxymethyl, or methoxy derivatives. In general, Race 1 was even more significantly affected by the aromatic acids. Glycosylated coumarins were inactive in the bioassay, compared to their aglycones.

Key Words—*Phytophthora parasitica* var. *nicotianae*, black shank, aromatic acids, bioassay, growth inhibition.

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INTRODUCTION

Phenols inhibit the growth of microorganisms. Several reports have shown that phenolic compounds of tobacco increase in tissues infected by tobacco mosaic virus (Fritig and Hirth, 1981), *Peronospora tabacina* (blue mold) (Cohen and Kuc, 1981), *Pseudomonas solanacearum* (bacterial wilt) (Sequeira, 1969), and *Thielaviopsis basicola* (black root rot) (Gasser et al., 1988). Preliminary studies have shown that the major phenolic compounds of tobacco roots, 3-*O*-caffeoylquinic acid (chlorogenic acid) and the coumarins, scopolin and scopoletin, are also increased in healthy tissue adjacent to tissue infected with the black shank fungus (*P. parasitica* var. *nicotianae;* Snook et al., 1991). Chlorogenic acid inhibited the growth of the fungus in a laboratory bioassay, and the activity resided in the caffeic acid moiety of the molecule. Scopoletin was also shown to inhibit *P. parasitica* var. *nicotianae* growth. The objectives of this research were to further elucidate this structure–activity relationship by testing a number of aromatic acids and coumarins for inhibition of growth of *P. parasitica* var. *nicotianae* (Races 0 and 1).

METHODS AND MATERIALS

All aromatic acids and coumarins (except scopolin, see below) were obtained commercially. Except for 7-hydroxycoumarin (which was sublimed), all were used as received. The laboratory bioassay of compounds for inhibition of growth of *P. parasitica* var. *nicotianae* (PPN) was similar to that described (Csinos et al., 1986). Each concentration of test material and untreated controls was replicated 10 times. Cork borer plugs (5 mm, No. 2 cork borer) of the fungus stock culture, were placed with fungal culture side down on the edge of the test plates. Plates were placed in Ziploc bags and incubated at 27°C for 14 days or until fungal growth had nearly reached the opposite edge of the agar control plates. Radial mycelial growth was measured, and the percentage inhibition of growth was determined relative to the control. Averages of the 10 replications at each compound concentration and the controls were used in the calculations.

Effective dose for 50% growth inhibition (ED_{50}) was calculated for each compound tested by plotting percent inhibition versus concentration (straight lines were drawn between each data point) and determining from the graph the concentration (mM) of the compound that would produce a 50% inhibition of fungal growth. An example is given in Figure 1 and shows a plot of the percentage inhibition of growth versus concentration of monohydroxybenzoic acids. The shape of the concentration–activity curves shown was characteristic for all of the compounds tested. From the curves in Figure 1, ED_{50} can be determined to be 78, 179, and 246 mM for *o*-, *p*-, and *m*-hydroxybenzoic acid respectively.

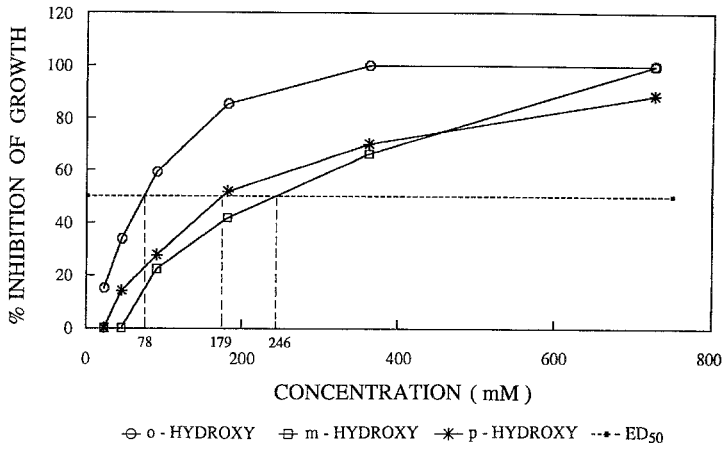


FIG. 1. Percent inhibition of growth of *P. parasitica* by hydroxybenzoic acids.

The lower the ED_{50}, the more active the compound. For several compounds, only the highest concentration of compound gave any inhibition of growth, and ED_{50}s were estimated by a simple proportion from that value to 50% inhibition.

Scopolin was isolated by the following new procedure from freeze-dried, flue-cured tobacco roots. The ground roots (230 g) were extracted with MeOH (3 × 400 ml), and the extract was filtered with the aid of celite. The solvent was reduced to about 200 ml with a rotary evaporator and 30 g of silicic acid (Mallinkrodt 100 mesh, washed with MeOH and heated at 150°C for 16 hr) and 100 ml isooctane added. The solvent was removed and the sample–silicic acid mixture was deposited onto the top of a silicic acid column (100 g packed in a 3 × 33-cm column with methylene chloride, CH_2Cl_2). The column was eluted with the following solvents: 50% ethyl acetate (EtOAc)–CH_2Cl_2, 1 liter; 75% EtOAc–CH_2Cl_2, 1 liter; EtOAc, 1 liter; 20% acetone–EtOAc, 1 liter; 40% acetone–EtOAc, 1 liter; 60% acetone–EtOAc, 1 liter; 80% acetone–EtOAc, 8 liters. The last solvent eluted the scopolin, was evaporated to dryness, and the residue dissolved in 40 ml MeOH–H_2O (1:1). This mixture was divided into two equal portions and each chromatographed on a preparative reverse-phase column (Waters PrepPak 500 material repacked into a Cheminert LC column, 2.54 × 58 cm) and eluted at 2 ml/min with 35% MeOH–H_2O to yield 388 mg of >95% pure scopolin with mp 222–224°C; Lit. mp 226–228°C (Pollock and Stevens, 1965). Apparently, due to tightly bound water, the scopolin isolated from the reverse-phase column would not crystallize. A convenient method of displacing this water of hydration was to dissolve the scopolin in MeOH and add an equal volume of acetonitrile. After this solution was evaporated to dryness, the solid residue could readily be crystallized from MeOH.

RESULTS AND DISCUSSION

Laboratory Bioassays of Aromatic Acids against Race 0 PPN. Our previous work on the activity of chlorogenic acid and its component parts, caffeic acid and quinic acid, against inhibiting the growth of *P. parasitica* var. *nicotianae* (PPN) showed that the activity of chlorogenic acid lies in the caffeoyl moiety of the molecule (Snook et al., 1991). This prompted us to examine other substituted aromatic acids for activity against *P. parasitica* in our laboratory bioassay. The effect of alkyl chain length on activity was investigated by examining the series: benzoic, phenylacetic, phenylpropionic, and phenylpropenoic acids. The results of this study are given in Table 1 and allowed structure—activity relationships to be determined.

For the monohydroxy aromatic acids, the order of activity was: *o-* > *p-* > *m-*, except for the phenylpropenoic acids, where *m*-hydroxy was more active than *p*-hydroxy. In examining the effect of chain length on the activity of the monohydroxy acids, it was found that phenylpropenoic acids were the most active in each series (*o-*, *m-*, or *p-*), while the phenylacetic acids were the least active. *p*-Hydroxyphenylpropenoic acid (*p*-coumaric acid) exists in either the *cis* or *trans* configuration; both isomers were tested. The data (Table 1) showed that the *trans* isomer was slightly more active than the *cis* isomer.

The six isomers of dihydroxybenzoic acid were tested for inhibition of growth of *P. parasitica* and the ED_{50}s are presented in Table 1. The 2,3-dihydroxy isomer was the most active, while the isomers without an *o*-hydroxyl group were inactive. Of the three trihydroxybenzoic acids tested, only the 2,3,4- isomer showed activity. Based on the results with the dihydroxybenzoic acids, expected activity for 2,4,6-trihydroxybenzoic acid, with two *o*-hydroxyls, was not found.

As mentioned before, caffeic acid (3,4-dihydroxyphenylpropenoic acid), is a component part of the tobacco root compound, chlorogenic acid. It exhibited an ED_{50} of 211 mM (Table 1). Of the 26 hydroxy acids tested, 11 were more inhibitory than caffeic in the laboratory bioassay. Dihydrocaffeic acid [β-(3,4-dihydroxyphenyl)propionic acid] was slightly more active than caffeic acid (198 mM vs. 211 mM), which is the opposite result of that found for the three monohydroxy acids. 2,5- and 3,4-Dihydroxyphenylacetic acids were inactive in the bioassay. Thus, the activity of the 3,4-dihydroxy acids was: phenylpropionic $\geq$ phenylpropenoic (cinnamic) >> benzoic >>>> phenylacetic.

Methoxy and dimethoxy aromatic acids were some of the most inhibitory compounds evaluated (Table 1). The methoxybenzoic acids were two to seven times more active than the corresponding hydroxy acids, while the activity of the methoxycinnamic acids ranged from one to four times the activity of their hydroxy counterparts. While the *p*-methoxybenzoic acid was found to be the

TABLE 1. INHIBITION BY AROMATIC ACIDS OF GROWTH OF *Phytophthora parasitica* VAR. *nicotianae*, RACE 0 AND RACE 1

Substituent	ED_{50} (mmolar)[a]			
	Benzoic acid	Phenylacetic acid	Phenylpropanoic acid	Phenylpropenoic acid (*trans*)
None	15 (60)[b]	110 (108)	17 (35)	144 (324)
OH				
o-	78 (67)	153 (136)	34 (24)	14
m-	246 (222)	307 (263)	241 (161)	63
p-	179 (164)	263 (210)	151 (60)	98
p-cis	—	—	—	118
DiOH				
2,3-	203 (212)	—	—	—
2,4-	381 (368)	—	—	—
2,5-	532 (550)	—[c] (—)[c]	—	—
2,6-	571 (502)	—	—	—
3,4-	1300[d] (2160)[d]	—[c] (—)[c]	198	211 (137)
3,5-	1080[d] (2600)[d]	—	—	—
TriOH				
2,3,4-	439 (428)	—	—	—
2,4,6-	—[c] (—)[c]	—	—	—
3,4,5-	—[c] (—)[c]	—	—	—
MeO				
o-	35 (38)	—	—	15
m-	39 (45)	—	—	13
p-	22 (24)	—	—	24
DiMeO				
2,5-	49 (396)	60 (—)[e]	—	23 (112)
3,4-	46 (458)	320 (2830)[d]	—	59 (199)
OH + MeO				
4-OH-3-MeO	106 (88)	—	—	81 (41)
3-OH-4-MeO	—	—	—	21
4-OH-3,5-DiMeO	—	—	—	179
NH_2				
m-	146 (146)	—	—	—
p-	66 (199)	256 (234)	—	53 (49)
Cl				
o-	—	—	—	16 (17)
m-	—	—	—	12.6 (13.1)
p-	—	—	—	13.1 (13.7)
NO_2				
o-	—	—	—	58 (54)
m-	45 (39)	—	—	24 (49)
p-	40 (37)	129 (124)	—	97 (53)

[a] Variance around the concentration for ED_{50} is $\pm 10\%$ at the 95% confidence level.
[b] Race 1 in parenthesis.
[c] No activity at maximum concentration tested (DiOH 595 mM, TriOH 588 mM).
[d] Estimated by simple proportion from highest concentration and percent inhibition to 50% inhibition.
[e] Activity plateaued at 43% inhibition at concentrations between 255 and 510 mM.

most active of the three isomers, the *o*- and *m*-methoxycinnamic acids were about equal, but more active than the *p*-isomer.

2,5- and 3,4-Dimethoxyaromatic acids were also evaluated (Table 1). Except for the benzoic acids, the 2,5- isomers were much more active than the 3,4- isomers. 3,4-Dimethoxyphenylacetic acid was the least active, while 2,5-dimethoxycinnamic acid (phenylpropenoic) was the most active.

We also examined the activities of several hydroxymethoxy aromatic acids, including: vanillic (4-OH-3-MeO-benzoic), ferulic (4-OH-3-MeO-cinnamic), isoferulic (3-OH-4-MeO-cinnamic), and sinapic (4-OH-3,5-DiMeO-cinnamic) acids (Table 1). The introduction of the methoxy group at the *m*-position of *p*-hydroxybenzoic acid resulted in an acid (vanillic) that was more active (ED_{50} = 106 mM) than the corresponding hydroxy acid (ED_{50} = 179 mM), but not as active as *p*-methoxybenzoic acid (ED_{50} = 22 mM). The same trend was observed in a comparison of *p*-coumaric, ferulic, and *p*-methoxycinnamic acids. Isoferulic acid (3-OH-4-MeO-cinnamic) was much more active than ferulic acid (4-OH-3-MeO-cinnamic). This acid contains a *m*-hydroxyl and a *p*-methoxyl and was almost equal in activity to the *m*-methoxycinnamic acid and was more active than *m*-coumaric acid. Sinapic acid (4-OH-3,5-DiMeO-cinnamic) was much less active than either *p*-coumaric or *m*-methoxycinnamic acids.

The amino- and nitro-substituted aromatic acids that were tested are listed in Table 1. The order of activity was phenylpropenoic > benzoic >> phenylacetic for amino-substituted acids, while the activity of the nitro-substituted acids was benzoic >> phenylpropenoic > phenylacetic.

The ED_{50}s of hydroxy-, methoxy-, nitro-, and chlorocinnamic acids, for which all three positional isomers were available, are presented graphically in Figure 2. The most active acids for inhibiting the growth of *P. parasitica* were *o*-hydroxy-, *o*- and *m*-methoxy-, and the three chlorocinnamic acids.

In order to fully assess the contribution of substituents, it was necessary to determine the activity of the unsubstituted aromatic acids (Table 1). Benzoic and phenylpropanoic acid were found to have very high activities. Their activities were comparable to the highest activities of substituted acids (Table 1).

The high activity of benzoic acid prompted us to compare its activity to metalaxyl [*N*-(2,6-dimethylphenyl)-*N*-(2-methoxyacetyl)alanine methyl ester], which is the recommended fungicide for control of *P. parasitica* on tobacco. Accordingly, the percent inhibition of growth of *P. parasitica* versus concentration of metalaxyl and benzoic acid was plotted (Figure 3). Analysis of the data indicates that metalaxyl is about 130 times more active than benzoic acid at the 50% inhibition level (0.11 mM vs. 15 mM) but only 14 times more active at the 92–95% level (3.6 mM vs. 51.2 mM).

A summary of the activity of all the aromatic acids (except the hydroxymethoxy acids) tested is presented in Figure 4. For each substituent and chain length, only the most active isomer listed in Table 1 is plotted. In some cases,

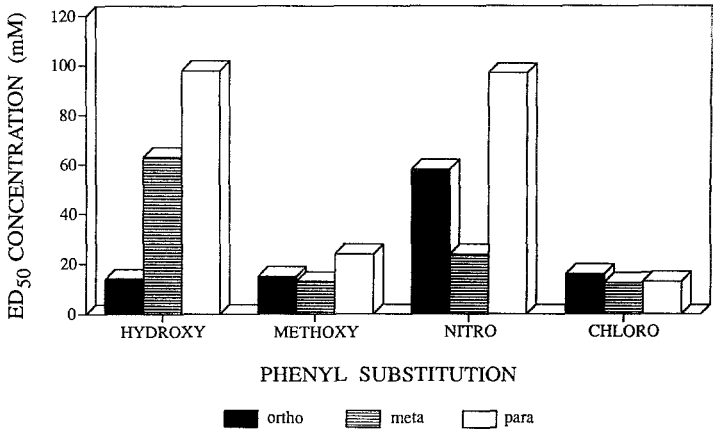


FIG. 2. ED_{50} for growth inhibition of *P. parasitica* by substituted cinnamic acids.

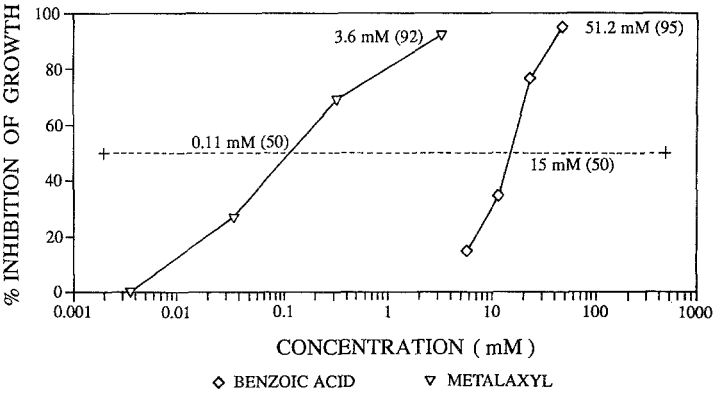


FIG. 3. Percent inhibition of growth of *P. parasitica* by benzoic acid and metalaxyl.

only one or two of many possible isomers were available for testing. Thus, the activities of *o*-hydroxy-, 2,3-dihydroxy-, *p*-methoxy-, and 3,4-dimethoxybenzoic acid are presented (Figure 4) to give a few examples of the most active isomer of a group. Consequently, the most active acids are *o*-hydroxycinnamic, *m*-methoxycinnamic, *m*- + *p*-chlorocinnamic, benzoic, and phenylpropionic acids.

Laboratory Bioassays of Aromatic Acids against Race 1 PPN. Race 1 of *P. parasitica* appears to be more susceptible to inhibition of growth (lower ED_{50}) by the tested aromatic acids than Race 0 (Table 1). Of the 49 aromatic acids tested against both races of *P. parasitica*, 33 (67%) exhibited ED_{50}s towards Race 1 that were lower or equal to those for Race 0. Further, the ED_{50}s of 22

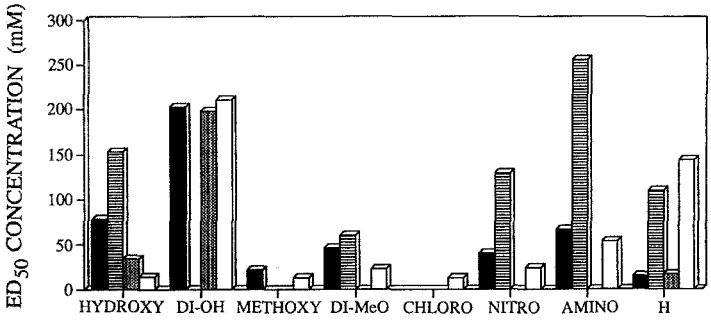


FIG. 4. ED_{50} for growth inhibition of *P. parasitica* by the most active aromatic acids. (Most active substituted phenyl isomer plotted.)

of the 33 compounds were significantly lower for Race 1. Several compounds, however, were considerably less toxic to Race 1 of the fungus. The ED_{50}s for the dimethoxyaromatic acids, benzoic, cinnamic, and *p*-aminobenzoic acids were 3–10 times higher than those for Race 0.

Laboratory Bioassays of Coumarins. Coumarins have been reported as inhibiting the growth of fungus (Batra et al., 1989; Jurd et al., 1970; Singh et al., 1987, 1988). As noted above, several coumarins in tobacco roots, scopoletin (6-methoxy-7-hydroxycoumarin) and scopolin (7-glucosylscopoletin), increase in concentration in root tissue adjacent to *P. parasitica*-infected tissue. Consequently, it was of interest to determine the activity of scopoletin, scopolin, and other substituted coumarins in this laboratory bioassay. The resulting data (Table 2) are given as percent inhibition of growth versus concentration. Due to the low activity of the coumarins, it was not possible to calculate ED_{50}s for most of these compounds. Scopolin, isolated from tobacco roots, was tested for activity along with esculetin (6,7-dihydroxycoumarin) and its glucoside, esculin. The dihydroxycoumarin, (esculetin) was as active as the methoxyhydroxy compound, scopoletin. Thus, the presence of a free hydroxyl group at position 6 is not required for activity. The substitution of a glucose on the hydroxyls of both compounds resulted in the loss of activity. Gasser et al. (1988) reported similar results with the activity of scopolin against *Thielavopsis basicola*. The substitution of methoxyls for hydroxyls in the aromatic acids, but not in coumarins, resulted in enhanced activity (Table 2). 6,7-Dimethoxycoumarin was slightly more active than dihydroxy- or monomethoxycoumarin, while 5,7-dimethoxycoumarin was completely inactive.

Coumarin itself was active in the laboratory bioassay, and an ED_{50} of 430 ppm (295 mM) could be calculated for the compound. 4-Hydroxycoumarin was about twice as active as coumarin (ED_{50} = 236 ppm, 162 mM). 7-Hydroxycoumarin was much less active than the 4-hydroxy isomer. Jurd

TABLE 2. PERCENT GROWTH INHIBITION OF *Phytophthora parasitica* VAR. *nicotianae* RACE 0 BY COUMARINS

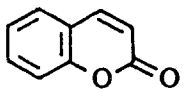
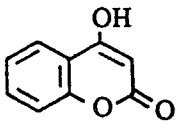
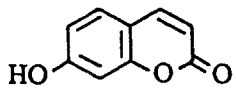
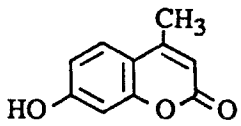
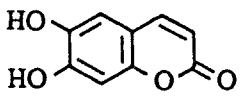
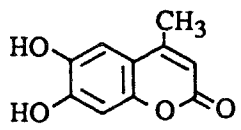
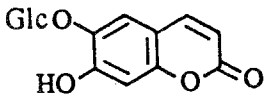
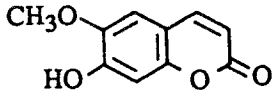
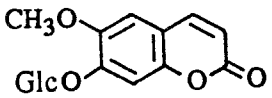
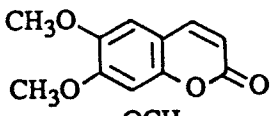
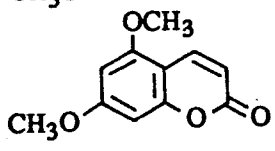
Coumarin		Inhibition of growth (%)						
Substituent		31.25 ppm	62.5 ppm	125 ppm	250 ppm	500 ppm	1000 ppm	2000 ppm
None		0	0	0	16	63	100	100
4-OH		30	34	36	53	59	100	NT[a]
7-OH		0	0	11	37	37	43	NT
4-Me-7-OH		0	24	36	64	76	80	74
6,7-DiOH		0	0	7	23	40	32	NT
4-Me-6,7-DiOH		0	0	0	0	0	14	NT

TABLE 2. Continued

Coumarin		Inhibition of growth (%)						
Substituent		31.25 ppm	62.5 ppm	125 ppm	250 ppm	500 ppm	1000 ppm	2000 ppm
6-Glc[b]-7-OH		0	0	0	0	0	16	22
6-MeO-7-OH (scopoletin)		0	0	0	17	38	39	NT
6-MeO-7-Glc (scopolin)		0	0	0	0	0	0	NT
6,7-DiMeO		0	0	0	28	45	49	37
5,7-DiMeO		0	0	0	0	0	0	0

[a]NT—not tested.
[b]Glc—glucosyl.

et al. (1970) tested 4- and 7-hydroxycoumarin against 15 different fungi and found both compounds inhibited the growth of only *Byssochlamys fulva*. 7-Hydroxycoumarin and 6,7-dihydroxycoumarin have similar activities. However, addition of a methyl group to the 4-position of 7-hydroxycoumarin doubled its activity, while the addition of a methyl to the 4-position of 6,7-dihydroxycoumarin resulted in almost a complete loss of activity.

In comparison to the aromatic acids, the coumarins are much less inhibitory towards the fungus and, thus, would probably only have a minor potential as antifungal candidates. The data for the aromatic acids should be of importance in the design and synthesis of new *P. paracitica* fungicides.

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HOST MARKING PHEROMONE OF *Rhagoletis cerasi*: FORAGING BEHAVIOR IN RESPONSE TO SYNTHETIC PHEROMONAL ISOMERS

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Abstract—We studied the foraging behavior of *Rhagoletis cerasi* females in trees treated with synthetic cherry fruit fly host marking pheromone (HMP) under seminatural conditions (potted trees enclosed in a screen cage). Results show that synthetic HMP (particularly the 8*RS*-15*R* isomer configuration (racemic mixture)) was highly effective in eliciting behavioral responses similar to those reported in studies using natural HMP. Flies exposed to synthetic pheromone exhibited short tree residence times (i.e., emigrated faster), increased flight frequency rates (measured as number of alightings per/minute), higher irritation indices while on a tree or a fruit, and oviposited fewer eggs per fruit visit than flies exposed to clean trees and fruit (not treated with synthetic HMP). Furthermore, we provide evidence showing that when flies were continuously exposed to an HMP-saturated environment, they exhibited an increased tendency to lay eggs in marked fruit.

Key Words—*Rhagoletis cerasi*, Diptera, Tephritidae, foraging behavior, host marking pheromone.

INTRODUCTION

Work on insect host marking pheromones (HMPs) [also referred to as oviposition deterring pheromones (ODPs)] was recently revolutionized when Hurter et al. (1987) succeeded, after 20 years of work, to chemically characterize and synthesize the HMP of the cherry fruit fly, *Rhagoletis cerasi*. The pheromone

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is a complex molecule {*N*[15(β-glucopyranosyl)oxy-8-hydroxypalmitol]-taurine} exhibiting four stereoisomers. This breakthrough opened up the possibility of testing and eventually incorporating synthetic HMPs as a novel element into fruit fly management schemes.

Fruit fly host marking pheromones were first reported by Prokopy (1972) and Cirio (1972), who described a pheromone deterring repeated oviposition in apple maggot (*Rhagoletis pomonella*) and walnut husk flies (*R. completa*), respectively. Shortly thereafter, a similar pheromone was described in the cherry fruit fly (*Rhagoletis cerasi*) (Katsoyannos, 1975). Subsequent work by a number of authors [reviewed by Prokopy (1981), Roitberg and Prokopy (1987) and Averill and Prokopy (1989a)] has revealed that HMPs are quite common among tephritid fruit flies: they have been reported in three of the five economically important genera: *Rhagoletis*, *Anastrepha*, and *Ceratitis* (the two remaining genera being *Dacus* and *Toxotrypana*). In the case of *Dacus*, studies on *D. oleae* have shown that the deterrent substances diverting females from ovipositing on already attacked fruit are contained in the juice that trickles from oviposition wounds (Girolami et al., 1981).

HMPs elicit a number of complex electrophysiological (Bowdan, 1984; Städler and Katsoyannos, 1979; Städler et al., 1987) and behavioral responses: oviposition deterrence (generally resulting in an even egg distribution within trees) (Prokopy, 1972; Averill and Prokopy, 1989b); male arrestment (Prokopy and Bush, 1972; Katsoyannos, 1975); increased emigration from heavily utilized resource sites (Roitberg et al., 1982, 1984); clutch size regulation (Papaj et al., 1990); and regulation of number of clutches oviposited per fruit visit (Papaj et al., 1989). Roitberg and Prokopy (1981, 1983) have further shown that experience is required for HMP recognition and that host deprivation influences responses to HMPs.

Here we describe certain behavioral responses of *R. cerasi* to the synthetic HMP (stereoisomers) identified by Hurter et al. (1987) both under laboratory and seminatural conditions. The isomers selected for testing were those eliciting the highest discrimination in laboratory studies (Boller and Aluja, 1992). Work reported here represents a stage-setting phase for subsequent studies aimed at testing synthetic HMPs in the field as a cherry fruit fly control mechanism (Aluja and Boller, 1992).

METHODS AND MATERIALS

Insects. Fly collection methods are described in Boller et al. (1987). Adults (ca. 60 females and 15 males) were kept in 30 × 30 × 40-cm Plexiglas cages on a diet of sucrose, enzymatic yeast hydrolysate, and water. Cherry tree branches with leaves were placed on both lateral sides of the cage to provide

natural resting sites. These branches were replaced every three days. Once sexual maturity was reached and mating activity commenced, ca. 25 freshly picked and washed cherries, in an optimal ripening stage for *R. cerasi* oviposition, were introduced into the cage to allow females to oviposit. Cherries were replaced twice daily to avoid high HMP concentrations.

Test Compounds. We tested two of the four synthetic stereoisomers of the cherry fruit fly HMP described by Hurter et al. (1987) and Ernst and Wagner (1989). The pheromone is a complex molecule {*N*[15(β-glucopyranosyl)-oxy-8-hydroxypalmitol]-taurine} containing two chiral centers at the C-8 and C-15 positions (thus allowing for the formation of four stereoisomers). Isomer configurations tested were: (1) 8*R*-15*R* (*RR*) and (2) 8*RS*-15*R* (racemic mixture). All isomers were diluted in MeOH and tested at a concentration of 10^{-3} mg/ml. In the case of isomer 8*RS*-15*R* (racemic mixture), a concentration of 10^{-2} mg/ml was also utilized (Table 1).

Research Arena. In the laboratory tests the bioassay arena consisted of a Plexiglas cage (30 × 40 × 40 cm) into which freshly picked cherry branches with leaves were placed on both lateral sides to provide natural resting sites for the test flies. Thirty freshly picked cherries were presented to the flies (12 per cage) on a mobile wooden platform. Of these, 15 cherries served as controls and 15 were treated with different HMP isomers (five fruits per treatment). Each bioassay lasted 60 min. Using a data sheet showing graphically the arrangement of the 30 numbered cherries, an observer recorded the following information: landings on each fruit, oviposition attempts (i.e., boring into fruit) and successful oviposition (i.e., boring followed by ovipositor dragging). The bioassay arena utilized, procedures followed during laboratory tests and type of flies utilized are described in more detail by Boller and Aluja (1992).

TABLE 1. DESCRIPTION OF FIVE DIFFERENT TREATMENTS TESTED DURING STUDY

Treatment	HMP characteristics	HMP concentration (mg/ml)	HMP application protocol
1	No HMP applied (control treatment)		
2	Racemic mixture 8*RS*-15*R*	10^{-3}	Fruit and foliage
3	Isomer 8*R*-15*R*	10^{-3}	Fruit and foliage
4	Racemic mixture 8*RS*-15*R*	10^{-3}	Only fruit
5	Racemic mixture 8*RS*-15*R*	10^{-2}	Fruit and foliage

The greenhouse experiments were conducted using six 3-year-old potted cherry trees whose canopies were ca. 1 m (diam.) × 2.5 m (height). Four trees (placed in corners corresponding to the four cardinal points) were enclosed in a 2.9-m-diam × 2.9-m-tall cylindrical clear Saran-screen cage. The roof of the cage was covered with commercial greenhouse shading material. The cage was built inside a greenhouse where temperature was controlled (experiments were conducted during April–May when ambient temperature is too low for flies to be active). Following a technique used by Aluja et al. (1989), we manipulated the architecture of each tree until an approximately even distribution of foliage throughout the tree was obtained. Distance between different tree canopies in the cage was ca. 0.8 m. We also clipped away ca. 35% of all leaves to allow the researcher to view clearly almost any point within the tree from any location in the cage. The tree canopy was divided into eight sectors, each of which was clearly tagged (numbers 1–8).

Each tree was sprayed with a different HMP solution with the aid of a garden mist sprayer (one separate sprayer was used for each test solution). All leaves (top and bottom parts) and branches were treated until runoff. Spraying was performed outside the cage at five-day intervals. To avoid wind drift contamination while applying the different isomers, trees were covered with a plastic sheet. The control treatment consisted of untreated trees with untreated fruit. Every morning, four trees were selected at random and introduced into the cage for testing. Eight fruit were hung in the tree (one per tree sector). All fruit used was picked in nearby orchards from early-fruiting cherry varieties and kept overnight in a cold room at 8°C.

Foraging Behavior Tests (Greenhouse Experiments). On the test day, a Plexiglas cage holding flies and ca. 25 cherries was transported from the laboratory to the field cage. Fruit in the fly-holding cage was replaced every 2 hr to avoid accumulation of HMP. Once a fly started to oviposit on one of the cherries (inside the Plexiglas cage), the fruit was removed and, after ovipositor dragging, the fly was transferred to a predesignated leaf in the lower half of the canopy of one of the four available test trees. Trees were selected for testing at random.

Once released, a fly was followed continuously for up to 1 hr as it foraged within the test tree. All behaviors (as well as times performing each behavior) exhibited by foraging flies were recorded. If a fly flew to a neighboring tree or to the cage wall before the 1-hr time limit, the test was concluded and another fly released.

Laboratory Tests. To ascertain if continuous exposure to HMP reduced fly sensitivity to HMP-treated trees or fruit (through habituation or sensory adaptation), we saturated several fly-holding cages with a HMP solution (including cherry leaves placed inside cages as resting sites). The HMP solution consisted of the racemic mixture (8*RS*-15*R*) diluted in water with 0.1% Ajoutol. Sexually mature flies were released (ca. 60 females and 15 males) into each cage and

kept there under continuous HMP exposure during 24 hr. After this period, 12 females were tested under the protocol described under "research arena" (also see Boller and Aluja, 1992). The control treatment consisted of flies held in clean cages (no HMP exposure) and tested under the conditions described above.

Data Collection and Analysis. All information was recorded using a Radio Shack TRS 80 (model 102) computer programmed in BASIC to function as an event recorder. Raw data files were later transferred to an IBM portable computer for summarization and statistical analysis. The following dependent variables were examined: mean time spent on tree (minutes), flight frequency (no. alightings per minute), mean time spent on fruit (minutes), mean number of ovipositions per fruit visit, irritation index while on tree, *sensu* Boller et al. (1987) (number of behavioral transitions while on tree; e.g., foraging–resting–foraging–cleaning–resting–foraging), irritation index (number of behavioral transitions) while on a fruit.

We tested for homogeneity of variances using Cochran's test and Bartlett's test (Statgraphics procedure; Statgraphics, 1986). Variables termed mean time spent on tree, flight frequency, and mean number of ovipositions per fruit visit were analyzed by a one-way analysis of variance since they passed the homogeneity of variance test (Statgraphics procedure; Statgraphics, 1986). All remaining variables that exhibited unacceptable inequality in variances were analyzed by a Kruskal-Wallis procedure. Where appropriate, multiple-range analysis by the 95% confidence intervals method was performed. In the case of the HMP saturation experiment, we computed the fruit discrimination index using the formula provided by Boller and Hurter (1985).

Environmental Conditions during Tests. Even though ambient temperature outside the greenhouse was very low (4–8 °C at midday), conditions inside the greenhouse were optimal. Flies were tested under the following mean temperatures: treatment 1, 24.9 $\pm$ 0.4, treatment 2, 26.1 $\pm$ 0.5; treatment 3, 25.4 $\pm$ 0.6; treatment 4, 26.2 $\pm$ 0.3; and treatment 5, 26.8 $\pm$ 0.3.

RESULTS

Effect of HMP on Tree and Fruit Residence Time. Overall, treatment effect on the variable termed residence time on a tree was marginally insignificant ($P = 0.067$; confidence level 95%). Nevertheless, important trends can be detected in Figure 1. For example, flies exposed to untreated trees (treatment 1, Figure 1) exhibited the longest mean tree residence time (30.3 min). This contrasts sharply with results obtained when flies were exposed to trees treated with the racemic mixture at concentrations of 10^{-3} and 10^{-2} mg/ml (treatments 2 and 5, respectively; Figure 1). Under these conditions fly residence time on a tree was cut by almost half. Importantly, flies foraging in a clean tree where only

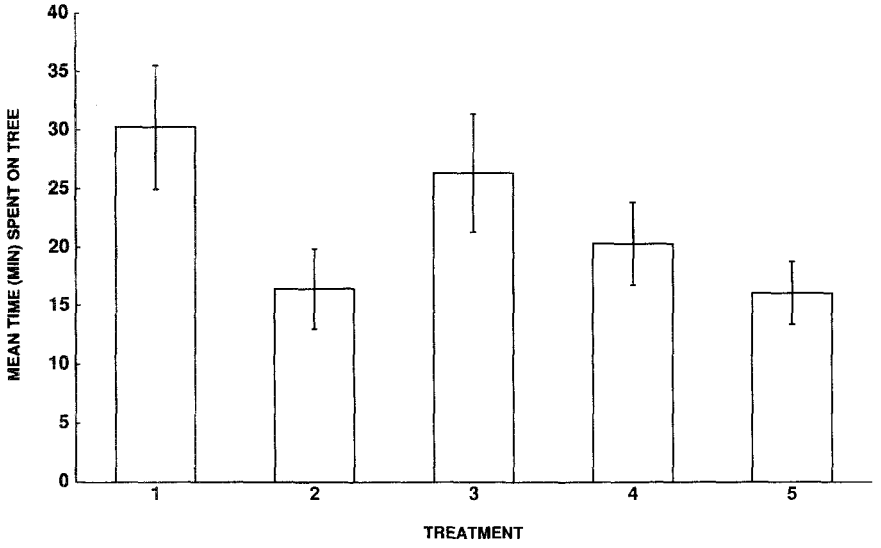

FIG. 1. Tree residence time exhibited by *R. cerasi* females exposed to an untreated cherry tree or a cherry tree sprayed with different HMP solutions at varying concentrations (see Table 1 for treatment description). $N = 20$ in every treatment. Differences between means marginally insignificant (one-way ANOVA, $P = 0.067$).

fruits were treated (treatment 4, Figure 1) spent on average 10 min less time in the tree than flies exposed to an untreated tree with clean fruit (treatment 1, Figure 1).

Flies spent the least time on cherries if fruit but not tree foliage was treated with HMP (treatment 4, Figure 2). The same effect was seen if HMP sprayed on both fruit and foliage was highly concentrated (treatment 5, Figure 2). In contrast, the time flies spent on cherries hung in an untreated tree was four times greater (treatment 1, Figure 2). Flies foraging in trees treated with HMP isomer 8*R*-15*R* and racemic mixture 8*RS*-15*R* at a concentration of 10^{-3} mg/ml, spent almost half as much time on cherries as flies foraging in clean trees (treatments 2 and 3, Figure 2).

Effect of HMP on Flight Frequency (Number of Alightings per minute). Flies alighted most often within the tree when exposed to trees treated with a high concentration of HMP racemic mixture 8*RS*-15*R* (treatment 5, Figure 3) and alighted least often when exposed to untreated trees (treatment 1, Figure 3). Intermediate values were observed with treatments 2, 3, and 4 (Figure 3).

Effect of HMP on Tree and Fruit Irritation Indicies. Irritation index while on a tree was lowest for flies foraging in trees treated with HMP in its 8*R*-15*R* isomer configuration and untreated trees (treatments 3 and 1 respectively; Figure

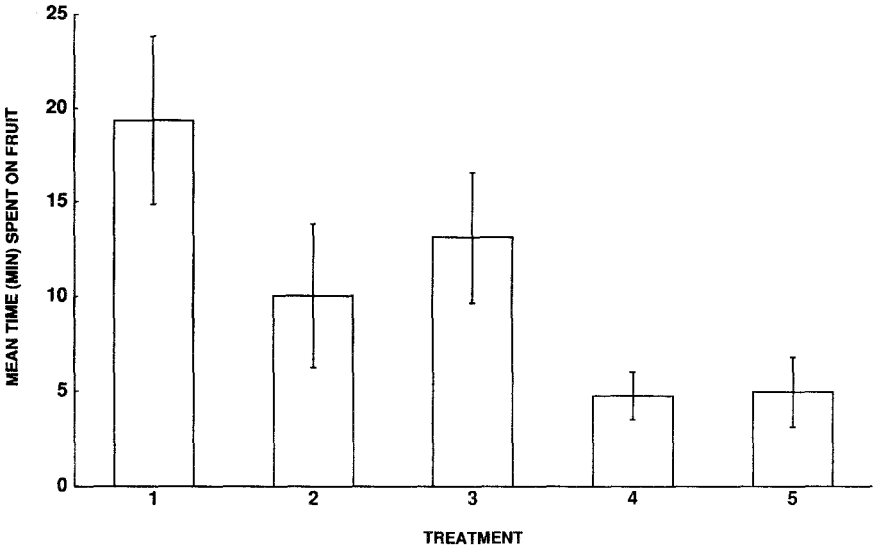

FIG. 2. Fruit residence time exhibited by *R. cerasi* females exposed to an untreated cherry tree or a cherry tree sprayed with different HMP solutions at varying concentrations (see Table 1 for treatment description). $N = 20$ in every treatment. Differences between means marginally significant (Kruskal-Wallis, $P = 0.047$).

4). The highest index was observed in flies treated with HMP in its 8*RS*-15*R* isomer configuration at a concentration of 10^{-2} mg/ml (treatment 5, Figure 4). Intermediate values were observed with treatments 2 and 4 (tree treated with 8*RS*-15*R* at a concentration of 10^{-3} mg/ml and only fruit (not foliage) treated with 8*RS*-15*R* at a concentration of 10^{-3} mg/ml) (Figure 4). Importantly, when only fruit (not foliage) was treated, the irritation index when flies were on fruit was significantly increased when compared to flies foraging in trees where both fruit and foliage were treated with varying HMP solutions (treatment 4, Figure 5).

Effect of HMP on Number of Ovipositions per Fruit Visit. Flies laid the least number of eggs per fruit visit when only fruit (not foliage) was treated with a low concentration (10^{-3} mg/ml) of the 8*RS*-15*R* HMP (treatment 4, Figure 6) or when exposed to trees and fruit treated with a high concentration (10^{-2} mg/ml) of the same HMP isomer mixture (treatment 5, Figure 6). The most eggs per fruit visit were laid when flies were exposed to clean trees and fruit (treatment 1, Figure 6). Intermediate values were obtained when trees and fruit were treated with HMP isomer 8*RS*-15*R* and racemic mixture 8*R*-15*R* at a concentration of 10^{-3} mg/ml (treatments 2 and 3, Figure 6).

Effect of Continuous HMP Exposure on Fly Discrimination Ability. We

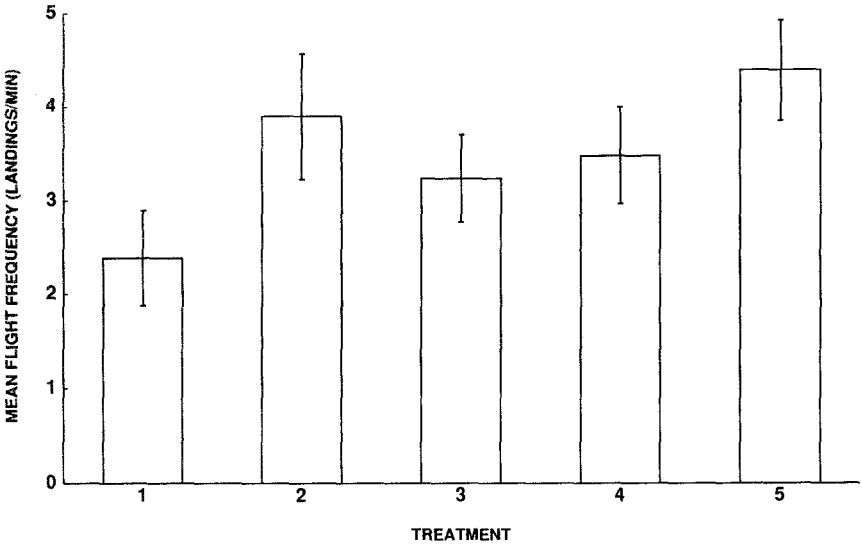

FIG. 3. Flight frequency (alightings per minute) exhibited by *R. cerasi* females exposed to an untreated cherry tree or a cherry tree sprayed with different HMP solutions at varying concentrations (see Table 1 for treatment description). $N = 20$ in every treatment. Differences between means significant (one-way ANOVA, $P = 0.017$).

found that flies continuously exposed to an HMP-saturated environment exhibited an increased tendency to lay eggs in marked fruit (Table 2).

DISCUSSION

We feel that the experiments reported here are significant for three reasons. First, we showed for the first time that synthetic HMPs are highly effective at eliciting behavioral responses similar to those reported in studies using natural HMPs (e.g., Roitberg et al., 1982). Second, we provide evidence that a racemic mixture of two synthetic stereoisomers of the cherry fruit fly HMP outperforms single isomers in causing a reduction in fruit infestation and in enhancing emigration from a host tree. Third, we document the fact that flies continuously exposed to synthetic HMP exhibited an increased tendency to lay eggs in marked fruit.

Our results indicate clearly that synthetic HMP isomers are highly effective at eliciting behavioral responses similar to those reported in studies using natural host marking pheromones. Roitberg et al. (1982) and Roitberg and Prokopy (1984) reported that the active search time of *Rhagoletis pomonella* females foraging in potted trees (similar to those used in this study) was reduced sig-

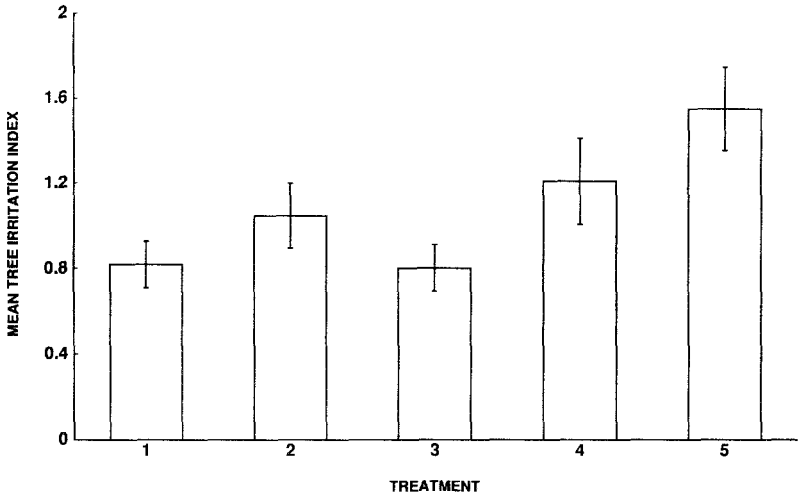

FIG. 4. Irritation indicies exhibited by *R. cerasi* females exposed to an untreated cherry tree or a cherry tree sprayed with different HMP solutions at varying concentrations (see Table 1 for treatment description). $N = 20$ in every treatment. Differences between means highly significant (one-way ANOVA, $P = 0.001$).

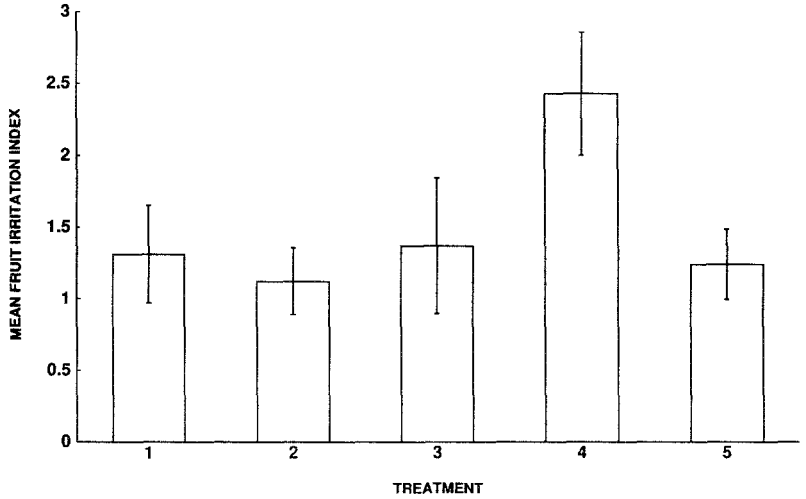

FIG. 5. Irritation index while on fruit exhibited by *R. cerasi* females exposed to an untreated cherry tree or a cherry tree sprayed with different HMP solutions at varying concentrations (see Table 1 for treatment description). $N = 20$ in every treatment. Differences between means marginally significant (Kruskal-Wallis, $P = 0.045$).

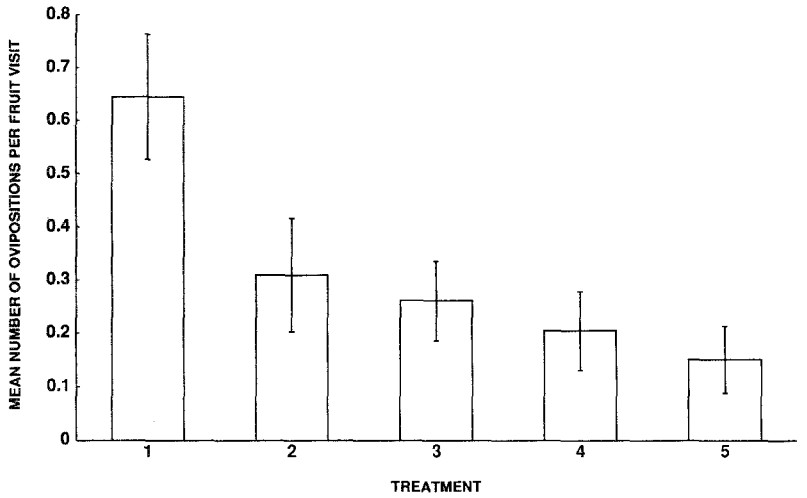

FIG. 6. Number of ovipositions per fruit visit by *R. cerasi* females exposed to an untreated cherry tree or a cherry tree sprayed with different HMP solutions at varying concentrations (see Table 1 for treatment description). $N = 20$ in every treatment. Differences between means highly significant (one-way ANOVA, $P = 0.002$).

TABLE 2. DISCRIMINATION COEFFICIENTS OF *Rhagoletis cerasi* FEMALES EXPOSED TO HMP-SATURATED ENVIRONMENT AND HMP-FREE ENVIRONMENT

Environment	Discrimination coefficient (±SE)
HMP-free	95.4 (±4.0)
HMP-saturated	18.0 (±8.5)

nificantly if, previous to release, females were exposed to HMP-marked fruit or encountered low-quality resources while foraging (i.e., HMP-marked fruit), when compared to flies that, previous to release, had been exposed to unmarked fruit or encountered high-quality resources while foraging (i.e., clean fruit). We observed a similar pattern in our studies (Figure 1, treatments 1 vs. 4).

The fact that the racemic mixture 8*RS*-15*R* proved highly effective at reducing fruit infestation, increasing flight frequency rates, and promoting emigration from host trees is significant because synthesis of this racemic mixture is not only easier to accomplish but also cheaper than the synthesis of individual isomers (Hurter, personal communication). Aluja and Boller (1992) report that the same racemic mixture reduced fruit infestation by approximately 90% under field conditions.

Two findings reported and discussed here could support the development of a cherry fruit fly control scheme using HMPs. First, continuous exposure to synthetic HMPs causes flies to exhibit an increased tendency to lay eggs in marked fruit and in general seems to reduce sensitivity to the HMP (presumably through habituation or sensory adaptation). Should HMPs ever be used as a large-scale management tool, the key to success will be the development of an effective HMP field deployment strategy. Based on our results, we suggest a discontinuous application, treating certain parts of the tree and leaving others untreated. This approach would allow foraging flies to encounter both treated and untreated tree parts without the concomitant increase in sensilla desensitization caused by constant HMP exposure. Second, synthetic HMP caused flies to move at a higher rate and emigrate faster when compared to flies foraging in untreated trees. We thus feel confident that discontinuous HMP deployment (i.e., leaving certain tree parts untreated), in addition to utilizing yellow sticky traps (Remund and Boller, 1975) to capture activated, host-deprived flies during the process of emigration will become an effective cherry fruit fly management strategy in organic cherry orchards (such as originally postulated by Boller, 1981; and Prokopy, 1981).

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LABORATORY COLONIZATION HAS NOT REDUCED CONSTITUTIVE OR INDUCED POLYSUBSTRATE MONOOXYGENASE ACTIVITY IN VELVETBEAN CATERPILLARS

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Abstract—We evaluated whether velvetbean caterpillars (*Anticarsia gemmatalis*) from a laboratory colony had reduced constitutive (basal) and/or induced activities of their polysubstrate monooxygenase (PSMO) detoxification enzyme system as a result of long-term rearing (>100 generations) on artificial diet without introduction of field-collected individuals. Larvae from the laboratory colony and those from a recently collected field strain were fed either a standard artificial diet (control), one containing the inducing allelochemical, flavone, or foliage of *Indigofera hirsuta* (a host plant of this species), and their midgut PSMO activity was assessed by measuring the in vitro rate of aldrin epoxidation. Compared with the field-strain larvae, caterpillars from the laboratory colony had 1.9-fold greater constitutive activity (standard artificial diet) and 2.3-fold greater induced activity (flavone-treated diet). In addition, the magnitude of induction was somewhat greater for the laboratory-colony larvae (induced activity was 2.0-fold greater than constitutive activity) compared with those from the field-strain (1.6-fold). In contrast, no difference in strain activity was found when larvae were fed *I. hirsuta* foliage. The lower PSMO activity of the field-strain larvae when fed artificial diet may have been caused by their reduced feeding and growth performance compared with laboratory-colony larvae, and it may explain their greater sensitivity to allelochemicals incorporated in the artificial diet, as we found previously. The results of this study indicate that long-term rearing of the *A. gemmatalis* laboratory colony on artificial diet, without the introduction of field individuals, apparently has not selected for low constitutive activity or decreased inducibility of PSMO, and thus these larvae provide a suitable model for studying xenobiotic detoxication. In addition, they suggest that using an arti-

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ficial diet to evaluate resistance to pesticides or other xenobiotics in field-collected insects, as is frequently done, may underestimate the level of resistance if the diet, through various causes, reduces the activity of detoxification enzymes contributing to the resistance.

Key Words—Allelochemical, *Anticarsia gemmatalis*, detoxification, field strain, flavone, *Indigofera hirsuta*, induction, laboratory colony, Noctuidae, Lepidoptera, polysubstrate monooxygenase.

INTRODUCTION

Polysubstrate monooxygenases (PSMOs) (also known as mixed-function oxidases or MFOs) are considered the most important detoxification enzyme system for the metabolism of pesticides and other lipophilic xenobiotic compounds into more easily excretable polar products (Brattsten, 1979; Hodgson, 1985). Insects feeding on plants or artificial diets that contain particular xenobiotics frequently have increased levels of PSMO and other detoxification enzyme activity compared with the constitutive (basal) activity of larvae consuming a control diet (Yu, 1986; but not always, e.g., Sheppard and Friedman, 1989). There are many studies demonstrating that induced enzyme activity can increase an insect's tolerance to several synthetic pesticides (Brattsten, 1988a; Soderlund and Bloomquist, 1990). In addition, induction by allelochemicals can increase the rate of metabolism of such compounds measured in vitro (Yu, 1987; Wadleigh and Yu, 1988; Cohen et al., 1989; Nitao, 1989; Harwood et al., 1990), but whether an insect's tolerance to an ingested allelochemical is increased following induction of detoxification enzymes by the same compound has been seldom studied (Kennedy, 1984; Lindroth, 1991; Slansky, unpublished data; Wheeler et al., unpublished data).

Clearly, additional research is essential if we are to understand the role of detoxification enzymes in the ability of plant-feeding insects to cope with allelochemicals (see discussion in Lindroth, 1991). Insects from laboratory colonies provide convenient subjects for such studies, but they may not be representative of insects in the field. Substantial changes can occur in the amount of genetic variation and biology of field-collected insects after they have been reared in laboratory colonies for various durations (Chambers, 1977; Berlocher and Friedman, 1981; Mason et al., 1987; Pashley et al., 1987; Guthrie and Jarvis, 1988; Henneberry and Clayton, 1990). Because of this, Huettel (1976) emphasized the need to monitor the quality of laboratory-reared insects, including their flight capacity, ability to locate host plants and mates, mating competitiveness, vision, and other traits.

Huettel (1976) described larval feeding and development as additional measurable traits, but he did not mention detoxification enzyme activity. It is likely that such activity will be reduced in individuals from laboratory colonies

compared with those from field populations, based on the following line of reasoning. If, as suggested by certain authors (Krieger et al., 1971; Whittaker and Feeny, 1971; Brattsten, 1979, 1988a,b), PSMOs are metabolically expensive to produce and maintain (but see Neal, 1987; Appel and Martin, 1992), or if higher levels of detoxification enzyme activity otherwise have deleterious pleiotropic effects, then continuous rearing of insect colonies for many generations on artificial diets (presumably with relatively low, more-or-less constant levels of allelochemicals) may select for insects with lower detoxification enzyme load and thus with increased sensitivity to xenobiotics. Although we are unaware of any studies examining intergeneration variation in detoxification enzyme activity for insects from laboratory colonies, such changes have been documented for pesticide-resistant insects. In the absence of a pesticide challenge, resistant individuals often have reduced fitness relative to susceptible ones (Roush and McKenzie, 1987; Campanhola et al., 1991), and the level of resistance can decline in a few generations (e.g., Brown et al., 1978; Leonard et al., 1988).

In contrast to laboratory colonies, insects and other plant-feeding arthropods in field populations may be selected for increased levels of detoxification enzymes because of their exposure to various allelochemicals in different host plants and to changes in the allelochemical content of any one host plant, such as associated with leaf maturation and with induction of the compound(s) caused by feeding damage (Karban and Myers, 1989). For example, when Gould et al. (1982) selected a strain of spider mite (*Tetranychus urticae* Koch) to survive on a previously toxic plant species, the mites became more resistant to certain pesticides, which suggests that their detoxification enzyme levels had been increased relative to the unselected strain (see Mullin and Croft, 1983). Furthermore, increased constitutive-level detoxification enzyme activity occurs often in field populations exposed frequently to synthetic pesticides (Brattsten, 1988a; Soderlund and Bloomquist, 1990), although the magnitude of induction of insects from resistant strains is not consistently greater, and indeed is often less, than that of individuals from susceptible strains (Yu and Terriere, 1973; Yu, 1984).

As part of an investigation on the effects of ingested allelochemicals on insects, we are studying caterpillars of several noctuid moths reared in laboratory colonies, including the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner. To assess the appropriateness of investigating allelochemical detoxification by *A. gemmatalis* larvae from a laboratory colony maintained on artificial diet for over 100 generations without infusion with field-collected individuals, we compared their PSMO activity with that of larvae from a recently collected field strain. Constitutive PSMO activity was measured for larvae fed the standard artificial diet used to rear the laboratory colony. In addition, induced PSMO activity was assessed for larvae fed the artificial diet to which was added flavone, an allelochemical known to induce several detoxification enzyme systems, including PSMO (Yu, 1984; Yu and Hsu, 1985; Christian and Yu, 1986). To

further investigate dietary influences on PSMO activity, an additional treatment consisted of larvae fed foliage of hairy indigo, *Indigofera hirsuta* L. (Leguminosae), a host plant of this species (Buschman et al., 1977) that caused a low level of PSMO induction in larvae from the same laboratory colony used in the present study (Christian and Yu, 1986).

METHODS AND MATERIALS

Collection and Rearing of Insects. Field-strain larvae (instars 3–6) were collected in September 1990 from soybean [*Glycine max* (L.) Merrill, var. Jupiter] in Gainesville, Florida. Larvae were brought back to the laboratory and fed fresh soybean leaves until pupation. Prior to feeding, the leaves were surface sterilized by washing in a dilute solution of sodium hypochlorite (1% v/v) and blotted dry. To maintain leaf moisture, petioles were inserted into water-filled vials and replaced every one to two days. Adults were fed a 10% honey–water solution on cotton and maintained (about 20/container) in 3.8-liter (1-gallon) cardboard containers topped with paper toweling and lined with white paper for oviposition substrate. Eggs from these adults provided the field-strain larvae used in the enzyme assays. The laboratory strain was acquired as eggs from the ARS-USDA Insect Attractants, Behavior and Basic Biology Research Laboratory in Gainesville, Florida, and reared en masse on an artificial diet following Greene et al. (1976), but lacking formalin and tetracycline. This laboratory colony (established from insects collected in Gainesville, Florida) had been reared continuously for more than 100 generations (about 10 years) without infusion with field-collected individuals. All rearing occurred at 27 ± 1°C, 50 ± 10% relative humidity, and 14:10 hr light–dark photoperiod.

Larvae used in the enzyme assays were fed *I. hirsuta* foliage selected from the upper half of field plants in the pod-filling stage (field strain) or standard artificial diet (laboratory colony) from the neonate stage until day 1 of the sixth instar. Individual larvae were then transferred to inverted 30-ml plastic cups with tightly fitting plastic caps and fed one of three foods for two days prior to dissection and enzyme analysis: (1) artificial diet alone (control); (2) artificial diet with flavone (Sigma Chemical Co., St. Louis, Missouri); and (3) *I. hirsuta* leaves. We used 0.08% fm (fresh mass) flavone because studies conducted with another noctuid species, the fall armyworm, *Spodoptera frugiperda* (J.E. Smith), indicated that at this concentration, flavone induced maximum PSMO activity with no increase in mortality of the latter instars and only a slight (ca. 12%) reduction in larval dry mass gain (unpublished data). Flavone was dissolved in a minimum of acetone, mixed with alphacel (1% fm), rotoevaporated to dryness (about 60°C), and mixed with the artificial diet as it cooled following cooking.

Aldrin Epoxidase Analysis. Polysubstrate monooxygenase activity was

determined by measuring the rate of epoxidation of aldrin to produce dieldrin using NADPH as a cofactor. Methods followed Yu (1982) and Christian and Yu (1986) for measurement of aldrin epoxidase activity from midgut crude homogenates. Ten to 15 larval midguts were dissected from 2-day-old sixth-instar larvae and washed in 1.15% KCl (1–4°C). Midguts were homogenized in 20 ml 0.1 M sodium phosphate buffer (pH 7.5) for 30 sec and filtered through cheesecloth. Homogenates (0.5–1.0 mg protein/ml) were incubated in duplicate for 15 min at 30°C, and the reaction was stopped by the addition of 10 ml of hexane. Samples were analyzed by gas chromatography on a 122-cm × 2-mm-ID glass column packed with 3% SE 30 on 100/120 Gas Chrom Q. Activity was expressed as picomoles of dieldrin per minute per milligram of protein.

Statistical Analysis. A factorial design was used for the enzyme activity experiment (three to five replicates per treatment combination) with strains (field and laboratory) and food (control diet, flavone-treated diet, and *I. hirsuta* foliage) as the main effects. Activity data were analyzed with PC/SAS (SAS Institute, 1987) by two-way analysis of variance (ANOVA; PROC GLM) with interaction. When the interaction was significant ($P < 0.05$), each strain was analyzed independently by a one-way ANOVA (Montgomery, 1984). When more than two means were compared, Tukey's HSD method was used.

RESULTS

Aldrin epoxidase activity was influenced significantly by both food and strain; the interaction of these two factors was also significant (Table 1). Larvae from the laboratory colony fed the control artificial diet and those fed the flavone-

TABLE 1. TWO- AND ONE-WAY ANALYSIS OF VARIANCE OF ALDRIN EPOXIDASE ACTIVITY OF FIELD AND LABORATORY STRAINS OF *A. gemmatalis* LARVAE FED CONTROL OR FLAVONE-TREATED ARTIFICIAL DIETS, OR *I. hirsuta* FOLIAGE

Source	*df*	*F*	*P*
Two-way ANOVA:			
Food	2,38	31.86	0.0001
Strain	1,38	33.82	0.0001
Food * Strain	2,38	12.61	0.0001
One-way ANOVA: Among-food comparisons for each strain			
Field	2,17	4.66	0.0244
Laboratory	2,21	33.99	0.0001
One-way ANOVA: Between-strain comparisons for each food			
Artifical diet	1,12	22.15	0.0005
Artifical diet + flavone	1,16	39.08	0.0001
I. hirsuta foliage	1,10	0.03	0.8609

treated diet had significantly greater activity (1.9- and 2.3-fold, respectively) than the field-strain larvae (Figure 1). However, enzyme activity of the larvae fed *I. hirsuta* leaves did not differ significantly between strains.

Within-strain comparisons indicated that activity was significantly greater for both the laboratory- and field-strain larvae fed the flavone diet compared with those fed the artificial diet alone or *I. hirsuta* foliage (Table 1 and Figure 1). Enzyme activities for larvae of either strain fed the latter two diets were comparable. The magnitude of induction (i.e., increase in PSMO activity for larvae fed the flavone diet compared with those fed the control diet) was somewhat greater for the laboratory strain (2.0-fold) compared with the field strain (1.6-fold).

DISCUSSION

Our results indicate that the field and laboratory strains differed in their PSMO activity when fed the artificial diet. Larvae from the laboratory colony had both higher constitutive (standard artificial diet) and induced (flavone-containing diet) activity and a somewhat greater magnitude of induction. Reduced PSMO (and possibly other detoxification enzyme) activity of the field-strain

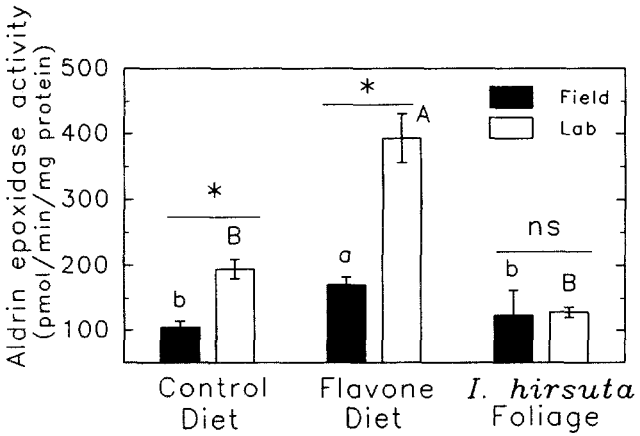

FIG. 1. Aldrin epoxidase activity (mean ± SE) of *A. gemmatalis* larvae from field and laboratory strains. Sixth instars were fed the control artificial diet alone and with flavone (0.08% fm), or *I. hirsuta* foliage for two days prior to enzyme analysis. An asterisk above a pair of bars (or ns = not significant) indicates a significant difference between strains within diets according to a one-way ANOVA. Different lowercase letters above solid bars and uppercase letters above open bars indicate significant differences (Tukey's HSD, $P \leq 0.05$) among foods for field and laboratory strains, respectively.

larvae fed the artificial diet may explain their increased mortality compared with larvae from the laboratory colony when caffeine or coumestrol were added to the diet, although little is known about the detoxification of these two allelochemicals by insects (see Slansky and Wheeler, 1992). In contrast to the differences found when the artificial diet was the food source, enzyme activity was similar between the strains when larvae were fed foliage of *I. hirsuta*.

Aldrin epoxidase activity for the *A. gemmatalis* sixth instars (2-days-old) from the laboratory colony fed artificial diet in our study was similar to that found previously using larvae of the same age from the same colony (Christian and Yu, 1986). Additionally, the induction occurring in the present study in the laboratory strain when fed diet containing flavone (0.08% fm) was similar in magnitude (2.0-fold) to that reported previously (1.8-fold; Christian and Yu, 1986), although the flavone concentration used in that study was slightly higher (0.1% fm). It is likely that PSMO activity increases with the ingested dose of an inducing substance (e.g., Brattsten and Wilkinson, 1973), but that it may already have peaked in *A. gemmatalis* larvae fed the 0.08% fm flavone diet, as found for caterpillars of *S. frugiperda* (unpublished data). These similarities between the results of our study and those of Christian and Yu (1986) indicate that, at least during the past five years (>60 generations), constitutive and flavone-induced PSMO activities of the laboratory colony larvae have not declined. The midgut aldrin epoxidase activities of *A. gemmatalis* found in our study also are comparable to those reported for many other foliage-feeding lepidopteran larvae (Krieger et al., 1971; Rose, 1985).

PSMO activity of the laboratory-colony larvae fed *I. hirsuta* foliage in our study was not significantly different from that of larvae fed the control artificial diet, whereas Christian and Yu (1986) found slightly but significantly higher activity in their *I. hirsuta* foliage-fed larvae (1.3-fold greater than the control). Variation in *I. hirsuta* leaf quality during leaf and plant maturation and under different growing conditions [e.g., the foliage we used was field-collected, whereas Christian and Yu (1986) used greenhouse-grown plants] may account for this difference between the two studies. At least one allelochemical (a flavonoid glycoside) has been reported from *I. hirsuta* (Harborne and Williams, 1988).

Other than in the context of pesticide resistance, there appear to be few studies of insects from laboratory colonies that have examined intergeneration changes in detoxification enzyme activity or sensitivity to xenobiotics that could reflect such activity. In one study, Salloum and Isman (1988) found that larvae of an F_1 generation of field-collected variegated cutworms, *Peridroma saucia* (Hübner), gained more weight when fed an artificial diet than larvae from a laboratory colony reared for over 12 generations, but larvae from both strains had their weight gain reduced about 75% by addition of an ethanolic extract of *Artemisia* to the artificial diet. Larvae of the European corn borer (*Ostrinia*

nubilalis Hübner) from a 6-year-old laboratory colony showed reductions in feeding and survival similar to those from a 1-year-old colony when both were fed resistant corn genotypes, compared with larvae fed susceptible genotypes (Manuwoto and Scriber, 1985). These results suggest that sensitivity to allelochemicals did not increase with long-term laboratory rearing.

There are at least two possible causes for our finding of lower PSMO activity of the field-strain larvae compared with the laboratory colony larvae when fed the artificial diet with or without added flavone. One is that larvae from the two sources differed genetically in their detoxification enzyme capacity (e.g., Scriber et al., 1989; Roush and Daly, 1990). However, our data do not appear to support this hypothesis, because under this scenario, we would predict that the field strain likely would have at least higher constitutive activity, as described in the Introduction. The lack of a significant difference between the strains when fed foliage of *I. hirsuta* also suggests there are no substantial interstrain genetic differences in constitutive detoxification capacity.

A second cause of the lowered enzyme activity of the field-strain larvae when fed the artificial diet may be a decrease in their ingested dose of any inducing allelochemicals occurring in the plant material ingredients of the control diet (i.e., soy protein, pinto beans and wheat germ) (Greene et al., 1976) and of the added flavone. We previously found that the mean biomass-relative consumption rate (RCR) of the field-strain *A. gemmatalis* larvae fed artificial diet was reduced over 30% compared with that of the laboratory-colony larvae (Slansky and Wheeler, 1992). Because the field-strain larvae used in the present study were from the same F_1 generation as those used by Slansky and Wheeler (1992), their RCR when fed the artificial diet, with or without flavone, probably also was lower than that of larvae from the laboratory colony. Dose-dependent induction of PSMO activity by ingested flavone has been demonstrated in *S. frugiperda* (unpublished data) and for other allelochemicals in other lepidopteran larvae (e.g., Brattsten and Wilkinson, 1973; Yu, 1984). The similarity in PSMO activity between the laboratory- and field-strain larvae fed *I. hirsuta* foliage may be explained by their similar RCR when fed this food plant (Slansky and Wheeler, 1992), because larvae of each strain would have ingested allelochemicals and nutrients at comparable rates.

An inadequate availability of nutrients for the diet-fed field-strain caterpillars resulting from their reduced consumption rate, as reflected in their decreased growth rate compared with the laboratory-colony larvae (Slansky and Wheeler, 1992), also may have contributed to their lower PSMO activity. The level of PSMO and other detoxification enzymes in insects can be reduced by nutrient deficiencies (Patil et al., 1990) and other apparent stresses (e.g., parasitism; Fix and Plapp, 1987), as in mammals (Boyd and Campbell, 1983), although activity is not always lowered on the reduced-quality diets (Lindroth et al., 1990, 1991). Switching the field-strain larvae from one diet (foliage of *I. hirsuta*) to another

(artificial diet) during the first day of the sixth instar may have contributed to their reduced performance (e.g., Scriber, 1979, 1981). However, the laboratory-colony larvae switched from artificial diet to *I. hirsuta* foliage showed no significant difference in PSMO activity compared with the colony larvae fed artificial diet continuously, suggesting that a dietary switch does not necessarily result in reduced PSMO activity.

Obviously, more research is required to determine the impact of nutritional influences on detoxification enzyme activity, but whatever the cause, the reduced PSMO activity of the field-strain larvae fed artificial diet suggests a need for caution when field populations of insects are evaluated for pesticide resistance. Frequently, individuals from the field are fed artificial diet in the laboratory and the LD_{50}s for pesticides are determined and/or their detoxification enzyme activity is measured relative to susceptible individuals from laboratory colonies. If feeding larvae the artificial diet results in reduced levels of detoxification enzymes that contribute to the resistance, then the degree of resistance may be underestimated.

The metabolic cost of detoxification enzyme load in insects has been hypothesized to be significant (Whittaker and Feeny, 1971; Brattsten, 1979). Reduced fitness of pesticide-resistant individuals (Roush and McKenzie, 1987; Campanhola et al., 1991), and a decline in the level of resistance in a few generations (e.g., Brown et al., 1978; Leonard et al., 1988) in the absence of a pesticide challenge, support the hypothesis that high detoxification enzyme loads have biologically significant metabolic costs or exert deleterious pleiotropic effects. Recent empirical studies, however, have failed to detect increased metabolic costs associated with a high level of PSMO induction (Neal, 1987) or with presumed increases in allelochemical detoxification (Appel and Martin, 1992). Moreover, fitness of pesticide-resistant insects is not always reduced relative to susceptible individuals in the absence of pesticides (Longstaff, 1991).

Our results suggest that these enzymes, at least at constitutive levels, are not metabolically expensive. If they were, it is likely that individuals with low enzyme levels would dominate the laboratory colony, which has been reared over many generations without infusion of wild-caught individuals on a more-or-less chemically constant artificial diet, probably with low levels of allelochemicals. Alternatively, metabolism of endogenous compounds (Hodgson, 1985) and exposure to allelochemicals in the artificial diet (e.g., isoflavones in the soybean flour; Liener, 1980) may select for maintaining a certain constitutive level of detoxification enzymes. Further, selection would not necessarily favor a reduced ability to induce these enzymes, unless there were negative pleiotropic effects, because inducibility likely conserves resources and limits metabolic costs until a higher level of the enzymes are needed for xenobiotic metabolism (Brattsten, 1988a). Whatever their cost, PSMO enzymes have not been reduced to an unusually low constitutive level in our laboratory-colony *A. gemmatalis* larvae,

and they can be induced to high levels of activity (present study and Christian and Yu, 1986). Thus, the laboratory-strain larvae provide a suitable model system for studying xenobiotic metabolism and detoxification enzyme induction, although the results may not be directly comparable to field-strain insects.

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INTRA- AND INTERSPECIFIC SEX PHEROMONE RESPONSES OF SCREWWORM AND SECONDARY SCREWWORM FLIES

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Abstract—A laboratory behavioral assay examined intra- and interspecific responses to sex pheromone by screwworms, *Cochliomyia hominivorax*, and secondary screwworms, *C. macellaria*, in relation to the duration of colonization of *C. hominivorax* test males. Females of *C. macellaria*, like those of *C. hominivorax*, were found to produce a pheromone that stimulates male copulatory attempts on contact. Newly colonized (<22 generations) *C. hominivorax* males did not respond to *C. macellaria* pheromone, indicating that pheromone contributes to reproductive isolation between these two closely related species. Although long-colonized (>200 generations) *C. hominivorax* males did respond to *C. macellaria* females or their extract, this behavior was infrequent and significantly less common than intraspecific responses. Depriving *C. macellaria* adults of dietary protein did not affect the potency of female extracts, but did reduce male responsiveness to pheromone. These results provided little evidence that colonization reduces the ability of *C. hominivorax* males to differentiate between *C. hominivorax* and *C. macellaria* females using sex pheromones.

Key Words—Screwworm, *Cochliomyia hominivorax*, *Cochliomyia macellaria*, Calliphoridae, Diptera, sex pheromone, laboratory adaptation, species recognition.

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INTRODUCTION

Sex pheromones in many Diptera act at close range or on contact, where they function in sex and species recognition (Haynes and Birch, 1985; Blomquist et al., 1987). Females of the screwworm, *Cochliomyia hominivorax* (Coquerel), also known as the primary screwworm because of its role as a primary agent of wound myiasis, likewise produce a pheromone that stimulates male copulatory attempts on contact (Mackley and Broce, 1981; Hammack, 1986). Sexually active male screwworms (Krafsur, 1978; Guillot et al., 1978) and other Diptera with similarly reduced mating flights (Downes, 1969) occupy waiting stations or territories, from which they take flight in pursuit of a variety of suitably sized moving targets. Upon contact, mating attempts ensue when the targets are female screwworm flies, otherwise mating flights are aborted and males return to their waiting stations. This behavior suggests that the screwworm sex pheromone functions on contact and is important for species discrimination.

Changes in screwworm mating behavior and sex pheromone communication occur during colonization. Male responsiveness to pheromone increases and potency of female extracts drops with time in colony (Hammack, 1987, 1991); these factors apparently combine to produce the asymmetric mating patterns that typify crosses between old and new screwworm strains. Mating is asymmetric in that copulatory attempts occur more readily between old-strain males and new-strain females than in the reciprocal direction (Alley and Hightower, 1966; Spates and Hightower, 1967; Hammack, 1987; Mangan, 1988).

Changes in sex pheromone communication with colonization are relevant to screwworm eradication programs in Central America and North Africa that are based on the sterile insect technique (SIT) (Krafsur et al., 1987; Beesley, 1991). Population suppression via SIT requires that sterile released males mate with wild females and thereby render their eggs infertile (Knipling, 1955). Even long-colonized screwworm males would be expected to vigorously attempt copulation with a wild conspecific female, but their ability to discriminate species has not been examined.

The closest relative of the screwworm (SW) is the secondary screwworm (SS), *Cochliomyia macellaria* (F.). Morphological differences are subtle, at least in the adult female, and the two were not recognized as separate species in this country until 1933 (Cushing and Patton, 1933). Although their larval habitats are distinct in that SW feeds on living tissue of homeotherms whereas SS breeds in carrion or necrotic flesh (Laake et al., 1936), the adults occur together. Indeed, SS greatly outnumber SW at baits used to monitor SW adults (Lindquist, 1938; Mackley, 1986).

This study compares the ability of SW males differing in duration of colonization to discriminate SW and SS females using sex pheromone cues. In doing so, it establishes the existence of a sex pheromone in SS females and

examines the effect of protein in the adult diet on SS pheromone production and reception.

METHODS AND MATERIALS

SS were collected in the state of Chiapas, Mexico, and tested after about 40–60 generations in the laboratory. Two SW strains were used. The 009 SW strain was collected in Texas in 1974–1975 and colonized for more than 200 generations. The OW-87 SW strain was collected in Belize in 1986 and tested within 21 generations. SS larvae were reared on ground beef that was aged at room temperature for several days before use. SW were reared on a beef-based larval diet (Melvin and Bushland, 1940) that was treated with formaldehyde and changed twice daily to retard putrefaction. Adults of both species were separated by sex within 24 hr of adult emergence to ensure virginity. They were held at 25± 1°C in a 12:12 hr light–dark photoperiod and provided corn syrup and separate water ad libitum. In addition, SS adults in protein-fed treatments had continuous access from emergence until 5 days of age to fresh beef liver changed daily. Females of both species were tested four to six days after adult emergence. Males were 1–2 weeks old.

Sex pheromone was extracted with *n*-hexane from lots of 100 females, unless stated otherwise. Extract, concentrated to 1.0 or 5.0 female equivalents (FE) in 5 μl hexane, was applied to hexane-washed males that served as decoy flies in bioassays. Controls were treated with 5 μl of hexane instead of extract. Whole females killed by freezing, and controls for whole dead females (previously frozen females washed in hexane and additionally treated with 5 μl hexane) were also used as decoys in some tests. Each decoy was mounted on a cork (No. 6) that was used to stopper glass test tubes containing individual test males. Inversion of the tubes forced the test males into contact with the decoys. Bioassays determined whether or not the test males attempted copulation with decoys during 1 min of forced contact. Decoys were tested with a series of 10 males and then discarded. Males were tested only once. More detailed descriptions of the extraction and assay techniques have been reported previously (Hammack, 1987).

Intraspecific and reciprocal interspecific responses to pheromone were initially measured with dead SW and SS female decoys tested with males of each species. A second test substituted 1.0 FE of extract on a washed male decoy for each dead female. A third also examined intraspecific and reciprocal interspecific responses, but decoys treated with SS extract received 5.0 FE, whereas those treated with SW extract again received 1.0 FE. These tests were first done with the newer OW-87 SW strain. They were then repeated with OW-87 females but test males of the longer-colonized 009 strain. The hexane-washed male

decoys to which extract was applied were in all cases the same species and strain as the responding males.

Fifty SS females that had been fed protein and 50 not fed protein (unfed) were extracted six days after emergence. Each extract was applied at 1.0 FE to separate washed decoys (unfed SS males) and assayed with protein-fed and unfed SS test males. The response to 1.0 FE of SS extract was also compared with that to control, using only protein-fed SS flies.

Statistical analysis was limited to treatments assayed simultaneously with males matched in age and larval rearing group. Except for the SS protein feeding test, analyses compared treatments with regard to the total numbers of flies responding and not responding using chi-square calculated from 2×2 contingency tables. Treatment variances were more uniform in the protein feeding test, where percentage response data ($N = 10$ decoys per treatment) were transformed (arcsin $\sqrt{}$) and analyzed by ANOVA and Student-Newman-Keuls multiple range test (SAS Institute, 1988).

RESULTS

In tests involving SS and OW-87, the more newly colonized of the two SW strains, females or female extract elicited copulatory attempts from significantly more conspecific males than did solvent controls (Table 1, tests 1–3). The response of males to conspecific females or their extract was at least 65% within SW and 23% within SS. No interspecific or control responses were observed with OW-87 SW or SS males. Males of the longer-colonized 009 SW strain reacted to SS females or their extract at rates significantly exceeding those to controls in two of three tests (Table 1, tests 4–6). However, no more than 16% of the 009 males reacted to SS extract, even when 5.0 FE was tested, whereas 97% of them responded to 1.0 FE of conspecific extract.

Feeding liver to SS adults had no effect on the potency of female pheromone extracts but did significantly enhance male responsiveness to pheromone (Table 2). About twice as many fed as unfed males responded to 1.0 FE of extract. The difference in response to female extract compared with control was more marked when SS flies fed protein were tested; 1.0 FE of extract elicited responses from 58% of test males, whereas solvent alone produced no responses ($\chi^2 = 38.1$, $df = 1$, $P < 0.001$).

DISCUSSION

The results show that SS females, like SW females (Mackley and Broce, 1981; Hammack, 1986), produce a mating-stimulant pheromone that elicits copulatory attempts from conspecific males. The absence of interspecific copulatory

TABLE 1. INTRA- AND INTERSPECIFIC MATING ATTEMPTS BY MALES OF SCREWWORM (SW), *C. hominivorax*, AND SECONDARY SCREWWORM (SS), *C. macellaria*, IN RELATION TO TIME IN COLONY OF SW MALES

Test	Time in colony of SW test males	Males tested (*N*)	Female stimulus	Males responding (%) to female stimulus[a]					
				SW males			SS males		
				SW	SS	Control[b]	SW	SS	Control[b]
1	Short[c]	150	Whole dead females	74	0	—	0	51	—
2	Short	100	Female extract (1.0 FE[d] SW or 1.0 FE SS)	65a	0	0b	0	23a	0b
3	Short	100	Female extract (1.0 FE SW or 5.0 FE SS)	68a	0	0b	0	37a	0b
4	Long[e]	150	Whole dead females	97	6a	0b	0	38	—
5	Long	150	Female extract (1.0 FE SW or 1.0 FE SS)	97	1a	3a	0	11a	0b
6	Long	150	Female extract (1.0 FE SW or 5.0 FE SS)	97	16a	4b	0	41a	0b

[a] Within tests and species of male, members of a pair followed by the same letter are not significantly different at $P = 0.05$ by contingency chi-square.
[b] Hexane washed insects treated with hexane in place of female extract.
[c] Belize SW strain colonized fewer than 22 generations.
[d] Female equivalent.
[e] 009 SW strain colonized more than 200 generations.

TABLE 2. EFFECT OF FEEDING PROTEIN TO *C. macellaria* ADULTS ON MEAN PERCENTAGE OF MALES ATTEMPTING COPULATION UPON EXPOSURE TO HEXANE EXTRACT OF FEMALES (1.0 FEMALE EQUIVALENT)[a]

	Female	
Protein feeding status/sex	Unfed	Fed
Unfed male	31a	27a
Fed male	61b	55b

[a] Values followed by the same letter are not significantly different at $P = 0.05$ by Student-Newman-Keuls multiple-range test following arcsin $\sqrt{}$ transformation of the percentage data and a significant ANOVA ($F = 5.51$; $df = 3, 36$; $P < 0.003$).

attempts when SS and the more newly colonized SW strain were tested indicates that SW and SS pheromones are distinct. Pheromonal differences may thus contribute to reproductive isolation between these two closely related species, which produce no hybrid progeny and only occasionally attempt interspecific matings when caged together (Chamberlain and Hopkins, 1978).

The SS protein feeding tests were done because SS, unlike SW, is anautogenous and SS fed only carbohydrate responded poorly in intrastrain comparisons. Protein in the adult diet has been shown to affect sexual behavior in other anautogenous blow flies (Parker, 1968; Bartell et al., 1969; Barton Browne et al., 1976). If dietary protein were to enhance the pheromone activity of SS females, as it does for *L. cuprina* females (Bartell et al., 1969), then protein-deprived SS females should not have been used to compare responses of old and new strain SW males. However, access to liver for five days after adult emergence had no effect on the pheromone activity of SS females, although it did double the number of males that responded to pheromone. Considering that males were forced to contact pheromone during bioassays, the effect on males appears to involve altered sensitivity to pheromone rather than just a generalized change in locomotory activity. Protein deprivation also vastly reduces oriented mounts by males of *Protophormia terrae-novae* R.-D., although reduction may be mediated by means other than pheromone responsiveness (Parker, 1968).

Although some 009 SW males did attempt mating in response to SS females or their extract, this behavior was infrequent, especially when compared with intraspecific responsiveness. As already noted, infrequency of the interspecific interactions was not attributable to low potency of the SS extracts caused by lack of dietary protein. Thus, in spite of a generalized increase in response levels, the present experiments provided little evidence that longer colonization detrimentally affects the ability of SW males to discriminate *C. hominivorax* from *C. macellaria* females using pheromone cues.

The degree of spatial and temporal overlap that occurs in the field between SW and SS mating activities needs to be clarified before the importance of pheromones in maintaining reproductive isolation can be fully evaluated. Although both species are trapped at proteinaceous baits, it has not been established that both mate at these locations. Many other blow flies do apparently mate near carrion, where females obtain the protein needed for oogenesis, and some species oviposit (Parker, 1968; Shorey et al., 1969; Barton Browne et al., 1976; Woodburn and Vogt, 1982). Meskin (1986) described the assumption of waiting stations on or around carrion by sexually active males of six calliphorid species that use the stations to intercept approaching females. The observation that carrion and wounds attract about equal numbers of SS males and females (Lindquist, 1938; Krafsur, 1978) suggests that protein resources also serve as mating aggregation sites in this species. In contrast, carrion baits attract at least five times more SW females than males (Lindquist, 1938; Coppedge et al., 1977), and few SW males are trapped on host wounds attractive to SW females (Parish, 1937; Guillot et al., 1977; Thomas and Mangan, 1989). The rarity of SW males at the host and their failure to respond to host odors attractive to females (Hammack et al., 1987, 1989) or to volatiles associated with virgin females (unpublished data) argue against SW mating at the host. The substantial predominance of inseminated over virgin females at the host (Guillot et al., 1977) and their monogamous habit (Knipling, 1955) are consistent with this view. Indeed, were males present on the host, their mating attempts could interfere with oviposition, considering that female sex pheromone activity is unaffected by insemination (Hammack, 1987). Evolution of parasitism by a presumed carrion-breeding ancestor of the screwworm apparently offered not only a more consistent larval habitat with reduced interspecific competition for limited food resources (see Hanski, 1987, for references), but also the opportunity for spatial separation of mating and ovipositional behaviors.

In spite of the success of SIT in controlling screwworms, their mating behavior has been observed only rarely in the field. Sterile released SW mate on flowering vegetation, where both sexes feed on nectar or honeydew and where, compared with host, the higher proportion of virgin females increases the opportunity for males to encounter receptive females (Guillot et al., 1978; Mackley and Long, 1983). Broce (1980) reported increased sexual activity among SW males exposed to components of swormlure-2, a chemical bait used for population monitoring (Mackley, 1986). Food odors associated with aggregation sites are known to stimulate male sexual activity in other Diptera, including *L. cuprina* and *Drosophila melanogaster* (Shorey et al., 1969). Although swormlure was originally formulated from likely decay products of proteins and fats (Jones et al., 1976) and attracts at least four times more females than males (Coppedge et al., 1977), Broce (1980) suggested that it mimics the odor of floral sites where screwworms aggregate. However, Krafsur (1978) reported aggre-

gation by sexually active wild SW males on nonflowering vegetation away from obvious food resources. Similar but isolated observations of sterile males on nonflowering vegetation led Mackley and Long (1983) to consider the possibility that SW mating at floral feeding sites is an artifact stemming from the abnormally high density characteristic of sterile released populations. Thus, it is uncertain whether screwworms mate mostly on flowers, around carrion, or away from food resources. The significance of the observed pheromonal divergence in maintaining reproductive isolation between SW and SS can be fully evaluated only when this important issue is resolved and the degree of spatial and temporal overlap between SW and SS mating activities clarified.

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ATTRACTION OF MALE TURNIP MOTHS *Agrotis segetum* (LEPIDOPTERA: NOCTUIDAE) TO SEX PHEROMONE COMPONENTS AND THEIR MIXTURES AT 11 SITES IN EUROPE, ASIA, AND AFRICA

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Abstract—Selected combinations of (Z)-5-decenyl, (Z)-7-dodecenyl, and (Z)-9-tetradecenyl acetates, the pheromone components of the turnip moth *Agrotis*

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segetum were tested for field attractancy at six, two, and three sites in Europe, Asia, and Africa, respectively. At all of the sites in Eurasia and in northern Africa the ternary mixture of the acetates captured most males, while at the sites south of the Sahara in Africa, (Z)-5-decenyl acetate alone was responsible for attraction. Differences in male attraction among the populations studied confirm the existence of significant population variation in the pheromone of *A. segetum*. Interpretation of the present results together with earlier studies suggests that this variation is more or less continuous in Eurasia and north Africa, while a clearly distinct pheromone type is present in the areas south of the Sahara desert.

Key Words—Sex pheromone, *Agrotis segetum*, Lepidoptera, Noctuidae, field trapping, (Z)-5-decenyl acetate, (Z)-7-dodecenyl acetate, (Z)-9-tetradecenyl acetate, geographical population variation.

INTRODUCTION

Several earlier reports have dealt with the chemical identity of the female sex pheromone of the turnip moth (*Agrotis segetum* Den. & Schiff.). Today it is generally accepted that (Z)-5-decenyl acetate (Z5–10 : Ac; names of compounds are abbreviated according to Arn et al., 1986), (Z)-7-dodecenyl acetate (Z7–12 : Ac) and (Z)-9-tetradecenyl acetate (Z9–14 : Ac) are the three behaviorally important components present (Bestmann et al., 1978; Arn et al., 1980; Tòth et al., 1980; Löfstedt et al., 1982). In a trapping study conducted in four European countries, the ternary mixtures of these compounds were generally more attractive to male moths than binary combinations or the single compounds (Arn et al., 1983). However, minor differences at some of the sites indicated possible geographical variation in the responses of males. In France, Z5–10 : Ac on its own captured a sizable number of moths, and these catches did not differ significantly from some catches of the ternary mixtures (Arn et al., 1983). Corresponding results were obtained with a binary Z7–12 : Ac/Z9–14 : Ac bait in Hungary (Arn et al., 1983) and Bulgaria (Subchev et al., 1986), and some combinations of Z5–10 : Ac/Z7–12 : Ac in Denmark (Arn et al., 1983).

Further evidence supporting the idea of pheromone differences between populations has been provided by analysis of female-extracted pheromone components, and investigations of olfactory sensilla on male antennae of turnip moth populations from various places in Europe (Löfstedt et al., 1986; Hansson et al., 1990).

The turnip moth is present within the entire temperate zone of Eurasia, in the Mediterranean, as well as in several regions of Africa, down to its southernmost parts (Cayrol, 1966). In the present study, selected combinations of the three pheromone components were tested at six, two, and three sites in Europe, Asia, and Africa, respectively, in order to provide further evidence for differ-

ences and similarities in the male response to pheromone components of the respective geographical populations.

METHODS AND MATERIALS

Compounds used in the tests were purchased from the Institute of Pesticide Research (Wageningen, The Netherlands). Gas chromatographic analysis revealed that *Z*5–10:Ac contained <3% of the corresponding *E* isomer and <5% of the corresponding alcohol; *Z*7–12:Ac <3% of the *E* isomer with no detectable alcohol, and *Z*9–14:Ac <8% of the *E* isomer with no detectable alcohol. Baits were prepared using 0.5 × 1-cm pieces of rubber tubing (Taurus, Budapest, Hungary; No. MSZ 9691/6; extracted three times in boiling ethanol for 10 min, then three times in dichloromethane overnight, prior to use). Baits were made with the required amounts of compounds from the same stock solutions in hexane administered to the surface of the dispensers. Prepared dispensers were wrapped individually in aluminum foil, and stored at −65°C until use, or sent air mail to the different test sites. Before setting up the test, the dispensers were stored at −20°C by each respective cooperator. Bait variations tested are summarized in Figure 1.

Unless stated otherwise, field tests were conducted by using sticky traps similar in shape and size to those described by Arn et al. (1979), but made from polyethylene sheets. Traps containing different baits were set up in pentagonal blocks. The distance between traps within a block was 4–5 m. The distances between blocks ranged from 100 to 1000 m. When checked, the traps were moved one position forward within a block. At the same time, captured males were recorded and sticky inserts changed to new ones. At each test site, five traps were operated from each bait variation. In statistical analyses, catches recorded at a trap inspection were regarded as replicates. Captured moth specimens from each test were sent to Budapest, and their species identity was determined on the basis of wing pattern characters by L. Ronkay (Hungarian Natural History Museum, Budapest).

Capture data were transformed to log $(x + 1)$ and differences between means were tested for significance by Duncan's new multiple-range test (NMRT). Cluster analysis of relative male responses to the five different blends in the 11 populations was performed using SYSTAT (Wilkinson, 1989). Clustering was accomplished by the Ward minimum-variance method.

Details of Testing Procedures According to Sites

Bulgaria. Polski Trumbesh, May 30–June 22, 1990; vineyard; old bait replaced by new one on June 6; traps inspected every two days.

Egypt. El-Tahrir region, Shibin El Kom, March 19–April 6, 1990; lime plantation; traps inspected every two days.

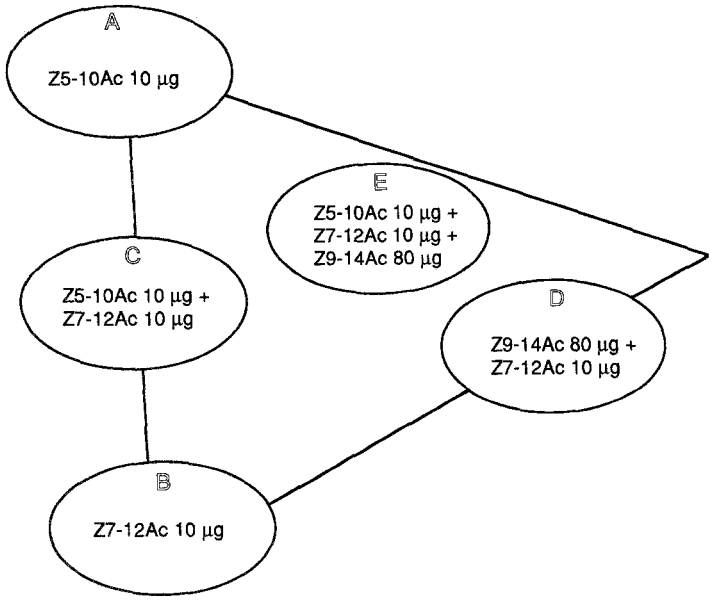

FIG. 1. Composition of baits used in field trapping tests for catching male *A. segetum* in various geographical regions. For ease of comparison, baits are presented according to the triangular arrangement scheme of Arn et al. (1983).

Hungary. Budakeszi, Pest county, May 30–June 9, 1989; vineyard; traps inspected every two days.

Italy. Bologna, September 8–22, 1989; mixed vegetable fields; traps inspected once weekly; from each bait variation only one trap was operated.

Kazakhstan (U.S.S.R.). Alma-Ata, July 9–23, 1990; apple orchard; traps inspected every three to four days.

Moldavia (U.S.S.R.). Kishinev, July 17–30, 1990; mixed vegetable fields; traps inspected every three to four days.

Republic of South Africa. Cathedral Peak, Forestry Research Station, February 22–April 10, 1990; extensive and partly abandoned fields, overgrown orchards; old baits replaced by new ones on March 12; traps inspected every three to four days.

Spain. Granada, July 17–August 2, 1989; fields of various vegetables; traps inspected every two days.

Sweden. Knästorp, outside the city of Lund, July 7–12, 1989; sugar beet field; traps inspected every two to three days; trap model Lund II (Anderbrant et al., 1989) was used.

Turkmenistan (U.S.S.R.). Bayram Ali, Mariyskaya oblast, August 14–23, 1989; alfalfa field; traps inspected every three to four days.

Zimbabwe. Kutsaga Research Station, Harare, December 28, 1989–January 26, 1990; tobacco field; traps inspected every three days; delta traps (made from white corrugated polyethylene; PLA Insect Monitoring and Control Systems, Southampton, U.K.) were used.

RESULTS

Catches of A. segetum. In all of the tests in Eurasia and the single test in north Africa (Egypt), the ternary mixture of the three acetates clearly caught better than any of the other combinations (Figure 2), substantially confirming results of previous trapping tests in four countries in Europe (Arn et al., 1983). The binary mixture of Z5–10:Ac/Z7–12:Ac caught significantly less than the ternary blend in all places except in Spain. The combination of Z7–12:Ac/Z9–14:Ac catches were close to nil in Sweden, Hungary, Kazakhstan, and Turkmenistan, whereas in Spain, Moldavia, Bulgaria, and Egypt the captures were somewhat greater, but still lower than catches with the ternary blend. The Z7–12:Ac/Z9–14:Ac blend generally caught less than the Z5–10:Ac/Z7–12:Ac blend, except in Moldavia, Kazakhstan, and Egypt. Z5–10:Ac, or Z7–12:Ac as single components hardly caught any moths.

In contrast, at the two sites south of the Equator in Africa, the largest number of males was captured by Z5–10:Ac alone (Figure 2). Smaller catches in other combinations could be attributed to the Z5–10:Ac content of the respective variations.

Cluster analysis suggested classification of the investigated populations into two major types (Figure 3). The two southern African populations form a clearly distinct group (I), in which more than 75% of the moths are trapped by the pure Z5–10:Ac bait. All the Eurasian populations form one cluster together with the population from Egypt (II). These populations are characterized by high responses to the three-component blend. However, two subgroups may be distinguished within this second cluster. All the European populations form one subgroup (a) together with the Egyptian population. These populations are characterized by the highest responses to the three-component blend (mean = 55%, range 45–68%), and relatively large responses to some of the binary blends. The populations from the Asian sites in Kazakhstan and Turkmenistan cluster together and form a second subgroup (b), which differs from the European populations by outstandingly high catches with the three-component blend (95% and 77%, respectively, for the two populations). The difference between clusters (a) and (b) is obviously small compared to the difference between clusters I and II, and its biological significance is not clear. However, it is interesting to note that the two Asian populations, which are geographically relatively close, actually cluster together.

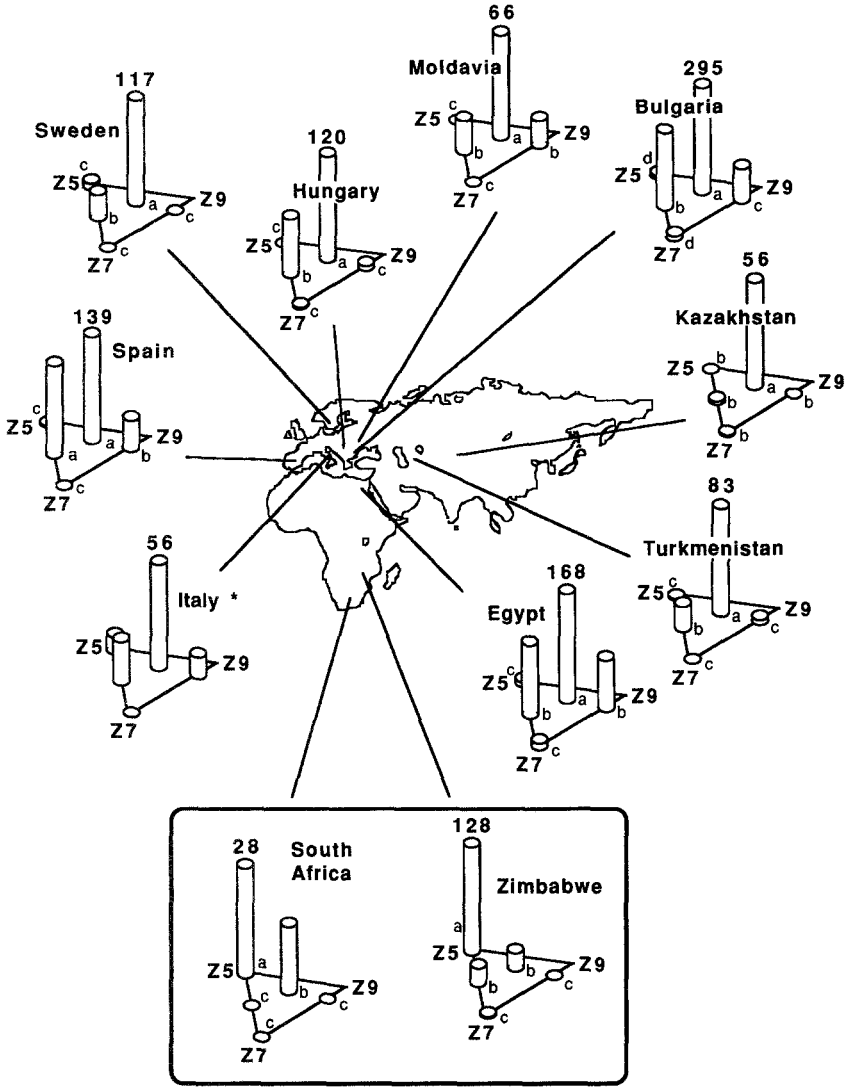

FIG. 2. Percentage catches of male *A. segetum* at 11 sites in Eurasia and Africa. Numbers above columns show total catch of best bait in the given test. Z5 = Z5–10:Ac; Z7 = Z7–12:Ac; Z9 = Z9–14:Ac. Columns with same letter within a diagram are not significantly different at $P = 0.05$ by Duncan's NMRT. Triangular arrangement of baits is the same as in Figure 1. *In Italy only one trap per bait variation was set up, and data were not statistically analyzed for significance.

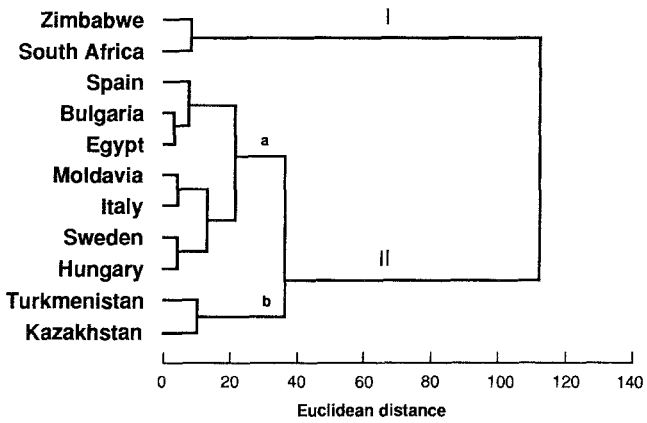

FIG. 3. Dendrogram resulting from cluster analysis of relative trap catches by five different pheromone blends in 11 populations. The analysis was performed on the data in Figure 2.

Catches of Other Species. Apart from male *A. segetum*, other species captured in the test included *Autographa gamma* L., and *Emmelia trabealis* Scop., attracted to Z7-12:Ac and Z5-10:Ac, respectively, at many of the sites in Eurasia and north Africa. Sizable numbers of male *Mythimna loreyi* Duponchel were caught by the two baits containing both Z7-12:Ac and Z9-14:Ac (i.e., D and E) in Spain.

A total of 51 *Agrotis clavis* Hfn. males was caught by Z5-10:Ac in the Hungarian test. No males were captured by any of the other treatments.

In the Zimbabwean trial, a total of 14 *Chrysodeixis acuta* Wlk. was caught by the bait containing Z5-10:Ac/Z7-12:Ac, while in Sweden 2 and 51 male *Cnephasia pasiuana* Hbn. (Tortricidae) were captured by Z5-10:Ac and Z5-10:Ac/Z7-12:Ac, respectively.

DISCUSSION

Results from South Africa and Zimbabwe in the present study clearly demonstrate the presence of a male type distinct from previously described ones, since here Z5-10:Ac alone caught more than any of the other baits. It is at variance with earlier data from France, where Arn et al. (1983) reported that Z5-10:Ac alone caught as many moths as the ternary blend. The addition of Z7-12:Ac or Z9-14:Ac seemed to decrease catches at both sites in southern Africa (Figure 2). Our results of pheromone extract analyses and of studies on male antennal receptors on insects from Kenya further confirm the occurrence of a distinct pheromonal type of *A. segetum* for Africa (to be published in detail

elsewhere). The Sahara desert appears to be an ecological barrier that has separated the Eurasian and sub-Saharan African populations.

Electrophysiological studies and analyses of female-extracted pheromone components of different *A. segetum* populations (Löfstedt et al., 1986; Hansson et al., 1990) have suggested the existence of at least three main pheromone populations in Europe: a population found in France, producing more Z5–10:Ac in the pheromone, and possessing many Z5–10:Ac-tuned receptors on the male antennae; a population found in Sweden, producing less Z5–10:Ac and possessing fewer receptors for this compound (but still more abundant than the Z7–12:Ac receptors); and a population found in Armenia/Bulgaria, producing very little Z5–10:Ac and possessing fewer receptors tuned to Z5–10:Ac than to Z7–12:Ac. The cooccurrence of males similar to the Swedish and Armenian/Bulgarian type has also been proven for Hungary (Hansson et al., 1990). When studying the antennal receptors of males captured by traps baited with the Z7–12:Ac/Z9–14:Ac blend, males belonged almost exclusively to the Armenian/Bulgarian type; while both Armenian/Bulgarian and Swedish types of males were caught in about equal numbers by ternary baits (Hansson et al., 1990). Consequently, the higher numbers caught with ternary baits in a former (Arn et al., 1983), as well as in the present study, could be attributed to this bait being able to attract both types of males, while binary combinations can be attractive mainly to one of the male types.

On the other hand, this explanation strongly suggests the cooccurrence of both male types not only in Hungary (Hansson et al., 1990), but also at the other Eurasian and north African sites (Figure 2). The coexistence of such pheromonal types also in Sweden is indicated in retrospect by the results of the analysis of pheromone extracts from single females (Löfstedt et al., 1985), where a high variation in ratios between the three acetate components was demonstrated.

In the present study, no catches similar to earlier results from a French site (Arn et al., 1983) were observed at any of the sites in Eurasia. Likewise, high catches with the Z7–12:Ac/Z9–14:Ac blend, as previously described from Bulgaria (Subchev et al., 1986), were not confirmed at the Bulgarian site in this study, nor by a preceding preliminary trapping trial conducted in 1989, where similar baits were tested with very much the same results as the ones presented here (Tòth and Subchev, unpublished). We suggest that the above deviations in capture data may result from local or temporal variations in relative abundance of the cooccurring pheromonal types. It could be that in the year and at the site of the study by Subchev et al. (1986), the Armenian/Bulgarian male type was much more abundant than the other one, due to unknown selection pressures or the effects of random fluctuations of the local conditions on the two pheromonal types.

Distinct pheromone differences between populations of the same or sibling species have been shown to depend on allelic differences at a small number of loci (Löfstedt, 1990). This might explain the distinct pheromone difference between the Eurasian and southern African populations of *A. segetum*. However, the more or less continuous variation in the pheromone communication systems among the Eurasian populations seems to involve either several different alleles at the response and production loci or quantitative inheritance variation.

In *Agrotis fucosa* Butler, a noctuid pest in Japan, two components, Z5–10:Ac and (Z)-7-decenyl acetate (Z7–10:Ac), have been identified as pheromone components (Wakamura, 1978, 1980). Some authors mention *A. fucosa* as synonymous with *A. segetum* (Dunkelblum and Gothilf, 1984; Steck et al., 1979; Wakamura et al., 1981) and suggest that *A. fucosa* may represent the Japanese pheromonal type of *A. segetum*. As for the taxonomists' opinion, a taxonomic checklist of lepidopterous species from Japan states that: ". . . *fucosa* Butler Trans. Ent. Soc. Lond. *1881*, 179 (1881) = *segetum* part., auct., nec. Schiffermüller et Denis, 1775 . . ." (Sugi, 1958), which means that some authors erroneously refer to *A. fucosa* as *A. segetum*. According to this checklist, *A. segetum* is not present in Japan (Sugi, 1958). However, in a later edition, *A. fucosa* is listed as being synonymous with *A. segetum* (Sugi, 1982).

During several years of extensive field-trapping tests in Hungary with binary mixtures of Z5–10:Ac and Z7–10:Ac, we never caught any male *A. segetum* with any of the baits (Tòth and Szöcs, unpublished). Consequently, based on differences of pheromone composition, it may be more probable that *A. fucosa* and *A. segetum* are separate species.

From an ecologist's point of view it is important to determine if the observed variation between populations is adaptive or not. If differences in blend attractivity between populations were accompanied by large catches of nonspecific moths in the suboptimal blends, this would be evidence for the importance of interspecific competition in the evolution of the communication channel. Continuous trapping during the whole flight season at all sites would have been necessary to allow any definite conclusions to be drawn. However, it is interesting to note that in Eurasia and north Africa several other species were attracted by Z5–10:Ac alone, whereas this compound only attracted *A. segetum* in Zimbabwe and South Africa. This supports an adaptive interpretation of the observed differences in attraction between the *A. segetum* populations.

Captures of *A. gamma*, *E. trabealis*, and *M. loreyi* in this study confirm earlier descriptions of the sex attractant of the respective species (Szöcs et al., 1983; Tòth et al., 1983; Takahashi et al., 1983). No male attractant has been described previously for *A. clavis*, *C. acuta*, and *C. pasiuana*. Of these, *A. clavis* is a species very similar to *A. segetum* in appearance and is recorded as a potential pest of vegetables in Eurasia (Cayrol, 1966).

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COMPARISON OF ANACARDIC ACID BIOSYNTHETIC CAPABILITY BETWEEN INSECT-RESISTANT AND -SUSCEPTIBLE GERANIUMS

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Abstract—The garden geranium (*Pelargonium xhortorum*) has been shown to secrete anacardic acids in the form of a viscous sticky exudate from tall glandular trichomes, and this exudate provides a sticky trap defense against small pest species. The anacardic acids from genetically related pest-resistant and -susceptible plants have been characterized, and resistance has been shown to depend upon the presence of $\omega 5$ unsaturated anacardic acids. In this study, the biosynthesis of these anacardic acids was comparatively investigated by incubating [^{14}C]methyl palmitate, margarate, stearate, oleate and linoleate on floral buds of resistant and susceptible plants. In addition, the incorporation of [^{14}C]valine, -isoleucine, and -leucine into anacardic acids was also studied. Nineteen anacardic acids were quantitated utilizing an improved HPLC technique. Fatty acids and, to a much lesser extent, amino acids were incorporated into anacardic acids. There are at least two pathways of biosynthesis operating: direct elongation, and β-oxidation with reincorporation of the [^{14}C]acetate, the latter being more prevalent in the resistant plant. The amino acids were processed into branched chain anacardic acids, isoleucine being the precursor of the anteiso compounds, and valine the iso branched ones. The major difference between resistant and susceptible plants was the ability of resistant plants, but not the susceptible plants, to synthesize $\omega 5$ unsaturated anacardic acids. Both types of plants were capable of directly incorporating ^{14}C-labeled fatty acid methy esters into anacardic acids regardless of the plant's normal anacardic acid composition, thus bypassing the plant's tightly controlled regulation of the chemical structures of anacardic acids. No evidence was found for $\omega 5$ desaturation of saturated anacardic acids. A revised biosynthesis scheme is presented.

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Key Words—Biosynthesis, anacardic acids, glandular trichomes, geraniums, *Pelargonium xhortorum*.

INTRODUCTION

Geraniums (*Pelargonium xhortorum*) have been shown to possess tall glandular trichomes, which secrete a viscous sticky exudate, providing sticky trap resistance against small pest species, such as the two-spotted spider mite (*Tetranychus urticae* Koch) and the foxglove aphid (*Acyrthosiphon solani* Kaltenbach) (Craig et al., 1986; Gerhold et al., 1984; Stark, 1975; Walters, 1988, Walters et al., 1988, 1989a,b, 1990a; Hesk et al., 1990). Chemical analysis of the exudate in both insect-resistant and -susceptible geraniums has shown it to be composed of anacardic acids, which are 6-alkyl salicylic acid derivatives possessing varying side chain lengths, degrees of side chain unsaturation, and branching (Walters et al., 1988; Hesk et al., 1990).

Analysis of the exudate from resistant plants has shown that it is predominantly composed of unsaturated anacardic acids, with C_{22} $\omega5$ and C_{24} $\omega5$ unsaturated anacardic acids accounting for over 80% of the exudate composition (Walters, 1988; Hesk et al., 1990). In addition, there are small but significant quantities of C_{23} $\omega6$, C_{25} $\omega6$, and C_{23} anteiso anacardic acids and the saturated C_{22} and C_{24} analogs. In contrast, the exudate from susceptible plants is predominantly saturated, with the C_{22} and C_{24} saturated anacardic acids being the major components and accounting for 50% of the total. The anacardic acids with an $\omega5$ or $\omega6$ double bond are all but absent in the susceptible plant. Instead, the remaining 50% is made up of a complex mixture of anacardic acids, of which the most notable are the C_{23} anteiso, the C_{23} saturated, and two unsaturated anacardic acids not seen in the resistant plant, the C_{24} $\omega6,9$ and the C_{24} $\omega9$ (Hesk et al., 1990). Anacardic acids have been shown to be the critical factor in small pest resistance of geraniums (Walters et al., 1989b), and resistance is dependent upon the presence of $\omega5$ unsaturated anacardic acids.

Geissman (1963) and Gerhold et al. (1984) proposed that these compounds are synthesized from fatty acid precursors in similar manner to fatty acid biosynthesis. The first experimental evidence for this mechanism was presented by Walters et al. (1990b), who studied the incubation of ^{14}C-labeled fatty acid methyl esters on the sepals of pest-resistant geraniums. It was found that saturated fatty acids were predominantly incorporated into saturated anacardic acids via the addition of three acetate units, thus supporting the hypothesis. This finding also gave rise to the conclusion that the $\omega5$ anacardic acids must be derived from $\omega5$ fatty acid precursors because no evidence could be found for the $\omega5$ desaturation of saturated anacardic acids. The appearance of ^{14}C label in the C_{22} $\omega5$, C_{24} $\omega5$, and C_{22} saturated anacardic acids, in addition to the

expected C_{24} saturated compound when [^{14}C]stearate was incubated, suggested that a second mechanism was operating. Hence, it was suggested that a portion of applied ^{14}C-labeled fatty acids was degraded into [^{14}C]acetate by β-oxidation, and then the [^{14}C]acetate was reincorporated into the anacardic acid biosynthesis. Thus this pathway was termed the indirect pathway (Figure 1).

While the biosynthesis of anacardic acids has been studied in the resistant plant (Walters et al., 1990b), no data are available on the biosynthesis in susceptible plants. This is particularly important since genetic experiments indicated susceptible plants differed from resistant plants by one or two genes (Winner, 1975, and unpublished data). Hence the present paper describes the biosynthesis of anacardic acids in the susceptible plant, with a wider range of fatty acids than previously used and with a more thorough analysis of the products using an improved HPLC separation technique, and compares the results to experiments carried out in parallel in the resistant plant. It was thought likely that the branched-chain and odd-chain-length anacardic acids were derived, in part, from

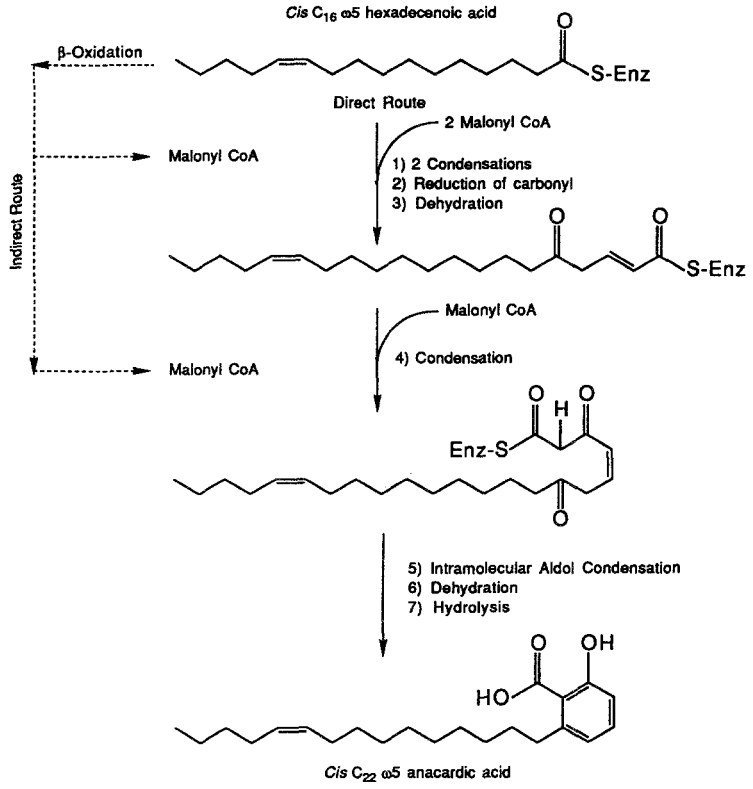

FIG. 1. Biosynthesis of an anacardic acid from a fatty acid precursor.

amino acids; thus three ^{14}C-labeled amino acids were incubated in both plant lines to test this hypothesis. The relative composition of anacardic acids, found within the resistant and susceptible plants, was determined by HPLC using UV detection to provide a base for comparison with the ^{14}C data and to determine the relative contribution of the two proposed biosynthetic pathways.

These studies are part of our continuing effort to further understand plant defense mechanisms with the expectation that some day we will be able to transfer these mechanisms to other plants.

METHODS AND MATERIALS

Radiochemicals. [1-^{14}C]palmitate, [1-^{14}C]margarate, [1-^{14}C]stearate, [10-^{14}C]oleate (C_{18} $\omega 9$ or $\Delta 9$), [1-^{14}C]linoleate (C_{18} $\omega 6,9$, or $\Delta 9,\Delta 12$), L-[U-^{14}C]isoleucine, L-[U-^{14}C]valine and L-[2-^{14}C]leucine were obtained from Research Products International Corporation, Mount Prospect, Illinois, or from ICN Radiochemicals, Irvine, California. All had a specific activity of 45–60 mCi/mmol. The purity of each was checked by TLC and autoradiography prior to use, and all were found to have a radiochemical purity in excess of 98%.

Plant Source. The geraniums used for the study were maintained in a growth chamber at 24°C using standard cultural practices, with a 16-hr photoperiod and an 8-hr nyctoperiod. The plant line 71-17-7 had previously been determined to be resistant to the two-spotted spider mite (*Tetranychus urticae* Koch) and the foxglove aphid (*Acyrthosiphon solani* Kaltenbach), and the line 85-26-8 had previously been determined to be mite and aphid susceptible (Gerhold et al., 1984; Stark, 1975; Walters et al., 1989b, 1990a). These two plant lines were used throughout the study, as chemical analysis of the anacardic acids produced by a number of resistant and susceptible plants showed that the anacardic acid profile was constant within each plant line (Walters et al., 1988, 1990c; Hesk et al., 1990).

Fatty Acid Incubation Experiments. The methyl esters of [1-^{14}C]palmitate, [1-^{14}C]margarate, [1-^{14}C]stearate, [10-^{14}C]oleate, and [1-^{14}C]linoleate were applied in turn to the sepals of the resistant and susceptible plants in a hexane solution. The incubation was performed on floral tissue, because earlier work had shown that there was a high density of glandular trichomes, and hence a high production of anacardic acids in these tissues (Walters et al., 1989b, 1990a). The labeled compound (2–4 μCi) was dissolved in a small volume of hexane (5–20 μl) and applied in 0.5- to 2-μl batches by a syringe to the surface of each unopened flower (sepal), which usually led to 10–12 flowers being treated. A small volume was used to minimize tissue damage by the solvent. In the early stages of the study, 2 μl of hexane solution was applied to the sepals of each flower, but it was subsequently found that the tissue damage could be further

reduced and the radiochemical incorporation greatly improved by applying only 0.5 μl of hexane solution. Hence this procedure was used for the remainder of the study.

The labeled acids were administered in two doses, half at the beginning of the experiment, and the second three days later. Each incubation was allowed to proceed for 7–10 days. At the conclusion of the incubation, the inflorescences (minus the petals) were soaked in methylene chloride for 1 hr. The extract was then dried over anhydrous sodium sulfate, filtered, and evaporated to leave the crude ^{14}C anacardic acid residue. This procedure had previously been shown to produce a representative extract of anacardic acids (Hesk et al., 1990; Gerhold et al., 1984; Walters et al., 1989a).

Amino Acid Incubation Experiments. Five microcuries of ^{14}C-labeled isoleucine, leucine, and valine were pipetted into 1-ml microvials, and freshly cut third node leaves and flowerheads were placed into the vials. On absorption of the amino acid solutions into the stems of the leaves and flowers, water was added in sufficient quantity to ensure that the leaf or flower stem remained in water. After the incubations had been run for one week, the leaves and flowers were removed from the vials, and the anacardic acids were extracted as described above.

TLC Analysis. The ^{14}C-labeled extracts were analyzed by TLC autoradiography to determine the extent of incorporation of the fatty acid precursor into anacardic acids and also to partially purify the anacardic acids prior to radio HPLC analysis. The carboxyl group in the anacardic acids was methylated (diazomethane-diethyl ether, 5-min treatment) (Walters et al., 1988) prior to TLC. Merck 20 × 20-cm silica gel F254 plates with a preconcentration zone were used, and the plates were developed in a two-step solvent system consisting of benzene–diethyl ether–ethanol–acetic acid (50:40:1:0.5, by volume) followed by hexane–diethyl ether–acetic acid (84:15:1, by volume). Development was allowed to proceed to about half the length of the plate in the first step and the entire length of the plate in the second. After the plates were dried, the plates were exposed to X-ray film for 1–10 days, depending on the radioactivity of the material. The monomethylated anacardic acid band was located by viewing at 254 nm and comparing the R_f (approximately 0.65) value with that of a monomethyl anacardic standard, before it was scraped from the plate and eluted with methylene chloride (3 × 2 ml). The methylene chloride extract was filtered through a Pasteur pipet containing a glass wool plug, and then evaporated to leave the anacardic acid extract. Finally the phenolic group on the anacardic acids was methylated (diazomethane-diethyl ether plus a few drops of methanol, 30-min treatment) (Walters et al., 1988) to give the dimethylated anacardic acids.

HPLC Analysis. The anacardic acids were analyzed by reverse-phase HPLC to determine those acids into which the fatty acid precursor had been incorpo-

rated. A 25-cm × 4.6-mm Supelcosil 5 μ LC8 DB column was used with a solvent system of isopropanol, acetonitrile, and 0.01 M acetic acid (490 : 147 : 343) run isocratically at 1 ml/min. The radioactive peaks were detected using a Packard Tricarb RAM 7500 radioactive monitor, with a solid fluor packed flow cell. The anacardic acids were also monitored at 212 nm using a Waters Lambda Max 480 UV detector.

In the case of the ^{14}C anacardic acids recovered from the ^{14}C amino acid incubations, the recoveries were too low to be quantified with the radioflow monitor. Hence, for each amino acid incubation, the eluent from the entire HPLC run was collected into scintillation vials, blended with 15 ml of liquid scintillator, and counted in a Beckman LS8000 counter to determine the distribution of the radioactivity in the anacardic acid profile.

These ^{14}C data are presented as analysis of pooled sample extracts from 10 to 12 separately treated flower heads.

RESULTS AND DISCUSSION

The results for the incubation experiments in the susceptible and resistant plants are given in Tables 1–4. Because of the improved HPLC technique and structural elucidation studies (Hesk et al., 1990), 19 anacardic acids were analyzed for the relative ^{14}C incorporation, as compared to only six anacardic acids in the previous biosynthesis experiments with resistant geraniums (Walters et al., 1990b). Approximately 10–25% of the administered dose was recovered in the methylene chloride extract and of that, 10–20% was found in anacardic acids, giving an overall incorporation of between 1 and 2%. This compares favorably with the earlier biosynthesis experiment (Walters et al., 1990b).

In the case of the amino acid incubations, the overall incorporation into anacardic acids was much lower, with only 0.05–0.6% of the administered dose recovered in the methylene chloride extract. This lower incorporation rate is presumably due to the amino acids principally being used in other biosynthetic pathways.

Palmitate Incubation. The results showed that [^{14}C]palmitate was primarily incorporated into the C_{22} saturated anacardic acid by the proposed direct chain elongation mechanism, approximately 70% in both plants. The remaining 30% in the resistant plant was represented by C_{22} $\omega 5$, C_{24} $\omega 5$, and C_{24} saturated anacardic acids. The two unsaturated acids are presumably derived from β-oxidation of the palmitate into [^{14}C]acetate units, which are then reincorporated into the ^{14}C anacardic acids and appear in a similar relative proportion to that seen in the UV profile. As the ^{14}C C_{24} saturated anacardic acid is present in larger ^{14}C relative amounts than would be expected from the β-oxidation mechanism (UV concentration), it is likely that a portion of the added

TABLE 1. ANACARDIC ACID BIOSYNTHESIS FROM INCUBATION OF ^{14}C FATTY ACIDS IN RESISTANT GERANIUMS

Name	Relative UV Areas (%)[a]	Radioactivity (%) from ^{14}C fatty acids				
		Palmitate	Margarate	Stearate	Oleate	Linoleate
C_{20} sat.	0.4					
C_{22} ω5	30.6	8.1	7.5	15.9	28.7	22.9
C_{21} anteiso	0.6					
C_{24} ω6,9	1.0				28.5	**47.0**
C_{23} ω6	3.4					2.6
C_{22} iso	0.5					
C_{22} sat.	5.2	**67.1**[b]	5.4		6.1	6.0
C_{24} ω9	0				**7.3**	5.2
C_{24} ω5	49.8	12.3	10.6	26.2	27.1	16.2
C_{23} anteiso	2.0					
C_{23} sat.	0.7		**76.4**			
C_{25} ω6	0.5					
C_{24} iso	0.4					
C_{24} sat.	2.6	12.5		**57.1**		
C_{26} ω5	1.5					
C_{25} antesio	0.9					
C_{25} sat	0					
nCi isolated		44	31	21	68	73

[a]Represents relative anacardic acids found in plant extracts.
[b]Boldface type indicates product expected from direct incorporation pathway.

[^{14}C]palmitate has undergone chain elongation to [^{14}C]stearate. This elongation mechanism is well recognized in plants (Stumpf, 1987). We propose that normally the palmitate undergoes a Δ11 desaturation followed by elongation to form ω5 anacardic acids; however, when we add [^{14}C]palmitate or any other acid externally, it bypasses the normal desaturation step in the resistant plant and gets directly incorporated, in part, into anacardic acids. It is conceivable that the plant has a sufficient pool of C_{16} ω5 fatty acid and, thus, does not need to form any more ω5 desaturated fatty acids.

Similarly, in the susceptible plant, the two minor radiolabeled peaks, the C_{24} ω6,9 and the C_{24} saturated acid, are present in lower relative amounts than that observed in the UV profile. The C_{24} saturated anacardic acid can be formed by chain elongation of palmitate to stearate, and the C_{24} ω6,9 from the β-oxidation of [^{14}C]palmitate to [^{14}C]acetate and reincorporation of the $^{14}CO_2$ mechanism. There is also the possibility that the C_{24} ω6,9 is formed from the Δ9,Δ12 desaturation of any stearate that is formed from elongation of the pal-

TABLE 2. ANACARDIC ACID BIOSYNTHESIS FROM INCUBATION OF ^{14}C FATTY ACIDS IN SUSCEPTIBLE GERANIUMS

Name	Relative UV Areas (%)[a]	Radioactivity (%) from ^{14}C fatty acids				
		Palmitate	Margarate	Stearate	Oleate	Linoleate
C_{20} sat.	3.3					
C_{22} ω5	0.5					
C_{21} anteiso	0.8					
C_{24} ω6,9	16.6	5.9	2.4	5.3	64.9	**89.8**
C_{23} ω6	0.4					
C_{22} iso	1.0					
C_{22} sat.	23.9	**70.0**[b]	3.7	4.4	7.6	3.9
C_{24} ω9	7.9				**20.6**	2.2
C_{24} ω5	0.1					
C_{23} anteiso	7.5					1.9
C_{23} sat.	5.6		**87.0**			
C_{25} ω6	0					
C_{24} iso	1.8					
C_{24} sat.	27.3	24.1	1.6	**90.3**	7.5	2.2
C_{26} ω5	0					
C_{25} anteiso	2.4					
C_{25} sat.	1.0		5.2			
nCi isolated		29	71	15	45	60

[a] Represents relative anacardic acids found in plant extracts.
[b] Boldface type indicates product expected from direct incorporation pathway.

mitate. However, in view of the fact that this does not happen in the stearate incubation, which is described later, this possibility can be discounted.

Figure 2 illustrates the various pathways of metabolism operating for palmitate, with the unbroken arrows showing the major pathway and the broken arrows showing minor routes of metabolism. The same pathways of degradation and biosynthesis are functioning for all the incubated fatty acids.

Margarate Incubation. [^{14}C]Margarate was incorporated in a straightforward manner in both the resistant and susceptible plants, with 76% in the resistant and 87% in the susceptible being processed by the direct elongation synthesis into the C_{23} saturated anacardic acid. As in the case of the palmitate experiment, the radio HPLC trace from the resistant plant showed a small amount of degradation of the [^{14}C]margarate had occurred, resulting in the minor peaks corresponding to C_{22} ω5, C_{24} ω5, and the C_{22} acids. In the susceptible plant, there is evidence for an even smaller amount of β-oxidation and reincorporation, as indicated by the very small amounts of the C_{24} ω6,9 and C_{22} and C_{24} acids showing up in the radio HPLC trace. The C_{25} anacardic acid, by analogy with

TABLE 3. ANACARDIC ACID BIOSYNTHESIS FROM INCUBATION OF ^{14}C AMINO ACIDS IN RESISTANT GERANIUMS

		Radioactivity (%) from ^{14}C amino acids					
		Isoleucine		Valine		Leucine	
Name	UV areas[a]	Flower	Leaf	Flower	Leaf	Flower	Leaf
C_{20} sat	0.4	0	0	1.6	2.3	0	4.1
C_{22} ω5	30.6	19.9	11.4	19.7	12.1	36.4	24.2
C_{21} anteiso	0.6	**6.5**[b]	**8.8**	0.5	0.6	0.4	1.5
C_{24} ω6,9	1.0	1.5	1.0	1.2	1.1	0.3	4.1
C_{23} ω6	3.4	10.2	12.7	4.0	5.1	3.4	3.5
C_{22} iso	0.5	0.8	0	**26.0**	**24.8**	1.4	1.0
C_{22} sat	5.2	3.5	1.5	8.5	14.7	4.2	9.2
C_{24} ω9	0	0	0	0	0	0	0
C_{24} ω5	49.8	33.3	17.5	26.5	19.6	46.9	44.1
C_{23} anteiso	2.0	**15.2**	**35.0**	1.2	0.9	2.0	1.0
C_{23} iso	0	0	0	0	0	**0.1**	**1.7**
C_{23} sat	0.7	1.0	0	1.0	0.7	0.5	1.0
C_{25} ω6	0.5	1.8	3.1	0.9	0.6	0.7	1.2
C_{24} iso	0.4	0	0	**8.1**	**16.3**	0.5	0
C_{24} sat	2.6	1.0	0	0.8	0.7	1.3	3.1
C_{26} ω5	1.5	0.8	0	0	0	1.0	0
C_{25} anteiso	0.9	**4.2**	**9.0**	0	0	0.8	0
C_{25} iso	0	0	0	0	0	0	0
C_{25} sat	0	0	0	0	0	0	0
nCi isolated		10.2	8	7.2	1.4	11.0	0.6

[a] Represents relative anacardic acids found in plant extract.
[b] Boldface type indicates expected branch chain product.

the palmitate experiments, very likely arises from chain elongation of [^{14}C]margarate to [^{14}C]nonadecylate (C19:0), prior to anacardic acid biosynthesis.

Stearate Incubation. This experiment gave an interesting contrast between the resistant and susceptible plants. In the resistant plant, only 57% of the [^{14}C]stearate was incorporated into C_{24} anacardic acid via the direct pathway. The remaining peaks in the radio HPLC trace were the C_{22} ω5 and C_{24} ω5 acids, formed from β-oxidation of [^{14}C]stearate (similar to UV profile). In contrast, in the susceptible plant over 90% of the ^{14}C label had been incorporated via the direct elongation mechanism into the saturated C_{24} anacardic acid. Only a small amount of the β-oxidation was operating as evidenced by the minor peaks corresponding to the C_{24} ω6,9 and C_{22} acids.

Oleate Incubation. The radiolabeled products from the [^{14}C]oleate incu-

TABLE 4. ANACARDIC ACID BIOSYNTHESIS FROM INCUBATION OF ^{14}C AMINO ACIDS IN SUSCEPTIBLE GERANIUMS

		Radioactivity (%) of ^{14}C amino acids					
		Isoleucine		Valine		Leucine	
Name	Relative UV areas (%)[a]	Flower	Leaf	Flower	Leaf	Flower	Leaf
C_{20} sat	3.3	1.0		4.1		4.6	
C_{22} ω5	0.5	0		0		0	
C_{21} anteiso	0.8	**9.0**		1.4		1.0	
C_{24} ω6,9	16.6	6.0		6.5		11.9	
C_{23} ω6	0.4	1.0		1.1		1.0	
C_{22} iso	1.0	1.3		**20.2**		4.9	
C_{22} sat	23.9	16.9		16.8		33.9	
C_{24} ω9	7.9	1.3		1.3		5.9	
C_{24} ω5	0	0		0		0	
C_{23} anteiso	7.5	**37.0**[b]		4.0		6.7	
C_{23} iso	0	0		0		0	
C_{23} sat	5.6	4.8		5.6		6.3	
C_{25} ω6	0	0		0		0	
C_{24} iso	1.8	1.7		**25.2**		1.2	
C_{24} sat	27.3	13.3		11.4		19.3	
C_{26} ω5	0	0		0		0	
C_{25} anteiso	2.4	**5.4**		1.0		0	
C_{25} iso	0	0		0		**1.7**	
C_{25} sat	1.0			1.0		1.2	
nCi isolated		1.3	0	1.4	0	1.2	0

[a] Represents relative anacardic acids found in plant extracts.
[b] Boldface type indicates expected branch chain product.

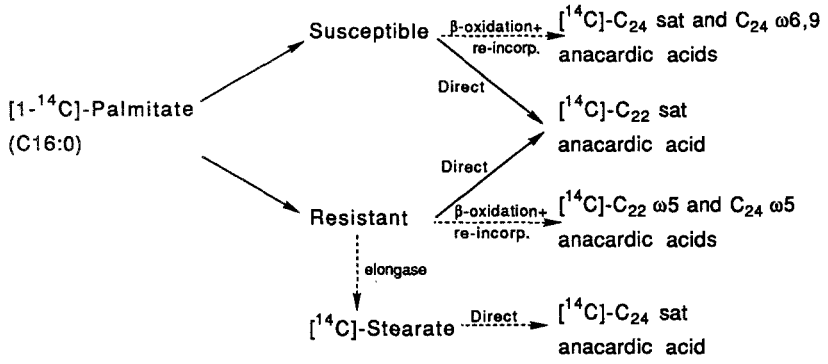

FIG. 2. Metabolism of palmitate in resistant and susceptible geraniums.

bation in the resistant plant showed a complicated pattern. The major peaks, which all contained about 28% of the label, corresponded to the C_{22} $\omega5$, C_{24} $\omega5$, and C_{24} $\omega6,9$ acids. In addition, there were small amounts of C_{22} and C_{24} $\omega9$ acids, the latter arising from the direct elongation of the oleate. The $\omega5$ and C_{22} saturated anacardic acids are readily accountable from the indirect synthetic pathway, involving β-oxidation. It should be noted that the label in the [^{14}C]oleate was in the 10 position, rather than in the carboxyl group, as was the case of the other radiolabeled fatty acids. The fact that a majority of the ^{14}C label was found in the C_{22} $\omega5$, C_{24} $\omega5$, and C_{22} anacardic acids, suggests that degradation of the [^{14}C]oleate proceeds along most of the length of the chain. The high amount of ^{14}C C_{24} $\omega6,9$ found in the resistant plant incubations is unusual, for the $\omega6,9$ is not normally found in resistant plants. Its biosynthesis does not fit either proposed pathway. Alternately, we propose the [^{14}C]oleate is, in part, desaturated to [^{14}C]linoleate and then processed to the C_{24} $\omega6,9$ unsaturated anacardic acid via the direct method. A $\Delta12$ desaturase is well known in plants (Jaworski, 1987).

The conversion of [^{14}C]oleate into C_{24} $\omega6,9$ is a major product (64.9%) in the susceptible plant. The UV trace shows it only represents 16.6% in the normal exudate. About 20% of [^{14}C]oleate is processed by the direct synthesis pathway into the C_{24} $\omega9$ anacardic acid. The remainder of the ^{14}C activity is located in the saturated C_{22} and C_{24} acids, which are presumably derived by the β-oxidation pathway or reduction of the oleate to sterate and subsequent incorporation. However, it should be noted that the degradation pathway of oleate is much less important in the susceptible plant when compared to the resistant plant.

Linoleate Incubation. The incubation of [^{14}C]linoleate in the resistant plant, like the incubation of [^{14}C]oleate, produced a complicated profile. Nearly half of the label was found in the C_{24} $\omega6,9$ peak and presumably formed via the direct elongation pathway. The remaining peaks in the radiochromatogram, the C_{22} $\omega5$, C_{24} $\omega5$, and saturated C_{22} anacardic acids, can be readily explained from the β-oxidation of the [^{14}C]linoleate to [^{14}C]acetate units followed by their reincorporation into the anacardic acids (consistant with UV profile). In the susceptible plant, the linoleate incubation was much more straightforward, with only about 10% of the [^{14}C]linoleate found incorporated into anacardic acids via the indirect pathway. The vast majority was processed by the direct elongation into the C_{24} $\omega6,9$.

L-Isoleucine Incubation. [^{14}C]Isoleucine was incorporated into ^{14}C anacardic acids in both resistant and susceptible flowers, and also in resistant leaves, although the amount of labeled anacardic acids from susceptible flowers was only about a tenth of that recovered from the resistant flower incubation. No labeled anacardic acids could be detected in the susceptible leaf incubation. It is possible that the lower rate of anacardic acid biosynthesis seen in the leaves compared to the flowers may be a reflection on the much lower number of

glandular trichomes seen on the susceptible leaves, or that the leaves are more completely utilizing the amino acid in biosynthesis of other biomolecules.

Looking first at the resistant plant incubations (Table 3), it can be seen that major labeled peaks were the C_{22} $\omega5$, C_{24} $\omega5$, and C_{23} $\omega6$ unsaturated anacardic acids and the C_{21}, C_{23}, and C_{25} anteiso compounds. The C_{22} $\omega5$ and C_{24} $\omega5$ are readily explainable by considering that it is well documented that isoleucine is degraded to succinyl CoA via the succinate pathway (Lehninger, 1975). Succinyl CoA is subsequently converted to acetyl-CoA via the Krebs cycle. In addition, the degradation of α-methylacetoacetyl-CoA gives rise to acetyl-CoA and propionyl-CoA. Thus, [^{14}C]isoleucine is degraded to [^{14}C]acetyl-CoA, and hence is incorporated into [^{14}C]anacardic acids at potentially each longation step. It would be expected that, if this mechanism was operating, the relative amounts of the radiolabeled C_{22} $\omega5$ and C_{24} $\omega5$ anacardic acids should closely match the anacardic acid UV profile as is found.

The propionyl-CoA formed during the degradation of isoleucine can give rise to C_{17} fatty acid and explains the relatively high incorporation of label into the C_{23} $\omega6$ anacardic acid, and to a lesser degree as the C_{25} $\omega6$ acids. The high degree of ^{14}C incorporation found in the anteiso branched chain anacardic acids, particularly the C_{23} anteiso acid, is readily explained by considering that α-methylbutyryl-CoA is a precursor (Lehninger, 1975). Despite the lower rates of incorporation of [^{14}C]isoleucine in the susceptible plant, the overall picture was much the same as in the resistant plant, with ^{14}C C_{22}, C_{23}, C_{24} saturated and C_{24} $\omega6,9$ peaks (Table 4) occurring in a similar proportion to that of the UV trace. Similar to the resistant plant experiment, much of the label was present in the C_{21}, C_{23}, and C_{25} anteiso peaks. As expected the susceptible plant did not form any $\omega5$ or $\omega6$ acids.

L-Valine Incubations. The valine incubation showed a similar pattern to that of isoleucine. Thus the overall incorporation was higher in the resistant experiments, and flowers gave a higher amount of labeled anacardic acids than leaves. In a similar manner to isoleucine, no detectable incorporation of valine into anacardic acid was seen in the susceptible leaf.

The pattern of labeled anacardic acids in the resistant leaves and flowers showed that the basic mechanisms described in detail for isoleucine were operating. Hence, the C_{22} $\omega5$, C_{24} $\omega5$, and C_{22} saturated anacardic acids arise from degradation of [^{14}C]valine to [^{14}C]succinate and ultimately [^{14}C]acetyl-CoA. The high amounts of activity in the two iso peaks, particularly the C_{22} iso, is explained from the formation of [^{14}C]isobutyryl-CoA via the degradation of [^{14}C]valine in the succinate pathway. In the susceptible flower, valine was incorporated into the iso anacardic acids. The C_{22} and C_{24} saturated and C_{24} $\omega6,9$ anacardic acids were derived as above from the formation of [^{14}C]acetyl-CoA. Again, no labeled $\omega5$ or $\omega6$ anacardic acids were seen in the susceptible plant.

L-Leucine Incubations. With leucine incubations, the resistant plants gave

higher recoveries of radiolabeled anacardic acids than that observed with susceptible plants, and the flowers gave much better incorporations than leaves. As was found in the other two amino acid incubations, no labeled anacardic acids were recovered from susceptible leaves. The profile of anacardic acids in both resistant and susceptible plants closely matched the profile in the UV trace, suggesting that virtually all the [^{14}C]leucine was degraded to [^{14}C]acetyl-CoA and subsequently reincorporated into fatty acids.

It is interesting to note that during the degradation of leucine, a branched chain CoA intermediate, isovaleryl-CoA is formed. If this were to be used in fatty acid biosynthesis, as the intermediates isobutyryl-CoA and α-methylbutyryl-CoA were, iso branched chained fatty acids with an odd-numbered chain length would result, and hence anacardic acids with an odd number of carbons and an iso branched product would be formed. The iso branched chain anacardic acids have not been found in the glandular trichome exudate from either resistant or susceptible plants, which is consistent with the fact that iso branched fatty acids with an odd chain length are not found to any great extent in nature (Baker and Holloway, 1975; Radunz, 1987).

Relevance of ω6 Anacardic Acids in Resistant Plants. The finding of C_{23} ω6 and C_{25} ω6 unsaturated anacardic acids provided a new insight into how the desaturation to form the C_{22} ω5 and C_{24} ω5 anacardic acids takes place (Hesk et al., 1990). It had been previously proposed that palmitate and stearate were desaturated via a ω5 desaturase, which counts in from the methyl end of the chain, to form the ω5 C_{16} and ω5 C_{18} unsaturated fatty acids (Walters, 1988; Walters et al., 1990b). These compounds were then thought to be selected preferentially from all the other fatty acids in the pool and synthesized into the corresponding C_{22} ω5 and C_{24} ω5 acids via anacardic acid synthetase, thus accounting for their high predominance in the exudate from resistant plants.

The mechanism for how ω6 unsaturation arises can be explained by considering that instead of the ω5 desaturase counting in from the methyl end of the chain, the desaturase counts in from the carboxyl end of the fatty acid. Hence it would be a Δ11 desaturase, rather than an ω5 desaturase. This mechanism has the attraction that, in the desaturation of the C_{17} fatty acid, the Δ11 enzyme would give rise to a double bond between carbns ω6 and ω7, thus giving rise to a C_{17} ω6 fatty acid and ultimately the C_{23} ω6 unsaturated anacardic acid. The C_{25} ω6 unsaturated anacardic acid could be formed by elongation of the C_{17} ω6 fatty acid to the C_{19} ω6, by analogy with the elongation of palmitate to stearate (Stumpf, 1987), followed by anacardic acid synthesis. Similarly the C_{24} ω5 unsaturated anacardic acid is likely synthesized by elongation of the C_{16} ω5 fatty acid, formed from the Δ11 desaturase, to the C_{18} ω5 unsaturated fatty acid, followed by anacardic acid synthesis.

Support for this idea can be seen from the work of Bjostad et al. (1987), where a Δ11 desaturase has been observed in insects during biosynthesis of sex

pheromones in *Trichoplusia ni*. Incubations with [^{14}C]acetate gave rise to significant quantities of a ^{14}C-labeled C_{16} ω5 fatty acid, along with smaller amounts of labeled C_{18} ω5, C_{14} ω5, and C_{12} ω5 analogs. A further experiment using [16-^{3}H]C_{16} ω5 was shown to produce a significant amount of the labeled C_{14} ω5 fatty acid. It was thus concluded that the C_{14} ω5 and also the observed C_{12} ω5 fatty acids were derived from the chain shortening of the C_{16} ω5 and not from desaturation of the C_{12} and C_{14} saturated analogs. Similarly, it was proposed that the C_{18} ω5 derived from chain elongation of the C_{16} ω5, rather than desaturation of the C_{18} saturated fatty acid. These data would be consistent and analogous with our proposed Δ11 desaturase in geraniums.

Resistant and Susceptible Differences. From the experimental data presented, it is clear that the two plant lines metabolize the fatty acid and amino acid precursors in a similar manner, but with some important differences. Both plant lines seem to be able to accept whatever precursor ^{14}C fatty acid and amino acid they are given and incorporate them into anacardic acids. However, resistant plants more readily β-oxidize and reincorporate the acetate of the applied fatty acids, as compared with the susceptible plant. For example, in the incubation of stearate, over 90% was converted directly to the C_{24} saturated anacardic acid in the susceptible plant, whereas in the resistant plant, nearly half of the applied ^{14}C label was degraded and reincorporated into the ω5 acids. It

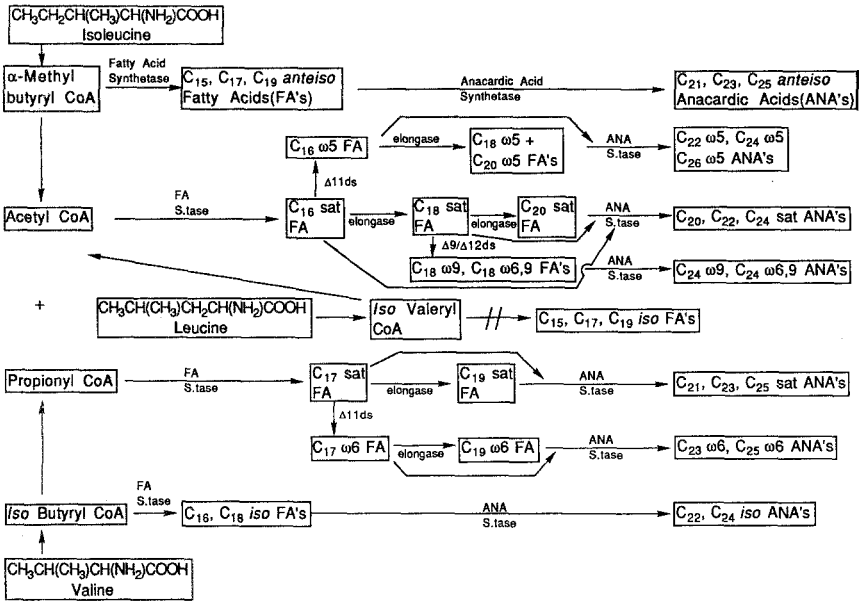

FIG. 3. Biosynthesis of anacardic acids from amino acid precursors.

is possible the β-oxidation and reincorporation is used by the resistant plant to convert non-ω5 fatty acids into ω5 anacardic acids via this indirect route. These biosynthesis data do support the earlier findings that ω5 anacardic acids are not found in susceptible plants. Hence, in the susceptible plant it is possible that the Δ11 desaturase enzyme is absent, or marginally active, and may be the potential genetic difference between the resistant and susceptible plants. It is clear that both susceptible and resistant plants similarly synthesize anacardic acids when presented with a common substrate fatty acid; however, only the resistant plant possessed the ω5 fatty acids, which are preferently incorporated into anacardic acids. Collectively, these data have resulted in a revision of the biosynthesis scheme for anacardic acids as presented in Figure 3. This scheme considers the biosynthetic differences between the resistant and susceptible plants, the presence of a Δ11 desaturase, the significant chain elongation step, and the formation of iso and anteiso anacardic acids.

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CHESTNUT BARK TANNIN ASSAYS AND GROWTH OF CHESTNUT BLIGHT FUNGUS ON EXTRACTED TANNIN

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Abstract—Tannins extracted from the green bark of each of two Chinese, Japanese, and American chestnut trees were assayed in a protein-binding test. Four levels of tannins were added to a buffered, minimal growth medium, and a standard, virulent strain of the chestnut blight fungus was grown. There were only slight differences in protein binding between the extracts from different species. Fungal growth was better with tannin than without, but there was no difference between species extracts in their ability to improve fungal growth rate. There was also no inhibition of blight fungus growth by any of the tree tannins, so tannin toxicity is not the reason for Asian chestnut tree resistance.

Key Words—chestnut, *Castanea*, tannin, chestnut blight, *Cryphonectria parasitica, fungus.*

INTRODUCTION

Tannins and their phenolic breakdown products have been implicated in the resistance of plants to their pathogens (Cruickshank and Perrin, 1964). When the chestnut blight epidemic began in the United States in 1904 (Anagnostakis, 1987), explanations were sought for the resistance of Asian chestnut trees to this fungus disease. Chestnut trees have high levels of tannin in their bark and wood, and these were implicated by early studies (Cook and Wilson, 1916; Graves, 1926) in Asian tree resistance. Attempts have been made to prove this hypothesis (Nienstaedt, 1953; Hebard and Kaufman, 1978, J.R. Elkins, unpublished), but no conclusive data have been published. In reviewing the subject,

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Roane et al. (1986) suggested that different tannin forms in different species, or different breakdown products might explain Asian tree resistance.

To further pursue the question, I have used Hagerman's (1987) protein-binding assay for the amount of total tannin (condensed and hydrolyzable) present in extracts. This was described by Hagerman and Butler (1989) as suitable for determining the potential biological activity of the tannin in a sample. Extracted tannins were mixed with a buffered growth medium, so that pH would not be a factor in affecting the blight fungus, and fungal growth rate was used as an index of effect.

METHODS AND MATERIALS

Assay Method. Radial diffusion plates were prepared as described by Hagerman (1987). A 1% agarose solution (w/v, SeaKem GTG, from FMC) was mixed and melted in a pH 5.0 buffer of 50 mM acetic acid and 60 μM ascorbic acid. When this solution had cooled to 45°C, bovine serum albumin (Sigma) was added (0.1% w/v). Aliquots of 9.5 ml were dispensed into sterile plastic Petri dishes (''100 × 15'' actually 87 × 13 mm) and allowed to cool overnight.

Uniform wells were punched in the agarose with a 4-mm-diameter cork borer. Solutions were added to the wells, and the plates were incubated at 30°C for 120 hr. The diameters of the resultant rings were measured to the nearest millimeter diameter, and when faint diffusion was evident beyond the ring only the dense white ring was measured. Baker Analyzed Tannic Acid was always included as a control.

Tannin standards were all dissolved in 70% acetone. Used were Baker Analyzed Tannic Acid, Mallinckrodt AR Tannic Acid, and quebracho (condensed) tannin purified as described by Asquith and Butler (1985) and modified by Hagerman (personal communication). The crude quebracho tannin was a gift of A.E. Hagerman.

Bark Extraction. The chestnut trees that were used for bark samples are growing at our Experiment Station Farm and our Chestnut Plantation, both in Hamden, Connecticut, and have been used in inoculation tests to compare their resistance to chestnut blight (Anagnostakis, submitted to *Can. J. Forest Res.*). Branches cut were all growing rapidly in an orchard setting (not suppressed). The two Americans [*Castanea dentata* (Marsh.) Borkh.] were R1T18 and Scientists Cliff, the two Chinese (*C. mollissima* Bl.) were R1T15 Mahogany and R9T3 Tiger Paw, and the two Japanese (*C. crenata* Sie. Zuc.) were R7T7 (an orchard type) and R34T6 (a forest type). Stems were harvested on August 9, 1990, and February 26, March 4, April 16, and June 20, 1991. Some stems were cut and stored in plastic bags in a freezer for later use.

Hagerman (1988) has shown that plant tannins are most efficiently extracted using a solution of 70% acetone. Chestnut stems with green bark (no dead bark tissue) were cut into 5-cm lengths and pieces with four or five growth rings were used. Bark was cut in strips about 3 mm wide and placed immediately into 70% acetone. Two grams of bark were extracted in 20 ml in a beaker sealed with parafilm ("M," American Can Co.) for 1 hr at room temperature. The solution was then decanted into another beaker and another 2 g of bark added and extracted for 1 hr. The extracts were filtered (Whatman No. 1 paper) and stored in tightly capped scintilation vials in a freezer.

Extract levels were calculated as "protein precipitation units," which were in square millimeters and were the squared diameters of the circles measured in the radial diffusion tests of the extracts. This number was corrected by subtracting the squared diameter of the wells, and means of replicates and the total standard error of the means calculated.

Fungal Growth Experiments. The tannin levels encountered by the fungus are grams of tannin per gram live weight of bark. Therefore, for each experiment, the number of grams of tannin per gram (fresh weight) of bark was calculated by comparing the sizes of the precipitation rings of the bark extract to the sizes of the rings formed by Baker Tannic Acid solution. The milligrams of tannin used per milliliter of agar medium ranged from 0.33 to 21 in the four experiments, with levels within each experiment about the same for the extracts used. The highest level used was equivalent to the level in fresh bark; i.e., all the extract from 1 g (fresh weight) of bark in each gram of agar medium. The second highest level was equivalent to two to five times the amount of tannin (1 mg/ml) used as a supplement in media (Anagnostakis, 1982). The other experiments used about 1 mg/ml and about half that amount. Baker (hydrolyzable) tannin, Mallinckrodt (hydrolyzable) tannin and quebracho (condensed) tannin were also used in media to test their effect on fungal growth.

Extracts and tannin standards in 70% acetone, and 70% acetone as a control, were placed in sterile 125-ml beakers and the liquid evaporated in a stream of sterile air in a laminar flow hood. Double-strength minimal medium (Puhalla and Anagnostakis, 1971) buffered with 1.0 M MES [2-(*N*-morpholino)-ethanesulfonic acid, Sigma] was added to the beakers, mixed to dissolve the dried, extracted materials, and the pH was adjusted to 6.0. These media were filter-sterilized (Nalgene disposable filters). Noble agar (Difco) was mixed as 4% in demineralized water in a separate solution and autoclaved at 18 lb of steam pressure for 50 min. The media and cooled agar solutions were mixed in equal amounts and poured into sterile plastic Petri dishes.

Rapidly growing cultures of *Cryphonectria parasitica* (Murr.) Barr strain 155 (ATCC No. 38755) on potato dextrose agar (PDA, Difco) were used for inoculum. Pieces were cut from the margin of cultures less than 7 days old with a 4 -mm - diameter cork borer and placed in the center of the media to be tested.

Cultures were grown at 28°C in 12 hr/day white fluorescent light. Colony diameters were measured to the nearest millimeter daily, and expansion per day (the slope of a linear regression line ± the standard error of the slope) was used to express growth rate (Anagnostakis and Aylor, 1984).

RESULTS

The precipitation circles formed by the control tannins were not the same in all experiments (Table 1). This is probably due to slight variations in the amount of tannin in the standard solutions or to oxidation of the tannins in solutions kept from one experiment to another. Within experiments, the results were quite consistent for control tannins and for bark extracts. A linear regression of square millimeters versus milligrams of tannin using all of the results from Baker Tannic Acid reported in Table 1 (total of 48 tests) resulted in an R^2 of 0.91, suggesting that even though there is a fourfold difference in PPT units per milligram in the six experiments, the results are comparable. The calculated total error factors for combined data (column "All" in Table 1) are low.

Amounts of tannin recovered from bark of the same trees harvested in late February, early March, mid-April, late June, and mid-August ranged from a high of 0.03 mg tannin per mg bark (August) to a low of 0.01 (June). This may have been due to the spring bark having more moisture, and thus 4 g of bark appeared to have less tannin. Samples harvested and extracted throughout the year and tannin levels compared to dry bark weights would answer this question. However, in two experiments the extracted bark was dried and weighed for comparison. Table 2 lists the amount of tannin calculated for the bark harvested in August, late February, or early March, and late June (calculation based on the PPT units of the Baker Tannic Acid in each test). With the exception of the August bark from American tree R1T18, differences are small. More tests of this are certainly needed.

Fungal growth rates in four experiments are listed in Table 3. There were no differences between fungal growth rates with three orders of magnitude difference in extract amount used. The fungus grew a little better with bark extract than with commercial tannins in two of three experiments and better with tannin than without in two of four experiments (statistics in Table 3). There was no indication of inhibition due to tannins from any of the trees, even at levels assumed to be like those in fresh bark. The small amount of purified quebracho tannin available was used in one experiment, and 0.01 mg/ml, two orders of magnitude less than the amount of tannic acid used to supplement media, supported good growth.

TABLE 1. PROTEIN PRECIPITATED (mm^2)[a] PER MILLIGRAM OF PURE TANNIC ACID AND PER MILLIGRAM (FRESH WEIGHT) OF EXTRACTED CHESTNUT BARK

Source	Test Dates						All
	25 February	6 March	8 March	16 April	26 June	5 July	
Controls							
BakTan[b]	490 ± 12	560 ± 19	530 ± 28	600 ± 39	2020 ± 64	960 ± 92	670 ± 60
MalTan[c]			530 ± 32		1800 ± 69	960 ± 58	870 ± 130
Quebracho	220 ± 7						220 ± 7
Chinese							
R1T15	13 ± 2	11 ± 1	9 ± 2	7.8 ± 1	26 ± 2	17 ± 3	12 ± 2
R9T3	17 ± 2	11 ± 2	12 ± 2		28 ± 3	22 ± 2	17 ± 2
American							
R1T18	8.1 ± 1	7.4 ± 1		5.6 ± 1	19 ± 2	16 ± 2	9.7 ± 2
SciCliff[d]	11 ± 2	6.7 ± 2			22 ± 2	16 ± 2	14 ± 2
Japanese							
R7T7	16 ± 2	13 ± 2			22 ± 2	22 ± 2	18 ± 2
R34T6	15 ± 2				22 ± 2	20 ± 2	19 ± 2

[a] Mean diameter squared (mm) minus the squared diameter of the well ± the total standard error of the mean (see text).
[b] Baker Analyzed Tannic Acid.
[c] Mallinckrodt AR Tannic Acid.
[d] Scientists Cliff.

TABLE 2. AMOUNTS OF TANNIN IN CHESTNUT BARK AS GRAMS OF TANNIN PER GRAM OF FRESH BARK AND PER GRAM OF DRY BARK

	Test Date			
	6 March		5 July	
Source	Fresh	Dry	Fresh	Dry
Chinese				
R1T15AM[a]	0.027	0.061		
R1T15FM[b]	0.018	0.036		
R1T15JJ[c]			0.016	0.040
R9T3AM	0.027	0.067		
R9T3FM	0.018	0.038		
R9T3JJ			0.021	0.058
American				
R1T18AM	0.009	0.024		
R1T18FM	0.018	0.035		
R1T18JJ			0.016	0.034
SciCliff			0.016	0.041
Japanese				
R7T7			0.021	0.050
R34T6			0.021	0.057

[a] Bark harvested in August and extracted in March 1991.
[b] Bark harvested in February or March and extracted in March 1991.
[c] Bark harvested and extracted in late June 1991.

DISCUSSION

Nienstaedt (1953) used old mycelium for his tests of toxicity (15 and 31 days old) and I used mycelium from the edges of rapidly growing colonies less than 7 days old. The older age of his cultures resulted in highly variable results, but there were still no significant differences between growth rates of a virulent culture on purified tannins from a Chinese, a Japanese, and an American tree in (unbuffered) PDA. A similar conclusion was reached by G. Farias (unpublished) in her study of tannases. Delen (1975) reported differences in the amount of tannin in bark of 11 varieties of European chestnut trees (*C. sativa* Ml.), but extraction and assay techniques are not detailed. He also found no inhibition of fungal growth using commercial tannin in the growth medium.

J.R. Elkins, P. Graham, and W. Pate (unpublished) have found that *C. parasitica* can use hamamelitannin extracted from chestnut bark as a carbon source. J.R. Elkins (unpublished) extracted (with water) large amounts of hamamelitannin from American chestnut bark, but little from the bark of Asian trees. He suggests that this form of tannin encourages the growth of the blight fungus

TABLE 3. GROWTH OF *Cryphonectria parasitica* ON COMMERCIAL TANNINS AND TANNINS EXTRACTED FROM CHESTNUT BARK

Experiment/source	Tannin (mg/ml medium)	Growth rate (mm/day)[a]
25 February		
R1T15	0.33	11.2 ± 0.6 ab
R9T3	0.36	12.0 ± 0.4 a
R1T18	0.51	11.4 ± 0.2 a
SciCliff[b]	0.41	12.0 ± 0.3 a
R7T7	0.43	11.4 ± 0.5 a
R34T6	0.47	11.0 ± 0.4 ab
BakTan[c]	0.50	10.0 ± 0.2 cd
Quebracho	0.01	10.3 ± 0.2 bc
Acetone	0	9.1 ± 0.2 de
Control	0	8.9 ± 0.1 e
13 March		
R1T15AM	0.79	10.7 ± 0.3 b
R1T15FM	0.78	10.8 ± 0.7 b
R9T3AM	0.74	12.2 ± 0.4 a
R9T3FM	0.78	11.2 ± 0.3 ab
R1T18AM	0.43	12.2 ± 0.3 a
R1T18FM	0.75	11.7 ± 0.4 ab
BakTan	1.00	11.4 ± 0.1 ab
MalTan[d]	1.00	10.9 ± 0.2 b
Acetone	0	10.6 ± 0.1 b
26 June		
R1T15	2.5	12.3 ± 0.2 a
R9T3	2.9	12.6 ± 0.7 a
R1T18	1.2	8.5 ± 0.4 b
SciCliff	2.4	11.9 ± 0.2 a
R7T7	2.9	11.9 ± 0.2 a
R34T6	2.9	11.3 ± 0.9 a
BakTan	5.0	8.6 ± 0.4 b
MalTan	5.6	7.0 ± 0.5 c
Acetone	0	7.5 ± 0.4 bc
Control	0	7.8 ± 0.5 bc
5 July		
R1T15	16	11.7 ± 0.4 bc
R9T3	21	13.5 ± 0.3 a
R1T18	16	10.5 ± 0.6 d
SciCliff	16	10.7 ± 0.4 cd
R7T7	21	12.0 ± 0.1 b
R34T6	21	12.5 ± 0.3 ab
Acetone	0	7.5 ± 0.4 e
Control	0	6.2 ± 0.5 f

[a] Rate from a linear regression of daily measurements of colony diameter in mm ± the standard error of the slope. Rates followed by the same letter are not significantly different at the $P = 0.05$ level (Duncan's multiple-range test).

[b] Scientists Cliff.

[c] Baker Analyzed Tannic Acid.

[d] Mallinckrodt AR Tannic Acid.

in American bark but not in Asian bark. Since both condensed and hydrolyzable tannins are detected by the plate diffusion method used in my study, this cannot be confirmed. However, the fact that no differences were seen in fungal growth rates on media with extracts used at bark levels suggests that tannins from American chestnut bark are not a better food source for the blight fungus than those from Asian chestnut bark.

Factors other than tannins have been implicated in the resistance of Asian chestnut trees to the chestnut blight fungus. A preliminary report by Samman et al. (1978) described a long-chain unsaturated fatty acid that inhibited fungal growth, and McCarroll and Thor (1985) described a protein isolated from the inner bark of Chinese chestnut trees that strongly inhibited the activity of polygalacturonase from the blight fungus. It is possible that the water extracts used by Cook and Wilson (1916) and by Nienstaedt (1953) contained some of these compounds. Tannin toxicity does not appear to supply the answer.

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GENETIC COMPONENT OF VARIATION IN CHEMICAL DEFENSE OF *Oreina gloriosa* (COLEOPTERA: CHRYSOMELIDAE)

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Abstract—Defensive secretions of adult *Oreina gloriosa*, liberated at the surface of the pronotum and elytra, contain a complex mixture of cardenolides, and ethanolamine. Proportions and concentrations of constituents determined by reverse-phase HPLC show considerable variation among individual beetles. Heritabilities of proportions of five main components were estimated by mother–offspring regression providing a validation of the less reliable full-sib correlation estimates. Average heritabilities based on the two methods were 0.51 and 0.58, respectively, estimated by using offspring of two age groups. Regression estimates of 2- and 10-week-old offspring differed significantly for one secretion constituent (RT16). Heritability estimates of concentrations of 16 secretion components were calculated by full-sib correlation analysis. Average heritability was 0.45, indicating a significant genetic component. Estimates did not differ significantly between the two age groups. We also estimated heritabilities of concentrations by a two-way model including data from offspring of both age groups. Heritability estimates based on this model are thought to correspond approximately to estimates based on samples from natural populations. The average of these estimates was lower ($h^2 = 0.31$) than the average heritability of each age group separately ($h^2 = 0.45$), suggesting a developmental effect on variation in chemical defense of *O. gloriosa*.

Key Words—*Oreina gloriosa*, Coleoptera, Chrysomelidae, chemical defense, allomone, cardenolides, quantitative variation, heritability.

INTRODUCTION

The alpine beetle *Oreina gloriosa* belongs to one of the most conspicuous families of Coleoptera, the Chrysomelidae. Adult *O. gloriosa* are slow-moving,

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brightly colored, and they often densely aggregate on their host plant *Peucedanum ostruthium* (Apiaceae), resulting in high visual exposure to predators. Nevertheless this chrysomelid shows a high survival rate (0.73–0.96 per week) (Eggenberger, 1989) and can live as long as three years (Rowell-Rahier, unpublished data), suggesting an effective protection by its chemical defense (Pasteels et al., 1988). The defensive strategy of adult *O. gloriosa* is based on the exocrine secretion of a complex mixture of cardenolides and ethanolamine at the surface of the pronotum and elytra (Van Oycke et al., 1988). Since the host plant of this monophagous beetle is known to contain no cardenolides, *O. gloriosa* produces its defensive chemicals de novo. The ability of leaf beetles to synthesize cardenolides de novo has been confirmed by labeling experiments (Van Oycke et al., 1987).

Chemical defense is an important component of the interaction between prey and predator, which is thought to be under strong selection for efficiency, since production, particularly de novo biosynthesis, of defensive chemicals is costly (Rowell-Rahier and Pasteels, 1986). In order to produce evolutionary change, however, selection requires heritable variation, which is measured and expressed as additive genetic variance. The ratio of additive genetic variance to phenotypic variance is defined as the heritability (h^2), which is estimated from the degree of resemblance between relatives by regression of offspring on parents or correlation between sibs (Falconer, 1989).

Although genetic sources of variation in exocrine secretions were shown to exist in pheromones of butterflies (Sappington and Taylor, 1990) and hypothesized for the kairomones of a prey–predator interaction (Herms et al., 1991), this is the first report dealing with heritabilities of chemical defense characters.

We recently reported on variation in chemical defense between populations of *O. gloriosa* (Eggenberger and Rowell-Rahier, 1991). Interpopulational variation in the constituents of the defensive secretion proved to be significantly correlated with genetic variation of six enzyme loci, suggesting that a considerable part of the total phenotypic variation in chemical defense is caused by genetic differences.

In this study we demonstrate a significant genetic component to the intrapopulational variation in chemical defense of *O. gloriosa*.

METHODS AND MATERIALS

Collection and Culture of Beetles. One hundred fifty adult *O. gloriosa* were collected randomly in July 1989 in Saas (Wallis, Swiss Alps) and brought in cooled boxes to the laboratory. The beetles were maintained individually in separate plastic containers on their food plant *P. ostruthium*, which was shipped weekly from the original locality. The offspring of the 48 collected females,

which had mated in the field with unknown males, were reared in plastic containers at constant temperature (17°C) and a constantly changing light–dark regime corresponding to natural conditions. In September 1989 all larvae buried themselves in the substrate provided: soil covered with regularly moistened spruce bark. Pupation was not completed by November, and the temperature was decreased to 2°C. At the end of June 1990, the temperature was raised to 17°C, triggering pupation and the emergence of 553 adults. The offspring of 27 females were maintained separately under the conditions previously described. The 27 families were selected so that there were at least five offspring per family.

Sample Preparation and Chromatographic Analysis. Pronotal secretion liberated as a result of mechanical irritation was collected in a capillary tube (0.2 mm ID) and quantified. The parental generation was "milked" in July 1989, a few days after arrival in the laboratory. Samples were dissolved in 50 μl acetonitrile–water 1 : 10 and stored individually at −70°C. The secretions of one group of offspring were sampled in July 1990 (two weeks after emergence) and those of the other group in September 1990 (10 weeks after emergence). Individual samples were dissolved in 50 μl acetonitrile–water 1 : 10 plus 2 μg ouabain (Merck), as internal standard. After filtration (0.2 μm pore size), 20-μl aliquots were analyzed by HPLC, [two-pump system (Waters 510); detector: photodiode array (Waters 994), 220 nm; column: Macherey-Nagel cartridge, C-18, 3 μm, 4 × 130 mm; eluent: chromatography grade acetonitrile (Baker) and water (Merck), 15–42% acetonitrile linear in 36 min, 0.45 ml/min; data analysis system: Maxima 820 data station] (Figure 1). The proportions of the main secretion components (RT3, RT9, RT16, RT28, and RT32) were calculated by dividing the respective peak area by the total area of main peaks. Concentrations (micrograms ouabain equivalents per microliter secretion) of 16 secretion components were twice their peak area divided by the area of the internal standard peak (ouabain) and the secretion volume. Since samples of the parental generation were run without an internal standard, concentrations could be calculated only for offspring.

Statistical Methods. Data were analyzed using SAS (SAS Institute Inc., 1990) on a VAX 8840. Weighted regression of offspring on mother was carried out using PROC REG. Weighting factors for regression were calculated by the procedure of Falconer (1989). Heritability estimates were twice the slope of the regression with a precision of twice the standard error of the slope. Random effect model analyses of variance were performed using PROC GLM. Prior to analyses of variance, proportions were arcsine transformed (Sokal and Rohlf, 1981). Concentrations were square-root transformed since residual distributions showed a poorer fit to Gaussian distributions (PROC UNIVARIATE) if other commonly applied transformations (Sokal and Rohlf, 1981) were used. Variance components were estimated by the maximum likelihood method (PROC VARCOMP), which agreed better with heritability estimates based on mother–off-

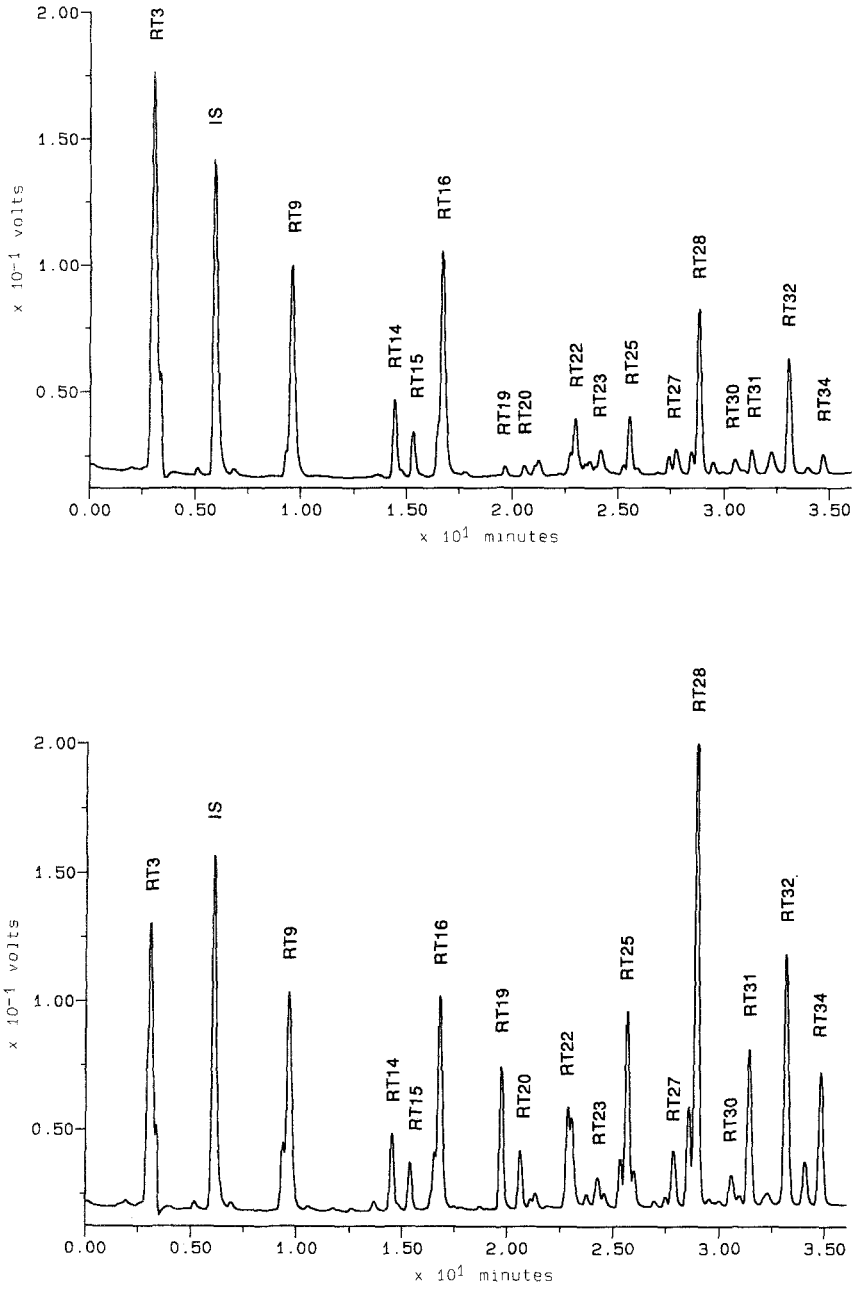


FIG. 1. Chromatograms obtained by reverse-phase HPLC of the defensive secretions emitted by individual beetles.

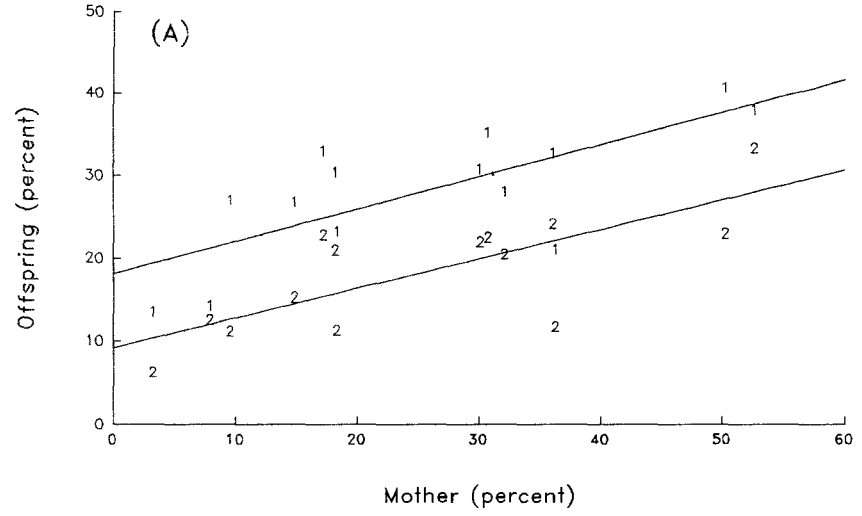
spring regression than either the restricted maximum likelihood method or the type-1 sum-of-squares method. Genetic and developmental effects were designated as random since we were interested in the variation that exists when genotype and age are randomly distributed as in natural populations. Heritability estimates based on full-sib analysis were twice the intraclass correlation coefficient (among-family variance component divided by total variance) with standard errors estimated by the delta technique (Bulmer, 1985).

Heritability estimates of proportions of secretion components were estimated using both parent–offspring regression and full-sib correlation analysis. Parent–offspring regression was carried out with data from 14 females and two different groups of their offspring, which were "milked" two and 10 weeks after emergence, respectively. The proportions of each of the five main components in the secretion of individual offspring were averaged per family and regressed for each age group separately on the proportions measured in their individual female parents. Full-sib correlation analysis was performed using data from the 138 living 2-week-old offspring belonging to 27 families and 92 10-week-old offspring of 25 females, respectively. (The numbers of families used for estimating heritabilities were different for parent–offspring regression and full-sib correlation because secretions of female parents were not always available.) The data for each group were tested for differences among families, which was assumed to be a random factor.

Heritabilities of concentrations of secretion components were estimated using full-sib correlation analysis. (Parent–offspring regression was not used since concentrations were not available for the parental generation.) Full-sib correlation was carried out on the same two age groups as used to estimate heritabilities of proportions. However, since some data on secretion volume were lost, the number of analyzed offspring was different. Data for each age-group were tested for differences among families.

RESULTS

Proportions of Five Main Components. Heritability estimates based on parent–offspring regression were significant ($P < 0.05$) for RT9 and RT16 of both age groups (Figure 2) and for RT3 of 2-week-old offspring (Table 1). Estimates for RT16 differed significantly ($P < 0.05$) between the two age groups. The among-family variance term was significant (Tables 2 and 3) for all secretion components in both age groups, with the single exception of RT3 in 10-week-old offspring. Heritabilities estimated by full-sib correlation analysis were significant for RT9, RT16, and RT28 in both age groups and for RT3 in 2-week-old offspring (Tables 2 and 3). Mother–offspring estimates were slightly lower than full-sib estimates, which are known to be biased by dominance and



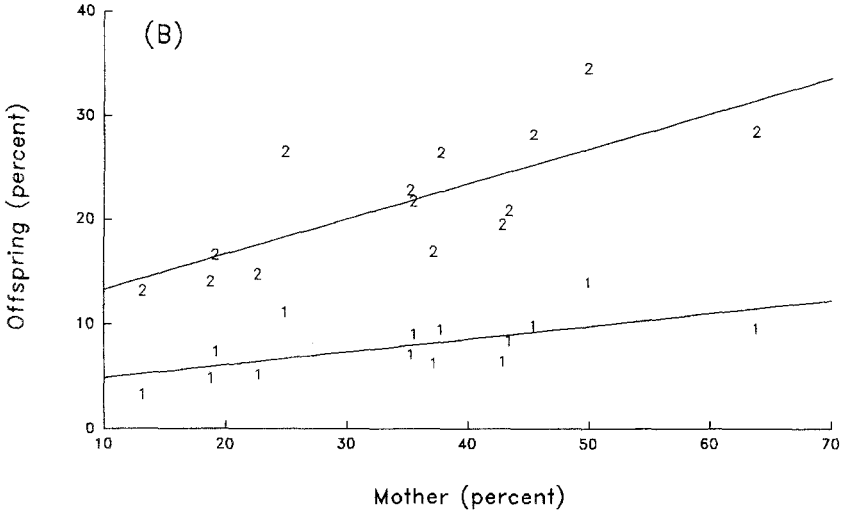


FIG. 2. Proportions of constituents in the secretion of offspring (averaged per family) in relation to those from the secretion of their mothers. Regression were carried out with 2-week-old (1) and 10-week-old (2) offspring. Slopes did not differ significantly for RT9 (A) but were significantly ($P < 0.05$) different for RT16 (B).

TABLE 1. MEAN PERCENTAGES (±SE) OF 5 MAIN SECRETION COMPONENTS OF TWO GROUPS OF OFFSPRING AND THEIR MOTHERS[a]

Secretion component	Mother (N = 14) Percent	Offspring 1 (age 2 weeks, N = 69) Percent	Offspring 1 h^2	Offspring 2 (age 10 weeks, N = 56) Percent	Offspring 2 h^2
RT3	22.2 ± 3.0	36.2 ± 0.9	0.52 ± 0.17**[b]	31.7 ± 0.9	0.23 ± 0.14 ns
RT9	25.5 ± 4.0	27.6 ± 1.1	0.78 ± 0.21**	19.4 ± 1.2	0.74 ± 0.18**
RT16	35.0 ± 3.7	8.1 ± 0.5	0.24 ± 0.09*	21.0 ± 1.0	0.67 ± 0.18**
RT28	6.1 ± 0.8	14.0 ± 0.5	0.57 ± 0.54 ns	11.3 ± 0.6	0.83 ± 0.49 ns
RT32	11.2 ± 1.2	14.1 ± 0.6	0.21 ± 0.35 ns	16.6 ± 0.6	0.22 ± 0.28 ns
Average			0.47		0.54

[a] Heritabilities (h^2 ± SE) were estimated by mother–offspring regression.
[b] $^{**}P < 0.01$; $^{*}P < 0.05$.

TABLE 2. MEAN PERCENTAGES (±SE) OF 5 MAIN SECRETION COMPONENTS OF 2-WEEK-OLD BEETLES[a]

Secretion component	Offspring 1 (age 2 weeks, N = 138) Percent	Offspring 1 h^2	ANOVA (effect of family) df = 26 Type III MS	F value	P value
RT3	37.1 ± 0.6	0.37 ± 0.16*[b]	0.0096	2.213	0.0023
RT9	27.7 ± 0.7	0.97 ± 0.18**	0.0289	5.932	0.0001
RT16	7.7 ± 0.3	0.63 ± 0.19**	0.0109	3.431	0.0001
RT28	13.6 ± 0.4	0.66 ± 0.19**	0.0087	3.660	0.0001
RT32	13.9 ± 0.4	0.26 ± 0.15 ns	0.0073	1.757	0.0234
Average		0.58			

[a] Arcsine transformed data were tested for differences among families by random effect model analysis of variance. Heritabilities (h^2 ± SE) were estimated by full-sib correlation analysis.
[b] $^{**}P < 0.01$; $^{*}P < 0.05$.

environmental effects (Falconer, 1989). However, the heritabilities estimated by the two methods did not differ significantly.

Concentrations of 16 Components. Fifteen of the 16 among-family variance components of 2-week-old offspring were significant, whereas with 10-week-old offspring five among-family variance components were not significant (Tables 4 and 5). Heritability estimates were significant for five secretion components (RT9, RT16, RT27, RT30, and RT34) of both age groups; the estimates of seven other secretion components (RT14, RT15, RT19, RT20, RT23, RT25,

TABLE 3. MEAN PERCENTAGES (±SE) OF 5 MAIN SECRETION COMPONENTS OF 10-WEEK-OLD BEETLES[a]

Secretion component	Offspring 2 (age 10 weeks, $N = 92$)		ANOVA (effect of family) $df = 24$		
	Percent	h^2	Type III MS	F value	P value
RT3	33.6 ± 0.9	0.15 ± 0.17 ns	0.0100	1.328	0.1816
RT9	19.0 ± 0.9	0.80 ± 0.24**[b]	0.0288	3.525	0.0001
RT16	19.9 ± 0.8	1.05 ± 0.22**	0.0204	5.155	0.0001
RT28	11.4 ± 0.4	0.60 ± 0.25*	0.0084	2.781	0.0005
RT32	16.1 ± 0.5	0.25 ± 0.26 ns	0.0063	1.998	0.0140
Average		0.57			

[a] Arcsine transformed data were tested for differences among families by random effect model analysis of variance. Heritabilities (h^2 ± SE) were estimated by full-sib correlation analysis.
[b] $^{**}P < 0.01$; $P < 0.05$.

TABLE 4. MEAN CONCENTRATIONS (±SE) OF 16 SECRETION COMPONENTS OF 2-WEEK-OLD BEETLES[a]

Secretion component	Offspring 1 (age 2 weeks, $N = 131$)		ANOVA (effect of family) $df = 26$		
	Amount ($\mu g/\mu l$)	h^2	Type III MS	F value	P value
RT3	52.7 ± 1.3	0.22 ± 0.15 ns	1.4345	1.624	0.0454
RT9	40.2 ± 1.5	0.47 ± 0.19*[b]	3.4884	2.497	0.0006
RT14	4.0 ± 0.2	0.31 ± 0.16 ns	0.3878	1.876	0.0138
RT15	3.2 ± 0.2	0.30 ± 0.17 ns	0.4401	1.892	0.0128
RT16	11.2 ± 0.6	0.41 ± 0.18*	1.5020	2.291	0.0017
RT19	4.8 ± 0.4	0.94 ± 0.20**	1.8060	5.033	0.0001
RT20	7.9 ± 0.5	0.75 ± 0.20**	2.0203	3.903	0.0001
RT22	7.4 ± 0.3	0.32 ± 0.18 ns	0.6219	2.015	0.0069
RT23	5.9 ± 0.3	0.30 ± 0.18 ns	0.5525	1.905	0.0119
RT25	12.7 ± 0.5	0.68 ± 0.20**	1.5402	3.568	0.0001
RT27	5.8 ± 0.3	0.56 ± 0.19**	0.7618	2.904	0.0001
RT28	20.1 ± 9.8	0.35 ± 0.18 ns	1.8225	2.122	0.0040
RT30	2.1 ± 0.1	0.43 ± 0.19*	0.3045	2.385	0.0010
RT31	4.2 ± 0.3	0.36 ± 0.18 ns	0.8610	2.118	0.0041
RT32	20.4 ± 0.9	0.13 ± 0.15 ns	1.5275	1.388	0.1255
RT34	4.7 ± 0.3	0.45 ± 0.19*	0.9557	2.472	0.0007
Average		0.44			

[a] Square-root transformed data were tested for differences among families by random effect model analysis of variance. Heritabilities (h^2 ± SE) were estimated by full-sib correlation analysis.
[b] $^{**}P < 0.01$; $^{*}P < 0.05$.

TABLE 5. MEAN CONCENTRATIONS (±SE) OF 16 SECRETION COMPONENTS OF 10-WEEK-OLD BEETLES[a]

Secretion component	Offspring 2 (age 10 weeks, $N = 90$) Amount ($\mu g/\mu l$)	h^2	ANOVA (effect of family) $df = 24$ Type III MS	F value	P value
RT3	66.0 ± 2.2	0.19 ± 0.20 ns	2.0143	1.481	0.1076
RT9	37.8 ± 2.0	0.59 ± 0.24*[b]	5.0083	2.621	0.0011
RT14	10.7 ± 0.5	0.60 ± 0.25*	0.7510	2.670	0.0009
RT15	5.6 ± 0.3	0.66 ± 0.23**	0.4719	2.858	0.0004
RT16	39.5 ± 1.8	0.58 ± 0.25*	3.4536	2.580	0.0013
RT19	1.8 ± 0.1	0.14 ± 0.17 ns	0.2932	1.155	0.3153
RT20	2.9 ± 0.2	0.41 ± 0.21 ns	0.5139	1.856	0.0257
RT22	7.5 ± 0.4	0.29 ± 0.19 ns	0.5766	1.561	0.0803
RT23	5.2 ± 0.3	0.60 ± 0.22*	0.7469	2.671	0.0009
RT25	9.6 ± 0.4	0.21 ± 0.18 ns	0.5660	1.344	0.1736
RT27	5.2 ± 0.3	0.57 ± 0.23*	0.5553	2.430	0.0025
RT28	22.7 ± 1.0	0.30 ± 0.25 ns	1.6703	1.704	0.0466
RT30	3.3 ± 0.2	0.74 ± 0.23**	0.4929	3.152	0.0001
RT31	8.7 ± 0.5	0.64 ± 0.26*	1.6849	2.858	0.0004
RT32	31.8 ± 1.2	0.15 ± 0.23 ns	1.3938	1.544	0.0855
RT34	5.8 ± 0.4	0.74 ± 0.26	1.1570	3.220	0.0001
Average		0.46			

[a] Square-root transformed data were tested for differences among families by random effect model analysis of variance. Heritabilities (h^2 ± SE) were estimated by full-sib correlation analysis.
[b] $^{**}P < 0.01$; $^{*}P < 0.05$.

and RT31) were significant for one age group only (Tables 4 and 5). Although average heritabilities of the two age groups were almost identical, some estimates showed apparent differences that were not significant when tested using Fisher's z-transformation (Zar, 1984). Heritabilities for both age groups together were estimated by a two-way model designating family, age, and the interaction term as random effects. P values for among-family variance components are listed in Table 6, showing 13 significant among-family variance components of which nine were also significant for each age group separately (Tables 4 and 5). Heritability estimates were significant for seven secretion components (RT9, RT15, RT20, RT22, RT28, RT31, and RT34). However, only two estimates (RT9 and RT34) were also significant for each age group when considered separately. Average heritability was lower (0.31) than the estimates of each age group considered separately (0.44 and 0.46), because variance associated with age increased the total variance and because some genetic variance was accounted for by the interaction term.

TABLE 6. MEAN CONCENTRATIONS (±SE) OF 16 SECRETION COMPONENTS OF 2- AND 10-WEEK-OLD BEETLES[a]

	Offspring 1 and 2 (age 2 and 10 weeks, $N = 221$)		ANOVA (effect of family) $df = 26$		
Secretion component	Amount ($\mu g/\mu l$)	h^2	Type III MS	F value	P value
RT3	58.1 ± 1.2	0.11 ± 0.11 ns	2.0056	1.409	0.2000
RT9	39.2 ± 1.2	0.57 ± 0.16**[b]	7.0131	5.411	0.0001
RT14	6.7 ± 0.3	0.20 ± 0.14 ns	0.8824	3.491	0.0014
RT15	4.2 ± 0.2	0.38 ± 0.16*	0.7023	5.477	0.0001
RT16	22.7 ± 1.2	0.19 ± 0.14 ns	3.8835	3.384	0.0018
RT19	3.6 ± 0.2	0.43 ± 0.21 ns	1.2864	2.827	0.0062
RT20	5.9 ± 0.4	0.44 ± 0.20*	1.7442	4.142	0.0004
RT22	7.4 ± 0.2	0.36 ± 0.13*	0.9279	3.937	0.0006
RT23	5.6 ± 0.2	0.05 ± 0.16 ns	0.6769	1.142	0.3733
RT25	11.4 ± 0.4	0.30 ± 0.16 ns	1.2827	2.140	0.0321
RT27	5.5 ± 0.2	0.32 ± 0.18 ns	0.9163	2.343	0.0195
RT28	21.2 ± 0.7	0.39 ± 0.14*	2.4607	3.360	0.0018
RT30	2.6 ± 0.1	0.13 ± 0.18 ns	0.4507	1.355	0.2281
RT31	6.0 ± 0.3	0.38 ± 0.15*	1.9829	2.971	0.0044
RT32	25.0 ± 0.8	0.20 ± 0.11 ns	2.2194	2.975	0.0043
RT34	5.2 ± 0.2	0.53 ± 0.15**	1.6891	3.692	0.0009
Average		0.31			

[a] Square-root transformed data were tested for differences among families by random effect two-way model analysis of variance. Heritabilities ($h^2 \pm$ SE) were estimated by full-sib correlation analysis using a random effect two-way model.
[b] $^{**}P < 0.01$; $^{*}P < 0.05$.

DISCUSSION

Heritable variation is required for evolutionary adaptation. Chemical defense is thought to be the result of evolutionary adaptation. In this study we found significant heritabilities for quantitative traits of chemical defense as estimated by mother-offspring regression and full-sib correlation analysis. Hence, predators may be directing the evolution of the chemical defense of *O. gloriosa*.

Since heritability estimates based on full-sib correlation are likely to be inflated by variance due to common environment (Falconer, 1989), we tried to minimize the environmental variance component by experimental design: all adult beetles were kept randomly distributed under the same controlled environmental conditions. Full-sib estimates are further biased by dominance variance (Falconer, 1989), a part of nonadditive genetic variance due to dominance (heterozygote not intermediate between homozygotes) or overdominance (hetero-

zygote more extreme than either homozygote). Mother–offspring regression estimates are known to be less biased and therefore more reliable than full-sib estimates (Falconer, 1989). Because data on secretion concentrations in the parental generation were not available, mother–offspring estimates could be calculated only for proportions. However, full-sib estimates were not significantly different from mother–offspring estimates, providing a validation of the less reliable full-sib estimates.

Standard errors of heritability estimates based on full-sib correlation are rather large, due to the relatively low power of maximum likelihood estimates (Scheiner and Lyman, 1989). This is obvious, since many heritability estimates are not significantly different from zero, even though the ANOVAs show significant among-family variance components.

We have suggested previously that quantitative variation in chemical defense between populations of *Oreina gloriosa* is partly genetically determined (Eggenberger and Rowell-Rahier, 1991). The present study shows the average heritability of proportions to be 54% of the intrapopulational variation, confirming this hypothesis.

There were no significant differences between the full-sib estimates of 2-week-old and 10-week-old offspring, not even for RT16, which shows significantly different mother–offspring regression estimates (Figure 2). Since heritability is the ratio of additive genetic and phenotypic variance, heritability change during development is a consequence of drift of this ratio, which can be due to change in both genetic and environmental variance. Change in genetic variance is thought to be the result of different gene expression. Genetic control of physiological response is reviewed by Humphreys (1991). Change in phenotypic variance may be caused by environmental or interaction effects. The pattern of change in the relative magnitude of phenotypic and additive genetic variance during growth can be rather complex (e.g., Henrich, 1989).

We also present heritabilities of concentrations based on a two-way model including data from both age groups. These estimates are thought to correspond more closely to estimates of populations with natural age structure than do estimates based on data from each age group separately. However, it should be emphasized that heritabilities depend on the magnitude of all variance components and therefore are valid only for the population for which they are estimated.

The biological significance of the complexity of exocrine secretion mixtures is obscure. It is thought that different components of the secretion may exert different effects or that the proportions of different components may confer specificity (Chapman, 1982). In a three-component allomone system investigated by Dettner and Grümmer (1986), the ratio of constituents was shown to correlate with the maximal penetration rate through the integument of a predatory arthropod. The secretion of *O. gloriosa* may be even more complex than pre-

sented here, since only major components detectable at UV 220 nm were quantified. All constituents are highly polar and, with the exception of RT3, show UV spectra typical of cardenolides. Because of the similarity of the constituents, the complexity of the mixture may be the inevitable consequence of its biosynthesis rather than of significance for chemical defense (or both). The exocrine secretion of *O. gloriosa* may also act as a pheromone (e.g., sex pheromone) and the mixture of constituents may therefore be a component of reproductive fitness. However, to our knowledge, there is no evidence supporting pheromonal function of the secretion.

In summary, heritability estimates show a considerable part of the variation in chemical defense of *Oreina gloriosa* to be genetically determined. Physiological and environmental sources of this variation will be discussed in following papers.

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(*E*)- AND (*Z*)-6-NONEN-2-ONE: BIOSYNTHETIC PRECURSORS OF *endo*- AND *exo*-BREVICOMIN IN TWO BARK BEETLES (COLEOPTERA: SCOLYTIDAE)

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Abstract—The ability of (*E*)- and (*Z*)-6-nonen-2-one to serve as precursors of the common scolytid pheromones *endo*- and *exo*-brevicomin was examined in vivo. When mountain pine beetles (MPB), *Dendroctonus ponderosae* Hopkins, or western balsam bark beetles (WBBB), *Dryocoetes confusus* Swaine, were exposed to [6,7-D_2](*E*)-6-nonen-2-one, the *endo*-brevicomin produced was enriched with two deuterium atoms per molecule (as determined by GC-MS), indicating that (*E*)-6-nonen-2-one served as a precursor of this pheromone. Similarly, when the beetles were exposed to [4,4-D_2](*Z*)-6-nonen-2-one, the *exo*-brevicomin produced was enriched with two deuterium atoms per molecule. Evidence in support of biological relevance of the latter observation include: (1) (*Z*)-6-nonen-2-one was found in the volatiles of male MPBs and WBBBs, indicating that this is a natural metabolite; (2) the *exo*-brevicomin produced by MPB was shown to be of natural (+) chirality by complexation chromatography; and (3) female WBBBs and MPBs (which are not known to produce *exo*-brevicomin) produced significantly less *exo*-brevicomin when exposed to the precursor than did the males.

Key Words—Bark beetles, *exo*-brevicomin, *endo*-brevicomin, Coleoptera, deuterium, (*E*)-6-nonen-2-one, (*Z*)-6-nonen-2-one, pheromone biosynthesis, Scolytidae.

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INTRODUCTION

Scolytid bark beetles are well known as economically important forest pests. There is little doubt that effective communication between conspecific individuals during activities such as host-colonization and mate-finding has contributed to the reproductive success of these beetles. Beetles use visual and vibrational cues for communication, but olfactory signals (pheromones) may be most important (Borden, 1985). Most of the scolytid pheromones identified to date are either terpenoid alcohols, the corresponding ketones, or bicyclic oxygen heterocycles (Borden, 1985; Vanderwel and Oehlschlager, 1987). Most terpenoid pheromones appear to be derived from host monoterpenes (Vanderwel and Oehlschlager, 1987), while the biosynthetic origins of the other pheromones are unknown. Silverstein (cited by White et al., 1980) speculated that the biosynthetic pathways to the bicyclic compounds may be similar to laboratory routes to these pheromones. Francke (1981) suggested that (*Z*)-6-nonen-2-one may serve as the natural precursor of *exo*-brevicomin. (*Z*)-6-Nonen-2-one is produced in conjunction with brevicomin by males of the ash bark beetle, *Leperisinus varius* F. (Francke, 1978, 1981). The two ketoalkenes (*Z*)- and (*E*)-6-nonen-2-one have served as precursors of *exo*- and *endo*-brevicomin, respectively, in laboratory syntheses (Rodin et al., 1971).

Our objective was to test the hypothesis that (*Z*)- and (*E*)-6-nonen-2-one serve as precursors to the brevicomins in vivo (Figure 1), in two species of bark beetles: mountain pine beetles (MPB), *Dendroctonus ponderosae* Hopkins, and western balsam bark beetles (WBBB), *Dryocoetes confusus* Swaine. Newly emerged, adult male MPB and WBBB produce both *exo*- and *endo*-brevicomin (Borden, 1985; Borden et al., 1987). In the past, investigations of the biosynthesis of these pheromones were limited by the technical difficulties involved in working with small quantities of volatile compounds. However, these difficulties

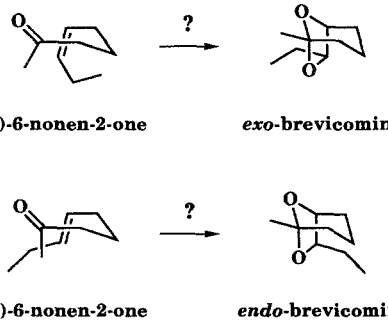

FIG. 1. (*Z*)- and (*E*)-6-nonen-2-one, logical precursors of *exo*- and *endo*-brevicomin, respectively.

were overcome by the use of stable isotope-labeled precursors, and detection of their incorporation by gas chromatography–mass spectroscopy (GC-MS).

METHODS AND MATERIALS

Insects

Lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann, infested with MPB was collected near Princeton, British Columbia. The cut ends of the logs were sealed with hot paraffin wax to prevent desiccation. The logs were either used fresh or stored at 4°C until they were placed in cages held at approximately 28°C. Emergent beetles were collected daily, and their sex was immediately determined by the shape of the seventh abdominal tergite (Lyon, 1958). The insects were either used the same day or were stored on moistened paper at 4°C in loosely capped jars for a maximum of one week. Only healthy appearing individuals were selected for experiments.

Subalpine fir, *Abies lasiocarpa* (Hook.) Nutt., infested with WBBB was collected near Merritt, British Columbia. The logs and beetles were treated as described for MPB. The sex of the emergent beetles was determined by the presence (females) or absence (males) of the setal brush on the frons (Bright, 1976).

Treatment of Insects

The approach taken to determine if (*E*)- and (*Z*)-6-nonen-2-one could serve as precursors of *endo*- and *exo*-brevicomin, respectively, in vivo was to expose insects to vapors of the deuterated analogs of (*E*)- and (*Z*)-6-nonen-2-one ([6,7-D_2] and [4,4-D_2], respectively) and to check (by GC-MS analysis) for the incorporation of deuterium into the brevicomins produced by the insects.

Newly emerged male MPB or WBBB were individually caged in 3.5 ml screw-cap vials (Gries et al., 1990), into which either 0 (control), 0.1 (for MPB) or 0.05 (for WBBB) μl of the labeled hypothesized precursor was placed. The sealed vials were stored at room temperature in the dark. After 24 hr insects were either: (1) extracted immediately (whole beetles were individually extracted with hexane); or (2) individually aerated. Purified, moisturized air was drawn over the beetles, and the volatiles emanating from them were trapped on Porapak Q (50–80 mesh, Applied Science Laboratories). After 48 hr, the aerated insects were individually extracted to give a second whole beetle extract. Captured volatiles were eluted from the Porapak Q with diethyl ether.

Analyses of exo- *and* endo-*Brevicomin in Extracts*

Analyses by GC were performed on a Hewlett-Packard 5890 GC equipped with a capillary inlet system and an FID. Samples were introduced by splitless injection onto a DB-1 column (15 m × 0.25 mm ID, 0.25 μm film thickness). The oven temperature program was: 50°C for 1 min, then 4°C/min to 80°C for 10 min, followed by an increase of 25°C/min to a final temperature of 275°C for 10 min. The brevicomins were identified by comparing their GC retention times and MS fragmentation patterns with authentic standards and were quantified using 3-octanol as an internal standard.

Mass spectra were recorded on a Hewlett-Packard 5985B GC-MS system by electron impact (EI, 70 eV) or by chemical ionization (CI, using isobutane as the ionizing gas). GC–Selected Ion Monitoring–Mass Spectroscopy (GC-SIM-MS) was performed as described by Vanderwel (1991).

Determination of Chirality of exo-*Brevicomin*

The chirality of *exo*-brevicomin produced by MPB was determined by complexation chromatography (Schurig et al., 1983).

Prior to analyses nonvolatile residues were removed from the extracts. The crude extracts from control (untreated) and [4,4-D_2](*Z*)-6-nonen-2-one-treated male and female MPBs (15 per treatment) were pooled and soaked onto a glass wool plug placed at the front end of a glass tube (30 × 0.8 cm), which had been bent into an ω shape, leaving the first 2 cm straight and horizontal. The bottom half of the ω-shaped tubing was cooled with liquid nitrogen, thus forming two traps. The crude beetle extract was volatilized by passing a gentle stream of nitrogen gas through the glass wool plug, which was finger-warmed through the glass. Volatiles were collected in the glass traps, concentrated to 30 μl, and purified by preparative GC on a Varian model 1200 gas chromatograph equipped with a 10:1 effluent splitter, FID, thermal gradient collector, and a stainless-steel column (3.0 m × 3.2 mm ID) packed with 10% SP-1000 on Chromosorb W (100/120). The oven temperature program was: 70°C for 2 min, then 6°C/min to 180°C. The chirality of the brevicomin produced by the insects was determined by comparison of the retention times with authentic standards on a 25-m × 0.25-mm (ID) fused silica capillary column coated with nickel-*bis*(1*R*)-3-heptafluorobutyrylcamphorate (50°C for 2 min, then 2.5°C/min to 85°C for 30 min) (Schurig et al., 1983).

Collection of Airborne Volatiles from Male MPB and WBBB

Three hundred male MPB or 180 male WBBB were inserted into a "tube aeration apparatus" (Gries et al., 1992). Purified air was drawn through the tube for 10 days, while the volatiles were trapped on Porapak Q. Captured

volatiles were eluted from the Porapak Q with pentane–diethyl ether (95:5), and analyzed by GC-MS.

Statistical Analyses

Data were analyzed by analysis of variance (ANOVA). Where more than two treatments were used, means were separated by Tukey's multiple range test ($P = 0.05$).

Purchased/Donated Chemicals

$LiAlD_4$ (98 atom % D) and $NaBD_4$ (98 atom % D) were purchased from Sigma Chemical Company; deuterium oxide (D_2O, 99.9 atom % D) was purchased from Merck-Sharp-Dohme. Leaf alcohol [(*Z*)-3-hexen-1-ol] was purchased from Bedoukian Research Inc. Palladium on activated carbon (10%) was purchased from Strem Chemicals, Inc. Phero Tech, Inc. donated (*E*)- and (*Z*)-6-nonen-2-one.

Synthesis of Stable Isotope-Labeled Chemicals

All reactions were performed in flame-dried glassware with magnetic stirring. Where necessary, inert atmospheres of either Ar or N_2 were employed. After quenching, the usual workup involved: extraction of the aqueous phase three times with diethyl ether; combination of the organic extracts and washing once with brine; drying the organic layer with anhydrous $MgSO_4$; filtration; and removal of solvent by rotary evaporation under reduced pressure (without heating).

[^{1}H]NMR spectra were recorded at 400.13 MHz in $CDCl_3$ (unless stated otherwise) on a Bruker WM-400 Spectrometer. Signal positions are in δ; coupling constants (J) are in Hz.

Preparation of [4,4-D_2](Z)-6-Nonen-2-one

(Z)-3-Hexenoic Acid. Chromic acid, 2.5 M, was added dropwise to a solution of leaf alcohol [(*Z*)-3-hexen-1-ol, 60 mmol] in acetone (200 ml) at 0°C, until the yellow color persisted (about 80 mmol). The solution was stirred for an additional 15 min at room temperature, then 2-propanol was added dropwise until the green color persisted. The reaction mixture was then filtered through Celite. The acetone was removed in vacuo. The remaining oil was dissolved in diethyl ether, washed with brine, and dried over anhydrous $MgSO_4$. Removal of the solvent in vacuo gave a green oil. Distillation under vacuum left a colorless oil (4.15 g, 61% yield), bp 104–110°C (ca. 10 mm Hg), 79% pure by GC analysis. [^{1}H]NMR: 0.97 (3H, t); 2.04 (2H, dt); 3.10 (2H, d); 5.43–5.52

(1H, m); 5.54–5.63 (1H, m); 10.25–10.90 (br s). MS (EI), *m/z* (relative abundance): 114 (M^+, 56); 68 (82); 60 (58); 55 (100); 41 (90).

[*1,1-D*$_2$] *Leaf Alcohol.* (*Z*)-3-Hexenoic acid (26 mmol) was dissolved in anhydrous diethyl ether (20 ml), and the solution was added dropwise to a cooled (0°C) suspension of $LiAlD_4$ (21.7 mmol) in anhydrous diethyl ether (50 ml). After the addition was complete, the reaction mixture was stirred at 0°C for an additional 1 hr. The reaction was quenched by pouring into ice-cold 2% HCl and worked up in the usual fashion to yield a colorless oil (2.21 g, 82% yield), 93% pure by GC analysis. [^{1}H]NMR: 0.96 (3H, t); 1.50 (1H, br s); 2.07 (2H, p); 2.30 (2H, d); 3.64 (0.3H, t); 5.27–5.37 (1H, m); 5.50–5.60 (1H, m). MS (EI) *m/z* (relative abundance): 102 (M^+, 34); 84 (58); 69 (100); 68 (45); 67 (48); 41 (90).

[*4,4-D*$_2$](Z)-*6-Nonen-2-one.* The [1,1-D_2] leaf alcohol thus formed was subjected to the reaction conditions described by Rodin et al. (1971) to form [4,4-D_2](*Z*)-6-nonen-2-one in an overall yield of 26%. The final product was 96% pure by GC analysis. [^{1}H]NMR: 0.95 (3H, dt, J = 1 Hz, J = 8 Hz); 1.57–1.67 (0.5H, m); 1.96–2.07 (4H, m); 2.12 (3H, s); 2.40 (2H, br s); 5.23–5.31 (1H, m, J = 1); 5.35–5.44 (1H, m, J = 1). MS (EI) *m/z* (relative abundance): 142 (M^+, 11); 84 (86); 69 (100); 68 (50); 67 (63); 43 (78). By GC-SIM-MS analysis the product was 88% enriched with two deuteriums and 12% unenriched.

Preparation of [*4,4-D*$_2$](Z)-*6-Nonen-2-ol*

$NaBH_4$ (ca. 3 mmol) dissolved in 95% ethanol (1 ml) was added dropwise to [D_2](*Z*)-6-nonen-2-one dissolved in 95% ethanol (1 ml). The reaction mixture was stirred at room temperature for 1 hr, before it was quenched with 2% HCl (10 ml). The usual workup yielded a colorless oil (86 mg, 85% yield), 98% pure by GC analysis. [^{1}H]NMR: 0.94 (3H, t, J = 7.5); 1.18 (3H, d, J = 6.0); 1.39–1.47 (2H, m); 1.50–1.54 (0.7H, br s); 1.97–2.07 (4H, m); 3.74–3.83 (1H, sx, J = 6.0); 5.27–5.41 (2H, m). MS (EI) *m/z* (relative intensity): 144 (M^+, 10); 84 (100); 73 (53); 69 (79); 68 (60); 67 (44).

Preparation of [*6,7-D*$_2$](E)-*6-Nonen-2-one*

1-Tetrahydropyranyloxy-3-hexyne. To a mixture of 4-tetrahydropyranyloxy-1-butyne (100 mmol) in THF (200 ml) was added dropwise *n*-butyllithium (44 ml, 110 mmol) at −78°C. After addition the mixture was stirred for 30 min. 1-Bromoethane (150 mmol) followed by HMPA (30 ml) were added at −78°C, and the mixture was allowed to warm to room temperature over a period of 18 hr. After adding brine, the reaction mixture was worked up as usual to yield 16 g (88% yield) of product which was 90% pure by GC analysis. [^{1}H]NMR: 1.04

(3H, t, J = 6.75); 1.16–1.83 (6H, m); 1.91–2.21 (2H,); 2.43–2.64 (2H, m); 3.22–3.64 (2H, m); 3.72–4.1 (2H, m); 4.76 (1H, t, J = 4).

3-Hexyn-1-ol. The above product and *p*-toluenesulfonic acid (2–3 crystals) in methanol (100 ml) were stirred for 2 hr at room temperature. Methanol was removed and the product purified by silica gel column chromatography to give the product in 68% yield. [^{1}H]NMR: 1.0 (3H, t, J = 7.98); 1.42 (1H, bs); 1.9–2.08 (2H, m); 2.16–2.34 (2H, m); 3.5 (2H, t, J = 7.98). MS (EI) *m/z* (relative intensity): 98 (65); 83 (24); 68 (100); 53 (22).

[3,4-D_2](E)-3-Hexen-1-ol. $LiAlH_4$ (119 mmol) in a mixture of dry diglyme (50 ml) and THF (10 ml) was heated and nearly 7 ml of low boiling fraction was distilled off. 3-Hexyn-1-ol (60 mmol) in diglyme (10 ml) was added at 0°C. After addition the mixture was heated at 140°C for 48 hr. The mixture was hydrolyzed with 20% DCl in D_2O at 0°C. The aqueous layer was extracted with pentane (8 × 25 ml) and the combined organic layers washed with brine (8 ×), dried, and the solvents removed as usual to provide 4.6 g (75% yield) of the product, 92% pure by GC analysis. [^{1}H]NMR: 1.0 (3H, t, J = 7.9); 1.47 (1H, t, J = 6.5); 1.89–2.15 (2H, q, J = 7.9); 2.26 (2H, t, J = 6.5); 3.55–2.72 (2H, q, J = 6.5).

[3,4-D_2](E)-3-Hexenyl Tosylate. To [3,4-D_2](*E*)-3-hexen-1-ol (45.5 mmol) was added *p*-toluenesulfonyl chloride (49.8 mmol) and powdered KOH divided into five portions that were added over 20 min. The solution was stirred for 1 hr and quenched by pouring into ice water (200 ml). The usual workup yielded 10 g of product (86% yield), which was 95% pure by GC analysis. [^{1}H]NMR (C_6D_6): 0.83 (3H, t, J = 6.55); 1.77–1.81 (2H, q, J = 6.55); 1.86 (3H, s); 2.06 (2H, t, J = 6.55); 3.86 (2H, t, J = 6.55); 6.72 (2H, d, J = 9.8); 7.77 (2H, d, J = 9.8).

[6,7-D_2](E)-6-Nonen-2-one. To a mixture of sodium salt of ethyl acetoacetate (46.87 mmol) in anhydrous ethanol (60 ml) at 0°C was added the tosylate prepared above (39 mmol) in ethanol (10 ml). The reaction mixture was refluxed (100°C) for 16 hr. The ethanol was removed, and brine was added to the syrupy residue. The usual workup, followed by silica gel column chromatography (CH_2Cl_2) gave the semipure product (4.8 g). The semipure ester was subjected to basic hydrolysis. After stirring with aqueous NaOH (3 g in 50 ml) for 72 hr at room temperature, the basic solution was washed once with ether and acidified with 18 N sulfuric acid, and heated for 6 hr at 90°C to complete the decarboxylation. The aqueous solution was extracted with pentane (5 × 30 ml). The organic layers were combined and washed successively with saturated $NaHCO_3$ and brine, dried over anhydrous $MgSO_4$, and the solvents removed at atmospheric pressure to give the product (1.6 g, 29% yield), which was 96% pure by GC. [^{1}H]NMR: 0.92 (3H, t, J = 6.55); 1.57–1.64 (2H, q, J = 6.55); 1.91–2.08 (4H, m); 2.11 (3H, s); 2.37 (2H, t, J = 6.55). MS (EI) *m/z* (relative intensity): 142 (M^+, 5); 127 (4); 113 (4); 98 (4); 84 (73); 69 (100); 58 (17);

43 (73). The final product was >98% enriched in two deuteriums as determined by GC-SIM-MS analysis.

Preparation of [6,7-D_2](E)-*6-Nonen-2-ol*

The alcohol was prepared by reduction of [6,7-D_2](*E*)-6-nonen-2-one with $NaBH_4$ as described above. The product was obtained in 90% yield, and was >99% pure by GC analysis. [^{1}H]NMR: 0.89 (3H, t, J = 7.5); 1.16 (3H, d, J = 6.0); 1.30–1.45 (4H, m); 1.78 (1H, br s); 1.93–2.00 (4H, m); 3.72–3.81 (1H, sx, J = 6.0). MS (EI) m/z (relative intensity): 144 (M^+, 11); 97 (30); 84 (100); 71 (58); 69 (85); 68 (48).

RESULTS AND DISCUSSION

Biosynthesis of endo-*Brevicomin from* (E)-*6-Nonen-2-one*

(*E*)-6-Nonen-2-one served as a precursor of *endo*-brevicomin in both MPBs and WBBBs. As hypothesized (Figure 2), male MPBs and WBBBs exposed to vapors of [6,7-D_2](*E*)-6-nonen-2-one produced *endo*-brevicomin enriched in two deuteriums (Figure 3). The incorporation of two deuterium atoms is clearly shown by the shift in the base peak from m/z = 157 to m/z = 159. The MS of deuterium-enriched *endo*-brevicomin isolated from WBBBs (not shown) was virtually identical.

endo-Brevicomin production was significantly enhanced in male and female MPBs exposed to the precursor (Table 1). In addition to *endo*-brevicomin, volatiles extracted from beetles exposed to vapors of (*E*)-6-nonen-2-one included unused precursor, as well as (*E*)-6-nonen-2-ol, which the insects likely produced through reduction of the ketone.

Biosynthesis of exo-*Brevicomin from* (Z)-*6-Nonen-2-one*

We hypothesized that changing the geometry of the olefinic bond in 6-nonen-2-one from *E* to *Z* would lead to the production of *exo*-, rather than *endo*-, brevicomin in vivo (Figure 4). This hypothesis was verified by the fact that MPB and WBBB exposed to [6,7-D_2](*Z*)-6-nonen-2-one produced *exo*-brevicomin that was enriched in two deuteriums (Figure 5). The MS of deuterium-

FIG. 2. Expected incorporation of [6,7-D_2](*E*)-6-nonen-2-one into [D_2]-*endo*-brevicomin in vivo.

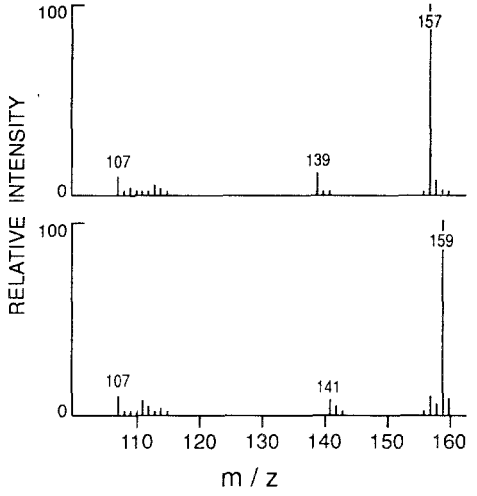

FIG. 3. MS (CI) of *endo*-brevicomin extracted from unexposed MPBs (top panel) and from male MPBs exposed to [6,7-D_2](*E*)-6-nonen-2-one (bottom panel).

TABLE 1. *endo*-BREVICOMIN CONTENT IN EXTRACTS OF MALE AND FEMALE MPB AND WBBB, EXPOSED (OR NOT EXPOSED) TO (*E*)-2-NONEN-6-ONE FOR 24 HR.

		Males[a]		Females[a]		
Species	Treatment	No. of samples[b]	*endo*-Brevicomin per beetle (ng, $\bar{X} \pm$ SE)	No. of samples[b]	*endo*-Brevicomin per beetle (ng, $\bar{X} \pm$ SE)	*P* of significant difference between males and females[c]
MPB	Unexposed	8	7.3 ± 1.4a	>10	n.d.	*
	Exposed	19	33.0 ± 5.0b	11	4.2 ± 0.9	*
WBBB	Unexposed	>10	n.d.	>10	n.d.	—
	Exposed	3	139.0 ± 76.0	1	27.9	*

[a] Means within a column for each experiment followed by the same letter are not significantly different, ANOVA, $P < 0.05$; n.d. = not detectable.
[b] Samples were normally prepared from three beetles.
[c] $*P < 0.05$; dash signifies comparisons not performed.

FIG. 4. Expected incorporation of [4,4-D_2](*Z*)-6-nonen-2-one into [D_2]-*exo*-brevicomin in vivo.

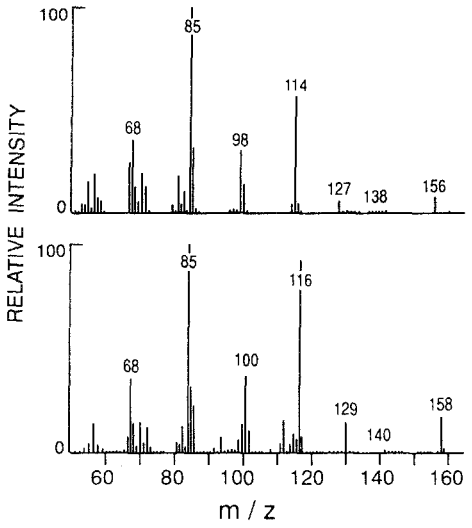

FIG. 5. MS (EI) of *exo*-brevicomin extracted from unexposed male MPBs (top panel) and from males exposed to [4,4-D_2](Z)-6-nonen-2-one (bottom panel).

enriched *exo*-brevicomin isolated from MPB and WBBB were virtually identical. As with (*E*)-6-nonen-2-one, extracts of both MPB and WBBB exposed to (*Z*)-6-nonen-2-one also contained excess precursor and the corresponding alcohol, (*Z*)-6-nonen-2-ol.

It is not clear from previous work if female MPBs and WBBBs produce *exo*-brevicomin. According to Pitman et al. (1969) emergent and fed female MPBs contained trace amounts of *exo*-brevicomin, but Pierce et al. (1987) failed to detect *exo*-brevicomin in fed female MPBs. *exo*-Brevicomin was not detected in female WBBBs (Borden et al., 1987). In our study, emergent unfed female MPBs and WBBBs did not contain detectable levels of *exo*-brevicomin unless they were exposed to (*Z*)-6-nonen-2-one. As in males, the *exo*-brevicomin produced by females exposed to the [4,4-D_2]-(*Z*)-6-nonen-2-one was enriched in two deuteriums. Although the quantity of *exo*-brevicomin produced varied from experiment to experiment (likely due to such factors as the health of the beetles, temperature and atmospheric conditions), female MPBs and WBBBs exposed to (*Z*)-6-nonen-2-one invariably produced less *exo*-brevicomin than exposed males, although this difference was not always statistically significant (Table 2).

Control (unexposed) male MPBs, and male and female MPBs exposed to [4,4-D_2](*Z*)-6-nonen-2-one, continued to emit *exo*-brevicomin after the initial treatment (Figure 6). *exo*-Brevicomin emitted by exposed male MPBs during the 48-hr aeration period was as enriched in two deuteriums as the first whole beetle extract. However, the *exo*-brevicomin extracted from these beetles after

TABLE 2. *exo*-BREVICOMIN PRODUCTION BY MALE OR FEMALE MPB OR WBBB, EXPOSED (OR NOT EXPOSED) TO (*Z*)-6-NONEN-2-ONE

Species and Exp.	Treatment	Males[a]		Females[a]		*P* of significant difference between males and females[b]
		No. beetles individually extracted	*exo*-Brevicomin per beetle (ng, $\bar{X} \pm$ SE)	No. beetles individually extracted	*exo*-Brevicomin per beetle (ng, $\bar{X} \pm$ SE)	
MPB						
Exp. 1	Unexposed (24 hr)	8	70.0 ± 14.0a	>10	n.d.	*
	Exposed (24 hr)	21	246.0 ± 30.0b	10	59.0 ± 12.0	*
Exp. 2	Unexposed (24 hr)	4	93.0 ± 18.0a	>10	n.d.	*
	Exposed (24 hr)	7	714.0 ± 163.0b	7	451.0 ± 142.0	N.S.
Exp. 3	Unexposed (16 hr)	4	20.4 ± 2.0a	>10	n.d.	*
	(24 hr)	9	47.0 ± 7.0a	>10	n.d.	*
	Exposed (16 hr)	4	71.9 ± 4.0a	4	14.3 ± 2.0a	N.S.
	(24 hr)	4	398.0 ± 136.0b	1	19.6a	*
WBBB	Unexposed (24 hr)	>10	n.d.	>10	n.d.	—
	Exposed (24 hr)	4	237.0 ± 57.0	2	50.0 ± 3.0	*

[a]Means within a column for each experiment followed by the same letter are not significantly different, ANOVA, $P < 0.05$; n.d. = not detectable.
[b]*$P < 0.05$; N.S. = not significant; dash signifies comparisons not performed.

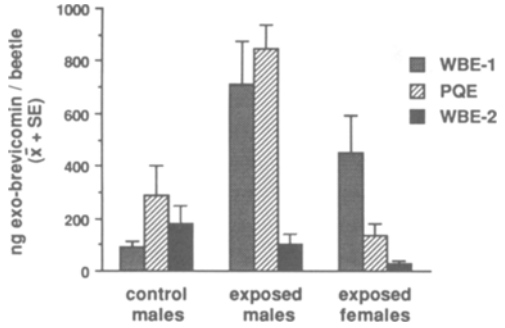

FIG. 6. Content of *exo*-brevicomin in extracts of male and female MPB exposed, or not exposed, to (*Z*)-6-nonen-2-one. WBE-1, whole beetle extract prepared after the 24-hr exposure period; PQE, Porapak Q extract; WBE-2, whole beetle extract prepared after the aeration period. Content of *exo*-brevicomin in exposed-male PQE is significantly greater than in exposed-female PQE, ANOVA, $P < 0.05$. *exo*-Brevicomin was not detected in extracts of unexposed female MPB.

TABLE 3. ENHANCEMENT OF $(M + 2)^+$ PEAK IN *exo*-BREVICOMIN ISOLATED FROM VARIOUS EXTRACTS OF MALE WBBB OR MPB, EXPOSED (OR NOT EXPOSED) TO [4,4-D_2](*Z*)-6-NONEN-2-ONE[a]

Treatment	No. insects individually extracted	Relative abundance ($\bar{X} \pm$ SE)	
		M^+	$(M + 2)^+$
Unexposed MPB			
Whole beetle extract 1	4	100.0	1.0 ± 0.0
Porapak Q extract	4	100.0	2.0 ± 0.5
Whole beetle extract 2	3	100.0	0.8 ± 0.0
Exposed MPB			
Whole beetle extract 1	2	21.9 ± 1.0	100.0
Porapak Q extract	7	20.8 ± 0.4	100.0
Whole beetle extract 2	7	28.5 ± 4.0	100.0
Exposed WBBB			
Whole beetle extract	4	18.0 ± 0.5	100.0
Precursor			
[4,4-D_2](*Z*)-6-nonen-2-one	—	13.9	100.0

[a]The relative abundance of the $(M + 2)^+$ peak in the MS of the putative precursor [4,4-D_2](*Z*)-6-nonen-2-one is provided for comparison.

the aeration period was less enriched in deuterium (Table 3), indicated by a larger proportion of unenriched molecules (M^+) relative to the enriched molecules [$(M + 2)^+$]. During the 48-hr aeration following the nonenone exposure, the exogenously supplied labeled precursor may have dropped to low enough levels to be significantly diluted by endogenously produced unlabeled precursor.

Male and female MPBs exposed to (Z)-6-nonen-2-one produced enough *exo*-brevicomin to determine its chirality by complexation chromatography (Schurig et al., 1983). Beetles of both sexes produced (+)-*exo*-brevicomin (Figure 7), as is naturally produced by male MPBs (this study; Johnston and Oehlschlager, 1982).

Although (Z)-6-nonen-2-one was undetectable in extracts of whole male MPBs and WBBBs, it was present in airborne volatiles of both beetles (Figure 8), indicating that it is a natural metabolite.

The biosynthetic origin of (Z)-6-nonen-2-one is not known. The compound (or a precursor thereof) may be supplied by the host tree or by fungi associated with the beetles (Borden, 1985; Vanderwel and Oehlschlager, 1987). Alternatively, the beetles themselves may produce (Z)-6-nonen-2-one, either through de novo synthesis or through the derivatization of compounds that contain a double bond in the ω-2 position. W. Francke (personal communication[4]) has

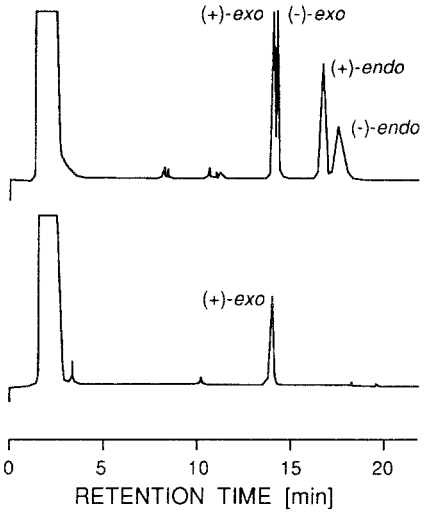

FIG. 7. Determination of chirality of *exo*-brevicomin produced by male MPB exposed to (Z)-6-nonen-2-one: GC trace of the standards (top panel) and purified extract from male MPB (bottom panel).

[4]Present address of Professor W. Francke: Institut für Organische Chemie und Biochemie der Universität, 2000 Hamburg 13, Martin-Luther-King-Platz 6, Germany.

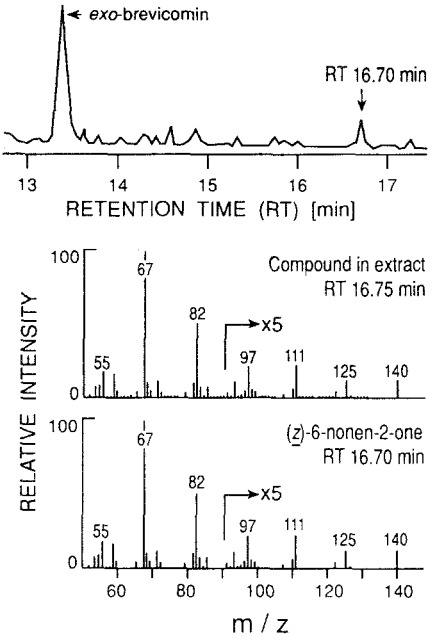

FIG. 8. GC-MS trace of a compound emitted by male WBBBs (top panel) with identical GC retention time and mass spectral characteristics as authentic (Z)-6-nonen-2-one (bottom panel).

suggested that (Z)-6-nonen-2-one might be derived from a 3-keto-(Z)-7-decenoyl intermediate, which could be biosynthesized through the oxidation of a (Z)-7-decenoyl intermediate. This 10-carbon fatty acyl intermediate might, in turn, be derived from a longer-chain fatty acyl precursor such as a (Z)-11-tetradecenoyl intermediate through limited chain shortening, as has been proposed to occur in tortricid moths (Roelofs and Bjostad, 1984).

Incorporation of (Z)-*6-Nonen-2-one into* exo-*Brevicomin: Biologically Relevant?*

A plausible mechanism for the observed conversion of (Z)-6-nonen-2-one into *exo*-brevicomin is through the epoxidation of the keto-alkene, followed by the cyclization of the thus-formed keto-epoxide, as hypothesized by Francke (1978) for *L. varius*. It is conceivable that poly-substrate monooxidases (PSMOs) might nonspecifically epoxidize exogenously supplied (Z)-6-nonen-2-one. Once formed, 6,7-epoxynonan-2-one is known to cyclize to brevicomin in the absence of an enzyme catalyst. Spontaneous cyclization of the keto-epoxide is known to be catalyzed by acid (Bellas et al., 1969), or it can be induced thermally under

neutral conditions (Wasserman and Barber, 1969). Thus, in the absence of other factors, one might not conclude that the conversion of (*Z*)-6-nonen-2-one to *exo*-brevicomin reported herein is biologically relevant.

Several facts provide compelling evidence that (*Z*)-6-nonen-2-one is indeed a natural precursor of *exo*-brevicomin in MPBs and WBBBs. (*Z*)-6-Nonen-2-one is a natural metabolite in male MPBs and WBBBs (Figure 8). The *exo*-brevicomin produced by MPBs exposed to the precursor was of the natural (+) chirality (Figure 7). Finally, although female MPBs weighed about 1.5 times as much as males, and would be expected to have PSMOs at least as active as the males, they did not "out-produce" males. To the contrary, female MPBs exposed to (*Z*)-6-nonen-2-one invariably produced less *exo*-brevicomin than males (Table 2) and, unlike males, did not continue to emit large quantities of *exo*-brevicomin during the 48-hr aeration period (Figure 6).

In conclusion, exogenously applied (*Z*)- and (*E*)-6-nonen-2-one are incorporated into *exo*-and *endo*-brevicomin, respectively, by both sexes of MPB and WBBB. The biochemical origin of these precursors and the biochemical mechanisms by which they are transformed to the brevicomins remain to be determined.

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ONTOGENIC VARIATION OF BIOLOGICALLY ACTIVE METABOLITES OF *Pteridium aquilinum* (L. KUHN), PTEROSINS A AND B, AND PTAQUILOSIDE IN A BRACKEN POPULATION OF THE TROPICAL ANDES

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Abstract—A negative correlation has been found between the amounts of pterosins A and B and ptaquiloside per biomass unit, and the growth stage of the blade of bracken. Their concentration decreased progressively from the crozier to the mature frond, where it attained less than 5% of the initial value. The growth was measured following the total blade length, its height, moisture content, and time of emergence from the soil surface. Quantitation of these compounds was achieved by HPLC using a water extraction, methylene chloride treatment, and silica gel microcolumn cleanup sequence. Pterosins were unevenly distributed in the blade, whereas ptaquiloside maintained a constant concentration throughout. Rhizomes contained only minor amounts of these compounds. Their possible role as semiochemicals in bracken is discussed.

Key Words—Bracken fern, *Pteridium aquilinum* var. *caudatum*, tropics, ptaquiloside, pterosin A, pterosin B, quantitation, HPLC, blade growth, semiochemicals.

INTRODUCTION

It has been known for nearly a century (Penberthy, 1893; Storrar, 1893) that bracken fern is the cause of various diseases in cattle (W.C. Evans, 1986), horse

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(W.C. Evans et al., 1951), sheep (W.C. Evans et al., 1973) and other farm and laboratory animals (W.C. Evans, 1986). Of major importance is the cancer scenario caused by the ingestion of bracken that is expressed as bovine enzootic hematuria in cattle (Rosenberger, 1971; I.A. Evans, 1979), in addition to carcinomas that affect liver, kidney, urinary bladder, mammary gland, and intestine of rats (Hirono et al., 1984a,b; Pamukcu and Price, 1969) and mice (Villalobos-Salazar et al., 1988). Furthermore, the milk produced by cattle exposed to feeding on *P. aquilinum* may be responsible for the high incidence of stomach cancer among people in Costa Rica (Villalobos-Salazar, 1985; Villalobos-Salazar et al., 1987, 1989a,b) and other countries (Galpin and Whitaker, 1989).

Nearly 100 metabolites of diverse structure have been isolated from bracken (Fenwick, 1988; Jones, 1983). Of these, ptaquiloside, an unstable illudane glucoside (Van Der Hoeven et al., 1983; Niwa et al., 1983; Ojika et al., 1987), was found to possess most of the carcinogenicity of the fern (Hirono et al., 1984a,b, 1987; Ojika et al., 1989).

Bracken is a cosmopolitan plant that grows in large thickets in the mountainous areas of the tropics (Ortega, 1990) in addition to the temperate regions of the world (Tryon, 1941). The dominance of *P. aquilinum*, reflected in its quick infestation of vast tracts of grazing land and its negative economic impact, provided impetus to the study of its ecological chemistry. In particular, it was of interest to evaluate the risk of exposing mammal herbivores to bracken in terms of ptaquiloside content as a function of frond development stage. While larger fronds are more easily perceived by grazing animals, they are also tougher and less palatable. In turn, smaller, younger fronds, albeit more difficult to find, are more tender and therefore may be more attractive as food to these animals.

The semiochemical composition and concentration of many plants are known to vary, inter alia, with the plant part and age (McKey, 1979). In bracken and other polypodiaceous ferns, the concentration of some sesquiterpenoids (Murakami and Saiki, 1989), flavonoids (Cooper-Driver, 1976), cyanogenic glycosides and phenolic acids (Cooper-Driver and Swain, 1976), pterosin F (Jones and Firn, 1979), and other bioactive compounds (Jones, 1983) have been found to vary significantly along the growing season. In the case of ptaquiloside, it is also known that it appears in higher concentration in the crozier than in the mature frond (Saito et al., 1989; Santos et al., 1989; Smith et al., 1989). However, this conclusion was based on plant samples collected nonsystematically in several regions and at different times of the year.

Hence, it was deemed desirable to obtain data on the systematic appearance of ptaquiloside in bracken in order to determine the cancerous risk potential of the plant for herbivorous mammals as a function of frond development. Pterosins A and B were also examined since their biological activity has also been reported (Saito et al., 1975). The results would also be an addition to the little known

chemistry of the carcinogenic var. *caudatum* (Villalobos-Salazar et al., 1988) that is the subject of the present study.

METHODS AND MATERIALS

Chemicals. A sample rich in ptaquiloside was isolated by water extraction of freshly cut fronds at room temperature following the method of Ojika et al., (1985). Pterosin B was obtained by extraction of fresh fronds with 0.03 N aqueous sodium hydroxide to convert the ptaquiloside into the desired pterosin B and increase its yield. This was followed by extraction with chloroform without neutralization to keep organic acids in the aqueous layer as sodium salts. The organic layer was dried over anhydrous magnesium sulfate, filtered, and evaporated over silica gel 60 (70–230 mesh) to prepare a column head for vacuum chromatography (Targett et al., 1979; Pelletier et al., 1986). Elution with hexane–ethyl acetate (EtOAc) 4:1 yielded 11 fractions of which fractions 3–6 contained pterosin B. Analytical samples were obtained by preparative thick-layer chromatography on silica gel 60, 2 mm thick, 20 × 20-cm glass plates using acetone–dichloromethane (DCM) 7:3 as developing solvent. Elution of the $R_f = 0.8$ band with warm EtOAc, evaporation, and recrystallization from hexane–DCM gave pure pterosin B as white needles, mp 106–107°C. This material was compared (IR, [^{1}H]- and [^{13}C]NMR) with an authentic sample that was recrystallized from the same solvent mixture.

Pterosin A was obtained from the *n*-butanol extraction of the initial frond water extract. The alcoholic layer was diluted with water previously saturated with *n*-butanol and washed in succession with petroleum ether and diethyl ether. The latter was evaporated to dryness and chromatographed through a silica gel column. Fractions eluted with hexane–EtOAc 7:3 contained pterosin A, which was purified further with the aid of thick-layer chromatography using DCM–acetone 3:7. Elution of the $R_f = 0.75$ band with warm EtOAc, evaporation, and recrystallization with hexane–DCM yielded pure pterosin A, mp 129–130°C (Yoshihira et al., 1971). Comparison of spectral data as above with an authentic sample provided confirmation of its identity.

Plant Sampling. Three flat 100-square-meter plots located at La Hechicera, Mérida, western Venezuela, at 1850 m altitude with low-density tree clusters of 8- to 10-m-high canopies and sparsely vegetated undergrowth, except for bracken, were chosen as study sites. As soon as the croziers could be seen to emerge from the soil surface, they were marked with sticks 30 cm from the stipe to avoid damage to the rhizome or roots. Growth was then followed through maturation by measuring the length of the stipe, the height of the blade by the length of the central rachis, and the total length of the laminae (see Figure 5).

Thirty fronds in each plot were measured weekly. Samples for chemical analysis were drawn weekly between 8:00 and 9:00 AM from January to April (dry season in the area) 1990 and the same period of 1991. Three fronds of the same age and approximately the same size were divided in two halves along the symmetry axis of the blade, one for moisture determination by drying to constant weight in an oven at 105°C and the other for extraction and chemical assay. Samples were clipped off with scissors, placed in previously weighed plastic clip bags, immediately stored in an ice box in the field, and carried to the laboratory within 15–20 min of collection. Typically fresh samples weighed 8–10 g (1.2–2.0 g dry weight). The bag contents were weighed, pooled in groups representing three fronds, and extracted with water in triplicate as described below. Rhizomes were sampled by cutting them approx. 10 cm from the base of the stipe. Collection, moisture content determination, and extraction was performed similarly.

Quantitative Extraction Procedure. Only nonreproductive fronds devoid of visible sporangia were used. After weighing, the fresh frond clippings were blended in distilled water (250 ml) with occasional stirring at room temperature for 2 hr. During this time little if any decomposition of ptaquiloside was expected to occur. The suspension was filtered through cheesecloth and around 60 ml of the filtrate was centrifuged (3000 rpm, 15 min). An aliquot (40.0 ml) of the clear supernatant was extracted exhaustively (5 × 25 ml) with DCM (extract 1). Emulsions were broken by centrifugation (3500 rpm, 10–15 min). The organic and aqueous phases were carefully divided. Aqueous sodium hydroxide (0.003 N, 10 ml) was added to the latter and the slightly alkaline mixture was warmed at 40–45°C for 2 hr to decomposed ptaquiloside quantitatively. The brownish solution was cooled to 10–15°C and extracted (5 × 25 ml) with DCM. The organic layer was centrifuged as above, dried over anhydrous magnesium sulfate, and filtered through DCM-soaked Whatman No. 1 filter paper into a 100-ml volumetric flask. The volume was corrected to 100.0 ml with chloroform and homogenized; four aliquots (20.0 ml) were drawn with the aid of a volumetric pipet and transferred to each of four pear-shaped 50-ml evaporation flasks, each containing 50 mg of silica gel 60 (70–230 mesh). Solvents were evaporated and the powdery residue was quantitatively transferred as column head to a Pasteur pipet fitted with a small cotton plug at the narrowing bottom neck that was previously filled with 250 mg of the same silica gel support. The procedure was repeated with an additional 25 mg of silica in all four replicates. The four microcolumns thus prepared (LeRoy and Goerlitz, 1970) were eluted first with 2.0 ml of hexane–EtOAc, 9:1, followed by 2.5 ml of the same mixture in a 1:2 ratio. The latter contained the ptaquiloside-derived pterosin B and was collected directly into a 2.0-ml volumetric flask. Solvents were evaporated under a current of dry argon or nitrogen gas, redissolved in HPLC grade methanol, and centrifuged to settle residual solids in suspension. The volume was corrected

to 2.0 ml with methanol and evaluated for pterosin content by HPLC. Extract 1 containing naturally occurring pterosins A and B was prepurified similarly and its quantitation achieved by HPLC. The recovery yields of pure analytes along the extraction–prepurification sequence was: 93 ± 2.1% for pterosin A; 94 ± 2.0% for pterosin B, and 93.8 ± 0.6 for ptaquiloside.

HPLC Analysis and Quantitation. Eight standard solutions of pterosin B were prepared in methanol in the 4.6×10^{-5} to 9.17×10^{-8} molar concentration range and were kept in tightly sealed volumetric flasks at 5°C until used. Optimal HPLC conditions were worked out using a LDC Milton Roy HPLC system equipped with two Constametric II pumps, a Waters Associates 490E, four-channel UV detector set at 220 and 260 nm (the UV maxima of pterosin), and a Waters Associates 10-cm reverse-phase radial microbondapack C-18 column of 10 μm particle size fitted to a 5-cm precolumn of the same stationary phase (40 μm). A water–methanol 35:65 mixture running at 1.0 ml/min at room temperature was found to give the best peak resolution. Retention times were 6 min 40 sec for pterosin A and 10 min 10 sec for pterosin B. Quantitation was derived from a minimum of seven 20-μl (loop size) injections per sample and coinjection with standards of the closest peak height range.

RESULTS

Frond Growth and Age. From the moment of emergence from the soil surface to the fully developed blade, bracken fronds in the site of this experiment reached maturity in around 70 days. Age (days from emergence) and the percentage of blade growth could be correlated as shown in Table 1. Growth measurements were made using a number of parameters such as length of the

TABLE 1. CORRELATION BETWEEN PERCENTAGE OF BLADE GROWTH AND TIME OF CROZIER EMERGENCE FROM SOIL SURFACE OF *Pteridium aquilinum* VAR. *caudatum* ($N = 30$, $P < 0.05$)

Days of emergence	Growth (%)
6	5.1 ± 1.5
12	11.3 ± 3.9
20	28.0 ± 14.3
26	41.9 ± 9.5
33	60.7 ± 9.9
40	80.2 ± 20.4
47	96.0 ± 29.6
68	100.0 ± 35.4

stipe from the ground level to the first division, L_s; length of the rachis, L_r, representing height attained by the frond; and total length of the frond L_t. Results are portrayed in Figure 1a–c. Moisture content was also found to decrease smoothly with age (Figure 1d).

The frond size spread over a wide range, depending on sunlight and competing vegetation, including earlier bracken blades. In order to avoid the chemical variance that frond size might introduce, only croziers and fronds representing the median population were chosen. However the median measurements cannot be determined until the frond has completed its growth, and it is essential to learn whether specimens for analysis are members of this median

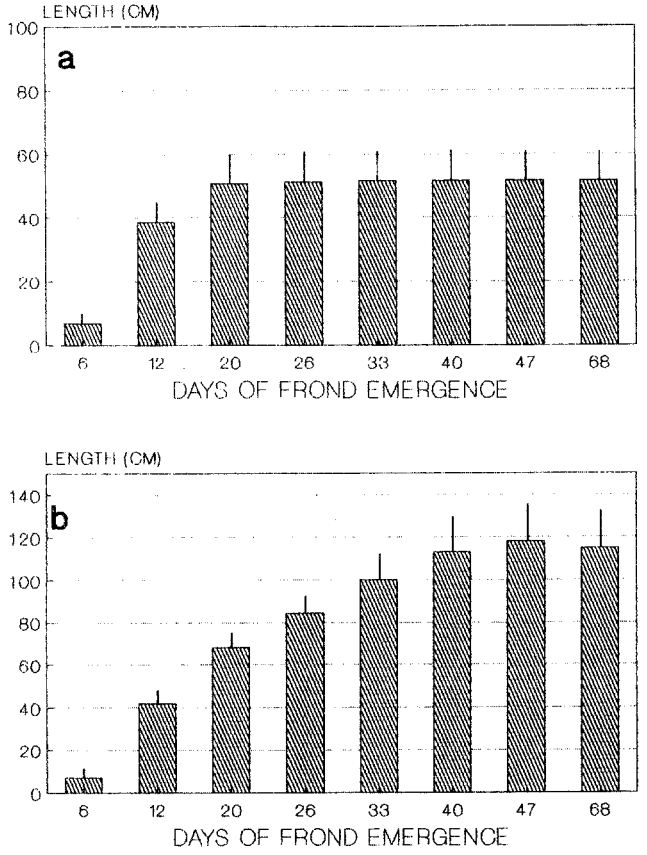


FIG. 1a–d. variation of blade growth parameters of *Pteridium aquilinum* var. *caudatum* with time of crozier emergence from the ground level, in the understory of a tropical cloud forest in the Andes of western Venezuela, during the dry season: (a) length of stipe; (b) length of rachis; (c) total blade length; (d) % moisture.

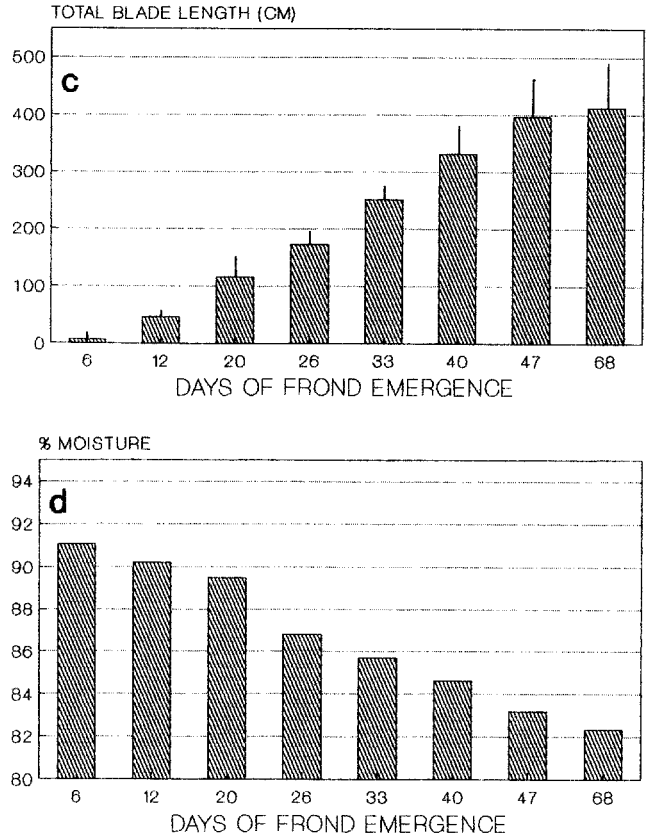


FIG. 1. Continued

population at all growth stages. To circumvent this problem, the length of the stipe to the first ramification L_s was employed. It was found that after two weeks of crozier emergence L_s reaches a maximum value that is maintained throughout frond development (Figure 1a). In addition, L_s correlated positively ($r = 0.80$) with the total blade length and expansion (Figure 2a). Hence, L_s is a nondestructive parameter for predicting at an early stage of development future frond size and allows sampling within the population of desired size range. L_r also correlated well ($r = 0.906$) with the frond expansion (Figure 2b).

Attempts to use leaf area as a criterion for growth was obstructed by the fact that croziers begin to develop unfolded leaflets only after 15–20 days, and thereafter the blade continues to grow in long, cylindrical shoots, sometimes reaching 60 cm upward, that do not contain segments or leaflets until several days later. Therefore, leaf area would not be representative of real growth. Data

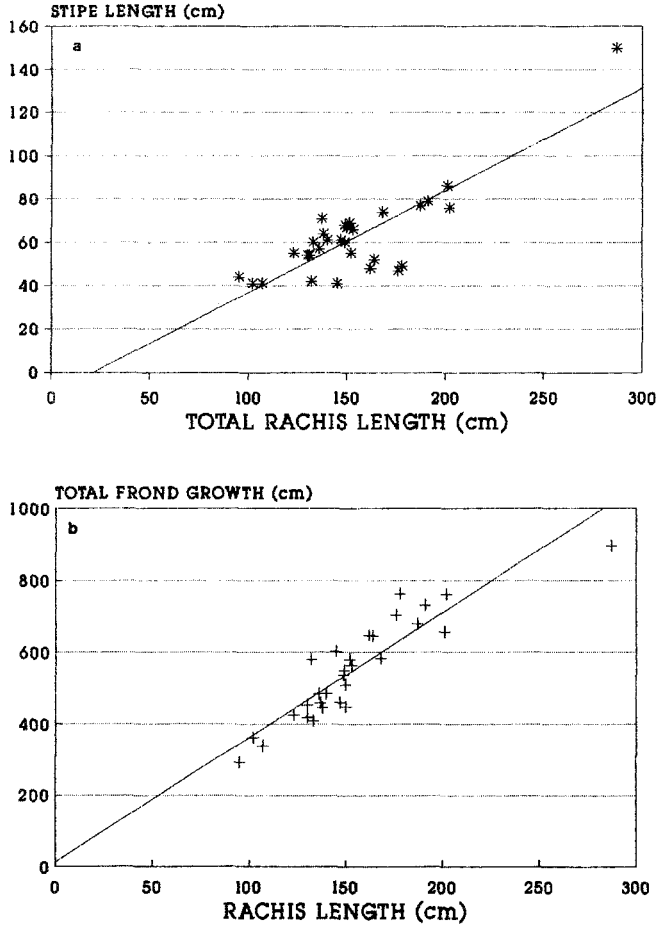


FIG. 2. Linear correlation of growth parameters: (a) rachis vs. stipe length; (b) rachis length vs. total frond growth.

on leaf area development in the literature (Jones, 1983) would be of little use for the present study as it refers to bracken varieties other than *caudatum* and a disparate environment.

Variation in Chemical Composition with Growth. Liquid chromatograms of extracts 1 and 2 containing naturally occurring pterosins A and B, and pterosin B derived from ptaquiloside, respectively, appear sufficiently well resolved for quantitation (Figure 3). Only mature, 68-day-old fronds produced an undetermined metabolite that interfered with the pterosin B peak in extract 1 and consequently could not be measured. Conversion of the base-produced pterosin B

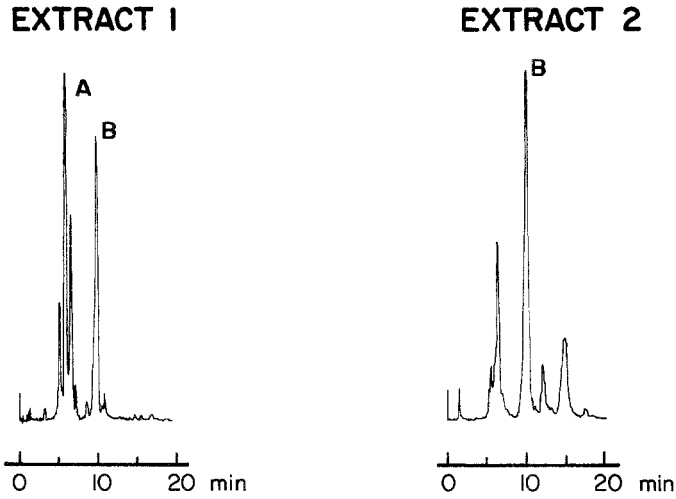


FIG. 3. HPLC chromatograms of extract 1 (naturally occurring pterosins A and B), and extract 2 (base hydrolysate of ptaquiloside) of bracken fern. Peak A refers to pterosin A and peak B to pterosin B. For instrument conditions, see text.

to ptaquiloside equivalents was achieved by multiplying the observed concentration by the molecular weight ratio of ptaquiloside to pterosin (1.8257).

The concentration of all three compounds examined varied markedly with blade growth. The highest amount was found in the 12-day-old crozier, typically representing a 41.8 ± 6.5-cm shoot without ramification or leaflets. The content of pterosin A rapidly contracted from 0.272 ± 0.06 ($P < 0.05$) to 0.012 ± 0.001 mg/g of dry frond weight during the first 33 days of crozier emergence from the ground and then underwent a sharp increase to 0.289 ± 0.004 by the 47th day when the frond was fully developed (Figure 4). The concentration of pterosin B followed a different course since it decreased progressively with growth from 0.66 ± 0.04 mg/g of dry fern tissue in the 12-day-old crozier to 0.084 ± 0.005 mg/g by the 47th day but showed no significant increase thereafter (Figure 4). Ptaquiloside exhibited the highest concentration of the three components, at 1.67 ± 0.31 mg/g at the crozier stage and was reduced to 0.113 ± 0.03 mg/g in the mature frond (Figure 4). On the other hand, the rhizomes around the base of the blade contained only a small fraction of the pterosins and ptaquiloside in the plant, being 0.0029 ± 0.0004 mg/g for pterosin A, 0.0044 ± 0.0007 mg/g for pterosin B, and 0.0046 ± 0.0009 mg/g for ptaquiloside.

Variation in Chemical Composition with Plant Part. Three fronds of equal size and grade of development (42–45 days old, 80–85% of development) were sampled as indicated in Figure 5. Being at an intermediate stage, these fronds featured three distinct structures: the lower base pair with fully developed, albeit

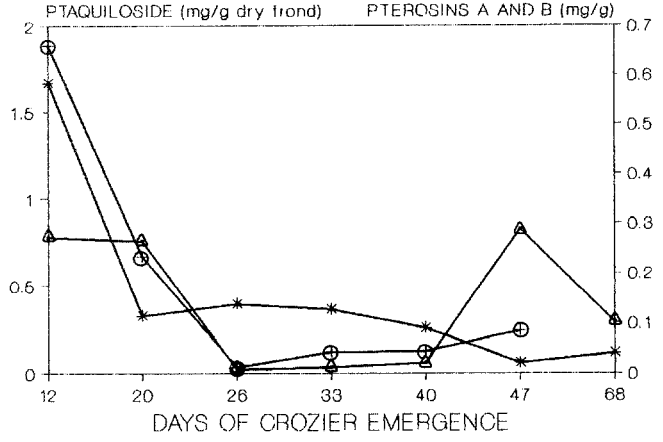


FIG. 4. Variation in chemical composition of bracken fern blades in milligrams of compound per gram of dry frond, with frond age: ★: ptaquiloside (left ordinate scale); △: pterosin A (right ordinate scale); ⊕ ; pterosin B (right ordinate scale).

tender leaflets; the second middle pair with unfolding leaflets; the upper part composed of unfolding, curled tops only. Results appear in Figure 5. While pterosins A and B are unevenly distributed, with the highest concentration in the middle pair, ptaquiloside concentration remains constant in all parts examined.

DISCUSSION

Ptaquiloside is so unstable that its isolation and characterization proved to be a task of utmost difficulty (Hirono, 1986). This quality renders its direct quantitation nearly impossible, although a recent attempt has been reported (Agnew and Lauren, 1991). Nevertheless, ptaquiloside is known to undergo transformation into pterosin B under heat, acid, or base (Matoba et al., 1987) according to Scheme 1. The stable 1-indanone obtained therefrom has a strong UV active chromophore (261 nm, log e = 4.19) as opposed to the weakly absorbing ptaquiloside. The base decomposition of ptaquiloside was preferred over the direct quantitation (Agnew and Lauren, 1991) or the thermal transformation (Saito et al., 1989), owing to its mildness (pH 11.5, 40°C) and completeness within a short period of time. In addition, HPLC offered higher sensitivity over TLC photodensitometry (Saito et al., 1989) for the quantitation of the derived pterosin B.

Bracken, like many other ferns, may be a large organism (Shefield et al., 1989) of which only the blades are visible. The complex array of rhizomes

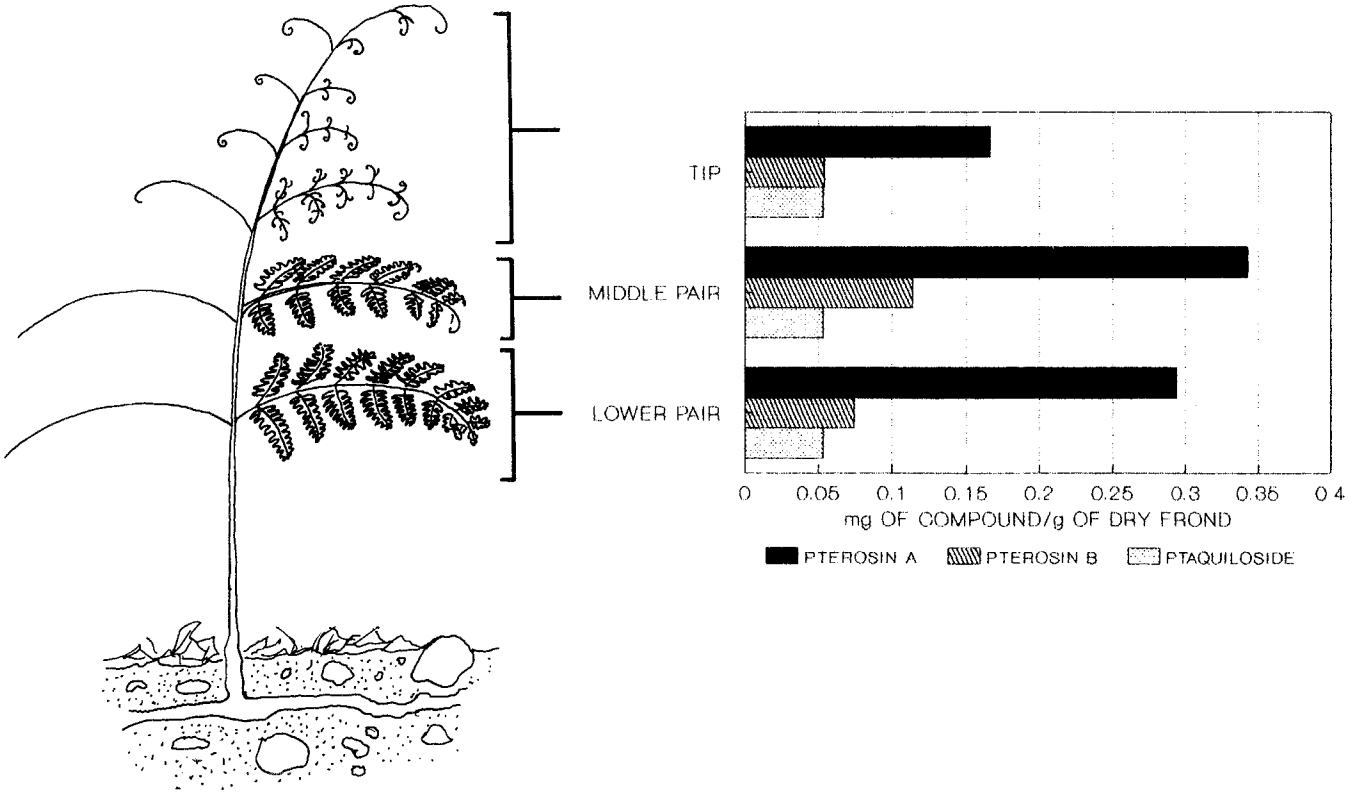
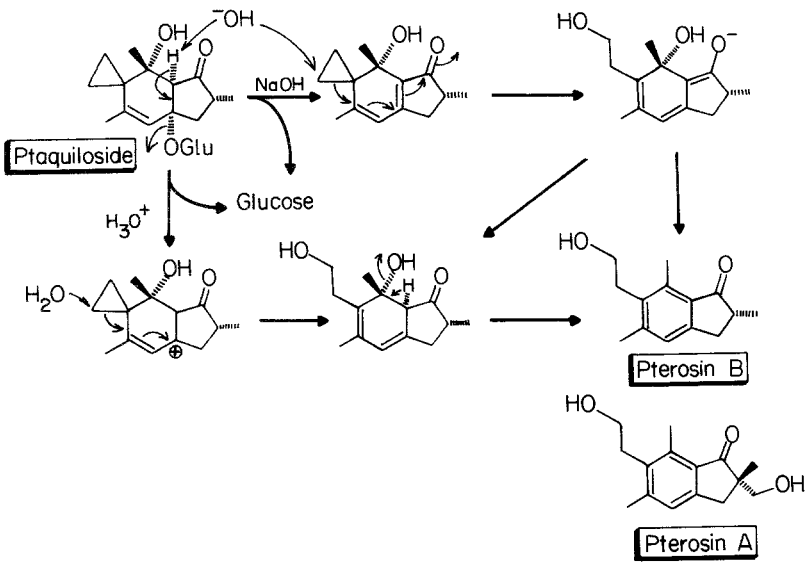


FIG. 5. Variation of chemical composition in bracken fronds, in milligrams of compound per gram of dry frond, with blade part.



SCHEME 1.

overwinters or remains active after fire to yield new frond growth when conditions become appropriate. Therefore blade development represents the growth of only part of the fern. In spite of this, metabolites in the fronds may be expected to vary with the age of the fronds as in many other plants because photosynthesis and a major portion of the metabolic process occur there.

While season is a crucial parameter to take into account to measure plant development in temperate climates, it is of less importance in tropical areas because temperature and photoperiod show very little variance. Only rainfall shows a consistent yearly cycle, although the dry season is usually short (60–90 days) in the tropical Andes above 1000 m altitude, and soil undergoes desiccation only in the first few centimeters of depth except in steep slopes and well-drained soils. In fact, bracken in the Mérida area, either in the open savanna or in the forest understory, develops viable croziers without interruption all year round. Therefore, chronological age of the frond rather than stage of the growing season was found more appropriate to follow plant growth in our location.

The present results show that even under the tree canopy where sunlight is partially blocked, bracken fronds grow very fast, reaching the mature stage in only 60–70 days. By comparison, competing grasses in the open sunlight require much longer periods, extending from three months to one year (Baruch et al., 1989). This fast development becomes a source of perennial food for herbivores, particularly during the dry season when planted or natural grasses become scarce.

Cattle frequently wonder along the edges of forest or into the understory where moisture is better preserved and bracken develops softer blades, thus becoming more palatable to them. The combination of frond availability and the higher concentration of ptaquiloside found in younger, savory tissue increases considerably the risk of poisoning of farm animals and other mammalian herbivores during the dry season.

The concentration pattern of pterosins A and B and ptaquiloside with frond development stage found here runs in parallel with earlier findings (Saito et al., 1989; Santos et al., 1989; Smith et al., 1989). In particular, Jones and Firn (1979) observed a gradual seasonal decrease in pterosin F concentration and deterrence activity of bracken (var. *aquilinum* from England) leaves against several nonadapted phytophagous insects between May and October. However, the frond development stage of plant specimens was not specified, and the growth rate was conceivably different owing to climatic and soil dissimilarity, so care must be exercised when comparing their results with the present report. Their study revealed, on the one hand, that the seasonal variation in pterosin F concentration was similar to the seasonal variation in deterrence activity of crude extracts of the fronds. On the other, not all the deterrence activity could be attributed to pterosin F alone; other active, noncharacterized fractions were present as well. The dynamics of pterosins A and B and ptaquiloside found here show the same pattern, thus suggesting a protective role against herbivory by these compounds also. In spite of its small apparency (Feeny, 1976) the tender crozier requires the highest protection because damage at this stage is more likely to be deleterious to plant fitness. In burned land around Mérida where bracken is the first and dominant growth, and thus the only food source available to insects or rodents in a relatively large area, crozier stumps with the growing tips removed are observed. Croziers sustaining such damage do not develop further and die. Therefore bracken may have evolved metabolic mechanisms to concentrate qualitative chemical defenses (Feeny, 1976) in these fragile tissues.

The disparate concentration of pterosins A and B as opposed to the uniform distribution of ptaquiloside in blades of intermediate development suggests different allocation mechanisms for these compounds. While the water-soluble ptaquiloside may be concentrated and transported in the vascular system, the less soluble pterosins may be deposited in appropriate structures within the fresh segments. Pterosin B results from light-induced decomposition of ptaquiloside (Saito et al., 1989). Hence, it is conceivable that the latter is a relocalizable source of pterosin B in the frond in addition to the light-stable pterosides (pterosin glycosides) also present in bracken. By the same token, pterosin A may have a ptaquiloside-like precursor of its own. In fact, such a compound, dennstoside A, has just been reported (Koyama et al., 1991) in *Dennstaedtia scabra*, a fern of the pteridaceae family to which *P. aquilinum* belongs, but still awaits discovery in bracken. This may be part of a complex array of chemical defenses

that makes *P. aquilinum* one of the most resistant and successful species in the plant kingdom (Harper, 1977; Page, 1979, 1982).

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CHEMICAL COMPOSITION OF SEX PHEROMONE OF ORIENTAL FRUIT MOTH AND RATES OF RELEASE BY INDIVIDUAL FEMALE MOTHS

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Abstract—The sex pheromone emitted by individual calling females of the oriental fruit moth, *Grapholita molesta*, was trapped within glass capillaries, and the composition and release rates were determined by gas chromatography–mass spectrometry. Aerial release of (*Z*)-8-dodecenyl acetate ranged up to 25.3 ng/hr, while the mean release rate was 8.48 ± 7.26 ng/hr (±SD). The proportion of (*E*)-8-dodecenyl acetate to (*Z*)-8-dodecenyl acetate was remarkably constant (4.20 ± 0.60%). Significant amounts of dodecyl acetate were also recovered but, contrary to previous reports, only trace quantities of (*Z*)-8-dodecenol were detected in the effluvium.

Key Words—*Grapholita molesta*, Lepidoptera, Tortricidae, sex pheromone, effluvium, release rates, GC-MS, (*E*/*Z*)-8-dodecenyl acetate.

INTRODUCTION

Control of the Oriental fruit moth, *Grapholita molesta* (Busck), (OFM), by dissemination of synthetic sex pheromone has been operational in Australia for several years (Vickers, 1990), but little is known about how the synthetic pheromone affects moth behavior to bring about mating disruption. An understanding of this process might lead to improved efficacy and also help in the application of mating disruption for other pest species.

In any study of mating behavior, including mating disruption, it is important

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[3]Work carried out while on work transfer to CSIRO, Canberra.

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to have accurate measurements of the chemical composition of the pheromone released by the female moths and of the amount of pheromone released by the moths under natural conditions. The major component of the sex pheromone of OFM was identified by Roelofs et al. (1969) as (Z)-8-dodecenyl acetate (Z8–12:OAc). Subsequently, Cardé et al. (1979) identified three additional components, (*E*)-8-dodecenyl acetate (*E*8–12:OAc), (Z)-8-dodecenol (Z8–12:OH) and the saturated alcohol dodecanol (12:OH), although 12:OH may be redundant in the communication system (Linn et al., 1986). Previous studies have also been made of the composition and quantity of sex pheromone released by female OFM (Cardé et al., 1979; Baker et al., 1980), but these determinations were from material collected on the inside surfaces of containers housing groups of insects. The capillary trapping method of Shani and Lacey (1984) enables determination of pheromone released by individual female moths and correlation with calling behavior (Barrer et al., 1987). The technique used here is a refinement of this for making simultaneous collections from individual females and is designed to improve the efficiency and accuracy compared to previous methods.

METHODS AND MATERIALS

Insects. The female moths were from a laboratory colony maintained at the Division of Entomology, CSIRO, Canberra. The colony was established from insects collected from a peach orchard near Cobram, Victoria. A few insects were collected between January 15 and 17, 1990, but survival was poor, and a further 15 female moths and a surplus of males were collected from bait pails (Rothschild et al., 1984) between February 15 and 17, 1990. The colony was maintained on green apples. Pupae were segregated by sex and the females kept at 25°C on an advanced 16:8 light–dark cycle (scotophase 1200–2200), and variable relative humidity (between 30 and 60%). Emerged moths were collected each morning and kept in 8-oz waxed cardboard cups (85 mm diameter, 50 mm high) and supplied with a 5% sugar solution.

Calling Behavior. We carried out limited observations on the calling behavior of female moths to ensure that the behavior of the Australian population is similar to that reported previously for the North American population (Baker and Cardé, 1979a). About 5 hr before lights-off on the day of observation, female moths were placed individually in glass vials (2 cm diameter, 5 cm high), which were covered by fine mesh cloth. The individual moths were then observed at 15-min intervals and their behavior recorded. Observations were begun 30 min after the females were placed in the vials and ended at lights-off.

Pheromone Collections. The collection apparatus consisted of six inverted filter flasks (50 ml), each supported by a retort stand. Air (Medical Grade, CIG,

Australia) was introduced through a glass wool plug in the side arm of each flask at 100 ml/min to preclude the entry of volatile contaminants during the collection.

Approximately 5 hr before lights-off, a female moth was placed in each flask and the opening was covered by a steel gauze. The moths were from collections of emerged moths made two days previously, i.e., they had experienced two, or possibly three, scotophases before the start of the pheromone collection. A glass capillary (60 cm long, 0.9 mm ID, 2.0 mm OD) supported by a retort stand, was inserted into the flask through the gauze cover and carefully positioned with the tip close (< 1 mm) to the tip of the female's abdomen. In most instances, it was possible to position the capillary orifice so that it cupped the tip of the abdomen, although care was taken to ensure that at no stage was there any contact between capillary and insect. Air was then drawn through each capillary at a rate of 25 ml/min, and the effluvium from each calling female was trapped on the glass walls of the capillaries.

Behavior of each female was noted every 5 min. Collection of the effluvium was begun from each female when she started calling and continued for as long as she remained stationary. In some instances, where the females maintained the calling position for several hours, the capillaries were changed at hourly intervals (maximum 3 hr). On a few occasions, collections were made from female moths that were not visibly calling, but that had remained stationary for over 1 hr. At the end of the collection period, the inner surface of each ice-cooled capillary was washed by gravity flow with 5×2 μl quantities of pentane (Mallinckrodt nanograde redistilled) from the inlet to outlet end (previously sealed to retain the washings). Known quantities of internal standard [(Z)-10-dodecenyl acetate] were then added to each solution and mixed thoroughly. The pheromone solutions were sealed within the capillary tubes and stored at -20°C until required for mass spectroscopic analysis.

Pheromone Determinations. Gas chromatography–mass spectrometry (GC-MS) was carried out on a VG 70-70 mass spectrometer (VG Analytical, Manchester) interfaced directly to an HP 5792A capillary gas chromatograph (Hewlett Packard) and a VG 11-250 data system. Solutions for analysis were introduced by way of an HP 19290A on-column injector. The cross-linked chromatography columns used (DBWax, J&W, 30 m length, 0.32 mm ID, 0.5 μm phase thickness; BP20, SGE, 25 m length, 0.33 mm ID, 1.0 μm phase thickness) were preceded by retention gaps of deactivated fused silica (SGE, 4 m length, 0.33 mm ID).

The on-column GC methodology used here to analyze the effluvia enabled transfer of mixtures through the gas chromatograph with minimal molecular discrimination (Grob, 1987). The use of a retention gap as a fore-column preserved the column's integrity and permitted the injection of large volumes without seriously jeopardizing the chromatographic resolution. Large injection

volumes allow the full dynamic range of sensitivity to be exploited because the concentration of solutions to very small volumes often leads to major loss of the solutes on surfaces or by evaporation. While very long retention gaps are normally prescribed for accommodating large injections (Grob, 1987), it was found in the present work that equivalent results could be obtained by injecting small aliquots (2–3 μl) in sequence onto a short retention gap such that the solvent evaporated between injections. The high conductance of the source diffusion pump was adequate to dispose of the vapor without electrical discharge.

The GC columns and retention gaps were reserved for effluvium analyses. Helium (ultra-high purity, CIG Australia) was used as a carrier gas, and a typical temperature program for elution of the pheromone constituents was 30°C isothermal followed by a gradient of 30°/min to 165°C isothermal (DB Wax). Injections of samples could be accomplished at 20- to 25-min intervals under such conditions.

The ionization mode adopted for determination of the pheromone components in the present study was negative-ion chemical ionization (NICI) using hydroxyl ions as the reagent. The OH^- ions were formed in the source by the addition of nitrous oxide (instrument grade, CIG Australia) to methane (ultra-high purity, CIG Australia) at a combined pressure of 40 Pa. The ionization energy was 100 eV, the source temperature 190°C, and the filament emission 1 mA. Selected ion monitoring of $(M - H)^-$ ions was accomplished at constant magnetic field by alternation of the accelerating and electric sector voltages under data system control, with dwell times of 75 msec per channel.

The origins of the reference chemicals were as follows: Acetates and alcohols were synthetic standards (Shin-Etsu, Tokyo, Japan) while aldehydes were prepared as required by Jones oxidation of the analogous alcohols. Purities were assessed by gas chromatography and gas chromatography–mass spectrometry. The compounds were stored at −80°C and stock solutions were stored at −20°C.

RESULTS

Calling Behavior. Postures of female OFM during the 5 hr before scotophase varied considerably. Of 148 observed females, 130 (88%) visibly extruded their ovipositors at some stage, although the degree to which they did this varied. Of these, 23 (16% of the total) spread or raised their wings for at least part of the time that they had their ovipositors extruded. The remaining 18 (12%) showed no visible calling behavior during the observation period, although they did remain stationary for most of the time, with their antennae raised and curved back over the body in an "alert" position. If undisturbed, most females that were visibly calling remained in the calling position for 2–3 hr.

At constant temperatures of 21–22°C, the first females started to call about

$4\frac{1}{2}$ hr before lights off (Figure 1) and the maximum rate (75% of the females) was achieved $2\frac{1}{2}$ hr before lights off and continued until lights off. At 24°C, the onset of calling was delayed about $1\frac{1}{2}$ hr and the maximum rate was not achieved until just before lights off.

Efficacy of Capillary Traps. The capacity of the capillary trap to retain the main pheromone component, Z8–12:OAc, under the conditions in this study was assessed by collecting the effluvia over an extended period of time from females that were clearly calling throughout the collection period. In one case, 46.3 ng of Z8–12:OAc was trapped over a period of 110 min, in another, 60.2 ng over 170 min. In both cases, <0.1% of the total amount of Z8–12:OAc was found in the downstream 30 cm of the 60-cm-long capillary. Although these data indicate that 30-cm capillaries would be sufficiently long to retain the pheromone constituents if they are replaced at hourly intervals, we preferred 60-cm-long capillaries to minimize possible breakthrough of more volatile potential minor components.

Individual Release Rates of Z8–12:OAc. Among individuals that exhibited

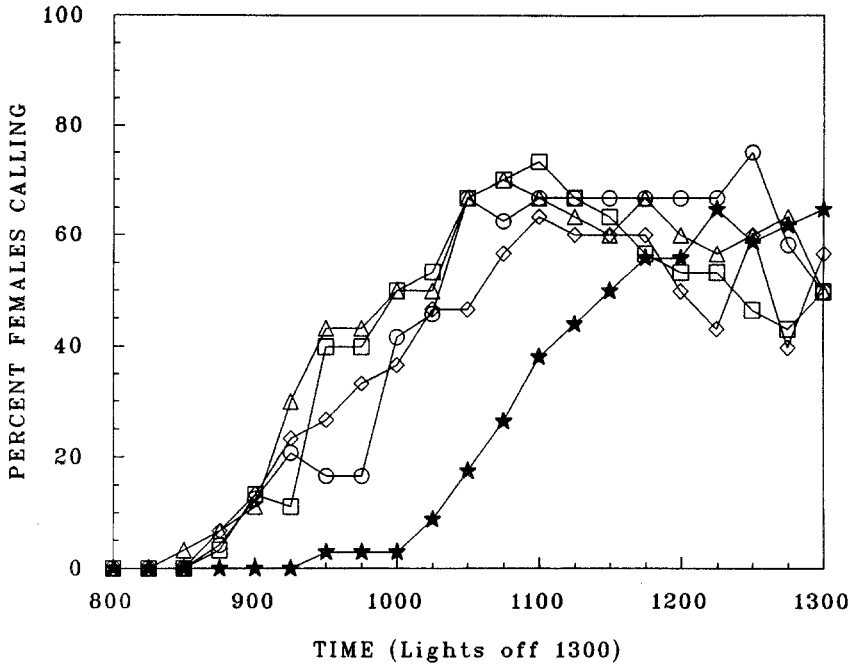

FIG. 1. Percentage of female oriental fruit moths calling, showing effects of age and temperature. Circles, 1-day-old females (N = 24); triangles, 2-day-old (N = 30); square, 3-day-old (N = 30); diamond, 4-day-old (N = 30); all kept at 21°C. Solid stars, 1- to 5-day-old females kept at 24°C (N = 34).

calling behavior during at least some of the collection period, the mean release rate (±SD) of Z8–12:OAc was 8.48 ± 7.26 ng/hr (N = 40, range 0.0–25.3 ng/hr) (Figure 2). Those females that did not exhibit any calling during the collection period released little if any Z8–12:OAc (range 0.0–0.12 ng/hr, N = 6).

For some females that exhibited prolonged calling behavior, effluvia were trapped over several successive intervals of about an hour each. The results (Table 1) indicate that most females maintain about the same rate of release throughout their calling period.

Ratio of Z *to* E*8–12:OAc in Effluvium.* The E:Z8–12:OAc ratios were measured in the effluvia from 32 individual females (Figure 3). The mean was 4.20% E isomer (SD = 0.596) to 95.8% Z isomer, with a range of 3.00 to 5.30% E isomer.

Additional Constituents of Effluvium. The alcohol, Z8–12:OH, was detectable in the effluvia of all strongly calling females, but the amount was only 0.45 ± 0.19% of the Z8–12:OAc (N = 11). Another minor constituent of the effluvia was (Z)-8-dodecenal (Z8–12:Ald) with a relative abundance of 0.42 ± 0.17%, similar to that of the Z8–12:OH. E8–12:Ald was also present in the effluvia

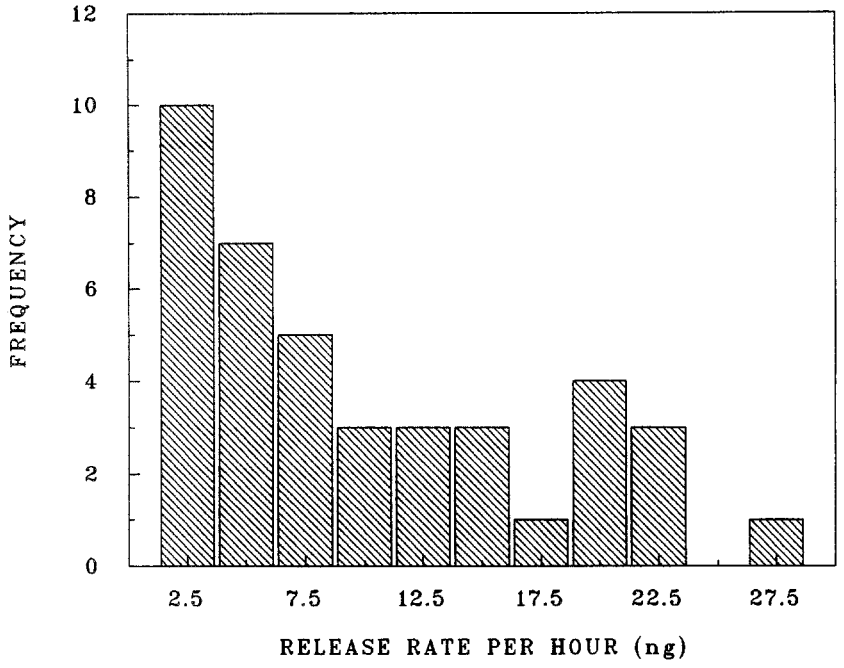

FIG. 2. Frequency distribution of release rates of (Z)-8-dodecenyl acetate (ng/hr) by female oriental fruit moths (N = 40).

TABLE 1. RELEASE RATE OF (Z)-8-DODECENYL ACETATE BY CALLING OFM FEMALES OVER SUCCESSIVE COLLECTION PERIODS SHOWING DURATION OF COLLECTION AND PERCENT *E*-ISOMER IN COLLECTED EFFLUVIA

Ref. # of female moth	Collection period								
	A			B			C		
	Collection time (min)	ng/h	%*E*	Collection time (min)	ng/h	%*E*	Collection time (min)	ng/h	%*E*
1	140	8.9	4.6	30	9.6	4.6	—		
2	115	6.9	3.7	50	6.2	4.3	—		
3	60	13.8	4.5	100	17.2	4.8	—		
4	60	14.2	4.9	60	18.9	5.0	60	27.2	5.1
5	63	8.5	4.3	60	9.0	4.2	55	14.1	4.1
6	60	15.6	4.5	60	14.0	4.0	55	11.8	4.3
7	60	21.9	4.9	60	23.4	4.9	50	15.2	5.0
8	60	9.6	3.9	45	3.0	3.3	—		
9	40	16.9	4.4	55	22.0	4.7	—		
10	65	8.9	2.9	80	11.3	3.0	—		
11	60	0.5	—	75	5.6	3.6	—		
12	60	6.4	3.9	55	7.7	4.1	—		

of some calling females, but only in trace amounts.

Another identified constituent was the saturated acetate, dodecyl acetate (12:OAc), which had, on average, an abundance of $3.44 \pm 1.16\%$. Also detectable in the effluvia were the saturated alcohol and aldehyde, dodecanol and dodecanal. These are present in only trace amounts, and blank experiments demonstrated that the presence of both can be attributed predominantly, if not entirely, to trace impurities in the hydrocarbon solvent (pentane).

DISCUSSION

The calling behavior and the periodicity of calling of OFM female moths in our experiments are similar to those described by Baker and Cardé (1979a) for OFM in the United States. From these two studies we conclude that during the first generation of moths in each field season in Australia, when temperatures are in the low 20°C range (Rothschild and Minks, 1974), sexual activity starts about $4\frac{1}{2}$ hr before sunset, and if temperatures drop below the threshold of 15°C, calling ceases (Rothschild and Minks, 1974; Baker and Cardé, 1979a). In subsequent generations, when temperatures rise into the 30s, the onset of calling is

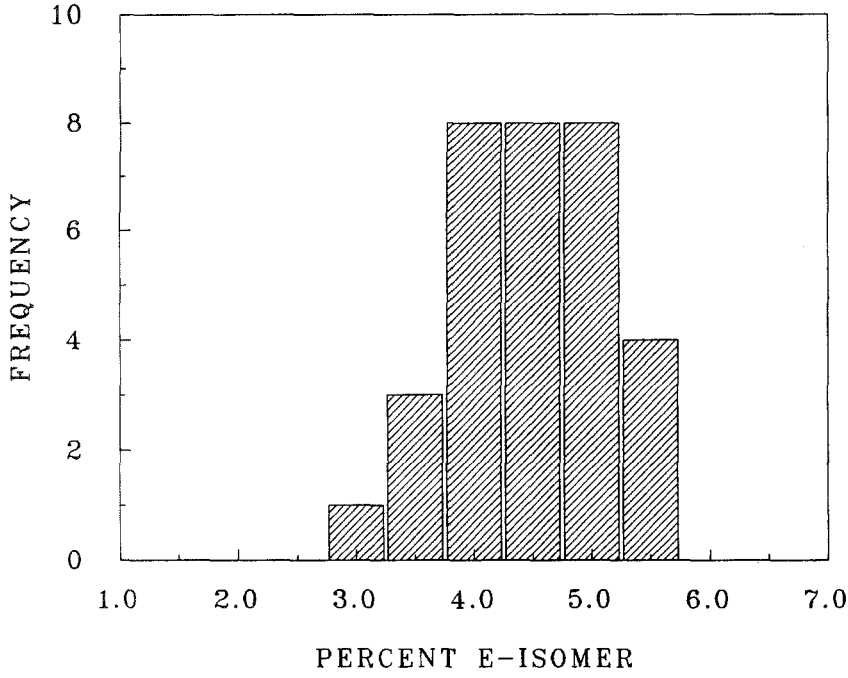

FIG. 3. Frequency distribution of percent *E* isomer of Δ-8-dodecenyl acetate in pheromone collected from individual female oriental fruit moths ($N = 32$).

delayed, and because all sexual activity ceases soon after dark, the period of sexual activity is restricted to only one or two hours (Rothschild and Minks, 1974; Vickers et al., unpublished data). Considerable variation was found in the postures adopted by calling female moths. Conner and Best (1988) have shown that airflow is faster around female moths that adopt a raised-wing calling posture, which in turn increases the volatilization rate of pheromone from the gland surface. Some of the highest release rates in the present study were from females with raised wings, although these could not be attributed to air currents because there was little air movement inside the flasks. Under field conditions, however, where moving air would be a factor, even greater differences in release rate among females can be expected.

Several investigators have used glass-adsorption techniques, involving either static or moving air, to collect the pheromone released by calling female moths. Placing moths in a closed container with static air is a simple and convenient technique, and recovery rates of synthetic standards up to 90% have been reported. However, 75% of the pheromone emitted by the moths can be lost due to adsorption onto the bodies of the insects themselves (Baker et al., 1980;

Ramaswamy and Cardé, 1984; Robacker and Johnson, 1989) and by enzymatic breakdown by the moth scales (Morse and Meighen, 1984), which introduces a major source of error. The situation can be improved by introducing an airflow (Weatherston et al., 1981), but some losses due to adsorption by the moths and by enzymatic breakdown can still occur. Furthermore, the results provide only average figures for groups of moths. This problem can be avoided by confining individual insects in glass tubes and forcibly extruding the gland into an airstream (Haynes et al., 1983, 1984; Haynes and Hunt, 1990). However, forcibly confining the female interferes with normal behavior and precludes the determination of natural release rates of pheromone, which was the main objective of the present study.

In a significant improvement in technique, the female is allowed to call spontaneously in a glass container while its effluvium passes through a small trap (Bjostad et al., 1980; Barrer et al., 1987; Schal et al., 1987; Witzgall and Frérot, 1989). However, the methods of Bjostad et al. (1980) and Schal et al. (1987), which require relatively large amounts of solvent in order to recover pheromone from Porapak Q adsorbent in the traps, are inappropriate for identification and quantification of subnanogram quantities of components and can vitiate accurate assessments of fractional composition. Moreover, while these limitations are easily obviated by methods that use bare glass capillaries as individual traps (Barrer et al., 1987; Witzgall and Frérot, 1989), their design does not guarantee an unimpeded passage of aerially released pheromone from gland to trap (Shani, 1990). The capillary method used in the present study solves all these problems. The female moth is free to adopt a natural calling position, and the emitted effluvium is drawn immediately into the glass capillary before it can come into contact with the body of the moth and from where it can be removed with small amounts of solvent. The technique is particularly well suited to insects such as the OFM, where the female moths will call in a small container, adopt a characteristic calling position and remain stationary for long periods of time. Loss of material from the air drawn into the capillaries was unlikely. At a flow rate of 25 ml/min there was no breakthrough with a 30-cm length of glass tubing (ID 0.9 mm), and for most of the collections a 60-cm length was used. However, the quantity of pheromone collected will be affected by the positioning of the capillary. This was dependent upon the position adopted by the female, which, when vertical with a well-extruded gland, allowed precise positioning of the capillary.

It was from females such as these that successive collections were made. This involved the insertion and positioning of new capillaries for each collection. Similar results were obtained on each occasion (Table 1), which demonstrates the reliability of the technique when the females are positioned conveniently. However, it was difficult to position the capillary accurately on females that adopted horizontal positions either on the sides or the roof of the flasks. For

this reason it is probable that the estimates for some females are low and the distribution of release rates is probably biassed towards the low end of the range.

The average release rate of 8.5 ng of Z8–12:OAc/calling hour is nearly three times the rate per insect (3.2 ng/hr) recorded from groups of female OFM by Baker et al. (1980). The reason for this discrepancy probably lies in the methods of collection. As pointed out above, the capillary method appears to be highly efficient when the capillary can be well positioned and, because the effluvium is drawn into the capillary immediately, there is little chance for it to be adsorbed onto the moth's body or to be broken down enzymatically. Baker et al. (1980) placed groups of 50 females in flasks, left them for 3 hr in static air, and then rinsed the walls of the flasks with hexane to collect the effluvia adsorbed onto the glass. They estimated that 90% of the material was adsorbed onto the moths, and they had to correct their estimates accordingly, which introduces a large uncertainty. Furthermore, it is very probable that there was considerable disturbance among the 50 moths in each flask and, although estimates were adjusted for the numbers of females calling, disturbed females probably produce less pheromone per hour because of delays in start-up.

Comparisons with other species of Lepidoptera are difficult because of the different collection techniques used and the different durations of collection. However, the release rates for OFM are similar to those recorded for other Tortricidae. Female spruce budworm, *Choristoneura fumiferana* (Clem.), release about 10 ng/calling female per hour (Silk et al., 1980; Ramaswamy and Cardé, 1984) with bursts of up to 50 ng/hr (Morse et al., 1982). Female carnation tortrix moths, *Cacoeciomorpha pronubana* Hbn., average 27 ng/hr with peaks close to 50 ng/hr (Witzgall and Frérot, 1989). The lowest rates reported from female Lepidoptera are a few nanograms per hour from the almond moth, *Ephestia cautella* (Wlk.) (Barrer et al., 1987; Shani, 1990). Forcibly extruded glands of the artichoke plume moth, *Platyptilia carduidactyla* (Riley) (Haynes et al., 1983), the pink boll-worm, *Pectinophora gossypiella* (Saunders) (Haynes et al., 1984), *Heliothis virescens* (F.) (Pope et al., 1982), and *H. zea* (Boddie) (Pope et al., 1984) all gave release rates of around 1–3 ng/min. Unconfined female *H. virescens* released about 400 ng/day, with a peak of about 140 ng/2 hr, while *H. subflexa* (Guenée) released about 870 ng/day with a peak of 335 ng/2 hr (Heath et al., 1991). A release rate of 880 ng/day was recorded from *Trichoplusia ni* (Hübner), with bursts of up to 50 ng/5-min period (Bjostad et al., 1980). Measurements of the release rate of pheromone from female gypsy moths, *Lymantria dispar* L., vary from 28 ng/2 hr (Charlton and Cardé, 1982) to as much as 4 μg/day (Ma and Schnee, 1983) with a peak rate of about 840 ng/30 min (Richerson and Cameron, 1974). The highest release rates recorded are from females of the arctiid, *Holomelina lamae* (Free.), which achieve a rate of 350 ng/10 min in short pulses (Schal et al., 1987).

These different release rates are associated with different calling behaviors.

The high rates achieved by the Arctiidae are associated with frequent extrusions and retractions of the gland, a strategy that presumably increases the effective range of threshold amounts of pheromone for male perception, but that provides an intermittent signal. Tortricids, such as OFM, spruce budworm, and carnation moth, which leave the gland extruded for long periods, release less pheromone per unit time, but provide a continuous signal. These different strategies will influence the efficacy of attempts to control populations by mating disruption and illustrate the need for a full understanding of how mating disruption works. If disruption is achieved by "false-trail following" (Bartell, 1982), then massive amounts of pheromone will have to be released to disrupt the arctiids, compared to species such as OFM. However, if adaptation and/or habituation are important, then the continuous release of lower amounts of pheromone may be equally effective for both types of insect.

Even though the release rates varied quite widely among the OFM females, the isomeric ratio remained remarkably constant. The standard deviation of 0.6% is much lower than those recorded for other species, 2.3–5.4% for the pink boll-worm (Haynes et al., 1984; Collins and Cardé, 1985) and 2.6% for *Argyrotaenia velutinana* (Walker) (Miller and Roelofs, 1980). One possible explanation is that low standard deviations for isomeric ratios are, in fact, normal for most species and that higher variation is the result of artifacts, such as differential destruction of isomers through enzymatic action, which is avoided by the improved collection procedure of the present study. Another possibility is that OFM, at least in Australia, is under considerable selection pressure from other insects using *E*8- and *Z*8–12:OAcs, resulting in a precise *E*:*Z* isomer ratio. A further possible explanation for the low variability is that the laboratory population originated from only a few insects from one location. The mean figure of 4.2% *E* isomer is lower than that reported for OFM from elsewhere. Biwer et al. (1979) reported the proportion of *E*- to be 8% in Europe, while Cardé et al. (1979) reported 6.5% in the United States. Possibly this difference is because of genetic drift in the Australian population, although logically this should also lead to a less precise *E*:*Z* ratio. A more likely explanation is that the Australian population has come under more intense selection pressures due to other species using the same compounds and which do not occur in Europe or North America.

The amount of the alcohol, *Z*8–12:OH, found in the present study (0.45% of the *Z*8–12:OAc) is considerably lower than the 17% reported by Cardé et al. (1979). Possibly this again reflects a difference between Australian and North American populations. Experiments in North America show that *Z*8–12:OH has a profound effect on behavior. Baker and Cardé (1979b) obtained a 10-fold increase in trap capture of OFM males when *Z*8–12:OH was added to the acetates. In wind-tunnel assays, Linn and Roelofs (1983) showed that the addition of *Z*8–12:OH over the range 2–100% of the acetates increased responses.

However, there was little difference due to different loadings over this range, although the durations of the responses were affected. Linn and Roelofs (1983) concluded that a loading of around 10% Z8–12:OH was optimum [which will produce a release rate from the rubber septa of around 30% that of the acetates (Baker et al., 1980; Heath et al., 1986)]. The role of Z8–12:OH in the behavior of OFM in Australia has not been thoroughly addressed, but its presence at loadings of 5% of the acetates on rubber substrate enhances wind-tunnel responses and trap-catches (Sanders, unpublished data).

The role of the saturated alcohol, 12:OH, is questionable. Cardé et al. (1979) reported that it represented about 4.5% of the total pheromone titer, but Biwer et al. (1979) were unable to detect any above their detection threshold of 2%. Early bioassays (Cardé et al., 1975) indicated that 12:OH had a synergistic effect when present at high dosages (three times the amount of the acetates), but later work suggested that 12:OH is redundant, showing synergistic effects only when in blends containing reduced amounts of the Z8–12:OH (Baker and Cardé, 1979b; Linn et al., 1986). In the present analyses we found traces of 12:OH, but attribute these to impurities in the solvent. We also found traces of the saturated aldehyde, 12:Ald, but also attribute these to impurities. An homologous series of saturated normal alcohols and aldehydes is present at trace levels in petroleum-derived hydrocarbon solvents such as pentane and hexane, which probably arise by reforming and oxidation during the refining.

The presence of the saturated acetate, 12:OAc is perhaps of greater significance. It has not been reported previously in the pheromone of OFM, but we found fairly constant amounts (3.44 $\pm$ 1.66%). Rothschild (1974) found that the presence of large amounts of 12:OAc (exceeding the amount of the unsaturated acetates) reduced trap catch. The 12:OAc therefore has biological activity, which could be expressed differently at lower concentrations. Possibly its role in the pheromone of OFM has been overlooked. It is a common impurity in Δ8–12:OAc and may well have been present in small amounts in pheromone blends tested previously in laboratory and field.

It is generally believed that disruption of mating behavior is most efficiently achieved by using the blend that most closely approximates that of the virgin female moth (Minks and Cardé, 1988). Although disruption of OFM can be achieved by a binary blend of the acetates, E8- and Z8–12:OAc, apparently with or without the addition of Z8–12:OH (Vickers and Rothschild, 1985), it is possible that this could be achieved more efficiently, and perhaps more cheaply, by blends containing Z8–12:OH and additional minor pheromone components. Mating disruption, as a method of controlling OFM populations, aims at preventing the male moths from locating the calling female moths. To understand how disruption works, it is therefore essential to know how potent the virgin females are, and for this the maximum release rate must be known, not merely

the average. This study has established that the maximum release rate of Z8–12:OAc by calling female OFM is at least 25 ng/female/hr.

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REASSESSMENT OF INTERACTION BETWEEN GUT DETERGENTS AND TANNINS IN LEPIDOPTERA AND SIGNIFICANCE FOR GYPSY MOTH LARVAE

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Abstract—Surface-active lysophospholipids are thought to interfere with tannin–protein interactions and may comprise an adaptation in Lepidoptera to tannin-rich diets. We found several lysophospholipids and phospholipids at about eight times the critical micelle concentration (CMC) in the midguts of gypsy moth larvae, which exhibited appropriately reduced surface tensions. We confirmed the interfering activity of lysophosphatidylcholine (lysolecithin) in a model astringency assay using tannic acid and hemoglobin (hemanalysis), but discovered that tannic acid and red oak tannin extracts precipitate the lipid from simulated midgut solutions quantitatively, even at pH 10. Leaf tannin extracts from trees on which gypsy moths grew and reproduced poorly precipitated lysolecithin more effectively than did extracts from trees on which gypsy moths performed well. Adding tannic acid to midgut fluid elevated surface tension, and about 25% of larvae feeding on oak leaves exhibited elevated midgut surface tension, suggesting a loss of surfactants. Larvae appear able to replace lost surfactants to a limited degree. An important effect of leaf tannins, and perhaps other phenolics, may be to reduce concentrations of surface-active phospholipids in the midgut and produce lipid or other dietary deficiencies in insects.

Key Words—Tannins, phenolics, surface tension, surfactants, lysolecithin, lysophosphatidylcholine, lipids, detergents, protein, *Lymantria dispar* L., gypsy moth, Lepidoptera, Lymontriidae, red oak, *Quercus rubra* L.

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INTRODUCTION

Tannins and other phenolics that occur widely in plants are capable of binding to proteins to form soluble (Hagerman and Robbins, 1987) or insoluble (Sumere et al., 1975; Hagerman and Butler, 1981) complexes, via hydrogen (Hagerman, 1988) or covalent (Felton et al., 1989a) bonding. This affinity for proteins can result in enzyme inhibition (Goldstein and Swain, 1965; Felton et al., 1989b), the repellent taste sensation called astringency or digestion inhibition (Feeny, 1970; Felton et al., 1989a). However, although tannin activity against pathogens and vertebrate herbivores has been demonstrated, there have been few successful attempts to show a negative impact of plant tannins on herbivorous insects (Bernays, 1981; Martin and Martin, 1984; Schultz, 1989).

As is the case for many insect species that feed on plants containing toxins, natural selection should favor traits that reduce negative tannic influences on individuals. Traits such as alkaline midgut pH (Feeny, 1970; Berenbaum, 1980; Schultz and Lechowicz, 1986), high cation concentrations (Martin et al., 1985), reducing midgut conditions (Appel and Martin, 1990), and the presence of detergents (Martin and Martin, 1984; Martin et al., 1985) have been proposed as influences on the activity of dietary tannins in insects (Appel and Schultz, 1992). Each of these factors can reduce the affinity of tannins for proteins (Felton and Duffey, 1991).

Martin et al. (1985) suggested that midgut detergency represents a widespread adaptation to dietary tannins in Lepidoptera larvae. They showed that detergency caused by the presence of lysophosphatidylcholine (lysolecithin) is more effective than high alkalinity in reducing the amount of plant protein precipitated by tannins under simulated midgut conditions in vitro. Other work done in vitro (Felton and Duffey, 1991) suggests that lysolecithin enhances protein solubility and diminishes oxidative activation of tannins by polyphenol-oxidase. These results have led these authors to call into question the assertion (Feeny, 1970) that plant tannins can actually have a negative impact on insects in a general, dose-dependent way.

In this study we confirmed that lysolecithin, which commonly occurs in insect guts, does interfere with a model tannin–protein binding–precipitation system. However, at least part of the interference is due to binding between the detergent phospholipid and tannin, ultimately resulting in precipitation. As a result of this interaction, dietary tannins or other phenolics could deplete surfactant phospholipids in the gut, increasing gut surface tension. Because surfactant phospholipids such as lysolecithin are critical to the bioavailability of dietary lipids in Lepidoptera (Turunen, 1983; Turunen and Kastari, 1979), and because high concentrations are necessary to inhibit tannin–protein binding, tannins could act as dose-dependent antidigestive factors under commonly occurring gut conditions. We present evidence to suggest that these factors influence

the performance of the gypsy moth (*Lymantria dispar* L.), an herbivorous lepidopteran feeding primarily on tannin-rich hosts.

METHODS AND MATERIALS

Phospholipid Content of Larval Midgut Fluid. We attempted to determine whether surfactant lysophospholipids were present in the midgut lumens of larvae of the gypsy moth using two-dimensional, thin-layer chromatography (Harborne, 1984). This organism, as larvae, feeds primarily and successfully on tannin-rich tree species worldwide (Barbosa and Krischik, 1988). All larvae for this and subsequent experiments were reared from eggs obtained from the USDA/ARS Otis Methods Development Laboratory, Otis AFB, Massachusetts.

Midguts were surgically removed from several diethylether-anesthetized fifth-instar larvae reared on artificial diet (Odell and Rollinson, 1966). The midgut fluid was removed and centrifuged at 13,000*g* for 2 min. Lipids were extracted from the supernatant according to the method of Bligh and Dyer (1959). The lipid extract was applied to a 20 × 20-cm silica gel glass TLC plate (Sigma). The solvent system in the first direction was $CHCl_3$–methanol–glacial acetic acid–water, 170:25:25:4. After the lipids were separated in the first dimension, the plate was rotated 90 degrees and subjected to a second solvent system, the composition of which was $CHCl_3$–Methanol–NH_4OH, 65:30:4. The spots were visualized using the phospholipid reagent of Fried and Sherma (1982). Spots were identified by cochromatography with phospholipid standards. The standards used were lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylglycerol, lysophosphatidylinositol, lysophosphatidylserine, lysophosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylserine, and phosphatic acid (Sigma).

Surface Tension and Critical Micelle Concentration of Gypsy Moth Larval Midgut Fluid. Surface tension of midgut fluid was determined using an inclined-tube manometer (Fisher) according to the method of Ferguson (1943) for microvolume quantities. Midgut fluid from 100 larvae fed on artificial diet (Odell and Rollinson, 1966) was individually measured to determine mean surface tension expressed in dynes per centimeter. Midgut contents were isolated from diethylether-anesthetized larvae as above and subjected to a 2-min, 13,000*g* centrifugation. The supernatant was drawn into a 25-μl micropipet. The surfactant concentration, in multiples of the critical micelle concentration, was also determined using the method of Martin et al. (1987). The critical micelle concentration (CMC) is that concentration of a surfactant at which there is a transition between the surfactant in the free, disassociated state and the micellar state (Martin et al., 1987). This transition is characterized by an abrupt change in

the surface tension of the solution. For this determination, the midgut fluids of 78 caterpillars were combined and a midgut fluid dilution series was prepared using distilled water. Surface tension was determined as above.

In vitro Inhibition of Tannin–Protein Precipitation by Lysolecithin. The impact of lysolecithin on astringency (Bate-Smith, 1973) was quantified using hemoglobin as the protein substrate and tannic acid (Sigma, batch T-1025) solutions (Bate-Smith, 1973; Schultz et al., 1981). For this and subsequent experiments, tannic acid was purified using Sephadex LH-20 columns as described by Hagerman and Klucher (1986). Hemoglobin solutions were diluted with either distilled water or a solution of lysophosphatidylcholine (lysolecithin; Sigma) in water to give a blank absorbance of 2.0 at 578 nm. This wavelength was used because it is an absorption maximum for hemoglobin. One milliliter of tannic acid in distilled water was added to 2 ml of these hemoglobin solutions while vortexing. Final concentration of tannic acid solutions ranged from 0 to 10 mg/ml. Hemoglobin–lysolecithin–tannin solutions had final lysolecithin concentrations of either 0.06% or 0.09% (w/v); these concentrations were chosen to represent likely physiological levels in larvae (Martin and Martin, 1984). The final pH of the solutions was found to be about 7 throughout the range of tannic acid added. In all cases, vortexed solutions were centrifuged at 13,000*g* and the absorbance of the supernatant read at 578 nm to quantify protein (hemoglobin) precipitation (Schultz et al., 1981).

In vitro Precipitation of Lysolecithin by Tannic Acid and Oak Leaf Extracts. Tannic acid or red oak (*Quercus rubra* L.) leaf extracts were used to precipitate lysolecithin from a radiolabeled lysolecithin solution. A lysolecithin solution having a specific activity of 17 μCi/mM was prepared by mixing 53 mCi/mM of [^{14}C]lysopalmitoylphosphatidylcholine (New England Nuclear) in methanol–toluene (1 : 1, v/v) with unlabeled lysopalmitoylphosphatidylcholine (Sigma). Aliquots of this lysolecithin solution, containing 1.2 μmol, were then placed in 1.5-ml polypropylene microcentrifuge tubes.

After the organic solvents were evaporated to dryness, 0.5 ml of a stock saline solution and 0.25 ml of a buffer solution were added to the tubes and vortexed for 2 min. Final composition of the saline solution was 2.5 mM NaCl, 131 mM KCl, 16 mM $CaCl_2$, and 25 mM $MgCl_2$. These were chosen to approximate the conditions in the midgut of lepidopteran larvae (Martin et al., 1985). It is known that the pH of a gypsy moth gut can vary over a wide range, depending upon larval age, larval diet, and gut region (Schultz and Lechowicz, 1986). Because of this, several different buffers were used, over a range of pHs, to determine the effects of pH on the ability of tannin to precipitate lysolecithin. The buffers used were 50 mM MES [2(*N*-morpholino)ethanesulfonic acid, pH 6.0], 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.0), 40 mM CHES [2(*N*-cyclohexylamino) ethanesulfonic acid, pH 9.0], and 50 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid, pH 10.0).

For precipitation assays, 0.25 ml of either the tannic acid (Sigma, T-1025) solutions or aqueous red oak leaf extracts were added to the lysolecithin solutions. Final lysolecithin concentration in both cases was 0.06% (w/v) (1.2 mM). This concentration of lysolecithin is about 15 times the critical micelle concentration (Martin and Martin, 1984), which was calculated to be 80 μM. Final tannic acid concentrations used ranged from 0 to 0.60 mg/ml.

Leaf extracts were obtained from the mature leaves of two red oak individuals as in Rossiter et al. (1988). The two trees used were numbered 34 and 43. These two trees were selected because they had the lowest and highest hemoglobin-binding capacity and supported the best and worst growth by gypsy moth larvae, respectively, in a concurrent study (Rossiter et al., 1988). An extract dilution series in 10% increments from 0 to 100% full strength was prepared for the two extracts using distilled water. The astringency of the extracts was determined in the absence of lysolecithin using hemoglobin as the protein substrate (Schultz et al., 1981).

After the addition of either the tannin or oak leaf extract solutions, the tubes were vortexed for 1 min, allowed to stand at room temperature for 15 min, and then centrifuged at 13,000*g* for 4 min. The supernatant was carefully and completely removed from each microcentrifuge tube, placed in a scintillation vial with 10 ml of scintillation cocktail (Hydrocount, Baker) and vortexed for 15 sec. Disintegrations per minute (dpm) were determined in a Beckman scintillation counter (model LS8000) with automatic quench correction. The amount of lysolecithin remaining in the supernatant after tannic acid or leaf extract addition was determined from the disintegrations per minute and expressed as micromoles per milliliter.

Impact of Tannic Acid on Surface Tension of Larval Midgut Fluid and Lysolecithin Solutions. Midgut fluid of gypsy moth larvae reared to the fifth instar on artificial diet was collected as described above and pooled. Nonbuffered tannic acid solution was added to subsamples of this midgut fluid in a ratio of 1:3 tannic acid solution to midgut fluid. The final concentration of tannic acid ranged from 0 to 2.4 mg/ml. Surface tension of the resulting solution was measured. The influence of tannic acid on the surface tension of buffered, saline, lysolecithin solutions, simulating midgut fluid, was also determined. Final composition of the saline solutions was as before, 2.5 mM NaCl, 131 mM KCl, 16 mM $CaCl_2$, and 25 mM $MgCl_2$. The final lysolecithin concentration was 0.06% (w/v). The pH of the solutions was either 6 (buffer: 50 mM MES) or 10 (buffer: 50 mM CAPS). Tannic acid, dissolved in water, was added to the above, yielding final concentrations ranging from 0 to 0.60 mg/ml. The solution was vortexed and the surface tension measured. Significance of differences among treatments was determined using general linear models (GLM) procedures (SAS Institute, 1985).

Impact of Oak Leaf Diets on Midgut Surface Tension. To determine the

effects of dietary tannins on midgut fluid surface tension, 77 fifth-instar gypsy moth larvae reared on artificial diet were switched to randomly selected, fresh red oak leaves. Other red oak leaves from the same tree were harvested and an astringency profile was determined for the tree (see below). The larvae were allowed to feed freely for 24 hr, at which time the surface tension of their midgut fluid was measured.

Astringencies of Red Oak Leaves. We randomly collected 64 leaves from the same red oak tree used for the effects of dietary tannins on midgut surface tension to ascertain the frequency with which gypsy moth larvae might encounter and ingest leaves having astringencies high enough to influence midgut surface tension. Four hundred milligrams of lyophilized powder from each leaf were extracted with acetone. The astringency of the extract was measured as its ability to precipitate hemoglobin from solution (Bate-Smith, 1973; Schultz et al., 1981; Rossiter et al., 1988). Astringency of the extract is expressed as percent tannic acid equivalents (% TAE) using purified tannic acid as a standard (Sigma, batch T-1025). The % TAE is therefore the equivalent fraction of the dry weight of the original leaf that would have to be tannic acid to produce a measured affinity for hemoglobin, although tannic acid per se is not likely to be present in red oak leaves. This measure of astringency was then converted to an equivalent concentration of tannic acid in water for comparison with other experimental results.

Impact of Variation in Dietary Lipids on Surface Tension. To determine what effect dietary lipids had on the surface tension of midgut fluid, 150 larvae were reared on a lipid-rich artificial diet (Odell and Rollinson, 1966). Upon reaching fifth instar, half were switched to a low-lipid diet in which wheat germ was replaced with casein, on a gram for gram basis. A lipid extraction (Bligh and Dyer, 1959) of the components of the diet, followed by gravimetric determination, indicated that the original artificial diet was about 6.6% dietary lipid by dry weight, whereas the lipid-reduced diet was approximately 0.8% lipid. At intervals of 24 hr, 10 larvae were randomly harvested from each group and the surface tension of their midgut fluids measured individually.

Significance of variation in midgut surface tensions among treatments (diets) and dates was determined using GLM procedures (SAS Institute, 1985).

RESULTS

Phospholipid Content of Larval Midgut Fluid. Two-dimensional, thin-layer chromatographic analysis of the phospholipids of midgut fluid of fifth-instar gypsy moth larvae revealed three principal phospholipids in addition to several minor ones (data not shown). The three were identified, by cochromatography with known standards, as lysolecithin, phosphatidylcholine, and phosphatidyl-

ethanolamine. Some of the minor phospholipids cochromatographed near the position of some of the other lysophospholipid standards, but they could not be positively identified.

Surface Tension and Critical Micelle Concentration of Gypsy Moth Larval Midgut Fluid. The mean surface tension of undiluted midgut fluid from larvae reared on artificial diet was 32.4 ± 1.4 (standard deviation) dynes/cm. The surfactant concentration of pooled midgut fluid from moth larvae reared on artificial diet was estimated to be eight times that of the CMC.

In vitro Inhibition of Tannin–Protein Precipitation by Lysolecithin. The relationships among protein precipitation, lysolecithin concentration, and tannic acid concentration are illustrated in Figure 1. At 0% lysolecithin, there was a threshold of 1.0 mg/ml tannic acid before significant amounts of hemoglobin were precipitated. Once this concentration was reached there was a linear relationship between percent hemoglobin precipitated and tannic acid concentration (slope = 0.586; r^2 = 0.989). The addition of lysolecithin raised the threshold concentrations of tannic acid required to precipitate hemoglobin. As the tannic acid concentration was increased to 4.0 mg/ml in the presence of 0.06% lysolecithin, more hemoglobin was precipitated, but to a lesser extent than without lysolecithin. Only at tannic acid concentrations greater than 4.0 mg/ml were significant amounts of hemoglobin removed from solution in 0.06% lysolecithin.

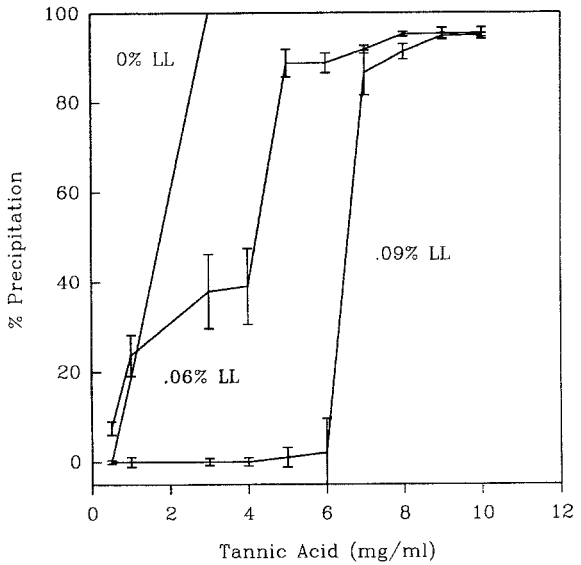

FIG. 1. Precipitation (as percent of maximum) of hemoglobin by purified tannic acid with 0%, 0.06%, and 0.09% lysolecithin (w/v) present. Vertical bars are one standard deviation.

In 0.09% lysolecithin, tannic acid concentrations greater than 6.0 mg/ml were required for hemoglobin precipitation.

In vitro Precipitation of Lysolecithin by Tannic acid and Oak Leaf Extracts. Tannic acid precipitated lysolecithin from solution quantitatively (Figure 2). At pH values of 6.0 and 7.0, there was a linear relationship between tannin concentration and lysolecithin precipitation above a threshold concentration of 0.24 mg/ml. Tannic acid, at a concentration of 0.60 mg/ml, precipitated lysolecithin to the point that it was below its CMC at both pH 6.0 and 7.0. For pH 9 and 10, a threshold tannic acid concentration was not evident; some lysolecithin was removed at the lowest tannic acid concentration, although more tannic acid was required per unit lysolecithin precipitated. As a result, more lysolecithin remained in solution at high tannin concentration at pH 9 and 10 than at 6 or 7. However, the lysolecithin remaining in solution at 0.60 mg/ml tannic acid at pH 9 was still near the critical micelle concentration of 80 μM.

Oak leaf extract also precipitated lysolecithin from solution (Figure 3). The extract from tree 43, which had an estimated astringency (affinity for hemoglobin) of 30.1% TAE, was more effective at precipitating lysolecithin than was the extract from tree 34, which had an astringency of 10.5% TAE (Figure 3). Both extracts precipitated lysolecithin more effectively at pH 6 than at pH 10.

Impact of Tannic Acid on Surface Tension of Larval Midgut Fluid and Lysolecithin Solutions. The effect of tannic acid on the surface tension of midgut fluid is illustrated in Figure 4. The pH of the gut fluid was 8.0. Tannic acid

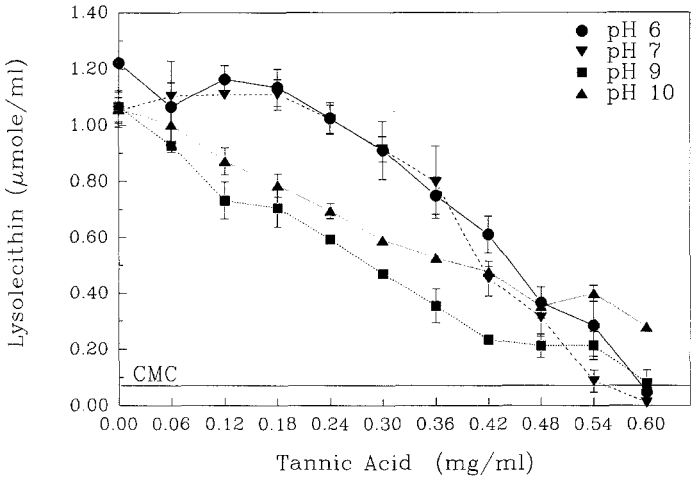

FIG. 2. Precipitation of radiolabeled lysolecithin by purified tannic acid at 4 pH values. CMC = critical micelle concentration of lysolecithin. Vertical bars are one standard deviation.

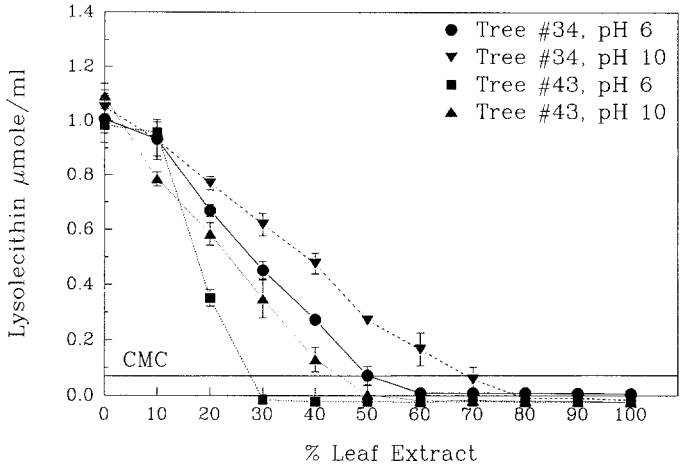

FIG. 3. Precipitation of radiolabeled lysolecithin by a dilution series of red oak leaf extracts; 100% leaf extract = undiluted extract. Extracts from tree 43 had three times the astringency of those from tree 34 and supported gypsy moth growth half as well (Rossiter et al., 1988). CMC = critical micelle concentration of lysolecithin. Vertical bars are one standard deviation.

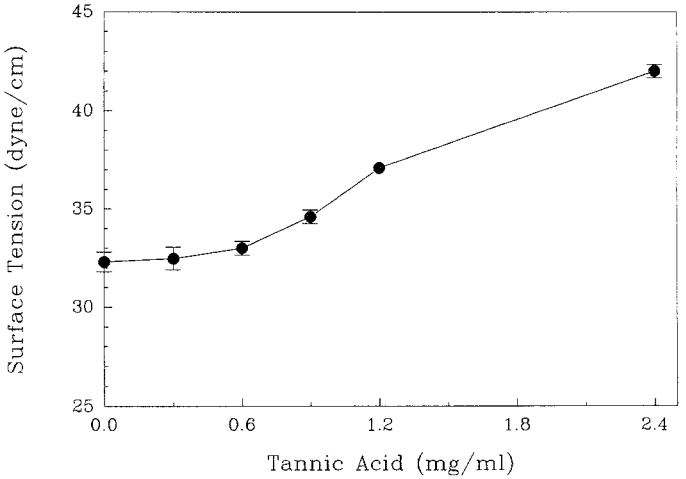

FIG. 4. Effect on surface tension of adding tannic acid to gypsy moth midgut fluid. Tannic acid concentrations are final concentrations. Vertical bars are one standard deviation. Surface tension is significantly greater above 0.9 mg/ml tannic acid (ANOVA, $P < 0.01$).

concentration of about 1.2 mg/ml in the midgut fluid caused an increase in surface tension. Tannic acid also caused an increase in the surface tension of a 0.06% lysolecithin solution in a dose-dependent, pH-sensitive fashion (Figure 5). At pH 6.0, tannic acid concentration in a range of 0–0.54 mg/ml had no effect on the surface tension of a lysolecithin solution. However, there is a distinct rise in surface tension at a tannic acid concentration of 0.60 mg/ml. Tannic acid, in the range of 0–0.60 mg/ml, had no effect on surface tension of a 0.06% lysolecithin solution at pH 10.0.

Impact of Oak Leaf Diets on Midgut Surface Tension. The consequences of switching larvae from artificial diet to red oak leaves can be seen in Figure 6. The 100 larvae reared on artificial diet had a mean midgut fluid surface tension of 32.4 ± 1.4 dynes/cm. Although the mean midgut fluid surface tension of the larvae that had consumed oak leaves for 24 hr was not significantly different (33.2 ± 2.5 dynes/cm; $P > 0.05$), the shapes of these two frequency distributions were significantly different (chi-square analysis, $P < 0.05$). This difference was due mainly to greater variation in midgut fluid surface tension among leaf-fed larvae (coefficient of variation = 7.5%) than in artificial diet-fed larvae (coefficient of variation = 4.3%). In our experiments, midgut fluid ranged between 32 and 33 dynes/cm. Approximately 22% of leaf-fed, but only 9% of diet-fed, larvae had midgut surface tensions above 33 dynes/cm; 36% of leaf-fed and 23% of diet-fed larvae had midgut surface tension below 32 dynes/cm.

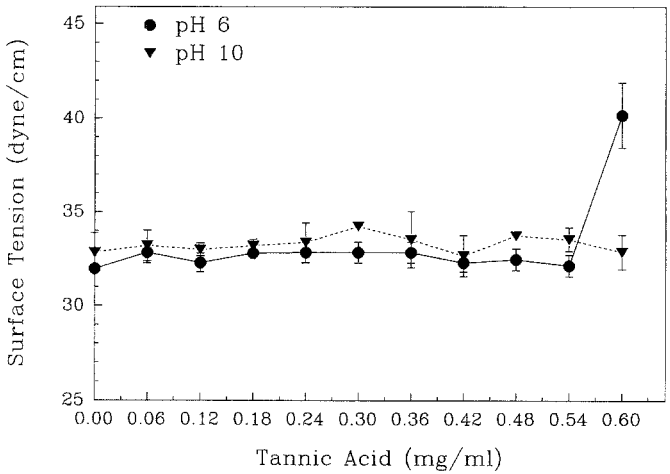

FIG. 5. Effect on surface tension of adding tannic acid to simulated midgut fluid with 0.06% (w/v) lysolecithin at 2 pH values. Surface tension was different only at tannic acid concentration of 0.60 mg/ml, pH 6 (ANOVA, $P < 0.05$). Vertical bars are one standard deviation.

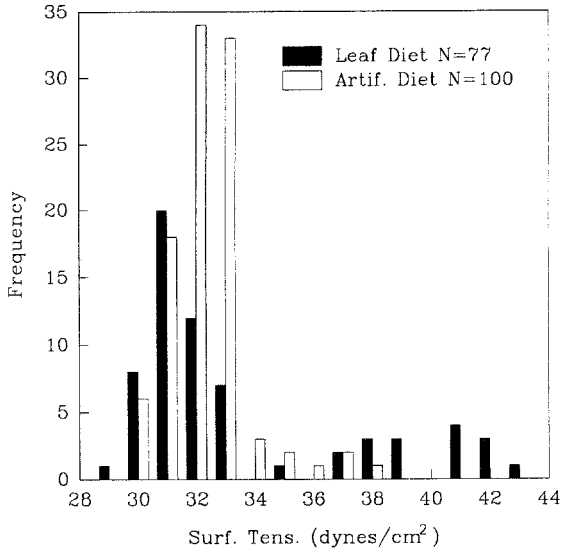

FIG. 6. Frequency distribution of midgut fluid surface tensions of fifth-instar gypsy moth larvae fed artificial diet (light bars) or red oak leaves (dark bars) for 24 hr.

Astringencies of Red Oak Leaves. The majority of the red oak leaf extracts assayed exhibited astringencies high enough to permit protein binding with physiological concentrations (0.06%) of lysolecithin present. In the protein-binding inhibition studies described above (Figure 1), hemoglobin precipitation in a 0.06% lysolecithin solution proceeded above tannic acid concentrations of 4 mg/ml. An extract from leaf powder having an astringency of 10% TAE has the same ability to precipitate hemoglobin as if it was 10% tannic acid by weight (Schultz et al., 1981). Because we used 400 mg dry weight of leaf powder and concentrated the extract to 10 aqueous ml, an extract from leaf material exhibiting 10% TAE has the same astringency as a 4 mg/ml tannic acid solution. Hatched bars represent leaves having TAE values above this level in Figure 7.

Impact of Variation in Dietary Lipids on Midgut Surface Tension. Larval midgut surface tension on normal and a reduced-lipid diet are shown in Figure 8. Initially, the mean midgut surface tension was 33.1 dynes/cm. The midgut fluid surface tension from larvae that remained on the lipid-rich diet did not vary significantly from this value over a seven-day period. After 24 hr, there was no significant change in the midgut fluid surface tension of larvae eating the reduced-lipid diet. However, at 48 hr and beyond, there was a significant increase in the surface tension of the midgut fluid (ANOVA, GLM procedure $P < 0.01$). The size of the larval fat bodies decreased over several days (unpublished observation). Midgut contents of larvae on reduced-lipid diet included exoskeleton parts,

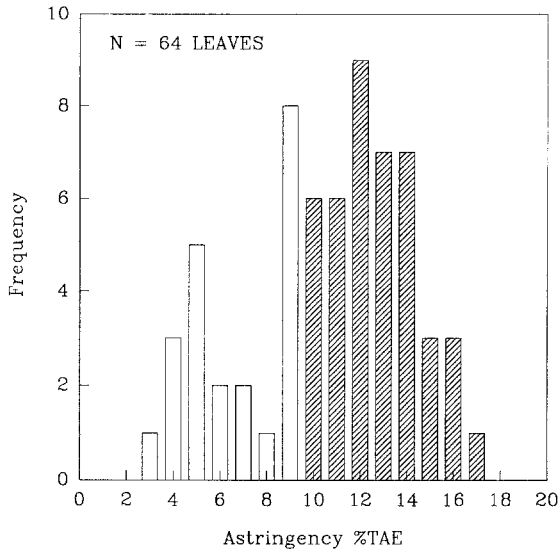

FIG. 7. Frequency distribution of the astringencies of 64 leaves from a single red oak tree (used to feed larvae in Figure 6). Astringencies greater than 10% (hatched) are calculated to be high enough to increase midgut surface tension of gypsy moth larvae.

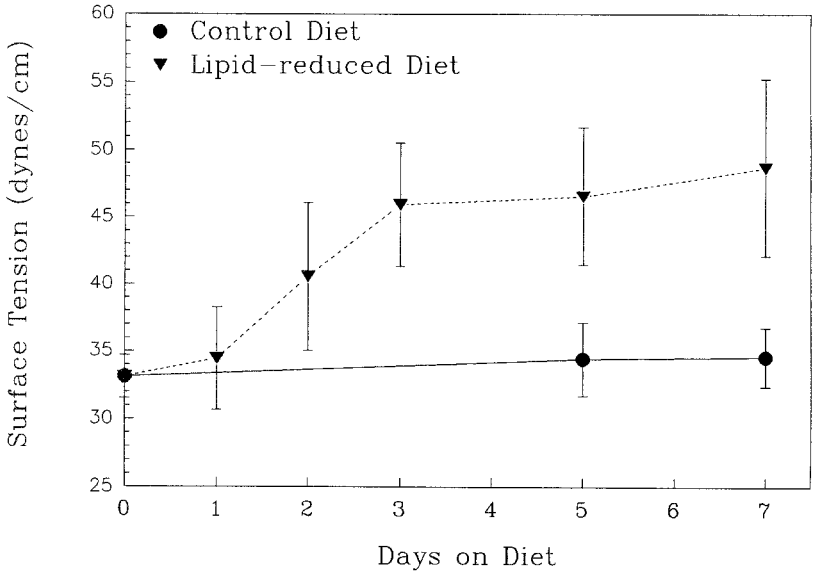

FIG. 8. Surface tension of midgut fluid from fifth-instar gypsy moth larvae reared on normal and reduced-lipid artificial diets. Values differ significantly after two days (ANOVA, $P < 0.02$). Vertical bars are one standard deviation.

and the number of caterpillars remaining in the common feeding container decreased at a rate greater than could be accounted for by harvest (unpublished observations). This strongly suggests that the surviving larvae were engaging in cannibalistic behavior. No such behavior was noted for larvae fed lipid-rich diet.

DISCUSSION

We found that surfactant phospholipids, including lysophosphatidylcholine (lysolecithin), occur in the midguts of gypsy moth larvae, where they appear to alter the surface tension of gut fluid. Larvae on tannin-free, lipid-containing diets had gut fluid surface tensions indicating lysolecithin concentrations up to eight times the critical micelle concentration. These results are similar to those of other workers (Martin and Martin, 1984, Felton and Duffey, 1991) for several other lepidopteran species. Martin and Martin (1984) pointed out that such gut fluid surfactants have been found in every insect species that has been studied. For this reason, and because the gypsy moth focuses much of its feeding on tannin-rich host plants (Barbosa and Krischik, 1988), it is not surprising to find detergents present in its midgut.

Larvae feeding on astringent red oak leaves exhibited more variable midgut surface tension than did larvae fed artificial diet. This is not surprising, given the numerous ways in which oak leaves must differ from the homogenous lab diet, and the fact that we could not measure the actual components of the leaf portions consumed and did not know how recently they had been consumed. These results suggest that dietary variation can influence concentration of surface active phospholipids. About 25% of leaf-fed larvae exhibited elevated midgut surface tensions, suggesting a reduction in surfactant concentrations. We hypothesized that surfactant activity might be lost (and surface tension increased) on diets having high tannin and/or low lipid concentrations.

As predicted, adding tannic acid to gypsy moth larval midgut fluid did increase surface tension. A significant rise in surface tension occurred above 1.2 mg/ml tannic acid, suggesting the existence of a threshold. A sharp threshold increase in surface tension was found above 0.54 mg/ml tannic acid in aqueous lysolecithin solutions buffered to pH 6. Although no such increase was seen in lysolecithin solutions buffered to pH 10, we only worked with tannic acid concentrations up to 0.60 mg/ml. This amount is insufficient to reduce the lysolecithin concentration at pH 10 below its CMC and half the amount seen to increase surface tension in midgut fluid. These results support our hypothesis that dietary tannin can reduce or eliminate surface activity of midgut phospholipids. More tannin is required for this impact at higher pH levels.

The mechanism by which tannin reduces midgut surfactance apparently involves tannin–lipid complexing. Takechi and Tanaka (1987) reported that

gallotannins were able to bind to basic lipids, such as lecithin and lysolecithin, although no precipitation was noted. We showed that the ability of lysolecithin to inhibit precipitation of hemoglobin (astringency) is lost above threshold tannin concentrations. These results, plus the observation of precipitates formed in tannin–lysolecithin solutions at low protein concentrations, indicated that tannins might actually be able to remove phospholipids from solution.

Indeed, we found that both tannic acid and red oak tannin extracts precipitate lysolecithin quantitatively from solution under conditions that simulate midgut cation concentrations and over a range of pH values. At lower pH values, this effect exhibited a prominent threshold; the threshold is less evident at higher pH values. Although we have not carried out a competitive binding study, our results lead us to hypothesize that the anticomplexing activity of midgut surfactants may be due to competitive binding with dietary tannins.

Surfactant concentrations in simulated gut fluid were reduced below the CMC by leaf extracts as dilute as 50% of full strength, even at pH 10. Larvae consuming these leaves are likely to experience a loss of surfactance. Extracts from tree 43 had a stronger effect on surfactant loss than did those from tree 34. These two trees were chosen for these experiments because extracts from tree 43 had three times the astringency of extracts from tree 34, and female gypsy moths reared on tree 43 had 50% lower pupal mass and fecundity than did females reared on tree 34 (Rossiter et al., 1988). Individual egg dry weights were also significantly lower on tree 43 than on tree 34 (Rossiter, personal communication).

We suggest that the ability of higher concentrations of leaf phenolics to reduce phospholipid concentrations below midgut CMC may be responsible for reduced growth and reproduction in gypsy moth larvae. Surface active lysophospholipids play a direct role in glyceride transport across mucosal cells in *Pieris brassicae* (Lepidoptera; Pieridae) (Turunen and Kastari, 1979) and facilitate lipid solubilization in many insects, activities analogous to those of bile in vertebrate digestive systems (Turunen and Kastari, 1979; Turunen, 1983; Turunen and Chippendale, 1989). Tannin-induced loss of surfactants in the midgut therefore could produce lipid deficiency, reduced adult weight, and reduced egg production and egg weight.

Dietary tannins might also be able to bind to the lipid component of epithelial cell membranes. This could lead to the disruption of membrane integrity and/or function. Such a phenomenon would explain why gut lesions are often observed in insects fed on high tannin diets (Steinly and Berenbaum, 1985). If epithelial cell membranes are vulnerable to tannin and/or phenolic binding, lysolecithin in the gut fluid may play a role in protecting these cells.

Two lines of evidence suggest that gypsy moth larvae may be able to "recharge" lost midgut lysolecithin from some source other than the diet for at least a limited time. First, caterpillars did not exhibit elevated midgut surface

tensions for 48 hr after being deprived of any phospholipid substrates. Second, fewer larvae than expected exhibited elevated midgut surface tensions when fed oak leaves that probably had high enough astringencies to reduce midgut surfactant concentrations.

The possibility that larvae may "recycle" or synthesize lysophospholipids to "recharge" midgut surfactants is inconsistent with present views of the sources and fate of these molecules in Lepidoptera. The lysolecithin in lepidopteran midguts is thought to arise from ingested lysolecithin and the hydrolysis of ingested dietary lecithin (Turunen, 1983). Lysolecithin is then absorbed by midgut mucosal cells, where it is resynthesized into the corresponding phosphatide (lecithin), which passes into the hemolymph (Turunen, 1983). Turnover time for lecithin in midgut cells is estimated at under 11 hr, and there is at present no known pathway by which it is returned to the midgut lumen (Turunen, 1983); in *P. brassicae*, "... dietary lecithin was found to be utilized by the larvae almost completely" (Turunen and Kastari, 1979). The means by which gypsy moth larvae maintained elevated midgut surface tensions 48 hr after their last meal containing phospholipids is therefore unknown. Some of the observed fat body depletion in these insects could have been caused by the use of fat reserves in maintaining midgut surfactant levels.

We have shown that gypsy moth larvae can suffer reduced midgut surfactance if they consume diets with moderate to high tannin concentrations. This effect appears to be due to precipitation of detergent lipids from solution by tannins and maybe other phenolics and is pH dependent. Reducing the midgut phospholipid concentration below its CMC should produce lipid deficiencies or reduced protein availability, depending on the tannin concentrations actually consumed. Because leaf tannin concentrations high enough to reduce lysolecithin concentrations below the CMC occur frequently in nature and seem to have a negative impact on gypsy moth growth and reproduction (e.g., Rossiter et al., 1988), we postulate that the loss of surfactant activity might comprise an important impact of plant tannins on this insect.

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INFLUENCE OF *Artemisia princeps* VAR. *orientalis* COMPONENTS ON CALLUS INDUCTION AND GROWTH

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Abstract—An in vitro study was performed to determine the potential application of tissue culture in determining allelopathic potential of *Artemisia princeps* var. *orientalis* (wormwood). Aqueous extracts and volatile substances of *A. princeps* var. *orientalis* were tested to determine their effects on callus induction and growth of several tested species. Extracts of 5% *A. princeps* var. *orientalis* caused some reduction in concentration, induction, and growth of callus, although they looked normal, whereas the explants of most receptor plants did not develop callus at higher concentration. Lettuce and *Eclipta prostrata* were the most sensitive species, and *A. princeps* var. *orientalis* was affected by its own extracts. The growth of calluses in MS 121 medium treated with essential oil of *A. princeps* var. *orientalis* was inhibited, and the degree of inhibition was proportional to the concentration of the essential oil.

Key Words—*Artemisia princeps* var. *orientalis*, allelopathy, aqueous extract, essential oil, callus induction, callus growth

INTRODUCTION

Several species of the genus *Artemisia* have been identified as having allelopathic potential (Funke, 1943; Halligan, 1976), based on seed germination and seedling growth bioassays. These findings were shown from aqueous extracts and volatile substances of *Artemisia*. Inhibition was due to various allelopathic chemicals

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(Yun, 1990). Microtests for allelochemicals using culture medium or extracts were first devised by Gressel and Holm (1964). Hogan and Manners (1990) reported that media and media extracts from callus cultures of small everlasting (*Antennaria microphylla*) inhibited leafy spurge callus tissue growth in suspension culture. The chronic involvement of hydroquinone, a previously isolated allelochemical from small everlasting, was implicated in the allelopathic interaction.

The present work deals with the effect of extracts and volatile substances of *A. princeps* var. *orientalis* on in vitro callus induction and growth of several plants.

METHODS AND MATERIALS

Preparation of Tissue Explants. Seed coats from the healthy seeds of *Artemisia princeps* var. *orientalis*, *Raphanus sativus* var. *hortensis* for. *acanthiformis*, *Eclipta prostrata*, *Zoysia japonica*, *Oryza sativa*, *Oenothera odorata*, and *Lactuca sativa* were removed. Seeds were rinsed in 75% ethyl alcohol for 30 sec and then placed in a 5% sodium hypochlorite solution for 15 min. The sterilized seeds were then washed with sterile distilled water three times. Two to three seeds were germinated on the surface of Murashige and Skoog's agar (MS) medium (Murashige and Skoog, 1962) in test tubes (2 × 15 cm). Callus culture medium containing 0.8% agar was adjusted to pH 5.6, and was autoclaved for 20 min at 121°C (Hogan and Manners, 1990).

Induction and Subculture of Callus for Testing. Leaf (5 mm^2) or hypocotyl (5 mm) explants of *A. princeps* var. *orientalis*, *R. sativus* var. *hortensis* for. *acanthiformis*, *E. prostrata*, *O. odorata*, and *L. sativa* were obtained from 7- to 12-day-old seedlings. Twelve segments were placed on the surface of 20-ml MS medium supplemented with 1 mg/liter of 2,4-D, 2 mg/liter of NAA and 1 mg/liter of kinetin (MS 121 medium) in a Petri dish (10 × 1.5 cm). The calluses of *Z. japonica* and *O. sativa* were directly induced from sterilized seeds on MS 121 medium. Induced calluses were subcultured every 30 days on the MS 121 medium to maintain continued growth, and the subcultured calluses were used for all experiments. The calluses were cultured in the dark at 25 ± 2°C (Lee et al., 1979).

Induction of Callus in Medium Containing Extracts. Leaf explants (5 mm^2) of *A. princeps* var. *orientalis* and lettuce were cultured in four-compartment Petri dishes containing MS 121 medium supplemented with different concentrations of the *A. princeps* var. *orientalis* water extracts. Water extracts were obtained as follows: One liter of distilled water was added to 200 g of the leaf at 20°C. After 24 hr, the aqueous extract was filtered through a 0.5-mm sieve, and the filtrates were either diluted to 5, 10, 20, 30, and 50% extracts or used

as an undiluted 100% extract. Three explants were put into each compartment with 5 ml of medium. The Petri dishes were sealed with parafilm (Whatman Co.) and incubated at 25°C in the dark to initiate callus induction. The callus induction was estimated and graded according to callus volume; good, bad, and none (Krikorian and Katz, 1968).

Callus Growth in Medium Containing Extracts. Calluses obtained from *A. princeps* var. *orientalis*, *R. sativus* var. *hortensis* for. *acanthiformis*, *Z. japonica*, *O. sativa*, *E. prostrata*, *O. odorata*, and *L. sativa* were cultured in the four-compartment Petri dishes containing MS 121 medium supplemented with different concentrations of *A. princeps* var. *orientalis* water extracts. The initial calluses were about 0.5 mm in diameter, and three callus masses were cultured in each compartment. The biomass of calluses was monitored by measuring dry weight (80°C, 48 hr) at 30–50 days. Dry weight of 10 calluses per treatment were obtained: Biomass as % of control = (Dry weight of callus on the extract medium/Dry weight of callus on the control medium) × 100.

Callus Growth Exposed to Essential Oil of Wormwood Plants. Essential oil was obtained as follows: Crushed wormwood leaf was placed in a glass flask (2000 ml) and water distillation was performed in Karlsruker's apparatus (Stahl, 1973). The water distillate was saturated with sodium sulfate and then extracted with ether. The ether extract was concentrated in an evaporator. Calluses obtained from *A. princeps* var. *orientalis*, radish, *E. prostrata*, and *Z. japonica* were placed in four-compartment Petri dishes containing 20 ml of MS 121 medium. Circles of filter paper with different amounts of essential oil from wormwood were put in the central well of the solid medium (0, 5, 10, 20, 30, 50, 100 μl per Petri dish). The Petri dishes were sealed with parafilm and incubated at 25°C in the dark. The biomass expressed as percent of control of calluses was monitored by the same method mentioned above.

RESULTS

Effect of Aqueous Extract on Induction and Growth of Callus. The induction of callus of *L. sativa* and *A. princeps* var. *orientalis* was affected by concentrations of *A. princeps* var. *orientalis* extracts as low as 5%. Growth of leaf explant was initiated after five to seven days, and induction of calluses from injured tissue occurred in about 10 days. The fresh callus tissue stayed white to pale yellow in color for 30 days. The induction rate of callus of *A. princeps* var. *orientalis* and *L. sativa* in the control was 96% and 100%, respectively (Table 1 and 2). At lower concentrations of the extract, callus induction recurred, but leaf explants at the higher concentrations did not form callus. The callus induced at the higher concentrations were very small and dark brown, and their biomass was very reduced. The induction rate of the callus of *A. princeps* var. *orientalis* was higher than that of *L. sativa*.

TABLE 1. CALLUS INDUCTION OF *Lactuca sativa* ON MS 121 MEDIUM WITH *A. princeps* VAR. *orientalis* EXTRACTS AFTER 30 DAYS OF CULTURE

Extract concentration (%, 200 g/1000 ml)	Callus induction (%)[a]		
	++	+	−
0 (control)	100	0	0
5	70	18	12
10	66	22	12
20	62	16	22
30	22	30	48
50	0	0	100
100	0	0	100

[a] ++, good; +, bad; −, none.

TABLE 2. CALLUS INDUCTION OF *Artemisia princeps* VAR. *orientalis* ON MS 121 MEDIUM WITH *A. princeps* VAR. *orientalis* EXTRACTS AFTER 30 DAYS OF CULTURE

Extract concentration (%, 200 g/1000 ml)	Callus induction (%)[a]		
	++	+	−
0 (control)	96	0	4
5	62	18	20
10	48	16	36
20	36	26	38
30	28	36	36
50	4	40	56
100	0	28	72

[a] ++, good; +, bad; −, none.

The biomass of subcultured callus of the receptor plants was inhibited by the *A. princeps* var. *orientalis* aqueous extract (Table 3). The biomass of *L. sativa* was the most inhibited of the receptor plants.

Effect of Essential Oil on Growth of Callus. The biomass of callus was reduced when the MS 121 medium was supplemented with any of the amounts of *A. princeps* var. *orientalis* essential oil that were tested (Table 4). *A. princeps* var. *orientalis* had the highest biomass. The biomass of *E. prostrata* was reduced the most among test species. The callus mass of *Z. japonica* was good for 5 μl and 10 μl/60 ml of essential oil, but it was greatly inhibited at higher concentration of the essential oil. In general, the callus biomass or size of receptor plants was inversely proportional to the concentration of the essential oil.

TABLE 3. BIOMASS OF CALLUS EXPRESSED AS PERCENT OF CONTROLS ON MS 121 MEDIUM AT DIFFERENT CONCENTRATIONS OF *A. princeps* VAR. *orientalis* EXTRACTS AFTER 30 DAYS OF CULTURE[a]

Receptor plant	Extract concentration (%, 200 g/1000 ml)						
	0	5	10	20	30	50	100
Artemisia princeps var. *orientalis*	100a	96.2a	91.3a	72.0b	70.8b	25.8c	22.9c
Oryza sativa	100a	94.9b	84.0c	67.0d	ND	ND	ND
Zoysia japonica	100a	85.2b	69.6c	59.9d	ND	ND	ND
Raphanus sativus var. *hortensis* for. *acanthiformis*	100a	79.4b	54.3c	47.8d	ND	ND	ND
Eclipta prostrata	100a	ND	ND	ND	60.4b	30.4c	16.5c
Oenothera odorata	100a	ND	ND	ND	50.7b	41.2b	29.9c
Lactuca sativa	100a	ND	ND	ND	40.1b	28.0c	21.8c

[a]Values in rows having the same letters are not significantly different at the 5% level by Duncan's multiple-range test. ND: not determined.

TABLE 4. BIOMASS OF CALLUS EXPRESSED AS PERCENT OF CONTROL IN PETRI DISH (MS 121 MEDIUM) FILLED WITH *A. princeps* VAR. *orientalis* ESSENTIAL OIL AFTER 50 DAYS OF CULTURE[a]

Receptor plant	Essential oil concentration (μl/60 ml)						
	0	5	10	15	25	50	100
Artemisia princeps var. *orientalis*	100a	69.4b	65.7bc	62.2bc	58.9bc	50.2c	48.7c
Raphanus sativus var. *hortensis* for. *acanthiformis*	100a	47.2b	44.8bc	40.1bc	39.3bc	34.2c	32.1c
Eclipta prostrata	100a	46.4b	30.3c	26.1c	22.4c	20.7c	20.6c
Zoysia japonica	100a	77.4b	62.5bc	49.7cd	38.9d	35.9d	32.5d

[a]Values in rows having the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

DISCUSSION

Helgeson (1984) suggested that tissue culture systems may be grown under controlled environments, in the absence of pathogens, and this allows for observation at the tissue and/or cellular level. The water extract and essential oil obtained from *A. princeps* var. *orientalis* affected the induction and growth of callus derived from the several species used in the bioassay. The color of calluses that were treated with low concentrations of extract turned from white and/or yellow to light brown but were changed to dark brown by high concentrations of extract. The calluses of *A. princeps* var. *orientalis* and rice were the most resistant, and those of lettuce and radish were the most sensitive. We suggest that the tissue of *A. princeps* var. *orientalis* explants are resistant due to their hard seed-coat texture and their metabolism, which overcomes the toxins of the extracts. Our results are similar to those of Wolf and Earle (1990), who found growth of susceptible callus from corn inhibited at 5 μg/ml of a *Helminthosporium carbonum* toxin, while that of resistant callus was inhibited only at the 50 μg/ml concentration.

Although induction and growth of *A. princeps* var. *orientalis* callus was more resistant than other receptor plants to the extracts or essential oil, they still showed autotoxicity and did much more poorly than controls. In vitro, this phenomenon was reported in the microspore culture of oilseed rape (Kott et al., 1988) and in cultures of tobacco or rice (Tsay, 1982).

We suggest that susceptible and resistant plant tissue cultures, i.e., callus induction and growth, are more sensitive than seed germination and plantlet growth bioassays and field experiments (Yun, 1990). Yet, we have no direct information explaining these differences in comparing the bioassay of tissue culture to other bioassays of allelopathic activity from *A. princeps* var. *orientalis*.

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Book Review

Plant Kairomones in Insect Ecology and Control. Contemporary Topics in Entomology 1. Robert L. Metcalf and Esther R. Metcalf. New York and London: Chapman and Hall, 1992, 168 pages. ISBN 0-412-01991-4.

This is a monograph written by two members of one of the most illustrious families in the history of North American entomology. Robert Metcalf has been a pioneer in research on the structure and activity of synthetic organic insecticides. He was also one of the early critics of the dangers of overeliance upon and overuse of pesticides in agriculture. His foresight led him to shift his multiple talents toward investigating basic chemical and ecological aspects of plant kairomones (= plant chemicals that "convey adaptive advantages to the insect receiver") and their potential practical value in reducing the need for insecticide use in agroecosystems. For more than two decades, he and his wife Esther (recently deceased) have been exploring plant kairomones that have powerful stimulating effects on two of our most economically important groups of insect pests: Diabroticite rootworm beetles and tephritid fruit flies. The Metcalfs and their coworkers have written numerous journal articles about their findings on these two groups. The major thrust of this monograph is a broad treatment of these findings.

The monograph is comprised of six chapters. The first two chapters on the chemical ecology of plant kairomones and volatile kairomones as lures for insects provide sufficient background information to enable a relatively uninitiated reader to appreciate the meat of the monograph: the next three chapters on basic and applied chemical ecology of Japanese beetles, rootworm beetles, and tephritid flies. The sixth and final chapter is a brief treatment of plant-produced chemicals that lure solitary bees as pollinators, particularly of orchids. To my knowledge, the Metcalfs have conducted little if any research on Japanese beetles and solitary bees. Their reasons for highlighting the chemical ecology of these two groups, as opposed to other insect groups in which plant kairomone effects have been studied in some depth (e.g., bark beetles, hymenopterous parasitoids), may not seem readily apparent. It is based on an underlying theme that surfaces in every chapter of the monograph: the prominent role of phenylpropanoids as plant kairomones in the evolutionary history of Japanese beetles, rootworm beetles, tephritid flies, and solitary bees.

In my judgement, the greatest strength of the monograph lies in the chapters on rootworm beetles and tephritid flies and the opportunity for side-by-side

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comparison of the chemical ecology of these two groups. For both, an attempt is made to paint an evolutionary scenario that traces present-day kairomonal insect preferences to ancestral roots. The extensive chemical characterization presented of kairomones associated with an array of plant species visited by rootworm beetles and tephritid flies affords a considerably greater level of knowledge-based speculation on the evolutionary history of the chemical ecology of these two groups than can be had for most other groups of insects. Even so, the Metcalfs rightly point out that the evolutionary basis of some of the insect–plant chemical relationships dealt with remains a mystery. For example, Diabroticite beetles are highly attracted to eugenol-type phenylpropanoid plant compounds, even though considerable search has failed to turn up such compounds either in blossoms of cucurbits or in corn silk, both of which are presently highly attractive resources to the beetles. In the case of Dacine tephritid flies, males (but not females) of some species are extraordinarily highly attracted to plant blossoms containing methyl eugenol, feeding on methyl eugenol to the point of extreme engorgement. Yet, the value of methyl eugenol to Dacine males is unknown. Interestingly, the Metcalfs point out more than once that the beginning of modern chemical ecology of insects can be traced to the discovery by Howlett in 1915 of the high attractiveness of two species of Dacine flies to methyl eugenol that Howlett isolated from lemon grass oil. This chemical characterization of a plant kairomone (albeit of undetermined benefit to the responding insect) predates by more than 40 years the first characterization of an insect pheromone (bombykol).

Besides the insights into evolutionary relationships portrayed, another major thread of the chapters on rootworms and tephritid flies is the practical value of plant kairomones and parakairomones (= synthetic analogs of kairomones) in monitoring and controlling species that are pests. Over the years, a variety of compounds attractive to rootworm and tephritid adults has been discovered through mass screening of shelf chemicals or by serendipidity. Here, the Metcalfs compare molecular structures of, and receptor sites for, such attractants with those of known plant kairomones, often with illuminating results. Of particular value to pest management is the painstaking process described for chemically altering the architecture of parakairomones and natural kairomones to render them yet more attractive to pest adults by, for example, increasing release rate and atmospheric transport while at the same time diminishing potential disadvantages, such as carcinogenicity.

Each of the chapters on Japanese beetles, rootworm beetles, and tephritid flies concludes with specific examples of how parakairomones and kairomones for these groups have been employed in pest management. Here is where the Metcalfs' strong desire for judicious insecticide use comes prominantly into play. In several examples, the Metcalfs illustrate how only minuscule amounts

of insecticide combined with attractants and feeding stimulants can achieve effective pest control as baits in agricultural situations.

The monograph should be of most value to those who have an interest in the application of basic elements of insect chemical ecology to pest management. It is especially illustrative of how a strong appreciation for evolutionary aspects of chemical interactions of insects with plants can be turned to applied advantage. It is written by a team whose research is exemplary of this approach. If there are shortcomings, they lie in the Metcalfs' emphasis of their own research (understandable) but omission of several recent relevant studies by others (therefore falling short of completely fulfilling the concept of a monograph as a thoroughly documented treatise on a limited area of biology). In addition, the concept of coevolution is sometimes applied in an overly loose fashion, and there are instances of overoptimism of the success rate to date of using parakairomones and kairomones in control of Japanese beetles, rootworms, and tephritids. On the whole, however, these shortcomings are relatively minor in relation to the value of having in one volume a compilation of the insights and accomplishments of such important contributors as Robert and Esther Metcalf.

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LEAF SURFACE CHEMICALS FROM *Nicotiana* AFFECTING GERMINATION OF *Peronospora tabacina* (ADAM) SPORANGIA

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Abstract—A bioassay was used to evaluate the effects of cuticular leaf components, isolated from *N. tabacum*, *N. glutinosa* (accessions 24 and 24a), and 23 other *Nicotiana* species, on germination of *P. tabacina* (blue mold). The leaf surface compounds included α- and β-4,8,13,-duvatriene-1,3-diols (DVT-diols), (13-*E*)-labda-13-ene-8α-,15-diol (labdenediol), (12-*Z*)-labda-12,14-diene-8α-ol (*cis*-abienol), (13-*R*)-labda-8,14-diene-13-ol (manool), 2-hydroxymanool, a mixture of (13-*R*)-labda-14-ene-8α,13-diol (sclareol), and (13-*S*)-labda-14-ene-8α,13-diol (episclareol), and various glucose and/or sucrose ester isolates. The above in acetone were applied onto leaf disks of the blue mold-susceptible *N. tabacum* cv. TI 1406, which was then inoculated with blue mold sporangia. Estimated IC_{50} values (inhibitory concentration) were 3.0 μg/cm^2 for α-DVT-diol, 2.9 μg/cm^2 for β-DVT-diol, 0.4 μg/cm^2 for labdenediol and 4.7 μg/cm^2 for the sclareol mixture. Manool, 2-hydroxymanool, and *cis*-abienol at application rates up to 30 μg/cm^2 had little or no effect on sporangium germination. Glucose and/or sucrose ester isolates from the cuticular leaf extracts of 23 *Nicotiana* species and three different fractions from

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N. bigelovii were also evaluated for antimicrobial activity at a concentration of 30 $\mu g/cm^2$. Germination was inhibited by >20% when exposed to sugar esters isolated from *N. acuminata, N. benthamiana, N. attenuata, N. clevelandii*, and *N. miersii*, and accessions 10 and 12 of *N. bigelovii*. These results imply that a number of compounds may influence resistance to blue mold in tobacco.

Key Words—*Nicotiana*, *Peronospora tabacina*, blue mold, leaf surface chemistry, diterpenes, sugar esters.

INTRODUCTION

The leaf surfaces of most higher plants have trichomes, which produce natural secretions that serve as mitigating factors in herbivory and disease development in some plant species (Behnke, 1984). In some tobacco (*Nicotiana tabacum* L.) and *Nicotiana* species, chemical compounds found in trichome exudates affect host plant resistance to several insect pests (Severson et al., 1985, 1991), but their role in disease resistance in tobacco is less clear. Removal of the leaf surface chemicals by washing the leaves with water or acetone increased the plants' susceptibility to *Peronospora tabacina* Adam (Hill, 1966; Reuveni et al., 1987), the obligate fungal pathogen responsible for tobacco blue mold, a disease of major economic importance (Main, 1991). In other studies, specific trichome exudate constituents have been reported to have fungicidal properties against germination of *P. tabacina* sporangia (Bailey et al., 1974; Cruickshank et al., 1977; Cutler et al., 1986; Lawson et al., 1988). These constituents include the diterpenes α- and β-4,8,13-duvatriene-1,3,-diols (DVT-diols), and the labdane, (12-*Z*)-labda-12,14-dien-8α-ol, which are synthesized in the gland cells of tobacco trichomes (Keene and Wagner, 1985; Kandra and Wagner, 1987).

In in vitro experiments, Menetrez et al. (1990) found that germination of *P. tabacina* sporangia was completely inhibited when exposed to 10 $\mu g/cm^2$ of the DVT-diols and was stimulated at much lower concentrations. Inhibition of germination of *P. tabacina* sporangia by *cis*-abienol was also reported, but this compound was less active than the DVT-diols (Menetrez et al., 1990). Sucrose esters, another exudate constituent common to many tobacco genotypes (Severson et al., 1991), reportedly had no effect on *P. tabacina* sporangium germination (Menetrez et al., 1990). The sucrose ester fraction tested in that experiment was isolated from a single tobacco genotype. It is possible that sucrose esters isolated from other genotypes may have antimicrobial activity as genetic variation for sugar ester type, and ester moiety composition does exist among *N. tabacum* genotypes and *Nicotiana* species (Severson et al., 1991). The objective of our research was to determine the effects of certain trichome exudate components on *P. tabacina* sporangium germination using an in vivo system. This included further characterization of the fungicidal activity of the

DVT-diols and *cis*-abienol and testing of additional exudate compounds, including several labdanes and a number of sugar esters isolated from *Nicotiana* species.

METHODS AND MATERIALS

Plants. Seed of Tobacco Introduction (TI) 1406 were germinated in a commercial soil mix in the greenhouse. Plants were transferred into plastic pots (8.5 cm diameter) containing steam-sterilized soil mixture (soil–sand–peat, 2:2:1 v/v/v) and then moved to a growth chamber kept at 24°C with a 12-hr photoperiod of fluorescent light. Plants were fertilized twice a week with Peters 20:20:20 (N/P/K) water-soluble fertilizer.

Isolation and Purification of Leaf Surface Compounds. Cuticular components were extracted from *N. tabacum* cultivars NC 2326 and NFT, *N. glutinosa* (accessions 24 and 24a), and 24 other *Nicotiana* species grown in field plots at Georgia Coastal Plain Experiment Station, Tifton, Georgia, and the Crops Research Laboratory, Oxford, North Carolina. Tops cut from plants six to eight weeks after transplantation were extracted with methylene chloride (Baker Resi-analyzed reagent grade) as previously described (Jackson et al., 1986; Severson et al., 1988). *cis*-Abienol (from NFT), α-DVT-diol, and β-DVT-diol (from NC 2326) were isolated in 98+% purity as described by Severson et al. (1988). The (13-*E*)-labda-13-ene-8α,15-diol (labdenediol) (from *N. glutinosa* no. 24a) and the sclareols, a mixture of (13-*R*)- and (13-*S*)-labda-14-ene-8α,13-diol (sclareol and episclareol), and (13-*R*)-labda-8,14-diene-2α,13-diol (2-hydroxymanool) (from *N. glutinosa* no. 24) were obtained in 98+% purity as described by Jackson et al. (1986). (13-*R*)-labda-8,14-diene-13-ol (manool) was obtained from Aldrich Chemical Co.

The cuticular extracts from the other *Nicotiana* species in Table 1 were taken to dryness on a rotoevaporator, and the residue was partitioned between hexane and 80% methanol–water as described by Severson et al. (1988). The methanol–water-soluble fraction (100–200 μg) containing the sugar esters was dissolved in 0.5 ml of 90% methanol–water, loaded into an injection loop, and chromatographed on a Bio-Sil C-18 (HL Silica, fully endcapped; 40–63 μm; Bio-Rad Laboratories) preparative reverse phase column (1.25 cm ID × 15 cm). The column was eluted with 225 ml 60% methanol–water, 90 ml 90% methanol–water, and 125 ml 100% methanol, and 5-ml fractions were collected. The solvent was removed on a Savant SpeedVac and fractions with observable residue were analyzed by capillary gas chromatography (Severson et al., 1984; Jackson et al., 1986). Fractions containing glucose and/or sucrose esters were combined to yield the sugar ester isolates tested. The chromatography cleanly separated the glucose and sucrose ester fractions in the *N. attenuata* sample, and they were combined separately.

TABLE 1. EFFECT OF GLUCOSE AND/OR SUCROSE ESTERS PARTITIONED FROM LEAF WASHES OF DIFFERENT *Nicotiana* SPECIES (ACCESSIONS) ON *P. tabacina* SPORANGIUM GERMINATION

Treatment (30 $\mu g/cm^2$)	Spores (N)[a]	Inhibition (%)
Experiment 1		
Control	110.8 ± 28.6	(62.4)[b]
N. noctiflora	96.7 ± 23.1	−0.6
N. nudicaulis	93.3 ± 22.6	3.4
N. pauciflora	115.7 ± 30.8	3.7
N. acuminata	105.9 ± 20.0	23.3[c]
N. miersii	101.1 ± 22.0	33.9[c]
N. clevelandii	102.5 ± 24.6	39.8[c]
Experiment 2		
Control	157.0 ± 35.5	(72.6)
N. langsdorfii	155.6 ± 39.3	4.6
N. palmerii	177.1 ± 45.4	5.9[c]
N. velutina[d]	168.9 ± 39.6	12.7[c]
N. rustica	172.0 ± 43.2	14.2[c]
N. bonariensis	173.4 ± 27.4	15.8[c]
N. maritima[d]	179.3 ± 30.8	16.3[c]
Experiment 3		
Control	51.3 ± 16.7	(31.0)
N. cavicola[d]	45.2 ± 9.0	−13.5
N. gossei	45.3 ± 10.5	−9.0
N. simulans[d]	45.8 ± 13.7	−6.0
N. plumbaginifolia	53.6 ± 20.6	−0.6
N. occidentalis	46.4 ± 11.5	4.7
N. bigelovii (13)	52.9 ± 17.3	13.2
Experiment 4		
Control	119.1 ± 37.3	(39.6)
N. hesperis	137.9 ± 16.7	−12.0
N. umbratica	105.6 ± 22.9	6.1
N. debneyi	116.7 ± 25.2	7.1
N. amplexicaulis	108.4 ± 28.6	10.3
N. benthamiana	113.2 ± 32.7	20.5
N. bigelovii (12)	108.4 ± 14.6	23.9[c]
N. attenuata	114.4 ± 29.9	27.9[c]
N. bigelovii (10)	116.1 ± 29.3	33.2[c]

[a] Data are the means ± standard deviation of 10 replications.
[b] Figures in parentheses are the mean percent germination.
[c] Means do not differ significantly from control treatment ($P < 0.05$) by paired t test.
[d] Blue mold-resistant species (Rufty et al., 1990).

Inoculum Production. P. tabacina inoculum was maintained following a procedure adapted by Cohen and Kuć (1980). Six-week-old plants of the blue mold-susceptible tobacco cultivar KY 14 were sprayed to runoff with a fresh sporangial suspension (2×10^5 sp/ml) of *P. tabacina* isolate KY-79. Plants were held at 22–24°C in a dark chamber with 100% relative humidity for 18 hr. Plants were then transferred to a growth chamber with 75% relative humidity and a 12-hr photoperiod for disease development. Sporangium formation was induced when chlorotic lesions developed (seven to eight days after inoculation) by placing plants in darkness for 24 hr at 20°C and 100% relative humidity. Fresh inoculum for each experiment was obtained by lightly brushing the sporulating leaf surface with cold sterile distilled water. To remove any germination inhibitors, sporangium suspensions were filtered and rinsed thoroughly using a 3.0-μm Millipore filter system. Sporangia were resuspended in sterile distilled water, and sporangium concentration was calculated with the aid of a hemacytometer.

Bioassay. Leaf disks (50 mm^2) excised from interveinal areas of leaves from 5- to 6-week-old TI 1406 plants were placed adaxial side up on wet filter paper in a Petri dish. A stock solution of each leaf surface compound was prepared by dissolving each compound in acetone. Chemical concentrations were prepared by diluting portions of the stock solution with acetone. A 10-μl aliquot of each treatment solution was applied evenly to the entire surface of each leaf disk using an Eppendorf repeater pipet. Leaf disks were then set aside to dry prior to inoculation. The following control treatments were included in each experiment: (1) no chemical or acetone was applied and (2) an acetone blank in which there was no leaf surface chemical.

Using an airbrush sprayer (Paashe Airbrush Co., Harwood Heights, Illinois), leaf disks were uniformly inoculated with a 500-μl sporangium suspension of *P. tabacina* (10×10^4–15×10^4 sp/ml) at 50 psi. To maintain a high humidity, the inner surface of the Petri dish covers were sprayed with distilled water. Plates were incubated for 24 hr at 15°C in the dark. Leaf disks in each plate were then sprayed with 500 μl of a 0.01% solution of the fluorescent stain CalcoFluor White MR2 (Polysciences, Inc., Warrington, Pennsylvania), removed from the filter paper, and arranged on glass slides. Slides were fixed by applying drops of ACCU-Mount 60 Mounting Medium (Stephens Scientific, Denville, New Jersey) and topped with cover slips. Sporangium germination was evaluated by fluorescence microscopy differentiation as developed by Wigglesworth et al. (1990). The study included a series of separate experiments. First, those chemicals reported previously to have antimicrobial activity were tested using a dilution series. Second, sclareol, labdenediol, manool, and 2-hydroxymanool were tested at a relatively high concentration. A dilution series experiment was performed for any of these compounds that displayed activity at the high concentration. Third, glucose and/or sugar ester mixtures from 24

Nicotiana species were tested at a concentration of 30 $\mu g/cm^2$. A completely randomized design was used for each experiment with 10 replications per treatment.

Analysis. Percent inhibition was calculated individually for each experiment by using the following formula: % inhibition = [(% germination of control treatment − % germination at each concentration tested)/% germination of control treatment] × 100. The log dose (concentration)–response relationship for each of the treatments was linearized using probits and inhibitory concentrations (IC_{50}) were estimated (SAS Institute Inc., 1985).

RESULTS

The in vivo bioassay was used to approximate the natural infection process and to permit observation of germ tube growth and infection plug formation. An integral part of this bioassay is the leaf tissue to which we applied the test chemicals and inoculum of the obligate pathogen, *P. tabacina*. We selected the blue mold-susceptible TI 1406 to provide the leaf tissue for the bioassay because this genotype is classified as having nonsecreting glandular trichomes, and it lacks the trichome exudate mass typically found on other tobacco genotypes such as those from which the test chemicals were extracted. To further reduce any possible complicating exudate compounds that may be produced by TI 1406, we used leaf tissue from juvenile plants grown under fluorescent lighting (Severson et al., 1985). Young plants produce little exudate and synthesis of trichome secretory product is low for plants grown under fluorescent lamps. Although TI 1406 has only minimal exudate compounds under optimum growth conditions, it does have cuticular waxes. Capillary gas chromatograms of the leaf surface washes of TI 1406 and several genotypes that have secreting glandular trichomes have similar profiles except for those compounds found in the trichome exudate (Nielsen and Severson, 1990). Thus, TI 1406 would likely have few if any leaf surface compounds that could influence detection of the activity of the test compounds.

Microscopic evaluation using fluorescence revealed that sporangia and germ tubes fluoresced bright blue against a red leaf surface background; thus, germination assessments were readily attainable. Sites of germ tube penetration of the leaf were apparent, and in some cases appressoria formation was visible. No morphological differences were observed among sporangia, and germ tube length did not appear to be affected when exposed to any of the chemical or control treatments. In all experiments, the acetone control had little or no effect on sporangium germination when compared to the untreated leaf disks, and thus only the acetone control was used for comparison purposes. A different batch of fresh inoculum was used for each experiment, and this resulted in some

variation in sporangium germination from one experiment to the next. Germination of sporangia for the control treatments was from 31% to 70% among the different experiments with a mean of 49%. Data from these different experiments were not compared directly as these experiments were performed separately.

IC_{50} values were 3.0 $\mu g/cm^2$ for α-DVT-diol and 2.9 $\mu g/cm^2$ for β-DVT-diol (Table 2). A similar inhibitory response was observed when sporangia were exposed to the α- + β-DVT-diol mixture. Percent inhibition after exposure of sporangia to α- + β-DVT-diol at the highest concentration tested was similar to inhibition levels of sporangia when exposed to the individual isomers at the same concentration. Sporangia were not completely inhibited from germinating at concentrations up to 120 $\mu g/cm^2$ of α-DVT-diol or β-DVT-diol. However, sporangium germination was reduced nearly 80% at DVT-diol concentrations of 30 $\mu g/cm^2$. There appeared to be little additional effect of increasing DVT-diol beyond 30 $\mu g/cm^2$. Furthermore, at very low concentrations of DVT-diols, including the mixture of the two isomers, there was no indication of any consistent stimulatory effect of these chemicals on sporangium germination.

The next leaf surface chemical tested was *cis*-abienol, which also had been reported previously to affect *P. tabacina* sporangium germination. In a dilution series experiment (data not shown), *cis*-abienol was tested for inhibitory effects at concentrations ranging from 3.75×10^{-4} $\mu g/cm^2$ to 120 $\mu g/cm^2$. Sporangium germination was not greatly reduced when exposed to any of the 10 concentrations of *cis*-abienol when compared to the control treatment. At the two lowest concentrations of *cis*-abienol (3.75×10^{-4} and 3.75×10^{-3} $\mu g/cm^2$), sporangium germination was greater than for the acetone control treatment.

Sclareols, manool, labdenediol, and 2-hydroxymanool, were initially tested at a single concentration of 30 $\mu g/cm^2$ to determine if any of these inhibited *P. tabacina* sporangium germination. Manool appeared to have no activity at this concentration. Although 2-hydroxymanool significantly reduced sporangium germination, the magnitude of the reduction was much less than that observed for the sclareol mixture and labdenediol. Thus, dilution series experiments were conducted only for the latter two chemicals.

Labdenediol and the sclareol mixture had IC_{50} values of 0.4 $\mu g/cm^2$ and 4.7 $\mu g/cm^2$, respectively (Table 3). Percent inhibition after exposure to additional amounts of the sclareol mixture (>15 $\mu g/cm^2$) was similar to inhibition levels of sporangia when exposed to 15 $\mu g/cm^2$. A similar response in sporangium germination was observed among concentrations of labdenediol ranging from 3.75 $\mu g/cm^2$ to 30 $\mu g/cm^2$. However, 60 $\mu g/cm^2$ of labdenediol caused nearly a 90% reduction in sporangium germination.

A mixture of DVT-diols, labdenediol, and sclareols were tested to determine if there was a synergistic effect among these compounds. The chemicals were mixed in a 1 : 1 : 1 ratio at all the concentrations. The ratio of the α and β isomers of DVT-diol was 2 : 1 within the DVT-diol component of the mixture.

TABLE 2. PERCENT INHIBITION OF *P. tabacina* SPORANGIA AFTER EXPOSURE TO α-DVT-DIOL, β-DVT-DIOL, AND α- + β-DVT-DIOL MIXTURE IN SEPARATE EXPERIMENTS

Concentration ($\mu g/cm^2$)	α-DVT-diol		β-DVT-diol		α- + β-DVT-diol	
	Spores (N)[a]	Inhibition (%)	Spores (N)	Inhibition (%)	Spores (N)	Inhibition (%)
Control	187.4 ± 31.1	(50.7)[b]	88.2 ± 19.2	(35.4)	136.3 ± 27.8	(70.2)
3.75×10^{-4}	184.6 ± 25.4	2.1	93.1 ± 22.0	21.5	154.0 ± 47.6	4.6
3.75×10^{-3}	183.7 ± 26.5	−5.3	88.5 ± 23.4	−3.9	141.4 ± 30.3	−0.7
3.75×10^{-2}	184.7 ± 15.5	1.2	94.9 ± 30.0	1.8	140.6 ± 33.9	1.9
3.75×10^{-1}	172.3 ± 37.7	11.8	96.3 ± 23.4	28.3	143.2 ± 35.6	3.2
3.75	188.4 ± 29.7	59.0	90.4 ± 20.7	50.3	132.7 ± 38.0	52.9
7.5	174.8 ± 15.0	68.8	84.8 ± 20.0	56.2	172.2 ± 78.8	59.8
15.0	166.8 ± 19.6	78.6	83.2 ± 16.2	68.3	127.3 ± 37.0	65.6
30.0	176.3 ± 23.6	78.5	83.7 ± 22.0	81.4	132.4 ± 46.1	78.1
60.0	163.1 ± 20.5	83.1	76.3 ± 21.9	81.8	116.4 ± 42.8	79.8
120.0	165.2 ± 20.4	85.7	79.6 ± 18.2	83.7	103.0 ± 14.3	82.3
IC_{50}[c]		3.0		2.9		5.0

[a] Data are the means ± standard deviation of 10 replications.
[b] Figures in parentheses are the mean percent germination.
[c] Inhibitory concentration ($\mu g/cm^2$) estimated by probit analysis.

TABLE 3. PERCENT INHIBITION OF *P. tabacina* SPORANGIA AFTER EXPOSURE TO DIFFERENT CONCENTRATIONS OF LABDENDIOL AND SCLAREOL MIXTURE

Concentration ($\mu g/cm^2$)	Labdenediol		Sclareols	
	Spores (N)[a]	Inhibition (%)	Spores (N)	Inhibition (%)
Control	176.0 ± 48.0	(38.5)[b]	183.9 ± 61.5	(41.8)
9.38×10^{-1}	171.6 ± 31.7	47.5	210.2 ± 64.0	22.2
1.88	192.6 ± 33.8	63.0	211.5 ± 65.9	36.4
3.75	193.2 ± 35.1	76.8	222.3 ± 55.0	56.0
7.5	204.5 ± 47.8	78.2	193.8 ± 62.2	57.0
15.0	187.4 ± 48.9	74.0	202.3 ± 83.4	65.1
30.0	180.5 ± 63.5	77.7	194.2 ± 50.7	67.1
60.0	180.0 ± 57.8	89.3	178.3 ± 54.5	67.2
IC_{50}[c]		0.4		4.7

[a] Data are the means ± standard deviation of 10 replications.
[b] Figures in parentheses are the mean percent germination.
[c] Inhibitory concentration ($\mu g/cm^2$) estimated by probit analysis.

TABLE 4. PERCENT INHIBITION OF *P. tabacina* SPORANGIA AFTER EXPOSURE TO MIXTURE OF α- + β-DVT-DIOLS, LABDENEDIOL, AND SCLAREOLS

Concentration ($\mu g/cm^2$)	Mixture[a]	
	Spores (N)[b]	Inhibition (%)
Control	220.4 ± 57.8	(50.7)[c]
4.69×10^{-1}	207.6 ± 54.8	11.6
9.38×10^{-1}	227.5 ± 67.6	21.4
1.88	212.9 ± 46.3	28.4
3.75	221.4 ± 67.2	67.0
7.5	193.7 ± 70.3	46.5
15.0	171.1 ± 41.1	71.4
30.0	192.9 ± 53.6	82.7
IC_{50}[d]		4.2

[a] Compound mixture of (2/3 : 1/3) : 1 : 1 (α- + β-DVT-diols)–labdenediol–sclareols at all concentrations.
[b] Data are the means ± standard deviation of 10 replications.
[c] Figures in parentheses are the mean percent germination.
[d] Inhibitory concentration ($\mu g/cm^2$) estimated by probit analysis.

Sporangium germination was not inhibited completely following exposure to the mixture at any concentrations tested. The IC_{50} value was 4.2 $\mu g/cm^2$ for this mixture (Table 4).

Sugar ester mixtures from 23 *Nicotiana* species and three different fractions from *N. bideglovii* were tested for antimicrobial activity at a single concentration of 30 $\mu g/cm^2$. Compared to the control treatments, leaf washes from five of the species, *N. acuminata*, *N. attenuata*, *N. benthamiana*, *N. clevelandii*, and *N. miersii*, and accessions 10 and 12 of *N. bigelovii* inhibited sporangium germination by > 20% (Table 1).

DISCUSSION

The data presented provide further support for the hypothesis that certain tobacco leaf surface components may influence resistance to blue mold in tobacco. Menetrez et al. (1990) observed in in vitro experiments total inhibition by the DVT-diols at 10 $\mu g/cm^2$ and a stimulation in germination as the DVT-diols were diluted.

Using an in vivo bioassay, we found that although sporangium germination was never inhibited completely, a 78% reduction in germination was observed when sporangia were exposed to 30 $\mu g/cm^2$ of the individual DVT-diol isomers or an isomer mixture. The concentrations at which we found α- and β-DVT diols inhibited sporangium germination were well within those levels reported to be found on leaves of field-grown plants of *N. tabacum* (Severson et al. 1985). IC_{50} values of 5.0 $\mu g/cm^2$ for the DVT-diols were less than is often applied for commercial fungicides. Cruickshank et al. (1977) reported the β isomer of the DVT-diols was more inhibitory than the α isomer, and recently Menetrez et al. (1990) suggested that the α isomer was more toxic. No relative differences were observed between the individual isomers in separate experiments of our study. On tobacco leaves these compounds are often found together as part of the exudate mass in a 2:1 ratio of α and β isomers, and therefore their individual activity may be of minor importance.

Menetrez et al. (1990) applied *cis*-abienol to the surface of water agar and found that *P. tabacina* sporangium germination was significantly inhibited. However, they found *cis*-abienol activity was suppressed when it was combined with other compounds including hydrocarbons and sucrose esters, which are also found in the leaf surface. We did not find *cis*-abienol to have any inhibitory activity at any of the concentrations tested. Possible interactions between *cis*-abienol and other leaf surface components not produced by trichomes may explain the lack of biological activity from *cis*-abienol in this in vivo system. Moreover, purified *cis*-abienol in acetone readily recrystallizes upon solvent reduction, and

crystalline masses on the leaf surface may not interact with the blue mold sporangium.

Sclareol, a leaf surface diterpene isolated from *N. glutinosa*, also has been reported to have biological activity. Bailey et al. (1974) exposed a wide range of fungal colonies and sporangia to sclareol and found that sclareol did not affect germination, but it did inhibit mycelial development on agar media. We found that when sporangia were exposed to 3.75 $\mu g/cm^2$ of a mixture of sclareol and episclareol, germination was inhibited by 56%. We believe this is the first report to find that sclareol inhibits sporangium germination.

In dilution series experiments, labdenediol, having a IC_{50} value of 0.4 $\mu g/cm^2$, was the most active leaf surface component tested. Higher concentrations of labdenediol further reduced sporangium germination, and at the highest concentration tested germination was nearly 90% inhibited.

Cutler et al. (1986) found sucrose esters inhibited the growth of gram-positive bacteria such as *Bacillus subtilis* but were not effective against the growth of gram-negative bacteria and the fungi *Curvularia lunata* and *Aspergillus flavus*. *P. tabacina* sporangia were not affected by sucrose esters in in vitro experiments (Menetrez et al., 1990). In our experiments, sugar esters from the different *Nicotiana* species showed less antimicrobial activity than the terpenoid compounds. Germination was reduced from 20% to 40% by the different sugar esters from six *Nicotiana* species, none of which have been reported to have blue mold resistance. This anomaly may have several explanations. Among these are that the sugar esters exist with a matrix of other compounds in the trichome exudate and their antimicrobial activity may be obscured by this matrix in evaluations for blue mold resistance. Additionally, tests identifying the relative resistance of *Nicotiana* species to blue mold may not have been conducted under plant growth conditions suitable for optimal biosynthesis of exudate compounds. These glucose and sucrose esters vary with the type of acid substitution on the glucose or sucrose moieties and most often consist of mixtures of these different esterified compounds. Because these sugar esters make up a small portion of the leaf surface profile, their individual significance in blue mold resistance needs further investigation.

In addition to genetic background, other factors have been reported to influence the leaf surface composition, including age of development and agronomic and environmental factors (Severson et al., 1985). While fungi-toxic leaf surface chemicals may not provide complete resistance to blue mold, we believe that they may have a significant role in the epidemiology of the disease. Elucidation of inheritance patterns for specific exudate constituents and trichome traits in tobacco permits plant breeders and geneticists to manipulate the composition and concentration of trichome exudate (Nielsen and Severson, 1990). This capability, coupled with the knowledge of antimicrobial or insecticidal

activity of exudate compounds, could enable the development of genotypes with enhanced pest resistance.

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TRENDS IN CHEMICAL ECOLOGY REVEALED WITH A PERSONAL COMPUTER PROGRAM FOR SEARCHING DATA BASES OF SCIENTIFIC REFERENCES AND ABSTRACTS

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Abstract—A compiled program, JCE-REFS.EXE (coded in the QuickBASIC language), for use on IBM-compatible personal computers is described. The program converts a DOS text file of current B-I-T-S (BIOSIS Information Transfer System) or BIOSIS Previews references into a DOS file of citations, including abstracts, in a general style used by scientific journals. The latter file can be imported directly into a word processor or the program can convert the file into a random access data base of the references. The program can search the data base for up to 40 text strings with Boolean logic. Selected references in the data base can be exported as a DOS text file of citations. Using the search facility, articles in the *Journal of Chemical Ecology* from 1975 to 1991 were searched for certain key words in regard to semiochemicals, taxa, methods, chemical classes, and biological terms to determine trends in usage over the period. Positive trends were statistically significant in the use of the words: semiochemical, allomone, allelochemic, deterrent, repellent, plants, angiosperms, dicots, wind tunnel, olfactometer, electrophysiology, mass spectrometry, ketone, evolution, physiology, herbivore, defense, and receptor. Significant negative trends were found for: pheromone, vertebrates, mammals, Coleoptera, Scolytidae, *Dendroctonus*, lactone, isomer, and calling.

Key Words—BIOSIS Previews, key word search algorithm, data base, reference manager, word processing, personal computer software, semiochemical, pheromone

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INTRODUCTION

The field of chemical ecology is interdisciplinary since chemistry, including analytical methods of separation and chemical identification as well as organic synthesis, is merged with ecology, including areas of behavior, physiology, genetics, and evolution. This diverse subject area becomes even more complex as studies of animals, plants, and microorganisms are reported. The number of articles published in the field of chemical ecology has continued to expand yearly, and it becomes increasingly difficult for the scientist to assimilate these results. However, the personal computer can be used to store, manipulate, and retrieve knowledge from data bases to aid in managing this ever expanding base of knowledge. Commercial services such as B-I-T-S (BIOSIS Information Transfer System) can provide the latest references on computer disk for viewing by personal computers. BIOSIS Previews also can often be used at a university library for retrospective searches on a particular topic. The located references from such a search of the many bioscience journals and publications, from the present back to 1969, can be downloaded to disk for a fee.

I have developed a compiled BASIC program, JCE-REFS.EXE, which runs on IBM-compatible computers from the DOS command line. The program can take DOS text files of either current monthly B-I-T-S listings or of retrospective searches of BIOSIS Previews and make a data base. This data base can then be searched for text strings (key words) and the located references/abstracts marked for export to a word processor in a format resembling a standard scientific citation. Other commercial data bases can be imported, such as MEDLINE (National Library of Medicine) and CA Search (American Chemical Society), but exported citations may not be formatted entirely accurately due to differences in punctuation.

In addition to describing the program and its general use, the objective was to download the entire 2133 references (October 1991) of the *Journal of Chemical Ecology (JCE)* with abstracts and make a data base. The data base then was searched repeatedly for many different key word phrases for each year (1975–1991) in order to illustrate certain trends in chemical ecology. The general areas covered were use of terms describing: (1) semiochemicals, (2) taxonomic groups, (3) methods, (4) chemical classes, and (5) general or specific subjects used in chemical ecology.

METHODS AND MATERIALS

An IBM-DOS text file from BIOSIS Previews (retrospective search) or B-I-T-S (monthly update) is required for processing by the program. Files from retrospective searches can be downloaded from a number of commercial com-

puter data bases such as Data-Star, ESA-IRS, DIALOG, and STN. References in a BIOSIS Previews text file may contain the subject identification codes (field tags) of AN (accession number), AU (author), TI (title), SO (source), IN (author affiliation), KW (key words), BC (biosystematic codes), and ST (supertaxa). Other field codes may include CD (coden), LG (language), YR (year), CC (concept codes), and AB (abstract). Monthly updates of recently published articles are formatted slightly differently by BIOSIS, such that the AN field code is replaced with an ID (identification) code and the CS (corporate source, author address) may substitute for the IN code above. The program can be adapted to use different field codes in BIOSIS files, as well as codes in other data bases (as will be explained subsequently).

The program JCE-REFS.BAS was developed with the QuickBASIC programming language (version 4.5, Microsoft). The source code was compiled to JCE-REFS.EXE, a binary, machine-coded, executable file, by the Microsoft compiler version 4.50 and overlay linker version 3.69. IBM-compatible personal computers can execute the program in monochrome or color using character-based monitors and DOS 2.1 or higher.

The major facilities and operation of JCE-REFS.EXE are diagrammed in Figure 1. The arrows between boxes indicate relationships between program

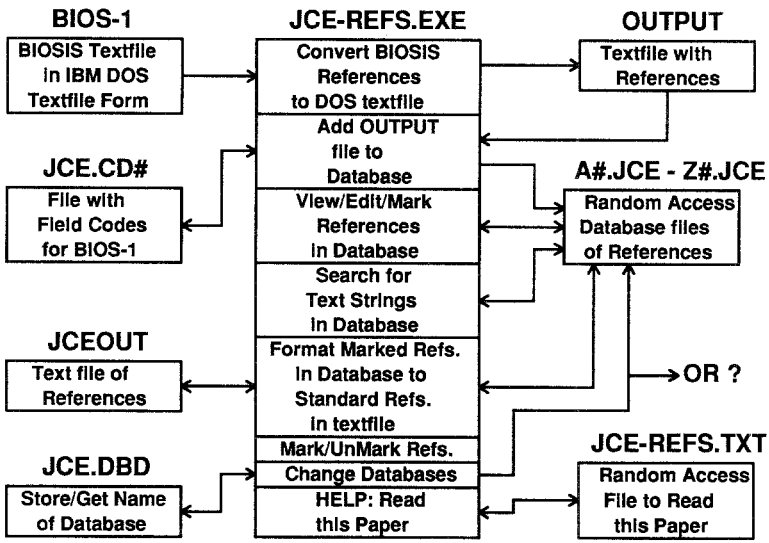

FIG. 1. Relationships of program parts of JCE-REFS.EXE, a BIOSIS file conversion and data-base searching utility for scientific reference management on the IBM-compatible personal computer.

parts but do not necessarily denote program flow. JCE-REFS.EXE is used by typing *jce-refs* at the DOS command line. The program loads and checks to see if a name of a data base is in the file JCE.DBD. If no name is present, then the default extension name (#.JCE) is placed in the file. The menu has seven options to choose from (Figure 1) besides a help section, DOS shell (DOS commands), or exiting from the program.

The first option is to convert BIOSIS files to a standard scientific reference format in a DOS textfile, always given the name OUTPUT. This is done by entering the name of the BIOSIS Previews file (e.g., named BIOS-1 and rep-

```
(A)
AN 85088636 8800.
AU BYERS-J-A.
TI NOVEL DIFFUSION-DILUTION METHOD FOR RELEASE OF SEMIOCHEMICALS TESTING
   PHEROMONE COMPONENT RATIOS ON WESTERN PINE BEETLE.
SO J-CHEM-ECOL 14 (1). 1988. 199-212.
IN DEP ANIMAL ECOL, UNIV LUND, S-223 62 LUND, SWED.
LG EN.
YR 88.
KW DENDROCTONUS-BREVICOMIS    TEMNOCHILA-CHLORODIA    PREDATOR    MYRCENE
   EXOBREVICOMIN PESTS.
BC 75304 Coleoptera.
ST ANIMALS#, INVERTEBRATES#, ARTHROPODS#, INSECTS#.
AB Each of the pheromone components of the Western pine beetle,
   Dendroctonus brevicomis LeC. (Coleoptera: Scolytidae), exo-brevicomin
   (E) and frontalin (F), were released in the forest at various ratios
   etc...


(B)
Byers, J.A. 1988. novel diffusion-dilution method for release of
semiochemicals testing pheromone component ratios on western pine beetle. J.
Chem. Ecol. 14 (1). 199-212.==dendroctonus-brevicomis temnochila-chlorodia
predator myrcene exobrevicomin pests. 75304 coleoptera. animals#,
invertebrates#, arthropods#, insects#. ab each of the pheromone components of
the western pine beetle, dendroctonus brevicomis lec. (coleoptera: scolytidae),
exo-brevicomin (e) and frontalin (f), were released in the forest at various
ratios etc...
dep animal ecol, univ lund, s-223 62 lund, swed


(C)
Byers, J.A. 1988. novel diffusion-dilution method for release of semiochemicals
testing pheromone component ratios on western pine beetle. J. Chem. Ecol. 14 (1)
. 199-212.==dendroctonus-brevicomis temnochila-chlorodia predator myrcene exobre
vicomin pests. 75304 coleoptera. animals#, invertebrates#, arthropods#, insects#
. ab each of the pheromone components of the western pine beetle, dendroctonus b
revicomis lec. (coleoptera: scolytidae), exo-brevicomin (e) and frontalin (f), w
ere released in the forest at various ratios etc...
dep animal ecol, univ lund, s-223 62 lund, swed
```

FIG. 2. The JCE-REFS.EXE program processes a BIOSIS text file (A) into a DOS text file (OUTPUT) as shown in (B), which is imported into the random access database file (B#.JCE) as in (C) that can be searched. The reference in the data base also can be exported as a DOS text file (B) or Pro-Cite style (not shown) for use with MacIntosh and IBM-compatible personal computers.

resented in Figure 2A). The user may look at the BIOS-1 file to see if the formatting is such that all text is indented three spaces except for the field codes. Rarely, the file can be received from BIOSIS in a corrupted condition; in this case a reformatting facility is selected to correct the file. Otherwise the user continues and is presented with a list of files (ending in .CD#) that contain various field codes appropriate to the commercial data base. The user enters one of the names or a new name, and the field codes are loaded and presented on a menu for editing. The field codes can be used without editing or new codes added for the respective authors, title, journal, and so forth. These codes make it possible to accommodate any of several types of BIOSIS formats as well as other data base styles such as CA Search and MEDLINE. The program then reformats the BIOSIS file (BIOS-1) using the field codes. The first letters of authors are capitalized and appropriate punctuation is added. The date (1900s or 2000s) is extracted from the journal string. The title and journal letters are uncapitalized and then the first letter of each word in the journal is capitalized. This saves time in the word processor as most letters need to be lowercase in a standard citation. The abstract text is also uncapitalized so that all text searches can be in lowercase. The resulting sequential file, OUTPUT (Figure 2B), is overwritten each time, so one must process the contents, as described subsequently, before another BIOSIS file is reformatted.

After reformatting the BIOSIS file, the second option is used in which the OUTPUT file is entered as the source of references for alphabetical placement in the 26 data base files (e.g., A#.JCE to Z#.JCE). Each reference is formed into a long text string of 36 lines by 80 characters, so the abstract can be included (Figure 2C). These 26 files are random access in which references from additional BIOSIS Previews files can be added by running options one and two again. Alternatively, another name can be chosen (option seven) in order to create additional data bases (e.g., for another journal).

The third option "View/Edit/Mark References in Data Base" allows one to scroll through the data base and view the references. Any references that are desired for word processing can be exported as a citation in the file JCEOUT by marking them (pressing the Insert key). References are unmarked with the Delete key. A global unmarking of all references also can be done (in option six). Facilities for searching for a text string and viewing the located reference and highlighted string are provided while viewing the data base.

The fourth option, "Search for Text Strings in Data Base," allows complex logic searches of the data base fields for up to two sets of 20 text strings simultaneously. The searches employ Boolean logic in one of four ways. Up to 20 text strings can be searched for in either an: (1) *OR* or (2) *AND* way. These also can be combined into: (3) *AND/OR* or (4) *AND+OR* searches with a total of up to 40 text strings. For example, an *AND+OR* search might look for

("pheromone" AND "Scolytidae") and ("1980." OR "1981." OR "1982.") in each reference. Note the use of the space and period in the dates in order to avoid finding page numbers.

Prior to searching for the text strings, the user is asked whether he or she wants to save this search profile for later recall. If the search profile is saved, then the next time a search is made the user may select option five to load the search profile and begin the search without the need for entering the text strings. During the search of the data base, the program displays any located references on screen and waits for keyboard input to mark (or unmark) or continue without marking until all references in the data base are searched. Options include searches of various combinations of authors, date, title, key words, abstract, and address. A search record showing the number of references found out of the total number in the data base is displayed at the end of the search (searches take only some seconds for 2133 references).

The fifth option, "Format Marked Refs. in Data Base to Standard Refs. in Text File," is used to take the author, date, title, journal, and, optionally, the key words, abstract (if present), and address strings and export them. These are saved in a DOS text file called JCEOUT (style as in Figure 2B), which is overwritten each time this option is chosen. This file can then be imported into any word-processing program (e.g., WordPerfect, WordPerfect Corp., Orem, Utah) as well as into the scientific reference manager, CardCat (Byers, 1991).

A sixth option includes either a global unmarking or global marking of all references in the data base. The "Change Data Bases" option allows for an almost unlimited number of databases, 208 available ASCII characters for file names $= [\Sigma_{i=1}^{8} P_i^{208}][\Sigma_{i=1}^{3} P_i^{208}] = 2.7 \times 10^{25}$. Each time the program is started, it gets the current data base, which is permanently stored in the file JCE.DBD until changed by the user. The user can receive help from within the program by reading the file JCE-REFS.TXT, which includes the contents of this paper with figures. This file appears to be a standard sequential DOS text file but is actually a random access file that allows scrolling the text forward and backward on the screen.

BIOSIS Previews (from Data-Star, Radio-Suisse) was searched for all articles published in the *Journal of Chemical Ecology* (including abstracts and addresses) from 1975 until October 1991. The resulting search file was downloaded into 16 files of about 200K each to a personal computer. The files were imported into the JCE-REFS program, which made a data base of 26 files with a total of 6 megabytes. This data base was searched by year, as described below, for certain key words as shown in Figures 3–5 and Tables 1–5 below. Except for the super taxonomic designations of BIOSIS (ending in #, as in Table 2 and Figure 4), it should be noted that the incidence of various key words found in the titles, key words, and abstracts are minimal estimates since the entire article was not searched. Furthermore, certain key words are more likely to be men-

tioned in the abstract than others due to the nature of the words. However, key words merely mentioned in the body of the paper, and not in the title, key words, or abstract, quite often are not of primary relevance to the article.

RESULTS AND DISCUSSION

It is commonly accepted that the number of scientific publications is increasing annually, although opinion would vary as to whether the function was geometric, exponential, or linear. The actual quantities of publications available to scientists as reflected in the BIOSIS data base is also not well known. In Figure 3A, the trend in publication rate is increasing, and a linear function ($r^2 = 0.89$) describes the relationship about as well as a geometric ($r^2 = 0.88$) or exponential equation ($r^2 = 0.90$). In 1990 more papers were published than ever before, with over 540,000 publications from more than 9000 journals available to the chemical ecologist (BIOSIS Previews). The number of papers in the *Journal of Chemical Ecology (JCE)* in 1990 also was highest (281). Here too a positive linear equation ($r^2 = 0.90$) describes the trend of increasing publication rate as well as do other curvilinear functions, although an exponential curve fits the data slightly better ($r^2 = 0.94$). However, from Figure 3B it can be seen that the number of papers in *JCE* has increased relatively faster than the publication rate in general in BIOSIS ($r^2 = 0.65$, positive slope, $P < 0.001$).

The number of "pheromone" papers in BIOSIS has increased linearly during the period from 1975 to 1990 ($r^2 = 0.92$) at the same rate as the increase in publications in BIOSIS (same percentage, Figure 3B, slope not significantly different from 0). On the other hand, the rate of pheromone papers in *JCE* has decreased (Figure 3B, Table 1). The uses of other semiochemicals such as "allomone," "allelochemic," and "semiochemical" have increased, although use of any combination of these in papers has not changed significantly over the period (Table 1). Uses of the words "deterrent" and "repellent" have increased (Table 1).

Most papers in *JCE* concern "animals" (average yearly percentage of 89.4), and "insects" make up the majority of these (79%) while "vertebrates" involve 13.9% of all papers, and "microorganisms" only 7.4% (Table 2). The percentage of animal papers has remained relatively constant, as has that for insects and "Lepidoptera," while the trend in vertebrate papers has decreased (Table 2), although the vertebrate trend over the last 15 years has been stable (Figure 4). In addition to vertebrates, other taxa in Table 2 show significant negative trends: "mammals" and "Coleoptera" (including "Scolytidae" and "*Dendroctonus*"). Significant positive trends are shown for plants (only "vascular") and "angiosperms" and "dicots," but "gymnosperms" and "monocots" show no significant relative changes (Table 2, Figure 4). The increase in

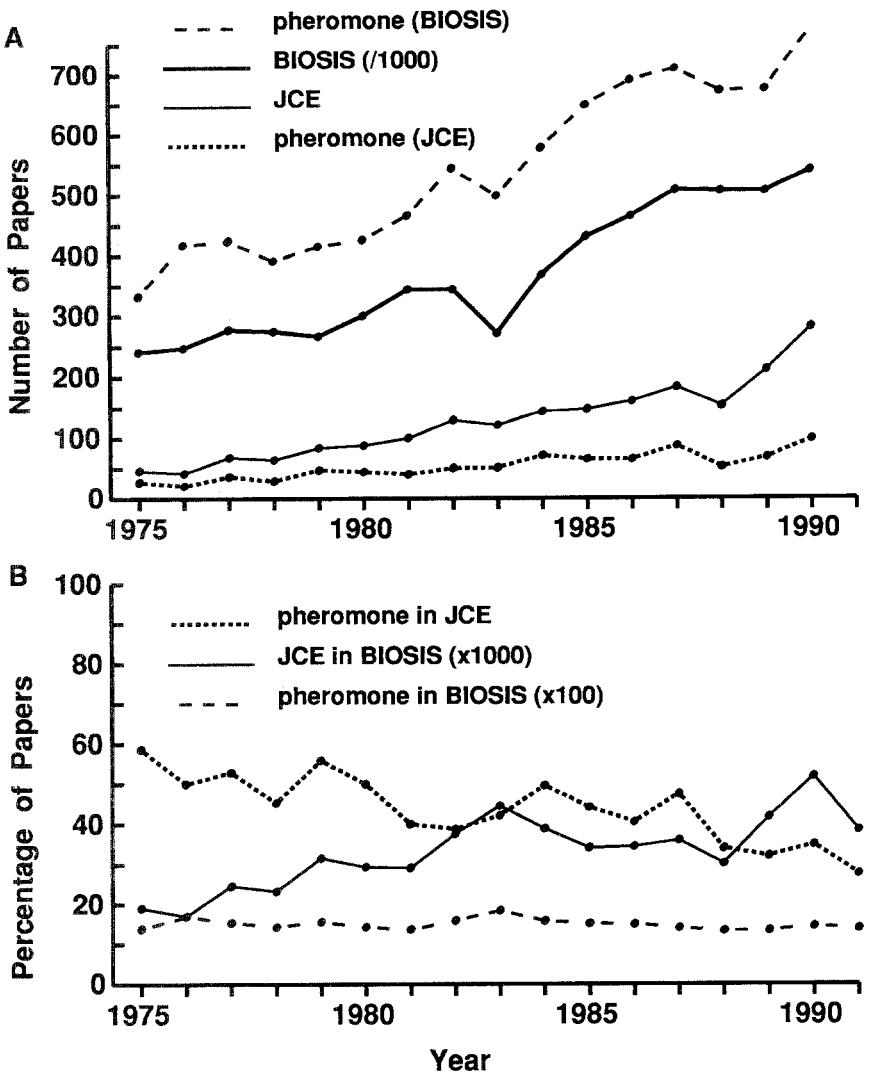

FIG. 3. (A) Number of papers with key word "pheromone" and total papers published per year in BIOSIS or *Journal of Chemical Ecology* (JCE), and (B) percentage of papers with "pheromone" in JCE or BIOSIS, and percentage of JCE papers in BIOSIS.

papers on plants may be real in part, but part of the increase also may be due to more often mentioning the "plant–insect" relationship than was done earlier.

There has been no significant change in the percentage of papers mentioning a "method" or "technique" useful to chemical ecology (Table 3). The use of

TABLE 1. POSITIVE AND NEGATIVE TRENDS FROM 1975 TO 1991 IN PERCENTAGE OF PAPERS IN *Journal of Chemical Ecology* USING VARIOUS SEMIOCHEMICALS IN TITLE, KEY WORDS, OR ABSTRACT (LINEAR REGRESSION WITH ARCSIN SQUARE ROOT TRANSFORMATION OF PERCENTAGES)[a]

Semiochemical	N	Mean % ± SD	r^2	Slope ± 95% CL	P (slope = 0)
Semiochemical	69	2.27 ± 2.79	0.72	1.9 ± 0.6	<0.001*
Pheromone	882	43.79 ± 8.68	0.68	−1.4 ± 0.5	<0.001*
Kairomone	85	4.12 ± 2.72	0.07	−0.4 ± 0.7	0.3
Allomone	33	1.36 ± 1.51	0.23	0.7 ± 0.7	0.05*
Allelochemic	114	4.11 ± 4.33	0.66	2.1 ± 0.8	<0.001*
Any of above	1105	52.81 ± 9.17	0.18	−0.8 ± 0.9	0.09
Deterrent	72	3.04 ± 2.16	0.40	1.0 ± 0.7	0.006*
Inhibitor	148	6.66 ± 2.32	0.09	0.3 ± 0.5	0.25
Repellent	53	2.12 ± 1.53	0.48	1.0 ± 0.6	0.002*
Attractant	249	12.43 ± 3.47	0.13	−0.4 ± 0.5	0.15

[a] P values with asterisks indicate slopes are significantly different from zero ($\alpha = 0.05$).

"wind tunnel" appears to have increased (although found in only 1.6% of the papers), while "bioassay" use has not changed but is used by 15.4% of *JCE* papers. The use of "olfactometer," "electrophysiology" (and related words), and mass spectrometry (GC-MS) have increased, while no significant trends were found for gas chromatography (including "GLC"), thin layer chromatography ("TLC"), capillary GC, high (pressure or performance) chromatography ("HPLC"), and nuclear magnetic resonance spectroscopy ("NMR," Table 3).

There were no significant trends in the frequency of use of several chemical groups (Table 4) except for lactone and isomer (negative trends) and a positive trend for ketone. Surprisingly, the use of "enantiomer" or "optical isomer" has not changed significantly over the period. Even when all chemical groups are searched for in an *OR* way, there is no significant change in relative frequency, with about 47% of the papers mentioning one or more chemical group. Searches of various prefixes indicating carbon chain length from 11 to 19 (undec, dodec, tridec, tetradec, pentadec, hexadec, heptadec, octadec, and nonadec) substantiated the commonly held view that even-carbon chains are more frequently part of semiochemicals than are odd-carbon chains (Figure 5). The most common chain unit mentioned was 14 carbons (Figure 5) and was found in 22.3% of the Lepidoptera papers, or 86.3% of the papers with "tetradec" (e.g., tetradecenyl acetate) also included "Lepidoptera."

Chemical ecology combines the biological fields of ecology, behavior, evolution, genetics, and physiology with chemistry. There are no significant trends in the use of the words "ecology" or "behavior," probably since these

TABLE 2. POSITIVE AND NEGATIVE TRENDS FROM 1975 TO 1991 IN PERCENTAGE OF PAPERS IN *Journal of Chemical Ecology* INVESTIGATING VARIOUS TAXA FOUND IN TITLE, KEY WORDS, OR ABSTRACT (LINEAR REGRESSION WITH ARCSIN SQUARE ROOT TRANSFORMATION OF PERCENTAGES)[a]

Taxa	*N*	Mean % ± SD	r^2	Slope ± 95% CL	*P* (slope = 0)
Microorganisms#	157	7.37 ± 2.62	0	−0.03 ± 0.5	0.92
Plants#	938	41.61 ± 10.54	0.46	1.5 ± 0.9	0.003*
Vascular plants#	859	38.21 ± 9.84	0.45	1.4 ± 0.8	0.003*
Nonvascular plants#	102	4.93 ± 1.9	0	−0.04 ± 0.5	0.85
Gymnosperms#	157	7.48 ± 3.25	0	0.05 ± 0.7	0.87
Angiosperms#	691	30.03 ± 9.45	0.58	1.6 ± 0.7	<0.001*
Dicots#	574	25.27 ± 7.91	0.45	1.2 ± 0.7	0.003*
Monocots#	194	8.58 ± 3.78	0.21	0.6 ± 0.7	0.06
Animals#	1888	89.38 ± 6.05	0.23	−0.9 ± 0.9	0.054
Vertebrates#	274	13.88 ± 4.3	0.27	−0.6 ± 0.5	0.03
Mammals#	199	10.85 ± 4.04	0.51	−1.0 ± 0.5	0.002*
Fish#	43	2.34 ± 1.5	0.06	−0.3 ± 0.8	0.36
Invertebrates#	1646	77.19 ± 6.68	0.02	−0.2 ± 0.8	0.57
Arthropods#	1566	73.84 ± 7.02	0.06	−0.4 ± 0.9	0.32
Insects#	1488	70.2 ± 6.52	0.08	−0.4 ± 0.7	0.28
Lepidoptera	619	28.05 ± 4.6	0.13	0.4 ± 0.5	0.16
Diptera	138	6.41 ± 2.8	0	0.1 ± 0.9	0.83
Hymenoptera	263	12.58 ± 3.74	0.09	−0.3 ± 0.6	0.25
Coleoptera	368	18.73 ± 4.6	0.44	−0.8 ± 0.5	0.004*
Scolytidae	69	4.0 ± 2.6	0.36	−0.8 ± 0.6	0.01
Ips	67	3.37 ± 1.7	0.05	−0.2 ± 0.5	0.38
Dendroctonus	61	3.46 ± 2.77	0.37	−0.9 ± 0.6	0.009*

[a] *P* values with asterisks indicate slopes are significantly different from zero ($\alpha = 0.05$). Number (#) indicates supertaxa designations of BIOSIS (ST codes).

fields were well established as central to chemical ecology by 1975. In fact, "behavior" is by far the most commonly mentioned field (22.9% of the papers) with "physiology" and "ecology" next at only 3.6% (Table 5). There is a positive trend in the use of "evolution" and "physiology," while "genetic" increases but is not quite significant.

Electrophysiological studies account for the majority of the physiology papers since 61% of these had "antennogra" or "electro" (*AND* + *OR* search). The word "receptor" also increases in frequency during the period due to the increase in electrophysiological studies. Uses of "herbivore" and "defense" also show increasing trends. The regression line for the percentage of studies mentioning "synergism," "synergist," or "synergistic" (only 3.6% average) does increase but the slope was not statistically significant from zero (Table 5). It is surprising that synergism is mentioned so sparingly, since it is well estab-

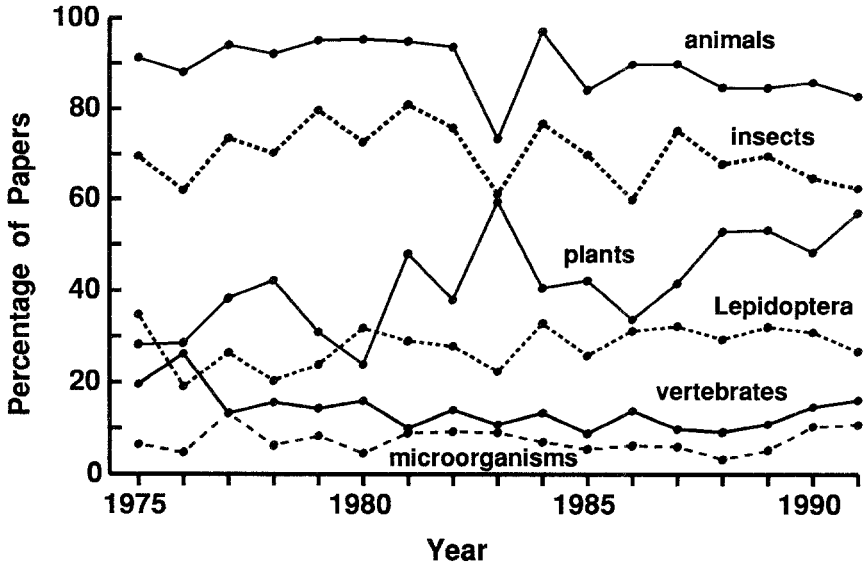

FIG. 4. Percentage of annual papers published in the *Journal of Chemical Ecology* that concern various supertaxa (as classified by BIOSIS Previews) from 1975 to October 1991.

lished that most pheromones, and probably most semiochemicals, are blends of several synergistic components (Silverstein, 1981; Byers, 1992a).

Deitz and Osegueda (1989) discuss the effectiveness of retrieving entomological literature from several data bases including AGRICOLA, AGRIS International, BIOSIS Previews, CAB Abstracts, Dissertation Abstracts Online, Life Sciences Collection (Cambridge Scientific Abstracts), SCISEARCH, and Zoological Record Online. They show that pertinent literature for a particular subject is likely to be found in any of these data bases in varying proportions and redundancy. While the cost effectiveness may vary depending on the blend of data bases used, it was apparent that BIOSIS Previews had the most relevant references. I have searched CA Search and BIOSIS Previews for all references in the *Journal of Chemical Ecology* and found that the former contained only about 86% of the articles while BIOSIS had all of them. Several key word searches and reference retrieval strategies have been proposed (Marshall, 1979; Harvey, 1979; Chiang and Chiang, 1984; Eyers and Taylor, 1988; Resh, 1988; Deitz and Osegueda, 1989). The results of these articles indicate that an exhaustive search of the literature is more costly and difficult than one might anticipate due to the scattering of references and the complexity of designing a key word search profile.

The advantage of building one or more personal data bases from a com-

TABLE 3. POSITIVE AND NEGATIVE TRENDS FROM 1975 TO 1991 IN PERCENTAGE OF PAPERS IN *Journal of Chemical Ecology* INVESTIGATING VARIOUS METHODS FOUND IN TITLE, KEY WORDS, OR ABSTRACT (LINEAR REGRESSION WITH ARCSIN SQUARE ROOT TRANSFORMATION OF PERCENTAGES)[a]

Method	*N*	Mean % ± SD	r^2	Slope ± 95% CL	*P* (slope = 0)
Wind tunnel	37	1.56 ± 1.79	0.43	1.0 ± 0.7	0.004*
Bioassay	332	15.37 ± 3.27	0	0.05 ± 0.5	0.83
Olfactometer[a]	50	2.15 ± 1.49	0.26	0.6 ± 0.6	0.04
Fraction	138	6.54 ± 1.87	0	0.04 ± 0.4	0.83
Antennogram[b] or electrophysiology[b,c]	145	6.16 ± 2.79	0.45	1.1 ± 0.6	0.003*
Method/technique	157	7.66 ± 3.06	0.02	−0.2 ± 0.6	0.58
Gas chromatography[c]	306	13.91 ± 3.63	0.10	0.3 ± 0.6	0.24
Capillary GC[c]	62	2.78 ± 1.52	0.08	0.3 ± 0.6	0.29
Mass spectrometry[b,c]	205	9.21 ± 2.36	0.24	0.4 ± 0.4	0.04*
HPLC[c]	44	1.90 ± 1.79	0.21	0.7 ± 0.7	0.06
TLC[c]	60	2.81 ± 1.30	0	0.05 ± 0.6	0.87
NMR[c]	35	1.52 ± 1.05	0.05	0.3 ± 0.6	0.37

[a] *P* values with asterisks indicate slopes are significantly different from zero ($\alpha = 0.05$).
[b] A truncation of the word was used, e.g. antennogra(m, phy), to include several possible related words.
[c] Several related words were searched using a multiple *OR* search, e.g., gas chromatograph, gas-liquid chromatograph, gas liquid chromatograph, GC-MS, GLC, or GCMS.

mercial data base such as BIOSIS is that the references in the personal data bases can be searched in many different ways over the course of one's career. This may be much less costly and more convenient than to search the commercial data bases multiple times. The drawback is that some references will be missed but this problem minimizes as the personal data base is enlarged. The located references in the data base usually will contain most of the other references in their "literature cited" sections. Of course, articles dated before 1969–1970 can only be found by searches of these sections.

BIOSIS (2100 Arch St., Philadelphia, Pennsylvania 19103-1399) has informed me that they would like presented the "fair use and copyright" issue concerning the use of their data bases. Their policy on downloaded information is that it "may be freely used without further payment to BIOSIS when temporarily stored in lieu of hard copy or printout form which is the normal medium delivered by a VENDOR." However, the temporary storage/use ceases when USER alters the archival characteristic and incorporates BIOSIS information into an "active" system of any type. Currently, a payment of 15 cents per reference per year is due BIOSIS when one has more than 500 references in an active system.

TABLE 4. POSITIVE AND NEGATIVE TRENDS FROM 1975 TO 1991 IN PERCENTAGE OF PAPERS IN *Journal of Chemical Ecology* MENTIONING VARIOUS TYPES OF CHEMICALS FOUND IN TITLE, KEY WORDS, OR ABSTRACT (LINEAR REGRESSION WITH ARCSIN SQUARE ROOT TRANSFORMATION OF PERCENTAGES)[a]

Chemical type	*N*	Mean % ± SD	r^2	Slope ± 95% CL	*P* (slope = 0)
Terpene	144	6.17 ± 2.40	0.08	0.3 ± 0.5	0.28
Acetate	313	15.25 ± 3.90	0.14	−0.4 ± 0.5	0.14
Hydrocarbon	102	4.76 ± 2.45	0	0.03 ± 0.8	0.94
Alcohol	146	7.17 ± 1.88	0.06	−0.2 ± 0.4	0.33
Aldehyde	132	6.43 ± 2.24	0.01	−0.1 ± 0.5	0.71
Ester	114	5.41 ± 1.57	0.01	−0.1 ± 0.4	0.65
Ketone	55	2.16 ± 2.18	0.33	1.0 ± 0.8	0.02*
Lactone	43	2.37 ± 1.66	0.36	−0.6 ± 0.4	0.01*
Tetradec	160	7.85 ± 3.36	0.14	−0.5 ± 0.6	0.13
Phenol	144	6.62 ± 3.81	0.22	0.7 ± 0.7	0.06
Any above or[b]	996	47.00 ± 4.24	0	−0.02 ± 0.5	0.94
Isomer	141	7.39 ± 2.66	0.49	−0.7 ± 0.4	0.002*
Enantiomer[c]	70	3.23 ± 1.71	0.05	0.3 ± 0.6	0.37

[a] *P* values with asterisks indicate slopes are significantly different from zero ($\alpha = 0.05$).
[b] epoxy, tannin, glycoside, quinone, furan, flavone or carbox.
[c] Or optical isomer.

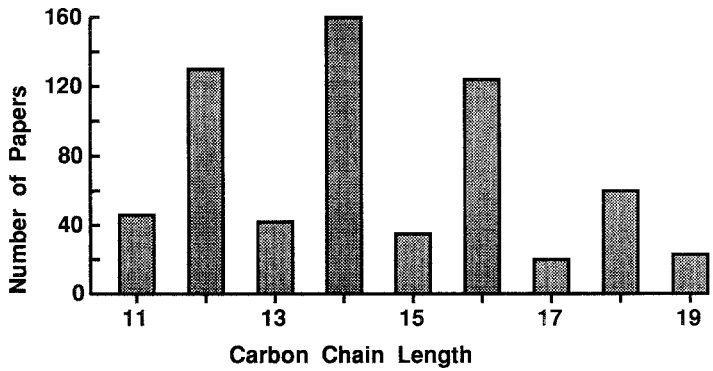

FIG. 5. Number of papers published in the *Journal of Chemical Ecology* concerning carbon chain lengths of 11 to 19 (1975 to October 1991).

The JCE-REFS.EXE program system can be obtained from the author (send a formatted disk and mailer). Donations of $5 or 35 Swedish kronor for shipping would be appreciated. The system can be used to condition BIOSIS Previews files prior to importing them into the scientific reference manager CARD-

TABLE 5. POSITIVE AND NEGATIVE TRENDS FROM 1975 TO 1991 IN PERCENTAGE OF PAPERS IN *Journal of Chemical Ecology* (1975–1991) USING VARIOUS CHEMICAL ECOLOGY TERMS IN TITLE, KEY WORDS, OR ABSTRACT (LINEAR REGRESSION WITH ARCSIN SQUARE ROOT TRANSFORMATION OF PERCENTAGES)[a]

Term	*N*	Mean % ± SD	r^2	Slope ± 95% CL	*P* (slope = 0)
Ecology[b]	87	3.60 ± 2.54	0.12	0.6 ± 0.9	0.18
Evolution	55	2.26 ± 1.64	0.33	0.8 ± 0.7	0.02*
Genetic	37	1.55 ± 1.24	0.22	0.6 ± 0.6	0.005
Behavior	498	22.89 ± 3.74	0.10	2.8 ± 0.5	0.21
(Top three)	168	6.66 ± 3.76	0.39	1.0 ± 0.7	0.007*
(Top four)	636	28.47 ± 6.03	0.32	0.7 ± 0.6	0.02*
Physiology[b]	88	3.63 ± 2.18	0.44	1.1 ± 0.7	0.004*
Habitat	30	1.21 ± 1.31	0.17	0.6 ± 0.8	0.10
Mating	85	4.32 ± 1.89	0.15	−0.3 ± 0.5	0.13
Olfactory (tion)	123	5.82 ± 1.73	0	0.02 ± 0.5	0.92
Attraction	187	8.77 ± 2.12	0.01	−0.1 ± 0.4	0.68
Orientation	44	2.15 ± 1.09	0.05	0.2 ± 0.5	0.38
Herbivore	63	2.43 ± 1.90	0.41	1.0 ± 0.7	0.006*
Defense	128	5.44 ± 2.36	0.48	0.7 ± 0.6	0.002*
Calling	42	2.20 ± 1.38	0.29	−0.7 ± 0.6	0.03
Social	29	1.45 ± 0.83	0.08	−0.2 ± 0.4	0.27
Synergist (m, tic)[b]	82	3.61 ± 1.13	0.19	0.3 ± 0.3	0.08
Receptor	82	3.41 ± 1.91	0.36	0.9 ± 0.7	0.01*
Allelopathy[b]	134	6.15 ± 4.74	0.05	0.4 ± 0.9	0.41
Competition	29	1.58 ± 1.51	0.16	−0.5 ± 0.7	0.12
Adaptation	37	1.75 ± 1.06	0	−0.01 ± 0.5	0.97

[a] *P* values with asterisks indicate slopes are significantly different from zero ($\alpha = 0.05$).
[b] A truncation of the word was used, e.g. ecology(y, ical), to include several possible related words.

CAT.EXE (Byers, 1991). Another program system, BIOSIS.EXE (Byers, 1992b), also is used to reformat BIOSIS data base files and is compatible with CARDCAT, but it can make reprint request cards. However, the BIOSIS.EXE program cannot handle abstracts while JCE-REFS.EXE can.

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INFLUENCE OF MONOTERPENE VAPORS ON SPRUCE SPIDER MITE, *Oligonychus ununguis*, ADULT FEMALES

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Abstract—Adult female spruce spider mite, *Oligonychus ununguis* (Jacobi), were exposed to various concentrations of four host conifer monoterpene vapors (limonene, β-pinene, α-pinene, and Δ^3-carene) for 24 hr to determine the lethal and sublethal effects. All four compounds were toxic to the mites. Further, at concentrations below the calculated LC_{50}s, all four compounds decreased oviposition by the mites and three of the compounds (limonene, β-pinene, and α-pinene) influenced movement. Whereas *O. ununguis* populations may not normally be exposed to high concentrations of host monoterpenes, trees continuously emit some monoterpene vapors, and when trees are damaged or under stress, oleoresin may accumulate at points on the external surface of tree tissues thereby exposing mites to the influence of monoterpene vapors.

Key Words—Arachnida, Acari, Tetranychidae, *Oligonychus*, conifer, monoterpene, toxicity, oviposition, dispersal

INTRODUCTION

The spruce spider mite, *Oligonychus ungungis* (Jacobi) (Acari: Tetranychidae) is a serious pest of conifers throughout the world. It is capable of attacking most genera of conifers including spruce (*Picea*), pine (*Pinus*), fir (*Abies*), larch (*Larix*), and juniper (*Juniperus*) (Löyttyniemi, 1970; Jeppson et al., 1975; Johnson and Lyon, 1976; Drooz, 1985). Spruce spider mite is probably the most destructive conifer-feeding spider mite in the United States (Johnson and Lyon,

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1976) and has been suggested to be the only mite capable of causing economic injury in nurseries and plantations of coniferous trees (Kielczewski, 1966). Damage by spruce spider mite includes yellowing or browning of needles (Garman, 1923; Kielczewski, 1966), resulting in potential premature needle abscission (Peterson and Hildahl, 1969) and possible reductions in tree shoot growth (Löyttyniemi, 1971).

As with many herbivores, spider mite performance on a host plant may be influenced by the plant's chemical content. Researchers working with other spider mite species have demonstrated that nutritional components located in leaf tissue, especially nitrogen and amino acids directly impact spider mite biology (i.e., Cannon and Connell, 1965; Tulisalo, 1971; Dabrowski and Bielak, 1978; Hanna et al., 1982). Furthermore, there was a positive correlation reported between the essential amino acid content in spruce needles and the occurrence of spruce spider mite (Löyttyniemi and Tulisalo, 1972). However, along with nutritional compounds, plants have secondary metabolites within leaf tissues that may have negative impacts on the attacking mite population. Many terpenoid compounds may be placed within this category of potential plant protectants. For example, some components of the essential oils of spice plants are oxygenated monoterpenes, and when these essential oils are sprayed on the spider mite *Tetranychus cinnabarinus* (Boisd.), the result was an increase in mortality and repellancy (Mansour et al., 1986). Furthermore, sesquiterpenes present in tomato have been demonstrated to be both toxic and repellent to the spider mite *T. urticae* Koch (Patterson et al., 1975), and exposure of female *T. urticae* to the three principal monoterpenes in the essential oil of peppermint leaves (pulegone, menthone, and menthol) resulted in reduced oviposition and increased mortality (Larson and Berry, 1984) and spider mite injury increased the relative amounts of menthone and menthol in peppermint leaf essential oils (DeAngelis et al., 1983).

The potential influence of conifer needle monoterpenes on spruce spider mite has not been investigated. However, conifers usually have many terpenoid compounds present within their tissues, and these have been demonstrated to be toxic to several bark beetle species including *Dendroctonus frontalis* Zimmermann, *D. brevicomis* LeConte, *Ips calligraphus* (Germar), and *Scolytus ventralis* LeConte (Smith, 1965; Coyne and Lott, 1976; Raffa et al., 1985; Cook and Hain, 1988). Further, needle monoterpene content has been suggested to be important in tree resistance to the western spruce budworm, *Choristoneura occidentalis* Freeman (Cates et al., 1983). As part of an investigation of the interaction between spruce spider mite and its host conifers, this experiment was conducted to examine potential lethal and sublethal effects of four common conifer monoterpenes on adult female *O. ununguis*. Specifically, monoterpene influences on adult mortality, oviposition, and movement were examined.

METHODS AND MATERIALS

Experimental work was conducted from April 1990 through July 1991. Adult female *O. ununguis* were removed from an insectary-reared colony maintained on cuttings of Fraser fir, *Abies fraseri* (Pursh) Poir. Each adult female mite was placed on a single fir needle. The needles were individually placed either in a 1.0-ml glass vial that was sealed with a cotton plug (just enough to prevent mite escape) or on a circular (diameter = 16 mm) piece of black construction paper affixed with Tanglefoot, five per dish, to the bottom of an open-topped Petri dish (diameter = 9.0 cm). Vials and/or Petri dishes were placed in a 2-liter desiccator with an NaCl slurry to maintain relative humidity at ca. 75% (Winston and Bates, 1960). Desiccators were maintained for 24 hr at 26°C in a constant-light environment.

Monoterpene treatments were applied within each desiccator by suspending, from the top of the desiccator just prior to sealing, a piece of round no. 1 filter paper (diameter = 5.5 cm), which had the appropriate concentrations of the given monoterpene applied topically to it. In this manner, as the monoterpene volatilized, mites were exposed to various levels of monoterpene vapors. The monoterpenes tested were obtained from Aldrich Chemical Company and were (*S*)-(−)-limonene (92% purity), 1(*S*)-(−)-β-pinene (98% purity), 1(*S*)-(−)-α-pinene (98% purity), and Δ^3-carene (95% purity). The concentrations injected onto the filter paper were 0, 5, 10, 25, 50, 70, and 100 μl, which corresponds to ca. 0, 2.5, 5.0, 12.5, 25.0, 35.0, and 50.0 ppm within the sealed containers, respectively.

At 24 hr the mites were removed from the desiccators and those in vials were examined under a dissecting microscope for mortality at all monoterpene concentrations and oviposition at the 0-, 5-, 10-, 25-, and 50-μl injection levels. Mites on the round black arenas were examined to determine if they had moved beyond the 16 mm disk and were stuck in the surrounding Tanglefoot. This test was conducted at monoterpene injection concentrations of 0, 10, 50, 70, and 100 μl. Mites were exposed to the various treatments five per replicate with four replicates conducted at the 5-, 70-, and 100-μl injection levels, eight replicates at the 10-, 25-, and 50-μl injection levels, and 31 zero microliter (control) injections.

The influence of the monoterpenes on survival was determined using probit analyses to obtain LC_{50} estimates for each monoterpene. Regression analyses were conducted to determine the influence of the individual monoterpenes on oviposition (eggs per live female) and dispersal tendency (percentage of mites moved off the 16-mm disks). Correlation analyses were conducted between percent survival and oviposition and between percent survival and dispersal tendency for the mites exposed to each monoterpene. All statistical analyses

were conducted using the SAS statistical package (Statistical Analyses System 1982).

RESULTS AND DISCUSSION

All four monoterpenes tested were toxic to adult female *O. ununguis* (Table 1). The order of toxicity based upon the LC_{50} calculations was limonene $\geq$ β-pinene $\geq$ α-pinene $\geq$ Δ^3-carene. Limonene is also one of the most toxic host monoterpenes to several conifer-feeding scolytid bark beetles (Smith, 1965; Coyne and Lott, 1976; Raffa et al., 1985; Cook and Hain, 1988). Host monoterpene vapors from peppermint have been demonstrated to be toxic to the mite *T. urticae* (Larson and Berry, 1984) as have topical applications of tomato sesquiterpenes (Patterson et al., 1975).

There was a significant decrease in oviposition (eggs per live female) as the concentration of each monoterpene increased {limonene, $F(4,26) = 6.16$, $[P > F] = 0.0013$; β-pinene, $F(4,26) = 4.49$, $[P > F] = 0.0068$; α-pinene, $F(4,26) = 4.43$, $[P > F] = 0.0080$; Δ^3-carene, $F(4,26) = 4.59$, $[P > F] = 0.0061$} (Figure 1). It is not surprising therefore to find a positive correlation between oviposition and survival of *O. ununguis* females for each of the monoterpenes {limonene, $r = 0.5687$, $[P > r] = 0.0008$; β-pinene, $r = 0.6631$, $[P > r] = 0.0001$; α-pinene, $r = 0.2403$, $[P > r] = 0.1930$; Δ^3-carene, $r = 0.4912$, $[P > r] = 0.0050$} . The decrease in oviposition began at monoterpene concentrations less than those resulting in significant mortality. Similarly sublethal affects of host monoterpene vapors have been reported for *T. urticae* exposed to peppermint monoterpenes (Larson and Berry, 1984).

There was also a significant relationship between monoterpene concentration and dispersal tendency (movement off of the circular disk) for *O. ununguis* females exposed to limonene $\{F(4,27) = 4.73, [P > F] = 0.0051\}$, β-pinene, $\{F(4,27) = 6.22, [P > F] = 0.0011\}$, and α-pinene $\{F(4,27) = 37.46, [P > F] = 0.0001\}$ but not for those mites exposed to Δ^3-carene $\{F(4,27) = 0.61, [P > F] = 0.6589\}$ (Figure 1). Again, given these results, it is not surprising to find a significant, positive correlation between dispersal tendency and survival of *O. ununguis* for three of the monoterpenes {limonene, $r = 0.4997$, $[P > r] = 0.0036$; β-pinene, $r = 0.5673$, $[P > r] = 0.0007$; α-pinene, $r = 0.7993$, $[P > r] = 0.0001$} but not for Δ^3-carene ($r = 0.0599$, $[P > r] = 0.7447$). The decreased movement at higher monoterpene concentrations for the three compounds may indicate a rapid effect of the toxic vapors with little movement by the mites being possible at these higher concentrations. The one monoterpene that did not demonstrate this result, Δ^3-carene, is the compound that had the highest LC_{50} concentration (Table 1). The increased movement in the presence of relatively low concentrations of host monoterpene

TABLE 1. PERCENT SURVIVAL ($\bar{X} \pm$ SD) OF ADULT FEMALE SPRUCE SPIDER MITES EXPOSED TO VARIOUS CONCENTRATIONS OF FOUR MONOTERPENE VAPORS FOR 24 HOURS

Monoterpene	Concentration (μl)[a]								
	0[b]	5	10	25	50	70	100	LC_{50}[c]	95% CI[c]
Limonene	89.0 ± 14.4	86.7 ± 16.3	87.5 ± 14.9	77.5 ± 9.6	57.5 ± 24.9	30.0 ± 11.5	0.0 ± 0.0	49.0	41.7–59.0
β-Pinene	89.0 ± 14.4	81.3 ± 23.9	95.0 ± 14.1	74.2 ± 10.7	44.4 ± 19.5	60.0 ± 16.3	0.0 ± 0.0	51.7	36.1–86.3
α-Pinene	89.0 ± 14.4	85.0 ± 19.1	100 ± 0.0	92.5 ± 9.6	62.5 ± 25.9	55.0 ± 30.0	0.0 ± 0.0	61.3	41.1–121.1
Δ^3-Carene	89.0 ± 14.4	90.0 ± 11.5	87.5 ± 18.3	87.5 ± 5.0	68.1 ± 22.4	58.8 ± 14.4	0.0 ± 0.0	62.5	44.9–113.8

[a] Monoterpene concentration applied to filter paper suspended from ceiling of desiccators: equivalent ppm measurements are 0 μl = 0 ppm, 5 μl = 2.5 ppm, 10 μl = 5.0 ppm, 25 μl = 12.5 ppm, 50 μl = 25.0 ppm, 70 μl = 35.0 ppm, and 100 μl = 50.0 ppm.

[b] 0 μl concentration = control desiccators. One control desiccator was used during each replicate. Therefore, all control measurements were pooled for these analyses.

[c] Estimated concentration and 95% confidence interval for 50% mortality calculated using probit analysis (Statistical Analysis System, 1982).

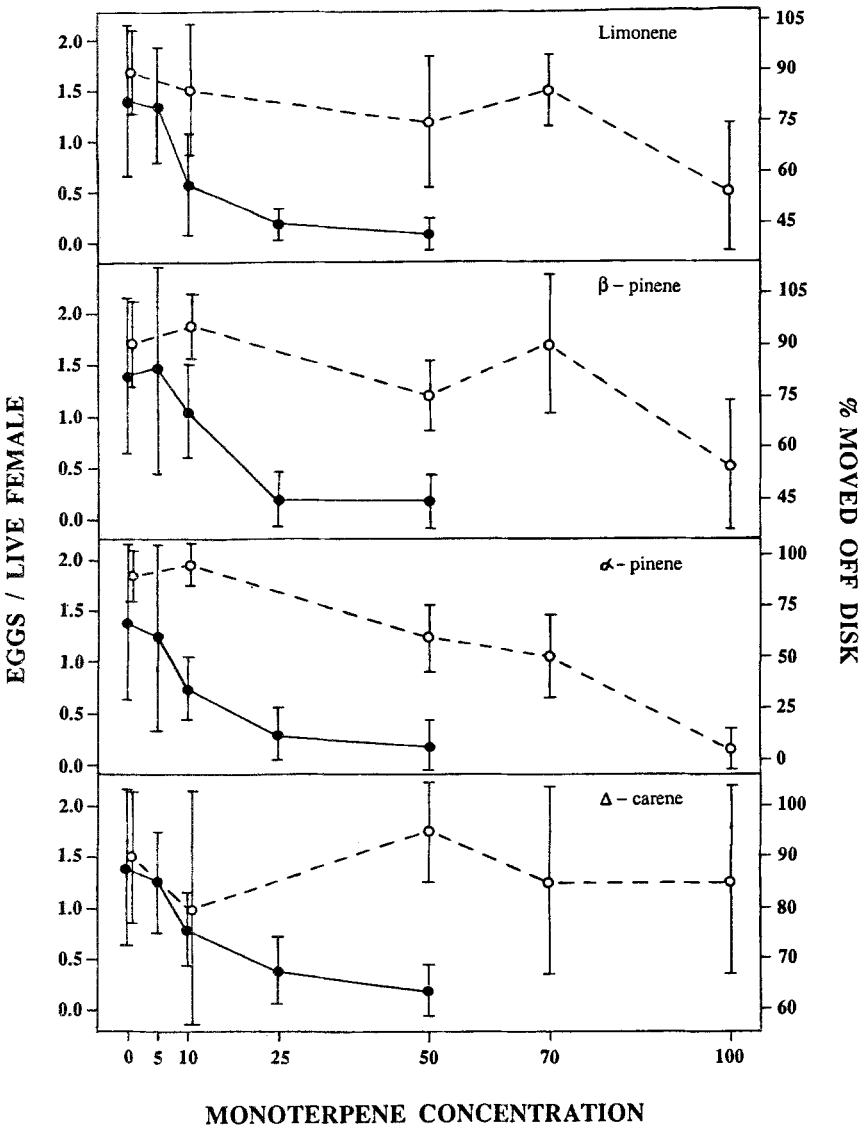

FIG. 1. Sublethal effects ($\bar{X} \pm$ SD) of monoterpene vapors on oviposition (●—●) and movement (○—○) of adult female spruce spider mites exposed to the vapors for 24 hr. 0 μl concentrations = control desiccators, one per replicate, which were pooled for these analyses.

vapors as was observed in the β-pinene and α-pinene exposures may be beneficial in the field by acting to disperse mites away from sites of needle or limb damage and the subsequent increased release of resin monoterpenes. However, a more accurate measure of dispersal tendencies in the presence of host monoterpene odors could possibly be obtained by placing mites on small twigs and monitoring movement in the presence of added host odors.

Although they feed by piercing leaf tissue and sucking out cell contents, *O. ununguis* may not normally be exposed to high concentrations of the monoterpenes contained within needle tissue. These compounds usually occur in specialized structures within the plant but they are continuously lost from tree tissue (Kramer and Kozlowski, 1960), which would constantly expose mites to a low dosage. Furthermore, under water stress, conifers may experience premature needle abscission with an accompanying deposition of resin droplets at the abscission point (Heikkenen et al., 1986). Since the leaf (or needle) is the basic microhabitat exploited by *O. ununguis* for population growth as opposed to the whole plant (Wanibuchi and Saitô, 1983), limited dispersal ability would expose the mites to higher monoterpene vapor concentrations released from these resin droplets and trapped within the canopy of the tree. Even if the concentration of these higher vapor concentrations was not great enough to cause mortality, populations of *O. ununguis* may experience some sublethal affects such as the decreased oviposition and movement observed during this experiment.

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CHEMICAL DEFENSE OF *Populus balsamifera*: A CLARIFICATION

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Abstract—A previous report that the relative palatabilities of juvenile versus mature *Populus balsamifera* towards browsing snowshoe hares is due to the presence of 2,4,6-trihydroxydihydrochalcone in the juvenile growth form has been shown to be incorrect. Specifically, 2,4,6-trihydroxydihydrochalcone is present in internodes of *Populus balsamifera* in exceedingly low concentrations (<0.02% wet wt) and its concentrations are not elevated in internodes of the juvenile developmental stage. Its high levels in the buds (20–25% wet wt) of *Populus balsamifera*, however, probably makes it a deterrent substance for herbivores that cannot remove buds from twigs during foraging.

Key Words—2,4,6-Trihydroxydihydrochalcone, balsam poplar, *Populus balsamifera*, snowshoe hare, *Lepus americanus*, herbivory, plant defense.

INTRODUCTION

Recently two groups (Reichardt et al., 1990; Jogia et al., 1989) have reported upon the chemical defense of balsam poplar (*Populus balsamifera*) parts and growth stages against feeding by snowshoe hares (*Lepus americanus*). Of interest was the fact that these reports do not agree on the defensive agents respon-

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sible for the selective utilization of balsam poplar growth stages (juvenile vs. mature). Reichardt et al. (1990) reported that hares avoid internodes of juvenile balsam poplar due to an interaction between three volatile secondary metabolites—salicaldehyde, 6-hydroxycyclohexenone, and 1,2-cyclohexadione. Jogia et al. (1989), however, attributed this avoidance to a single compound, 2,4,6-trihydroxydihydrochalcone (2,4,6-TDC), which Reichardt's group failed to report. Recently, however, Sinclair (personal communication) has verified that salicaldehyde, a substance reported by Reichardt et al. (1990), is a feeding deterrent to snowshoe hares using the bioassay protocol described by Jogia et al. (1989).

Hence, the relative importance of 2,4,6-TDC compared to the other three reported defensive substances of *P. balsamifera* as a snowshoe hare antifeedant has yet to be established. We developed three hypotheses to explain the omission of 2,4,6-TDC by Reichardt et al. (1990).

The first hypothesis is that balsam poplar from Kluane Lake in the study of Jogia et al. (1989) is different in its secondary chemistry than balsam poplar found in interior Alaska (Reichardt et al. 1990).

The second hypothesis is that while 2,4,6-TDC may be present in balsam poplar internodes at concentrations [0.8% according to Jogia et al. (1989)] low enough to have escaped detection by Reichardt et al. (1990), it may still act as the primary defensive agent in juvenile poplar. This hypothesis is consistent with both reports because the presence of one defensive compound does not preclude the presence of other defensive agents.

Finally, it is possible that the presence of 2,4,6-TDC in the internodes of balsam poplar as reported by Jogia et al. (1989) was due to contamination of internodes by bud oils in spite of the fact that buds were removed from the twigs prior to processing. This latter hypothesis arises from several observations. First, buds from other populations of balsam poplar contain copious quantities of 2,4,6-TDC (ca. 50% of extractable material; Greenaway et al., 1989). Secondly, buds of balsam poplar are extremely resinous, and, upon thawing, this resin tends to stick to anything, including internodes, which contacts buds. Finally, the secondary chemistry of balsam poplar internodes appears to be qualitatively different from that of buds (Mattes et al., 1987).

METHODS AND MATERIALS

Collection of Plants. Current season's growth (CSG) twigs from both mature and juvenile winter-dormant balsam poplar were collected in 1991 from five sites near Fairbanks, Alaska, along with five sites at Kluane Lake. Prior to transporting the twigs, buds were removed. Then the twigs were cut into two sections: internodes containing no bud scars (Alaskan, Kluane) and short sec-

tions containing bud scars (Kluane population only). In addition, the older growth twigs of mature poplars from Kluane were sampled for buds and internodes that contained no bud scars. Twig diameters were all less than 4 mm. Voucher specimens (Clausen 2) of the Alaskan population are stored at the museum located at the University of Alaska, Fairbanks.

Preparation of Extracts for Chemical Analysis. Approximately 1 g of each collection was accurately weighed and extracted in 20 ml of acetone for 48 hr. The extracted material was removed and the extract was diluted to 25 ml. A carefully measured portion (ca. 1 ml) of the extract was removed, and the acetone was allowed to evaporate overnight.

Internode and scar extracts were reconstituted with 2 ml of 50% aqueous acetonitrile containing 1 ml of benzyl gentisate as internal standard. Bud extracts were diluted to 1 ml with 50% aqueous acetonitrile and 100 μl of this solution was withdrawn and added to 10 ml of the above internal standard solution. These final solutions were analyzed directly by HPLC (see below). The effective concentrations (milligrams of plant tissue extract per milliliter) were 0.0125 (internodes/scars) and 0.00025 (buds).

2,4,6-TDC Quantifications. Twenty microliters of the above solutions were injected onto a C-18 reverse-phase analytical column (25 cm $\times$ 4.6 mm) and eluted with acetonitrile–water–acetic acid (50:47.5:2.5). Flow rate was 1 ml/min and peaks were detected with a variable wavelength detector set at 285 nm. The retention times for 2,4,6-TDC and benzyl gentisate (internal standard) were about 5.6 and 6.7 min, respectively. Response factors were calculated by injecting a standard solution of 2,4,6-TDC (0.023 mg/ml; see below) and benzyl gentisate (0.25 mg/ml).

Synthesis of 2,4,6-TDC. To a 100-ml round-bottom flask equipped with a stirbar, hot water bath, and nitrogen bubbler, was added 750 mg of phloroglucinol, 25 ml nitrobenzene and 1 g aluminum chloride. After stirring for 3 min, 1 ml of dihydrocinnamoyl chloride (Aldrich) was slowly added. The temperature was raised to 80°C for 3 hr after which TLC (Si Gel; ether–chloroform 15:85) of the reaction mixture indicated the reaction was complete. The crude reaction mixture was diluted with 100 ml pentane and loaded onto a 200-ml flash chromatography column (silica gel) that had been prepared with chloroform. The column was eluted first with chloroform to remove the nitrobenzene followed by 15% ether in chloroform. After combining the appropriate fractions and removal of solvent, 430 mg (28% yield) of pure 2,4,6-TDC was isolated. Final purification was accomplished by recrystallization from chloroform. The ^{1}HNMR spectrum was identical to that reported in the literature (Itufford and Lasswell, 1978). The [^{13}C]NMR spectrum (d4-methanol), which to our knowledge has not been reported before, is as follows (ppm relative to TMS): 206.1, 166.2, 165.8 (two carbons), 143.2, 129.4 (four carbons), 126.9, 105.4, 95.8 (two carbons) 46.9, and 32.2.

Statistical Analyses. All comparisons within a population (Kluane, Yukon Territory, Canada, or interior Alaska) were analyzed for significance using a paired t test. Comparisons between populations were analyzed using a t test.

RESULTS

Table 1 shows the results of the analysis of plant parts for 2,4,6-TDC. Buds contained about 20% of 2,4,6-TDC (wet wt) in agreement with earlier reports (Greenaway et al., 1989). There was no significant difference ($P > 0.1$) between 2,4,6-TDC concentrations in juvenile and mature buds from CSG or between the two poplar populations. Mature older growth buds, however, did contain ($P < 0.1$; paired t test) less 2,4,6-TDC than buds from either of the other growth stages.

Levels of 2,4,6-TDC in internodes ranged between 0.25% (in one mature older growth sample) to less than 0.005% (detection limit of analyses), and no set of internodes we examined had an average 2,4,6-TDC concentration of greater than 0.06%. Mature CSG internodes contained on the average about twice as much 2,4,6-TDC as juvenile CSG internodes, but this difference was not significant ($P > 0.1$; paired t test). Again, there was no significant difference between the Alaskan and Kluane Lake populations or between growth stages.

Bud scars from Kluane samples contained more ($P < 0.1$; paired t test) 2,4,6-TDC than the other sections of internodes, but the levels were all less than 0.22%. There was slightly more, but insignificant ($P > 0.1$; paired t test), 2,4,6-TDC in the juvenile bud scars compared to the mature bud scars. No

TABLE 1. LEVELS OF 2,4,6-TDC (PERCENT WET WEIGHT) IN BALSAM POPULAR PARTS AND GROWTH STAGES[a]

	Alaskan	Kluane Lake
A. Juvenile current season growth		
Buds	19.5 (3.6)	20.0 (1.8)
Internodes	0.0034 (0.0022)	0 (0)
Bud scars	no data	0.168 (0.022)
B. Mature current season growth		
Buds	17.0 (3.8)	25.3 (4.5)
Internodes	0.0067 (0.0035)	0.0160 (0.0104)
Bud scars	no data	0.135 (0.032)
C. Mature older growth		
Buds	no data	16.0 (1.1)
Internodes	no data	0.0629 (0.0476)

[a]Numbers in parentheses represent standard errors ($N = 5$).

comparison could be made between the two populations because no bud scars were sampled from the Alaskan population.

DISCUSSION

The data of Table 1 show that the concentrations of 2,4,6-TDC in parts and growth stages of balsam poplar from both Kluane Lake and interior Alaska are very similar. Thus the differences between the previous conclusions of Jogia et al. (1989) and Reichardt et al. (1990) are probably not due to differences in the secondary chemistry of plants from these two sites (hypothesis 1).

The very low levels of 2,4,6-TDC present in balsam poplar CSG internodes ($<0.02\%$) makes it unlikely that it plays a significant role in the defense of balsam poplar internodes unless it is a very active deterrent. The rather remote chance that 2,4,6-TDC is sufficiently active even at such low concentrations (hypothesis 2), however, cannot explain the relative palatabilities of juvenile vs. mature growth stages of balsam poplar to snowshoe hares. Even if 2,4,6-TDC was extremely potent as a deterrent, the levels of 2,4,6-TDC would have to be greater in internodes of the juvenile growth stage to explain hares' preference for the mature growth stage, a situation not substantiated by our results in either population.

It is also unlikely that the levels of 2,4,6-TDC in the budscars of juvenile twigs can explain hare preference for the mature stage. First, the results of Jogia et al. (1989) and Reichardt et al. (1990) indicate that hares prefer the mature growth stage about 30-to 100-fold. Such a large discrimination between developmental stages of a single species seems to require a relatively large difference in defensive chemistry. This, however, is shown not to be the case in this study. For instance, if one assumes that bud scars comprise about 33% of a twig (a considerable overestimate), then a simple calculation using the results from the Kluane population (Table 1) shows that the amount of 2,4,6-TDC in the twigs (including bud scars) of the mature and juvenile growth stages are equal. In addition, the difference between concentrations of 2,4,6-TDC in the bud scars of juvenile and mature CSG was not significant ($P > 0.1$, paired t test).

Finally, the results of Reichardt et al. (1990) suggest that the chemical defense relating to the preference of mature over juvenile twigs resides in the internodes themselves; when buds and budscars are removed, hares still prefer mature over juvenile growth stages by roughly the same factor as whole twigs. Because mature and juvenile internodes contain similar levels of 2,4,6-TDC, hares must use some other means to differentiate between growth forms. This refutation of hypothesis 2, however, does not necessarily mean that 2,4,6-TDC has no effect on the overall palatability of balsam poplar relative to other plant species. This may be especially true for those herbivores such as moose that are incapable of selectively feeding on the internode portion of the twigs.

The elimination of hypotheses 1 and 2 leads us to believe that differences between the results of Jogia et al. (1989) and Reichardt et al. (1990) concerning the defense of winter dormant balsam poplar against snowshoe hares must be due to contamination of the internodes samples of Jogia et al. with bud oil.

It is interesting to note that the 1990 paper by Reichardt et al. also dealt with the secondary chemical defense of balsam poplar buds, but failed to report the role of 2,4,6-TDC or even its presence. The results of that study demonstrated that the hares' rejection of buds could be explained solely on the basis of the deterrent properties of several volatile bud metabolites. Nonvolatile metabolites were, therefore, never investigated. They did state in this earlier report that other defensive substances could also be present in buds but their presence would not make buds any less palatable in conventional feeding trials. The demonstration by Jogia et al. (1989) of the antifeedant properties of 2,4,6-TDC and its concentrations in buds of winter-dormant Alaskan balsam poplar indicate that the statement of Reichardt et al. (1990) about "defensive overkill" in poplar buds is supported in the sense that other chemicals may be active.

In a similar vein, hares' preference for mature over juvenile internodes may be caused by aspects of this plant's complex chemistry that have not yet been discovered. For instance, it is not known if the deterrent substances reported by Reichardt et al. (1990) result in physiological stress to browsing hares or whether these substances advertise the presence of other chemicals that are not deterrent, but nonetheless are noxious. Equally intriguing is the unknown role of 2,4,6-TDC in the defense of balsam poplar against herbivores. Since it is now clear that 2,4,6-TDC does not affect hares' discrimination between juvenile and mature balsam poplar, the function of the antifeedant properties of this chemical needs to be further investigated.

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LIPID COMPONENTS IN ANAL SCENT SACS OF THREE MONGOOSE SPECIES (*Helogale parvula*, *Crossarchus obscurus*, *Suricata suricatta*)

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Abstract—Anal sac secretions of three social mongoose species (*Helogale parvula*, *Crossarchus obscurus*, and *Suricata suricatta*) were chemically analyzed by means of gas chromatography–mass spectrometry. Compounds with high molecular weights (greater than 250) were found to distinguish the three species. Differences in composition suggest a possible species-specific role in scent marking. Male *H. parvula* had vitamin E present in the anal sac, whereas it was absent in the females. This suggests a possible sex-specific function of vitamin E in *H. parvula*. No differences in chemical composition were found between male and female *S. suricatta*. Ten compounds were found in female *C. obscurus* that were not present in the male.

Key Words—Mongoose, *Helogale parvula*, *Crossarchus obscurus*, *Suricata suricatta*, anal gland, lipids.

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INTRODUCTION

Mongooses are small to medium-sized carnivores that have a diverse array of social structures, from a solitary existence as in the small Indian mongoose, to a highly cooperative social structure as in the dwarf mongoose. Pocock (1916) related the size of mongoose scent sacs with the particular social structure they possess: gregarious mongooses have larger scent glands than more solitary mongooses, suggesting that more social species mark more frequently than more solitary species. Although this hypothesis has not been tested, it merits investigation. Pocock (1916) further described the morphology of the scent sacs of mongooses as distinctly different than other carnivores. There are several depressions and folds in the glands, which contain small glandular pits with several secreting pores. Secretions from these glands are thick waxy brown foul-smelling substances (Decker, personal observation).

Mongooses have very characteristic and stereotypical postures of scent marking (Gorman et al., 1974). Behavior patterns vary with the type of object being marked, especially with regard to its height above the ground (Rasa, 1973). Preferred objects for marking by dwarf mongooses are smooth, upright, horizontal, or sloping branches (Rasa, 1973). Such objects are marked from a "hand-stand" position (Gorman et al., 1974). Anal drag is a posture in which the animal strokes its anal surface across a very low or flat object. Rasa (1973) reports the anal drag to be used exclusively in allomarking. In the "leg lift" posture, the animal lifts its leg toward a low object. Again, this is most commonly used in allomarking (Rasa, 1973) but occasionally is used to mark low-lying objects such as the food dish.

Several functions of scent marking have been proposed (for reviews see Ralls, 1971; Eisenberg and Kleiman, 1972; Johnson, 1973; MacDonald, 1979). Conover and Gittleman (1989) have classified these potential functions into six general categories: (1) identity—information regarding individual, group, or sexual identification (Rasa, 1973; Gorman, 1976); (2) status—information on dominance status (MacDonald, 1979; Erlinge et al., 1982); (3) reproductive status—related to courtship and breeding (Gorman, 1980; Gorman and Trowbridge, 1989); (4) spatial information—serves a territorial function (Peters and Mech, 1975; Kruuk, 1978); (5) temporal information—reveals when individuals were at a particular location (Rasa, 1973); and (6) foraging—serves a bookkeeping function by informing an individual of whether it has previously looked for food in a particular area (Henry, 1977; MacDonald, 1979).

The objectives of this study were to establish methods of collection and analysis of mongoose secretions, to identify potential biologically active compounds present in the scent secretion of three species of mongooses, and to propose a potential function of scent marking in these mongooses.

METHODS AND MATERIALS

Materials. Solvents were chromatographic grade quality purchased from J.T. Baker Chemicals Co., Phillipsburg, New Jersey. Derivatizing agents were purchased from Supelco, Inc. (Bellefonte, Pennsylvania), Applied Science (State College, Pennsylvania), Aldrich, Inc. (Milwaukee, Wisconsin), Sigma Chemical Co. (St. Louis, Missouri), and Pierce Chemical Co. (Rockford, Illinois).

Collection. Anal gland secretions were collected from three species of social mongooses (*Helogale parvula*—two males, two females; *Crossarchus obscurus*—one male, two females; and *Suricata suricatta*—one male, one female) from the National Zoo, Washington, D.C., by swabbing the anal sacs with sterile cotton swabs. All chemical analyses were conducted at the Center for Environmental Biotechnology, University of Tennessee.

Extraction. The secretion was immediately extracted with a modified Bligh and Dyer solvent system (White et al., 1979), which consists of chloroform, methanol, and a phosphate buffer in a ratio of 1:2:0.8. The sample was left in the solvent system for a minimum of 4 hr. Subsequently, equal amounts of water and chloroform were added to the samples separating them into aqueous and organic phases. The aqueous layer was discarded, and the organic (lipid containing) phase was transferred to Teflon-lined screw-capped test tubes and dried under a stream of nitrogen at room temperature.

Lipid Isolation. Silicic acid columns were prepared using 0.5 g Unisil (100–200 mesh, Clarkson Chemical Co., Inc., Williamsport, Pennsylvania), activated at 100°C for 60 min and preextracted in a minimal volume of chloroform. Total lipid was applied to the top of the columns in a minimal volume of chloroform. Sequential washes of 5 ml of chloroform, acetone, and methanol eluted nonpolar, glyco-, and phospholipids. Each fraction was dried under a stream of nitrogen.

Mild Alkaline Methanolysis. The mild alkaline methanolysis procedure (White et al., 1979) was utilized to prepare methyl esters of the ester-linked fatty acids of each lipid fraction.

Gas Chromatography (GC). Dry methyl esters of nonpolar, glyco-, and phospholipids were dissolved in isooctane containing the internal standard of methyl nonadecanoate. Samples of 1 μl were injected onto a 50-m nonpolar, cross-linked methyl silicone fused silica capillary column (0.2 mm ID, Hewlett Packard) in a Shimadzu GC-9A GC. A 30-sec splitless injection at 270°C was used. Hydrogen at a linear velocity of 35 cm/sec was the carrier gas with a temperature program starting with an initial temperature of 100°C. The temperature was then increased at a rate of 10°C/min to 150°C. At 150°C the temperature was increased 3°C/5 min to 282°C. An equal detector response was assumed for all components. Peak areas were quantified with a program-

mable laboratory data system (Nelson Analytical 3000 Series Chromatography Data System, Revision 3.6).

Gas Chromatography–Mass Spectrometry (GC-MS). Tentative component identification prior to GC-MS was based on comparison of the retention times with authentic standards. GC-MS analysis was performed on a Hewlett Packard 5996A GC-MS fitted with a direct capillary inlet utilizing the same chromatographic system except for use of a helium carrier gas and the temperature program, which started at 100°C and increased to 280°C at 3°C/min for a total analysis time of 60 min. The electron multiplier voltage was between 1800 and 2000 V, the transfer line maintained at 300°C, the source 280°C, and analyzer 250°C, and the GC-MS was autotuned with DFTPP (decafluorotriphenylphosphine) at *m/z* 502 with an ionization energy of 70 eV. The data were acquired using the Hewlett Packard 6/VM data system. Compounds were identified using the GC-MS library, which provided an 80% or greater probability of being correct for all compounds.

Nonpolar Lipid Fractionation. Preliminary analyses suggested the importance of the heavier compounds in distinguishing the species. Therefore, to achieve greater separation of those compounds with high molecular weights (greater than 250), the nonpolar lipid portion was subsequently fractionated. In this procedure, silicic acid columns were prepared using 1.0 g Unisil (100–200 mesh, Clarkson Chemical Co., Inc., Williamsport, Pennsylvania) activated at 100°C for 60 min and preextracted with chloroform. Sequential washes of 10 ml of hexane, hexane–diethyl ether (90:10), and hexane–diethyl ether (80:20) eluted the compounds with smaller molecular weights (<250) so that better separation of the larger compounds (molecular weights >250) could be achieved. Ten microliters of BSTFA [*N,O*-bis(trimethylsilyl)trifluoroacetamide] were added to the fraction containing compounds with high molecular weights (>250). These samples were subsequently analyzed on the GC-MS. The temperature program initially began at 50°C for 1 min. The temperature was then raised 30°C/min until it reached 150°C. Subsequently, the temperature was raised 2°C/min to 250°C, then 5°C/min to 300°C with a final time of 30 min.

Statistical Analyses. A phenogram was generated from a similarity matrix using the Numerical Systems Program (Rohlf et al., 1982). Principal component analysis was performed to summarize variation among taxa.

RESULTS AND DISCUSSION

The Bligh-Dyer extraction was very effective in revealing a larger spectrum of compounds than previous analyses of carnivore scent secretions (Gorman et al., 1978; Davies et al., 1988; Apps et al., 1988, 1989). The primary difference between this study and previous studies is the choice of solvent systems used

to extract the compounds. Previous analyses have used *n*-hexane as the solvent, and this is only effective in revealing the straight-chain hydrocarbons, whereas the Bligh-Dyer system is effective in revealing more complex compounds.

An unweighted paired group cluster analysis showed differences among species in only the nonpolar lipid fraction (Figure 1). *Helogale parvula* males and females grouped together. Similar patterns were also detected in *C. obscurus* and *S. suricatta*. Separation of individuals within a single species was not apparent with the glyco- and phospholipid portions of the samples, and are therefore not reported here.

Although the sample sizes in this study are too small to assess significant differences in the chemical composition of the anal sacs between sexes and among species, we feel that it is worthwhile to present these differences as a descriptive measure.

Intraspecific Comparison of Helogale parvula. Sixty-six compounds were revealed in the neutral lipid fraction of *H. parvula* (Appendix 1). Other studies of carnivore scent secretions have revealed between 10 and 30 compounds (e.g., Gorman et al., 1978; Davies et al., 1988; Apps et al., 1989). This may suggest the complexity of scent marking in this species.

Eleven additional compounds were found in the dominant male *H. parvula*, which were not found in the subordinate male or females (see Appendix 1). Although Rasa (1973) has reported no differences in frequency of marking between dominant and subordinate males, the additional chemicals found in the dominant male suggests that the scent secretions of the dominant male may serve an additional or different function.

Vitamin E was present in the anal sac of male *H. parvula* and was absent in the female *H. parvula*. This compound may be sex specific in *H. parvula*.

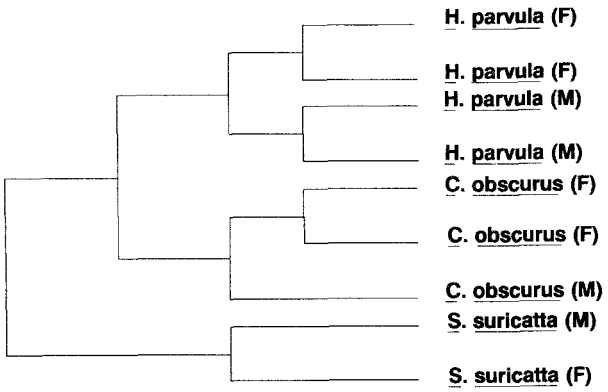

FIG. 1. A phenogram illustrating the similarity in nonpolar lipids among *Helogale parvula*, *Crossarchus obscurus*, and *Suricata suricatta*. M = males; F = female.

Although many studies (e.g., Silverstein et al., 1966) have shown that a combination of several chemicals are necessary for biological activity, some studies of carnivores have identified single compounds to be unique to the males (e.g., Apps et al., 1989). This may suggest that single compounds can play an important sex-specific role in some cases. A detailed study of the dwarf mongoose by Rasa (1973) has shown that they traverse their home range in 25–30 days. These mongooses were able to detect scent secretions from themselves or other mongooses for up to 30 days after the scent was deposited (Rasa, 1973). Rasa suggests that the primary function of anal marking, as animals traverse their home range, is to provide information on individual identity and to "date" the mark. Rasa also found that another mongoose can detect differences in intensity of a mark up to 1 hr after it has been deposited.

Intraspecific Comparison of Crossarchus obscurus. Eleven additional compounds were found in relatively large quantities in *C. obscurus* females, which were not found in *C. obscurus* males (Figure 2). Very little is known about the behavior and ecology of *C. obscurus* (for a review see Goldman, 1987). However, these results may suggest that the females rely more heavily on scent marking than do the males.

Intraspecific Comparison of Suricata suricatta. Few qualitative differences were found between the male and female meerkat (Figure 3). This may suggest

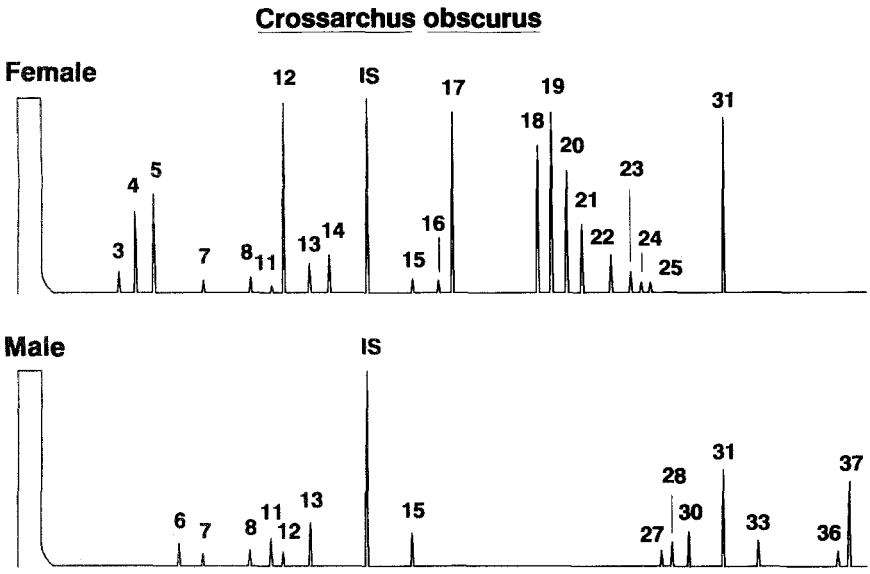

FIG. 2. Chromatogram of male and female *Crossarchus obscurus*. Numbers above peaks correspond to those in Appendix 1, where compounds and relative quantities of each are given. IS = the internal standard of methyl nonadecanoate.

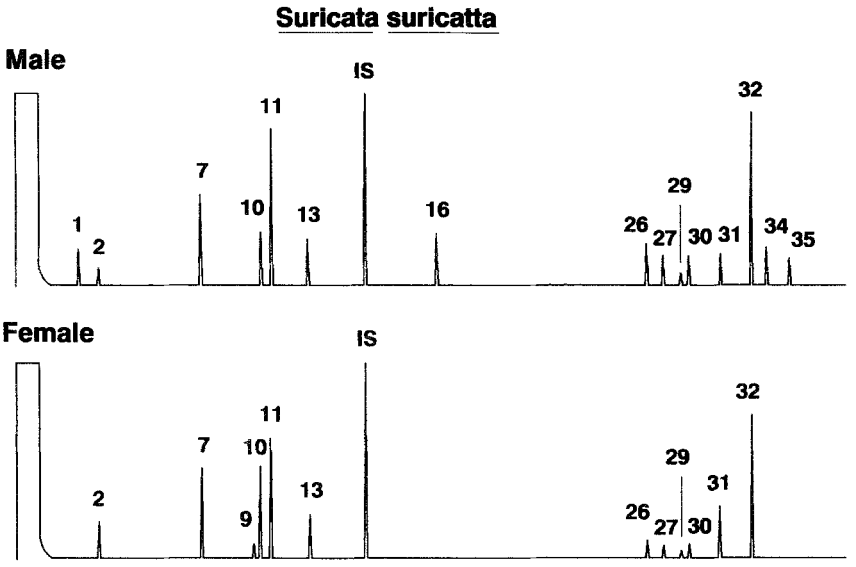

FIG. 3. Chromatogram of male and female *Suricata suricatta*. Numbers above peaks correspond to those in Appendix 1, where compounds and relative quantities of each are given. IS = the internal standard of methyl nonadecanoate.

that the function of scent marking in male and female meerkats does not differ. Additionally, Moran and Sorensen (1986) found no marked differences in the frequency of marking between male and female meerkats. Furthermore, large individual differences in scent marking were not tied consistently to sex or age.

Interspecific Comparison. Principal component analysis summarized 85% of the variation in the first two components (Figure 4). Figure 4 reveals that males and females of the same species are more similar to each other in the chemical composition of their scent sacs than with individuals of different species. Additionally, the species are clearly different from each other. If the "loadings" are overlayed onto the "scores" in Figure 4, we can see which compounds are best able to maximally separate the species. For example, cholesterol (denoted as sterol a) distinguishes the meerkats, the polybranched trienoic acid (denoted as 2464a) distinguishes the kusimanse, and a variety of compounds (18:0, sterol b, 713a, and 713d) distinguish the dwarf mongooses. The compounds that distinguish the species are relatively heavy compounds (molecular weights greater than 250) and, therefore, suggest a function that promotes long-term signaling of the scent mark. Female *C. obscurus* had a unique profile of compounds that were not present in the male (Figure 2). Few differences were found between male and female *H. parvula* or between male and female *S. suricatta* (see Figure 3 and Appendix 1).

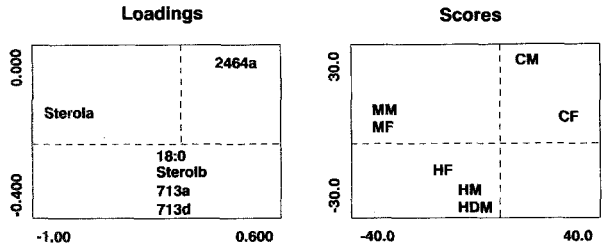

FIG. 4. Results of principal component analysis. "Loadings" represent those compounds which were maximally able to separate the species. "Scores" represent the relative separation of the species. CM = *Crossarchus obscurus* male; CF = *Crossarchus obscurus* female; MM = *Suricata suricatta* male; MF = *Suricata suricatta* female; HM = *Helogale parvula* male; HDM = *Helogale parvula* dominant male; HF = *Helogale parvula* female. 2464a = an unknown polybranched trienoic acid; sterol a = cholesterol; 18:0 = an 18-carbon saturated carboxylic acid; sterol b = cholestanol; 713a = pentadecanol; 713d = heptadecanol.

Three previous studies have chemically analyzed the anal sac secretions of mongooses. Gorman et al. (1974) found a series of short-chained (C_1–C_{10}) saturated carboxylic acids in the small Indian mongoose (*Herpestes auropunctatus*). Similar compounds were found in the yellow mongoose, *Cynictis penicillata*, by Apps et al. (1989). Hefetz et al. (1984) found heavier compounds, C_{10}–C_{22}, in the Egyptian mongoose, *Herpestes ichneumon*. Our results are most similar to those of Hefetz et al. (1984). Differences in results, to some extent, may be due to differences in collection regimes and analysis of secretions.

The short-chained (C_1–C_{10}) highly volatile fatty acids have been found to be present in the scent secretions of most carnivores analyzed (e.g., Gorman et al., 1974; Albone and Perry, 1976; Preti et al., 1976). These compounds are relatively simple and do not have a large number of possible structural isomers as opposed to more complex molecules (i.e., those with larger molecular weights). Since many animals have short-chain fatty acids in their scent secretions, it seems unlikely that these serve a unique communicative function. The short-chain fatty acids probably function in directing the animal to the scent, while the heavier compounds are actually the communicative part of the scent. The fact that animals actually go up to the scent to smell it (Decker, personal observation), lends some support to this argument.

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APPENDIX 1. RELATIVE QUANTITIES (PICOMOLES) FOR NONPOLAR LIPID FRACTION OF FATTY ACIDS PRESENT IN ANAL SACS OF DWARF MONGOOSE (*Helogale parvula*), MEERKAT (*Suricata suricatta*), AND KUSIMANSE (*Crossarchus obscurus*)[a]

#	Compound	(MW)	HDM	HM	HF	CM	CF	SM	SF
1	unknown	(?)	0	0	0	0	0	1,844	0
2	br pentadecanol	(229+)	28,399	35,924	5,113	0	0	717	1,771
3	unknown	(?)	0	0	0	0	1,335	0	0
4	br 2Me13:0	(214+)	7,929	2,740	3,528	0	6,378	0	0
5	br 2Me13:0	(214+)	4,960	5,203	217	0	620	0	0
6	16:1w9c	(256)	2,080	2,551	245	1,750	0	0	0
7	16:0	(258)	45,133	32,221	8,029	3,576	3,545	1,321	1,295
8	17:0	(272)	3,362	2,773	741	293	1,045	0	0
9	br 1Me18:0	(285+)	3,884	6,668	1,299	0	0	0	934
10	br heptadecanol	(258+)	8,483	8,487	3,153	0	0	2,257	1,687
11	i18:0	(300)	28,936	19,774	6,055	1,475	251	6,332	4,220
12	18:1w9c	(284)	3,647	3,809	0	398	10,747	0	0
13	18:0	(286)	10,009	8,897	2,568	2,862	1,157	2,653	1,958
14	unknown	(?)	0	0	0	0	1,478	0	0
15	20:0	(314)	1,634	1,340	617	2,024	1,175	0	0
16	unknown	(?)	0	0	0	0	789	2,962	0
17	?-methyl, ?-hydroxy benzene	(108+)	0	0	0	0	22,600	0	0
18	1,1-phenyl 1 propene	(194)	0	0	0	0	41,177	0	0
19	br benzene	(78+)	0	0	0	0	24,287	0	0
20	br benzene	(78+)	0	0	0	0	3,655	0	0
21	?-phenyl pentane	(148)	0	0	0	0	2,161	0	0
22	?-phenyl pentane	(148)	0	0	0	0	719	0	0

APPENDIX 1. CONTINUED

#	Compound	(MW)	HDM	HM	HF	CM	CF	SM	SF
23	?-phenyl pentane	(148)	0	0	0	0	817	0	0
24	?-phenyl pentane	(148)	0	0	0	0	1,027	0	0
25	?-phenyl pentane	(148)	0	0	0	0	4,826	0	0
26	unknown	(?)	7,365	3,563	1,806	0	0	1,367	859
27	26:1	(364)	7,776	23,380	8,203	161	0	1,098	254
28	26:1	(364)	0	0	0	846	0	0	0
29	26:1	(364)	5,490	10,697	3,036	0	0	329	61
30	26:1	(364)	28,456	76,044	24,779	469	0	2,713	409
31	polybr trienoic acid	(125+)	2,242	6,086	4,358	7,762	17,292	1,367	3,504
32	Cholesterol	(386)	406,422	529,061	133,481	0	0	4,599	3,964
33	21 dihydroxy desmosterol	(402)	10,927	9,969	2,733	261	0	0	0
34	28:1	(392)	0	0	0	0	0	1,967	0
35	28:1	(392)	0	0	507	0	0	540	0
36	29:1	(406)	0	0	0	335	0	0	0
37	29:1	(406)	0	0	0	3,881	0	0	0
	12:0	(202)	7,135	4,497	924	0	0	0	0
	br 2Me13:0	(214+)	3,319	2,547	0	0	0	0	0
	br 2Me13:0	(214+)	802	3,844	0	0	0	0	0
	br 2Me13:0	(214+)	4,346	6,974	375	0	0	0	0
	br 2Me13:0	(214+)	8,656	6,974	375	0	0	0	0
	br 13:0	(215+)	3,587	2,091	0	0	0	0	0
	a13:0	(230)	433	2,091	461	0	0	0	0

br 2 Me14:0	(229+)	1,394	2,164	133	0	0	0	0
br 2 Me14:0	(229+)	2,664	4,936	799	0	0	0	0
i14:0	(244)	38,104	19,456	3,551	0	0	0	0
br 14:0	(230+)	5,559	5,932	2,378	0	0	0	0
2,9 dimethyl 14:0	(258)	34,045	25,434	0	0	0	0	0
14:0	(230)	7,831	3,776	1,030	0	0	0	0
15:0	(244)	3,815	3,257	625	0	0	0	0
a15:0	(258)	38,657	17,101	2,746	0	0	0	0
pentadecanol	(231)	112,620	81,677	13,981	0	0	0	0
i16:0	(272)	123,541	57,501	0	0	0	0	0
br16:1	(255+)	10,637	2,749	559	0	0	0	0
16:1w7c	(256)	233	10,392	323	0	0	0	0
hexadecanol	(244)	50,274	33,985	5,629	0	0	0	0
br hexadecanol	(244+)	2,487	3,677	0	0	0	0	0
br hexadecanol	(244+)	6,576	3,740	0	0	0	0	0
br hexadecanol	(244+)	3,193	2,101	0	0	0	0	0
br 17:0	(271+)	643	4,032	1,071	0	0	0	0
br 17:0	(271+)	2,736	3,421	1,082	0	0	0	0
a17:0	(286)	20,683	9,018	2,321	0	0	0	0
heptadecanol	(258)	578,134	47,940	13,322	0	0	0	0
18:2	(282)	18,401	8,997	2,178	0	0	0	0
18:1w7c	(284)	1,359	9,785	935	0	0	0	0
octadecanol	(270)	9,233	4,389	1,315	0	0	0	0
a19:0	(302)	2,628	0	276	0	0	0	0
nonadecanol	(326)	18,355	6,194	2,160	0	0	0	0
22:0	(342)	2,561	0	605	0	0	0	0
26:1	(364)	7,849	0	0	0	0	0	0
28:1	(424)	2,562	1,950	0	0	0	0	0
28:1	(424)	2,967	1,468	0	0	0	0	0
28:1	(424)	918	0	607	0	0	0	0
28:1	(392)	0	0	1,256	0	0	0	0
Cholestanol	(250+)	98,573	18,442	1,442	0	0	0	0
Vitamin E	(379)	8,782	5,495	0	0	0	0	0

APPENDIX 1. CONTINUED

#	Compound	(MW)	HDM	HM	HF	CM	CF	SM	SF
	Unknown	(?)	4,599	0	0	0	0	0	0
	Unknown	(?)	2,815	0	0	0	0	0	0
	Unknown	(?)	233	0	0	0	0	0	0
	Unknown	(?)	3,044	0	0	0	0	0	0
	Unknown	(?)	2,960	0	0	0	0	0	0
	Unknown	(?)	2,032	0	0	0	0	0	0
	Unknown	(?)	2,860	0	0	0	0	0	0
	Unknown	(?)	5,824	0	0	0	0	0	0
	Unknown	(?)	701	0	0	0	0	0	0
	Unknown	(?)	167	0	0	0	0	0	0
	Unknown	(?)	4,599	0	0	0	0	0	0
	Unknown	(?)	8,126	5,985	0	0	0	0	0
	Unknown	(?)	5,989	2,377	0	0	0	0	0
	Unknown	(?)	0	319	204	0	0	0	0
	Unknown	(?)	0	0	505	0	0	0	0
	Unknown alcohol	(17+)	5,950	4,123	0	0	0	0	0
	Unknown alcohol	(17+)	9,308	4,749	0	0	0	0	0
	Unknown alcohol	(17+)	7,321	5,049	0	0	0	0	0

[a] # = peak numbers corresponding to Figures 2 and 3. Those compounds without peak numbers are only present in *H. parvula* and are listed at the end of this appendix; MW = molecular weight; HDM = Dominant *H. parvula* male; HM = *H. parvula* male; HF = *H. parvula* female; CM = *C. obscurus* male; CF = *C. obscurus* female; SM = *S. suricatta* male; SF = *S. suricatta* female. Fatty acids are designated as a total number of carbon atoms : number of double bonds with the position of the double bond nearest the aliphatic (w) end of the molecule indicated. This is followed by the suffix c for *cis* and t for *trans* configuration of monoenoic acids. The prefixes i, a, or br indicate iso-, anteiso-, or branched (position undetermined). Mid-chain branching is indicated by the number of carbon atoms from the carboxyl end of the molecule and Me for the methyl group (10Me 16:0 is a 17-carbon fatty acid).

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EFFECTS OF SALICYLIC ACID ON GROWTH AND STOMATAL MOVEMENTS OF *Vicia faba* L.: EVIDENCE FOR SALICYLIC ACID METABOLIZATION

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Abstract—The influence of salicylic acid on the growth and stomatal movements of *Vicia faba* L. was investigated. Whereas shoot length, fresh weight, and transpiration rates, which are directly correlated with stomatal pore widths, were only affected at salicylic acid concentrations higher than 3.5 mM after long-term treatments, guard cells in epidermal peels exhibited a high sensitivity at concentrations as low as 0.001 mM, resulting in stomatal closing. HPLC analysis of methanolic extracts from roots and leaves revealed the presence of free salicylic acid and a metabolite, whose amount increased with time in plants previously incubated with a medium containing salicylic acid. The possible ability of *Vicia faba* to detoxify the phenolic acid may be one explanation of the discrepancy between the stomatal reaction in epidermal peels directly treated with the phenolic acid and after application through the transpiration stream. The results indicate that, under natural conditions, salicylic acid will not act as an allelopathic compound whose toxic properties severely affect the growth of *Vicia faba*.

Key Words—Allelopathy, growth inhibition, detoxification, salicylic acid, stomatal movements, *Vicia faba*.

INTRODUCTION

Allelopathy, as defined by Molisch (1937), comprises interactions among plants, including microorganisms, by heterogeneous, mostly phenolic substances. Allelochemicals interfere with the biochemistry and physiological processes of the plant by interaction with plant hormones, enzyme functions, respiration, water

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status, and cell division. These effects result in an inhibition of plant development and growth, which is not only significant for the individual plant, but can influence an ecosystem by changing the pattern of vegetation (Muller and Chou, 1972; Rice, 1984).

Salicylic acid or its derivatives are naturally occurring substances, e.g., in the bark and leaves of *Salix alba* and *Quercus falcata* and in the rhizosphere of *Zea mays* and *Phaseolus vulgaris* (Harborne, 1977; Pareek and Gaur, 1973). During decomposition of plant residues from *Zea mays* and *Secale cereale*, salicylic acid is released into the soil (Chou and Patrick, 1976). This compound can also appear when flavonoids are degraded from decaying *Trifolium pratense* and can reduce growth and development of red clover (Chang et al., 1969). Evidence was obtained that salicylic acid may be synthesized in very low amounts by a larger number of plants than previously noted (Yalpani et al., 1991).

The influence of salicylic acid on stomatal movements was investigated by several research groups with conflicting results. Rai et al. (1986) reported that stomatal opening occurred when epidermal strips of *Commelina communis* were incubated with the phenolic acid, whether or not they were pretreated with ABA. Bhatia et al. (1986) observed stomatal closing in the presence of very low concentrations of salicylic acid. Patterson (1981) pointed out that allelochemicals such as salicylic acid may function on stomatal physiology, which, together with further effects, finally result in a general growth reduction. Inhibiting influences can be reduced, however, by various detoxifications. Involved reactions include oxidations, decarboxylations, methylations, and acylations of the allelochemical and/or its conjugation with amino acids or sugars, the latter being most widespread in the plant kingdom (Harborne, 1977). Detoxifications diminish the reactivity of harmful molecules as well as their permeability through membranes and allow the storage in suitable compartments such as the vacuole (Pridham, 1965).

In the present study, the influence of salicylic acid on the growth of *Vicia faba* L. was investigated with special regard to stomatal movements and a possible resistance of the plant against allelopathic attack by detoxification of the phenolic acid.

METHODS AND MATERIALS

Bioassay on Influence of Salicylic Acid on Growth of Vicia faba

Seeds of *Vicia faba* L. cv. Alfred (Lochow-Petkus, Bergen) were pretreated with methanol for 20 min and soaked in tap water overnight. The seeds were sown in five plastic pots, each filled with steamed soil. Seeds were allowed to germinate and grow for 18 days in a 12-hr photoperiod, provided by HQI400-lamps (Osram) with a light intensity of about 300 $\mu E/m^2$ at 21°C and 80%

humidity. Soil was kept moist for the first five days; afterwards the plants were treated either with 10 mM citrate buffer, pH 5.8, as control or with salicylic acid of defined concentrations (1, 2, 3.5, and 5 mM) dissolved in 10 mM citrate buffer. During the following 12 days, shoot length was measured, and at the end of this period, fresh weight of shoots and leaves was determined. For further experiments, plants were grown for maximally 21 days, normally watered or watered with salicylic acid solutions.

Effects of Salicylic Acid on Stomatal Apertures

Studies on Epidermal Peels. The abaxial epidermal tissue from leaves of 3-week-old plants was peeled off during the early light period and the cuticle incubated in one of the solutions described below for 3 hr. Illumination (170 $\mu E/m^2$) of the peels was provided by a cold light source (Schott, KL1500). Incubation solutions were prepared as follows: (1) 25 mM 4-morpholine-ethanesulfonic acid (MES)/Tris buffer, pH 6.5, supplemented with either 0, 0.001, 0.01, 0.1, or 1 mM salicylic acid (SA); (2) the same molarities were dissolved in distilled water; and (3) 10 mM citrate buffer, pH 5, 6, or 7, supplemented with 0.001, 0.01, or 0.1 mM SA. Controls were incubated only with citrate buffer pH 5, 6, or 7. All incubations were performed in Petri dishes covered by CO_2-depleted air. After treatments, 50 stomatal pore widths per peel were measured.

Measurements of Transpiration Rates. Fifteen plants were grown from seeds in a glasshouse at 20°C and a photoperiod of 12 hr. Light was provided by metal halide HQI400 lamps (Osram). The irradiance at plant level was 170–200 $\mu E/m^2$. Plants aged 14 or 21 days were then placed in an environmentally controlled chamber (volume 0.6 m^3) at a constant temperature of 20°C and 50% relative humidity. Partial pressure of CO_2 in the system was continuously monitored with an IR gas analyzer (URAS, Hartman & Braun) and maintained at 330 ppm CO_2. HQI400 lamps were used for illumination (170 $\mu E/m^2$). For measurements of transpiration rates, a steady-state porometer was used [PLC Parkinson leaf chamber and leaf chamber analyzer LCA2, Analytical Development Company (ADC)]. Leaves from whole plants were placed in the leaf cuvette (6.25 cm^2), continuously flushed with air. The flow rate (300 ml/min) of air, entering the leaf cuvette was monitored with a mass flowmeter (type ASU-MF, ADC). Transpiration rates (mmol $H_2O/m^2/sec$) were automatically recorded with a data logger 2 (Dl 2, ADC) and evaluated with an Epson HX10 computer (ADC).

Rates of transpiration were evaluated during treatments with salicylic acid. The compound was applied during watering with 10 ml of 10 mM citrate buffer, pH 5.8, containing 1.5, 3.5, or 7 mM salicylic acid. Applications with the buffer alone were performed as controls. When treatments were extended over

more than two weeks, plants were watered with salicylic acid solutions in the glasshouse for one week. Transpiration rates were monitored over five days (three data collections per day every 4 hr).

Preparations of Methanolic Extracts from Roots and Leaves

Roots and leaves from 3-week-old plants, placed in a 0.5 mM salicylic acid-containing medium (25 mM MES/Tris, 0.5 mM KCl, 0.25 mM $CaSO_4$ 0.1% Na ascorbate, 0.5 mM SA) or the medium alone for 14 hr, were harvested, washed thoroughly with distilled water, and ground to a powder in liquid nitrogen. The powder was extracted for 30 min with 10% methanol (1 ml/g plant material) at pH 4 and gently stirred. The mixture was filtered through eight layers of gauze and centrifuged for 10 min at 10,000*g*. The supernatant was evaporated to dryness at 30°C in vacuo. The residue was dissolved in 100 μl methanol/g plant material and centrifuged again for 10 min at 10,000*g*. The resulting supernatant was analyzed by HPLC.

High-Performance Liquid Chromatography (HPLC)

For HPLC analysis of the extracts, a Beckmann programmable solvent module 126 with UV/VIS detector 126 was used. Separation of phenolic compounds, detected at 280 nm, was performed with a RP-18 column (ODS type RP18, 46 × 250 mm, Beckman). Column temperature was kept at 20°C by a thermostat (WO, Industrial Electronic). Phenolics were eluted at a flow rate of 0.5 ml/min by the gradients described below, using 1% acetic acid in water and methanol as solvents A and B.

Gradient 1. 0–10 min, 100% A; 10–11 min, 92.4% A in B; 11–24 min, 77.3% A in B; 24–36 min, 77.3% A in B; 36–46 min, 50% A in B; 46–66 min, 100% B; 66–70 min, 100% B; 70–71 min, 100% A.

Gradient 2. 0–1 min, 100% A; 1–19 min, 71% A in B; 19–28 min, 55% A in B; 28–40 min, 44% A in B; 40–49 min, 24% A in B; 49–55 min, 100% B.

Gradient 3. 0–1 min, 77.3% A in B; 1–20 min, 50% A in B; 20–28 min, 32.8% A in B; 28–40 min, 25.8% A in B; 40–50 min, 100% B; 50–55 min, 100% A.

Data were evaluated by the system gold computer program (Beckman).

Isolation of Metabolite and Acidic Hydrolysis

The metabolite, detected by HPLC in methanolic extracts of incubated plant material, was collected after HPLC separation. Fractions of about 50 HPLC runs were combined, concentrated, and rechromatographed to prove purity of the collected substance. For acidic cleavage of the metabolite, 100 μl of the

pure concentrated fraction was mixed with the same quantity of 1 N HCl. The mixture was incubated at 60°C for 3 hr and analyzed for the decrease of the metabolite and the occurrence of salicylic acid by HPLC. The latter was identified by cochromatography with salicylic acid standards.

Statistics

Experiments were repeated three times. Significance of data was determined by Vierfelder test with Yates correction or by F test and Student's t test.

RESULTS

Salicylic Acid-Induced Growth Reduction

Seedlings of *Vicia faba* treated with the different concentrations of salicylic acid showed a significant reduction of shoot growth and fresh weight. The growth inhibition was dependent not only on the salicylic acid concentration, but also on the time of exposure. Significant differences between the controls and treated seedlings were obvious after 18 days of application (Figure 1). A 25% reduction of the growth was achieved by 5 mM salicylic acid after seven

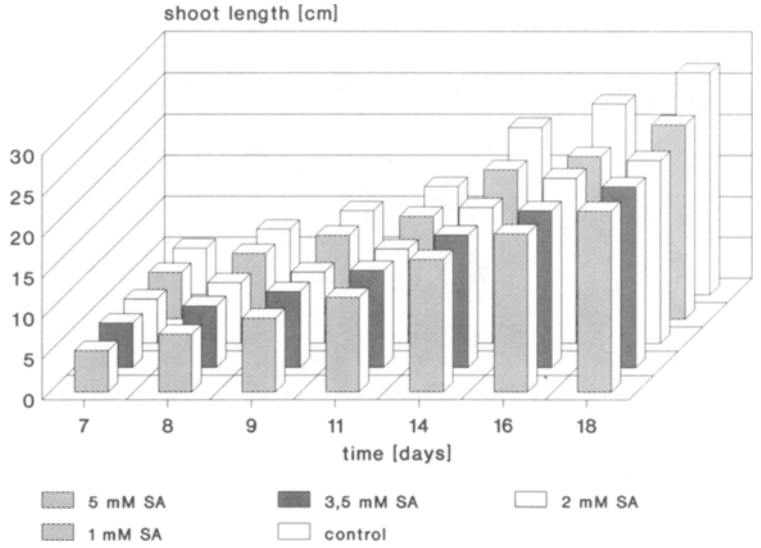

FIG. 1. Plants were watered with salicylic acid (SA) of the given concentrations over a period of 11 days. Highest SA concentrations inhibited shoot growth after two days of treatment. A significant inhibition was also achieved for the lower concentrations after 10 days.

days, indicating that the allelopathic action of the phenolic acid on *Vicia faba* is only developed at high salicylic acid concentrations or the uptake of the compound is insufficient. Thus an examination of plant material for the recovery of salicylic acid was necessary.

Effects of Salicylic Acid on Stomatal Movements

Incubations of epidermal peels in the presence of salicylic acid resulted in a concentration-dependent reduction of stomatal apertures. When incubations were performed with salicylic acid dissolved in distilled water, a concentration as low as 0.001 mM was sufficient to diminish stomatal pore widths more than 50%, whereas 0.1 and 1 mM caused a complete closure (Figure 2). Additionally, epidermal peels were incubated with MES/Tris (pH 6.5) to estimate pH influences. Under these conditions, the same effects were observed, but higher concentrations of the phenolic compound were necessary to induce stomatal closure, e.g., 0.001 mM caused only a 30% reduction of stomatal pore width and with 0.01 mM, a 76% reduction was noted (Figure 2). Stomatal closure in the presence of salicylic acid is therefore supposed to be an effect of the allelochemical, which may be superimposed on or intensified by low pH. The relatively large standard deviations may be due to the known and normally occurring heterogeneity in stomatal apertures appearing on the same leaf (Raschke et al., 1975).

The interaction of pH and salicylic acid was further studied by incubating

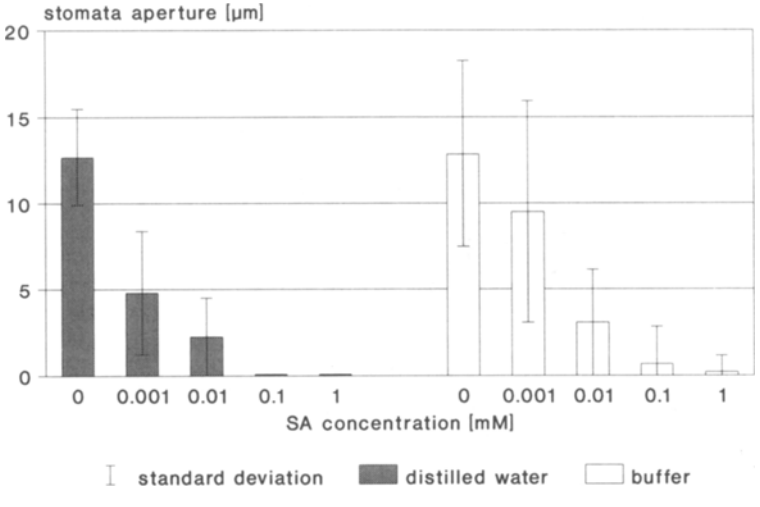

FIG. 2. When salicylic acid (SA) was dissolved in distilled water, 0.001 mM was sufficient to reduce stomatal pore widths to 50%, buffered SA solutions were effective at higher SA concentrations. Distilled water only or the buffer alone were used as controls.

epidermal peels in citrate buffer at pH 5, 6, and 7 alone or supplemented with 0.001, 0.01, or 0.1 mM of salicylic acid. At pH 5 and 6, stomatal apertures were reduced to about 46% compared to pore width at pH 7. In the presence of salicylic acid, however, reductions of apertures were higher at all three pH values, but SA was most effective at pH 5, at concentrations as low as 0.001 mM (pH 7: 52%; pH 6: 61%, pH 5: 67%) compared to the controls at corresponding pH (Figure 3). Although under low pH conditions alone, a reduction of the stomatal pore widths was observed, the results clearly indicate a further salicylic acid-induced stomatal closing. This is in accordance with the known fact that the nondissociated form of salicylic acid possesses a higher physiological activity than the dissociated one (Basund, 1962; Scharff and Perry, 1976).

Transpiration Rates after Treatments with Salicylic Acid

In subsequent studies, salicylic acid applications were performed by watering 3-week-old plants with 1.5, 3, 5, and 7 mM solutions of the allelochemical for five days. As illustrated in Figure 4, only the highest concentrations (5 and 7 mM) caused significant reductions (14%, 11%) of the transpiration rates at the fifth day of treatment. When 2-week-old plants were pretreated with salicylic acid solutions of the same concentrations over a period of six days, a remarkable reduction (between 60 and 40%) of the transpiration rate during the seventh and

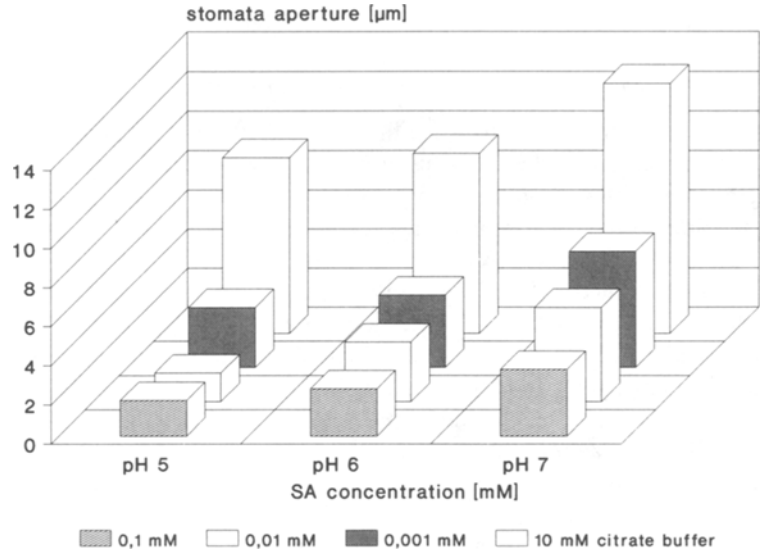

FIG. 3. Lower pH values of the solutions intensify the effect of salicylic acid (SA), inducing stomatal closure in epidermal peels.

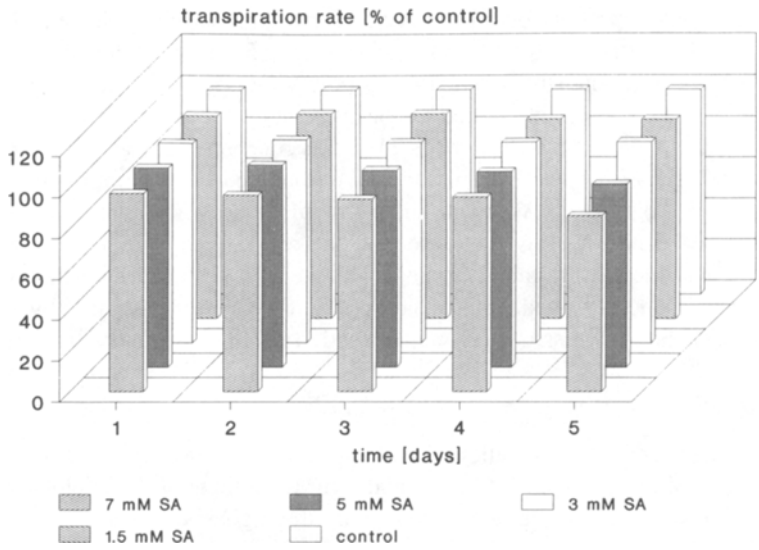

FIG. 4. Significant reduction of the transpiration rates (14%) was only achieved after the fifth day with the highest salicylic acid (SA) concentration used. Measurements were extended over a period of five days.

eleventh day of treatment was achieved for the highest salicylic acid concentrations used (Figure 5).

Appearance of Salicylic Acid and Its Metabolite in Roots and Leaves

The obvious differences between the reactions of guard cells in epidermal peels and the results of the bioassay on the allelochemical's effectiveness on plant growth as well as stomatal responses to salicylic acid treatments of whole plants required further studies on the appearance of salicylic acid in plant tissues and its possible metabolism. When whole plants were incubated for 14 hr in a medium supplemented with 0.5 mM salicylic acid, methanolic extracts of the roots and leaves, analyzed by HPLC (Figure 6B and C), contained free salicylic acid identified by cochromatography, and an additional new compound absent in controls (Figure 6A). In comparison with the root extracts, those of leaves contained a considerably higher amount of endogenous phenolics, but salicylic acid as well as the additional compound could be identified using gradient 2. Both compounds were also detectable in soil-grown, SA-treated plants (data not shown). When plants were incubated in the SA-containing medium, however, the amounts of salicylic acid and of the additional compound were higher.

The compound found in roots and leaves of treated plants was considered as a detoxification product of absorbed, exogenous salicylic acid. Showing a

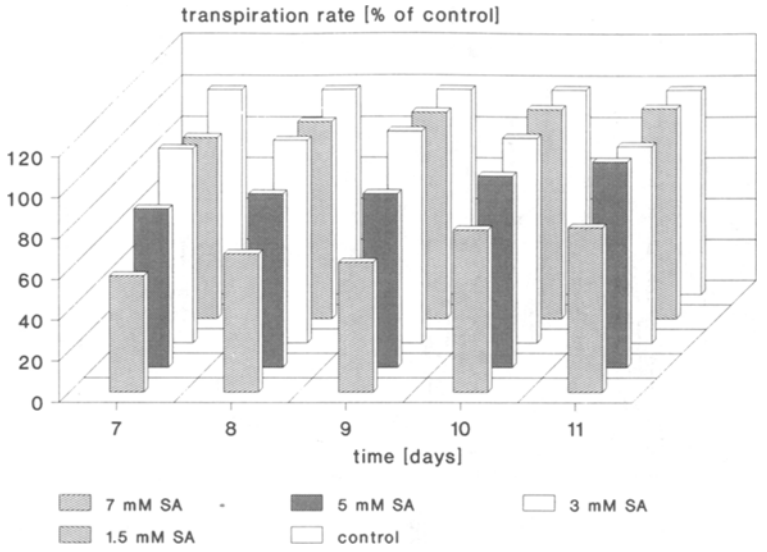

FIG. 5. Reduction (30%) of the transpiration rates after 11 days of treatment with 7 mM salicylic acid (SA).

lower retention time of 31.2 (gradient 2) or 37.5 (gradient 1) min compared to free salicylic acid (42.5 min, gradient 2/58.1 min, gradient 1), its increasing concentration with time is correlated with the presence of a decreasing amount of salicylic acid in the extracts (Figure 7). Especially for the purpose of isolating the assumed metabolite, further studies were performed with medium-incubated plants.

In a first attempt at identification, the new compound was collected after preparative HPLC and subsequently hydrolyzed with 1 M HCl. HPLC analysis of the hydrolyzed products revealed the release of salicylic acid (Figure 8). Thus an uptake of salicylic acid, its transport to leaves, and the appearance of a possible metabolite are evident.

DISCUSSION

The growth reduction of *Vicia faba* plants after treatment with salicylic acid is in agreement with the results of Kurogochi et al. (1978) and Leather and Einhellig (1985), who found similar effects in bioassays with *Oryza sativa* and *Sorghum bicolor*. The allelopathic action of the phenolic acid on *Vicia faba* occurred only at relatively high concentrations. This might be due to a small amount of uptake as a consequence of low availability in the soil, caused by

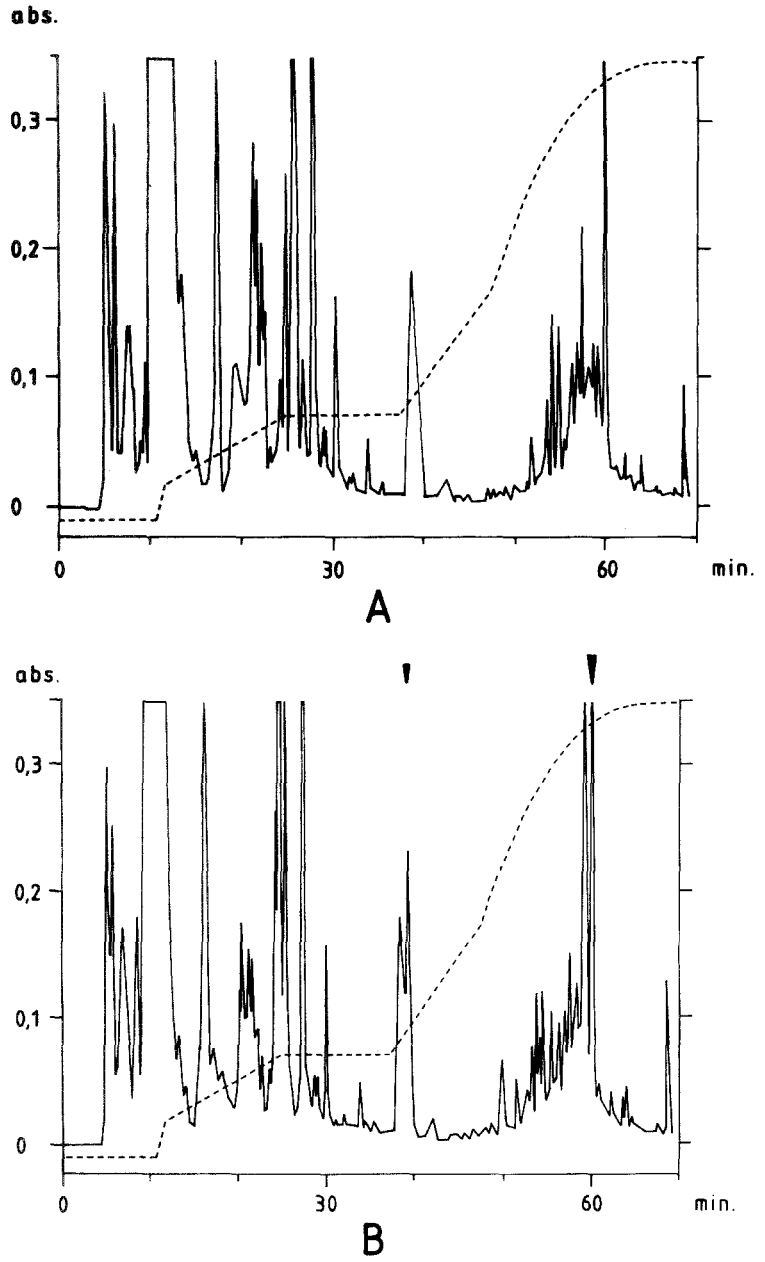

FIG. 6. HPLC chromatograms of (A) methanolic extract from roots, harvested from untreated plants (control), (B) root extract, prepared from salicylic acid (SA) -incubated plants, (C) leaf extract from SA-treated plants, illustrating free SA as well as the additional compound (retention time of salicylic acid: 42.6; retention time of metabolite: 32.1).

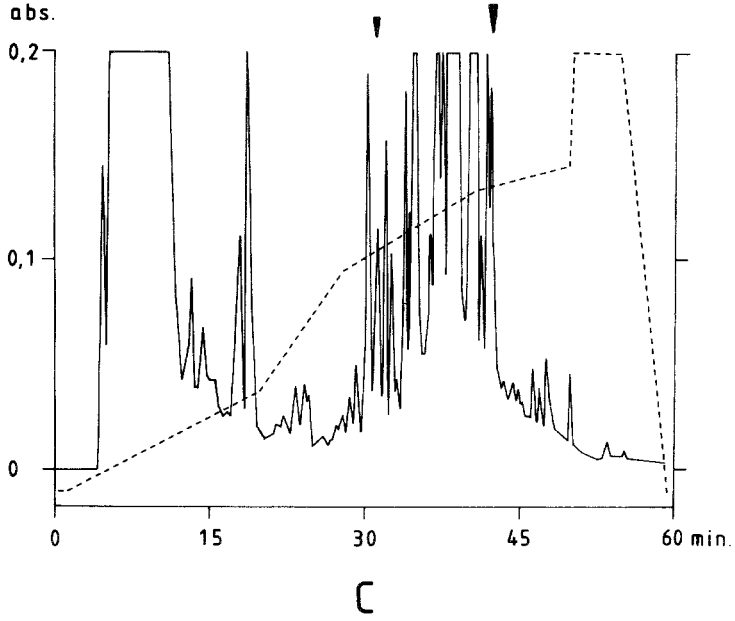

FIG. 6. Continued

adsorption to soil particles or decomposition of the allelochemical by microorganisms (Muller and Chou, 1972; Heisey, 1990). Therefore, the concentrations of salicylic acid used (0.1–5 mM) may not be identical with the amount available for uptake by the plant but, rather, should be lower (Dalton et al., 1983). The latter could be one explanation for the relatively late appearance of growth inhibition only at the highest concentration. A further reduction of inhibiting effects must be expected if the phenolic acid is detoxified after entering living cells.

Influences of allelochemicals on stomata, responsible for the regulation of gas exchange, are an important factor affecting growth and development of a plant (Patterson, 1981). One explanation for the growth inhibition observed is a possible effect on stomatal apertures. Guard cells in peels of abaxial epidermis were very sensitive to low concentrations of salicylic acid, resulting in stomatal closing. This is in agreement with the observation of Bhatia et al. (1986), who studied stomatal responses of *Euphorbia hirta* after salicylic acid applications of 5–25 ppm. Rai et al. (1986), however, described stomatal opening following salicylic acid treatments of epidermal peels from *Commelina communis*. The phenolic acid used here is assumed to produce membrane depolarization and to increase membrane permeability to inorganic ions (Macri et al., 1986; Balke, 1985). In different plants (*Hordeum vulgare:* Glass, 1973, 1974; *Avena sativa:*

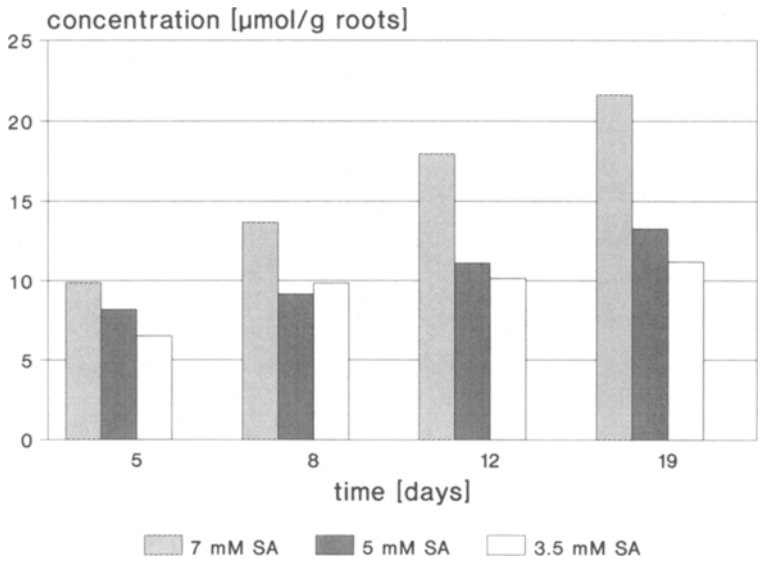

FIG. 7. Concentration of the assumed salicylic acid (SA) metabolite in extracts from roots of plants, watered with SA of the given concentrations. The amount of the metabolite increased with time and depended on the applied SA concentration.

Harper and Balke, 1981; *Saccharomyces cerevisiae:* Scharff and Perry, 1976; Hoeberichts et al., 1980), salicylic acid was responsible for an uncontrolled efflux of inorganic ions, especially K^+. Potassium, however, is known to be an essential factor in stomatal opening (Raschke and Schnabl, 1978; Schnabl, 1985, 1992; Michalke and Schnabl, 1990). A loss of K^+, due to changes of guard cell plasma membrane properties induced by salicylic acid, should therefore result in stomatal closing.

Measurements of transpiration rates after salicylic acid applications during plant watering were performed to avoid wounding effects, which may appear in isolated epidermal peels. As water loss through the cuticle layer was found to be negligible, transpiration rates are directly correlated with stomatal pore widths (Nonami et al., 1990). Additionally, applications during watering seem to simulate the more natural situation. A significant reduction of the transpiration rates occurred after five days of treatment, but again only for the highest concentrations used in this study. Thus stomata of intact plants showed much less sensitivity compared to those of isolated epidermal peels, coinciding with the result of the bioassay on growth inhibition. Similar effects are described by Patterson (1981) and Raschke et al. (1975). They found an impaired transpiration by allelochemicals for *Glycine max*, *Phaseolus vulgaris*, *Hordeum vulgare*, and *Xanthium strumarum*. For *Phaseolus vulgaris*, Larque-Saavedra (1978, 1979)

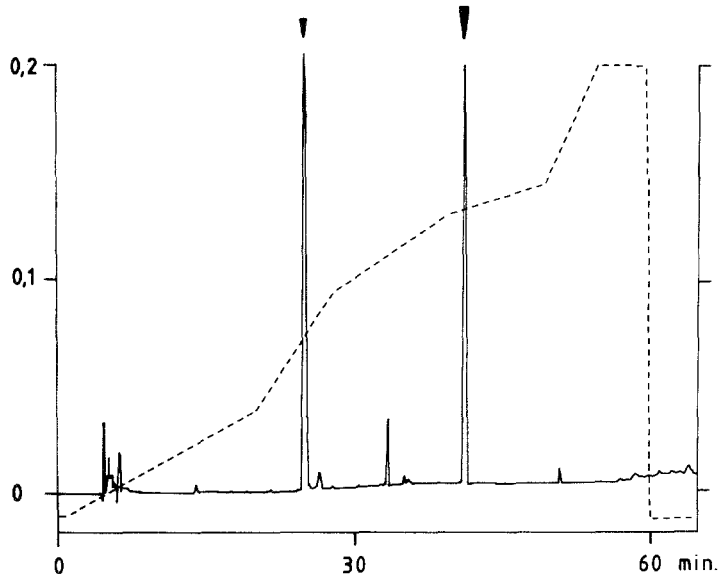

FIG. 8. Released salicylic acid showed a retention time of 41.47 min, the residual metabolite a time of 25.15 min.

demonstrated a 43% reduction of the transpiration rate after applications of 1 mM acetylsalicylic acid, when the compound was transported by the transpiration stream.

The results presented here indicate a growth reduction and a decrease of stomatal aperture and transpiration rate for *Vicia faba*. However, inhibiting effects were obvious only at high concentrations of the phenolic acid associated with long-term treatments. HPLC analysis of methanolic extracts prepared from roots and leaves contained salicylic acid and a metabolite, whose acidic hydrolysis released free salicylic acid. Hence, *Vicia faba* is able to absorb and to metabolize the allelochemical by altering its solubility, most probably by conjugation with a water-soluble nontoxic compound. The latter mechanism is a known strategy, existing in a large number of plants, to detoxify allelochemicals or endogenous phenolic compounds (Harborne, 1979; Balke et al., 1987; Tabata et al., 1988; Tanaka et al., 1990). It is therefore assumed that, under natural conditions, salicylic acid may be an allelochemical less harmful to the species studied here. The exact mechanism of detoxification as well as the identity of whole metabolite has to be elucidated in the future.

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EFFECT OF TEMPERATURE AND SUCROSE CONCENTRATION ON HYDROQUINONE TOXICITY IN LEAFY SPURGE SUSPENSION CULTURE CELLS

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Abstract—*Euphorbia esula* (leafy spurge) suspension culture cell bioassays were used to determine whether sucrose accumulation enhanced the glucosylation (detoxification) of hydroquinone in this noxious weed. The bioassay results indicate that cold temperatures and exogenous hydroquinone represent a dual stress to spurge cell growth that can be partially ameliorated by hydrolysis of sucrose. The persistent susceptibility of leafy spurge suggests that hydroquinone-producing forage plants (which are not toxic to animals) might be used as natural competitors.

Key Words—Leafy spurge, *Euphorbia esula*, small everlasting, *Antennaria microphylla*, hydroquinone, arbutin, sucrose, tissue culture, biotransformation, allelopathy.

INTRODUCTION

The perennial weed, leafy spurge (*Euphorbia esula* L.), is endemic to the upper Great Plains of the United States and the prairie provinces of Canada. Infestations are deleterious to rangeland operations due to spurge competition with palatable forage species and toxicity to grazing livestock. It has not proven economically feasible to control the weed by either cultural and/or chemical methods (Manners, 1987).

The competitive advantage of leafy spurge is enhanced by its extensive

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root system, which produces new vegetative shoots from adventitious crown and root buds (Coupland et al., 1955). Abundant root carbohydrate reserves are correlated with the overwintering strategy and high regenerative potential of the plants (Cyr and Beweley, 1989), and efforts to control perennial weeds have concentrated on altering seasonal fluctuations of carbohydrate accumulation patterns.

Recent observations have shown that, in contrast to many other perennial plants, leafy spurge accumulates large amounts of free sucrose in the roots at the close of the growing season, instead of the more typical accumulation and conversion of sucrose to starch (Lym and Messersmith, 1987). Others have suggested that this pattern is important in development of winter hardiness in leafy spurge roots and elongation and winter survival of root buds, perhaps through exploitation of cryoprotectant properties of colligative molecules such as sucrose (Sakai and Yoshida, 1968). Cyr and Beweley (1989) suggested that it is more likely that sucrose serves as a readily phloem-mobile substrate for rapid spring regrowth.

Hydroquinone has been isolated from the low-growing forb, small everlasting (Manners and Galitz, 1985), and assays have established its phytotoxicity to leafy spurge (Hogan and Manners, 1990). Biotransformation of hydroquinone to its monoglucoside, arbutin, is mediated by a uridine diphosphoglucose (UDPG) -dependent glucosyltransferase (Hogan and Manners, 1991). The activity of this enzyme is substrate-induced in leafy spurge and is affected by various metabolic, as well as abiotic, factors. Since glucosylation of hydroquinone requires a readily available carbohydrate pool, seasonal accumulation of sucrose in spurge roots may affect the rate of hydroquinone biotransformation in vivo. In this investigation, a tissue culture bioassay was developed to determine whether the unusual carbohydrate accumulation patterns of leafy spurge at reduced temperatures affect glucosylation of hydroquinone, thereby providing seasonal amelioration of allelochemical effects of that compound on plant growth. The effect of sucrose accumulation and low temperatures on the glucosylation of exogenous hydroquinone was simulated by supplying increasing amounts of exogenous sucrose simultaneously with hydroquinone to suspension culture cells of leafy spurge maintained at low temperatures.

METHODS AND MATERIALS

Tissue Cultures. Suspension and callus cultures of leafy spurge and small everlasting were grown as previously described (Hogan and Manners, 1990). Hydroquinone (1,4-dihydroxybenzene) solutions (500 μM) were prepared in absolute methanol and introduced aseptically into flasks of leafy spurge suspension culture cells at a final methanol concentration of 1% or less (Davis et al.,

1978). This concentration of hydroquinone had previously been determined to produce a sublethal phytotoxic response during all stages of cell growth (Hogan and Manners, 1990). High-pressure liquid chromatographic analysis of media extracts showed that all the hydroquinone was absorbed by the cells within 24 hr (data not shown). HPLC analysis of extracts from leafy spurge suspension culture cells treated once with 500 μM hydroquinone showed that 85% of this compound (on a mole per mole basis) was converted to arbutin during a short exposure time (six days). This suggests that very little, if any, hydroquinone is bound in the media. Test concentrations of arbutin (*p*-hydroxyphenyl-β-glucoside) (0.05, 0.1, 0.25, 0.5, 0.75, 1.0, and 5.0 μM) were dissolved directly in the hot growth media for callus culture experiments.

Cell Viability Tests. Exogenous sucrose was added directly to the culture media at 0, 2, 3, 4, 5, and 6% by weight, respectively. Erlenmeyer flasks (250 ml) containing 50 ml media were inoculated with 5 ml suspended cells (1.5 g fresh weight) from a common culture. After seven days of growth (25°C, dark), the cells were harvested by vacuum filtration. Fresh-filtered cells (0.2 g) were washed with 20 ml phosphate buffer (pH 7.0) and suspended in 3 ml triphenyltetrazolium chloride (TTC, 0.6% in phosphate buffer, pH 7.0). After incubation overnight (27°C, dark), the cells were washed twice with distilled water and extracted with hot ethanol (5 min). Reduced TTC (red supernatant) was measured by absorbance at 485 nm, using a Spectronic 20 spectrophotometer (Yokoyama et al., 1990). Media in the absence of cells was used as a control to rule out the presence of competing reductants.

HPLC Analysis of Sugars. Cells exposed to varying temperatures (10, 15, 17, and 25°C), high exogenous sucrose concentrations, and/or hydroquinone for one week (dark) were harvested by vacuum filtration, rapidly frozen under liquid nitrogen, and stored frozen until used. The cells were Soxhlet-extracted with 80% ethanol overnight. The ethanol was removed in vacuo, and the residual aqueous solutions were filtered and analyzed for sucrose, glucose, and fructose, using high-performance liquid chromatography (HPLC, Waters carbohydrate analysis column, 3.9 × 300 mm; acetonitrile–water, 75:25 v/v; 1.5 ml/min; RI detection with Waters differential refractometer model R401; 20 μl injection). Retention times (observed): fructose, 4.64 min; glucose, 5.14 min; sucrose, 6.57 min. Data are expressed as micrograms of carbohydrate per gram fresh weight of cells.

Statistical Analysis. All bioassays included at least three replications. Individual determinations of TTC reduction (Figure 2 below) and carbohydrate accumulation (Figure 3 below) were averaged and subjected to one-way analysis of variance. *F* values were calculated to determine significant differences between treatments at the 0.05% level. Multiple regression analysis was performed on the data from Table 1 (cell weight as a function of sucrose concentration, temperature and the presence of hydroquinone).

TABLE 1. EFFECT OF TEMPERATURE AND EXOGENOUS HYDROQUINONE ON GROWTH OF LEAFY SPURGE SUSPENSION CULTURE CELLS

	10°C		15°C		17°C		25°C	
Sucrose (%)	−HQ	+HQ[a]	−HQ	+HQ	−HQ	+HQ	−HQ	+HQ
0	3.15[b]	0.94	3.97	1.18	3.78	1.35	3.89	3.89
2	3.38	1.31	7.81	4.85	11.35	6.83	32.24	14.47
3	3.59	1.35	8.22	5.32	9.75	7.81	37.06	13.30
4	3.21	1.31	7.96	4.21	9.60	4.74	33.73	11.90
5	3.22	2.02	7.48	3.55	9.64	5.22	32.42	11.89
6	2.91	1.86	6.67	3.38	9.05	7.05	33.57	13.36

[a] Flasks treated with 500 μM hydroquinone 24 hr after inoculation.
[b] All data are expressed as grams fresh weight of cells at one week of growth and are the average of three representative experiments.

RESULTS AND DISCUSSION

Cell Viability. Sucrose supplied in excess (up to a concentration of 4% sucrose) to the growth media of leafy spurge suspension culture cells enhanced cell growth (Figure 1) during all phases of the cell cycle. At higher concentrations of sucrose (>5%), cell dry weight leveled off and cell respiration rates

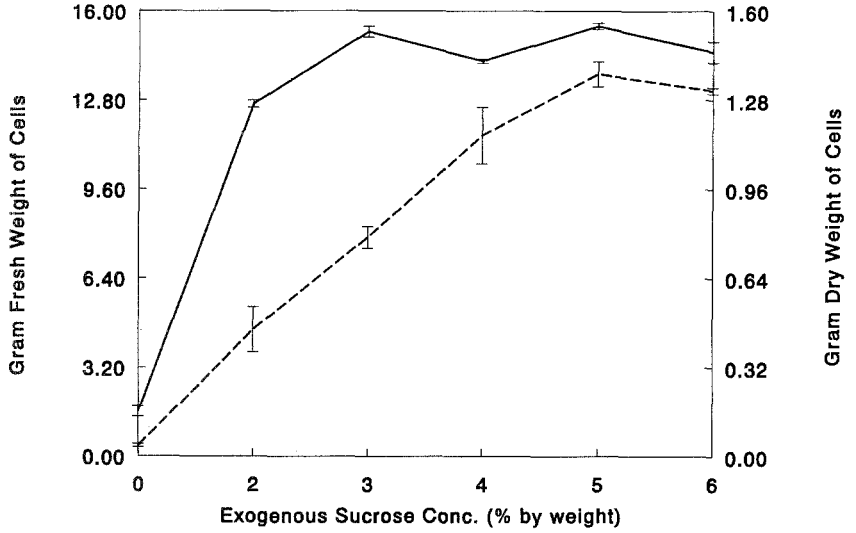

FIG. 1. Growth of leafy spurge suspension culture cells in the presence of excess sucrose: grams fresh weight of cells, solid line; grams dry weight of cells, dashed line.

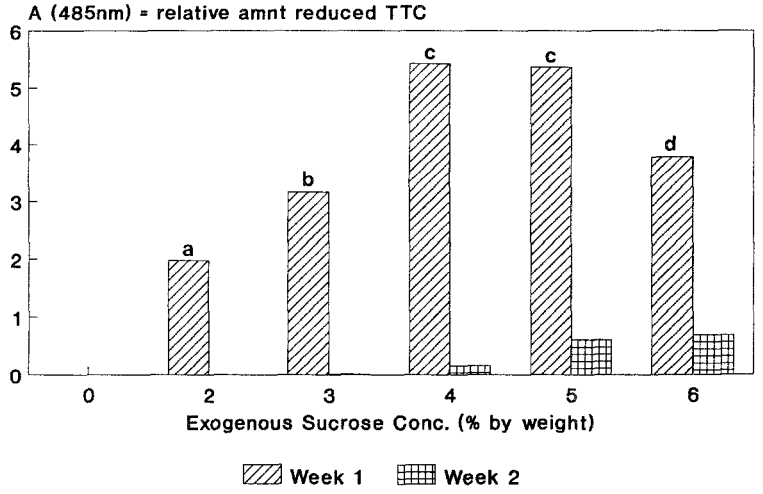

FIG. 2. Effect of sucrose on cell viability of leafy spurge suspension cultures. TTC = triphenyl tetrazolium chloride. Identical letters represent treatments that are not significantly different from each other at the 0.05% level.

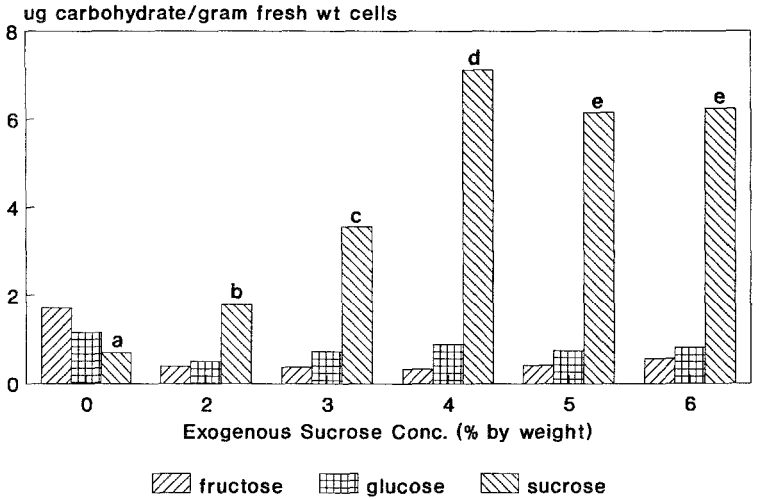

FIG. 3. Carbohydrate accumulation in leafy spurge suspension culture cells exposed to exogenous sucrose after one week of growth. Identical letters represent treatments that are not significantly different from each other at the 0.05% level.

declined. Cell respiration rate (TTC-reducing ability) and the ability to sequester free sucrose were reduced in the cells when exogenous levels of sucrose were greater than 4% and 5%, respectively (Figures 2 and 3). Although the cells were not plasmolyzed when exposed to levels of sucrose greater than 4%, it is clear that these supraoptimal levels impose an osmotic stress, resulting in a lack of cell dry weight increase beyond that observed in the 4% treatment (Figure 1). By the second week, most cells were dead due to media depletion (the cell cycle for this cell line is seven days). Supraoptimal levels of sucrose provided enough residual carbohydrate in the medium to support limited growth of cells exposed to 5% or 6% sucrose for two weeks (Figure 2).

Fructose and glucose levels remained steady over time, regardless of the amount of exogenous sucrose supplied in the media (Figure 3). No significant differences were found at the 0.05% level for fructose or glucose between any of the treatments, using one-way analysis of variance. Therefore, either the cells did not hydrolyze the free sucrose, or the free hexoses liberated in the cells were immediately metabolized to more conjugated forms not detected in the assay. The failure of cells to metabolize sucrose is consistent with the observations of Yokoyama et al. (1990), who did not detect metabolism of exogenous sucrose in cell cultures of *Catharanthus roseus*, and of Cyr and Beweley (1989), who observed only small winter increases in the glucose content of leafy spurge roots when fructose levels remained at or near detection limits throughout the year.

Suboptimal Temperature and Hydroquinone Effects on Suspension Culture Growth. Leafy spurge suspension cells are most efficiently cultured at 25–27°C (Hogan and Manners, 1990). At suboptimal temperatures plant cells are subjected to abiotic stress. Cell growth was reduced in leafy spurge suspension cultures when temperatures were lowered by 8–15°C and the typical cumulative increase in biomass was altered; i.e., the logarithmic phase of fresh weight increase occurred later and was less extensive (Figure 4). Additionally, the previously observed phytotoxicity of exogenous hydroquinone to spurge suspension cells (Hogan and Manners, 1990) was exacerbated at the lower temperatures (Table 1). The suboptimal temperatures slowed cell metabolism, thereby inhibiting pathways required for cell growth and hydroquinone biotransformation. The reduced temperature and increased hydroquinone therefore represent dual stresses to spurge cells that significantly lower cell growth and viability.

The observed late-season accumulation of sucrose in spurge roots was simulated in this investigation by addition of exogenous sucrose to the cell culture media and incubation at temperatures consistent with temperatures to which whole plants are exposed during the cool fall of the Northern Plains. The presence of sucrose enhanced cell biomass accumulation (as measured by fresh weight) at these reduced temperatures both with and without the addition of hydroquinone (Table 1). Sucrose (up to a concentration of 4%) also ameliorated

the phytotoxic effect of hydroquinone at lower temperatures (Table 1). However, there was no clear indication that sucrose sequestration was affected by hydroquinone (data not shown). The results of multiple regression analysis on these data predict that fresh weight is correlated with changes in sucrose concentration, temperature, and the presence of hydroquinone (HQ) by the following: cell wt = 1.225(temp) + 0.919(sucrose) − 6.419(HQ) − 11.594, $F_{3,44} = 29.6$, $P < 0.001$, $R^2 = 0.669$.

Accumulation of free sucrose in leafy spurge cells provides a potential source of glucose (via sucrose hydrolysis) for glucosylation of hydroquinone to form the less phytotoxic monoglucoside, arbutin. The detoxification of hydroquinone enlarges the arbutin pool, creating a shift in the equilibrium of the detoxifying reaction in favor of hydroquinone accumulation (Figure 5) (Hogan and Manners, 1991). In addition, arbutin and other structurally similar β-phe-

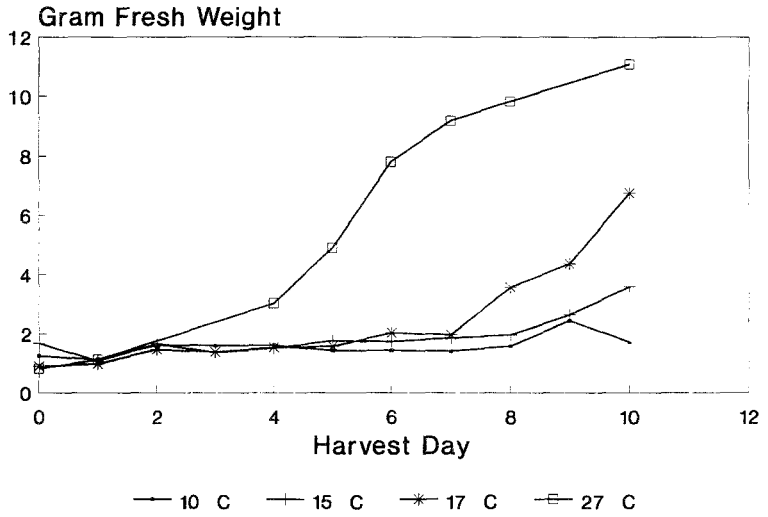

FIG. 4. Growth of leafy spurge suspension culture cells at reduced temperatures.

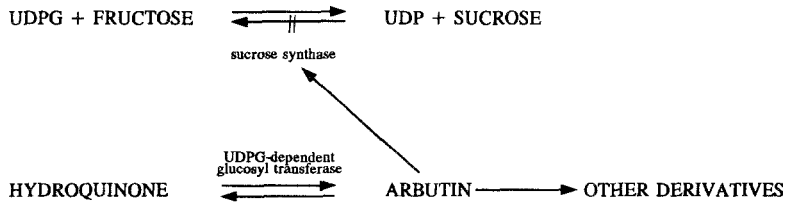

FIG. 5. Competitive inhibition by arbutin on sucrose synthase activity. UDPG = uridine diphosphoglucose.

nolic glycosides are competitive inhibitors of sucrose synthase, an enzyme responsible for sucrose degradation (Wolosiuk and Pontis, 1974). These factors represent a potential regulatory mechanism associated with the size of the arbutin pool in leafy spurge. Increases in arbutin concentration in spurge could both inhibit sucrose degradation (reduce detoxification of hydroquinone) and promote hydroquinone accumulation (increase autotoxicity).

Confirmation of the effect of arbutin was observed when it was exogenously supplied to leafy spurge callus. In comparison to small everlasting, spurge callus growth was significantly reduced in the presence of arbutin at concentrations above 100 μM (Table 2). This inhibition pattern continued until media depletion occurred after six weeks in continuous culture (data not shown), suggesting a constant turnover of arbutin to hydroquinone in leafy spurge. In contrast to the turnover observed in leafy spurge, small everlasting may divert arbutin into alternative pathways, to produce less phytotoxic derivatives (Figure 5) (Hogan and Manners, 1991).

The significance of this difference between the metabolism of the two species under field conditions could be exploited for weed control. Both small everlasting and leafy spurge can convert hydroquinone to arbutin (Hogan and Manners, 1991). However, the kinetics of the biotransformation reactions associated with hydroquinone detoxification differ in the two plants, resulting in the higher concentrations of hydroquinone found in leafy spurge (Hogan and Manners, 1991). The unusual sucrose accumulation by leafy spurge roots at the end of the growing season could also influence the equilibrium of these reactions and might exacerbate the effect of the allelochemical. Hydroquinone-producing forage species could act as natural competitors in spurge-infested areas, assum-

TABLE 2. EFFECT OF ARBUTIN ON CALLUS GROWTH[a]

Arbutin (μM)	Increase in grams of fresh weight (%)	
	Small everlasting[b]	Leafy spurge[b]
0.00	100	100
0.05	91.4	96.1
0.10	94.9	96.4
0.25	70.7	57.9
0.50	61.6	30.3
0.75	45.9	35.9
1.00	36.1	20.7
5.00	0	1.0

[a] After six weeks of continuous culture.
[b] Values at arbutin concentrations higher than 0.1 mM were shown to be significantly different between the two species using one-way analysis of variance.

ing that hydroquinone is released from the roots of living plants and can persist in the soil. These studies have not been done, although work by Selleck (1972) showed that the susceptibility of leafy spurge to encroachment by small everlasting in the field was attributable to chemical rather than competitive factors. Intervention in the ability of leafy spurge to accumulate and metabolize sucrose (in the presence of allelochemicals requiring carbohydrate for detoxification) could compromise the competitive advantage of this plant and render it more susceptible to other control methods.

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PHEROMONAL TRANSMISSION OF AN AVERSIVE EXPERIENCE IN DOMESTIC PIG

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Abstract—The process of spontaneous learning in an automatic food dispenser by a group of domestic female pigs was studied when one of the animals of the group had an aversive experience. Restraining a gilt in the dispenser without access to food resulted in later avoidance of the system by other gilts, especially when the reactions of the restrained animal had been especially violent and associated with urination. The hypothesis of a delayed transmission of an unpleasant experience was tested. The food dispenser was sprayed with urine collected from either a control gilt or from an animal undergoing stress. In half of the cases, the presence of urine of a stressed animal resulted in a long-lasting avoidance of the food dispenser, suggesting the existence of some kind of alarm pheromone produced in the urine of a sow during an unpleasant experience.

Key Words—Stress, urine, alarm pheromone, pig.

INTRODUCTION

The possibility for an animal to transmit information from an unpleasant experience is of high functional significance. It would inform another animal of the risks coming from the environment, thus inducing avoidance of potential danger. A delayed transmission of such information would make it possible to avoid a place where some kind of aggression has occurred. The production of alarm pheromones during aggression or an unpleasant experience has been observed in laboratory mice (Müller-Velten, 1966; Carr et al., 1970; Rottmann and Snowdon, 1972; Zalaquett and Thiessen, 1991) and rats (Valenta and Rigby, 1968;

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Stevens and Köster, 1972; Mackay-Sim and Laing, 1980). Furthermore, when avoidance is impossible, chronically exposing mice to the odor of a conspecific stressed by electrical foot shocks depresses the immune system (Thiessen and Cocke, 1990). No similar system of communication has been described in non-rodent mammalian species.

During a study on learning in an automatized food dispenser by a group of domestic sows, some observations suggested the transmission of an unpleasant experience.

A computer-controlled pelleted food dispenser provides rations adapted to the individual animals' feeding requirements, even when the sows are housed in groups. However, under practical husbandry conditions, some animals are difficult to train to use such a system, and a period of training is necessary before all the animals adequately use the feeder (Vieuille-Thomas and Signoret, 1991). To facilitate the process of learning, the influence of the presence of an already trained animal in a group of naive sows has been observed (Vieuille-Thomas and Signoret, 1989). No social facilitation occurred in the presence of a trained animal, but, on the contrary, in some cases there was a considerable increase in the delay of the first contact of any animal with the food dispenser. Such a surprising reaction appeared to be related to difficulties during the process of training the demonstrator animal that had showed a strong fear reaction. Consequently, a series of experiments were designed to assess the existence of transmission of such an unpleasant experience and to test the hypothesis of the existence of an alarm pheromone in this species.

METHODS AND MATERIALS

The experiments took place in a large pig farm (1200 sows) located in central France in which groups of 10 naive gilts had to be trained each week, or fortnight, to the use of a computer-controlled food dispenser (Codeporc). Young females, ages 7–10 months, were inseminated when tethered in their stall. After confirmation of pregnancy one month later by ultrasonic echography, they were moved to a 40-square-meter pen with an automatic feeder with which they had never been in contact. The sows were identified by ear tags and a collar equipped with a transponder allowing access to the feeder. The success of the learning process was assessed by measuring the delay for the first naive gilt to use the food dispenser.

The food dispenser is a double-gated complex system, with a steel partition in the middle, designed to allow a single sow to feed without being disturbed by the others. When an animal enters the feeder and is identified by the electronic system and the transponder, the rear gates automatically close and food is dispensed at the rate of 240 g/min until the animal moves back, or the total amount programmed for this animal has been dispensed. Gate locking, and falling of

pelleted food in the metal trough are noisy and might startle the sows during their first experience of the dispenser. Furthermore, the animal that reacts to such noises by moving back violently could prevent the gates from opening and thus become trapped in the dispenser. The ineffective escape reactions are then associated with apparent panic involving sound emission, redness of the entire body, and urination.

Experiment 1. Eight experimental groups of 10 naive gilts were studied. Each consisted of nine pregnant gilts plus a nulliparous female that was neither pregnant nor in estrus. In the afternoon of the day before the onset of the process of learning, this female was individually introduced to the experimental pen and guided by plywood panels into the food dispenser. The back doors were locked, and the animal was thus kept trapped in the dispenser for 20 min without receiving any food as reinforcement. Such a treatment in an unknown environment could be considered an unpleasant experience. It induces violent reactions: escape attempts associated with frequent grunts, squeals, and eventually urination and defecation. The gilt was then reintroduced to her group. On the next morning, after a minimum delay of 14 hr, the whole group had free access to the food dispenser. They had not been fed since the morning of the previous day.

The results of learning to use the food dispenser were compared with those of 13 groups of 10 naive gilts in which no animal had experienced any contact with the dispenser. In all cases, one group was observed per week.

Experiment 2. Twenty groups of 10 naive pregnant gilts were observed. In each case, the back doors and the inside of the food dispenser were sprayed during late afternoon with urine produced either by a stressed or a control female. In 10 experimental groups, urine was obtained from a nonpregnant, nonestrous gilt tethered for the first time. The process of tethering generally induces strong reactions of escape attempts, biting of the chain, squealing, and sweating. Urine produced after a minimum delay of 15 min was collected. When no urine could be collected within 30 min, another sow was used in the same way. For the 10 control groups, urine was obtained from a quiet nonpregnant, nonestrous sow. In both cases, urine was used immediately to be sprayed on the dispenser.

As in the first experiment, the groups of naive gilts after having been fed in the morning, were moved during the late afternoon to a waiting pen. The next morning they were introduced to the experimental pen to learn the use of the food dispenser. Consequently, the delay between spraying the urine and the beginning of the learning process was a minimum of 14 hr.

RESULTS

Experiment 1. The experience of having been restrained in the dispenser did not change the reactions of the naive females except in two groups in which the latency to the first contact was exceptionally long (more than 10 hr, i.e.,

during the first day of the experiment), whereas in control groups, the maximum was only 133 min (Figure 1). However, the difference in the average latencies to the first use of the food dispenser between experimental and control groups was not statistically significant. When comparing the ranges of variations in the latencies, they were significantly larger for experimental than for control groups (Snedecor test; $P < 0.01$).

In two experimental groups out of eight, the experience of one gilt having been restrained in the dispenser induced a long-lasting avoidance by the other animals, whereas in the other six, no difference appeared when compared with control groups. Such a discrepancy might be explained by the different degree of reaction of the experimentally restrained animal to this situation. In the two experimental groups where the contact latency was considerably increased, the reaction of the experimental female had been the strongest during restraining. The number of sound emissions was higher than in the other six experimentally restrained gilts ($P < 0.05$), and urination had taken place in both cases (Figure 2). The long delay in approaching the food dispenser shows that, in these cases, the group witnessed the stress of the restrained gilt. However, no obvious behavior of the stressed animal can account for the avoidance of the food dispenser by the group. One possible hypothesis would be the emission of some kind of alarm pheromone. In stressed rats, alarm pheromones have been observed to originate from body odors and urine, but not feces (Mackay-Sim and Laing, 1981a,b). As the occurrence of urination during stress was associated with avoidance by our group of animals, the hypothesis of the transmission of the aversive experience by means of a pheromone present in urine of the stressed female was tested (experiment 2).

Experiment 2. The latencies to the use of the food dispenser by the first gilt are significantly longer in experimental groups compared with controls (315.7

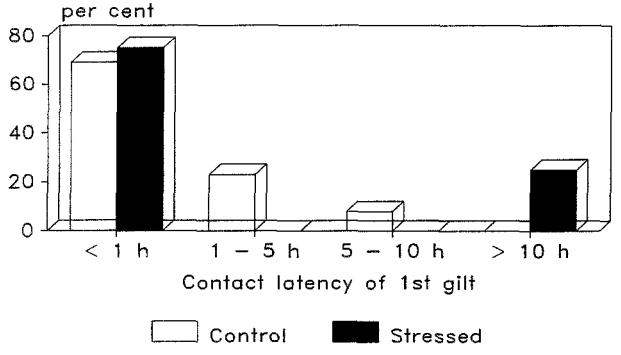

FIG. 1. Effect of the presence of a previously restrained animal (black) or control (no restraining) (open) on the contact latency of the other gilts with the food dispenser.

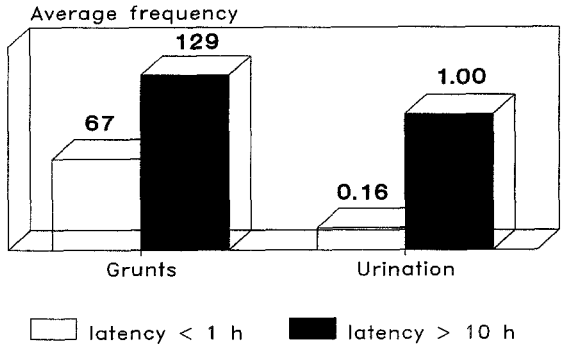

FIG. 2. Average numbers of grunts and urinations by restrained animal (ordinate) vs subsequent late (black) and short (open) contact latencies of the other gilts with the food dispenser.

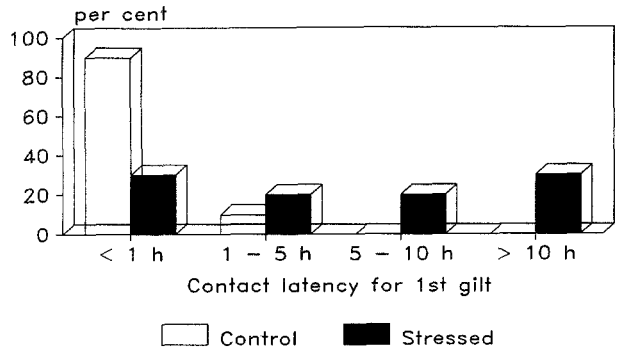

FIG. 3. Effect of spraying urine from a stressed (black) or control (open) animal on the contact latency for a group of gilts with the food dispenser.

±80.3 vs. 39.8 ±17.5 min) (Figure 3). As in experiment 1, the range of variation is extremely large among the experimental groups: In three cases no gilt had used the food dispenser during the first 10 hr, and only three used it during the first 3 hr. In the control groups the average latency was 39.8 min and the maximum 187 min. Finally, only 18% of the gilts in the experimental groups spontaneously learned to use the food dispenser during the first day, compared with 50% in controls.

The results demonstrate the avoidance of the place where the urine from a stressed sow has been sprayed, whereas the urine of a quiet female induced no delay in approaching the feeding device. In five groups, no animal approached the food dispenser during the first 6 hr, and for three of them this avoidance was still observed after 10 hr, although the animals had received no food since

the previous morning. The persistence of such an effect is remarkable as the urine had been sprayed more than 14 hr before the beginning of the observations. However, in half of the experimental groups, no avoidance was observed. In such cases no alarm pheromone appears to be present in urine. As different sows had been stressed in order to obtain urine for each experimental group, individual differences in reaction to restraining could account for such differences.

DISCUSSION

Olfactory communication is especially developed in pigs. Pheromones are involved in the organization of the sequence of sexual behavior in attracting estrous females and facilitating the display of the posture of receptivity (Signoret, 1970) and have been identified as androstenone (Melrose et al., 1971). A primer effect accelerating the occurrence of puberty has been described involving the interaction of pheromones and other sensory stimulations (Hughes et al., 1990). During aggressive encounters, urine and plasma from actively fighting pigs both efficiently reduce aggression in a group of test pigs (McGlone et al., 1987). This suggests that aggression-inhibiting pheromones could be produced under specific conditions of interspecific fighting. The results obtained in the present experimental series demonstrate the presence of an avoidance-inducing factor produced in the urine of the female domestic pig undergoing a stressful situation. It would logically result from metabolism of hormones secreted during acute stress. The identification of the chemical compounds involved and the conditions of their production would be of both theoretical and practical importance.

The relatively long persistence of this action allows an efficient spatiotemporal communication signaling those places that could be potentially dangerous. The functional significance of such a system of communication is evident in wild animals. Its persistence in a domestic species is interesting, and the consequences are of practical importance. The modern conditions of intensive animal production—restriction of movements and tethering—could result in stress reactions in at least some individuals, thus producing alarm pheromones. As large groups of animals are kept indoors, the occurrence of a stress situation in some of them would be sufficient to create a pheromonal environment of stress, which could influence the behavioral and physiological reactions of the whole group. It has been shown in mice (Cocke and Thiessen, 1986) that chronic exposure to such alarm pheromones could result in a decrease of immune defenses. The consequences of some kind of mismanagement could thus impair not only the welfare of some individuals, but influence the behavioral reactivity and the health of the whole herd.

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EFFECTIVENESS OF CARDENOLIDES AS FEEDING DETERRENTS TO *Peromyscus* MICE

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Abstract—I compared the feeding responses of five species of *Peromyscus* mice (*aztecus*, *polionotus*, *melanotis*, *leucopus*, and *maniculatus*) to three bitter-tasting cardenolides (ouabain, digoxin, and digitoxin) that differ greatly in lipophilic character. *Peromyscus*, like other muroid rodents, are unusual in that they can ingest relatively large amounts of cardenolides without adverse physiologic effects. In experiment 1, I determined avoidance thresholds for the three cardenolides with 48 hr, two-choice tests. Mice exhibited large interspecific differences in avoidance threshold, and the interspecific ranking of the thresholds (*maniculatus* = *leucopus* > *melanotis* > *polionotus* > *aztecus*) was the same for each of the cardenolides. In experiment 2, I re-evaluated the avoidance thresholds, but this time monitored the pattern of intake (i.e., bout lengths) during initial feeding encounters with cardenolide-laced diets. For each cardenolide, mice were subjected to three tests. In test 1, they received a control diet; in test 2, a diet containing the cardenolide at a concentration 1 $\log_e$ unit below the avoidance threshold (as determined in experiment 1); and in test 3, a diet containing the cardenolide at the avoidance threshold concentration. Results were similar across all species and cardenolide types: Bout lengths in tests 1 and 2 were statistically equal, whereas those in test 3 were significantly shorter than those in test 1. The rapid rejection of cardenolide-laced diets in test 3 is consistent with a preingestive (i.e., gustatory) mechanism underlying the avoidance thresholds. I conclude (1) that *Peromyscus* species differ substantially in taste sensitivity to cardenolides and that these differences may influence each species' respective ability to eat cardenolide-laced insects; and (2) that a species' relative taste sensitivity to one cardenolide predicts its sensitivity to other cardenolides.

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Key Words—Primary taste aversions, deterrency, taste thresholds, bitterness, lipophilic character, cardenolides, ouabain, digoxin, digitoxin, preference tests, feeding bouts, mice, *Peromyscus*, Muridae.

INTRODUCTION

As with many chemical defenses systems, the cardenolide defense of the monarch butterfly (*Danaus plexippus*) is effective against some but not all predators. For example, only two of the 25 insectivorous bird species that occur at the Mexican overwintering sites feed extensively on monarchs (Fink and Brower, 1981; Fink et al., 1983; Brower and Calvert, 1985; Arellano-G. et al., 1992). Similarly, among the potential mouse predators, only one of the five species (*Peromsycus melanotis*) eats large numbers of monarchs (Glendinning and Brower, 1990). Feeding studies show that at least two mouse species (*P. aztecus* and *Reithrodontomys sumichrasti*) are repelled by the unpleasant taste of the cardenolides (Glendinning, 1990, Glendinning et al., 1990). These species, like other muroid rodents, are unusually insensitive to the toxic effects of ingested cardenolides (Table 1). Thus, gustatory rather than toxicological sensitivity appears to determine how effectively these mice can exploit foods laden with cardenolides.

Based on the unexpected species differences in cardenolide responsiveness, I now describe experiments that further examine the gustatory response of *Peromyscus* mice to cardenolides. In particular, I address three questions: (1) how much interspecific variation in cardenolide taste sensitivity exists within the genus *Peromyscus*; (2) are the interspecific differences ecologically relevant; and (3) does a species' relative taste sensitivity to one cardenolide predict its sensitivity to cardenolides that differ greatly in lipophilic character?

METHODS AND MATERIALS

Experiment 1: Two-Choice Preference Tests

Subjects. *Peromyscus aztecus*, *P. melanotis*, and *P. polionotus* were obtained from outbred laboratory colonies at Florida State University. *Peromyscus maniculatus* and *P. leucopus* came from outbred colonies at the *Peromyscus* Stock Center, University of South Carolina (see Table 2 for sample sizes). (I will henceforth refer to mice in terms of their species designation only.) All mice were (1) reared in captivity on standard laboratory chow, (2) not used in previous experiments, and (3) adult males, owing to a shortage of females. Prior to testing, mice were housed individually in clear plastic cages (25 × 46 × 21 cm) with pine-shaving bedding, and maintained on pelleted

TABLE 1. TOXIC SENSITIVITY OF SEVERAL MAMMALS TO ORAL DOSAGES OF CARDIAC GLYCOSIDES

Species	Cardiac glycoside	Dosage (mg/kg)
Carnivora		
Felidae		
Felis sylvestris catus (domestic cat)	digoxin	0.2[a]
	digitoxin	0.2[a]
Rodentia		
Caviidae		
Cavia porcellus (domestic guinea pig)	digoxin	3.5[a]
	digitoxin	3.7[a]
Muridae		
Mus domesticus (domestic mouse)	digoxin	17.8[a]
	digitoxin	5.0–50.0[a]
Rattus norvegicus (Norway rat)	digitoxin	56.0[a]
Peromyscus maniculatus (deer mouse)	calotropin	>50.0[a]
Reithrodontomys sumichrasti (Sumichrast's harvest mouse)	digitoxin	>18.1[b]
P. aztecus (aztec mouse)	digitoxin	>72.2[b]
P. melanotis (black-eared mouse)	digitoxin	>107.0[b]

[a] Acute LD_{50} data from Tatken and Lewis (1983) and Marty (1983).
[b] Chronic LD_{50} data from Glendinning et al. (1990), where juvenile mice were maintained on a diet laced with digitoxin. The values indicate mean daily ingestion rates over 26 consecutive days. Despite the prolonged and high intake of digitoxin, no mice died or displayed signs of CG toxicity. In fact, the *P. melanotis* and *P. aztecus* grew slightly more rapidly on the digitoxin-laced diet than on a control diet.

chow (Purina lab chow pellets #5001) and tap water ad libitum on a 15:9 (light–dark) cycle.

Cardenolides and Artificial Diets. Three cardenolides of different lipophilic character were added to diets; ouabain was the least lipophilic and digitoxin the most (Figure 1). The diets were prepared by dissolving digitoxin, digoxin, or ouabain (Sigma Chemical Co.) in 95% ethanol, thoroughly mixing the solution with powdered chow (Purina lab chow meal #5001), and evaporating the ethanol for 14 hr at 60°C in a forced-draft oven (see Glendinning et al., 1990, for details). The control diet was prepared identically, except that no cardenolide was dissolved in the ethanol.

Test Conditions and Protocol. Mice were tested individually under the same housing and caging conditions described above, except that no pelleted chow was available. Two glass food cups (5 cm tall, 5.5 cm diameter, with a 3.0-cm-diameter hole in the plastic lid) were placed on the bottom of each cage, separated by about 7 cm; a stainless-steel water spout was positioned directly

TABLE 2. RANGE OF CARDIAC GLYCOSIDE CONCENTRATIONS USED IN TESTS

Peromyscus species	Mice tested (*N*)	Cardiac glycoside	Concentration[a]			
			mmol/kg diet		μg/0.1 g diet	
			Lowest	Highest	Lowest	Highest
aztecus	11	digitoxin	0.005	0.1	0.37	7.4
		digoxin	0.01	0.3	1.0	20.5
		ouabain	0.1	1.9	7.0	141
polionotus	12	digitoxin	0.1	1.9	7.4	148
		digoxin	0.1	1.9	7.6	152
		ouabain	0.3	5.3	19	383
melanotis	10	digitoxin	0.3	5.3	20	403
		digoxin	0.3	5.3	21	412
		ouabain	0.3	5.3	19	383
leucopus	10	digitoxin	0.3	5.3	20	403
		digoxin	0.3	5.3	21	412
		ouabain	0.7	14.3	52	1041
maniculatus	10	digitoxin	0.3	5.3	20	403
		digoxin	0.3	5.3	21	412
		ouabain	0.7	14.3	52	1041

[a] Two types of concentration units are provided: one based on the molecular density (mmol/kg diet) and the other on the mass (μg/0.1 g diet) of each CG. The latter concentration units help relate the findings of this study to those of other studies. Each mouse received an ascending series of four concentrations of each CG; the concentrations differed by whole, natural log increments. For example, *aztecus* received the following four concentrations (in mmol/kg diet) of digitoxin: 0.0048, 0.013, 0.036 and 0.097 (these values were rounded off for clarity).

between the food cups. One cup was designated the tastant and the other the control cup. Petri dishes were placed underneath each cup to recover spilled diet.

Each mouse was tested with all cardenolides. For each cardenolide, mice were subjected to five 48-hr, two-choice tests. In the tests, the control cup always contained control diet; the tastant cup contained control diet in test 1 and an ascending series of four concentrations of the cardenolide in tests 2–5. The four cardenolide concentrations spanned whole, $\log_e$ intervals (Table 2) and were selected (based on preliminary tests) so as to range from behavioral indifference to rejection. Owing to species differences in taste sensitivity, the concentrations of each cardenolide differed among species.

The order of testing the cardenolides was identical for all mice: ouabain, digoxin, and then digitoxin. Toxicity (i.e., oral, intraperitoneal, and intravenous LD_{50} values; Tatken and Lewis, 1983) and intestinal absorption (Lauterbach, 1981) data from laboratory rats and mice indicate that ouabain has the lowest

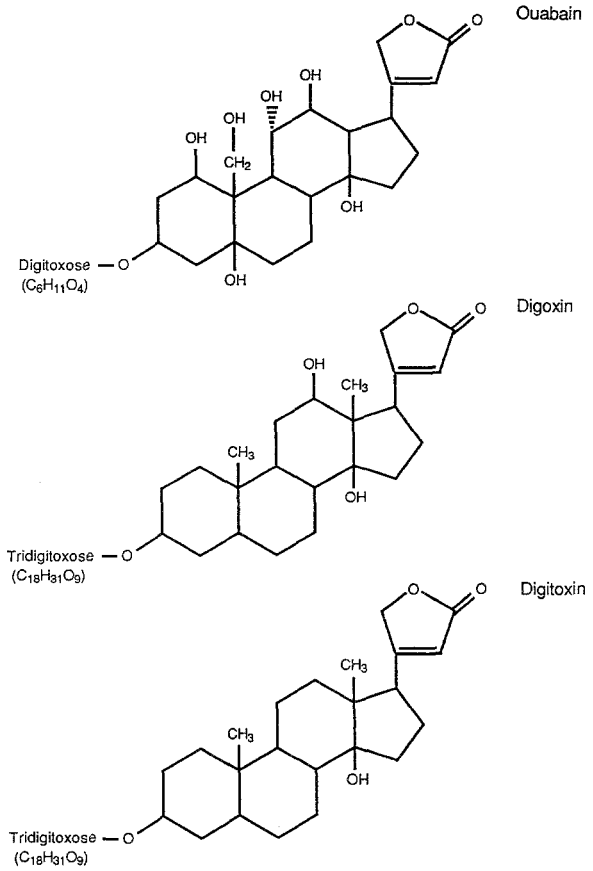

FIG. 1. Structure of the three cardenolides. Lipophilic character (log *P*), as indicated by octanol–water partition coefficient, of ouabain, digoxin, and digitoxin is −1.69, 1.25, and 2.80, respectively (Medicinal Chemistry Data bank, Pomona College). The difference in log *P* values can be attributed primarily to variable numbers of OH groups.

and digitoxin the highest potential toxicity in muroid rodents. Testing the cardenolides in an ascending order of toxicity minimized any potential order effects due to the formation of feeding aversions.

Each test lasted two days, and intake from both cups was determined gravimetrically after each day. At the onset of day 2, fresh cups and diet were provided, and the position of the cups was reversed to control for position preferences.

Data Analysis. For each test, the amount of diet consumed from the tastant cup was divided by the amount consumed from both cups to calculate the preference ratio. The taste avoidance threshold for each cardenolide was defined a

priori as the lowest cardenolide concentration at which the mean preference ratio was significantly below 0.5 [$P < 0.01$; one-sample (one-tail) t test].

To determine whether total food intake (i.e., control + tastant diet) changed across the 15 two-choice tests, I ran a two-factor, repeated measure ANOVA (Gagnon et al., 1991) with total food intake as the dependent measure and cardenolide concentration and cardenolide type as the main effects. This analysis was run separately for each species.

Even though muroid rodents are highly tolerant to ingested cardenolides (see Table 1), I nevertheless evaluated the mice for signs of sickness in two ways. First, I observed individual mice at the end of each 24-hr test period (in this and the next experiment) for signs of cardenolide toxicity such as cardiac failure, emesis, neurotoxicity, ataxia, dyspnea, severe tremors, and/or convulsions (Detweiler, 1967; Marty, 1983). Second, if negative postingestive feedback, rather than gustatory deterrence, was causing mice to limit intake of the cardenolide-laced diets, then I expected the nightly dosage of ingested cardenolide to remain constant across the range of test concentrations.

Experiment 2: Effect of Cardenolides on Feeding Bout Length

In laboratory rats, drinking bout length varies as a function of palatability. Thus, bout lengths are positively correlated with the concentration of sucrose (Smith, 1988) and negatively correlated with that of repellent tastants like quinine hydrochloride, sucrose octaacetate, caffeine, denatonium benzoate, and *n*-propyl-6-thiourea (Glendinning and Smith, 1990). In this experiment, I investigated whether diets containing threshold concentrations of ouabain, digoxin, or digitoxin (as determined in experiment 1) would shorten feeding bout length in the five *Peromyscus* species. To minimize involvement of postingestive (e.g., malaise) or experiential (e.g., gustatory habituation) effects, I considered only the initial feeding encounters with cardenolide-laced diets.

Subjects. Eight males of each of the five *Peromyscus* species were tested. The animals were caged and maintained as previously described.

Cardenolides and Artificial Diets. The same cardenolides and diets described previously were used here.

Test Conditions and Protocol. Each mouse was housed individually in a suspended stainless-steel cage (10 × 24 × 13 cm) equipped with a stainless-steel sipper spout and two adjacent feeding stations (each containing one food cup) (see Gannon et al., 1992, for details). To access powdered diet from either feeding station, a mouse had to insert its head through a restricted opening into a food cup. In so doing, it interrupted an infrared (IR) light beam. Beam breaks at each feeding cup were monitored by electronic circuitry, digitized, and transferred to a computer, which stored them in sequential 6-sec time periods (or sampling bins) over a 24-hr period. When a mouse's head broke the IR beam

for any part of a second during a sampling bin, a count of 1 was recorded. This resulted in a maximum of six counts per sampling bin. Later, the feeding records were parsed into distinct bouts, separately for each feeding cup. A bout began when ≥ 3 beam breaks occurred within a sampling bin and ended when no beam breaks occurred during a subsequent sampling bin.

Prior to testing, mice were housed in their respective test cages for five days to ensure they had acclimated to the feeding and drinking stations. Only one *polionotus* did not acclimate, and it was removed from the experiment.

Each mouse was tested with all cardenolides. For each cardenolide, individual mice were subjected to three 48-hr, two-choice tests. In test 1, control diet was present in both food cups. In test 2, the control diet was presented in one cup, while a diet containing a cardenolide at the concentration 1 $\log_e$ increment below the taste avoidance threshold (i.e., sub-AT diet) was presented in the other. In test 3, the control diet was presented in one cup, while a diet containing a cardenolide at the avoidance threshold concentration (i.e., AT diet) was presented in the other. The sub-AT and AT diets differed among mouse species and cardenolide type and were selected based on results from experiment 1. The order in which the three cardenolides were tested and the protocols for running the two-choice tests and preparing the diets were as in experiment 1.

Data Analysis. To assess the effect of cardenolide concentration on feeding bout length, I decided a priori to include in the analysis only a fraction of the data collected in each 48-hr test. First, I used only those feeding records from the tastant cup. Mice were offered both control and tastant cups merely to make the test situation (i.e., two-choice test) analogous to that of experiment 1.

Second, I attempted to include in the analysis only those feeding records from day 1. This was not always possible, however, as some mice showed strong sidedness in preliminary tests. Thus, a mouse may have avoided the tastant cup when it was in the right feeding station solely because the mouse habitually fed from the left station. To address this problem, I used records from day 1 only when a mouse fed from the tastant cup for ≥ 5 min during day 1 (i.e., exhibited ≥ 300 feeding counts), or for < 5 min during each of the test days. When a mouse did not meet these conditions (i.e., fed from the tastant cup for < 5 min during day 1 and ≥ 5 min during day 2), I used records from day 2. To quantify how often this latter situation occurred, I calculated the median number of times day 2 records were used across the nine tests (i.e., three two-choice tests for each of the cardenolides), separately for each species.

Third, I considered only the initial 10 feeding bouts from a given test day. I reasoned that this number of bouts was large enough to provide an accurate sample of the initial feeding encounters, but small enough to prevent postingestive or experiential effects from confounding the results. Preliminary feeding experiments indicated that mice of all five species routinely exhibited > 83 feeding bouts per test day when control diet was in both food cups.

For the analysis, I ran a two-factor ANOVA (separately for each species), using the mean length of the initial 10 feeding bouts as the dependent measure and cardenolide concentration and cardenolide type as main effects. Because cardenolide type and the interaction were nonsignificant for all five species, I collapsed the data across cardenolide type and employed orthogonal contrast analyses (Gagnon et al., 1991) to evaluate feeding bout lengths for the three diet types.

RESULTS

Experiment 1: Two-Choice Preference Tests

Concentration–response (C-R) functions for the three cardenolides are provided in Figure 2, and the avoidance thresholds derived from these functions are presented in Figure 3. Large differences in avoidance thresholds for each cardenolide were apparent among the five species, differing in one instance by over two orders of magnitude. In addition, the interspecific ranking of avoidance thresholds was the same for all three cardenolides: *maniculatus* = *leucopus* > *melanotis* > *polionotus* > *aztecus*. Thus, species with a relatively low avoidance threshold to one cardenolide (e.g., ouabain) also had a low threshold to the other two.

There were no significant effects of cardenolide type or cardenolide concentration on total food intake (for each species, $P > 0.05$ for the main effects and interaction). Thus, total food intake by individual mice remained constant across the 15 two-choice tests (mean grams ingested $\pm$ SE per 48-hr test by *aztecus* = 9.7 $\pm$ 0.2, *melanotis* = 6.8 $\pm$ 0.1, *maniculatus* = 5.5 $\pm$ 0.1, *leucopus* = 5.4 $\pm$ 0.1, and *polionotus* = 4.6 $\pm$ 0.1). Given this finding, the only way to explain the C-R functions in Figure 2 is that mice ate proportionally less tastant diet as the cardenolide concentration was increased.

Two lines of evidence argue against the hypothesis that aversive postingestive feedback caused mice to limit intake of the cardenolide-laced diets. First, no mice displayed any overt signs of cardenolide toxicosis in this or the next experiment. Second, in all species, the nightly cardenolide dosage (milligrams per kilograms mouse mass) actually increased as a function of cardenolide concentration in the tastant diet (Figure 4). There were only two exceptions to this pattern: *aztecus* and *melanotis* ingested relatively constant dosages of digitoxin across the range of test concentrations. However, it is unlikely that these exceptions can be explained by a postingestive mechanism, given that the highest nightly dosage of digitoxin for *aztecus* and *melanotis* was only 0.53 and 39.4 mg/kg, respectively. Both species can ingest substantially larger dosages with impunity (see Table 1).

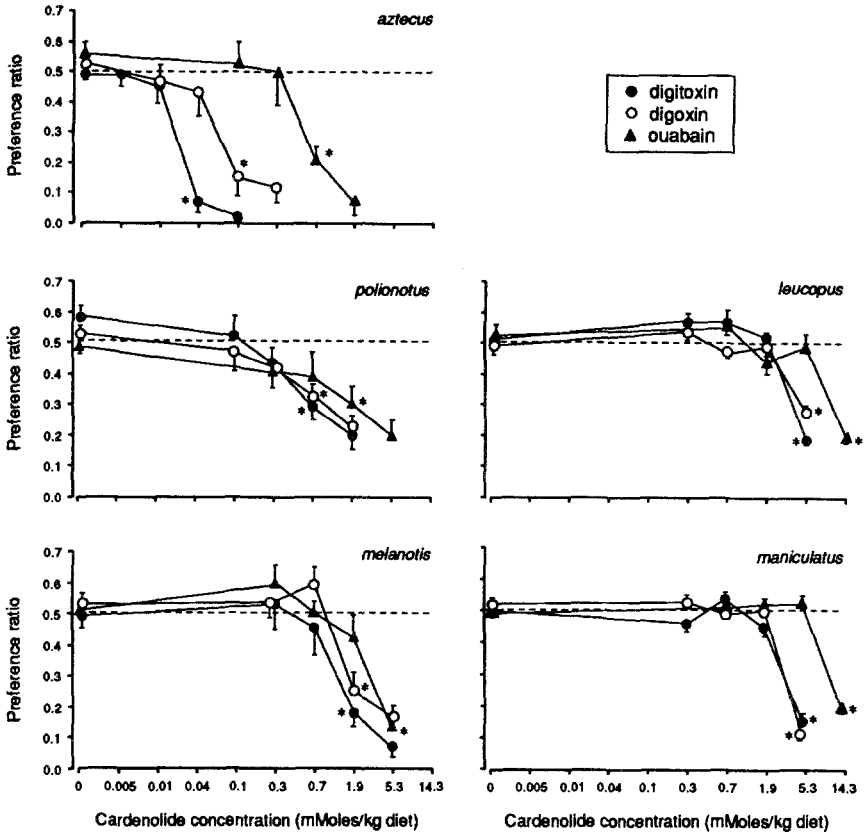

FIG. 2. Preference ratios (mean ± SE) across several concentrations of digitoxin, digoxin, and ouabain in five *Peromyscus* species. A preference ratio of 0.5 (indicated by the dotted line) represents no discrimination between the control or cardenolide-laced diet, whereas a ratio of 0.0 represents complete avoidance of the cardenolide-laced diet. Avoidance thresholds for each cardenolide are indicated with asterisks. The x axis tick marks span natural log increments.

Experiment 2: Effect of Cardenolides on Feeding Bout Length

There were significant cardenolide concentration effects for all species ($F_{2,28} \geq 27.15$; $P = 0.0001$). Otherwise, there were no significant effects of cardenolide type ($F_{2,28} \leq 2.41$; $P \geq 0.05$) or the interaction ($F_{4,28} \leq 1.82$; $P \geq 0.05$) in either species (Figure 5). Because the effect of cardenolide type on feeding bout duration was not significant, I collapsed the data across cardenolide type for contrast analyses. These analyses showed that, for all species, feeding bout length on the control diet was statistically identical to that on the sub-AT

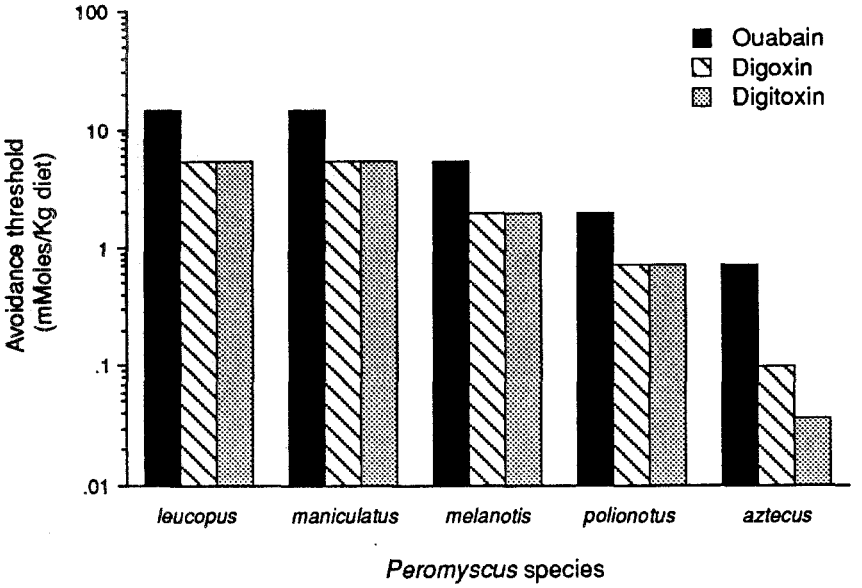

FIG. 3. Avoidance thresholds to ouabain, digoxin, and digitoxin in the five species of *Peromyscus*, as indicated by two-choice tests. The data from which the thresholds were derived are presented in Figure 2.

diet ($F_1 \leq 1.73$; $P \geq 0.05$), but significantly longer than that on the AT diet ($F_1 \geq 51.44$; $P = 0.0001$). For all species, the mean bout length on the control diet (after collapsing across cardenolide type) ranged between 31 and 41 sec; that on the AT diet ranged between 15 and 22 sec (see Figure 5). Thus, all five species rapidly detected and rejected diets containing cardenolide at the AT but not the sub-AT concentration.

Because the vast majority of mice fed from the tastant cup for ≥ 5 min during day 1, I rarely had to use day 2 records in the bout analyses. The median number of times day 2 records were used was 0 for *aztecus* and *polionotis* (range = 0–3 across the nine tests) and 1 for *melanotis*, *leucopus* and *maniculatus* (range = 0–3).

DISCUSSION

Two major findings are apparent. There are large interspecific differences in taste sensitivity to cardenolides within the genus *Peromyscus*, and taste sensitivity to one cardenolide predicts sensitivity to other cardenolides.

I conclude that a preingestive (i.e., gustatory) rather than postingestive

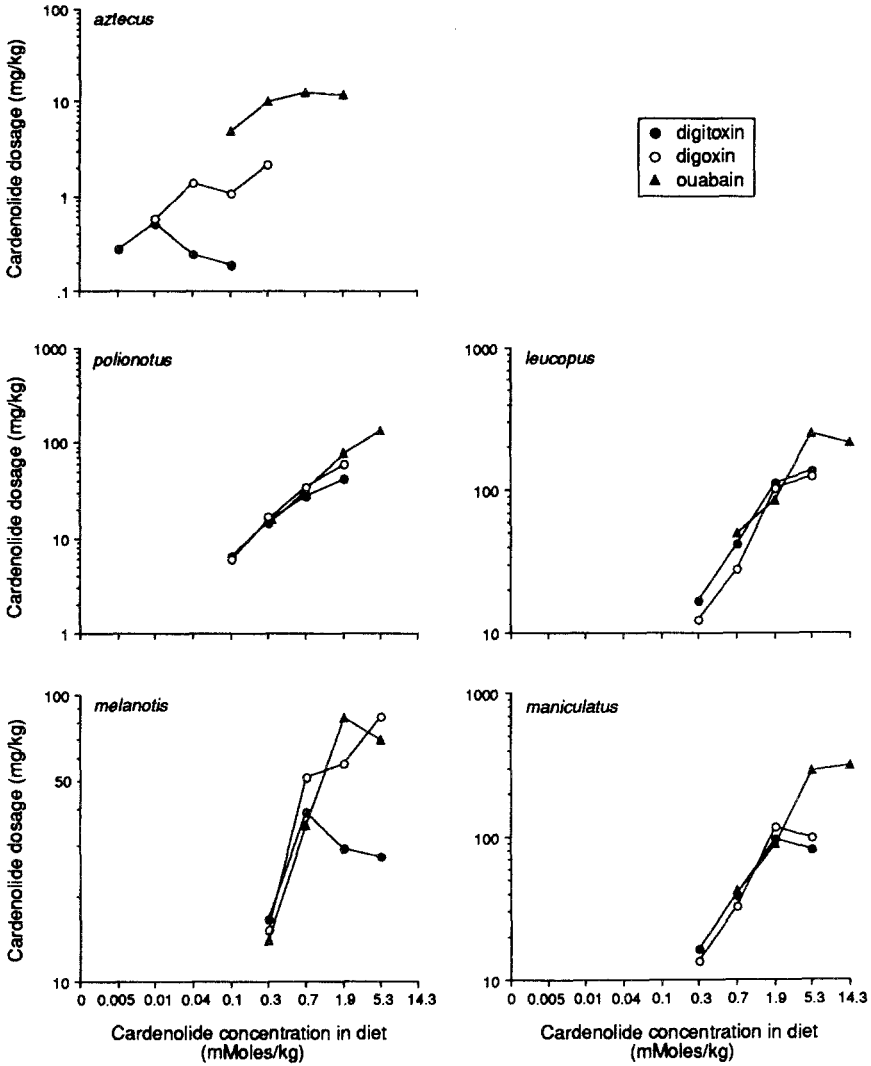

FIG. 4. Mean nightly dosage of digitoxin, digoxin, and ouabain ingested by the five *Peromyscus* species in experiment 1. In all species, nightly dosage increased as a function of cardenolide concentration in the tastant diet. Note that different scales were used on the *y* axis of the graphs.

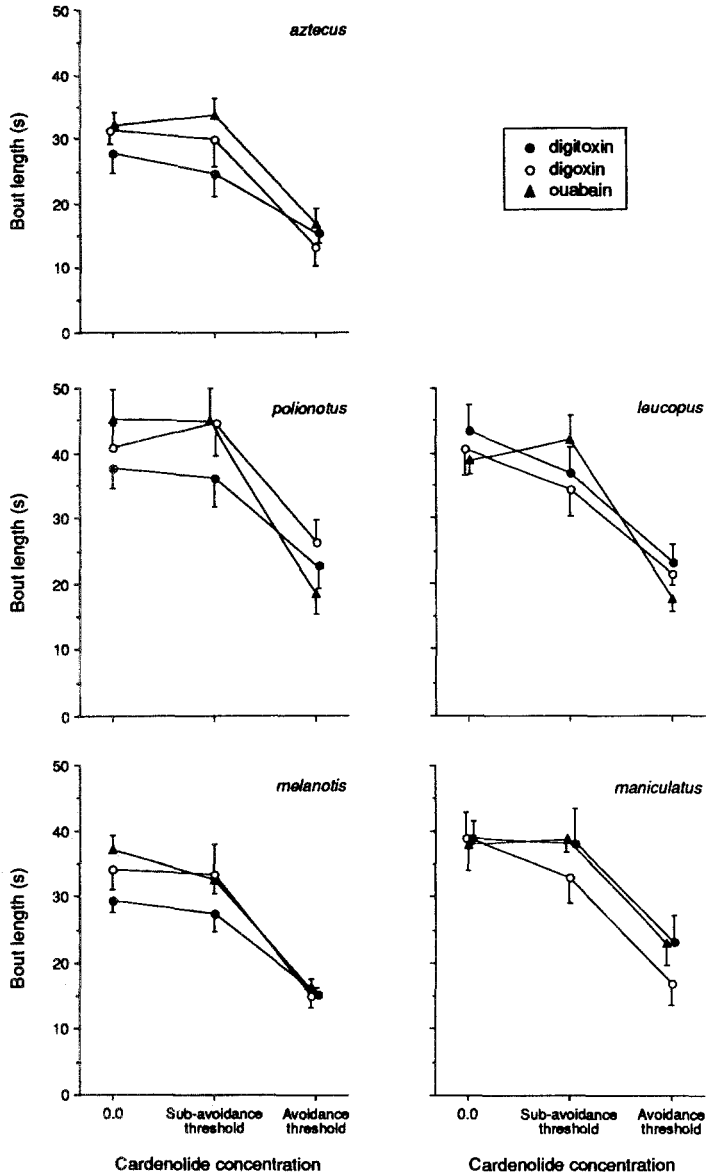

FIG. 5. Feeding bout lengths (mean ± SE) across several concentrations of digitoxin, digoxin, and ouabain in five *Peromyscus* species. Each cardenolide was presented at three concentrations: 0.0 mmol/kg diet, the subavoidance threshold (i.e., the concentration one $\log_e$ unit below the avoidance threshold), and the avoidance threshold. The actual subavoidance and avoidance threshold concentrations differed among the cardenolides and mouse species and were based on the results in Figures 2 and 3.

(i.e., toxicosis) mechanism underlies the avoidance thresholds in all species for several reasons. First, the AT diets (i.e., diets containing either of the cardenolides at the avoidance threshold concentration) caused an immediate decrease in feeding bout length. Second, I observed no evidence of aversive postingestive feedback from the cardenolide-laced diets. Third, cardenolides in general are absorbed extremely poorly across the intestinal wall of muroid rodents, including *maniculatus* (Lauterbach, 1981; Marty, 1983). Indeed, comparative studies of oral and intraperitoneal LD_{50} values within muriod rodent species show that oral values are invariably much higher (Tatken and Lewis, 1983; Marty, 1983).

Ecological Relevance of Avoidance Thresholds

To determine how the cardenolide concentration in the AT diets compared to the natural cardenolide concentration in potential insect prey, I first determined the median avoidance threshold for the three cardenolides (separately for each species). Then, I overlaid each species' median threshold value on: (1) the frequency distribution of cardenolide concentrations in Mexican overwintering monarchs, and (2) the range of cardenolide concentrations in milkweed bugs, *Oncopeltus fasciatus* (Figure 6). This comparison seemed valid given that the range of lipophilicity spanned by ouabain, digoxin, and digitoxin is similar to that spanned by cardenolides in monarchs and milkweed bugs (Moore and Scudder, 1985; Seiber et al., 1986; Malcolm et al., 1989).

Assuming results from artificial diets are relevant to insects, then it appears that more than 68% of the monarchs contain suprathreshold concentrations of cardenolides for *aztecus*, whereas only 29, 4, 0, and 0% do so for *polionotus*, *melanotis*, *leucopus*, and *maniculatus*, respectively. Extrapolation of these results to free-ranging mice supports earlier findings (Glendinning, 1990; Glendinning et al., 1990) that *aztecus* avoids eating monarchs, and *melanotis* eats them, because of their differential taste sensitivities to cardenolides. Even though *polionotus*, *leucopus*, and *maniculatus* do not naturally encounter overwintering monarchs, the results indicate that (like *melanotis*) they are preadapted, to varying degrees, for eating them.

A different story emerges with milkweed bugs: Nearly all of them contain cardenolide concentrations above the median avoidance thresholds for all five *Peromyscus* species. Thus, in contrast to the cardenolide defense of monarchs, that of milkweed bugs may effectively deter predation by all *Peromyscus* species.

These findings illustrate two important points often neglected in studies of predator responses to deterrents. First, even congeneric predators respond quite differently to deterrents. Consequently, one should use natural predators, rather than convenient or abundant predators that do not naturally encounter the focal prey species, because the latter may provide misleading results. Second, avoidance thresholds of predators to deterrents attain greater ecological significance

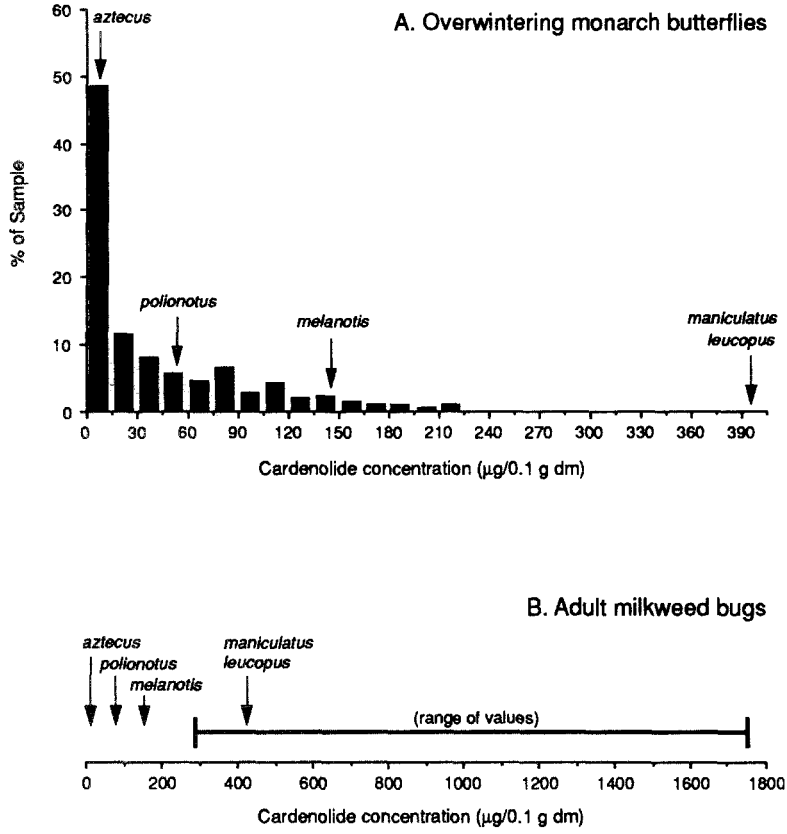

FIG. 6. (A) Frequency distribution of the whole body concentration of cardenolides in overwintering monarch butterflies on Sierra Chincua, a primary overwintering site in Mexico (data from Malcolm and Brower, 1989). (B) Concentration range of cardenolides in adult milkweed bugs, *Oncopeltus fasciatus*. For the milkweed bugs, only fresh mass concentrations of cardenolides were available (Moore and Scudder, 1985). Nevertheless, because adult milkweed bugs are on average 45% dry mass (Slansky, 1980), I was able to estimate the dry mass concentrations of cardenolides. The median avoidance thresholds for each of the *Peromyscus* species (i.e., the median of the digitoxin, digoxin, and ouabain avoidance thresholds) are indicated on graphs A and B. Since monarchs and milkweed bugs both contain several types of cardenolides, the units are represented as µg/0.1 g rather than mmol/kg. Note that different scales were used on the x-axis of the two graphs.

when they are related to the natural concentration range of the chemicals in prey.

Taste Sensitivity to One Cardenolide Predicts Sensitivity to Others

In all five species, the relative taste sensitivity to one cardenolide reliably predicted sensitivity to the other two. In related studies, the relative taste sensitivity of *aztecus* and *melanotis* to digitoxin was the same as that to cardenolides in both wild-caught and laboratory-reared monarch butterflies (Glendinning, 1990; Glendinning et al., 1990). It thus appears that a *Peromyscus* species with a relatively low taste sensitivity to a lipophobic cardenolide (e.g., ouabain) will be similarly insensitive to a lipophilic one (e.g., digitoxin).

I am presently examining the possibility that taste sensitivity to cardenolides may also predict sensitivity to other classes of deterrents. Studies with SWR inbred mice (*Mus domesticus*) indicate that taste sensitivity to the deterrent, sucrose octaacetate, accurately predicts sensitivity to structurally related (e.g., other acetlyated sugars) and unrelated (e.g., strychnine and denatonium benzoate) deterrents (Whitney et al., 1990, 1991). Likewise, in human populations, low taste sensitivity to three deterrents (PTC, PROP, and caffeine) predicts taste sensitivity to a host of structurally unrelated ones (Fischer et al., 1965; Hall et al., 1975; Tanimura and Mattes, 1989).

Effect of Lipophilic Character on Avoidance Threshold

Elsewhere, several investigators report significant inverse correlations between lipophilic character and bitter taste thresholds of numerous drugs, amino acids, and peptides in humans (Asao et al., 1987; Gardner, 1978, 1979). These correlations are thought to reflect the relative ease with which lipophilic chemicals contact or pass through lipid barriers (e.g., the mucous/lipid interphase and membrane of taste cells) and access physiological sites of action (Koyama and Kurihara, 1972; Wolkowski et al., 1977). The results of the present study are consistent with the human studies: (1) the cardenolide with the lowest lipophilic character, ouabain, elicited the highest threshold in all five species; and (2) in *aztecus*, the avoidance thresholds for the three cardenolides were inversely related to lipophilic character. In contrast, a study with blue jays suggests a positive relationship between avoidance threshold and lipophilic character of three cardenolides (digoxin, digitoxin, and digitoxigenin; Brower and Fink, 1985). To resolve these contradictory results, the mice and birds must be tested with a wider array of cardenolides.

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(*S*)-(+)-IPSDIENOL: INTERSPECIFIC INHIBITION OF *Ips latidens* (LECONTE) BY *Ips pini* (SAY) (COLEOPTERA: SCOLYTIDAE)

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Abstract—In south-central British Columbia, the attraction of *Ips latidens* (LeConte) to its pheromone, ipsenol, was inhibited by (*S*)-(+)-ipsdienol, a pheromone for *I. pini* (Say). (*R*)-(−)-Ipsdienol had no effect on *I. latidens*. (*S*)-(+)-Ipsdienol probably plays a role in interspecific communication between the two species, facilitating reductions in interspecific competition for breeding material and/or interspecific mating interference.

Key Words—Coleoptera, Scolytidae, *Ips latidens*, *Ips pini*, interspecific communication, synomone, pheromone, ipsenol, ipsdienol, chirality, enantiospecificity.

INTRODUCTION

Ecological and reproductive isolation among bark beetles can be facilitated by the use of pheromones (Wood, 1970; Lanier and Burkholder, 1974; West-Eberhard, 1984; Kohnle et al., 1986, 1988; Miller, 1991). Beetles are generally attracted to sources of pheromones produced by conspecifics while avoiding sources of pheromones produced by individuals of other species (Wood, 1970; Birch, 1978, 1984; Borden, 1982). In western North America for example, interspecific inhibition of responses to pheromones occurs between various sympatric species such as *Ips paraconfusus* Lanier and *I. pini* (Say) (Birch and Wood, 1975; Birch and Light 1977; Birch et al., 1977, 1980; Birch, 1978;

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Light and Birch, 1977) and *I. paraconfusus* and *Dendroctonus brevicomis* LeConte (Byers and Wood, 1980, 1981).

Ips pini and *I. latidens* (LeConte) are broadly sympatric in western North America and breed in large single-species aggregations, primarily in the phloem tissue of lodgepole pine (Bright, 1976; Furniss and Carolin, 1980; Wood, 1982). Both species use pheromones. In south-central British Columbia, *I. pini* produce ipsdienol with a mean chiral ratio of 66:34 (*S*)-(+):(*R*)-(−) (Miller et al., 1989). Both sexes are attracted to sources of ipsdienol with chiralities ranging from 30:70 to 80:20 (*S*)-(+):(*R*)-(−) (Miller, 1991). In the same region, male *I. latidens* produce ipsenol and both sexes respond to ipsenol regardless of chirality, although there is a slight preference for the (*S*)-(−) enantiomer (Miller et al., 1991).

Ipsenol inhibits the attraction of *I. pini* to infested material in Idaho (Furniss and Livingston, 1979) and California (Birch and Light, 1977). In south-central British Columbia, ipsenol inhibits the attraction of *I. pini* to ipsdienol-baited funnel traps (Borden et al., 1992). However, the effect of ipsdienol, produced by *I. pini*, on *I. latidens* has not been determined. We hypothesized that one or both enantiomers of ipsdienol inhibit the attraction of *I. latidens* to sources of ipsenol.

METHODS AND MATERIALS

Racemic ipsenol and racemic ipsdienol (chemical purities, 98% and > 95%, respectively) were obtained from Bedoukian Research Inc. (Danbury, Connecticut) and Borregaard, A.S. (Sarpsborg, Norway), respectively. E.K. Czyzewska (Department of Chemistry, Simon Fraser University, Burnaby, British Columbia) supplied chiral ipsdienols (optical purities, 98% (*S*)-(+) and 98% (*R*)-(−), respectively, chemical purities, > 98%).

Each lure consisted of 10 Microcap disposable pipettes (2 μl) (Drummond Scientific Co., Broomall, Pennsylvania), each sealed at one end and filled with either racemic ipsenol, racemic ipsdienol, or one of the chiral ipsdienols, and placed in a polyethylene, microcentrifuge tube (1.8 ml) (Evergreen Scientific, Los Angeles, California). The release rates of ipsenol and ipsdienol from such lures were both approximately 100 μg/day at 24°C (determined by weight loss).

In all experiments, replicates of four eight-unit, multiple-funnel traps (Lindgren, 1983) (Phero Tech Inc., Delta, British Columbia) were set in grids of 2 × 2 in stands of lodgepole pine near Princeton, British Columbia. Replicate grids were placed at least 100 m apart, and traps were spaced 10–15 m apart within each replicate. Each trap was suspended such that the top of each trap was approximately 1.3–1.5 m above ground level. No trap was within 2 m of

any tree. For each experiment, insects were collected only at the termination of the experiment.

Experiment 1 tested the effect of various chiralities of ipsdienol on the attraction of *I. latidens* to ipsenol. Ten replicates were set from June 26 to August 22, 1985. The treatments were as follows: (1) racemic ipsenol alone; (2) ipsenol and (*S*)-(+)-ipsdienol; (3) ipsenol and (*R*)-(−)-ipsdienol; and (4) ipsenol and racemic ipsdienol. Lures were replaced on July 25, 1985.

Experiment 2 tested the effect of (*S*)-(+)-ipsdienol on the attraction of *I. latidens*. Nine replicates were set from June 4 to 18, 1985. The treatments were as follows: (1) blank control; (2) racemic ipsenol alone; (3) (*S*)-(+)-ipsdienol alone; and (4) ipsenol and (*S*)-(+)-ipsdienol.

The data were analyzed using the SAS statistical package version 5.0 (SAS Institute Inc., Cary, North Carolina). Trap catch data, transformed by ln (Y + 1) to remove heteroscedasticity, were subjected to either one- or two-way analyses of variance (ANOVA). Ryan-Einot-Gabriel-Welsch (REGW) multiple-range tests were used when $P < 0.05$.

RESULTS AND DISCUSSION

(*S*)-(+)-Ipsdienol significantly inhibited the attraction of *Ips latidens* to sources of ipsenol. In experiment 1, catches of *I. latidens* to traps baited with either racemic or (*S*)-(+)-ipsdienol were approximately 20-fold lower than those to traps baited with ipsenol alone (Figure 1). Catches to traps baited with ipsenol and (*R*)-(−)-ipsdienol were not significantly different from those to ipsenol alone. In experiment 2, in which significant levels of (*R*)-(−)-ipsdienol were not present, (*S*)-(+)-ipsdienol again significantly reduced the capture of *I. latidens* (Figure 2).

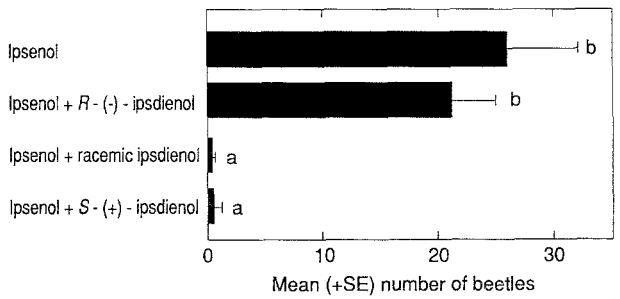

FIG. 1. The effect of chiral ipsdienol on the capture of *Ips latidens* in ipsenol-baited, multiple-funnel traps near Princeton, British Columbia, from June 26 to August 22, 1985 (N = 10). Means followed by the same letter are not significantly different at P = 0.05 [REGW multiple range test on data transformed by ln (Y + 1)].

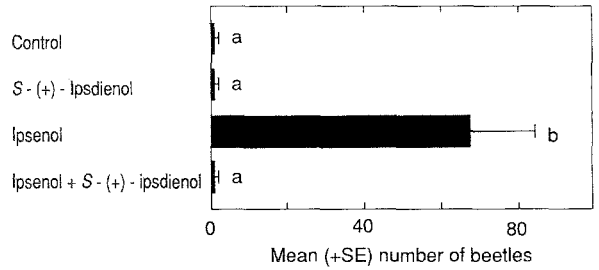

FIG. 2. The effect of the interaction between (*S*)-(+)-ipsdienol and racemic ipsenol on the capture of *Ips latidens* in multiple-funnel traps near Princeton, British Columbia, from June 4 to 18, 1985 ($N = 9$). Means followed by the same letter are not significantly different at $P = 0.05$ [REGW multiple range test on data transformed by ln $(Y + 1)$].

In south-central British Columbia, male *I. pini* produce substantial amounts of (*S*)-(+)-ipsdienol in their pheromone blend (Miller et al., 1989). Both *I. pini* and *I. latidens* breed in single-species aggregations in the phloem tissue of lodgepole pine, often in association with the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Bright, 1976; Furniss and Carolin, 1980; Wood, 1982). Both species seem to show ecological separation in their choice of breeding material; *I. latidens* seem to prefer drier phloem material than *I. pini* (Miller and Borden, 1985). It is probable, therefore, that (*S*)-(+)-ipsdienol plays a role in interspecific communication between *I. latidens* and *I. pini*, facilitating reduced interspecific competition for breeding material and/or reduced interspecific mating interference.

In contrast, *I. pini* in western United States and southeastern British Columbia use primarily (*R*)-(−)-ipsdienol as a pheromone (Stewart, 1975; Plummer et al., 1976; Birch et al., 1980; Miller et al., 1989). In these regions, (*R*)-(−)-ipsdienol may have a stronger effect on *I. latidens* than it does in south-central British Columbia. The response to (*S*)-(+)-ipsdienol is probably still present since other competing species such as *I. paraconfusus* utilize (*S*)-(+)-ipsdienol as a pheromone. Mutual inhibition between *I. pini* and *I. paraconfusus*, in these regions, is based, in part, on the chirality of ipsdienol (Light and Birch, 1977; Birch et al., 1980).

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OLFACTORY RECOGNITION OF NONHOSTS ASPEN AND BIRCH BY CONIFER BARK BEETLES *Tomicus piniperda* AND *Hylurgops palliatus*

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Abstract—The field response of *Tomicus piniperda* (L.) and *Hylurgops palliatus* (Gyll.) (Coleoptera: Scolytidae) to the attractant ethanol in combination with volatile wood constituents released from the nonhost tree species *Populus tremula* L. (Salicaceae) and *Betula pendula* Roth (Betulaceae) was studied using flight barrier traps. The attraction of both species decreased when aspen or birch wood was added to the ethanol bait. The same was true for *Rhizophagus depressus* (F.) (Coleoptera: Rhizophagidae), a predatory species associated with conifer bark beetles. *Glischrochilus quadripunctatus* (L.), *Epuraea bickhardti* St.-Claire Deville, *E. unicolor* (Oliv.) (Coleoptera: Nitidulidae), and *Rhizophagus parvulus* (Payk.) (Coleoptera: Rhizophagidae) were caught in higher numbers in traps baited with both ethanol and wood of aspen or birch than in traps baited with ethanol alone. In a separate experiment, landings of *T. piniperda* and *H. palliatus* on nonhosts (black plastic tubes) were demonstrated with sticky traps.

Key Words—*Tomicus piniperda*, *Hylurgops palliatus*, *Rhizophagus depressus*, *R. parvulus*, *Glischrochilus quadripunctatus*, *Epuraea bickhardti*, *E. unicolor*, Coleoptera, Scolytidae, Rhizophagidae, Nitidulidae, ethanol, nonhost volatiles, *Populus tremula*, *Betula pendula*, olfactory orientation, attraction, decreased attraction.

INTRODUCTION

Most scolytids, as well as many other bark- and woodboring insects, reproduce only in dead or weakened trees. This resource is generally scarce and tends to be distributed unevenly in space and time. Furthermore, host trees are often

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present in mixed stands with nonhost species. Thus, such insects need effective means for finding and recognizing suitable breeding material.

Many bark beetles are attracted by host volatiles, alone or in combination with pheromones (e.g., Borden, 1982). Host odors may be characteristic of individual tree species. The major components of the volatile essential oils in trees responsible for characteristic odors are the terpenoids (see Harborne and Turner, 1984, and references therein). Some bark beetles, including both conifer and broad-leaved breeding species, are attracted by mono- and sesquiterpenes present in their host trees (e.g., Byers et al., 1985; Millar et al., 1986; Schroeder and Lindelöw, 1989).

Volatiles may also provide information about tree vitality. High concentrations of monoterpenes in the air may indicate the presence of damaged trees (Ikeda et al., 1980; Strömvall and Pettersson, 1991). Degradation processes in dead or dying tree tissue result in the formation of substances that are absent or present in only small amounts in healthy trees. One such substance is ethanol, which is formed in dying tissue of both conifers (Moeck, 1970; Cade et al., 1970; Ikeda et al., 1980; Sjödin et al., 1989) and broad-leaved trees (Elliot et al., 1983). Ethanol attracts some scolytids reproducing in broad-leaved trees (Roling and Kearby, 1975; Montgomery and Wargo, 1983) as well as some scolytids breeding in coniferous trees (e.g., Moeck, 1970; Schroeder and Lindelöw, 1989).

Little is known about the mechanisms underlying bark beetle rejection of nonhost tree species. Rejection could be based on a lack of certain host characteristics and/or a negative response to some nonhost stimuli. Two ambrosia beetles reproducing in broad-leaved trees, *Trypodendron domesticum* (L.) and *Anisandrus dispar* (F.), respond negatively to α-pinene, one of the major monoterpene constituents of both Scots pine, *Pinus sylvestris* L., and Norway spruce, *Picea abies* (L.) Karst. Both beetles are strongly attracted by ethanol alone, while α-pinene-baited and unbaited traps catch a few or no individuals. When α-pinene is released along with ethanol, attraction to the latter compound is inhibited or decreases strongly (Nijholt and Schönherr, 1976; Schroeder and Lindelöw, 1989). Single cell responses to the odor from birch bark have been demonstrated in the conifer scolytids *Trypodendron lineatum* (Oliv.) and *Ips typographus* (L.) (Tømmerås, 1989; Tømmerås and Mustaparta, 1989). For some scolytids, laboratory studies demonstrate that the rejection of nonhost tree species can be based on gustatory cues. *Ips paraconfusus* Lanier selected a host over a nonhost only after the beetles had bored through the outer bark (Elkinton and Wood, 1980). In the presence of certain nonhost substances, *Scolytus multistriatus* (Marsh.) rejected host-tree twigs suitable for maturation feeding (Gilbert and Norris, 1968).

The present study was initiated to give information about the behavioral response of the two conifer bark beetles, *Tomicus piniperda* (L.) and *Hylurgops*

palliatus (Gyll.), to nonhost trees. Both species are common throughout Scandinavia. The flight periods of both species occur in early spring. In Scandinavia *T. piniperda* reproduces almost exclusively in Scots pine while *H. palliatus* reproduces in both Scots pine and Norway spruce, the two major conifers in this region. Both species are restricted to weakened or dead trees for their reproduction, *H. palliatus* preferring a somewhat more decayed breeding substrate than *T. piniperda*.

The olfactory orientation to breeding material has been studied in both beetles in recent years. *T. piniperda* lacks an aggregation pheromone (Perttunen et al., 1970; Byers et al., 1985; Lanne et al., 1987). The only study conducted with *H. palliatus* in this respect did not present any evidence of an aggregation pheromone (Kohnle, 1985). *T. piniperda* is strongly attracted by certain Scots pine monoterpenes and weakly attracted by ethanol, while the reverse is true for *H. palliatus* (Byers et al., 1985; Kohnle, 1985; Klimetzek et al., 1986; Schroeder, 1988; Schroeder and Lindelöw, 1989). Monoterpenes and ethanol can interact synergistically in attracting *H. palliatus* and *T. piniperda* (Vité et al., 1986; Schroeder and Lindelöw, 1989).

In an initial experiment in the present study, the landing rates of *T. piniperda* and *H. palliatus* on hosts and nonhosts were estimated. In subsequent field experiments, the responses of *T. piniperda* and *H. palliatus* to volatiles from wood of the nonhosts European white birch, *Betula pendula* Roth (Betulaceae), and aspen, *Populus tremula* L. (Salicaceae), were studied.

METHODS AND MATERIALS

All experiments were conducted in 30- to 45-year-old Scots pine stands in the provinces of Uppland and Gästrikland in central Sweden. The landing frequencies of scolytids on vertical, black plastic tubes; healthy Scots pines; top-cut pines; and upright-standing, newly cut logs of Scots pine were compared in 1981. The diameter of the experimental trees was 14–17 cm. The top-cut trees were 6 m long, the logs 3 m long, and the tubes 1.5 m long. The top-cutting, which removed all green branches, was conducted in February, and the logs were cut in March 1982. The tubes (diam. 12 cm) were attached 0.5 m above ground. The four treatments were arranged in 14 randomized blocks. The distance between the treatments in each block ranged from 4 to 15 m and between blocks from 20 to 50 m. Each experimental tree and tube was surrounded by a trap cylinder (0.5 m high) constructed out of Stickem-Special-coated, 6.5-mm mesh wire netting. The cylinders were attached 1.5 m above ground. The distance between the trap and the surface of the trees and tubes was about 2 cm. All scolytids caught on the traps from April 4 to June 17, 1982 were collected. After the experiment, the top-cut trees and the logs were debarked and inspected.

The number of scolytid egg galleries and the presence of emergence holes were recorded. The healthy trees were carefully examined for boring dust and pitch tubes without being felled and debarked.

The attraction of beetles to combinations of 95% ethanol (5% water) and wood of aspen or European white birch was compared with the attraction to ethanol alone in 1990 and 1991. The attractiveness of the two tree species was studied in separate experiments, three with aspen and four with birch (Table 1). The treatments in each experiment were set out in pairs. The distance between the two traps in each pair was 10–15 m, and the distance between pairs was 20 m. In experiments 1 and 2 (1990), and 5 and 6 (1991) (Table 1), the same arrangements were used, but the positions of the treatments in each trap pair were switched between experiments. In each day of baiting, healthy aspen or birch trees were felled and cut into bolts (10–20 cm long), which were split with an axe. The cut wood was put into containers and covered with fine netting to keep bark beetles out. Wood volume differed between experiments (Table 1).

TABLE 1. OVERVIEW OF EXPERIMENTS WITH ASPEN AND BIRCH

Year and exp.	Type of wood combined with ethanol bait[a]		Trapping period	Bait replacements during tests	Replicates (*N*)
	Tree species	Volume (liters)			
1990					
1	aspen	55	March 17–20	new ethanol each day	6
2	birch	55	March 31		8
3	aspen	55	April 6–27	April 11, new ethanol; April 21, new wood and ethanol	7
4	birch	45	April 11–27	April 22, new wood and ethanol	8
1991					
5	aspen	45	April 11–15		8
6	birch	45	April 25–May 13 May 17–June 19	(May 13–17, no trapping) May 17, new wood and ethanol; May 27, new wood and ethanol refilled; June 7, new wood and ethanol refilled	9
7	birch	8	April 29–May 13 May 17–June 19	(April 29–May 13, no trapping) May 17, new wood and ethanol refilled; May 27, new wood and ethanol refilled; June 7, new wood and ethanol refilled	11

[a] Ethanol release rates given in text (estimated in laboratory) and in Table 2 (estimated in the field).

Ethanol was included in the experiments since previous studies demonstrated that, generally, only low numbers of *T. piniperda* and *H. palliatus* are caught in unbaited flight barrier traps (Schroeder, 1988; Schroeder and Lindelöw, 1989). Hence, a repellent effect of volatiles from wood of broad-leaved trees would be difficult to detect using unbaited traps as a control. Ethanol attracts both beetles, and the substance may be present in dying broad-leaved trees as well as in dying conifers.

Beetles were caught in flight barrier traps modified from the type described by Chapman and Kinghorn (1955). The barriers consisted of 15-cm-high, transparent plastic sheets. Water-filled trap troughs were placed directly beneath the barriers, on top of the containers. Sex determinations were made by dissection for all species except *Epuraea bickhardti* St.-Claire Deville (Coleoptera, Nitidulidae), whose males can easily be recognized based on the appearance of the middle tibia.

The ethanol dispensers were placed in the containers just under the net lids. In experiments 1 and 2, 10-ml polyethylene vials (6 ml ethanol) with 9-mm-diam. openings were used. In all other experiments 200-ml glass jars (185 ml ethanol) with 45-mm-diam. openings were used. The release rates of ethanol from the dispensers in laboratory at 15°C were 3.0 ± 0.3 g/24 hr for the polyethylene vials and 9.1 ± 1.0 g/24 hr for the glass jars. In 1991, the field release rates during the trapping periods were estimated by measuring the amount of ethanol left in the dispensers at the end of each experiment, or before refilling during an experiment (Table 2). The mean air temperature varied between 5.0 and 8.4°C during the trapping periods in 1991.

TABLE 2. AMOUNTS OF ETHANOL RELEASED FROM DISPENSERS PLACED IN TRAPS BAITED WITH WOOD (E & W) AND IN TRAPS WITHOUT WOOD (E) IN 1991[a]

Expt.	Trapping period	Days (N)	Amount released per day ($\bar{X}$ ± SD, g)	
			E & W	E
5	April 11–15	4	7.3 ± 1.6	5.8 ± 0.7
6	April 25–May 13	18	3.5 ± 0.9	3.0 ± 0.4
	May 17–27	10	4.2 ± 0.7	4.0 ± 0.6
	May 27–June 7	11	3.2 ± 0.8	2.8 ± 0.4
7	April 29–May 13	14	2.0 ± 0.8	1.5 ± 0.7
	May 17–27	10	2.4 ± 1.4	1.4 ± 1.1
	May 27–June 7	11	1.4 ± 0.5	1.0 ± 0.4

[a]For number of replicates, see Table 1.

RESULTS

The scolytids *T. piniperda*, *H. palliatus*, *Hylastes brunneus* Er., *H. cunicularius* Er., and *T. lineatum*, were caught on the traps on the plastic tubes (Table 3). The numbers of beetles caught on the tubes did not differ significantly from the numbers caught on healthy and top-cut trees for any of the species. Much higher numbers of *T. piniperda* and *H. brunneus* were caught on the newly cut logs than in the other treatments, and the differences in catch were statistically significant for both species. At the end of the experiment, the only scolytid breeding attacks found were caused by *T. piniperda* on logs. Twelve of the 14 logs were attacked ($\bar{X}$ = 26 egg galleries/log), and in 10 of the logs the attacks resulted in the emergence of a new generation of beetles.

The addition of aspen or birch wood to the ethanol bait resulted in reduced catches of *T. piniperda*, *H. palliatus*, and *Rhizophagus depressus* (F.) (Coleoptera, Rhizophagidae) (Tables 4 and 5). The reduction in catch in the different experiments varied between 58% and 96% for *T. piniperda*, 8% and 87% for *H. palliatus*, and 44% and 90% for *R. depressus*. The difference in number of beetles caught between treatments with wood and treatments without wood was statistically significant at the $P = 0.05$ level in one experiment for *T. piniperda* and in five experiments each for *H. palliatus* and *R. depressus*.

In contrast to the species mentioned above, 1.6–3.4 times as many *Glischrochilus quadripunctatus* (L.) (Coleoptera, Nitidulidae), 2.7–8.3 times as many *Epuraea bickhardti*, 5.3–20 times as many *E. unicolor* (Oliv.) and about twice as many *Rhizophagus parvulus* (Payk.) were caught in the wood-baited traps as in the traps baited with ethanol alone. These differences were statistically significant for *G. quadripunctatus* in two experiments, for *E. bickhardti* in six

TABLE 3. MEAN NUMBER OF BEETLES CAUGHT PER STICKY TRAP ATTACHED TO HEALTHY, TOP-CUT, AND NEWLY CUT LOGS OF SCOTS PINE AND TO PLASTIC TUBES

Species	Host-tree species	Mean catch			
		Plastic tube	Healthy pine	Top-cut pine	Pine log
T. piniperda	Scots pine	2.2	1.5	3.9	31.4[a]
H. palliatus	Scots pine Norway spruce	3.9	4.0	4.1	10.6
H. brunneus	Scots pine	1.4	0.5	1.3	23.6[a]
H. cunicularius	Norway spruce	1.6	1.4	1.4	1.6
T. lineatum	Scots pine Norway spruce	0.9	1.9	0.9	2.5

[a] Significantly different from other treatments, $P < 0.05$, Newman-Keuls multiple-range test.

TABLE 4. SUMS OF BEETLES CAUGHT IN BARRIER TRAPS BATED WITH ETHANOL ALONE (E) OR ETHANOL AND CUT WOOD OF ASPEN (E & A)

Year	Test	Species	Catch		P value[a]	Sex ratio (% females)
			E	E & A		
1990	1	*T. piniperda*	96	14	0.067	47
		H. palliatus	40	10	0.012	31
		R. depressus	18	9	0.237	52
		G. quadripunctatus	22	56	0.058	67
	3	*T. piniperda*	27	4	0.056	69
		H. palliatus	89	22	0.036	33
		R. depressus	27	13	0.044	63
		G. quadripunctatus	62	98	0.008	52
		E. bickhardti	58	172	0.006	52
1991	5	*T. piniperda*	171	61	0.023	50
		H. palliatus	65	21	0.014	46
		R. depressus	82	28	0.029	82
		G. quadripunctatus	10	34	0.059	57
		E. bickhardti	240	797	0.010	47
		E. unicolor	14	280	0.013	76

[a] Two-tailed paired-sample t test.

experiments, for *E. unicolor* in three experiments, and for *R. parvulus* in the only experiment in which this species was present.

In all but one case there was no significant (χ^2 test, $P < 0.05$) difference in sex ratio between beetles caught in treatments with wood and those without wood for any of the beetle species in any of the tests (Tables 4 and 5). The exception was *R. depressus* in experiment 6, where the female percentage was significantly lower in the wood baited treatment (58%) than in the treatment with ethanol alone (69%).

As a result of the lower mean air temperature, the field release rates of ethanol (Table 2) are considerably lower than those measured in the laboratory at 15°C. Rates of release were somewhat higher from dispensers placed in traps with wood than from dispensers in traps without wood: 24% higher in experiment 5, 12% higher in experiment 6, and 48% higher in experiment 7.

DISCUSSION

The present study shows that rejection of the nonhosts aspen and birch by the bark beetles *T. piniperda* and *H. palliatus* may be based on volatiles emitted from these tree species. Both aspen and European white birch are among the

TABLE 5. SUMS OF BEETLES CAUGHT IN BARRIER TRAPS BAITED WITH ETHANOL ALONE (E) OR ETHANOL AND CUT WOOD OF BIRCH (E & B)

Year	Test	Species	Catch		P value[a]	Sex ratio (% females)
			E	E & B		
1990	2	*H. palliatus*	30	4	0.019	35
		R. depressus	29	3	0.068	77
		E. bickhardti	18	48	0.046	42
	4	*H. palliatus*	33	11	0.107	56
		R. depressus	18	5	0.010	61
		G. quadripunctatus	36	77	0.045	57
		E. bickhardti	21	175	0.001	60
1991	6	*T. piniperda*	50	21	0.337	63
		H. palliatus	97	41	0.002	51
		R. depressus	298	167	0.050	65
		R. parvulus	10	21	0.010	40
		E. bickhardti	43	198	0.001	52
		E. unicolor	10	95	0.001	75
	7	*H. palliatus*	53	49	0.758	45
		R. depressus	201	99	0.027	67
		E. bickhardti	44	159	0.006	53
		E. unicolor	10	53	0.025	53

[a]Two-tailed paired-sample t test.

most common of the broad-leaved tree species in Sweden. Furthermore, they are often found in mixed stands together with Norway spruce and Scots pine. Thus the probability of *T. piniperda* and *H. palliatus*, as well as other conifer bark beetles, encountering aspen and birch is quite high. An olfactory response allowing the beetles to quickly reject nonhosts after or just before landing would save them valuable time for colonizing suitable breeding substrate.

The much higher number of *T. piniperda* caught on the pine logs than on the healthy unattacked pine trees can be ascribed to the large amounts of host volatiles released from the initiated egg galleries (cf., Schroeder, 1987) and from the end surfaces of the logs cut just before the start of the beetle flight period. *T. piniperda*, *H. palliatus*, and some other scolytids were caught in low numbers on the plastic tubes, demonstrating that when searching for breeding substrate these species may also land on objects lacking characteristics specific to their host-tree species.

The decreased attraction of *R. depressus* to ethanol in the presence of aspen and birch wood demonstrates that beetles associated with bark- and woodboring species also may respond to volatiles from nonhost tree species. *R. depressus*

is a predatory species inhabiting the galleries of bark beetles such as *T. piniperda* and *H. palliatus* in Scots pine and Norway spruce (Nuorteva, 1956). *R. depressus* was earlier demonstrated to be attracted by the conifer monoterpene α-pinene and by ethanol (Schroeder and Lindelöw, 1989).

Substances causing insects to make oriented movements away from its source are termed repellents according to designations of Dethier et al. (1960). The response of beetles to wood alone was not tested in the present study. Hence, it was not possible to determine if wood of birch and aspen has a true repellent effect. Moreover, since no unbaited traps were included in the experiments, it was not possible to establish whether the traps baited with wood and ethanol caught lower numbers of bark beetles than what would have been caught in unbaited traps. Thus, "decreased attraction" is used to account for the observed results.

Only a few reports of release rates of ethanol from cut wood were found in the literature. No information on ethanol release rates from aspen or birch is available. Ikeda et al. (1980) reported a daily average ethanol release of 0.7 μl/kg from bolts of red pine, *Pinus densiflora*, during a 14-day period following cutting. This suggests that if any ethanol were formed in the wood used in the present study, the release rates should have been negligible compared with the amounts released from the dispensers.

The somewhat higher release rate of the ethanol dispensers in traps baited with wood was probably due to the heat-storing capacity of the wood. The rate of release of ethanol from the type of dispenser used in the present study increases with temperature. Thus, as temperatures fell in the evenings, heat stored in the wood could have acted as a temperature buffer, thereby preventing release rates from dropping as fast as in the dispensers in the treatments without wood. Nevertheless, the somewhat higher release of ethanol from wood-baited treatments could not have caused the lower catches of the two bark beetles and *R. depressus*. In previous dose–response field experiments with ethanol, the beetle attraction increased as release rates of ethanol were increased from 0.002 to 50.4 mg/24 hr for *H. palliatus* and from 0.002 to 3.1 mg/24 hr for *R. depressus* (Schroeder, 1988; Schroeder and Lindelöw, 1989). About the same numbers of *T. piniperda* were attracted by all release rates covered by the latter dose range.

The combination of birch or aspen volatiles and ethanol was more attractive than ethanol alone to the nitidulid beetles *G. quadripunctatus*, *E. bickhardti*, and *E. unicolor* and to the rhizophagid beetle *R. parvulus*. These species inhabit fungi-infected bark and galleries of bark- and woodboring insects (Nuorteva, 1956; Palm, 1959). *R. parvulus* and *E. unicolor* are found almost exclusively on broad-leaved trees, and *G. quadripunctatus* is common on both conifer and broad-leaved trees. *G. quadripunctatus* is also attracted by ethanol and α-pinene (Schroeder and Lindelöw, 1989). In contrast to the other species, *E. bickhardti*

has been recorded mainly from Scots pine and Norway spruce in Scandinavia, and only a few reports of this species from broad-leaved trees exist (Saalas, 1917; Nuorteva, 1956).

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AN EQUILIBRIUM AND STABILITY STUDY OF Δ^1-PYRROLINE[1]

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Abstract—Several properties of the compound 3,4-dihydro-2H-pyrrole (Δ^1-pyrroline), which has been reported as a component of the male-produced Mediterranean fruit fly pheromone, have been determined by [^{1}H]- and [^{13}C]nuclear magnetic resonance spectroscopy. The stability of Δ^1-pyrroline in several solvents and at moderately elevated temperatures has been investigated, and it has been established that it exists as both a monomer and trimer in solution. Although equilibrium studies indicate that the trimer is thermodynamically more stable than the monomer in solution, only the monomer was found in the vapor phase based on infrared analysis.

Key Words—3,4-Dihydro-2H-pyrrole, pheromone, Δ^1-pyrroline, [^{1}H]NMR spectroscopy, [^{13}C]NMR spectroscopy, equilibrium, Mediterranean fruit fly, *Ceratitis capitata*, Diptera, Tephritidae.

INTRODUCTION

The Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wied.), is considered to be one of the major economic pests of deciduous and tropical fruits in the world. Previous small infestations in California and Florida have resulted in

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[1]This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or recommendation for its use by USDA.

expensive eradication procedures that include the widespread release of large amounts of pesticide. The most effective monitoring tool available for the medfly is trimedlure (Beroza et al., 1961), and it is routinely used in California, Texas, and Florida. Trimedlure predominantly attracts males, although a small percentage of females do respond to this chemical (Nakagawa et al., 1970). In the wild it has been demonstrated that medfly males release a pheromone that attracts females for mating (Féron, 1962). Baker et al. (1985) identified nine volatile compounds emitted and/or extracted from sexually mature male Mediterranean fruit flies. The identified components included 3,4-dihydro-2H-pyrrole, (*E*)-2-hexenoic acid, dihydro-3-methylfuran-2(3H)-one, 2-ethyl-3,5-dimethylpyrazine, linalool, ethyl (*E*)-3-octenoate, geranyl acetate, (*E*,*E*)-α-farnesene, and ethyl acetate. It was claimed that the 3,4-dihydro-2H-pyrrole was active, but no data were reported to support pheromonal activity of this or other identified compounds. Subsequently, Jang et al. (1989) determined electroantennogram responses of male and female medflies to those nine compounds and to an additional 56 compounds collected from air passed over male medflies. Recently, we reported the qualitative and quantitative analyses of three principal components, ethyl (*E*)-3-octenoate, geranyl acetate, and (*E*,*E*)-α-farnesene, as volatiles emitted by laboratory-reared and wild male medflies during the photophase (Heath et al., 1991). Additionally, we synthesized these compounds, developed a method to formulate each of the compounds, and investigated the attractiveness of the pheromone blend to wild female medflies in the field.

Efforts to quantitatively collect and analyze the compound 3,4-dihydro-2H-pyrrole (Δ^1-pyrroline) released by male medflies were without success (see Baker et al., 1985; Jang et al., 1989). To further evaluate the potential synergistic effect of Δ^1-pyrroline to the existing three-component blend, it was apparent that additional knowledge of this compound would be required. The chemical characterization of Δ^1-pyrroline is complicated by the fact that in solution it irreversibly transforms to unknown compound(s) at temperatures less than 30°C above ambient conditions. Attempts to synthesize the material result in the isolation of a trimeric species of Δ^1-pyrroline and not the desired monomer (Nomura et al., 1977). Attempts to isolate the trimeric species in the gas phase for microwave or infrared analysis, however, fail, and only the monomer is observed in the gas phase (Edwards et al., 1985). It has previously been determined by [^{13}C]NMR spectroscopy that in dilute solution the monomer can be observed (Poisel, 1978). This suggests that there may be an equilibrium established in solution that can be characterized by a series of temperature dependent experiments.

In this work, the equilibrium of synthetic Δ^1-pyrroline with the trimer is established and characterized in DMSO-d_6. The stability of Δ^1-pyrroline in D_2O, $CDCl_3$ solutions, and neat was examined, and the thermal stability was deter-

mined in DMSO-d_6. GC-FTIR analysis confirmed the exclusive presence of the monomer in the vapor phase.

METHODS AND MATERIALS

Synthesis. The trimer of Δ^1-pyrroline was synthesized according to the procedure of Nomura et al. (1977). The final reaction mixture was light orange in color. The crude material was worked up with CH_2Cl_2 and ice water. The CH_2Cl_2 layer was washed with brine, dried overnight at 4°C over Na_2SO_4 and anhydrous K_2CO_3, filtered through a plug of Na_2SO_4–K_2CO_3, and the solvent was removed by rotary evaporation without heating. The last traces of solvent were removed by a stream of dry nitrogen resulting in a viscous, transparent orange oil. No impurities were observed in the NMR spectrum.

IR Spectra. Capillary GC-FTIR analysis was performed on neat Δ^1-pyrroline using a Hewlett-Packard model 5890 gas chromatograph (GC) equipped with a cool on-column injector and a Nicolet model 20SXC FTIR equipped with a light-pipe GC interface. Helium at a linear velocity of 18 cm/sec was used as the carrier gas, and a 30-m × 0.25-mm-ID Carbowax column was used for the analysis. Various oven temperature programs and light-pipe interface temperatures were used in an attempt to find both the monomer and trimer in the gas phase. The final analysis was performed using an isothermal oven program at 150°C with the interface temperature set at 210°C. A neat liquid analysis was also performed using NaCl plates.

NMR Spectra. NMR spectra were obtained on a Bruker AC-300 spectrometer equipped with an Aspect 3000 computer and a 5-mm dual $^1H/^{13}C$ probe. The operating frequency was 300.135 MHz for ^1H and 75.469 MHz for ^{13}C. Standard Bruker automated acquisition programs were used for all experiments. All one-dimensional spectra were from 64K data points. The spectral width for ^1H was set at 6024 Hz and for ^{13}C at 18519 Hz with acquisition times of 5.439 and 1.769 sec, respectively. The resolution of the spectra was 0.565 Hz per point for ^{13}C and 0.184 Hz/pt for ^1H. The numbers of scans for ^1H and ^{13}C were 16 and 128, respectively.

The initial spectra and assignments were obtained in $CDCl_3$ with solutions of 50 μl of sample dissolved in 0.35 ml of $CDCl_3$ with TMS as internal reference. The spectral assignments were based on ^1H and ^{13}C (^1H decoupled) chemical shifts, ^1H coupled ^{13}C spectra, ^1H–^1H coupling constants, proton-splitting patterns, and integrated proton intensities. The monomer absorptions could be distinguished from those of the trimer by their relative intensities. A DEPT analysis and ^{13}C–^1H HETCOR experiment were performed to confirm initial assignments. A thermal degradation study was performed with 100 μl of sample

dissolved in 0.4 ml of DMSO-d_6. The molal concentration scale was used for the equilibrium study. The solution was prepared with 0.052 g of sample dissolved in 0.44706 g of DMSO-d_6. A neat sample was analyzed using an 8-μl insert obtained from Wilmad (Buena, New Jersey). In order to determine the stability of the material in water, a series of experiments were conducted in D_2O over the course of several days.

For all temperature-controlled experiments (5°C increments), 20 min of equilibration time was allowed before data acquisition was initiated. After each 10-degree change in temperature, a ^1H spectrum was obtained after equilibration for an additional 30 min to confirm temperature stability.

RESULTS

GC-FTIR Analysis. Edwards et al. (1985) collected IR and microwave spectra of Δ^1-pyrroline vapor phase directly. They reported that no trimer could be detected in the gas phase. Our GC-FTIR analysis yielded the same results. Only the monomer was detected, and the spectra obtained in our analysis were identical to that of Edwards et al. The major absorptions for the gas sample were found at 2974.1, 2954.1, 1626.0, 1454.3, 1302.5, 1072.7, and 913.3 cm^{-1}. The major absorptions for the neat sample were 2956.3, 2872.4, 2785.6, 1673.8, 1458.7, 1393.1, 1338.9, 1233.2, 1206.3, 1194.7, 1178.7, and 1066.0 cm^{-1}. No overlap between the neat and gas phase spectra could be found.

NMR Analysis. The initial NMR spectral data for both the monomer and trimer are listed in Table 1 with specific assignments found in Figure 1. The carbon spectra contained eight absorptions supportive of the presence of both

TABLE 1. SPECTRAL ASSIGNMENT OF MONOMER AND TRIMER[a]

Monomer			Trimer		
^{13}C (ppm)	^1H (ppm)	Multiplicity	^{13}C (ppm)	^1H (ppm)	Multiplicity
166.95	7.61	M($J \approx 1$ Hz)	82.07	3.01	M(mixed J)
61.26	3.85	T(J = 7.5 Hz) of M(J = 2.4 Hz)	45.94	2.32	D(J = 6.1 Hz) of T(J = 8.7 Hz)
36.65	2.53	T(J = 8.3 Hz) of M($J \approx 1$ Hz)	27.95	1.7–1.9	overlapping M
20.53	1.7–1.9	overlapping M	20.33	1.7–1.9	overlapping M

[a]D = doublet, T = triplet, M = multiplet.

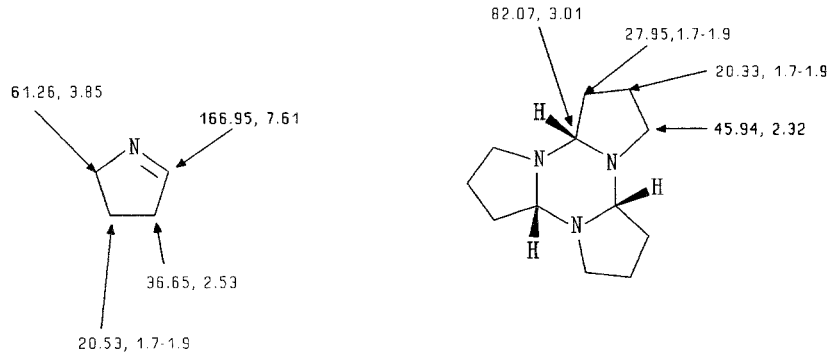

FIG. 1. Δ^1-Pyrroline monomer and trimer and chemical shift assignments in ppm ^{13}C, ^1H.

monomer and the assumed trimer of point group C_3. Nomura et al. (1977) reported only four ^{13}C resonances (20 MHz in $CDCl_3$) at 81.96, 45.79, 27.96, and 20.37 ppm. These are in agreement with our findings for the trimer. Initial small variations in absorption intensities with temperature indicated that the two species were in equilibrium. A neat sample was prepared in a capillary insert and ^{13}C spectra were recorded. There were only four absorptions present. If the absorption of lowest ppm magnitude in the neat sample is aligned with the trimer absorption at 20.33 ppm in $CDCl_3$, then the other three neat absorptions align with those of the trimer with a root mean square (RMS) difference of only 0.6 ppm. Based on this observation, the neat material is essentially purely trimer.

The material has been reported to be unstable in solution at moderate temperatures (Nomura et al., 1977). In order to determine the thermal stability, a sample was prepared in DMSO-d_6. The chemical shifts were identical to those observed in $CDCl_3$. The sample was studied at 5-degree increments from 295 to 340°K. From 295 to 320°K, only the original eight ^{13}C absorptions were apparent. The trimer absorptions decreased in intensity with increasing temperature, and the monomer absorptions increased for both ^{13}C and ^1H spectra. At 325°K, several additional low-intensity ^{13}C absorptions become evident. At 335°K numerous ^{13}C absorptions were apparent, and by 340°K the trimer ^{13}C absorption at 27.95 ppm was not detected. After cooling at ambient temperature for 16 h the pattern of ^{13}C absorptions observed at 340°K remained. From this experiment the original observation that the material is not stable at elevated temperatures is substantiated. The maximum temperature at which equilibrium is maintained without degradation was determined to be 320°K (47°C).

In order to establish the equilibrium and thermodynamic constants for the material, an equilibrium study was performed in DMSO-d_6 from 294 to 320°K. The integrated ^1H intensities for both trimer and monomer were tabulated for

the protons adjacent to the nitrogen (7.61 and 3.85 ppm for monomer; 3.01 and 2.32 ppm for trimer) and used to establish the ratio of monomer to trimer in solution. The preequilibrium concentration of trimer was $[T_i]$ = (0.5618 mol trimer)/(kg solvent) based on the fact that the neat material is $\approx 100\%$ trimer. Using the concentration ratio from experiment $[T_i]$, and the mass balance relationship between monomer and trimer (1 mol trimer $\rightarrow$ 3 mol monomer) allows the concentrations of monomer and trimer to be determined. The equilibrium constant can then be calculated at each temperature as $K_{eq}(T) = [M]^3/[T]$ where $[M]$ is the molal concentration of the monomer and $[T]$ is the molal concentration of the trimer. Using the standard thermodynamic relationships $\Delta G = RT \ln [K_{eq}(T)] = \Delta H - T\Delta S$ and assuming the changes in enthalpy and entropy are constant with temperature over the experimental range studied, the experimental data can be fitted to a linear model. The modeled enthalpy change is ΔH = 22.004 kcal/mol trimer. The modeled entropy change is ΔS = 0.0678 kcal/(mol-degree) trimer. The correlation coefficient for the fitting is 0.9827, and the data are tabulated in Table 2.

An attempt to study the rate of conversion from monomer to trimer was performed. The same sample used to perform the equilibrium study was equilibrated at 320°K and immediately cooled to 300°K and ^{1}H spectra were initiated. All spectra obtained at successive times were identical to the initial spectra, indicating that the reaction from excess monomer to trimer was faster than could be measured.

The stability of the material with time was also studied in the solvents used for analysis. These samples were maintained at laboratory ambient conditions

TABLE 2. EXPERIMENTAL AND MODEL EQUILIBRIUM AND THERMODYNAMIC DATA[a]

Temp (Kelvin)	$[M]/[T]$	$[M]$	$[T]$	K_{eq} (exp)	ΔG_{exp} (kcal/mol)	K_{eq} (model)	ΔG_{mod}
Increasing Temperature							
294	0.522	0.250	0.479	0.033	2.000	0.029	2.072
305	0.730	0.330	0.451	0.079	1.535	0.112	1.327
310	1.079	0.446	0.413	0.214	0.949	0.201	0.988
315	1.344	0.521	0.388	0.365	0.631	0.355	0.649
320	1.605	0.587	0.366	0.553	0.376	0.614	0.310
Decreasing Temperature							
315	1.528	0.569	0.372	0.494	0.441		
310	1.032	0.431	0.418	0.192	1.016		
305	0.792	0.352	0.444	0.098	1.406		
294	0.514	0.216	0.479	0.031	2.025		

[a] [M] = conc. monomer (mol/kg); [T] = conc. trimer; G = free energy; K_{eq} = molal equilibrium constant.

in 5-mm NMR sample tubes. They were not degassed, and the tubes were loosely covered with plastic NMR caps. Under these conditions solvent could escape and air could enter the tube. The volume of air to sample was approximately 10:1. Samples prepared in $CDCl_3$ showed no changes with time over the course of several weeks. A sample in DMSO-d_6 was stable during the four days it was used to record the thermodynamic and kinetic studies. However, analysis of the DMSO-d_6 solution after a three-week period resulted in the detection of approximately 20 additional ^{13}C absorptions. By the fourth week 30 absorptions were indicated, and the intensities of the new absorptions were of the same magnitude as those assigned to the monomer and trimer. In D_2O, the ratios of the intensities of the original carbon absorptions declined over the course of four days with the occurrence of several new ^{13}C absorptions. After two weeks, the trimer absorption at 27.95 ppm and the monomer absorption at 36.65 ppm were no longer observed. These two absorptions originate from the same carbon in the monomer and trimer. On the surface of the degraded samples, a rigid layer developed, suggesting that some type of polymerization occurred. The neat sample was stable with time at ambient temperature.

DISCUSSION

The purpose of this study was to characterize Δ^1-pyrroline so that mechanisms for a controlled release of volatiles could be developed and new test methods for analysis could be suggested. This has been accomplished. Δ^1-Pyrroline in neat form is stable and exists purely in the trimeric form. Although the neat material is purely trimer, only the monomer is found in the gas phase. This suggests that Δ^1-pyrroline can be formulated for release as neat material in capillary tubes similar to that described by Heath et al. (1991). The release rate is dependent on the diameter of the capillary and the height of the vapor–air column above the liquid (Weatherston et al., 1985).

The currently available methods often used for quantifying Δ^1-pyrroline involve the use of reagents that have a very short shelf life to form a stable oxidized derivative of Δ^1-pyrroline that is finally analyzed by GC (Sakamoto and Samejima, 1979). This method is not practical for the routine analysis of many samples, as is often the case with material collected in collaborating laboratories. Equilibrium between the monomer and trimer was established in all three solvents studied, and at the concentrations used for NMR analysis, the trimer was the predominant species. Since the equilibration of monomer and trimer is essentially instantaneous, we are developing techniques for direct chromatographic analysis of Δ^1-pyrroline that has been extracted from collections made using absorbent material. The present analysis indicates, however, that although water may be used for an extraction solvent, the short shelf life of

Δ^1-pyrroline in water would demand chromatographic analysis within one day of extraction. Although Δ^1-pyrroline is thermally unstable in solution, ambient temperatures are below the threshold of rapid degradation. The analysis of field-collected medfly pheromone and the formulation of synthetic medfly pheromone are currently under investigation.

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OPTIMAL FRACTIONATION AND BIOASSAY PLANS FOR ISOLATION OF SYNERGISTIC CHEMICALS: THE SUBTRACTIVE-COMBINATION METHOD

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Abstract—Studies of chemical ecology of an organism are founded on the isolation and identification of a semiochemical, often comprised of two or more synergistic compounds (each synergist alone has little activity, but presented together they are bioactive). Chromatographic fractionation and bioassay methods of binary splitting, additive combination, and subtractive combination are compared for efficiency in isolating synergists. Formulas are derived for the latter two methods that calculate the expected number of bioassay tests required for isolation of from two to five synergists from biological extracts with any number of compounds, depending on the number of initial (major) chromatographic fractions. A computer program based on the formulas demonstrates the superiority of the subtractive-combination method. Simulations with the program were used to determine the optimal number of initial fractions for the additive- and subtractive-combination methods when isolating two to five synergists from extracts of from 25 to 1200 compounds. Methods of bioassay, isolation, identification, and field testing of semiochemicals are discussed.

Key Words—Pheromone, semiochemical, behavioral bioassay, chemical isolation, gas chromatography, chemical fractionation, synergist.

INTRODUCTION

Biological studies of olfactory perception, receptor biochemistry, pheromone biosynthesis, orientation behavior, and communication as well as many other areas require the use of the purified semiochemicals. Chemical ecology, in fact, is founded on the identification of chemicals that mediate the behavioral or

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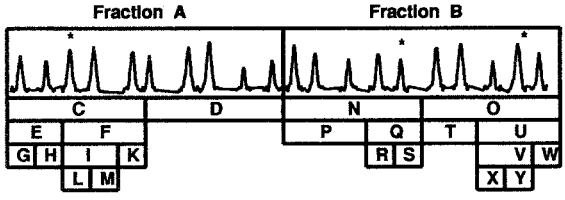
physiological phenomena. The first step, however, is not the identification of the chemicals present in extracts of organisms but rather observation of the behavioral or physiological changes induced by chemical emissions in a virtually natural environment. For example, a specific mating behavior that appears to be elicited by odors from others of the species would indicate the presence of a pheromone. The next step is development of a bioassay, which consists of describing a consistent set of behavioral reactions and movements stimulated by the release of a suspected pheromone under controlled conditions. The third step is then the isolation and identification of the chemicals comprising the pheromone. Most aggregation or sex pheromones of insects consist of two or more compounds that alone have moderate or no activity but released together are synergistic in eliciting maximum behavioral response (Silverstein and Young, 1976; Silverstein, 1981; Cardé and Baker, 1984; Byers, 1989). In Figure 1, a hypothetical chromatogram is shown of an insect extract in which three pheromone components (synergists) are depicted as eluting differently. The problem is to find efficient methods for locating them for subsequent chemical identification.

Behavioral tests (bioassays) can be coupled to chemical separation tech-



Fractionation	Tests	No. Tests	Activity	Conclusion
-	Extract	1	yes	proceed
Extract	A; B	2	none	A and B needed
A	CB; DB	2	CB	D inactive
C	EB; FB	2	FB	E inactive
F	IB; KB	2	IB	K inactive
I	LB; MB	2	LB	L active
B	NL; OL	2	none	N and O needed
N	POL; QOL	2	QOL	P inactive
Q	ROL; SOL	2	SOL	S active
O	TSL; USL	2	USL	T inactive
U	VSL; WSL	2	VSL	W inactive
V	XSL; YSL	2	YSL	Y active
11 GC passes		23 total		L; S; Y active

FIG. 1. Outline of the binary splitting method for isolation of synergistic chemicals. The peaks represent a chromatogram with three synergists indicated by the asterisks. Letters represent fractions, see text for details.

niques and fractionation of biological extracts with several methods. One common method could be described as binary splitting. The extract is fractionated into two parts and each tested for activity. This method either assumes that only one compound is active or that all active compounds are found in one fraction—so it would not work well for synergists. However, a variation could be used as in Figure 1, where successive binary fractionation would be tested in combination with other active fractions using a combinative scheme. This method will be discussed subsequently.

Another scheme, developed in the 1960s during the pioneering isolation of bark beetle pheromones, is here called the additive-combination method (Silverstein et al., 1966, 1967, 1968). In principle, the method uses chromatography, for example, gas chromatography (GC), to separate chemicals as they elute sequentially into fractions. The fractions then can be recombined and tested for behavioral activity in a bioassay. Silverstein et al. (1967) drew attention to the "notable feature" of the pheromone isolation work on the North American bark beetle, *Ips paraconfusus*, where attractant activity disappeared upon fractionation but reappeared upon recombination of appropriate fractions. They state that "no fraction can be discarded until it has been tested in combination with the other fractions." The exact methodology is unclear, but it can be assumed that all fractions would be tested individually first, then in two-way combinations, then three-way, and so forth in an additive-combination procedure. This method has been used on other important beetle species for isolation of their pheromone components: western pine beetle, *Dendroctonus brevicomis* (Silverstein et al., 1968), smaller elm bark beetle, *Scolytus multistriatus* (Pearce et al., 1975), and flat grain beetle, *Cryptolestes pusillus*, (Millar et al., 1985). However, because of the large number of combinations possible, relatively few other studies have attempted isolation of components using the additive-combination method due to the work involved.

A third method, subtractive-combination, also assumes that synergistic fractions must be tested in combination, but instead of combining smaller numbers of fractions together, it subtracts fractions from the whole blend of fractions. In 1976 L.E. Browne, while at the University of California, Berkeley, proposed that this method would be more efficient than the established method of additive combination of fractions (personal communication). My colleagues and I later used the subtractive-combination method for isolation of host (Scots pine) attractants of the pine shoot beetle, *Tomicus piniperda*, of Europe (Byers et al., 1985). However, the subtractive method was first used for isolation of synergistic pheromone components on the smaller spruce engraver of Europe and Asia, *Pityogenes chalcographus*, where a pheromonal structure not previously known for bark beetles, methyl (*E*,*Z*)-2,4-decadienoate, was identified (Byers et al., 1988, 1989, 1990a).

The objective of this paper is to describe and compare the three methods,

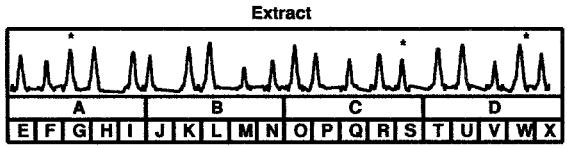
especially the additive-and subtractive-combination methods. The question is which of the methods is most efficient in terms of number of bioassay tests required and number of passes of compounds through the chromatograph. Assuming two fractionations, mathematical formulas will be derived for both the additive- and subtractive-combination methods that describe the probabilistic number of tests that would be required to ensure isolation of all active components given: (1) the number of fractions of the extract on the first fractionation, (2) the number of synergistic components, and (3) the number of total compounds in the extract. A computer program that uses the formulas will determine the optimal number of initial major fractions, which depends on the number of total compounds in the extract and the number of synergists.

METHODS AND MATERIALS

Descriptive examples of binary splitting and additive- and subtractive-combination procedures are presented to explain the methodology used for each. Hypothetically, an extract consisting of 20 compounds can be passed through a gas chromatograph (GC) resulting in the "chromatogram" shown in Figures 1 and 2. It is assumed that the third, fifteenth, and nineteenth eluting compounds are active only in synergism. The first method of binary splitting of fractions is outlined in Figure 1. Fractions A and B alone are not active; the conclusion is that both have synergists. Fraction A is divided into C and D and these fractions are combined with B in order to isolate the active compounds in A (Figure 1). Fraction C is active, D is not active, and further splitting of C into E and F (active), then F into I (active) and K, and finally I into L (active) and M reveals that L is a synergist. Fraction B is then fractionated similarly, with fractions tested with L (Figure 1) until the active compounds S and Y are found.

The second method, additive combination (Figure 2), fractionates the extract initially into four major fractions A–D. These are tested alone (none active), in binary combinations (none active) and then tertiary combinations (only ACD active). Next, fraction A is fractionated into five fractions and each tested with fractions C and D—showing that compound G is active. The same procedure is done with major fraction C, combining each minor fraction with G to find compound S active. Initially, major fraction D is fractionated and these minor fractions combined with G and S to find compound W active.

The third method, subtractive combination (Figure 2), also fractionates the extract into four initial fractions A–D, but the fractions are combined into four blends each with one of the fractions not present (subtracted). Since the removal of fraction B had no effect on the activity, it is concluded that this fraction does not contain synergists. Fractions A, C, and D when subtracted from the blend resulted in a loss of activity, so each has synergists. Fraction A is fractionated



ADDITIVE-COMBINATION

Fractionation	Tests	No. Tests	Activity	Conclusion
-	Extract	1	yes(+)	proceed
Extract	(A to D)	4	none(-)	
-	AB to CD	6	none(-)	
-	ABC to BCD	4	ACD active	ACD needed
A	(E to I)CD	5	GCD active	G active
C	(O to S)GD	5	GSD active	S active
D	(T to X)GS	5	GSW active	W active
2-4 GC passes		30 total		G; S; W active

SUBTRACTIVE-COMBINATION

Fractionation	Tests	No. Tests	Activity	Conclusion
-	Extract	1	yes(+)	proceed
Extract	Extract-A	1	no(-)	A needed
-	Extract-B	1	yes(+)	B inactive
-	Extract-C	1	no(-)	C needed
-	Extract-D	1	no(-)	D needed
A	(A-E to I)CD	5	++-++	G active
C	(C-O to S)GD	5	++++-	S active
D	(D-T to X)GS	5	+++-+	W active
2-4 GC passes		20 total		G; S; W active

FIG. 2. Outline of the additive-combination and subtractive-combination methods for isolation of synergistic chemicals. The peaks represent a chromatogram with three synergists indicated by the asterisks. Letters represent fractions, see text for details.

into five fractions, which are combined and tested with major fractions C and D in five tests, with a different minor fraction subtracted. Subtraction of minor fraction G results in a loss of activity, indicating that compound G is a synergist. Similar fractionation and testing of major fraction C with D and G shows S active, and fractionation of D reveals that W is active.

Using the schemes shown in Figure 2, two equations (1 and 2, see Results) were derived that describe the number of bioassay tests that are required to ensure that all possible bioactive compounds are isolated using the additive- or subtractive-combination methods. The formulas assume that the extract is fractionated once into any number of major fractions (from two to the number of compounds), that these fractions are bioassayed, and that any of the indicated active fractions are fractionated into individual compounds (the second fractionation). The two formulas, however, are somewhat unrealistic since most insects

do not have any number of possible synergists but usually from two to five. It also must be considered that the active compounds will have various probabilities of being together or separated in the major fractions depending on (1) the number of synergists, and (2) the number of these fractions.

Formulas were derived that describe the probabilities of from two to five synergistic compounds being contained together, each separate, or in various combinations given any number of initial major fractions (see Results). The probabilities that all synergists elute together or separately were obtained from McCall (1970). The combinatorial notation used here is the following:

$$\text{All combinations of three things selected from five} = C_3^5 = \frac{5!}{3!(5-3)!} = 10$$

$$\text{All permutations of three things selected from five} = P_3^5 = \frac{5!}{(5-3)!} = 60$$

The probability formulas were combined with equations 1 and 2 (Results) for calculating the number of bioassay tests for the additive- and subtractive-combination methods. The resulting formulas, shown in Table 1, describe the expected number of tests that would be required to isolate from two to five synergists for any number of initial major fractions, the indicated number of bioactive major fractions, and for any number of compounds in the extract. The formulas were implemented in a BASIC language computer program (output shown in Figure 3). The program was used to obtain the expected number of tests required to isolate from two to five synergists in an extract of 60 compounds (Figure 4), or three synergists in extracts of 60–240 compounds (Figure 5), for each method. The program also generated the expected tests required to isolate two to five synergists from extracts of 25–1200 compounds (in steps of 25) using fractionations of 3–80 major fractions (fraction simulation run) for a total of 77 $\times$ 48 $\times$ 4 = 14,784 comparisons for each method. The minimum values for each fraction simulation run (total of 48 $\times$ 4 = 192) for each method, which are the optimal number of major fractions, were plotted in Figure 6 using geometric least-squares regression.

RESULTS

In Figure 1, the binary splitting method required 23 tests to isolate, in theory, the three synergists, compounds L, S and Y. The additive-combination method required 30 tests to isolate the three synergists, while the subtractive-combination procedure needed only 20 tests (Figure 2). However, the binary splitting method required 11 GC fractionation passes, while the additive- and subtractive-combination needed only two GC passes (at most four passes if each active fraction is fractionated separately).

TABLE 1. EXPECTED NUMBER OF BIOASSAY TESTS REQUIRED FOR ISOLATION OF TWO TO FIVE SYNERGISTS IN EXTRACTS OF ANY NUMBER OF COMPOUNDS (n) AND WITH INITIAL FRACTIONATION INTO ANY NUMBER OF FRACTIONS (f)[a]

Two synergists

$$\text{Additive-combination tests} = 1 + p_2 C_1^f + p_{1+1} \sum_{i=1}^{2} C_i^f + p_2 \sum_{i=1}^{2} C_i^n + p_{1+1} 2C_1^n$$

$$\text{Subtractive-combination tests} = 1 + f + p_2 C_1^n + p_{1+1} 2C_1^n$$

Three synergists

$$\text{Additive-combination tests} = 1 + p_3 C_1^f + p_{2+1} \sum_{i=1}^{2} C_i^f + p_{1+1+1} \sum_{i=1}^{3} C_i^f + p_3 \sum_{i=1}^{3} C_i^n + p_{2+1} \left[\sum_{i=1}^{2} C_i^n + C_1^n \right] + p_{1+1+1} 3C_1^n$$

$$\text{Subtractive-combination tests} = 1 + f + p_3 C_1^n + p_{2+1} 2C_1^n + p_{1+1+1} 3C_1^n$$

Four synergists

$$\text{Additive-combination tests} = 1 + p_4 C_1^f + p_{3+1} \sum_{i=1}^{2} C_i^f + p_{2+2} \sum_{i=1}^{2} C_i^f + p_{2+1+1} \sum_{i=1}^{3} C_i^f + p_{1+1+1+1} \sum_{i=1}^{4} C_i^f +$$

$$p_4 \sum_{i=1}^{4} C_i^n + p_{3+1} \left[\sum_{i=1}^{3} C_i^n + C_1^n \right] + p_{2+2} \left[\sum_{i=1}^{2} C_i^n + \sum_{i=1}^{2} C_i^n \right] + p_{2+1+1} \left[\sum_{i=1}^{2} C_i^n + 2C_1^n \right] + p_{1+1+1+1} 4C_1^n$$

$$\text{Subtractive-combination tests} = 1 + f + p_4 C_1^n + p_{3+1} 2C_1^n + p_{2+2} 2C_1^n + p_{2+1+1} 3C_1^n + p_{1+1+1+1} 4C_1^n$$

Five synergists

$$\text{Additive-combination tests} = 1 + p_5 C_1^f + p_{4+1} \sum_{i=1}^{2} C_i^f + p_{3+2} \sum_{i=1}^{2} C_i^f + p_{3+1+1} \sum_{i=1}^{3} C_i^f + p_{2+2+1} \sum_{i=1}^{3} C_i^f +$$

$$p_{2+1+1+1} \sum_{i=1}^{4} C_i^f + p_{1+1+1+1+1} \sum_{i=1}^{5} C_i^f + p_5 \sum_{i=1}^{5} C_i^n + p_{4+1} \left[\sum_{i=1}^{4} C_i^n + C_1^n \right] + p_{3+2} \left[\sum_{i=1}^{3} C_i^n + \sum_{i=1}^{2} C_i^n \right] +$$

$$p_{3+1+1} \left[\sum_{i=1}^{3} C_i^n + 2C_1^n \right] + p_{2+2+1} \left[2 \sum_{i=1}^{2} C_i^n + C_1^n \right] + p_{2+1+1+1} \left[\sum_{i=1}^{2} C_i^n + 3C_1^n \right] + p_{1+1+1+1+1} 5C_1^n$$

$$\text{Subtractive-combination tests} = 1 + f + p_5 C_1^n + p_{4+1} 2C_1^n + p_{3+2} 2C_1^n + p_{3+1+1} 3C_1^n + p_{2+2+1} 3C_1^n + p_{2+1+1+1} 4C_1^n + p_{1+1+1+1+1} 5C_1^n$$

[a] The probabilities of various combinations of synergists occurring together in fractions are represented as p_{1+1} etc. (see Results). The letter C represents the notation for combinations (see Methods and Materials).

```
enter # fractions (2 to 100)? 20
enter # synergists (2 to 5)? 4
Enter total # compounds in ext.? 200
```

N	P:4	P:3+1	P:2+1+1	P:2+2	P:1+1+1+1	Subtractive	Additive
4	.016	.188	.563	.141	.094	141.95	9118.821
5	.008	.128	.576	.096	.192	124.08	2922.56
6	.005	.093	.556	.069	.278	109.597	1302.857
7	.003	.07	.525	.052	.35	101.438	788.257
8	.002	.055	.492	.041	.41	91.75	544.606
9	.001	.044	.461	.033	.461	84.404	464.649
10	.001	.036	.432	.027	.504	79.78	489.29
11	.001	.03	.406	.023	.541	74.802	566.853
12	.001	.025	.382	.019	.573	72.959	713.3441
13	0	.022	.36	.016	.601	67.4	903.35
14	0	.019	.341	.014	.625	65.246	1175.614
15	0	.017	.324	.012	.647	63.034	1526.326
16	0	.015	.308	.011	.667	64.372	1975.648
17	0	.013	.293	.01	.684	61.932	2514.588
18	0	.012	.28	.009	.7	59.502	3173.383
19	0	.01	.268	.008	.714	60.656	3968.498
20	0	.009	.257	.007	.727	58.11	4905.865

FIG. 3. Output of a computer program that calculates the tests required to isolate the number of synergists entered (4) for the number of compounds entered (200) for up to 100 major fractions (N) for the subtractive-combination and additive-combination methods. The columns headed by P:4, P:3 + 1, etc., represent the probabilities that all four synergists occur in one fraction, or three synergists in one and the other in another fraction, respectively.

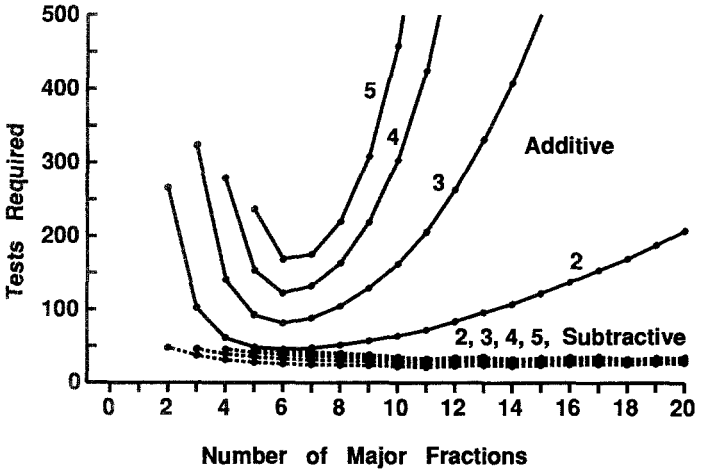


FIG. 4. The number of tests required to isolate from two to five synergists from an extract with 60 compounds with either the additive- or subtractive-combination methods, depending on the number of major fractions.

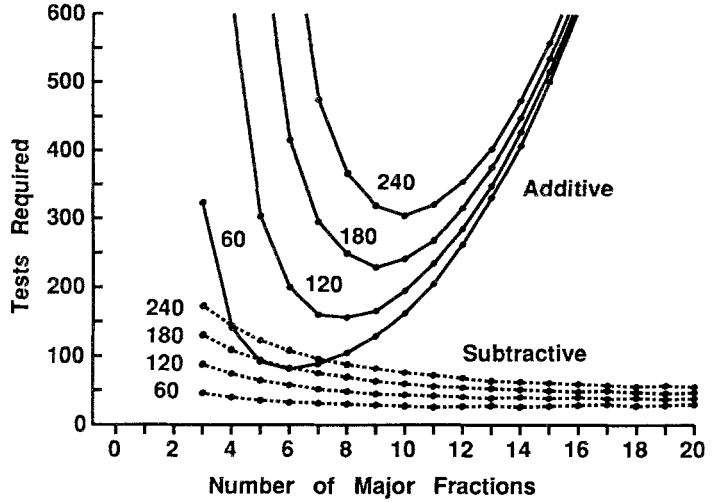


FIG. 5. The number of tests required to isolate three synergists from extracts with 60–240 compounds with either the additive- or subtractive-combination methods, depending on the number of major fractions.

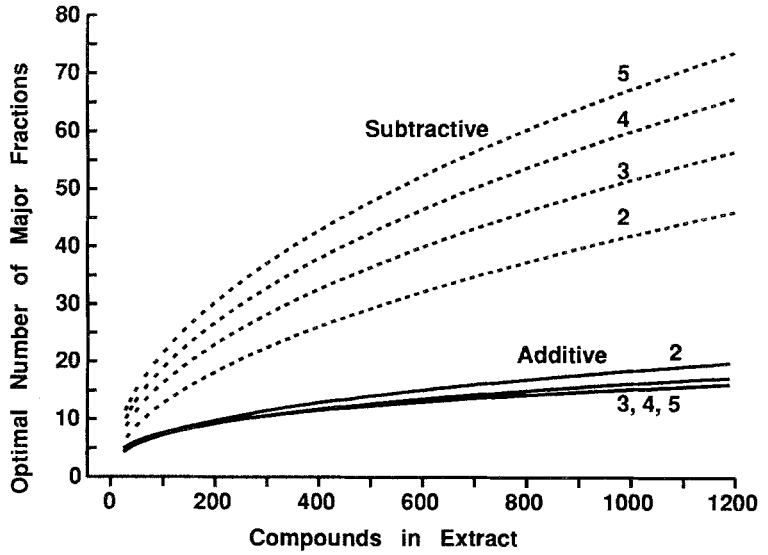


FIG. 6. The optimal number of major fractions for isolation of from two to five synergists from extracts with up to 1200 compounds using either the additive- or subtractive-combination methods.

The required number of tests to ensure the identification of all possible synergists using the additive-combination method and two fractionations is given by the following formula:

$$Tests = 1 + \sum_{i=1}^{a} \frac{f!}{i!(f-i)!} + a \sum_{i=1}^{v} \frac{v!}{i!(v-i)!} \quad (1)$$

where f = number of major fractions of the first fractionation, a = number of active major fractions, and v = average number of compounds per major fraction. The required number of tests to ensure identification of any number of possible synergists using the subtractive-combination method and two fractionations is:

$$Tests = 1 + f + v + a \quad (2)$$

The above formulas for calculating the number of bioassay tests assumes there is no limit on the number of synergists, since this is something that is not known prior to the study. However, it can be assumed that most studies of insect pheromones will encounter no more than two to five synergists responsible for a particular behavior. Thus, probabilities must be derived for the expected distribution of synergists among the major fractions. Intuitively, if the number of major fractions is small and the number of synergists is five, then the probability that several synergists would occur together in one fraction is large. If the number of fractions is large, then the probability is larger that each synergist is found alone in a fraction.

In the case of two synergists, they can occur either together in one fraction or each alone in a fraction. For three synergists there are three possibilities, each alone (1 + 1 + 1), two together and one alone (2 + 1) and all together (3). Four synergists can occur in five ways, (1 + 1 + 1 + 1), (2 + 1 + 1), (3 + 1), (2 + 2), and all together (4), assuming there are at least four fractions. Five synergists may be distributed in seven ways, (1 + 1 + 1 + 1 + 1), (2 + 1 + 1 + 1), (3 + 1 + 1), (4 + 1), (2 + 2 + 1), (2 + 3), and all together (5). The probability (p) that synergists occur each alone in a fraction is given by formula (a) below. The formula for the probability that all synergists occur together in a fraction is given in (b).

$$\frac{P_r^f}{f^r} \quad \text{(a)} \qquad \frac{P_1^f}{f^r} \quad \text{(b)}$$

where f is the number of major fractions and r is the number of synergists (P represents permutations). The probability that the synergists are distributed as (j + 1), (j + 1 + 1) or (j + 1 + 1 + 1), where j is 2, 3, or 4, is given by the general formula in (c), while the probability of the synergists occurring as (j + 2) is given in (d) below.

$$\frac{C_j^r P_k^{f-1} f}{f^r} \quad \text{(c)} \qquad \frac{C_j^r P_2^f}{f^r} \quad \text{(d)}$$

where C represents combinations, r is as above and k is the number of synergists alone (e.g., 1–3). For synergists distributed as (2 + 2), the probability can be calculated from equation (e) below. Finally, the probability that the synergists could be distributed as (2 + 2 + 1) is given by equation (f) below:

$$\frac{\frac{C_2^r P_2^f}{2}}{f^r} \quad \text{(e)} \qquad \frac{\frac{C_2^r C_2^f P_2^f}{2}}{f^r} \quad \text{(f)}$$

The probability formulas (a-f) above can be used with equations 1 and 2 to calculate the expected number of bioassay tests required to isolate from two to five synergists, for any number of major fractions, and for any number of compounds in the extract for the additive- and subtractive-combination methods (see Table 1). Output of a computer program (Figure 3) using the equations of Table 1 shows that the expected number of tests needed to isolate four synergists from an extract of $n = 200$ compounds and $f = 7$ major fractions with the additive method is 788 while the subtractive method requires only 101 tests.

The computer program was used to vary the number of major fractions and calculate the number of tests required to isolate from two to five synergists in an extract of 60 compounds for each method (Figure 4). From Figure 4 it can be seen that the minimum number of tests is most likely to occur at about six major fractions for the additive-combination method, while the minimum for the subtractive-combination method is ill-defined but occurs with higher numbers of major fractions. It is clear that in all cases the subtractive-combination method requires less tests than the comparable conditions with the additive method (Figure 4). The effect of varying the number of compounds in the extract when attempting to isolate three synergists is seen in Figure 5. Again there are optimal numbers of major fractions, i.e., for higher number of compounds in the extract the optimal number is larger. Again, however, in all cases, the subtractive-combination method is superior since it requires fewer biological tests.

The results of finding the minimum tests needed to isolate from two to five synergists for fractionations with from a few to 80 major fractions, for extracts from 25 to 1200 compounds, is shown in Figure 6. As the number of compounds per extract increases, the optimal number of major fractions increases for both methods. The number of synergists has little effect on the optimal number of fractions for the additive method, while an increasing number of synergists requires an increasing number of fractions for the subtractive method (Figure 6).

DISCUSSION

In regard to insect semiochemicals, there are two major bioassay methods in use today for isolation of components: (1) electrophysiological and (2) behavioral. The electronic recording of voltages at the electroantennogram, single-sensillum, or single-cell level has the advantage that it is consistently reproducible and more quantitative than behavioral observations. On the other hand, behavioral tests by their very nature are more descriptive of the actual phenomena of interest. Single-sensillum and single-cell electrophysiological recordings are dependent on the neuronal entities being tested. Of the literally thousands of sensillar cells on an insect (e.g., locusts: Greenwood and Chapman, 1984; Blaney and Simmonds, 1990), it is quite possible that various sensilla involved in a particular behavior could be overlooked and thus not probed. For example, in the moth, *Agrotis segetum*, of the receptor cells responding to one of the three pheromone components, only 2% were specialized to the Z9–14 : Ac component (Van Der Pers and Löfstedt, 1986).

Another disadvantage is that some insects such as locusts (Blaney and Simmonds, 1990) and bark beetles (Dickens et al., 1985; Lanne et al., 1987) contain either many generalist receptors or (more likely) many different kinds of more specialized receptors, since many chemicals elicit voltage potentials in the electroantennogram. This makes it more difficult to determine the importance of candidate chemicals to a specific behavior. Furthermore, synergism among pheromone components is not generally observed at the peripheral, antennal level, but in the realm of the brain (Mustaparta et al., 1980; cf. Byers, 1989; cf. Baker, 1989). The electrophysiological techniques thus are not truly rigorous when it comes to isolation of a pheromone that is usually a blend of synergistic chemicals (Silverstein and Young, 1976; Silverstein, 1981).

Using a computer program (Byers, 1992), I searched the titles, key words, and abstracts of all articles in the *Journal of Chemical Ecology* in 1990 (281 papers downloaded from BIOSIS Previews). Of the 182 papers on insects (65%), about 93 studies identified compounds in biological extracts. Of these papers, 34 studies primarily described chemicals found in extracts without ascribing bioactivity to individual compounds (e.g., Brown et al., 1990), eight studies used a combination of GC and electroantennogram (EAG) methods (e.g., Millar et al., 1990), while 51 studies identified one or more compounds and reported their biological activity. However, the majority of these latter studies appear to have selected compounds for bioassay based on whether they were: (1) major constituents of the extract (e.g., Riba et al., 1990; Sreng, 1990), (2) unique to male or female (e.g., Duffield et al., 1990), or (3) previously known to have activity in other species (e.g., Briggs, 1990). Thus many of these studies can be criticized as being biased since they do not treat all compounds equally but single out certain ones for special treatment—with the probability that many

ecologically relevant compounds are overlooked (until possibly a later study). Less than half the studies fractionated extracts, and it appears that very few reports used additive- or subtractive-combination methods to rigorously test for synergists. A search of all 281 papers also found that only 12 mentioned synergist, synergism, or synergistic. Of these, only six papers actually identified chemicals (2%)—three used additive or subtractive fractionation methods and three used comparative methods. Therefore, this indicates there is a need for a better understanding of the rationale and methods for isolation of semiochemicals so that more rigorous and complete studies can be done in the future.

The binary splitting method of fractionation has been used since the beginning of isolation studies. The fractions were not always split in two equal parts but into two solvent fractions with different polarities, or each "active" fraction was successively fractionated (e.g., Imai et al., 1990). The general assumption was that one chemical was responsible for the biological phenomenon (or alternatively that all synergists would fortuitously be contained in one fraction). This assumption is evident in work on isolation of insect host stimulants (e.g., in 1985: Dicke et al., 1985; Stubbs et al., 1985; McKibben et al., 1985; and in 1990: Shu et al., 1990) and ovipositional stimulants (e.g., in 1985: Maeshima et al., 1985; Kim et al., 1985; Hanula et al., 1985; and in 1990: Honda, 1990; Takahashi et al., 1990). The bioassay problem with host stimulants is not as severe as with pheromone components since quite often the former each have significant activity alone, although additive. Many times researchers have assumed, often correctly, that the chemicals with the largest quantities were bioactive, e.g., in isolation of locust pheromones (Nolte et al., 1973; Fuzeau-Braesch et al., 1988). The lack of tests for synergism may be one reason that locust pheromones have yet to be identified satisfactorily (cf. Byers, 1991).

With the finding that most insect pheromones consisted of two or more synergists, it becomes necessary to modify the binary method as in Figure 1. However, I was unable to find any use of the modified binary method in articles in the *Journal of Chemical Ecology*. The method is efficient in terms of bioassay tests required (Figure 1), but the large number of chromatographic passes (e.g., 11) is a disadvantage. At present, most insect pheromones are isolated with the use of GC, and multiple passes often cause the loss of biological activity due to gradual thermal breakdown of the compounds and to their adsorption on glass walls and chromatographic packing.

The additive- and subtractive-combination methods are both efficient in terms of fewer chromatographic passes (e.g., two to four passes, Figure 2). However, the subtractive-combination method requires fewer tests to isolate from two to five synergists in all possible ways to initially fractionate (Figure 4). There also is very little difference in the number of required tests regardless of the number of synergists that must be isolated, in contrast to the additive method where large differences occur (Figure 4). The number of compounds in

the extract affects the number of tests required in both methods, but the effect is much larger with the additive method (Figure 5).

The inflection points (troughs) in the curves are the optimal fractionation numbers, and they have been used in the power regressions in Figure 6. It is apparent that for the additive method there should be no less than five major fractions and no more than about the optimal number. For the subtractive method, the number of major fractions is not too important above five fractions. The optimal number (Figure 6) is, in fact, not critical since the curves are almost flat (Figure 5). The chemist must compromise between what is reasonable to fractionate (lower number of fractions) and the optimal number (usually a higher number). Of course, extracts with compounds that substitute for one another in activity will require fewer tests (with any method) to find a blend that is competitive in nature.

One of the more commonly used techniques for isolation of semiochemicals is called "differential diagnosis" (Vité and Renwick, 1971). With this method, the chemical elution patterns from the GC are compared between the sexes for unique differences. For example, the compounds found in male *Ips paraconfusus*, and which were not in the female, would be obtained commercially, by synthesis, or by up-scaling the isolation and then tested in the field. However, there are three major problems with this approach: (1) a unique pheromone component may not be discriminated from other compounds with the present GC column, (2) a pheromone component may be present in both sexes (e.g., *cis*-verbenol in *I. paraconfusus*; Renwick et al., 1976) and (3) the pheromone may consist of synergistic components, some of which are not unique or distinguishable, so that an incomplete blend would be tested with consequent failure to observe full activity.

In spite of these restrictions, the differential diagnosis method has allowed isolation of many pheromone components with a minimum of work, albeit with the consequence of some incomplete blends. Differential diagnosis can be used in conjunction with the subtractive-combination method. For example, Byers et al. (1990a) used subtractive tests to isolate two synergistic pheromone components of *P. chalcographus*, but one of the synergists could not be resolved from coeluting compounds on the GC column used. Thus the fraction, and adjoining fractions, containing the synergist (bioassay activity) was passed through a second GC column of different polarity in order to separate the compounds differently and obtain new fractions. Subtractive tests with the new fractions located the synergist in one of the fractions. Differential diagnosis using GC-MS extracted ion current profiles then was used to compare the two fractions with bioassay activity from both GC columns to pinpoint the synergist that was common to both of the fractions.

In addition to chromatographic fractionation or differential diagnosis in conjunction with behavioral or electrophysiological bioassay, there is the widely

used method of screening chemicals. The idea is to test previously identified pheromone components of closely related species on the species of interest (Booij and Voerman, 1985). This method is obviously the least rigorous of all but still can yield information, albeit often incomplete, on the pheromone components of a species. Of course the method relies on the use of the more rigorous chemical-fractionation and bioassay methods to identify the components in other related species. The screening method can be used in conjunction with chemical analyses of the insect to narrow the range of possibilities.

One problem with the screening method, and to a lesser extent with all methods, is that the quantities of component release must be close to natural rates in order to obtain an appropriate biological effect. Otherwise it is possible to either have too little (no response) or too much (confusion, inhibition, disruption). The ratio of the synergistic components may be very important as in the case of the *E* and *Z* isomers of straight chain hydrocarbons in moths (e.g., *Argyrotaenia velutinana*, Baker et al., 1976) or much less critical as in some bark beetles (*D. brevicomis*, Byers, 1988; *I. typographus*, Schlyter et al., 1987). De Jong (1987) describes a "direct search" method for determining the best blend of components given that the components are already identified. It is generally accepted that the optimal blend of components is that which approximates the ratio emitted by the organisms in nature (Cardé and Baker, 1984).

During the isolation study, or even when screening is done, the chemical amounts released should be known. The release rates of components in an extract or solvent fraction should be adjusted so that they reproduce approximately the rates of natural release that have been shown to be active. The diffusion-dilution method, where chemicals are released according to their mole percent in the solvent and the dimensions of the holding tube, is useful for laboratory and field bioassay (Byers, 1988). Other methods of adsorbents—wicks, capillary tubes, and semipermeable plastic membranes—have been used (cf. Byers, 1988; and review papers in Jutsum and Gordon, 1989; and Ridgway et al., 1990). Bengtsson et al. (1990) discusses a method for estimating the release of volatiles from adsorbents based on their vapor pressure.

Once the semiochemical components have been isolated and identified in the laboratory, it is important to verify their activity in the field. Ideally, the release rate should parallel the expected or measured rates of natural release (Byers et al., 1985). Usually, however, a range of rates that are expected to overlap the natural rate are used (Schlyter et al., 1987; Byers et al., 1988). As the release rate is increased, there is a point when activity begins (e.g., catch increases above the control), and this rate is assumed to be similar to the natural one. Of course, it is possible to erroneously conclude that a compound has activity when it does not if the compound is released at such high levels that minor impurities (such as pheromone components) elicit the behavior.

Field tests to confirm the activity of isolated semiochemical components

are also sometimes difficult to interpret due to the natural variation in population density with respect to trap position and time (Payne et al., 1978). In many previous studies a line of traps was fixed in the field and treatments were randomized to confirm the semiochemical identification (e.g., Byers et al., 1985). In spite of the replication and randomization, it is possible to have nonhomogeneous variation, thus often invalidating the statistical tests used. A recent field test method, the mechanical slow rotation of a pair of traps, evens the catch variation in position and time to allow more powerful statistics and more sensitive discrimination of semiochemical blends (Byers et al., 1990b).

The subtractive-combination method provides the most efficient fractionation and bioassay plan for the rigorous isolation and identification of semiochemicals. The program for determining the optimal number of major fractions based on assumptions of number of compounds and synergists is available from the author for IBM-compatible personal computers (send a formatted disk and $5 for shipping).

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COMPARISON OF VOLATILES FROM BEETLE-TRANSMITTED *Ceratocystis fagacearum* AND FOUR NON-INSECT-DEPENDENT FUNGI

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Abstract—*Ceratocystis fagacearum* (Bretz) Hunt is the causative agent of oak wilt disease, which is transmitted primarily by nitidulid beetles. This fungus was compared with four non-insect-dependent fungi for their volatile profiles using gas chromatography and mass spectrometry (GC-MS) and for their attractiveness to nitidulids using a wind-tunnel bioassay. The four additional fungi included *Xerula radicata* Sing, *Pluteus atricapillus* Kumm, *Tyromyces chioneus* Karst, and *Botrytis cinerea*. Nitidulids have been reported in association with each of these fungi, but unlike *C. fagacearum*, they are dispersed primarily by wind or rain. Significant attraction of three nitidulid species, *Carpophilus hemipterus* (Linne), *C. lugubris* Murray, and *Stelidota geminata* (Say) was elicited by *C. fagacearum* and to a lesser extent by *X. radicata*, but not by the others. A comparison of headspace volatile profiles showed that the odor of *C. fagacearum* was the strongest, both with regard to the number of components and in their rates of production. Chemical characterization of the headspace profile of *C. fagacearum* revealed 16 components: one aldehyde, one ketone, five alcohols, and nine esters. These components were all common fruit-odor constituents and many of them were previously shown to be attractive to nitidulid beetles. The results of this study suggest that, by mimicking food odors, *C. fagacearum* odor is an adaptation for attracting nitidulid and possibly other insect vectors.

Key Words—Oak wilt, *Ceratocystis fagacearum*, headspace volatiles, Coleoptera, Nitidulidae, *Carpophilus hemipterus*, *Carpophilus lugubris*, *Stelidota geminata*.

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INTRODUCTION

Oak wilt, caused by the fungus, *Ceratocystis fagacearum* (Bretz) Hunt, is a severe disease of oak trees and occurs over much of the United States (True et al., 1960; Gibbs and French, 1980; Appel et al., 1985). Insect vectors play an important role in its aboveground transmission by initiating new infection centers (Gibbs and French, 1980), and activity of nitidulid beetles is considered one of the three major factors responsible for the spread of oak wilt (Appel et al., 1987). Since the transmission of oak wilt by nitidulid beetles was first demonstrated (Dorsey et al., 1953; Norris, 1953), many studies have focused on the occurrence of nitidulid beetles in oak-wilt areas and the transmission of this disease to healthy trees (Dorsey and Leach, 1956; Skalbeck, 1976; Juzwik and French, 1983, 1985; Appel et al., 1986, 1987). *C. fagacearum* forms mycelial mats under the bark of oak trees that generate sufficient pressure to split the bark and expose their fruiting surface (Dorsey and Leach, 1956). The mycelial mats produce sticky spores that can not be transmitted by wind or rain (Dorsey and Leach, 1956; Collins and Kalnins, 1965); spores are characterized by a strong fruity odor, which is detectable around infected trees (Collins and Kalnins, 1965; Skalbeck, 1976). To better understand the relationship between *C. fagacearum* and nitidulids, we compared the volatile profile and attractiveness of *C. fagacearum* with those of four other wood-infecting, but non-nitidulid-dependent fungi.

METHODS AND MATERIALS

Fungal Cultures. A culture of oak-wilt fungus, *Ceratocystis fagacearum* (Bretz) Hunt, was initiated with an isolate from Department of Plant Pathology and Physiology, Virginia Polytechnic Institute and State University (Blacksburg, Virginia). *Xerula radicata* Sing, *Pluteus atricapillus* Kumm, and *Tyromyces chioneus* Karst were initiated with isolates from the Center for Forest Mycology Research, USDA (Madison, Wisconsin), and *Botrytis cinerea* was obtained from the Department of Plant Pathology, OARDC/The Ohio State University. All fungi were maintained on potato-dextrose agar (PDA) medium (BBL Microbiology System, Cockeysville, Maryland) at room temperature and were transferred every two to three weeks. Fungal cultures used for headspace-volatile collection and for bioassay were grown on the same medium in 240-ml Qorpak straight-sided jars at 24–26°C until at least $\frac{3}{4}$ of the medium surface was covered by fungal mycelia. Jars with PDA medium alone were incubated under the same condition to serve as the control. The lid of each jar was equipped with a 0.2-μm Bacterial Air Vent (Gelman Science, Ann Arbor, Michigan) for ventilation and a rubber septum (Thomas Scientific) for collection of headspace air samples.

Beetles and Wind-Tunnel Bioassay. Laboratory colonies of *Carpophilus hemipterus* (L.), *C. lugubris* Murray, and *Stelidota geminata* (Say) were maintained on artificial diet (Hall et al., 1978) under the conditions of 24 ± 1°C, 16:8-hr light–dark and 75–85% relative humidity. After eclosion from the pupae, both sexes were held with moisture in 360-ml plastic cups, but without contact with food or food odors. Six- to 7-day-old *C. hemipterus* and *C. lugubris* and 4-day-old *S. geminata* were used for bioassays, and beetles were discarded after each test.

Orientation of nitidulid beetles to fungal cultures and to PDA medium were evaluated in a wind tunnel as previously described (Lin and Phelan, 1991a). Conditions in the wind tunnel were 35–65% relative humidity, 24–27°C, 17 lx light at the wind-tunnel floor and wind speed of 0.5 m/sec. Two jars were placed on the tunnel floor 20 cm apart in the center of the upwind end of the tunnel. One jar was empty and the other contained the odor source; positions of the two jars were rotated between replicates. Ten beetles were released on the floor at the center of the downwind end of the tunnel at a distance of 180 cm from the jars, and those walking or flying to within 10 cm downwind of the jar in 10 min were counted as responders (Phelan and Lin, 1991). *S. geminata* was released at a distance of 120 cm from the jars because this beetle moved slower than the two *Carpophilus* species. Percent responses to the odorous and empty jars were compared by *t* test after arcsin-square root transformation.

Volatile Collection and Identification. Headspace-volatile collections followed the previously described method in which volatiles were trapped on Tenax GC and then thermally desorbed to a gas chromatograph (Phelan and Lin, 1991). Jars containing fungal cultures were opened for 3 min prior to collecting headspace samples. For a comparison of the volatile constituents of different fungi, a 20-ml headspace sample was injected into the Tenax trap using a gas-tight syringe, followed by a 2-min flush of the trap with helium (20 ml/min). The trap then was heated to 200°C and the helium flow through the trap was reversed, such that the retained constituents were desorbed and backflushed to the GC capillary column. This flushing method, used for qualitative comparisons, reduced the amounts of the highly volatile major components, ethanol and acetaldehyde, while enriching the levels of minor components of the profiles (Phelan and Lin, 1991). Volatile constituents were chromatographed using a Hewlett-Packard 5890A gas chromatograph (GC) with a direct interface to a Hewlett-Packard 5970C Mass Selective Detector. DB-FFAP column (30 m × 0.25 mm ID, 0.25 μm film thickness) was used in the among-fungi comparisons of headspace profiles. For a quantitative analysis of the *C. fagacearum* volatile profile, the trap was only flushed for 0.5 min, which removed air from the trap but retained all volatile constituents, as determined by breakthrough studies. Separation was carried out on a DB-1 capillary column (30 m × 0.32 mm ID, 5.0 μm film thickness). GC analyses were temperature-programmed from 30 to

200°C at 10°C/min, with a helium flow rate of 0.7 ml/min on DB-FFAP and 0.9 ml/min on DB-1. Volatile components were identified by comparison of mass spectra and retention times of authentic compounds. Amounts were peak areas with those of known quantities of authentic compounds. All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin).

RESULTS

Beetle Attraction. C. fagacearum elicited significant levels of up-tunnel attraction in all three nitidulid species (Figure 1). PDA medium alone did not attract any beetles, and of the four non-nitidulid-dependent fungi, only *X. radicata* evoked beetle response at a level greater than that to a blank jar (Figure 1).

Comparison of Headspace Volatile Profiles. The volatile profiles of *C. fagacearum*, four non-nitidulid-dependent fungi, and PDA medium are shown in Figure 2. Ten components were identified from the profile of *C. fagacearum* (Figure 2, Table 1). Four of these constituents also were detected from *X. radicata*, and at a lower concentration from *P. atricapillus*: ethanol, 2-methylpropanol, 3-methylbutanol, and 2-methylbutanol. Only ethanol was found in the profiles of *T. chioneus* and PDA medium, and *B. cinerea* emitted none of the *C. fagacearum* components but produced two unidentified later-eluting vol-

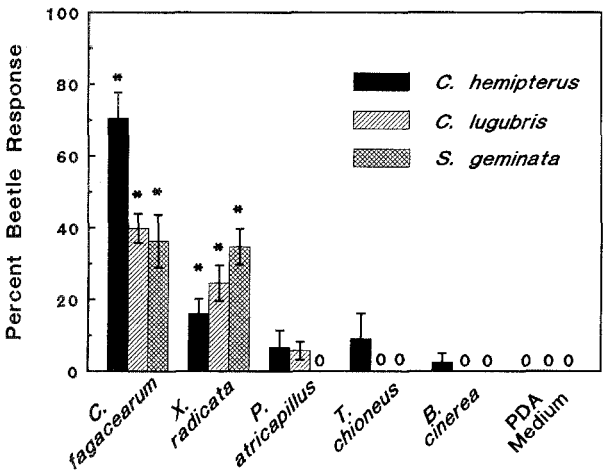

FIG. 1. Percent orientation ($\bar{X} \pm$ SEM) by *Carpophilus hemipterus*, *C. lugubris*, and *Stelidota geminata* to fungal cultures in a wind tunnel. N = four groups of 10 beetles. Bars marked with an asterisk indicate response significantly greater than that to a blank jar to which beetles never responded (Student's t test, $P < 0.05$).

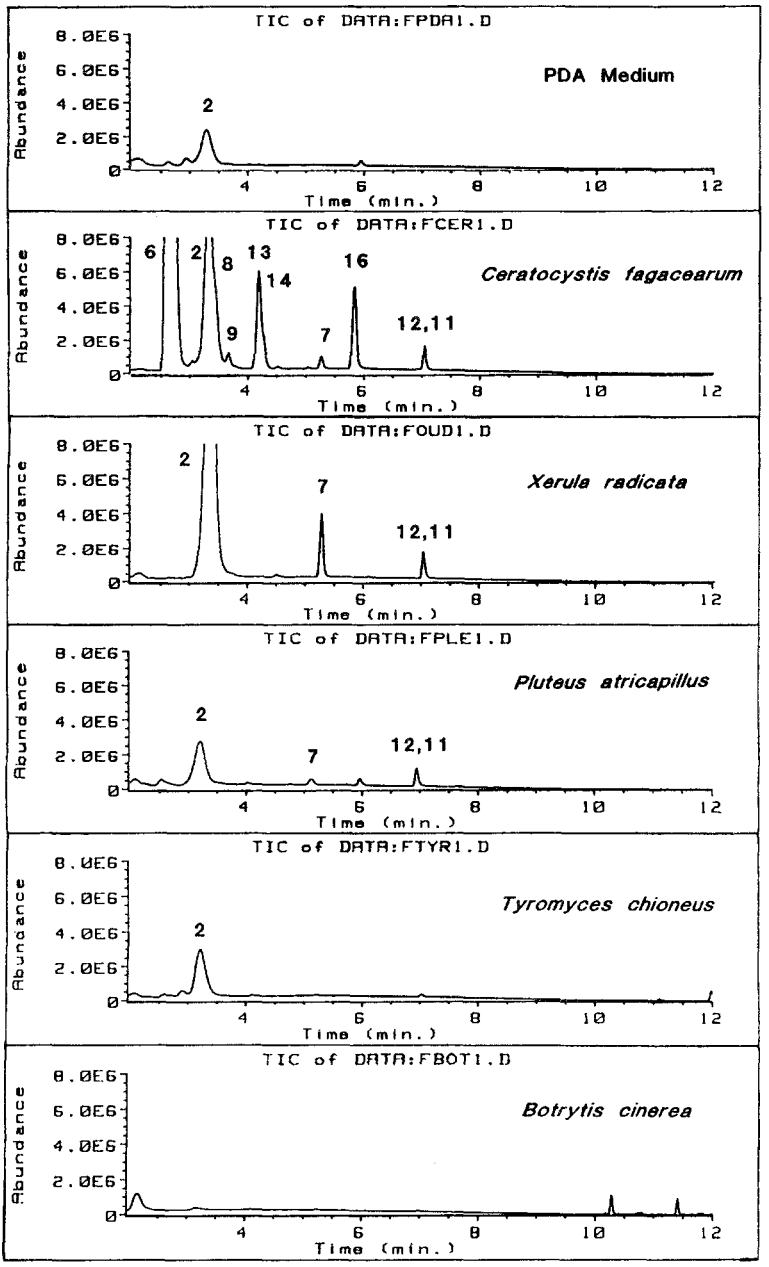

FIG. 2. Comparison of the volatile profiles of five fungi on PDA medium and of PDA medium alone. Note that the profiles were analyzed on DB-FFAP capillary column; component numbering follows Table 1, which was based on an analysis using DB-1. See text for explanation of methods for volatile collection and analysis.

TABLE 1. CHEMICAL CHARACTERIZATION AND QUANTIFICATION OF HEADSPACE ABOVE *Ceratocystis fagacearum* ON PDA

No.	Identity	*RT*[a]	Mass spectra (*m/z*)[b]	Amount (ng/ml)[c]	Percent[c]
1	Acetaldehyde	0.5	29, 41, 43, 44	<0.1	<0.1
2	Ethanol	1.4	27, 29, 31, 45, 46	4.0	2.8
3	Methyl acetate	2.9	29, 42, 43, 59, 74	0.4	0.3
4	1-Propanol	3.6	27, 29, 31, 42, 59, 60	0.1	0.1
5	2-Butanone	4.3	27, 29, 43, 57, 72	0.2	0.2
6	Ethyl acetate	4.9	43, 45, 61, 70, 73, 88	101.0	70.0
7	2-Methylpropanol	5.3	31, 33, 41, 42, 43, 55, 74	4.3	3.0
8	Ethyl propionate	7.3	29, 45, 57, 74, 75, 102	2.4[d]	1.7
9	Propyl acetate	7.4	27, 31, 41, 42, 43, 61, 73		
10	Methyl butyrate	7.6	27, 41, 43, 59, 71, 74, 87	0.1	0.1
11	3-Methylbutanol	7.9	41, 42, 43, 55, 56, 57, 70	8.7[d]	6.0
12	2-Methylbutanol	8.0	29, 41, 55, 56, 57, 70		
13	Isobutyl acetate	8.6	27, 29, 41, 43, 56, 62, 73	5.0	3.4
14	Methyl isovalerate[e]	9.0	39, 41, 43, 57, 59, 74, 85	6.1	4.2
15	Butyl acetate	9.8	27, 29, 41, 43, 56, 61, 73	0.3	0.2
16	Isopentyl acetate	11.2	39, 41, 43, 55, 61, 70, 87	11.9	8.2

[a] *RT* = retention time in minutes eluted from DB-1 column.
[b] Major ions of mass spectra, with the base ions underlined.
[c] Mean of three replicates (ng/ml headspace air sample, and percent of the total profile).
[d] Components were combined due to their unresolved GC peaks. Estimated ratio of ethyl propionate and propyl acetate is 1 : 1 and that of 3-methylbutanol and 2-methylbutanol is 4 : 1.
[e] Authentic compound was unavailable, identification based on mass spectrum alone.

atiles. Thus, the volatile profile of *C. fagacearum* appeared quantitatively and qualitatively richer than did those of the non-beetle-dependent fungi.

Characterization of Headspace Profile of C. fagacearum. A more extensive analysis of *C. fagacearum* odor aimed at quantifying individual components of the volatile profile revealed six additional minor components ($\leq 0.3\%$ of total profile; Table 1, Figure 3). Ethyl acetate was the most abundant component of *C. fagacearum* odor; its headspace concentration was 101.0 ng/ml and it made up 70.0% of the odor. Concentrations of other odor constituents ranged from <0.1 (acetaldehyde) to 11.9 ng/ml (isopentyl acetate).

DISCUSSION

Nitidulids have been reported in association with a number of plant pathogens. Because of their sap-feeding habit, those beetles that come to feed on fungal mycelial mats or infected tissues pick up pathogens and may transmit

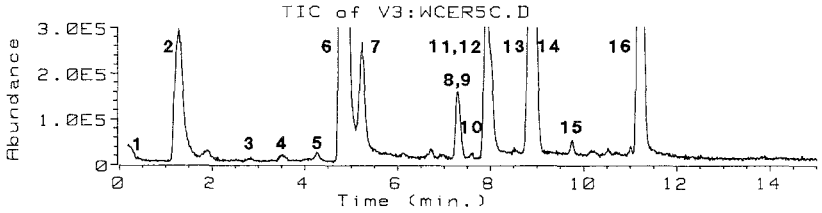

FIG. 3. Volatile profile from oak-wilt fungus, *C. fagacearum* on DB-1. See Table 1 for identity of peaks, and see text for explanation of methods for volatile collection and analysis.

them to healthy trees or fruits when they visit fresh wounds or ripening fruits. Nitidulids are important vectors of *Ceratocystis* canker of aspen in Colorado (Hind, 1972) and of almond in California (Moller and DeVay, 1968). In Hawaii, several species of nitidulids were proven to be important vectors of pineapple disease in sugarcane caused by *C. paradoxa* (Chang and Jensen, 1974). Nitidulids also were reported in association with *Monilinia fructicola*, the causative agent of brown rot in stone fruit in California (Tate and Ogawa, 1975), with *Fusarium* pathogens of corn (Windels et al., 1976; Attwater and Busch, 1983), and with yeasts responsible for diseases of figs (Miller and Mrak, 1953). Juzwik and French (1983) demonstrated that a seasonal peak for nitidulids coincided with the time of maximum mat production for *C. fagacearum*. Furthermore, both adults and larvae of *Glischrochilus* species have been observed feeding on mycelial mats of *C. fagacearum* in the field and adults may overwinter underneath these mats (Dorsey and Leach, 1956). *C. fagacearum* is hermaphroditic but self-sterile and, in addition to inoculating new hosts, nitidulids moving between mats bring about sexual reproduction by fertilizing receptive hyphae of one mat with endoconidia from another (Dorsey and Leach, 1956).

If this association represents a form of mutualism, it is not an obligatory one for the beetles. Nitidulids are not dependent on fungi as they can feed on a variety of ripening or decaying fruits and plant tissues; however, fungal infection does increase the attractiveness or even the nutritional value of food substrates for some nitidulids. *C. paradoxa*-infected sugarcane stalks were nine times more attractive to nitidulid beetles than healthy stalks in the field, and larvae of the nitidulid *Urophorus humeralis* reared on a diet of mixed sugarcane juice and the pathogen developed faster and gained more weight than those on sugarcane juice only (Chang and Jensen, 1974). Similarly, adults of *C. hemipterus* showed higher attraction to yeast-inoculated banana than to aseptic banana, and larvae feeding on yeast-inoculated banana developed faster and gained more weight than those on the aseptic substrate (Lin and Phelan, in preparation, a).

Our chemical characterization of *C. fagacearum* odor revealed 16 components including one aldehyde, one ketone, five alcohols, and nine esters (Table

1). Collins and Kalnins (1965) previously identified four aldehydes and one ketone from steam distillates of *C. fagacearum*; of these, only acetaldehyde was detected in the present study. All of the 16 components identified from the headspace profile of *C. fagacearum* have been found previously in the odors from various food substrates (Jennings, 1977; Macku and Jennings, 1987; Maarse and Visscher, 1988; Phelan and Lin, 1991; Lin and Phelan, 1991a), and 11 of them have been tested at natural release rates with one or more nitidulid species: *C. lugubris* (Lin and Phelan, 1991a), *C. hemipterus* (Phelan and Lin, 1991), *Glischrochilus quadrisignatus* and *G. fasciatus* (Lin and Phelan, 1991b), and *Stelidota geminata* (Lin and Phelan, in preparation, b). Acetaldehyde and ethyl acetate are essential components of attractive odor blends for all these species, except *C. lugubris*, for which ethyl acetate can be partially replaced by ethanol. The five alcohols present in the profile of *C. fagacearum* are attractive to these beetles except 2-methylpropanol and 3-methylbutanol, which have a negative effect on the response of *S. geminata*. Butyl acetate and isopentyl acetate are components of blends attractive to *S. geminata* , while isobutyl acetate has a negative effect on response (Lin and Phelan, in preparation, b). We have found ethanol to be attractive to all of the above species except *C. hemipterus* (Phelan and Lin, 1991). Alm et al. (1985, 1986, 1989) demonstrated that butyl acetate was attractive to *G. quadrisignatus* in the field but only at unnaturally high release rates (70–2000 mg/24 hr). Other components identified from the odor of *C. fagacearum* either have not been tested or have no significant effect on the nitidulid response.

In contrast to *C. fagacearum*, the volatile profiles of the other fungi in our studies were rather depauperate. These fungi were chosen for comparison because they are primarily dispersed by wind or rain, they can infect woody tissue, and they have been reported in association with nitidulids. *S. geminata* has been collected from *X. radicata*, *P. atricapillus*, and *T. chioneus* in nature (Weiss and West, 1921), and Wildman (1933) reported increased capture of nitidulids when cooked figs or peaches were inoculated with a *Botrytis* sp. However, the results of this study suggest that when these fungi are placed on agar, they elicit little or no attraction of nitidulids and generally do not produce attractive volatile blends. Of the non-insect-dependent fungi, only *X. radicata* elicited attraction at a level significantly greater than a blank jar, and this response was less than that to *C. fagacearum*.

We have contended previously that fungi generally enhance attraction of nitidulids through a simple increase in the release of volatiles common to fruit substrates rather through a qualitative change in the odor profile (Phelan and Lin, 1991). This quantitative change is likely brought about by some combination of increased fruit-cell lysis and/or fungal catabolism of fruit constituents that parallels processes characteristic of fruit ripening. Our results here support this contention for non-insect-dependent fungi inasmuch as such fungi involved

in the present study produced no or only small amounts of fruity volatiles in the absence of fruit. In contrast, *C. fagacearum*, a fungus dependent on insects for transmission of spores and for sexual reproduction, produced an odor blend that was both quantitatively and qualitatively rich when cultured on agar. We would submit that production of this more complex odor profile may represent an adaptation on obligatory dispersal by insects.

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HONEYBEE NESTMATE RECOGNITION: EFFECTS OF QUEEN FECAL PHEROMONES

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Abstract—Previous work has shown that queen honeybees, *Apis mellifera*, produce waxy esters composed of 8–14 carbon acids and 6–14 carbon alcohols in their feces. We tested these esters for effects on nestmate recognition; 11 of the 12 esters tested significantly modified the recognition characteristics of worker honeybees. Pairwise tests showed that workers can discriminate between at least some pairs of queen esters and that workers can discriminate between a queen ester and hexadecane (another known nestmate recognition cue). These results suggest that a queen may use the esters to enable workers to recognize her or to scent-mark her colony.

Key Words—*Apis mellifera*, honeybee, Hymenoptera, Apidae, queen, fecal pheromones, esters, kin recognition.

INTRODUCTION

We tested the hypothesis that esters found in the feces of queen honeybees can modify the recognitive characteristics of their workers. Queens produce these compounds, but their glandular origins and possible functions are unknown. Blum et al. (1983) found six waxy esters in association with the sting apparatus of mated queens. Page et al. (1988; see also Blum and Fales, 1988) found the

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same esters, plus a number of similar compounds, in virgin queen feces. The acid moiety of these esters varies from 10 to 16 carbons and the alcohol from 6 to 14 carbons; it is the long chain length of the alcohol groups that makes these compounds unusual natural products (Blum et al., 1983; Blum and Fales, unpublished). One of these esters, decyl decanoate, is found on the tergal glands of honeybee queens (Espelie et al., 1990). A nonester in queen feces, *o*-aminoacetophenone, repels caged workers during fights between virgin queens, but no function has been suggested for the esters (Page et al., 1988).

Social insect queens often produce chemical signatures that identify themselves individually or that label the worker population in the colony (Breed and Bennett, 1987; Hölldobler and Wilson, 1990). Colony labels produced by the queen have been suggested in a number of ants (e.g., Carlin and Hölldobler, 1986) but have not been demonstrated in bees, termites, or wasps. Honeybee queens can be individually discriminated by workers; unfamiliar queens are attacked by workers in the colony (Breed, 1981). The identity of a queen can be learned by young workers, regardless of the genetic identity between the queen and workers (Breed, 1981). The chemical cues carried by genetically related queens appear to be similar (Breed, 1981; Boch and Morse, 1982; Page and Erickson, 1986), indicating that queens produce recognitive cues themselves.

Moritz and Crewe (1988) found that extracts of compounds from queen heads and tergal glands were less variable among closely related queens. In a metabolic activity, bioassay workers could not distinguish among head extracts but could distinguish among tergal extracts. This finding led Moritz and Crewe (1988) to conclude that tergal gland secretions might be a source of queen recognition cues.

In our tests, we first exposed workers to individual esters that are produced by queens, and then used a bioassay to determine if the exposure had affected the recognitive characteristics of the workers (Breed, 1983; Breed et al., 1985, 1988). All of the esters known to be produced by queens were used. Because the esters are structurally quite similar (similar chain lengths, identical functional groups), we also tested whether workers could, in fact, discriminate among esters. Because the large number ($N = 12$) of candidate esters made testing of all possible pairs unrealistic;, we paired esters to determine if overall carbon number affected the detectability of differences.

METHODS AND MATERIALS

The effects of queen esters on worker recognition were tested following the methods of Breed (1981, 1983) and Breed and Stiller (1992). Upon emergence, worker bees were established in 0.45-liter cardboard containers in the

laboratory with food and water provided ad libitum. Compounds were synthesized in the laboratories of M.S. Blum or T.H. Jones. The following esters are present in queen feces and were used in these experiments: hexyl decanoate, hexyl dodecanoate, decyl octanoate, decyl decanoate, octyl dodecanoate, hexyl tetradecanoate, octyl tetradecanoate, decyl dodecanoate, dodecyl decanoate, tetradecyl decanoate, tetradecyl dodecanoate, and hexadecyl decanoate. Statistical analyses were done on a Macintosh II computer using the Statview statistical package.

Effects of Queen Esters. To test the effects of the queen esters on recognition, 10 μg per bee of a compound were absorbed on filter paper and placed into a container (termed the source container) with 10 workers. After five days the workers were removed from the source container, one at a time, and placed into recipient containers with 10 bees each. Each source bee was used only once, and each recipient container was used only once. All the workers used in any given experiment emerged from the same brood frame and were half- or supersisters. The behavioral interactions were observed between the source and recipient bees for 5 min. An "attack" was recorded if, during this time, the source bee was bitten or stung. If not, an "acceptance" was recorded. All experiments were conducted blind; the observer did not know whether the source group had been treated or was a control. Control containers received filter paper but no compound in either container. We attempted to obtain a sample size of 50 for each ester.

Discrimination between Hexadecane and a Queen Ester. These tests were conducted using the methodology described above, but the source container received 10 μg per bee of hexadecane (Sigma) and the recipient container 10 μg per bee of hexadecyl decanoate. This quantity of hexadecane has the same magnitude of effect on recognition as the esters reported in this paper (Breed and Stiller, 1992).

Discriminations among Queen Esters. Pairwise combinations of queen esters were tested to determine if workers could distinguish between esters. These tests were conducted using the methodology described above, except that the donor and recipient containers both received 10 μg per bee of compound. In the experimental containers, the source container received one compound on filter paper and the recipient another. Controls received the same compound in both source and recipient containers. As not all 144 pairs of esters could be tested, the experimental design included two pairs with identical alcohol moieties, four with identical acid moieties, some with similar molecular weights, and some with very different molecular weights. We attempted to obtain a sample size of 40 for each pair of esters tested.

Control for Concentration Effect. Because the same quantity of each ester was used in the preceding experiment, bees could have discriminated on the basis of concentration, rather than structural differences between compounds.

As a control for the effect of concentration, one ester at the lower end of the molecular weight range of the compounds (decyl decanoate) and one ester at the high end of the range (tetradecyl dodecanoate) were presented to groups of bees. Bees receiving decyl decanoate were treated with 10 μg per bee; bees receiving tetradecyl dodecanoate received the molar equivalent. The experimental methodology was the same as in the preceding experiments.

RESULTS

Effects of Queen Esters. All of the esters tested except octyl tetradecanoate had significant effects on the acceptability of sister bees (Table 1). There is some variation in the magnitude of the effect among those with significant effects. Hexyl tetradecanoate was most similar to the controls and tetradecyl decanoate was least similar. There is no relationship between molecular weight and magnitude of effect. However, the lower-molecular-weight esters caused some mortality among the workers at the concentration used in these experiments. Seventy-one percent of the bees exposed to hexyl decanoate died, as compared to a mortality rate of 0–10% of bees observed in treatments with esters of 20 or more carbons.

Discrimination between Hexadecane and a Queen Ester. A representative queen ester, hexadecyl decanoate, was tested to determine if it could be distin-

TABLE 1. EFFECT OF ESTERS ON RECOGNITION[a]

	N	Attacks (%)	*P*
Hexyl decanoate (16)	54	64.8	0.0001
Hexyl dodecanoate (18)	77	40.3	0.0001
Decyl octanoate (18)	48	45.8	0.0001
Decyl decanoate (20)	58	50	0.0001
Octyl dodecanoate (20)	71	40.8	0.0002
Hexyl tetradecanoate (20)	46	34.8	0.03
Octyl tetradecanoate (22)	75	29.3	NS
Decyl dodecanoate (22)	61	54.1	0.0001
Dodecyl decanoate (22)	56	44.6	0.0001
Tetradecyl decanoate (24)	52	67.3	0.0001
Tetradecyl dodecanoate (26)	77	44.2	0.0001
Hexadecyl decanoate (26)	67	64.2	0.0001
Control	1141	21.5	

[a]Percent of bees rejected when the donor group was treated with the indicated compound and the recipient group received no compound. In the controls neither group received a compound. The number of carbons in each compound is indicated in parentheses. Probabilities are based on χ^2 comparisons between treatments and controls.

guished from hexadecane, an alkane known to affect nestmate recognition in honeybees (Breed and Stiller, 1992). Sixty percent of 87 source bees treated with hexadecane were attacked when placed into containers of sister bees treated with hexadecyl decanoate. This is significantly different ($\chi^2 = 41.75$, $P = 0.0001$) from the controls (12%, $N = 83$).

Discriminations among Queen Esters. Significant differences were found in six of 13 pairs tested between experimentals, in which the source group received ester 1 and the recipient group received ester 2, and controls, in which both groups received ester 1 (Table 2). No patterns were found relating overall compound size or chain length of either the acid or alcohol groups in determining whether discriminations are made (Table 2). Some quite similar pairs, e.g., tetradecyl dodecanoate and tetradecyl decanoate, are discriminated, while some quite dissimilar pairs, e.g., decyl octanoate and tetradecyl dodecanoate are not discriminated.

There were relatively few attacks in the treatments in which both groups received the same ester. Pooled across compounds, only 13.5% ($N = 510$) of these pairings resulted in attacks, which is significantly less ($\chi^2 = 23.92$, $P = 0.0001$) than the percentage of attacks in a sample of controls (Breed et al., 1992) in which no compound was present in either group (24.8%, $N = 741$). It is also significantly less than the controls in Table 1 ($\chi^2 = 14.4$, $P = 0.0001$).

Control for Concentration Effect. Bees receiving one of the compounds

TABLE 2. DISCRIMINATION BETWEEN ESTERS[a]

		Experimental		Control		
Ester 1	Ester 2	*N*	Attacks (%)	*N*	Attacks (%)	*P*
Octyl dodecanoate	Decyl decanoate	42	9.5	41	7.3	NS
Tetradecyl dodecanoate	Hexadecyl decanoate	46	13.0	45	15.5	NS
Hexyl tetradecanoate	Decyl octanoate	44	29.5	45	11.1	0.03
Decyl octanoate	Decyl decanoate	30	16.7	29	10.3	NS
Tetradecyl dodecanoate	Tetradecyl decanoate	35	42.8	37	16.2	0.008
Octyl tetradecanoate	Tetradecyl decanoate	46	65.0	47	17	0.0001
Hexadecyl decanoate	Tetradecyl decanoate	30	13.0	30	13.0	NS
Decyl decanoate	Tetradecyl decanoate	32	21.9	33	27.3	NS
Decyl decanoate	Tetradecyl dodecanoate	33	70	52	17	0.0001
Hexyl decanoate	Tetradecyl decanoate	30	50	39	13	0.0007
Hexadecyl decanoate	Decyl octanoate	38	42.1	37	2.7	0.0067
Hexyl dodecanoate	Tetradecyl dodecanoate	41	24	40	12	NS
Decyl octanoate	Tetradecyl dodecanoate	38	16	35	12	NS

[a] Percent of bees injected when the donor group was treated with ester 1 and the recipient group received ester 2. In the controls both groups received the same compound. Probabilities are based on χ^2 comparisons between treatments and controls.

and introduced into a group that had received the other compound were rejected 59% of the time ($N = 54$) while the controls (Table 2) were rejected only 17% of the time ($N = 52$) ($\chi^2 = 19.7$, $P < 0.0001$). This indicates that the discrimination is made even when concentrations are equal.

DISCUSSION

These findings support the conclusion that queen-produced esters can be used by worker honey bees to make discriminations among other bees. The function of these esters is not clearly understood, but these results suggest two possibilities. First, they may be used by the workers to discriminate among queens. Queens are individually recognizable (Breed, 1981), and they produce at least small amounts of these esters throughout their lives (Blum, unpublished). Second, the esters may be used by queens to scent-mark their colonies. Transfer of esters from the queen to the wax combs, or directly to workers, would result in a colony-level recognition signature. Virgin queens seem to produce large amounts of feces (30–100 μl in 48 hr), much of which is composed of these esters (Page, unpublished), but an attempt to quantify the relative amount of each ester in queen feces was not successful (Page and Blum, unpublished). However, the low level of ester production by older queens (Blum, unpublished) supports the first hypothesis, as scent marking should require continuous production of cues as new workers and comb are produced.

While the esters are found in feces, they are probably not normal defecatory products. Worker honeybee feces do not contain the esters, and the compounds are metabolic products, rather than undigested food materials (Page and Blum, unpublished). Our data support a role in recognition for these compounds because of the ability of workers to discriminate among the compounds and the rejection behavior elicited by the compounds. Given the at least partial overlap between fecal and tergal production of these esters (Espelie et al., 1990), our results are potentially consistent with those of Moritz and Crewe (1988) who argued that the tergal glands could be the source of queen recognition pheromones.

Eleven of the 12 esters tested had significant effects on recognition. This contrasts sharply with the findings of Breed and Stiller (1992) that only four of 18 alkanes, methyl esters, and organic acids had such effects. This effect does not vary with overall carbon number, or with carbon numbers in the alcohol or acid groups, through a substantial range of molecular weights. Thus, this is the most consistently effective class of compounds yet discovered in a recognition system.

The mortality caused by the lower-molecular-weight esters suggests a dual role for these compounds. Young queens release feces during fights in cages. One compound in queen feces, *o*-aminoacetophenone, has been shown to repel

workers (Page et al., 1988). The low-molecular-weight esters may also deter workers from entering the fight, or they may function as a chemical weapon against the other queen, or other workers that are antagonistic to the queen.

One pairwise comparison was performed between a queen ester and one of the alkanes, hexadecane, known to affect recognition (Breed and Stiller, 1992). As would be expected with such different structures, workers have no difficulty in discriminating between the two compounds. Based on this, we conclude that the esters can provide recognition cues that are, in general, distinguishable from the cues contained in wax produced by the workers (Breed et al., 1988).

Enough pairs of esters could be discriminated in the pairwise comparisons to support the hypothesis that several distinguishable signals are available among the esters for recognition. The pattern of discrimination is puzzling, as there is no apparent relationship between structural differences and differences demonstrated in our bioassay. This will present an interesting problem for further work on the chemosensory system. There is a possibility that discriminations could be made between pairs of esters based on the amount used, rather than on structural differences in the compounds. All treatments were made at 10 μg per bee; because molecular weight varied among compounds, different numbers of molecules were delivered. However, the difference in concentration is not associated with the pattern of discrimination, and our control for these effects showed no variation due to concentration.

If these esters function in queen recognition, as suggested by these data, then they could provide the basis for discrimination among queens on the basis of relatedness. This could have some significance for intercolonial interactions during swarming (Getz et al., 1982) and/or for male–female interactions during mating. Smith (1983) has shown that recognition signals used for inbreeding avoidance in *Lasioglossum zephyrum*, a halictid bee, are also used in nestmate recognition. Finally, our data suggest a chemical system for genetic recognition of the queen as demonstrated by Breed (1981) and Boch and Morse (1982).

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ELECTROANTENNOGRAM RESPONSES OF THE CABBAGE SEED WEEVIL, *Ceutorhynchus assimilis*, TO OILSEED RAPE, *Brassica napus* ssp. *oleifera*, VOLATILES

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Abstract—Electroantennograms (EAGs) were recorded from male and female cabbage seed weevils (*Ceutorhynchus assimilis* Payk.) in response to volatiles isolated and identified from the odor of oilseed rape (*Brassica napus* ssp. *oleifera* DC. cv. Ariana). Relatively large EAGs were obtained on stimulation with volatiles produced by the oilseed rape crop at the time when seed weevils were actively searching for host plants. Artificial rape odor without certain key volatile compounds was in most cases significantly less stimulatory than odor containing these volatiles. There were significant differences in the EAG response of the sexes of *C. assimilis* to the green leaf volatiles of oilseed rape and several terpenes present in rape flower odor. The importance of the qualitative and quantitative composition of host-plant odor in host location by *C. assimilis* is discussed.

Key Words—*Ceutorhynchus assimilis*, Coleoptera, Curculionidae, *Brassica napus*, semiochemicals, plant volatiles, olfaction, host plant, attractant, electroantennogram.

INTRODUCTION

Previous studies of the analysis and identification of volatiles from brassicas by distillation/extraction are numerous (Buttery et al., 1976; Cole, 1976; Wallbank and Wheatley, 1976; Daxenbichler et al., 1979; Uda et al., 1982; Kondo et al., 1985; MacLeod and MacLeod, 1990). However, few studies have used

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porous polymers (such as Tenax and Porapak) to look at the volatiles actually in the headspace of intact or damaged plants (Finch, 1986). Tenax (or Porapak) headspace sampling of volatiles provides a true reflection of the volatiles an insect is exposed to in the atmosphere around a plant (Buttery et al., 1982) and has been used in many studies to identify plant volatiles that influence insect and mite behavior (Hamilton-Kemp et al., 1988; Auger et al., 1989; Liu et al., 1989; Lugemwa et al., 1989).

Previous studies of the chemistry of oilseed rape (*Brassica napus* ssp. *oleifera* DC.) volatiles have concentrated on single-low cultivars of rape which have a seed content low in erucic acid, but high in glucosinolates. Any changes in the volatiles associated with double-low cultivars of oilseed rape in which the seeds are low in erucic acid and glucosinolates, have not been assessed.

Earlier studies have shown that adult cabbage seed weevils, *Ceutorhynchus assimilis* Payk. (Coleoptera: Curculionidae), an important pest of oilseed brassicas in Europe (Lamb, 1989), are behaviorally responsive to the odor of its host plant (Görnitz, 1953; Finch, 1977; Free and Williams, 1978; Evans and Allen-Williams, 1989; Evans, 1990). Seed weevils have been shown to detect components of oilseed rape odor via antennal receptors both in electroantennogram (EAG) studies (Kozlowski, 1984) and single cell studies (Blight et al., 1989). Blight et al. (1989) have identified specific olfactory receptors on weevil antennae to components of oilseed rape odor.

This investigation was undertaken to identify components of the odor of double-low cultivars of oilseed rape that play a role in the perception and subsequent behavioral response to oilseed rape by *C. assimilis*.

METHODS AND MATERIALS

Chemistry. The volatile compounds emitted by a double-low cultivar of oilseed rape (*B. napus* ssp. *oleifera* cv. Ariana) were collected by adsorption onto Tenax GC (mesh size 60–80, Phase Separations Ltd., Queensferry, Clwyd, U.K.). Glass tubes containing 0.1–0.2 g of Tenax sandwiched between silanized glass wool were used to adsorb volatile compounds drawn through the tubes by a vacuum pump (Charles Royston Pumps Ltd., Byfleet, Surrey, U.K.). The Tenax traps were prepared for use by passing nitrogen through them at a flow rate of 10 ml/min in a Pye 104 gas chromatograph (Pye Unicam Ltd., Cambridge, U.K.) oven heated to 275°C for at least 5 hr (Vanhaelen et al., 1977). This process purges any contaminants from the Tenax, and allows the Tenax to be cleaned and reused.

Tenax entrapment of volatiles from rape flowers and macerated leaves was carried out by drawing air through a glass jar containing the plant material. Tenax entrapment of the volatiles released by a small caged plot of flowering oilseed rape (growth stage 4.5, 5.2; Sylvester-Bradley and Makepeace, 1984) was achieved by suspending a vacuum pump from the roof of the cage and drawing air through a larger Tenax trap (1 g of Tenax) suspended 0.5 m above the center of the crop (approx 2.5 m above the ground) for 12 hr from 0800–2000 hr. The volatiles adsorbed on the Tenax traps were eluted by infusing 500 μl of hexane (HPLC grade, BDH, Poole, England) through the trap in a flow of nitrogen, and the eluate concentrated to a volume of 10–20 μl in a Rotava por-R rotary evaporator (Büchi Laboratoriums-Technik, Switzerland).

Identification of Oilseed Rape Volatiles. The identification of the compounds obtained from plant material by Tenax entrapment was carried out using gas chromatography (GC) and coupled gas chromatography–mass spectrometry (GC-MS). A Pye 204 and a Pye GCD gas chromatograph were used, both equipped with a 1.5-m × 4-mm-ID glass column packed with 2.5% Carbowax 20 M on 100–120 mesh Diatomite CQ (J.J.'s Chromatography Ltd., Kings Lynn, Norfolk, U.K.). GC conditions were as follows: inlet temperature 250°C, column temperature isothermal for 1 min at 80°C and then programmed at 8°C/min to 200°C; flame ionization detector (FID) temperature 250°C, nitrogen carrier velocity 30 ml/min. The output from the FID was directed to a pen recorder and a Shimadzu C-R6A Chromatopac integrator (Dyson Instruments Ltd., Houghton-le-Spring, Tyne and Wear, U.K.). The integrator measured the peak area and retention times for each peak.

The GC-MS analyses were performed on a VG 16F mass spectrometer (VG Micromass Ltd., Winsford, U.K.) operated at an ionization potential of 70 eV, connected to a Pye 104 gas chromatograph, with a BP20 quartz capillary column 25 m × 0.22 mm ID and 0.25 μm film thickness. The GC conditions were as follows: inlet temperature 250°C, isothermal for 5 min at 80°C and then programmed at 8°C/min to 200°C, with a helium carrier velocity of 0.75 ml/min. The mass spectra were analyzed by a VG 2015 computerized Data system (VG Micromass Ltd., Winsford, U.K.).

Compounds obtained from Tenax trapping of plant material were provisionally identified by comparing their retention times to those of authentic compounds previously isolated from rape odor (Tollsten and Bergström, 1988). Confirmation of the identity of these compounds and of any unknown compounds was obtained by comparing the mass spectra of the compounds present in the extracts with previously published spectra (Kjaer et al., 1963; Jennings and Shibamoto, 1980; Spencer and Daxenbichler, 1980; Eagles et al., 1981; Kameoka, 1984, 1986) or with the spectra of authentic compounds.

Insects. Adult cabbage seed weevils (*C. assimilis*) were obtained from a

field of flowering oilseed rape by sweep-netting, segregated by sex, and placed in separate cages. Weevils were isolated from food and plant odor for at least 12 hr before being used.

Olfactory Stimuli. The choice of test compounds was based on those identified from oilseed rape odor (Table 1). Several chemically related compounds were also included. Most of the compounds used were obtained from commercial suppliers (Table 2). However, the compounds 4-pentenyl isothiocyanate and 3-butenyl isothiocyanate were prepared from precursor compounds, and the sesquiterpene hydrocarbon (*E,E*)-α-farnesene was obtained from the natural coating of apples, where it is a major constituent (Murray, 1969).

Both 3-butenyl and 4-pentenyl isothiocyanate were obtained by entrapment on Tenax after enzymatic treatment of the parent glucosinolates 3-butenyl glucosinolate (gluconapin) and 4-pentenyl glucosinolate (glucobrassicanapin) by the enzyme myrosinase (thioglucoside glucohydrolase). The volatiles adsorbed onto the Tenax traps were eluted with 500 μl of hexane and analyzed by GC-MS to confirm the identities of the isothiocyanates present.

The method of extraction of α-farnesene from apples was adapted from that used by Murray (1969) and involved the dipping of whole apples (total of 2–3 kg) in 150 ml of hexane for 2 min each. The hexane extract was filtered and concentrated in a Rotavapor-R rotary evaporator to a volume of approximately 3–5 ml. This extract was analyzed by GC-MS to confirm the identity of the major peak obtained as that of (*E,E*)-α-farnesene (>90% purity).

Rape leaf and flower odor obtained by Tenax entrapment were also used in the EAG studies. All test compounds and extracts were dissolved in hexane, a solvent that did not elicit any detectable EAG responses when tested against seed weevils.

Artificial equivalents of rape leaf and flower odor (Table 1) were based on the compounds and their relative proportions identified in this study and those of Tollsten and Bergström (1988). Several of the artificial rape leaf and flower extracts were made up without particular compounds to observe the effect of the absence of the compound(s) on the weevil EAG response.

Differences in volatility between test compounds was not corrected for, and, accordingly, comparisons between the responses elicited are relative.

EAG Setup. The recording of EAGs was based on the arrangement of Visser (1979) for recording Colorado beetle EAGs. The head of the insect was removed and mounted on one of the glass electrodes. The glass electrodes (tip diameter 10–20 μm) were filled with 0.1 M potassium chloride (KCl) in distilled water as an electrolytic medium (Guerin and Visser, 1980). Silver wire (OD 0.5 mm, FSA Laboratory Supplies, Loughborough, Leics, U.K.) coated with silver chloride (Ag–AgCl) in the glass electrodes connected the preparation to the recording instruments; a P-01 Universal probe connected to an AM-05 EAG Amplifier (both from Syntech, Hilversum, Netherlands). The output from the amplifier

TABLE 1. VOLATILE COMPOUNDS IDENTIFIED IN OILSEED RAPE ODOR AND PERCENT COMPOSITION OF NATURAL AND ARTIFICIAL RAPE ODOR USED IN EAG STUDIES

Compound	Present in odor of		Composition (%) in odor of					
				Rape leaf			Artificial rape	
	Flowers	Leaves	Rape crop[a]	Male EAG	Female EAG	Rape flower	Leaf	Flower
Terpenes								
Caryophyllene	+	–	–	–	–	–	–	1.5
α-Cedrene	+	–	–	–	–	0.2	–	1.4
Cineole	+	–	–	–	–	–	–	1.4
β-Elemene[b]	+	–	–	–	–	–	–	–
α-Farnesene	+	–	62.4	–	–	67.3[c]	–	66.6[d]
Limonene	+	–	1.2	–	–	0.9	–	6.6
Linalool	+	–	–	–	–	2.1	–	1.4
Myrcene	+	–	–	–	–	0.4	–	5.0
trans-β-Ocimene[b]	+	–	–	–	–	–	–	–
Perillene[b]	+	–	–	–	–	–	–	–
α-Pinene	+	–	–	–	–	3.0	–	1.5
β-Pinene	+	–	–	–	–	0.2	–	0.3
Sabinene	+	–	4.4	–	–	5.9	–	10.9
Fatty-acid derivatives								
cis-Hex-3-en-1-ol	+	+	10.7	53.5[e]	39.8[f]	8.3	56.5[g]	–
Hexan-1-ol	–	+	1.1	–	–	–	4.5	–
trans-Hex-2-enal	–	+	4.4	3.4	1.2	–	6.8	–
trans-Hex-3-en-1-ol[b]	–	+	–	–	–	–	4.5	–
cis-Hex-3-en-1-yl acetate	+	+	3.8	40.8	57.2	4.6	20.4	1.7
Pentan-1-ol[b]	–	+	–	–	–	–	0.1	–
Pent-2-en-1-ol[b]	–	+	–	–	–	–	0.5	–
trans,*trans*-Hepta-2,4-dienal[b]	–	+	–	–	–	–	–	–

TABLE 1. CONTINUED

Compound	Present in odor of		Composition (%) in odor of					
				Rape leaf			Artificial rape	
	Flowers	Leaves	Rape crop[a]	Male EAG	Female EAG	Rape flower	Leaf	Flower
Benzenoids								
Benzaldehyde[b]	+	–	–	–	–	–	–	–
4-Methoxybenzaldehyde[b]	+	–	–	–	–	–	–	–
Phenylacetaldehyde	+	+	–	1.1	0.4	5.0	0.2	0.7
2-Phenylethanol	+	+	3.9	0.1	0.3	2.2	0.2	0.5
Nitrogen containing								
3-Butenyl isothiocyanate	–	+	1.8	0.1	0.1		0.3	–
sec-Butyl isothiocyanate	–	+	1.0	0.7	0.9	–	4.5	–
4-Pentenyl isothiocyanate	–	+	5.3	0.3	0.2	–	0.8	–
2-Phenylethyl isothiocyanate	–	+	–	–	–	–	0.6	–
2-Phenylpropionitrile	–	+	–	–	–	–	–	–
Sulfides								
Dimethyl disulphide[b]	–	+	–	–	–	–	0.2	–

[a] Flowering rape crop, growth stage 4.2–4.5 (Sylvester-Bradley and Makepeace, 1984).
[b] Additional compounds identified by Tollsten and Bergström (1988).
[c] 0.22 μl in 10 μl of concentrated rape flower extract.
[d] 1.00 μl in 10 μl of concentrated artificial rape flower extract.
[e] 1.38 μl in 10 μl of concentrated rape leaf extract.
[f] 0.55 μl in 10 μl of concentrated rape leaf extract.
[g] 2.50 μl in 10 μl of concentrated artificial rape leaf extract.

TABLE 2. EAG RESPONSES OF CABBAGE SEED WEEVILS TO TEST COMPOUNDS AS PERCENT OF RESPONSE TO *cis*-3-HEXENOL 10^{-2} ($\pm$ SEM, $N \geq 5$)

Compound	Source[a]	Male weevils, dose[b]		Female weevils, dose[b]	
		10^{-3}	10^{-1}	10^{-3}	10^{-1}
Terpenes					
Caryophyllene	S	59 ± 11	69 ± 27	57 ± 12	93 ± 4
α-Cedrene[c]	F	73 ± 16	106 ± 22	65 ± 16	69 ± 12
α-Farnesene	X	174 ± 41[d]		144 ± 13[d]	
Limonene[c]	S	54 ± 5	74 ± 4	68 ± 11	120 ± 15
Linalool	A	55 ± 9	98 ± 12	74 ± 12	78 ± 10
Myrcene	S	75 ± 16	96 ± 16	78 ± 13	97 ± 18
α-Pinene	A	76 ± 10	112 ± 14	71 ± 10	110 ± 11
β-Pinene[c]	A	37 ± 7	58 ± 11	56 ± 13	80 ± 19
Sabinene[c]	A	51 ± 5	85 ± 7	81 ± 10	125 ± 25
Green leaf volatiles					
cis-Hex-3-en-1-ol[c]	A, I	72 ± 5	144 ± 3	78 ± 6	119 ± 14
Hexan-1-ol[c]	A	72 ± 14	96 ± 14	130 ± 25	201 ± 78
trans-Hex-2-enal[c]	A	58 ± 11	119 ± 18	90 ± 7	154 ± 4
trans-Hex-3-en-1-ol[c]	A	70 ± 16	131 ± 19	54 ± 8	67 ± 6
cis-Hex-3-en-1-yl acetate[c]	E, I	66 ± 7	108 ± 12	51 ± 7	83 ± 7
Isothiocyanates					
3-Butenyl[c]	G	141 ± 28[e]		207 ± 39[e]	
sec-Butyl	M	53 ± 6	80 ± 13	67 ± 12	82 ± 14
4-Pentenyl	G	112 ± 10[f]		110 ± 7[f]	
2-Phenylethyl	F	64 ± 8	108 ± 14	87 ± 9	108 ± 15
Nonrape compounds					
Allyl isothiocyanate	F	116 ± 7	189 ± 36	132 ± 29	198 ± 23
Butyl isothiocyanate	A	110 ± 12	258 ± 35	114 ± 28	168 ± 10
tert-Butyl isothiocyanate	A	118 ± 20	96 ± 15	101 ± 10	89 ± 10

[a] Source of compounds: A - Aldrich, E - Extrasynthese, F - Fluka, G - Glucosinolate precursor (see Methods), I - International Flavors and Fragrances, M - Maybridge Chemical Co., S - Sigma, X - Extracted from apples.
[b] Dose of the compound in hexane (v/v).
[c] Significant difference between sexes (two-way analysis of variance on transformed data, $P < 0.05$).
[d] Actual dose of α-farnesene 1.5×10^{-3}.
[e] Actual dose of 3-butenyl isothiocyanate 2.2×10^{-3}.
[f] Actual dose of 4-pentenyl isothiocyanate 6.4×10^{-4}.

was directed to a chart recorder (Chemlab, Hornchurch, Essex, U.K.) and a VELA data logger (Data Harvest, Leighton Buzzard, U.K.). The preparation, manipulators, probe, microscope, and light source were all housed in a Faraday cage.

Odor Delivery System. If an adequate signal (a steady baseline and low noise) was obtained from the preparation, the air flow over the preparation was adjusted to 500 ml/min using a flowmeter (Rotameter) and the end of a glass tube (OD 5 mm) delivering the air placed to within 5 mm of the preparation. Tenax filtered and humidified compressed air was passed over the antenna. A small air pump (Charles Austin Pumps Ltd., Byfleet, Surrey, U.K.) removed the air from around the preparation.

Experimental Procedure. Ten microliters of the test solution was pipetted onto an approximately 0.25-cm^2 piece of glass microfiber filter paper (GF/C, Whatman, Maidstone, Kent, U.K.), which was placed in a 1-ml disposable plastic syringe (Gallenkamp, Loughborough, Leics, U.K.). The test chemical was allowed to evaporate and equilibrate in the syringe before use. For many of the compounds, the responses of antennae to doses ranging from 10^{-6} to 10^{-1} (0.001 μl/ml–100 μl/ml in hexane) was recorded to observe any dose–response relationship differences between male and female weevils. The tip of the syringe needle was inserted into the air flow 20 cm from the preparation. The syringe plunger was quickly depressed to pass 1 ml of the syringe contents into the airstream passing over the preparation.

The EAG was recorded on the chart recorder and on the VELA data logger; the data logger was programmed to sample the signal from the amplifier every 50 msec. Typically, the amplifier multiplied the signal from the preparation $\times$ 1000. As the antennal responses of a preparation tend to decline with time, the responses to the test compounds (measured as maximum amplitude in mV) were expressed as a percentage of the EAG response to a standard compound (Visser, 1979). The standard compound used was 10^{-2} *cis*-hex-3-en-1-ol (C3H), a compound found in the general green leaf volatile complex (Visser et al., 1979) and used as a standard for other EAG studies of phytophagous insects (Visser, 1979; Guerin et al., 1983) including seed weevils (Kozlowski, 1984). The application of a standard 10^{-2} C3H stimulus (10 μl/ml of hexane) to the weevil antenna elicited a response from the preparation in the range 0.3–0.5 mV. The antenna was treated every two to three stimulations with the standard, and the amplitude of the response of the test compound expressed as a percentage of the mean of the two adjacent standard response amplitudes (Guerin and Visser, 1980). The time between stimulations was 90 sec.

The EAG responses were converted to percent response compared to the standard of 10^{-2} C3H and transformed using arcsin square root (Snedecor, 1956) to meet the assumptions of the analysis of variance (Sokal and Rohlf, 1981). A two-factor analysis of variance was used to determine the effect of sex (male

vs. female) and dose on the EAG responses obtained (Zar, 1984). For each test compound or extract, at least five individual weevils of each sex were used.

RESULTS

Chemistry. The volatiles emitted from Ariana, the double-low cultivar of oilseed rape (Table 1), were little different from those emitted by single-low cultivars (Finch, 1978; Drozdowska and Rogozinska, 1982; Tollsten and Bergström, 1988; Evans, 1991). Several compounds isolated by Tollsten and Bergström (1988) in minor amounts from single-low cultivars of oilseed rape were not found in this study (Table 1). However, one compound, *sec*-butyl isothiocyanate, was found in rape leaf odor (Table 1). It has not previously been reported in *B. napus*, although it is found in other brassicas such as *B. campestris* and *B. juncea* (Tollsten and Bergström, 1988).

The majority of rape leaf odor consists of fatty acid derivatives (Table 1) that constitute the green leaf volatile complex (Visser et al., 1979; Buttery, 1981). Isothiocyanates, which are breakdown products of glucosinolates, are characteristic of most cruciferous plants (Fenwick et al., 1983) and are present in rape leaf odor (Table 1).

Rape flower odor mainly consists of terpene compounds (Table 1) with the sesquiterpene α-farnesene the dominant volatile in rape flower odor and the odor of the rape crop (Table 1). The odor sampled from a small crop of flowering oilseed rape was made up of volatiles associated with the leaves and flowers (Table 1).

EAG Response. For most test compounds and extracts, there was a significant effect of dose on the EAG response of weevil antennae (Table 2), demonstrating a typical dose–response effect (as shown in Figures 1–4 below). There were also significant differences between male and female responses to many of the compounds tested (Table 2). The EAG amplitudes obtained were in the range of 0.01–1.5 mV.

Of the compounds identified from rape leaf odor (Table 1), hexan-1-ol, 3-butenyl isothiocyanate, and 4-pentenyl isothiocyanate elicited the strongest EAG responses (Table 2). For hexan-1-ol and 3-butenyl isothiocyanate, the response of female weevil antennae was significantly stronger than the males. The response to 4-pentenyl isothiocyanate by both sexes was virtually identical (Table 2). Female antennae were particularly sensitive to relatively low doses of 3-butenyl isothiocyanate and hexan-1-ol (Table 2), but there was a relatively weak EAG response to the *sec*-butyl and 2-phenylethyl isothiocyanates identified from rape leaf odor. The response to the isothiocyanate compounds not present in rape odor was comparatively strong in the case of allyl isothiocyanate and butyl isothiocyanate (Table 2).

There were significant differences in the response of male and female weevils to all of the green leaf volatiles identified from rape leaf odor (Table 2).

Female antennae were significantly more responsive to rape flower extract than the male antennae (Figure 1). There were significant variations in the response of male and female antennae to several of the compounds in rape flower odor (Table 2). α-Farnesene was the only compound in rape flower odor that evoked strong EAGs from weevil antennae at relatively low doses (Figure 2). The responses obtained at a concentration of 1.5×10^{-2} of α-farnesene (Figure 2) were the strongest EAGs observed to any compound at any concentration tested. The EAGs obtained from other terpenes were weak compared to α-farnesene (Table 2).

The response of weevil antennae to the artificial equivalents of rape leaf and flower extracts indicated that the absence of particular compounds can significantly reduce the EAGs (Figures 2–4). Both male and female weevil antennae had significantly reduced EAGs when stimulated with artificial rape flower extract with no α-farnesene compared to when α-farnesene was present (Figure 2).

Male antennae were significantly less sensitive to artificial rape leaf extracts without 3-butenyl and 4-pentenyl isothiocyanates (Figure 3), and female antennae were less sensitive to artificial rape leaf extract without *cis*-hex-3-en-1-ol and *cis*-hex-3-en-1-yl acetate (Figure 4).

DISCUSSION

The compounds that constitute the green leaf volatile complex are commonly found in the foliage of numerous plant species and contribute to the "green odor" of leaves (Visser et al., 1979). The green leaf volatiles constituted over 97% of the natural rape leaf odor used in these EAG studies (Table 1). The green leaf volatiles *cis*-hex-3-en-1-ol and *cis*-hex-3-en-1-yl acetate were found to constitute over 90% of the volatiles present in rape leaf odor (Table 1), and omission of these compounds from artificial rape leaf odor significantly reduced the EAGs of female seed weevils and considerably reduced the EAGs of the males (Figure 4). These two compounds, either by their presence or by their relatively high concentration in natural and artificial rape leaf odor influenced the olfactory signal obtained from weevil antennae. These compounds also elicit EAGs from other phytophagous insects including the Colorado potato beetle, *Leptinotarsa decemlineata* Say. (Visser, 1979), carrot fly, *Psila rosae* F. (Guerin and Visser, 1980), and boll weevil, *Anthomonus grandis* Boh. (Dickens, 1984). For the Colorado potato beetle, the ratio of these and other green leaf volatiles to each other determined the quality of the olfactory signal and ultimately the behavioral response of the beetle to the odor (Visser and De Jong, 1988). Because of the widespread occurrence of green leaf volatiles, it was

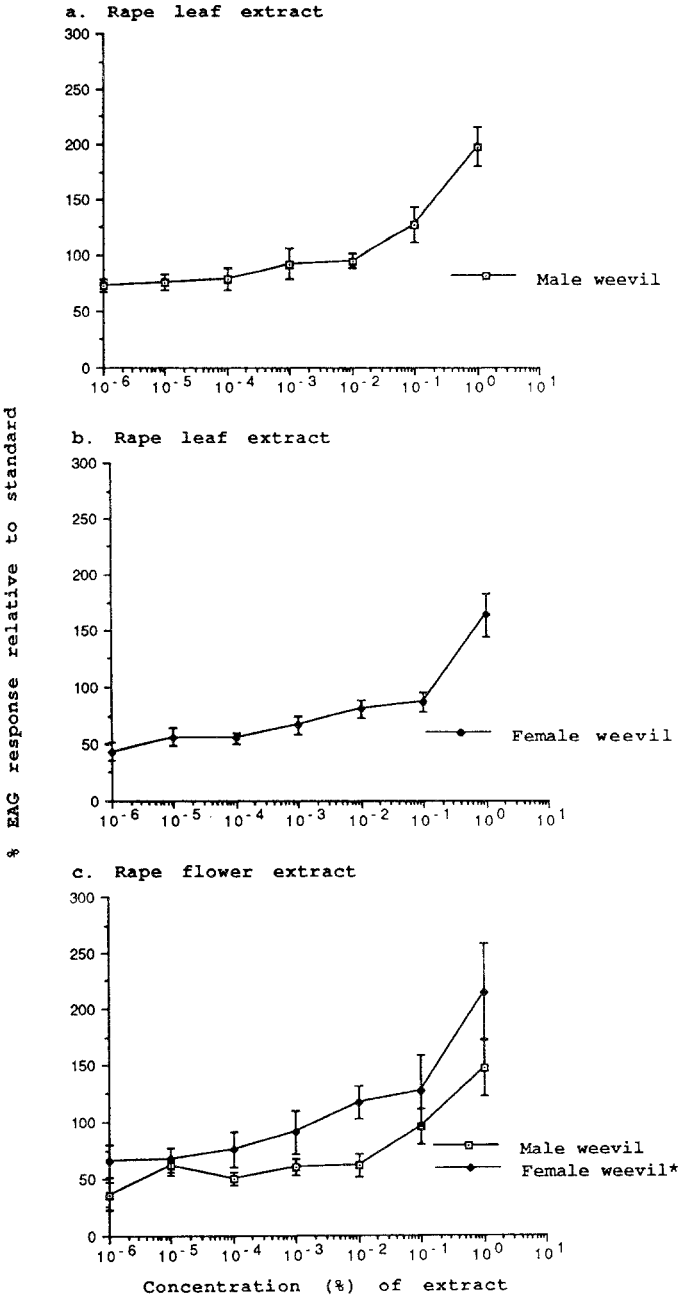

FIG. 1. Mean EAG response of male (a) and female (b) *C. assimilis* to dilutions of oilseed rape leaf and flower odor (c). Vertical lines represent standard errors, $N \geq 5$, and asterisk represents significant difference in responsiveness between the sexes (two-way analysis of variance, $P < 0.01$).

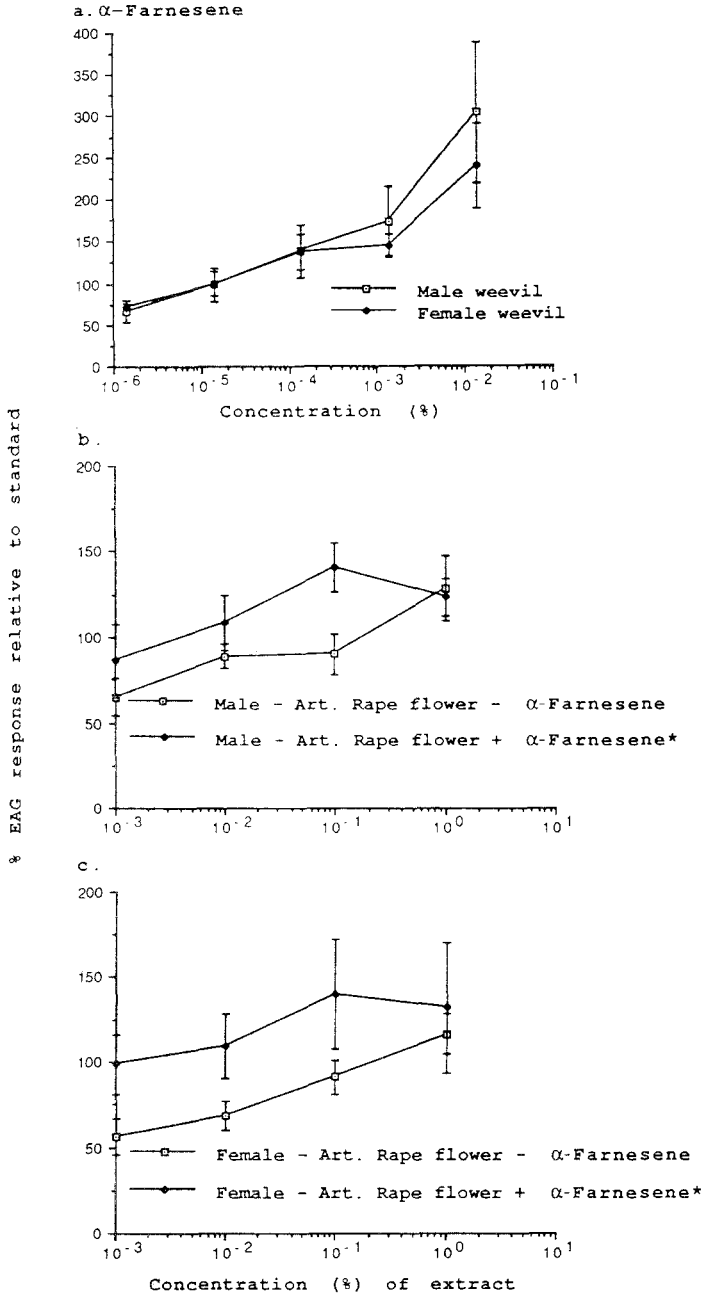

FIG. 2. Mean EAG response of *C. assimilis* to dilutions of α-farnesene (a) and an artificial rape flower extract (b and c) with and without α-farnesene. Vertical lines represent standard errors, $N \geq 5$, and asterisk represents significant difference in responsiveness (two-way analysis of variance, $P < 0.01$).

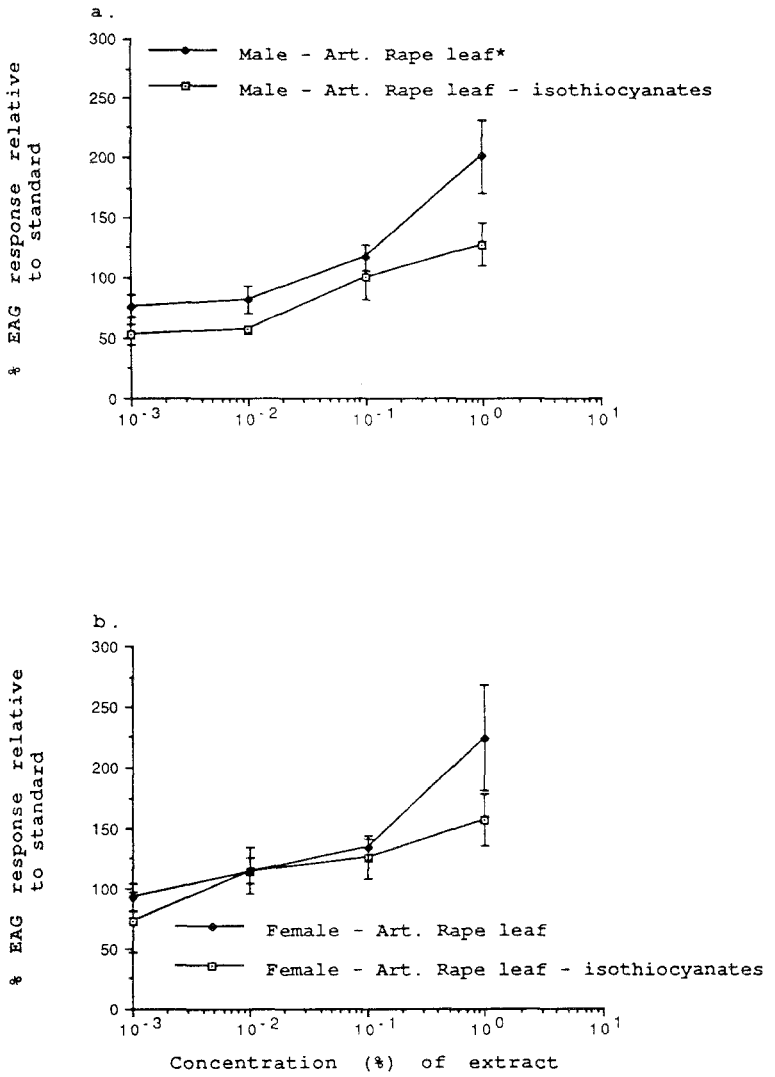

FIG. 3. Mean EAG response of *C. assimilis* to dilutions of an artificial rape leaf extract with and without 3-butenyl and 4-pentenyl isothiocyanate. Vertical lines represent standard errors, $N \geq 5$, and asterisk represents significant difference in responsiveness (two-way analysis of variance, $P < 0.01$).

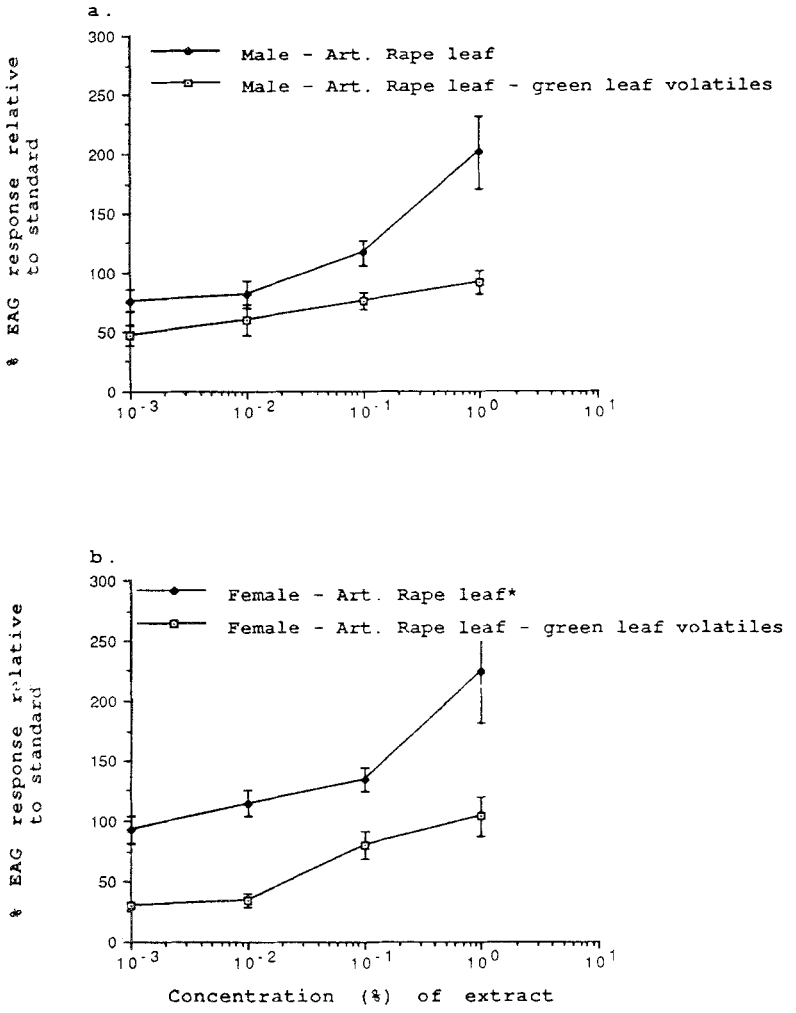

FIG. 4. Mean EAG response of *C. assimilis* to dilutions of an artificial rape leaf extract with and without the green leaf volatiles *cis*-hex-3-en-1-ol and *cis*-hex-3-en-1-yl acetate. Vertical lines represent standard errors, $N \geq 5$, and asterisk represents significant difference in responsiveness (two-way analysis of variance, $P < 0.01$).

considered unlikely that perception of their qualitative presence could be used to discriminate between host and nonhost plants (Light et al., 1988). The degree of responsiveness of seed weevil and other insect antennae (Guerin and Visser, 1980; Van Der Pers, 1981; Guerin and Städler, 1982; Dickens, 1984) to green

leaf volatiles suggests that the sensory investment in perception of these compounds may be adaptive.

The compounds 3-butenyl and 4-pentenyl isothiocyanate only accounted for $<0.5\%$ of natural rape leaf odor and 1% of the artificial rape leaf odor (Table 1), but a considerable reduction in EAG response was obtained when they were absent (Figure 3). They elicited relatively strong EAGs when tested individually (Table 2). Blight et al. (1989) have identified receptors on the antennae of seed weevils that specifically respond to these isothiocyanate compounds. In the EAGs of artificial rape leaf odor with neither of the above isothiocyanates, none of these specific isothiocyanate receptors are contributing to the overall sensory output, which may explain the reduced EAG response. 2-Phenylethyl and *sec*-butyl isothiocyanate, the other isothiocyanate compounds present in artificial (and natural) rape leaf odor elicited relatively weak EAGs from weevil antennae when tested singly (Table 2), despite the presence of receptors that Blight et al. (1989) showed responded strongly to the 2-phenylethyl isothiocyanate in single cell studies.

Kozlowski (1984) observed strong EAGs to allyl and butyl isothiocyanate (which are not found in oilseed rape odor) from female seed weevil antennae, as did this study (Table 2), but the specific isothiocyanate receptors identified by Blight et al. (1989) appeared relatively unresponsive to allyl isothiocyanate at the concentration used.

The detection of low amounts of host-specific compounds such as isothiocyanates would be important in contributing to the overall EAG, as appeared to be the case in the perception of 3-butenyl and 4-pentenyl isothiocyanate in seed weevils. Carrot flies, for example, (Guerin et al., 1983) were shown to exhibit strong EAGs to minor constituents of carrot leaf odor, as did bark beetles to minor constituents of bark odor (Tommerås and Mustaparta, 1987), which would be important in host location for both these insects. However, the magnitude of an EAG is not always indicative of the behavioral response of the insect to the stimulating compound. For example, the strongest EAG responses obtained in this study were to a $>90\%$ pure sample of α-farnesene (Figure 2), but this compound when presented alone in an olfactometer did not elicit any discernible behavioral response (Evans, 1991). Similarly, yellow water traps baited with α-farnesene did not catch significantly greater seed weevils than control traps (Evans, 1991). However when α-farnesene was omitted from an artificial rape flower extract, it considerably reduced the EAGs obtained (Figure 2) and the attractive behavioral response elicited by artificial rape flower odor in an olfactometer was absent (Evans, 1991). The quality of an EAG, determined by the presence of other compounds, appears to be as important as the magnitude of the EAG.

Similarly, a large EAG response to a compound, as in the case of the female weevil response to hexan-1-ol (Table 2), did not necessarily imply that

the compound is attractive. The behavioral responses of both male and female seed weevils to hexan-1-ol in an olfactometer indicated that within the range of doses tested (0.01%–5%), hexan-1-ol was a strong repellent (Evans and Allen-Williams, 1989; Evans, 1990). This example indicates the need for behavioral experiments to be carried out in conjunction with electrophysiological studies. Hexan-1-ol is usually found in very low or undetectable amounts in rape leaf odor (Evans, 1991), so its repellent effect on seed weevils in the field may be limited or nullified by the perception of other compounds in rape odor.

Sexual dimorphism in EAGs to plant volatiles has not been reported for many other insect species, but male turnip moths, *Agrotis segetum* Schiff., are more sensitive to plant volatiles than females (Hansson et al., 1989). There were significant sexual differences in the EAG response of seed weevils to several host-plant compounds, including each of the green leaf volatiles tested (Table 2). This difference was especially apparent in the responses to hexan-1-ol and *trans*-hex-2-enal, where female weevils were most sensitive. Female antennae were also significantly more sensitive to 3-butenyl isothiocyanate (Table 2), to rape flower extract (Figure 1), and to sabinene, a compound found in rape flower odor (Table 2). The increased sensitivity to sabinene was also apparent in the behavioral response of female weevils in an olfactometer (Evans and Allen-Williams, 1989; Evans, 1990), where attraction to sabinene by males was absent at low doses, but females were attracted.

Behaviorally, rape flower odor was most attractive to seed weevils (Evans and Allen-Williams, 1989), yet only the rape flower volatile α-farnesene strongly stimulates seed weevil antennae of both sexes (Figure 2). This compound is the major component of rape flower odor (Table 1), and seed weevils are usually searching for host-plants such as rape when they are in flower (Free and Williams, 1979). α-Farnesene was also shown to be the dominant volatile in the flower odor of other weevil host-plants such as turnip rape (*B. campestris* L.) and black mustard [*B. nigra* (L.) Koch.] (Tollsten and Bergström, 1988). The odor of flowering rape plants was dominated by α-farnesene (Table 1), but other compounds were also present that strongly stimulate weevil antennae such as 3-butenyl and 4-pentenyl isothiocyanate and *trans*-hex-2-enal (Table 1). All the compounds that elicit strong EAGs from seed weevil antennae are liberated from the rape crop at the time when weevils are actively searching for a host plant.

Cabbage seed weevil antennal receptors are capable of detecting a wide range of compounds associated with the odor of its host plant. These receptors are also able to perceive changes in the concentration of these compounds. The perception of green leaf volatiles and specific host-related compounds, in particular, appear to have a significant role to play in the overall perception and recognition of host odor by seed weevils.

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Magnolia virginiana NEOLIGNAN COMPOUNDS AS CHEMICAL BARRIERS TO SWALLOWTAIL BUTTERFLY HOST USE

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Abstract—The role of toxins and deterrents in preventing *troilus* group species (Lepidoptera, Papilionidae) from feeding on magnoliaceous hosts was investigated using bioassay-directed isolation of *Magnolia virginiana* allelochemicals. A fraction consisting of three neolignan compounds significantly reduced survival of first instar *P. palamedes*, a *troilus* group member. Two of these compounds, magnolol and a biphenyl ether, were tested individually and were both toxic to *P. palamedes*. The larval survival of *P. troilus*, another *troilus* group species, was also significantly reduced by magnolol but not by the biphenyl ether. In contrast, *P. glaucus*, a polyphagous *glaucus* group species that feeds on magnoliaceous hosts, was not affected by either compound. The effect of these compounds against a polyphagous nonpapilionid was examined using the fall webworm (*Hyphantria cunea*, Lepidoptera: Arctiidae). The biphenyl ether but not magnolol significantly lowered webworm first-instar survival, demonstrating that polyphagy does not preadapt lepidopterans to feeding on this neolignan. These results demonstrate that although phagostimulants play a role in the specialization of the *troilus* group on the Lauraceae, the presence of toxins and/or deterrents in nonhosts is also important in determining food plant patterns in these species.

Key Words—*Papilio palamedes*, *Papilio troilus*, *Papilio glaucus*, *Hyphantria cunea*, Papilionidae, Arctiidae, Lepidoptera, swallowtail butterfly, magnolol, 5,5′-diallyl-2,2′-dihydroxybiphenyl, 4,4′-diallyl-2′,3′-dihydroxybiphenyl ether, herbivore-plant, toxins, deterrents.

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INTRODUCTION

The *troilus* and *glaucus* species groups are closely related lineages in the swallowtail butterfly genus *Papilio* (Lepidoptera: Papilionidae) (Munroe, 1961; Hagen and Scriber, 1991, Sperling, 1991). Species within the *troilus* group are restricted in host use to the Lauraceae (Scriber et al., 1991a; Lederhouse et al., 1992). In contrast, members of the *glaucus* group have greater diet breadth and feed on plants from several plant families. One species, *Papilio glaucus* L. (formerly *P. glaucus glaucus*; Hagen et al., 1991), is especially polyphagous and uses plants in the Rosaceae, Tiliaceae, Oleaceae, Rutaceae, and Magnoliaceae (Scriber et al., 1991a). The use of magnoliaceous hosts is particularly noteworthy since only a few of the 220 *Papilio* species are able to grow on these plants (Scriber, 1984).

Scriber et al. (1991a) suggested that the polyphagous *glaucus* group evolved from an ancestor with a narrower range of host families similar to the *troilus* group species. Thus, the evolutionary divergence of the *glaucus* and *troilus* lineages has been accompanied by fundamental differences in host use behavior and physiology. To evaluate the importance of plant chemistry as a constraint on host-use evolution in swallowtail butterflies, we have sought to identify the phytochemical mechanisms of toxicity/deterrency underlying current host use patterns in *glaucus* and *troilus* lineages. If chemical traits of hosts present significant evolutionary barriers to herbivores such as *P. palamedes*, comparisons of the responses of phylogenetically related species to these traits may reveal patterns of adaptation in herbivore lineages.

We focused on the chemistry of sweetbay magnolia, *Magnolia virginiana* L. (Magnoliaceae), a host of *P. glaucus* but unsuitable for *troilus* group species. Phagostimulatory chemicals appear to be necessary for initiation of larval feeding in *troilus* group species (Scriber et al., 1991a; Nitao, 1992), but toxins and deterrents may also act as barriers to colonization of *M. virginiana* by these unadapted papilionids. A number of biologically active secondary compounds have been identified from magnoliaceous plants, including sesquiterpene lactones, alkaloids, and phenylpropanoids (Doskotch and Flom, 1972; El-Feraly and Yee-Ming, 1978; Kijjoa et al., 1989; Barbosa et al., 1990). In addition, Lindroth et al. (1986) hypothesized that toxic sesquiterpene lactones prevent *Papilio canadensis* Rothschild & Jordon larvae from feeding on another magnoliaceous host of *P. glaucus*, *Liriodendron tulipifera* L. (tuliptree). However, specific compounds active against *Papilio* species have not been identified.

Through bioassay-directed isolation of plant extracts, we tested the hypothesis that allelochemicals in *M. virginiana* prevent two *troilus* group species, *Papilio palamedes* Drury and *Papilio troilus* L., from using this as a food plant. We also bioassayed these compounds against *P. glaucus*, a *glaucus* group species that in certain parts of its geographic range uses *M. virginiana*. Finally, we

tested the compounds against *Hyphantria cunea* (Dru.) (Lepidoptera: Arctiidae), the fall webworm, to determine if these compounds possess activity against nonpapilionid lepidopterous herbivores. *H. cunea* has been reported to feed on over 230 plant species in at least 20 families, including the Magnoliaceae (Tietz, 1972; Johnson and Lyon, 1988), and serves as a model species to test for bioactivity against a polyphagous phytophage.

METHODS AND MATERIALS

Plant and Insect Material. Persea borbonia (L.) Spreng. and *Lindera benzoin* (L.) Blume are highly suitable hosts for *P. palamedes* and *P. troilus*, respectively (Lederhouse et al., 1992), whereas *Prunus serotina* (Rosaceae) is a host for *P. glaucus* and *H. cunea* (Scriber et al., 1991a; Tietz, 1972). Leaves of these plants were used as feeding substrates for bioassays. *P. borbonia* leaves were from potted trees originally purchased from Herren Nursery (Florida Division of Forestry) and subsequently grown in a greenhouse at Michigan State University for two to three years. *L. benzoin* and *P. serotina* leaves were collected from wild plants in Ingham Co., Michigan. *M. virginiana* leaves for chemical extraction were from trees of background similar to the *P. borbonia* plants.

P. palamedes were one generation removed from wild females (Highland County, Florida; Echols County, Georgia). *P. troilus* (Adams County, Ohio) and *P. glaucus* (Lawrence County, Ohio) were direct offspring of wild females. *H. cunea* were offspring of individuals originally collected as larvae from sweet gum, *Liquidambar styraciflua* L. (Hamamelidaceae) (Berkeley County, South Carolina).

Initial Separation and Bioassay of Crude Fractions. Fresh *M. virginiana* leaves (150 g) were homogenized and extracted with cold 80% methanol in water (3 × 500 ml) (Figure 1). The leaf residue was further extracted with 100% methanol (500 ml), followed with petroleum ether (35–60°C bp) (2 × 500 ml). The petroleum ether extract was concentrated in vacuo to dryness (1.1 g). The aqueous and absolute methanol extracts were combined, concentrated to remove the methanol, and the resulting aqueous mixture was extracted with dichloromethane (3 × 350 ml). The aqueous fraction was lyophilized (8.2 g) (fraction aqueous-1), and 5.5 g of this residue was dissolved in 150 ml water and extracted with ethyl acetate (3 × 150 ml). Dry masses of the ethyl acetate extractables and of the aqueous residue (aqueous-2) were 0.35 g and 4.6 g, respectively. The dichloromethane fraction was concentrated to dryness (4.2 g) (CH_2Cl_2 fraction), and 2.5 g of this residue was fractionated by flash chromatography (silica gel, 40–63 μm, Sigma, St. Louis, Missouri; 3:1 petroleum ether–ethyl acetate, 100% ethyl acetate, 100% methanol); after examination by

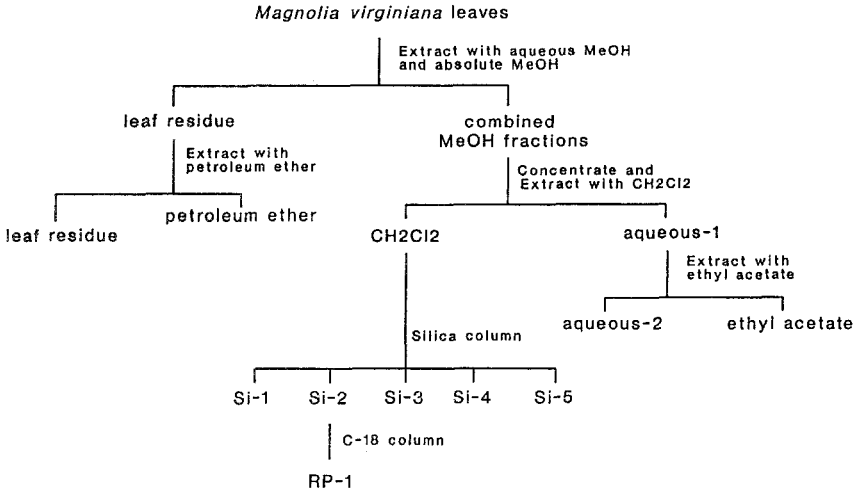
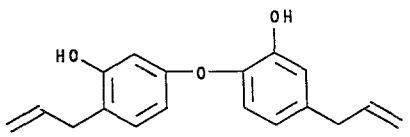


FIG. 1. Chemical fractionation scheme of *Magnolia virginiana* extracts.

thin-layer chromatography, fractions were combined to produce Si-1 through Si-5. Pigments were removed from fraction Si-2 with a reverse-phase flash column (octyldecyl, 40 μm, J.T. Baker, Phillipsburg, New Jersey; 80% methanol in water) (RP-1 fraction).

For initial identification of *M. virginiana* active fractions, we bioassayed all fractions using *P. palamedes* first instars. Extracts were applied to the top surfaces of detached host plant leaves in a manner similar to that of Lindroth et al. (1986). Extract residues were dissolved in appropriate solvents (water, MeOH, $CHCl_3$, or hexane) and uniformly applied with a microliter syringe to *Persea borbonia* leaves at concentrations equivalent to those occurring in *M. virginiana* leaves (grams of extract per gram of leaf). Each control leaf was treated with all solvents used for a particular round of bioassays. Bioassays were conducted at 24°C, 18:6 light–dark photoperiod. Neonate larvae in groups of 9–32 were fed treated leaves and their survival measured after three days. Newly treated leaves were added each day. Each bioassay was replicated two to three times.

Bioassay of Individual Compounds from Active Fraction RP-1. We bioassayed two of the major components of active fraction RP-1 individually against *P. palamedes*, *P. troilus*, *P. glaucus*, and *H. cunea* larvae. Components of fraction RP-1 were separated using preparatory thin-layer chromatography (PTLC) (silica gel, 2000 μm tapered Uniplate, Analtech, Newark, Delaware; developed twice in hexane–ether 4:1) (**1, 2,** and **3** in Figure 2). Compounds were identified using spectral means (NMR, MS, UV, and IR) (Nitao et al., 1991a). After identification, larger quantities of the two major components (**1,**



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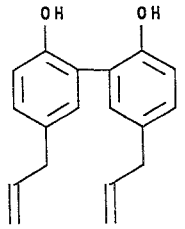

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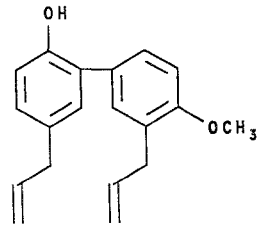

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FIG. 2. *Magnolia virginiana* neolignans identified in the fraction active against *Papilio palamedes* first instars. **1** = 4,4′-diallyl-2′3′-dihydroxybiphenyl ether (a biphenyl ether); **2** = 5,5′-diallyl-2,2′-dihydroxybiphenyl (magnolol); **3** = 3,5′-diallyl-2′-hydroxy-4-methoxybiphenyl (4-methoxyhonokiol).

a biphenyl ether, and **2,** magnolol) were isolated by extraction of lyophilized leaves with hexane and purification using centrifugal partition chromatography and PTLC (Nitao et al., 1991a).

The biphenyl ether and magnolol were tested individually at 0.125%, 0.25%, and 0.5% (grams of compound per gram of fresh leaf) against neonates of *P. palamedes*, *P. troilus*, and *P. glaucus* in groups of 8–15 larvae. These two compounds were also tested against *H. cunea* neonates in groups of 11–27 larvae. Compounds were applied in methanol to leaves of *Persea borbonia* for *P. palamedes* bioassays, *Lindera benzoin* for *P. troilus*, and *Prunus serotina* for *P. glaucus* and *H. cunea*. Leaves treated with methanol served as controls.

Larval survival was recorded after three days. Bioassays were replicated three to five times.

Because *H. cunea* neonates feed on the leaf surface rather than the edge, it was necessary to prevent larvae from behaviorally avoiding the treated side of the leaf. Prior to bioassaying, leaves were embedded in warm agar solution in Petri dishes such that only the treated side was exposed after the agar cooled and solidified. *Papilio* larvae feed from the leaf edge so such precautions were unnecessary for the swallowtail bioassays.

Statistical Analysis. Effects of *M. virginiana* extracts on larval survival were tested using analysis of variance (ANOVA) (SAS Institute, 1985). Dose-dependent effects of purified compounds on survival of *Papilio* larvae were analyzed using a polynomial regression ANOVA model (Mize and Schultz, 1985) (SAS PROC GLM, lack-of-fit model; Freund et al., 1986). Prior to analysis, data were transformed to equalize variances; *P. glaucus* survival data on the biphenyl ether compound were square-root transformed, whereas all other *Papilio* data were square-root arc-sine transformed (Sokal and Rohlf, 1981). Transformations failed to yield equal variances among treatments for *H. cunea* bioassays; dose-dependent effects for these data were analyzed by calculating the Kendall tau correlation coefficient on ranks of percent survival and concentration of compound.

RESULTS

Initial Separation and Bioassay of Crude Fractions. Crude extracts of *M. virginiana* leaves significantly decreased *P. palamedes* larval survival (one-way ANOVA, $F = 20.96$; $df = 3, 4$; $P < 0.01$). Greatest mortality was found on the aqueous-1 and CH_2Cl_2 fractions (Table 1A). The aqueous phase (aqueous-2) retained activity after extraction with ethyl acetate (Table 1B; $F = 13.51$; $df = 2, 3$; $P < 0.05$) but was not processed further.

The CH_2Cl_2 extract was fractionated by silica flash chromatography and resulted in five fractions, of which Si-2 possessed highest activity (Table 1C; $F = 5.17$; $df = 5, 6$; $P < 0.05$). This activity was retained after decolorization with a reverse-phase column (fraction RP-1) (Table 1D; $F = 23.94$; $df = 1, 4$; $P < 0.01$). Isolation and identification of RP-1 constituents revealed that this fraction consisted of three biphenylpropenoid neolignans (Figure 2) (Nitao et al., 1991a). Compounds **1** (the biphenyl ether), **2** (magnolol), and **3** (4-methoxyhonokiol) (Figure 2) comprised 56%, 31%, and 13%, respectively, of the 300-mg total obtained in RP-1 (62.4 g leaf equivalents); thus, these three compounds constituted 0.27%, 0.15%, and 0.06% (g/g), respectively, of *M. virginiana* leaf wet weight. Subsequent isolation of these compounds with cen-

TABLE 1. PERCENT SURVIVAL OF FIRST-INSTAR *Papilio palamedes* AFTER 72 HR ON LEAVES TREATED WITH *Magnolia virginiana* EXTRACTS[a]

Fraction	*N*	Survival (%)
A. Control	2	86.0 ± 6.0
Aqueous-1	2	18.0 ± 7.0
CH_2Cl_2	2	0 ± 0
Petroleum ether	2	89.0 ± 11.0
B. Control	2	67.0 ± 10.0
Aqueous-2	2	6.0 ± 6.0
Ethyl acetate	2	63.5 ± 2.5
C. Control	2	46.5 ± 1.5
Si-1	2	21.5 ± 13.5
Si-2	2	0 ± 0
Si-3	2	49.0 ± 1.0
Si-4	2	55.0 ± 15.0
Si-5	2	37.5 ± 22.5
D. Control	3	74.7 ± 4.6
RP-1	3	8.0 ± 8.0

[a]Data are untransformed means ± SE.

trifugal partition chromatography resulted in slightly higher yields (0.46%, 0.31%, and 0.08% for the biphenyl ether, magnolol, and 4-methoxyhonokiol, respectively).

Bioassay of Individual Compounds from Active Fraction RP-1. When fed the biphenyl ether or magnolol, larvae of both *troilus* group species were significantly affected by either the latter or both. *P. palamedes* survival decreased linearly in relation to the concentration of magnolol (linear response: $F = 107.33$; $df = 1, 16$; $P < 0.0001$) and the biphenyl ether ($F = 26.02$; $df = 1, 8$; $P < 0.001$) (Figure 3A). *P. troilus* larvae were significantly affected by magnolol (linear response: $F = 32.97$; $df = 1, 8$; $P < 0.001$) but not the biphenyl ether (linear response: $F = 2.28$; $df = 1, 8$; $P = 0.17$) (Figure 3B). In contrast, survival of *P. glaucus*, a *glaucus* group member that normally feeds on magnoliaceous hosts, was not significantly affected by either magnolol (linear response: $F = 0.24$; $df = 1, 8$; $P = 0.24$) or the biphenyl ether (linear response: $F = 0.08$; $df = 1, 8$; $P = 0.78$) (Figure 3C). Although the polyphagous fall webworm, *H. cunea*, can feed on certain magnoliaceous plants, larval survival was significantly reduced on the biphenyl ether (Kendall tau $= -0.667$, $N = 16$, $P < 0.05$). Survival of fall webworm larvae also tended to decrease linearly with magnolol concentration, but the effect was not statistically significant (Kendall tau $= -0.152$, $N = 16$, $P > 0.05$) (Figure 3D).

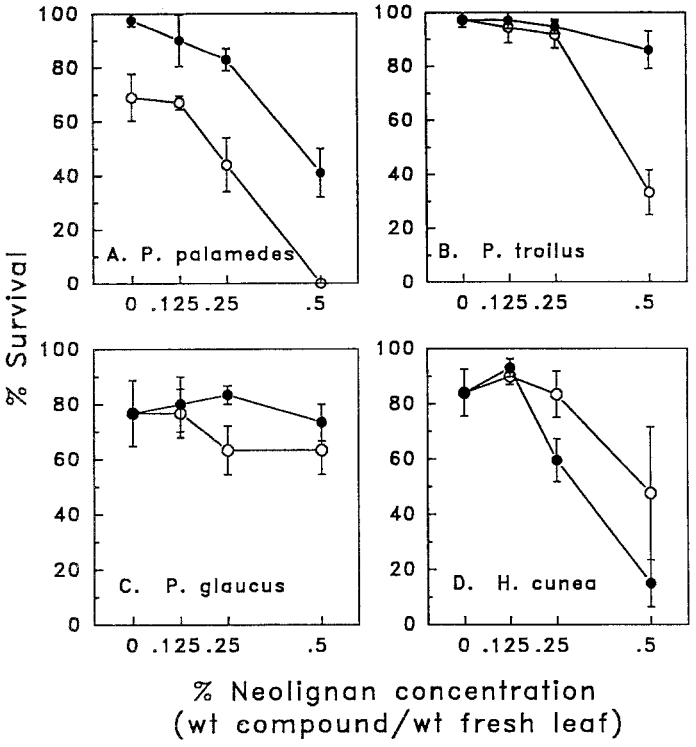

FIG. 3. Percent survival after 72 hr of first instars on various concentrations of magnolol and the biphenyl ether. (A) *Papilio palamedes*, (B) *P. troilus*, (C) *P. glaucus*, and (D) *Hyphantria cunea*. Open circles are magnolol treatments; closed circles are the biphenyl ether treatments. Data are untransformed means ± SE.

DISCUSSION

Host suitability for larvae is one of many factors determining host usage patterns of *troilus* group species (Scriber et al., 1991a; Nitao et al., 1991b; Lederhouse et al., 1992). This study indicates that the unsuitability of *M. virginiana* to *P. palamedes* and *P. troilus* larvae is in part due to neolignan compounds. Thus, allelochemicals in nonhosts, in addition to stimulatory cues necessary for host recognition (Scriber et al., 1991a; Nitao, 1992), serve to constrain the host range of *troilus* group larvae. In contrast, larval survival of *P. glaucus*, a closely related polyphage, was unaffected by *M. virginiana* neolignans.

Based on host use patterns and phylogenetic information, Scriber et al. (1991a) suggested that the polyphagous *glaucus* group species may have been

derived from a more specialized *troilus*-like ancestor. The authors proposed that evolutionary "release" from behavioral constraints characteristic of the *troilus* group freed the *glaucus* group species to expand their host range. This host range expansion was hypothesized to have been facilitated by generalized physiological adaptations capable of detoxifying chemicals shared by many of the new host families, such as those in the Rosaceae and Oleaceae. However, polyphagy by itself does not preadapt insect herbivores to feeding on the magnoliaceous neolignans described here; survival of another polyphagous species, *H. cunea*, was negatively affected by the biphenyl ether. Successful use of *M. virginiana* therefore appears to require specialized behavioral or physiological larval traits not associated with wide host breadth per se. Such specialization is indicated at a finer level by the existence of *P. glaucus* populations differentially adapted to *Magnolia* neolignan compounds (Nitao et al., in preparation).

These results are consistent with the suggestion of Scriber and coworkers (1991a) that more specialized mechanisms have evolved in certain *glaucus* group species, enabling the colonization of hosts with more unique chemistry, such as the Salicaceae, Rhamnaceae, as well as the Magnoliaceae. Similarly, *P. canadensis* and *Papilio rutulus* have been shown to possess esterase enzymes capable of detoxifying phenolic glycosides in their salicaceous hosts (Lindroth et al., 1988; Lindroth, 1989; Scriber et al., 1991b).

The mode of action of these neolignan compounds has yet to be determined. Postingestive toxicity is likely to play a part, since these allelochemicals possess broad biocidal activity against fungi, bacteria, mosquito larvae, and brine shrimp (Clark et al., 1981; El-Feraly et al., 1983; Nitao et al., 1991a). Phagodeterrency may also be involved, although in the feeding trials reported here, *P. palamedes*, *P. troilus*, and *H. cunea* larvae readily fed on neolignan-treated leaves, indicating that absolute gustatory deterrency was not the sole cause of mortality.

Neolignans, lignans, and other biphenylpropenoids are widely distributed in swallowtail host families, including the Lauraceae and Magnoliaceae (MacRae and Towers, 1984). Butterfly species are no doubt adapted to specific types of compounds within these broad phytochemical classes. The ability of *P. troilus* larvae to survive on the biphenyl ether but not magnolol illustrates such specificity. Similar chemicals occurring in the hosts of *P. troilus* may preadapt these larvae to feeding on plants containing biphenyl ether compounds. Behavioral and physiological preadaptation such as this may serve as "chemical facilitation" in the colonization of novel hosts by swallowtail butterflies (Feeny, 1991).

Compounds other than neolignans may also be involved in the inability of some *troilus* group members to use the Magnoliaceae. Unidentified compounds in the aqueous phase reduced *P. palamedes* larval survival. Polar constituents such as quaternary alkaloids and glycosides of lignans and phenols have been identified from other *Magnolia* species (Rao and Juneau, 1975; Rao and Davis, 1982) and are potentially responsible for the bioactivity against swallowtail

larvae. Sesquiterpene lactones have also been isolated from *Magnolia* species (El-Feraly and Yee-Ming, 1978); however, these probably were extracted into the ethyl acetate fraction of our protocol and therefore were unlikely to have caused the larval mortality.

The extent to which biphenylpropenoids affect host patterns within the *glaucus* group lineage has yet to be determined. Sesquiterpene lactones are thought to be responsible for the inability of *P. canadensis* to use *Liriodendron tulipifera*, a host for *P. glaucus* (Lindroth et al., 1986). However, biphenylpropenoids may also play a role. Although magnolol and the biphenyl ether have not been reported from *L. tulipifera*, another biphenylpropenoid has recently been identified from the leaves of this tree (Barbosa et al., 1990). That neolignans and lignans may influence host suitability for *glaucus* group species is suggested by the susceptibility of *P. canadensis* to low concentrations of magnolol and the biphenyl ether (unpublished data). More detailed study should elucidate the extent to which biphenylpropenoids have enhanced or restricted the radiation of swallowtail butterflies, such as those in the *troilus* and *glaucus* groups, onto their hosts.

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CHEMISTRY OF DEFENSIVE SECRETIONS IN NYMPHS AND ADULTS OF FIRE BUG, *Pyrrhocoris apterus* L. (HETEROPTERA, PYRRHOCORIDAE)

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Abstract—The defensive secretions from the nymphs and the adults of both sexes in *P. apterus* were chemically investigated. Forty components were identified from the nymphal posterior dorsoabdominal glands and 35 from the adult metathoracic glands of both sexes. Within the 43 identified chemicals, we found 23 aldehydes, five saturated hydrocarbons, five alcohols, three ketones, three lactones, two terpenes, one phenol and one ester. Thirteen of these compounds had never been reported within the Heteroptera. Whereas eight components are specific to the nymphs, methyl pentenal, (*E*)-2-hexenol, and heptadecanal are the only adult-specific components. The biological role of all the identified chemicals is almost unknown in *P. apterus* and is discussed comparatively with regard to other Heteroptera.

Key Words—*Pyrrhocoris apterus*, Heteroptera, Pyrrhocoridae, bug, exocrine glands, pheromones, defense.

INTRODUCTION

Pyrrhocoris apterus L. is a phytophagous bug usually occurring in dense populations concentrated around linden trees (*Tilia* species) and feeding on them. Despite the well-known defensive gland in nymphs (posterior dorsoabdominal

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glands) and in adults (metathoracic glands) (Stein, 1966a, b, 1967), the chemical nature of the exocrine compounds was only investigated superficially in nymphs. Staddon and Daroogheh (1982) reported that the volatile constituents of the posterior dorsoabdominal gland of nymphs contained three compounds: (*E*)-2-octenal and 4-oxo-(*E*)-2-octenal are the major compounds, while the third component (molecular weight, 154) remains unidentified. In his study on the aggregative behavior of *P. apterus*, Schmuck (1987) showed that the nymphal posterior dorsoabdominal gland contains components typical of a defensive secretion. The secretion would contain only four chemicals. The major one (66.5% of the secretion) was unidentified, whereas the three others were (*E*)-2-octenal (23.5%), heneicosane (10%), and tridecane (3.5%). Nevertheless, these percentages, with a total of 103.5, are rather questionable. Except for *P. apterus* and *Dysdercus intermedius* (Calam and Youdeowei, 1968; Calam and Scott, 1969), *Dysdercus superstitiosus* (Daroogheh and Olagbemiro, 1982), and, more recently, *Dysdercus cingulatus* (Farine et al., 1992), no other Pyrrhocoridae species have been chemically investigated.

It is well known that communication through pheromones requires only minute amounts of compounds. Unfortunately, chemical data of the so-called defensive secretion in Heteroptera are generally devoted to the identification of the major chemicals. Within the Pyrrhocoridae, *D. cingulatus* is the only one in which exocrine secretions were extensively studied (Farine, 1989). The location of the exocrine glands encountered in *P. apterus* and *D. cingulatus* is the same (Farine, 1989): the nymphs possess three dorsoabdominal glands (DAG) (abdominal terga 3, 4, 5). The small anterior (DAG 1) and median (DAG 2) glands are still functional in adults, whereas the posterior defensive gland (DAG 3) is only active in nymphs. Paired metathoracic glands (MTGs), opening at the base of the hind legs, are observed in adults of both sexes and a sternal glandular epithelium (sterna 2–6) only occurs in males. Regarding these observations, it was interesting to compare the chemicals of the defensive secretions identified in the tropical *D. cingulatus*, which is able to fly in order to escape predators, with those identified in the wingless European *P. apterus*.

This paper deals with the chemical analysis of the exocrine defensive secretions in nymphs (DAG 3) and adults (MTGs) of both sexes, with special attention to the minor exocrine components.

METHODS AND MATERIALS

Insects. A laboratory culture of *P. apterus* was developed from specimens collected in the field near Dijon, France. The insects were fed on sunflower (*Helianthus* sp.) and mallow (*Malva* sp.) seeds and water-soaked on cotton pads. Development without diapause was induced by a long photophase (14 hr) at a constant temperature of 26°C and 70% relative humidity.

Preparation of Extracts. The extraction technique used was described and discussed in a previous paper (Farine et al., 1992). Extracts of about 1000 nymphs of the fifth instar, 1000 males, and 1000 females were necessary to identify the numerous minor compounds (less than 3% of the total secretion).

Chemical Analysis. Gland extracts were analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS). The gas chromatograph was a Packard 437 A instrument with a flame ionization detector. Two different fused silica capillary columns were used: a DB 5 (30 m × 0.32 mm ID, J. and W. Scientific) and a CP Wax 58 CB (30 m × 0.22 mm ID, Chrompack). Injector and detector temperatures were, respectively, 250°C and 270°C. One microliter of each sample was injected in split–splitless mode with the oven at 50°C for 5 min, then programmed to 250°C at 2°C/min, using helium as carrier gas (5 ml/min). The relative proportions of the compounds were ascertained from GC peak-area integration using a CR 3 A Shimatzu recorder (Kyoto, Japan).

GC-MS was carried out on a Nermag R 10-10 C quadrupole mass spectrometer coupled with a Girdel 31 gas chromatograph. A fused silica capillary column coated with DB 5 (30 m × 0.32 mm ID, J. and W. Scientific) was used for analysis; the GC conditions were those described above. Electron impact mass spectra were obtained at 70 eV.

Identifications were made by comparison of the compound mass spectra to those of the EPA-NIH Mass Spectral Data Base (Heller and Milne, 1980) and the mass spectra from the library of the Laboratoire de Recherches sur les Arômes de l'I.N.R.A. (Dijon, France). Commercial compounds were purchased (Interchim, Montluçon, France) or were obtained as gifts (I.N.R.A., Dijon, France). Correct identification was confirmed if the GC characteristics of the identified compound were the same as those of the reference compound analyzed in the same conditions on DB 5 and CP Wax 58 CB columns. Identification was accomplished by coinjections of glandular extracts and pure compounds on the polar and apolar columns. Identification of 4-oxo-(*E*)-2-hexenal, 4-oxo-(*E*)-2-octenal, and 4-oxo-(*Z*)-2-octenal was matched with those published by Gilby and Waterhouse (1965) and MacLeod et al. (1977), respectively. Identification of other oxo-alkenals was conducted as described previously (Farine et al., 1992).

RESULTS

Posterior Dorsoabdominal Gland (DAG 3) of Nymphs. The 40 compounds identified in the secretion are listed in Table 1. Ninety-three percent of the secretion is composed of four aldehydes [4-oxo-(*E*)-2-octenal (57%), 4-oxo-(*E*)-2-decenal (15%), (*E*)-2-decenal (13%), (*E*)-2-octenal (8%)]. Thirty-six

TABLE 1. *Pyrrhocoris apterus* VOLATILE COMPOUNDS IDENTIFIED BY GC-MS IN SECRETION OF POSTERIOR DORSOABDOMINAL GLAND FROM NYMPHS (*N*), AND IN METATHORACIC GLANDS FROM MALES (*M*) AND FEMALES (*F*)[a]

Compound	Mol wt	%		
		N	M	F
1-Pentanol	88	0.05	0.12	0.18
Hexanal	100	0.03	0.10	0.14
Methylpentenal[b,c]	98		0.29	0.39
(*E*)-2-Hexenal	98	0.02	76.09	76.94
(*Z*)-2-Hexenal	98	0.05	1.86	1.99
(*E*)-2-Hexenol	100		1.92	1.87
Heptanal	141	0.03	0.06	0.07
α-Thujene[c]	136	0.01	0.19	0.17
(*Z*)-2-Heptenal	112	0.08	0.20	0.25
4-Oxo-(*E*)-2-hexenal	112	0.01	0.04	0.02
Methyl 2-hydroxy-3-methylpentanoate[c]	146	0.07	1.24	1.32
Octanal	128	0.01	0.07	0.01
2-Nonen-4-one[c]	140	0.05	0.06	0.04
5-Ethyl-2[5H]-furanone	112	0.60	0.05	0.04
(*E*)-2-Octenal	126	7.85	9.94	9.64
(*E*)-2-Octenol	128	0.04	0.51	0.51
2-Methoxyphenol	124	0.06	0.15	0.11
Nonanal	142	0.02	0.07	0.04
Benzeneethanol[c]	122	0.01	0.11	0.09
4-Oxo-(*E*)-2-octenal	140	57.08	0.15	0.11
2-Ethylcyclohexanone	126	1.82		
(*E*)-2-Nonenal	140	1.07		
4-Oxo-(*E*)-2-octenal	140	0.04	0.04	0.06
p-Cymen-8-ol	150	0.08	0.07	0.03
Dodecane	170	0.14	0.82	0.77
4-Oxo-(*E*)-2-nonenal	154	0.11		
4-Oxo-(*Z*)-2-nonenal[c]	154	0.75		
(*E*)-2-Decenal	154	13.37	0.14	0.12
(*Z*)-2-Decenal	154	0.01		
5-Butyldihydro-2[3H]-furanone	142	0.22		
Tridecane	184	0.30	3.77	3.49
4-Oxo-(*E*)-2-decenal	168	15.08	0.01	0.06
4-Oxo-(*Z*)-2-decenal[c]	168	0.52		
Tetradecane	198	0.02	0.22	0.11
1,4-(1-Methylethyl)-phenylethanone[c]	160	0.08		
5-Heptyldihydro-2[3H]-furanone[c]	184	0.02	0.07	0.04
Pentadecane	212	0.03	0.36	0.40
2-(3′-Isopropylphenyl)-1-propanol[c]	178	0.03	0.02	0.04
Hexadecane[c]	226	0.01	0.28	0.25
Tetradecanal	212	0.02	0.10	0.05
Pentadecanal[c]	226	0.06	0.31	0.39
Hexadecanal[c]	240	0.02	0.01	0.03
Heptadecanal	254		0.27	0.18

[a] The compounds are listed in order of elution on DB 5 capillary column. The percentages (%) of each compound were calculated as indicated in methods and materials. Mol wt: molecular weight.
[b] Methyl-branched pentenal, configuration not determined.
[c] Newly identified compound in Heteroptera.

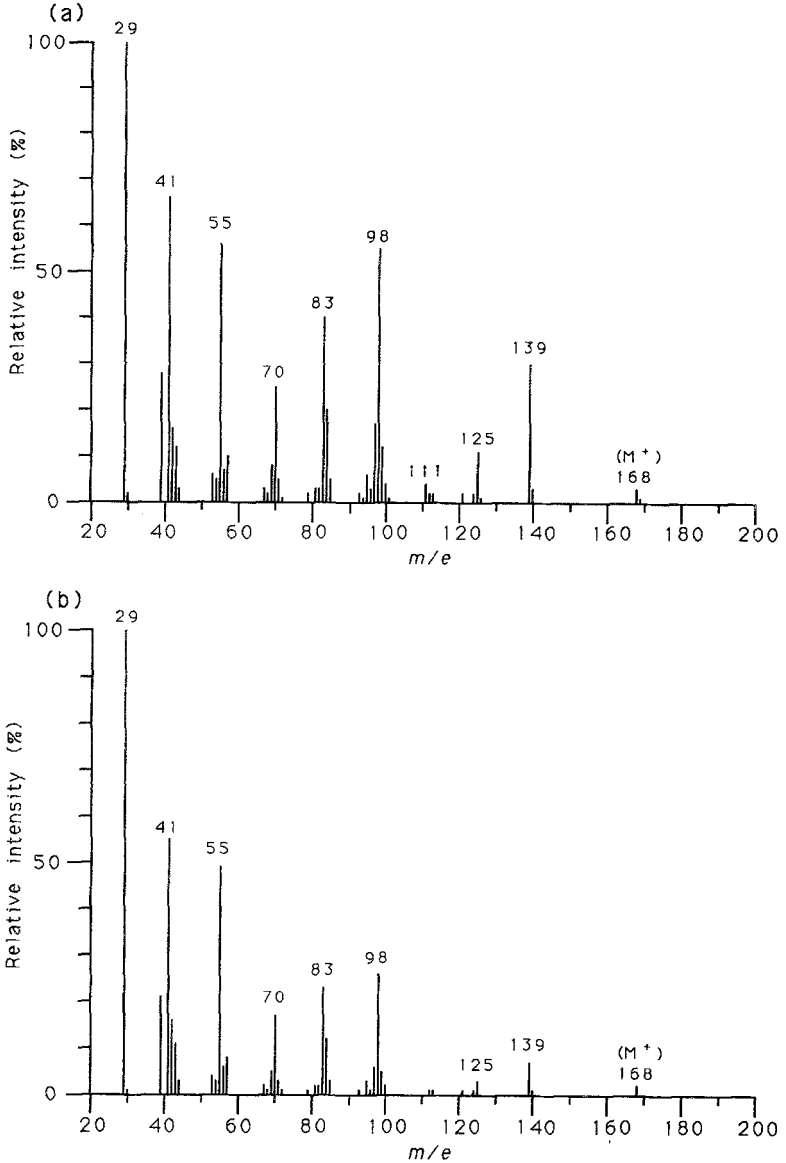

FIG. 1. EI mass spectra of 4-oxo-(*E*)-2-decenal (a) and 4-oxo-(*Z*)-2-decenal (b).

minor compounds were identified: 17 aldehydes, five saturated hydrocarbons, four alcohols, three lactones, three ketones, two terpenes, and two other compounds. It is the first time that spectra of 4-oxo-(*E*)-2-decenal (Figure 1a) and 4-oxo-(*Z*)-2-decenal (Figure 1b) have been published.

Metathoracic Glands (MTGs) of Males and Females. Thirty-five compounds were identified from the MTGs of both sexes (Table 1), and no noticeable difference was observed between males and females. Three compounds represent 90% of the secretion: (*E*)-2-hexenal (76%), (*E*)-2-octenal (10%), and tridecane (4%). The remainder of the secretion is a mixture of 16 aldehydes, four saturated hydrocarbons, five alcohols, two lactones, two terpenes, one ester, and two other compounds.

DISCUSSION

P. apterus is "warningly" colored and lives in dense and conspicuous aggregations (Bongers, 1968). Coincidence of aposematic coloration with a tendency to aggregate is the norm for insects (Pasteels et al., 1983; Borden, 1984), and it is well known that Pyrrhocoridae species form aposematic aggregations (Giles, 1958; Gupta, 1979; Farine and Lobreau, 1984). As in *D. cingulatus* (Farine, 1989), when the individuals are disturbed, the chemicals produced by the MTGs of adults and the DAG 3 of nymphal (*P. apterus*) deter predators (e.g., ants), elicit an alarm response for only a very short time, and induce the dispersion of the bugs (Schmuck, 1987; Farine, 1989). The exocrine secretion may also be considered as defensive, the ants not only being repelled but also paralyzed (Farine, 1989).

Most of the defensive secretions in the Heteroptera are highly volatile compounds of low molecular weight (mol wt) (Dazzini and Pavan, 1978; Staddon, 1979; Blum, 1981; Aldrich, 1988). In the nymphal DAG 3 of *D. cingulatus* (Farine et al., 1992), the molecular weight of the 34 identified compounds varies from 88 to 212, including 12 components below mol wt 120. In adults of both sexes, the 21 identified compounds in the MTGs vary from mol wt 92 to 184 and seven of them are less than mol wt 120. In nymphal and adult *P. apterus*, the molecular weight of the 43 identified compounds varies from 88 to 254. Only seven nymphal and nine adult components were determined to be less than mol wt 120. Consequently, the exocrine chemicals in *P. apterus* are enriched with high-molecular-weight constituents compared to their counterparts in *D. cingulatus*. The fact that *P. apterus* is essentially a terrestrial species and that the wingless adults cannot fly may explain the presence of a larger number of high-molecular-weight compounds. These chemicals, with a slow evaporation rate, could induce longer-lasting protection.

As in the other Pyrrhocoridae—*D. intermedius* (Calam and Youdeowei,

1968), *D. cingulatus* (Farine et al., 1992), and *D. superstitiosus* (Daroogheh and Olagbemiro, 1982)—the secretion of the nymphal DAG 3 of *P. apterus* is mainly a mixture of aldehydes. The nymphal secretions of *P. apterus* are characterized by the abundance of (*E*)-2-octenal, (*E*)-2-decenal, 4-oxo-(*E*)-2-octenal, 4-oxo-(*E*)-2-decenal, and by the lack of (*E*)-2-hexenal. 4-Oxo-(*E*)-2-decenal was first reported as a trace compound from *D. cingulatus* (Farine et al., 1992), but it is the first time that this oxo-alkenal has been found as a major constituent of an insect exocrine secretion. Besides the four major compounds, we determined that 7% of the secretion consisted of minor components. Our results differ from those of Schmuck (1987), who found tridecane and heneicosane as major compounds. Tridecane is effectively present in nymphal secretions, but as a minor component, whereas heneicosane has never been detected by us and certainly belongs to the cuticular hydrocarbons. To our knowledge, it is the first time that the adult defensive secretions have been investigated in *P. apterus*. Besides the major components (*E*)-2-hexenal, (*E*)-2-octenal, and tridecane, about 10% of the identified chemicals are minor compounds. Whereas the (*E*)-2-alkenals were identified as major components in *Dysdercus* species, tridecane was only found as a major compound in *P. apterus*. On the other hand, linalool, a major compound of the MTG secretions in *Dysdercus* species (Calam and Youdeowei, 1968; Daroogheh and Olagbemiro, 1982; Farine et al., 1992), has never been identified in *P. apterus*.

Many of the identified chemicals in *P. apterus* are considered to have a defensive function. Short-chain saturated and unsaturated aldehydes are very effective chemical irritants (Remold, 1963). The originality of the *P. apterus* secretions consists of abundant aldehydic compounds (23 of the 43 identified compounds are aldehydes). Hydrocarbons have defensive properties of their own (Blum, 1981) and in several insects serve to facilitate the penetration of the reactive aldehydes through the cuticle of arthropod predators (Remold, 1962). The five identified saturated hydrocarbons always appear as minor compounds (except tridecane in adults of both sexes) in *P. apterus*, and it is difficult to assert that these components have only a physicochemical action in this species. The same should be said concerning the identified esters in *P. apterus*. The ester could act as a wetting agent to facilitate the penetration of the main toxicant through the cuticle of the predators, but about 20% of the esters identified in the defensive secretions of insects (Blum, 1981) are produced by adult true bugs and are generally lacking in the nymphal defensive secretions. The only identified ester in *P. apterus* represents only 1% of the adult defensive secretions and appears as a minor compound in the nymphs. Volatile monoterpenes are common repellents in insects, particularly in the alarm-defense system of ants (Herout, 1970; Karlson, 1970). Within the identified terpenoid compounds in *P. apterus*, *p*-cymen-8-ol was described for the first time in *D. cingulatus* (Farine et al., 1992), and α-thujene was only reported in the defensive secretions of Termitidae (Everaerts et al., 1988).

The function of the aldehydes and hydrocarbons found in the defensive glands of the Heteroptera varies according to their concentration. The dual function of the aldehydes was first reported in *Cimex lectularius* (Cimicidae) (Levinson and Bar Ilan, 1971). In *Eurydema rugosa* (Pentatomidae), large amounts of (*E*)-2-hexenal act as an alarm pheromone, whereas small amounts of the same compound play an important part in the re-formation of the aggregation (Ishiwatari, 1974, 1976). In the nymphs of *Nezara viridula* (Pentatomidae) the dual function is played by various amounts of tridecane (Lockwood and Story, 1986). In *D. cingulatus* (Farine, 1989), large or small amounts of (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-decenal, 4-oxo-(*E*)-2-hexenal, 4-oxo-(*E*)-2-octenal and C_{11}–C_{15} saturated hydrocarbons have the same dual function.

In nymphal *P. apterus*, Schmuck (1987) showed that the aggregative behavior results mainly from an interindividual attraction guided by visual and chemical stimuli and that extracts of the DAG 1 and DAG 2 act as attractants. The nymphs are repelled when using high concentrations (10 individual equivalents) of C_{14}–C_{17} saturated hydrocarbons, while an arrestant effect is only shown when using low concentrations (0.1–1 individual equivalents) of the same compounds.

At the present time, numerous questions concerning chemical communication within the Heteroptera remain unanswerable and much theorizing is possible on the complexity of the defensive secretion. In *D. cingulatus* (Farine, 1989), we demonstrated the importance of most chemicals identified in the so-called defensive glands in nymphs and adults and proved that the trace components encountered are of primary importance in aggregation, alarm, defense, or sexual behavior. Compared to this species, the extensive chemical data in *P. apterus* also revealed the complexity of the defensive secretion in nymphs and adults of both sexes and the biological role of each compound still needs further study.

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SOIL TRANSFORMATION OF 2(3H)-BENZOXAZOLONE OF RYE INTO PHYTOTOXIC 2-AMINO-3H-PHENOXAZIN-3-ONE

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Abstract—Nonsterile soil transforms the rye metabolite 2(3H)-benzoxazolone (BOA) into 2-amino-3H-phenoxazin-3-one, which is an order of magnitude more toxic to barnyard grass than benzoxazolone. Benzoxazolone was recovered unchanged from sterile soil. However, *o*-aminophenol is converted to aminophenoxazinone by both sterile and nonsterile soil in the presence of air. Aminophenoxazinone is probably produced by microbial hydrolysis of benzoxazolone into *o*-aminophenol, which is oxidized to aminophenoxazinone in both sterile and nonsterile soil. No 2,2′-oxo-1,1′-azobenzene was found in any incubations of soil with benzoxazolone, *o*-aminophenol, or *o*-azophenol.

Key Words—2-Amino-3H-phenoxazin-3-one, 2(3H)-benzoxazolone, BOA, *o*-aminophenol, rye, *Secale cereale*, soil transformation, phytotoxicity, allelopathy, barnyard grass, *Echinocloa crus-galli*.

INTRODUCTION

The use of allelopathic mulches in reduced or no-till cropping systems has received attention in recent years. The practical and chemical aspects of using winter rye, *Secale cereale* L., mulch are perhaps the best studied (Worsham, 1989). Phytotoxic activity of rye mulch has been attributed largely to the cyclic hydroxamic acid 2,4-dihydroxy-1,4(2H)-benzoxazin-3-one (DIBOA) and its decomposition product 2(3H)-benzoxazolone (BOA) (**1**, Figure 1) (Barnes et al., 1987). A crystalline, red transformation product of BOA has recently been isolated from Michigan soil, and the structure 2,2′-oxo-1,1′-azobenzene (**2**) has

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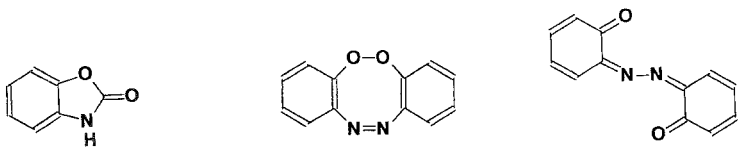


1 2 3

FIG. 1. Structures of 2(3H)-benzoxazolone **(1)**, 2,2′-oxo-1,1′-azobenzene **(2)**, and *o*-benzoquinone azine **(3)**.

been proposed for this pigment (Nair et al., 1990). This red transformation product proved more phytotoxic than BOA in vitro (Chase et al., 1991a) and is therefore a further possible allelochemical of rye mulch. The soil transformation of BOA was later determined to be mediated by the microorganism *Acinetobacter calcoaceticus* (Chase et al., 1991b).

Soil microorganisms are known to transform substituted anilines into azo compounds (Bartha and Pramer, 1972). However, microbial formation of an oxygen–oxygen bond between phenols is unprecedented. In fact, chemical attempts to generate phenolic peroxides by dimerization of ortho-, para-blocked phenolic radicals does not lead to stable phenolic peroxides (Pummerer et al., 1952), and yet the red soil transformation product appeared to be stable and to have a very high melting point with no decomposition reported. We have considered possible alternative structural interpretations of the data used to arrive at the 2,2′-oxo-1,1′-azobenzene structure. The published [^{1}H]- and [^{13}C]NMR data indicated an element of symmetry in the pigment leading to six carbon and four hydrogen signals for $C_{12}H_8N_2O_2$. All isomeric structures with the required symmetry can be rejected based on published melting point and spectral data except one, *o*-benzoquinone azine **(3)** reported to be the product obtained by silver(II) oxide oxidation of *o*-aminophenol (Ortiz et al., 1972). The ultraviolet–visible spectrum reported for this red oxidation product is the same as that reported for 2,2′-oxo-1,1′-azobenzene, and its high melting point is similar to that reported for the soil transformation pigment. It appeared that the red soil transformation product and the product of silver(II) oxide oxidation of *o*-aminophenol might be identical. Therefore we reinvestigated the properties of these red pigments.

METHODS AND MATERIALS

Soil Sample. Cecil and Appling gravelly sandy loam (Typic Kanhaludult), was collected from a fallow corn field at the North Carolina State University research farm at the Dorothea Dix property (plot number 163/H-2). Soil was stored in a sealed plastic bag at 4°C until needed.

Soil Transformation of BOA. A 2-g sample of BOA **(1)** was mixed into 400 g soil and incubated in the dark at 28°C. After 10 days, the soil was extracted twice with 400 ml methanol. The extract was concentrated to dryness (1.2 g), and the residue was redissolved in a small amount of ethyl acetate. After two days, crystals were collected from this dark red solution, which was allowed to stand for further crystallization. A final yield of 311 mg of red crystals was obtained.

Effect of Sterile and Septic Soil. To test whether soil microorganisms are required for transformation of BOA into the red pigment, sterile and nonsterile soil was supplemented with BOA and two potential intermediates, *o*-aminophenol and *o*-azophenol. A 50-g portion of soil was mixed with 50 mg of the test compound and 5 ml deionized water. The mixture was transferred to an Erlenmeyer flask, stoppered with a foam plug, and incubated in the dark at 25–28°C. In cases where sterile soil was used, the test compound was added aseptically to double-autoclaved soil. After 10 days the soil was extracted by stirring twice with 100 ml of methanol. The extracts were concentrated under vacuum and compared to the silver oxide oxidation product by thin-layer chromatography (TLC) in two solvent systems: toluene–ethyl acetate (50:40) and chloroform–methanol (95:5).

Silver(II) Oxide Oxidation of o- *Aminophenol.* Silver(II) oxide, AgO, was prepared according to the method of Hammer and Kleinberg (1953). The silver(II) oxide oxidation of *o*-aminophenol was carried out according to the method of Ortiz et al. (1972). A mixture of 6 g *o*-aminophenol and 31 g freshly prepared silver(II) oxide was stirred in benzene for 4 h. Insoluble silver oxides were removed by vacuum filtration. The dark red pigment in the filtrate was purified by flash column chromatography (chloroform–methanol 80:20). Preparative TLC (chloroform–methanol 9:1) yielded a dark red product (R_f 0.65 in chloroform–methanol 95:5), which was triturated with water to remove traces of *o*-aminophenol. The red solid was dissolved in the minimal amount of dimethylsulfoxide. Slow evaporation of some of the solvent from an open beaker produced fine, dark red crystals which were homogeneous by TLC in three solvent systems: chloroform–methanol 95:5 (R_f 0.6), hexane–acetone 70:30 (R_f 0.7), and toluene–ethyl acetate 50:40 (R_f 0.5). The dark red product diluted for TLC appeared as a bright orange spot, which was the only spot detected visually, by fluorescence quenching or by treatment with phosphomolybdic acid (Peña-Rodriguez et al., 1988).

Synthesis of Aminophenoxazinone. 2-Amino-3H-phenoxazin-3-one **(4)** was synthesized by the air oxidation of *o*-aminophenol as described previously (Kehrmann, 1906). In this procedure, 4 g of *o*-aminophenol were stirred in aqueous methanol at room temperature for three weeks. Production of the dark red aminophenoxazinone was monitored by TLC (chloroform–methanol 95:5). Solids that precipitated from the solution were collected and recrystallized from meth-

anol to give clusters of reddish black needlelike crystals. The aminophenoxazinone was further purified by washing with hot water and recrystallizing from methanol. The crystallized product was analyzed by TLC, UV, NMR, and MS. The yield of aminophenoxazinone after three weeks was 1.1 g. There was still some unoxidized *o*-aminophenol in the original methanolic solution after three weeks.

Spectroscopy. Ultraviolet spectra were measured on a Perkin-Elmer Lambda 4B UV/VIS spectrophotometer. [^{1}H]NMR was measured in DMSO-d_6 at 300 and 500 MHz on a General Electric Omega spectrometer. Proton-coupled and proton-decoupled [^{13}C]NMR spectra were measured at 125 MHz. Low-resolution mass spectral analysis was performed using a Hewlet-Packard 5985-B spectrometer.

Bioassay. Phytotoxicity was tested by a radicle elongation assay. A 1 mg/ml stock solution of the test compound was prepared in methanol. Volumes corresponding to 12.5, 25, 50, 100, 175, and 250 mg of test compound were applied evenly to 6-cm filter paper circles, and the solvent was allowed to evaporate completely. Filter paper wet with methanol and dried was used as a control. The dried paper impregnated with test compound was wet with 1.5 ml water in a 60 × 15-mm Petri dish, and 10 seeds of barnyardgrass (*Echinocloa crus-galli* L.) were added. After incubation in the dark for 72 h, the radicle lengths (mm) were measured and expressed as a percentage of the control radicle length.

RESULTS

Transformation of BOA and Other Compounds by Soil. BOA incubated with moist, nonsterile soil at 28°C was converted within 10 days into a dark red substance extractable by methanol. TLC showed the presence of a major orange pigment (red on concentration), higher R_f, very weak yellow and brown pigments, and a very small amount of unreacted, colorless BOA, detected by ultraviolet fluorescence quenching and by development with phosphomolybdic acid. Soil that had not been treated with BOA contained no extractable pigments.

The red pigment, recrystallized from methanol, had mp 252–258°C; UV maxima in methanol at 236 (31,000) and 432 nm (26,000); EIMS *m/z* 212 (100%, M^+), 185 (58%), 184 (28%); [^{1}H]NMR in DMSO-d_6 at 300 MHz: 6.34 ppm (s, 1H), 6.35 (s, 1H), 6.81 (broad, NH_2), 7.48 (m, 3H), 7.71 (dd, J = 1, 8 Hz, 1H). The singlets at 6.34 and 6.35 ppm were not resolved in pure DMSO but did resolve after addition of a drop of D_2O. The three-proton multiplet at 7.48 ppm was resolved at 500 MHz: 7.38 (dt, J = 1, 8 Hz,), 7.45 (dt, J = 1, 8 Hz,), and 7.48, (dd, J = 1, 8 Hz). Carbon resonances were assigned in the [^{13}C]NMR spectrum (Table 1) consistent with heteronuclear multiple bond

correlation spectra, multiplicities observed in [^{1}H]- and [^{13}C]NMR spectra, and model compounds.

Ten days after addition of BOA to doubly autoclaved soil, unreacted BOA was detectable in the methanolic extract, but no pigment was present. The concentration of BOA in sterile soil was undiminished judging from TLC spot intensity. On the other hand, TLC analysis showed that *o*-aminophenol was completely converted to the red pigment and no other product within 10 days in both sterile and non-sterile soil. *o*-Azophenol, a conceivable precursor to 2,2′-oxo-1,1′-azobenzene, or *o*-benzoquinone azine, was unaltered after 10 days of incubation in nonsterile soil.

Red Pigment Produced by Silver(II) Oxidation. The pigment produced by silver(II) oxide oxidation of *o*-aminophenol was purified by column chromatography and preparative TLC, followed by crystallization from dimethylsulfoxide. The red crystals had mp 268°C; UV maxima in methanol at 236 (23,000) and 432 nm (20,000); EIMS *m/z* 212 (100%, M^+), 185 (50%), 184 (21%); [^{1}H]NMR in DMSO-d_6 at 300 MHz: 6.42 ppm (s, 1H), 6.44 (s, 1H), 6.87 (broad, NH_2), 7.54 (m, 3H), 7.78 (dd, J = 1, 8 Hz, 1H); [^{13}C]NMR as in Table 1.

Comparison of Pigments to Aminophenoxazinone. 2-Amino-3H-phenoxazin-3-one **(4)** (Scheme 1) was prepared by air oxidation of *o*-aminophenol (Kehrmann, 1906) and recrystallized twice from methanol, mp 253–267°C (lit. mp 255–257°C). The red crystals had visible–ultraviolet maxima in methanol at 237 nm (22,000), 432 (19,600); EIMS *m/z* 212 (100%, M^+), 185 (53%),

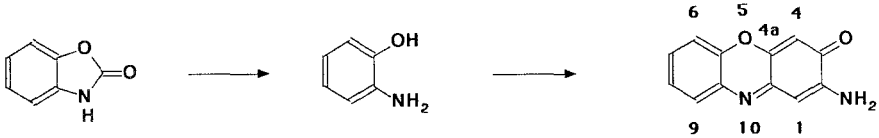
TABLE 1. [^{13}C] NMR SPECTRUM OF SOIL TRANSFORMATION PRODUCTS FROM BENZOXAZOLINONE

C, H#	Aminophenoxazinone from soil transformation	2,2′-Oxo-1,1′-azobenzene (Nair et al., 1990)
1	103.43, doublet[a]	104.86, doublet[b]
2	147.38, singlet	
3	180.24, singlet	
4	98.34, doublet	99.87, doublet[b]
4a	148.88, singlet	
5a	141.91, singlet	
6	115.93, doublet	117.25, doublet[b]
7	128.81, doublet	130.34, doublet[b]
8	125.28, doublet	126.60, singlet[b]
9	127.96, doublet	129.29, singlet[b]
9a	133.73, singlet	
10a	148.23, singlet	

[a] Proton decoupled [^{13}C]NMR spectra were measured in DMSO-d_6 at 125 MHz.
[b] Spectrum measured in DMSO-d_6; No C# assignments intended.

184 (22%). The [^{1}H]- and [^{13}C]NMR agreed with spectra previously reported for 2-amino-3H-phenoxazin-3-one (Hasegawa and Ueno, 1985). The red pigments from soil transformation of BOA and from silver(II) oxidation of *o*-aminophenol were indistinguishable from 2-amino-3H-phenoxazin-3-one by UV, EIMS, and [^{1}H]- and [^{13}C]NMR. The red pigments prepared by soil transformation and by Ag(II) oxide oxidation also comigrated with aminophenoxazinone on silica gel TLC at R_f 0.5 in toluene–ethyl acetate, 50:40 and at R_f 0.6 in chloroform–methanol (95:5).

Phytotoxicity of Aminophenoxazinone. The radicle elongation assay on barnyard grass showed that aminophenoxazinone was more phytotoxic than its precursor BOA (Figure 2). Root elongation was inhibited 50% by 0.1 mM aminophenoxazinone, 0.7 mM BOA, and 1.2 mM *o*-aminophenol, the probable hydrolytic intermediate between BOA and aminophenoxazinone.



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SCHEME 1. Soil transformation of benzoxazolone into 2-amino-3H-phenoxazin-3-one (4).

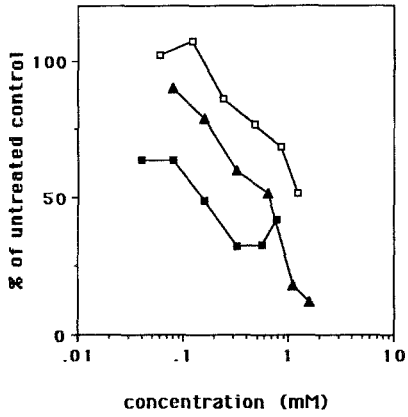

FIG. 2. Effects of aminophenoxazinone (—■—), *o*-aminophenol (—▲—), and benzoxazolone (—□—) on radicle elongation of barnyard grass.

DISCUSSION

In an earlier study silver(II) oxide was found to oxidize 7 substituted anilines to azoarenes (Ortiz et al., 1972). Based on this analogy and elemental composition, but no NMR data, the structure *o*-benzoquinone azine **(3)** was proposed for the red crystals produced by oxidation of *o*-aminophenol with silver(II) oxide. We synthesized and characterized this compound by NMR and other spectroscopic techniques because this product has a similar high melting point, the identical elemental composition and ultraviolet spectrum, and the symmetry of the reported soil transformation product of 2(3H)-benzoxazolone (BOA) **(1)** previously assigned the structure 2,2′-oxo-1,1′-azobenzene **(2)** (Nair et al., 1990). We found that the product of silver(II) oxidation of *o*-aminophenol is in fact not *o*-benzoquinone azine, but rather 2-amino-3H-phenoxazin-3-one **(4),** from which it is indistinguishable by thin-layer chromatography, mass spectral fragmentation, [^{1}H]- and [^{13}C]NMR, and UV spectra. Aminophenoxazinone and its derivatives are the only known products of oxidation of *o*-aminophenol by air (Kehrmann, 1906), photoirradiation (Ikekawa et al., 1968), inorganic oxidants (Ikekawa et al., 1968; Hishida et al., 1974), mammalian tissue (Tomoda, 1986), and purified enzymes (Nagasawa, 1959; Barry et al., 1989). Aminophenoxazinone, also known as the antibiotic questiomycin A from *Streptomyces thioluteus* (Gerber, 1967), is produced by a number of fungi (Turner and Aldridge, 1983) and bacteria (Gerber and Lechevalier, 1964).

We have also identified the red pigment produced from BOA by nonsterile soil as 2-amino-3H-phenoxazin-3-one. We note the identity of the ultraviolet and mass spectra and near identity of the proton and [^{13}C]NMR spectra of aminophenoxazinone obtained from transformation of BOA by North Carolina soil to that of the red pigment obtained by transformation with Michigan soil. Aminophenoxazinone contains all of the ^{13}C signals reported for the Michigan transformation product plus six low-intensity quaternary carbon signals not previously reported (Table 1). The [^{1}H]NMR of aminophenoxazinone contains all of the signals reported for 2,2′-oxo-1,1′-azobenzene **(2),** with the same chemical shifts and multiplicities, but, in addition, contains two singlets (6.34 and 6.35 ppm) and a broad singlet due to an NH_2 (6.81 ppm) not reported for the Michigan soil transformation product.

Although nonsterile soil converts the allelochemical BOA into the phytotoxic pigment aminophenoxazinone, sterile soil does not. The probable route of transformation of BOA is its hydrolysis to *o*-aminophenol, followed by oxidation to aminophenoxazinone (Scheme 1). Since hydrolysis of BOA in the laboratory requires prolonged treatment with aqueous alkali or acid at 100°C or higher (Graebe and Rostovzeff, 1902) and does not occur in sterile soil, the hydrolysis of BOA to *o*-aminophenol must be a step requiring soil microorganisms. Sterile soil in contact with air is capable of accomplishing the subsequent oxidation of

o-aminophenol into aminophenoxazinone. Some microorganisms are also known to oxidize *o*-aminophenol to aminophenoxazinone, but their presence has not been demonstrated in soil samples capable of converting BOA or *o*-aminophenol into aminophenoxazinone. The present study does not exclude the possibility that microorganisms contribute to the rate of oxidation of aminophenol to aminophenoxazinone. The transformation product, aminophenoxazinone, is an order of magnitude more phytotoxic than BOA. It is significant that this compound has recently been identified as the phytotoxin of the plant pathogen *Acrospermum viticola* (Kinjo et al., 1987). Thus, the microbially produced aminophenoxazinone has the potential for increasing the allelopathic effect of rye mulch.

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FIELD RESPONSE OF PREDATOR *Rhizophagus grandis* TO PREY FRASS AND SYNTHETIC ATTRACTANTS

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Abstract—A lure based on the proportional composition of monoterpenes in *D. micans* larval frass and deployed in Theysohn slot traps was highly attractive to *R. grandis* released in the field. The relative response to frass and lure was consistent over a range of doses, and behavior close to traps baited with either lure or frass appeared to be similar. The monoterpenes, formulated with antioxidant, appear to be stable over several weeks when released from proprietary reservoir and wick "air fresheners." The lure may be of value in monitoring predator populations.

Key Words—Frass, kairomone, monoterpenes, traps, *Dendroctonus micans*, *Rhizophagus grandis*, Coleoptera, Scolytidae, Rhizophagidae.

INTRODUCTION

Rhizophagus grandis (Gyll.) (Coleoptera; Rhizophagidae) appears to play an important role in the natural control of its prey, larvae of the spruce bark beetle, *Dendroctonus micans* (Kug.) (Coleoptera; Scolytidae) (Grégoire, 1988). This predator has been used with some success in biological control programs against *D. micans* in France, Soviet Georgia, Turkey, and Britain (King and Evans, 1984; Grégoire, 1988; N. Uslu, Ormancilik Arastirma Institusu, Ankara, Turkey, personal communication).

Two strategies have been adopted for predator release, both of which have

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resulted in successful establishment. In France, 50–1000 pairs were released per hectare in infested forests (Grégoire, 1988; Grégoire et al., 1985), whereas in Britain only 10–50 pairs were released at "infested sites" in and around a scheduled quarantine area (Wainhouse, 1987; Evans and King, 1989; Fielding et al., 1991). Establishment of *R. grandis* has been verified at some sites in Britain by felling trees in the year following release and searching *D. micans* galleries (N. J. Fielding, Forest Authority Research Station, personal communication). Despite the release of relatively few individuals per unit area, successful establishment is in part due to the kairomone response of the adult predator, which appears to be mediated by a blend of host-derived monoterpenes released from *D. micans* larval frass (Wainhouse et al., 1991).

In this paper, we report results of the release and recapture of unmarked laboratory-reared *R. grandis* adults (Evans and King, 1989) in experiments to determine the dose response to both *D. micans* larval frass and artificial lures based on a blend of monoterpenes.

METHODS AND MATERIALS

Release and Recapture of Unmarked R. grandis *Adults*

Four experiments were done in a 0.25-ha compartment of predominantly Sitka spruce (*Picea sitchensis*) planted in 1963 (OS ref. SO 500 734) near Ludlow, Shropshire, U.K. in which both *D. micans* and *R. grandis* were present at low density. In the compartment, thinned to about 2.5 m between trees, a 50-m/square trapping arena was formed using Theysohn slot traps (Speight and Wainhouse, 1989) at five positions about 10 m apart arranged on each of the four sides. The trap lines were either parallel or perpendicular to tree rows, which were oriented approximately WNW–ESE. Traps were at least 1 m from the nearest tree.

Frass and synthetic trap baits were used in experiments. Frass baits came from a mixed sample of *D. micans* larval frass (stored for several weeks at −18°C) from Norway and Sitka spruce. The frass was held in nylon 0.5-mm-mesh sachets, usually in units of 5 g occupying 5 × 2 × 1 cm. Smaller quantities of frass were packed to a similar density so that volume was approximately proportional to weight. Baits of liquid synthetic lures were dispensed, respectively, from: (1) 2.5 g of dried, diethylether-extracted Norway spruce bark powder, in nylon sachets, whose volume approximated that of 5 g of frass; and (2) a wick system using proprietary air freshener technology with the odor dispersing from a release pad (max. diam. 48 mm) supplied by a reservoir holding about 80 g of lure.

Experiments were carried out during July–September in both 1990 and 1991. Treatments of different trap baits were allocated at random to each trap-

ping position on each side of the arena. In each experiment, 2000–2500 laboratory-bred *R. grandis* (Evans and King, 1989) were released in moist sand in a plastic tray from which they could only disperse by flight. Females were assumed to outnumber males by about 1.8:1 (Wainhouse et al., 1991). Wire mesh over the tray prevented predation by birds. Releases were made from the center of the arena, usually around midday, when winds were light and variable. Following releases, temperatures subsequently rose to at least 19°C. Catches were collected at dusk on the day of release or the following morning before any further flight had occurred. The number of beetles in each trap and those remaining in the release tray were counted.

Experiment 1: Effect of Trap Number. At each trapping position, one to three traps were used, although only one was baited. Each trap was approximately 10 cm thick × 50 cm wide × 60 cm tall and occupied an upper, middle, or lower position. The tops of traps in the upper position were 2 m above the ground and traps were suspended one below the other to increase the vertical component of the visual silhouette. The arrangement of traps and the position of 5-g frass baits in this experiment are shown in Figure 1A. There were three trials using traps 1–5 and two using traps 6–10.

Experiment 2: Dose Response to Frass. Except for two trials with three traps per trapping position, this and subsequent trapping experiments employed

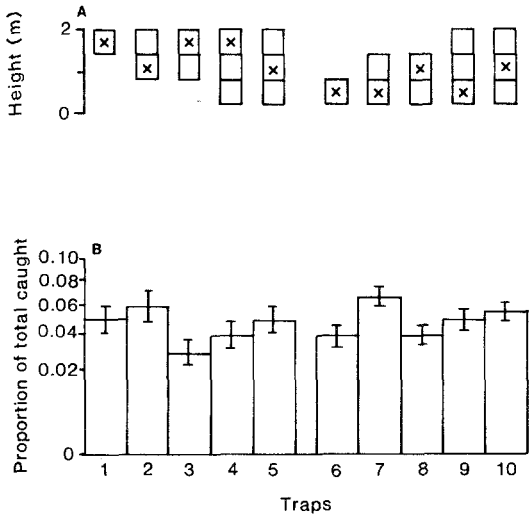

FIG. 1. (A) Arrangement of Theysohn slot traps and frass bait (×) in experiment 1. (B) Catch of *R. grandis* in each trap arrangement (A) as a proportion of the total caught in each trial. Means ± common SE. The total numbers caught were: traps 1–5 (three trials), 112, 247, 78; traps 6–10 (two trials), 198, 68.

two traps with the lower one baited (Figure 1A, trap 2). The following frass treatments were used in separate trials: (1) 4 and 10 g; (2 and 3) 2.5, 5, 10, and 15 g; and (4) 1, 2.5, 5, 10, and 15 g. Frass doses were made up in 2-g units (trial 1) or 1-, 2.5-, or 5-g units (trials 2–4). A control treatment of 2.3 ml of diethylether on 2.5 g of bark powder was included in trials 1–3.

Experiment 3: Response to Different Synthetic Lures. Lures were based on a mixture of monoterpenes, present in frass, that was highly attractive to *R. grandis* in laboratory wind-tunnel experiments (Wainhouse et al., 1991). Commercially available monoterpenes (95–99% purity) were used except for β-phellandrene, which was extracted from Sitka spruce resin or supplied by Glidco Organics, Jacksonville, Florida. The synthetic β-phellandrene was tentatively identified as the (−) enantiomer (D.R. Kelly, M.P.O. Orozco, unpublished observations).

Treatments comprised four lures with minor variations in composition (Table 1) and 5 g of frass. Lure 1, consisting of the five monoterpenes found in frass extracts, and lure 2 correspond to those used by Wainhouse et al. (1991). In lure 1a, *dl*-α-pinene and *dl*-limonene were used, and in 1c, (+)-α-pinene and (−)-limonene were used, which corresponded to the enantiomeric composition found in frass extract. Lure 2 contained myrcene as an impurity in the β-phellandrene extracted from Sitka spruce resin. Lure 3 was similar to lure 2 but was made using synthetic myrcene and β-phellandrene.

One-gram aliquots of each lure (+ approx. 1.2 g ether) were added to bark powder sachets for use in the field. All lures in this and subsequent experiments were formulated with 1% BHT by weight to retard oxidative reactions. There were two trials of this experiment.

Experiment 4: Dose Response to Lure 1a. Using the wick system, different release rates of the lure were obtained by using dispensers with different amounts

TABLE 1. PERCENT COMPOSITION OF LURES[a] USED IN TRAPPING EXPERIMENTS

	Lure 1a	Lure 1c	Lure 2	Lure 3
(+)-α-Pinene	14.0[b]	14.0	13.4	13.4
(−)-β-Pinene	28.0	28.0	26.8	26.8
3-Carene	14.0	14.0	13.4	13.4
dl-Limonene	11.5	11.5[c]	11.0	11.0
β-Phellandrene	32.5	32.5	31.2[d]	31.2
Myrcene			4.2[d]	4.2

[a] Lures 1a, c, and 2 correspond to those of Wainhouse et al. (1991).
[b] *dl*-α-Pinene.
[c] (−)-Limonene.
[d] Synthetic monoterpenes were used except these were extracted from Sitka spruce resin.

of the release pad exposed or by varying the number of dispensers used in traps. In each of seven trials of this experiment, treatments comprised four release rates of lure and a standard bait of 5 g of frass. Release rates of the lure, determined by weight loss during the trial, varied in each trial as follows (1) 0.006–0.05 g/hr, (2) 0.06–0.31 g/hr; (3–5) 0.07–0.26 g/hr, (6) 0.06–0.6 g/hr, and (7) 0.05–0.43 g/hr.

Temporal Changes in Release Rate and Composition of Lure 1a

Experiment 5: Release Rate from Wick Dispensers at Constant Temperature. The daily release rate of the lure was determined in the laboratory at temperatures of 15 ± 1°C and 20 ± 2°C from two or three wick dispensers with half the pad exposed in separate traps for 11 days. During experiments, relative humidity varied from 52% to 64%.

Experiment 6: Temporal Changes in Chemical Composition in the Field. Three wick dispensers in separate traps with a nominal initial release rate of 1–1.5 g/day at 20°C were deployed at three spruce forest sites near Ludlow for 32 days. Samples of lure were taken for chemical analysis at intervals.

A portion of each sample (0.05 ml) was added to 10-bromodecan-1-ol in methylene chloride (0.95 ml, 0.01 g/100 ml), and this mixture further diluted with methylene chloride (1 or 2 ml). The concentration of monoterpenes was determined relative to the 10-bromodecan-1-ol by GC-MS (Hewlett Packard 5890/VG Trio 1) using an EI source at 70 eV. A DB17 column (30 m × 0.322 mm) with a precolumn (10 m) was used for the separation under the following conditions: splitless 1 min, injector 170°C, program 45°C start, 1°C/min to 75°C then 10°C/min to 180°C for 11 min, helium 8 psi, 70 ml/min. The retention times (after a 3.7-min solvent delay) were α-pinene (5.50 min), β-pinene (7.80 min), myrcene (8.42 min), 3-carene (9.20 min), limonene (10.47 min) and β-phellandrene (11.07 min). The retention times were matched with standards and 3-carene and myrcene were confirmed by spiking.

RESULTS

Results from experiments 1–4 suggested that the majority of beetles caught were from releases made on the same day. Nevertheless, because beetles were unmarked, percent recapture could not be determined with certainty. In analyses of the recapture data, we have therefore used the proportion of the total caught responding to each treatment or, when treatments differed between trials, we used the actual number caught in traps. Total catches in the different trials of each experiment varied from 38 to 247.

Release and Recapture of Unmarked R. grandis *Adults*

Experiment 1: Effect of Trap Number. For each trial, the catch for each treatment (Figure 1A) was expressed as a proportion (p) of the total catch over all treatments in that trial. Data from the different trials of traps 1–5 were combined as were those for traps 6–10 (Figure 1B). The two sets of trials were then analyzed independently by analysis of variance of p transformed to angles (degrees) by the arcsin ($\sqrt{p}$) transformation. Differences between treatments were not significant, but in both sets of trials, more insects were caught in the two-trap arrangement in which the lower trap was baited (Figure 1A, traps 2 and 7). The prominence of the visual silhouette appeared to have no effect on trap catches. There was a significant difference between blocks (sides of the arena) ($F_{3,43} = 11.21$, $P < 0.001$) in the trials with traps 1–5.

Although most insects were caught in baited traps, some were also caught in the unbaited ones at the same trapping position. The overall mean for all trials (traps 1–10) was 6.1 *R. grandis* per baited trap and 0.9 for adjacent unbaited ones. The number of insects in the unbaited traps appeared to depend on the trap's position relative to the baited one. The total number of insects in traps above or below baited traps when there were two (Figure 1A, traps 2, 3, 7 and 8) or three traps (Figure 1A, traps 5 and 10) at each position are shown in Tables 2 and 3, respectively. The totals in Tables 2 and 3 were tested (χ^2 test) on the null hypothesis that the position of unbaited traps in relation to the baited trap has no effect on the probability of catches in unbaited traps. Significantly ($P < 0.01$–$P < 0.001$) more insects were trapped in unbaited traps above than below the baited trap in both the two- and three-trap arrangements (Tables 2 and 3). Possible interaction between rows and columns in Tables 2 and 3 was examined using Fisher's exact test. There was no significant evidence of inequality of probabilities.

Experiment 2: Dose Response to Frass. For each trial, the mean number of *R. grandis* caught relative to each dose of frass was determined and the pooled data analyzed by linear regression of log (ln) catch (+0.001) on log

TABLE 2. TOTAL CATCH IN TRAPS ABOVE OR BELOW A FRASS-BAITED TRAP IN TWO-TRAP ARRANGEMENTS IN FIGURE 1a[a]

	Traps 2 and 3	Traps 7 and 8	Total
Above baited trap	14	7	21
Below baited trap	2	3	5

[a]Data from experiment 1. $\chi^2_1 = 9.85$, $P < 0.01$ for departure of total from expectation of 13 in each cell. Fisher's exact test for interaction between rows and columns: probability of this table $P = 0.22$; P in the smaller tail of the distribution = 0.055.

dose. The regression, shown in Figure 2, was highly significant ($t_{13} = 3.89$, $R^2 = 53.81\%$, $P < 0.01$). No insects were caught in the control traps in this or any other experiments in which blank controls were used. Results from control traps were, therefore, omitted from the analysis.

Experiment 3: Response to Different Synthetic Lures. The catch for each treatment in the two trials was transformed as for experiment 1. Results from the two trials were combined in an analysis of variance, which revealed a significant difference between treatments ($F_{4,28} = 5.35$, $P < 0.01$) (Figure 3). Response to the frass was significantly different from that to the lures ($P < 0.05$ LSD test), but no significant differences in catch were detected between the four variants of the lure.

TABLE 3. TOTAL CATCH IN TRAPS ABOVE OR BELOW CENTRAL FRASS-BAITED TRAP IN THREE-TRAP ARRANGEMENT IN FIGURE 1a[a]

	Trap 5	Trap 10	Total
Above baited trap	23	17	40
Below baited trap	6	3	9

[a]Data from experiment 1. $\chi^2_1 = 19.6$, $P < 0.001$ for departure of total from expectation of 24.5 in each cell. Fisher's exact test for interaction between rows and columns: probability of this table $P = 0.26$; P in the smaller tail of the distribution = 0.19.

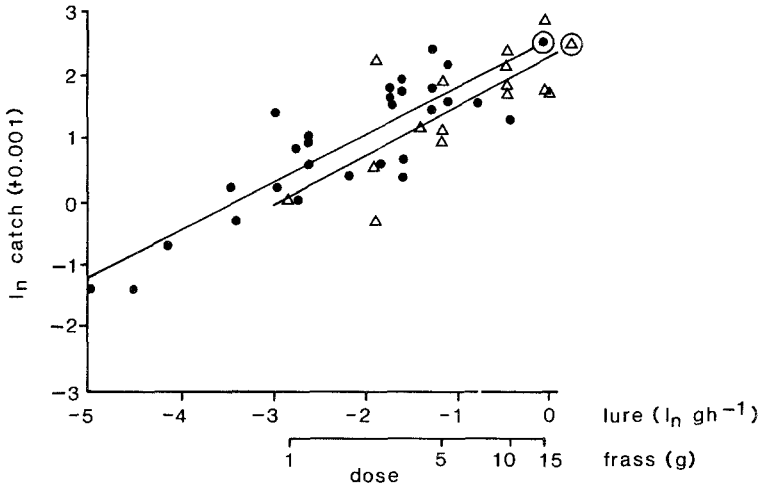

FIG. 2. Dose response to frass (△) ($y = 0.073 + 0.802x$) and lure 1a (●) ($y = 2.624 + 0.770x$) released from liquid dispensers. Relative position of axes adjusted assuming 5 g frass ≡ 0.29 g/hr of lure (see text).

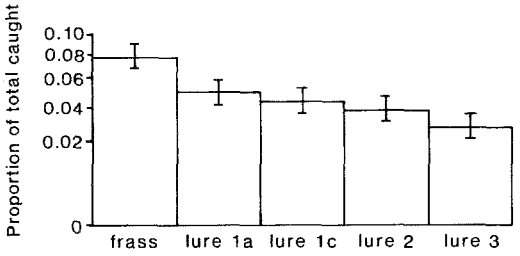

FIG. 3. Catch of *R. grandis* as a proportion of the total caught in each trial. Traps were baited with 5 g of frass or 1 g of lure on 2.5 g of bark powder. Means ± common SE. The total numbers caught (two trials) were: 118, 111.

Experiment 4: Dose Response to Lure 1a. The mean catch of *R. grandis* for each dose of lure was determined for each trial and the pooled data analyzed by linear regression of the log catch (+0.001) on log release rate (+0.001). The regression, shown in Figure 2, was highly significant ($t_{26} = 8.44$, $R^2 = 73.24\%$, $P < 0.001$). The log mean catch for the 5-g frass treatment was 1.67. From the regression of the dose response to lure, $y = 2.624 + 0.770x$, the response to the frass ($y = 1.67$) was estimated by substitution to be equivalent to a release rate of 0.29 g/hr of lure. This equivalence was used to determine the relative position of the x axis for frass and lure doses in Figure 2.

Using data from all the trials (Table 4), we compared the proportion of the total catch in the lower baited and upper unbaited trap in the frass and lure treatments (χ^2 test). There were no significant differences.

Temporal Changes in Release Rate and Composition of Lure 1a

Experiment 5: Release Rate from Wick Dispensers at Constant Temperature. There was a significant linear decrease in mean daily release rate from the wicks over a period of 11 days at 15°C ($t_8 = 8.46$, $R^2 = 89.96\%$, $P < 0.001$) and 20°C ($t_9 = 5.58$, $R^2 = 77.60\%$, $P < 0.001$) (Figure 4).

Experiment 6: Temporal Changes in Chemical Composition in the Field.

TABLE 4. TOTAL CATCH OF *R. grandis* IN BAITED AND UNBAITED TRAPS FROM ALL TRIALS OF DOSE–RESPONSE EXPERIMENT (EXPERIMENT 4)[a]

	Frass	Lure
Upper unbaited trap	36	73
Lower baited trap	136	311

[a] $\chi^2_1 = 0.17$, $P = 0.68$ (with Yates' correction for continuity).

The composition of the lure (Table 5) was estimated as the percentage of the total identified monoterpenes relative to the internal standard. The initial sample (day 0) shows good correspondence with the calculated concentration (Table 1). There was no evidence of oxidative changes in the monoterpenes over the 32-day period. An unidentified minor component in the sample was tentatively assigned as an isomer of limonene or β-phellandrene. Small amounts of other components totaled <3% of the sample.

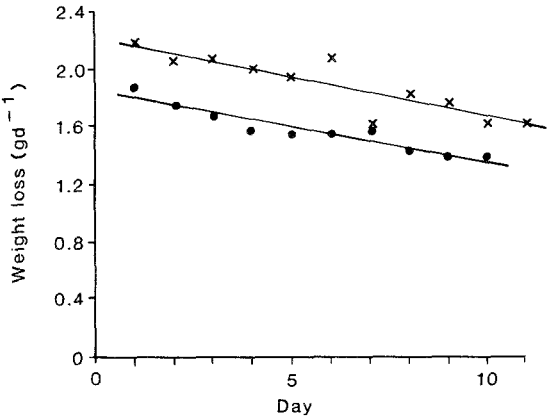

FIG. 4. Mean daily release rate of lure 1a from liquid wick dispensers at 20°C (×) ($y = 2.213 - 0.055x$) and 15°C (●) ($y = 1.841 - 0.048x$).

TABLE 5. COMPOSITION OF LURE 1a IN RESERVOIR OF LIQUID WICK DISPENSERS IN THEYSOHN SLOT TRAPS[a]

	DAY						
	0	2	4	8	16	32	± SE
(dl)-α-Pinene	14.5	14.6	14.5	14.9	15.0	14.7	0.15
(−)-β-Pinene	26.4	26.6	26.7	27.2	27.1	27.1	0.17
3-Carene	15.2	15.4	15.5	15.3	15.1	15.4	0.09
dl-Limonene	15.7	15.3	15.5	14.8	14.5	15.2	0.24
β-Phellandrene	26.1	26.2	25.8	25.9	26.4	25.7	0.18
Unknown	2.0	1.9	1.9	1.9	1.9	2.0	0.06

[a] A single dispenser, with an initial release rate of 1–1.5 g/day, was used at each of three field sites. Values are means of the three sites except for initial value (day = 0) based on one sample of the newly formulated lure. SE estimated from the pooled variation within sites.

DISCUSSION

Results from wind-tunnel experiments (D. Wainhouse, T. Wyatt, unpublished observations) suggest that a distinct silhouette at the source of attractive odors stimulates a landing response. In our field experiment (experiment 1), there is no evidence that increasing the number of unbaited traps, and so enhancing the visual silhouette at a single trapping position, results in higher captures of *R. grandis* when a standard frass bait is used. This suggests that chemical rather than visual cues are the main factors governing the approach of *R. grandis* to traps. Only one trap was baited at each position, and this caught most of the insects. Some, however, were caught in unbaited traps, more entering those positioned above than below the baited trap (Tables 2 and 3). Based on wind-tunnel observations (D. Wainhouse, T. Wyatt, unpublished observations), we suggest this arises from an upward component to the flight close to traps as the predator searches for the source of the odor, followed by a landing response when the odor is lost or concentration declines. The relative numbers of insects caught in the lower baited and upper unbaited traps from the lure and frass treatments in experiment 4 (Table 4) are very similar, and this clearly suggests that the behavior of *R. grandis* in approaching and entering traps containing lure is comparable to that of the natural attractant. We attributed the difference in numbers caught between the sides of the arena for trials with traps 1–5 (experiment 1) to the effect of wind direction during trapping.

Fewer insects (Figure 3) were caught with lures containing myrcene (Table 1, lures 2 and 3) than those without, although the difference was not significant. In laboratory experiments, (Wainhouse et al., 1991), the presence of myrcene caused insects responding to lure to make more approaches to the target before eventually landing. This behavior may have contributed to the reduced catches in traps baited with lures containing myrcene. Myrcene was a minor component of our frass. The enantiomeric composition of α-pinene and limonene does not appear to be important in determining response to the lure, confirming results of laboratory experiments (Wainhouse et al., 1991).

In our traps, a release rate of 0.29 g/hr of lure (1a in Table 1) was estimated to give a catch similar to that of the odor released by 5 g of frass. Results from wind-tunnel experiments (Wainhouse et al., 1991) suggest that approximately 1 mg of lure was equivalent to 50 mg of frass, i.e., 0.1 g of lure to 5 g of frass. Comparison between results from the field and laboratory is not straightforward because, in the wind tunnel, response was assessed over a relatively short period (8 min), and we have no detailed information on relative release rates from frass or the bark powder used as a medium for the lure. Results suggest, however, that the lure is less active in the field by a factor of at least 3 (0.29 g/hr $\equiv$ 5 g frass in the field, 0.1 g $\equiv$ 5 g frass in the laboratory). Nevertheless, the data

presented in Figure 2 show clearly that the relative response to lure and frass in the field is consistent over a wide range of doses.

Oxygenated monoterpenes, quantitatively minor components of *D. micans* frass used in our experiments (Wainhouse et al., 1991), are attractive to flying *R. grandis* in wind-tunnel experiments but only at high concentrations, equivalent to 13–259 times their concentration in frass (Grégoire et al., 1992; D. Wainhouse and D. Kelly, unpublished observations). These chemicals probably function as oviposition stimulants (Grégoire et al., 1991) but could affect orientation close to prey galleries and so may affect trap entry.

The decline in the release rate of the lure from the wick dispensers (Figure 4) may be due to polymerization of the monoterpenes on the surface of the release pad, but the monoterpenes (with the antioxidant BHT) in the reservoir remain unchanged after several weeks of exposure in the field.

The attractiveness of the *R. grandis* lure, its relative stability in the field, and the results of preliminary trapping experiments in "natural" field populations (unpublished) suggest that the lure may be of value in IPM programs. Semiochemical lures for natural enemies have a number of suggested uses such as (1) monitoring activity and population size, (2) inducing host-seeking behavior, (3) reducing the innate tendency of released individuals to disperse, or (4) increasing rates of parasitism by released parasitoids (Lewis et al., 1972, 1982; Gross et al., 1975; Gross, 1981; Nordlund et al., 1981; Lewis and Nordlund, 1985; Powell, 1986).

The *R. grandis* lure could be used to confirm the establishment of the predator following release, to attract *R. grandis* to *D. micans*-attacked trees and to estimate dispersal following release. It may also be possible to monitor indirectly for *D. micans*, which is difficult to detect at low population density. Trapping *R. grandis* would be most useful around quarantine areas within which the predator is well established and where spread of the bark beetle could be prevented by destruction of infested trees.

Some female *D. micans* were caught in our frass- or lure-baited traps, usually when maximum temperatures were 21–25°C. This is probably a response to the monoterpenes, some of which have been shown to be attractive in laboratory experiments (Vasechko, 1978). A response to conspecific frass may explain the observation that on occasion several *D. micans* females may attack the same tree (D. Wainhouse, unpublished). Further work is needed to determine whether direct monitoring of *D. micans* would be possible.

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FIELD RESPONSE OF THE PINE SAWFLY *Neodiprion sertifer* TO CONTROLLED RELEASE OF DIPRIONYL ACETATE, DIPRIONYL PROPIONATE AND *trans*-PERILLENAL

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Abstract—Two dispenser types for pine sawfly (Hymenoptera: Diprionidae) pheromones—glass capillaries and dental cotton rolls—are described, and release rates reported. Glass capillaries of different diameters were used in field tests to determine dose–response relationships of (2*S*,3*S*,7*S*)-3,7-dimethylpentadecan-2-yl acetate (diprionyl acetate), diprionyl propionate, and *trans*-perillenal on the trap catch of male *Neodiprion sertifer*. A linear relationship between the logarithm of the capillary cross-sectional area and the logarithm of the release rate was found. However, capillaries of the same diameter could vary in the amount released by as much as a factor of 10. Both the acetate and propionate were attractive alone, but no synergistic effect was found. *trans*-Perillenal did not show any behavioral activity. Dental cotton rolls have been used extensively in diprionid pheromone research without knowledge about release rates. The release rate from rolls loaded with different amounts of diprionyl acetate was determined by extracting rolls after different times under constant temperature and wind conditions or after use in field tests. Quantification of diprionyl acetate was done on GC. The release rate was found to be proportional to the amount applied over several orders of magnitude, and persisted for several weeks. Capillaries and rolls with similar release rates caught similar numbers of sawflies.

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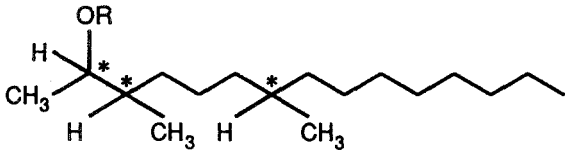
INTRODUCTION

Two factors are of vital importance when investigating the biological activity of semiochemicals: the chemical composition (including the purity of components) and the amounts of the various components released. Often, the first factor receives much more attention, while release rates and ratios are approximated or even unknown, especially under field conditions. Much effort has been made to determine and control release rates from commercial pheromone formulations, e.g., hollow fibers, plastic laminates, and microcapsules, mainly used for lepidopterous pests (see review chapters in Kydonieus, 1980; Mitchell, 1981; Leonhardt and Beroza, 1982; Kydonieus and Beroza, 1982; Jutsum and Gordon, 1989; Ridgway et al., 1990).

The pheromone system of several North American diprionid species has been investigated by e.g. Coppel et al. (1960), Jewett et al. (1976), and Olaifa et al. (1987, 1988). Most efforts have been made to identify the pheromone of different species, and to understand how reproductive isolation between sympatric species can be achieved by the use of different pheromone blends. For the 12 species studied so far, the main component of the sex pheromone has always been found to be the acetate (R=$COCH_3$) or propionate (R=COC_2H_5) of 3,7-dimethyl-2-pentadecanol (diprionol, R=H):



Diprionol has three optically active carbon atoms (*), and thus eight possible stereoisomers. In many species only one of the stereoisomers, the (2*S*,3*S*,7*S*)-, is active, but in some the addition of another stereoisomer synergizes or inhibits attractiveness (Kraemer et al., 1979, 1981, 1983; Kikukawa et al., 1982, 1983; Olaifa et al., 1984, 1987, 1988). In all field studies reported, female extracts or synthetic chemicals have been released from dental cotton rolls. However, so far there have been no published reports on release rates or on persistence of release ratios over time. Dental cotton rolls have also been used in several studies of lepidopteran pheromones, but the major drawback is a too rapid release of the pheromones (see review by Weatherston, 1989).

In an earlier paper we showed the importance of composition and purity

of optical isomers of 3,7-dimethylpentadecan-2-yl (diprionyl) acetate for the attraction of male pine sawflies, *Neodiprion sertifer* (Geoffr.) (Anderbrant et al., 1992). One basis of that study was a thorough knowledge about the relative release rates of the isomers when applied at different doses. In the present study we present release rates measured both in the field and in the laboratory and report results from field tests on *N. sertifer* using different release rates. The field tests were conducted to establish dose–response relationships for the known attractants (2*S*,3*S*,7*S*)-diprionyl acetate and propionate and, for a monoterpene, (*E*)-2-methyl-5-(3-furyl)-2-pentenal (*trans*-perillenal), found in the females (Ahlgren et al., 1979; Wassgren et al., 1992). Possible synergistic effects were also investigated.

METHODS AND MATERIALS

Chemicals

For the field tests in 1981 and 1982 (2*S*,3*S*,7*S*)-diprionyl acetate and propionate were prepared by Byström et al. (1981). These esters contained 14% of the inactive 2*S*,3*S*,7*R*-isomer and less than 0.02% of the inhibitory 2*S*,3*R*,7*R*-isomer. In 1982 (tests 82:1 and 3), release measurements were done using a *threo*-mixture, i.e., the 2*S*,3*R*,7*R*, 2*S*,3*R*,7*S*, 2*R*,3*S*,7*S*, and 2*R*,3*S*,7*R* isomers (Magnusson, 1978). In the 1988 tests the (2*S*,3*S*,7*S*)-diprionyl acetate was prepared by Högberg et al. (1990). This contained 2.3% of the 2*S*,3*S*,7*R* isomer, and less than 0.05% of the other isomers (Högberg et al., 1990; Anderbrant et al., 1992). The *trans*-perillenal (Baeckström et al., 1982) contained less than 2% of the *cis* isomer.

Measurements of Pheromone Release Rates

Two types of dispensers were used, glass capillaries of different inner diameters, and 4 × 1-cm dental cotton rolls (Celluron No. 2, Paul Hartmann S.A., France). The release from capillaries was measured after they had been used in field experiments, whereas measurements of release rates from cotton rolls were done both in the laboratory under defined conditions and after they had been used in the field.

Rate of Pheromone Emission from Capillaries in Field. Capillaries were purchased (Microcaps, Drummond Scientific Co.) or made by ourselves. Their inner diameters ranged from 0.0045 mm to 0.91 mm. Capillaries were filled by dipping one end into the neat chemical. Only part of the capillary was filled and was then tilted to move the substance off the end. The end that had been dipped was then cut off so no substance could emit from the outer capillary surface. The amount of substance was measured under a microscope before and after

field experiments. Capillaries were either open at both ends to give a relatively high release rate, or sealed with glue in one end after filling. Measurements were made of capillaries used in the tests in 1982 (82:1, 3, 4, and 6) and 1988 (88:1 and 2). For the tests done in 1981 the release rates were estimated based on the relationships found in the 1982 tests.

Rate of Pheromone Emission from Cotton Rolls in the Laboratory. Emission of (2*S*,3*S*,7*S*)-diprionyl acetate from dental rolls was studied at three different doses: 6, 60, and 600 μg per roll, and at two different wind speeds: 0.1 and 1.0 m/sec. The acetate was added to the rolls as a hexane solution (600 μl of 1 μg/μl, 0.1 μg/μl, and 0.01 μg/μl solutions, respectively). The dispensers were placed horizontally in a holder, well separated from each other, in a mini wind tunnel (20 cm $\times$ 12.5 cm diam.) at constant temperature (range: 19.5–20.1°C). After 1, 2, 4, or 5, 8, and 15 or 16 days (for 0.1 m/sec after 2, 8, and 16 days) three rolls were removed and each was placed in a round flask. To each flask 10 ml pentane (analytical grade) was added, the flask was stoppered, and left over night. The pentane portion was removed by pipet and known amounts of 18:OAc were added as internal standard. The rolls were extracted with two additional 10-ml portions of pentane, and the combined rinse solution was condensed to a volume suitable for GLC analysis. Capillary gas chromatography with flame ionization detection was performed on a Hewlett Packard model 5890A GC, equipped with a 25-m Carbowax column. Conditions of chromatography were: starting temp. 60°C, split valve opened after 1 min, temperature maintained at 60°C for 2 min following injection, then programmed at 30°C/min to 230°C.

To measure the recovery efficiency of diprionyl acetate by the described method, dispensers of each load were also extracted immediately after application of the pheromone.

Rate of Pheromone Emission from Cotton Rolls in the Field. Dental rolls loaded with 6, 60, 600, or 6000 μg diprionyl acetate were collected after about one month in the field (tests 88:1 and 2). The dispensers were kept frozen in sealed vials until extracted according to the procedure described above.

In addition, rolls loaded with 600 μg diprionyl propionate were hung under rain and sun shelter during the summer of 1988. Three rolls were collected after 3, 5, 9, 20, and 30 days, and then subjected to extraction as described above.

Tests of Behavioral Activity

The experiments in 1981 and 1982 were done in a young lodgepole pine, *Pinus contorta* Dougl., plantation near Ekshärad, province of Värmland, Sweden, from the end of August to the beginning of October. In 1988 the tests were done from August to October in young Scots pine, *Pinus sylvestris* L., plantations near Gryt, province of Östergötland, Sweden.

In 1981 cross-barrier traps made from two 35 × 35-cm white plastic-covered cardboard sheets were used. All sides were covered with Stikem Special (Seabright Enterprises, Emeryville, California). In 1982 and 1988, Lund-I sticky traps were used. These consist of two white, horizontal plastic-covered cardboard sheets approximately 6 cm apart with the upper surface of the bottom sheet covered with Stikem Special. Both traps are depicted in Anderbrant et al. (1989). Traps were hung in young pines about 2 m above the ground and at least 30 m apart. In order to reduce positional effects, traps were moved forward one step after each collection (in 1981 and 1982) or to a new random position restricted so that all baits should be in all positions at least once (in 1988).

In 1981 and 1982 substances were released from capillaries only, whereas a comparison between traps with capillaries or dental rolls was done in the 1988 tests.

RESULTS

Measurements of Pheromone Release Rates

Rate of Pheromone Emission from Capillaries in the Field. The release of diprionyl acetate was measured in tests 82:1, 82:3, and 82:4. In test 82:1 capillaries open at both ends were used, and the logarithm of the release rate showed a linear relationship with the logarithm of the cross-sectional area of the capillary (Figure 1A). Approximately the same capillary sizes were used in 82:4, resulted in a similar relationship (Figure 1A). In both experiments release rates from capillaries of a certain diameter varied over about one order of magnitude. In 82:3, one end of the capillaries was sealed. Twelve of 34 capillaries did not release any measurable substance, but if these zero-release capillaries were excluded from the analysis, a linear relationship appeared, with smaller intercept than for capillaries open at both ends (Figure 1A).

In test 82:6 the release of *trans*-perillenal from capillaries open at both ends showed a similar relation to capillary cross-sectional area as did diprionyl acetate (Figure 1B).

Rate of Pheromone Emission from Cotton Rolls in the Laboratory. The recovery efficiency of pheromone from dental roll dispensers by the described extraction procedure was high at all three doses investigated. For 600, 60, and 6 μg diprionyl acetate applied, 575 ± 25 (SD), 54.5 ± 2.7, and 5.5 ± 0.22 μg was detected in the rinse solution.

Two regression models, $y = a \exp [b \ln (x + 1)]$ and $y = a - b \ln (x + 1)$, where y is the remaining amount and x is the time in days, were fitted to the data. The models gave a similar fit for all data sets, and in Figure 2 the equations with largest r^2 value are given. The decrease in recovered amount over time for the three different doses applied showed the same type of rela-

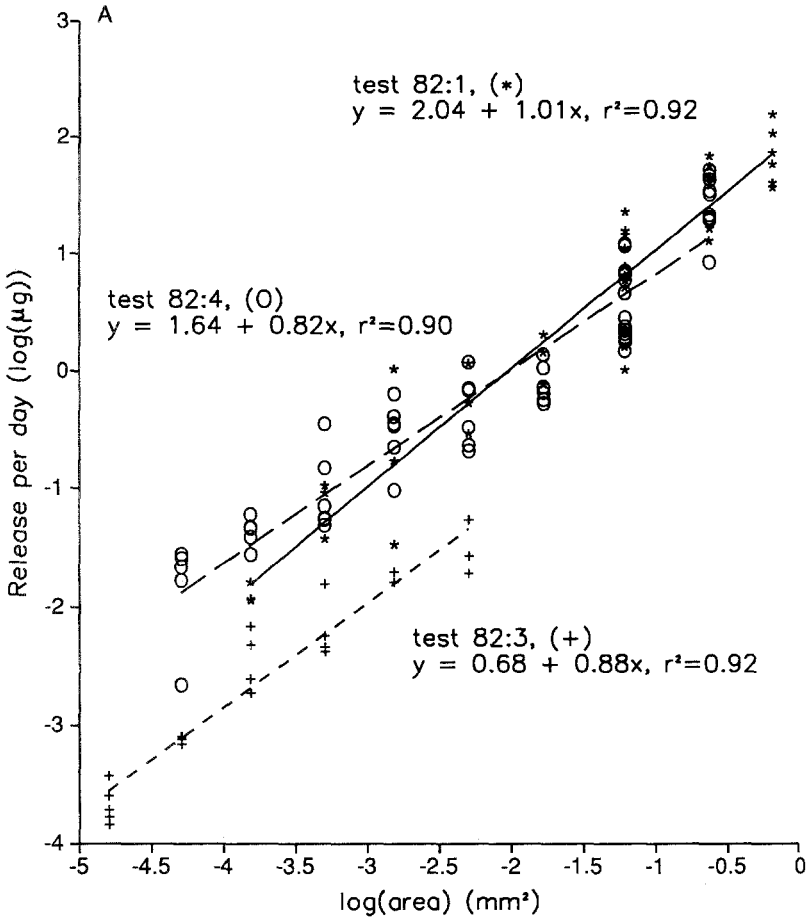


FIG. 1. Release of diprionyl acetate (A) and *trans*-perillenal (B) in the field from capillaries of different cross-sectional area. In tests 82:1, 4, and 6 capillaries were open at both ends, and in test 82:3 they were sealed at one end. The released amount was measured under a microscope after the experiment was finished, 82:1 after 5 days, 82:3 after 15 days, 82:4 after 7 days, and 82:6 after 9 or 17 days (see Table 3).

tionship when wind and temperature conditions were the same (Figure 2A, 1 m/sec and Figure 2B, 0.1 m/sec). This implies that the ratio between different doses persists for a long time. The derivatives of the regression equations in Figure 2 were used to compute the release rates over time, as shown in Figure 3. The release at both wind speeds decreased exponentially over time: rapidly during the first days but then the rate remained fairly stable, and decreased from

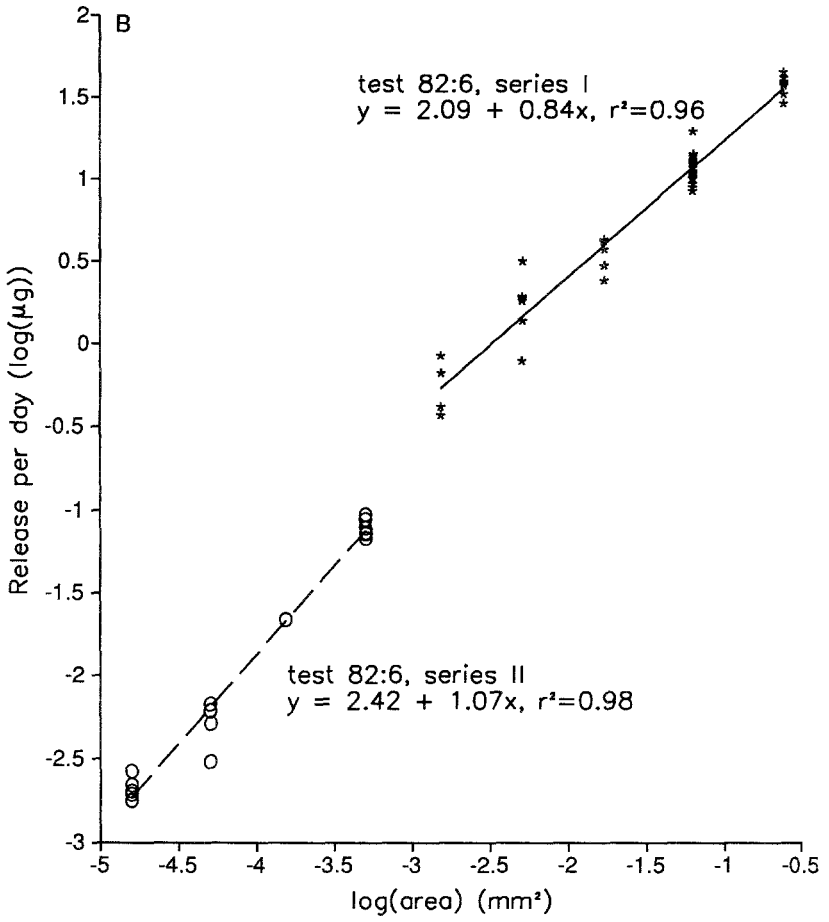


FIG. 1. Continued

about 3%/day after eight days to about 2%/day after 16 days when wind speed was 0.1 m/sec.

Rate of Pheromone Emission from Cotton Rolls in the Field. The described extraction technique was used also for dispensers from field experiments. The amounts of diprionyl acetate released during a month under field conditions were proportional to amounts applied (Table 1). When 10 times more pheromone was applied to a dispenser, the released amount increased approximately 10 times.

The release over time was calculated by using the regression equations from the laboratory data ($y = a \exp[b \ln (x + 1)]$, Figure 2A), and solving for

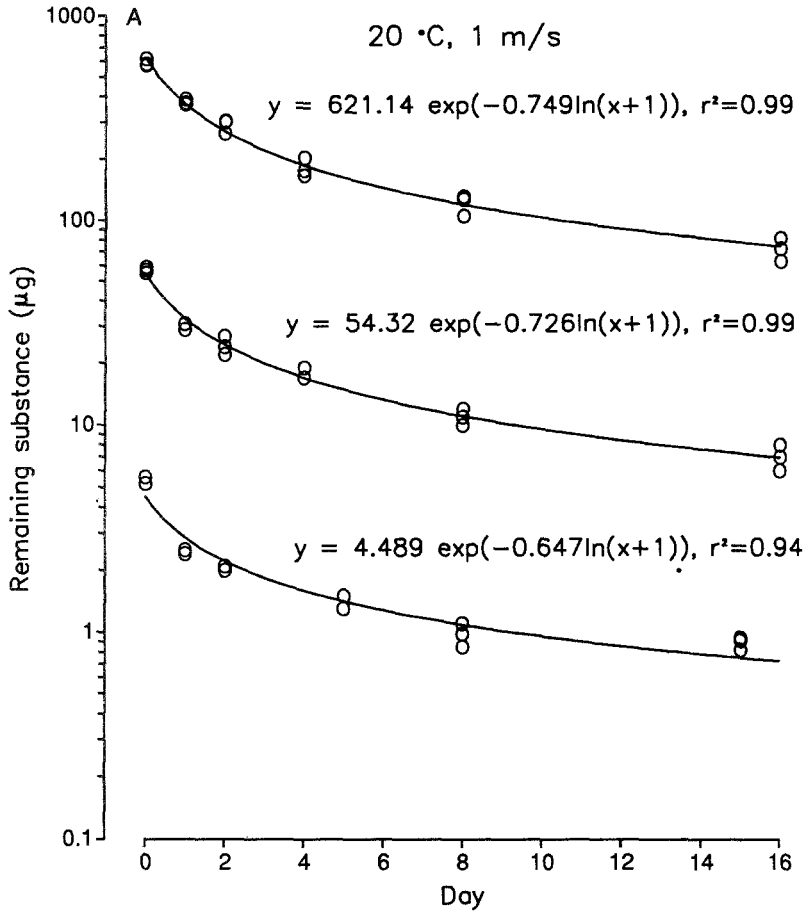


FIG. 2. The amount of diprionyl acetate remaining in dental rolls after different times in a mini wind tunnel at 20°C and 1.0 m/sec (A), or at 20°C and 0.1 m/sec (B).

the constant b. As y (remaining amount) the averages for each load from tests 88:1 and 2 were used (Table 1), and x was put to 33.5 days (average duration of the two experiments). For the highest amount used in the field (6000 μg), the constant a was put to 6000 since no regression equation from the laboratory was available. These calculations resulted in the curves shown in Figure 4.

The extracted amounts from dispensers containing 600 μg diprionyl propionate after different times in the field are also shown in Figure 4. A similar relationship as for the acetate was found. It should be mentioned that the propionate release test was done in the summer and thus under a higher average temperature than the acetate field test.

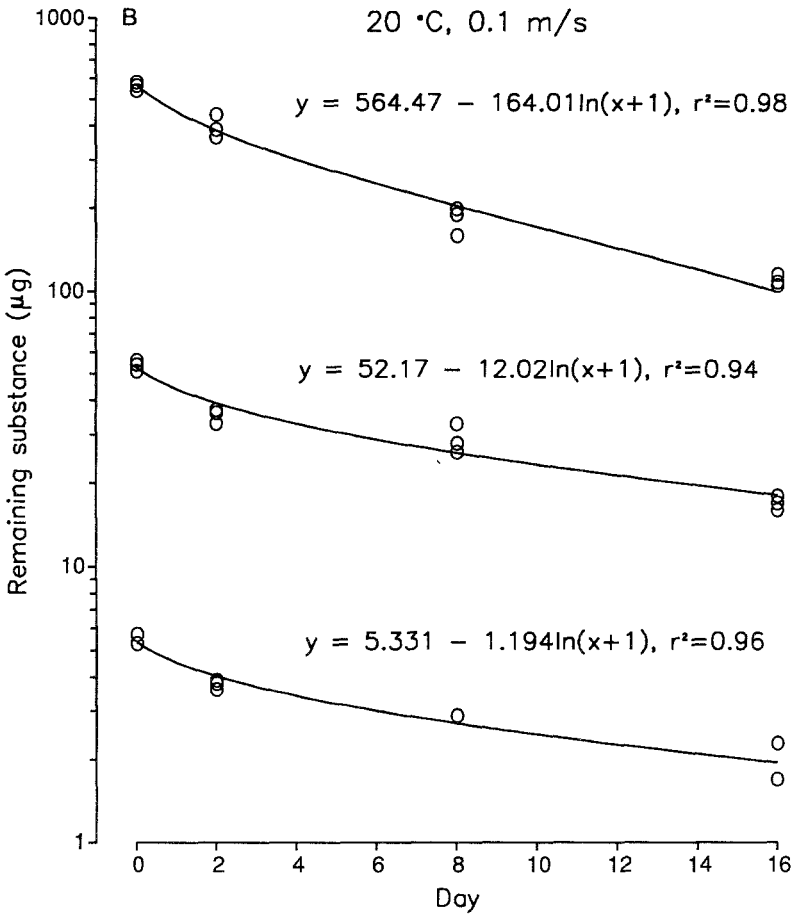


FIG. 2. Continued

Tests of Behavioral Activity

Tests 82:1 and 82:3 were part of an extensive study where the role of the different optical isomers of diprionyl acetate was investigated (Anderbrant et al., 1992).

Tests of Dose-Response Relationships (81:1, 82:4, 88:1, 88:2). In test 81:1, *trans*-perillenal, (2*S*,3*S*,7*S*)-diprionyl acetate, and (2*S*,3*S*,7*S*)-diprionyl propionate were released from different traps with approximate (estimated from the relationship found in the 82:1, 3, 4, and 6 tests, see Figure 1) release rates of 0.4–11.2 µg/day for the acetate and propionate, and 0.8–19.8 µg/day for *trans*-perillenal. No clear dose-response relationships were found, although for

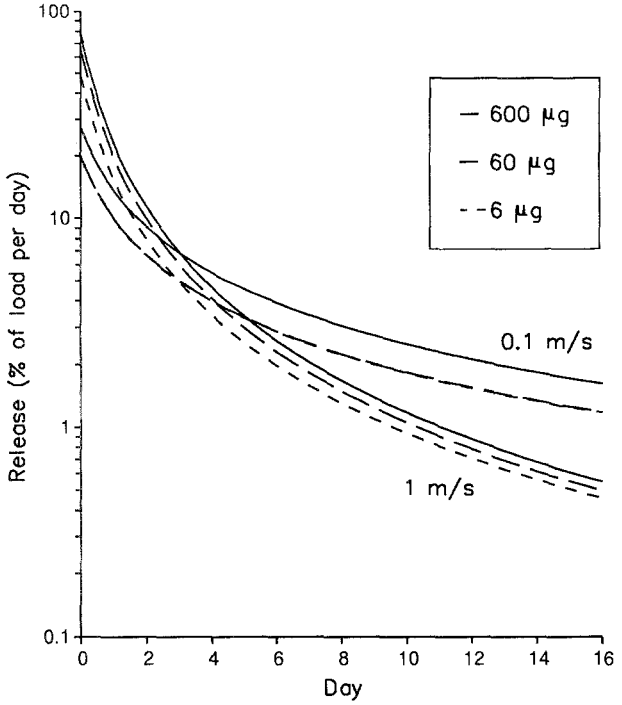


FIG. 3. Calculated instantaneous release of diprionyl acetate from dental rolls, expressed as percent of applied amount, at different wind speeds. Curves are the derivatives of the regression equations in Figure 2.

TABLE 1. AMOUNT OF DIPRIONYL ACETATE EXTRACTED FROM DENTAL ROLLS (ONE PER LOAD AND TEST) AFTER FIELD EXPOSURE IN TESTS 88:1 (31 DAYS) AND 88:2 (36 DAYS), AND THE CALCULATED RELEASE[a]

Amount applied to dispensers (μg)	Extracted amount (μg)		Released amount (μg d^{-1})	
	Test 88:1	Test 88:2	Test 88:1	Test 88:2
6	1.1	1.2	0.16	0.13
60	7.6	3.0	1.68	1.58
600	184	49	13.4	15.3
6000	2670	2027	107	110

[a]For the number of male sawflies caught, see Figure 6.

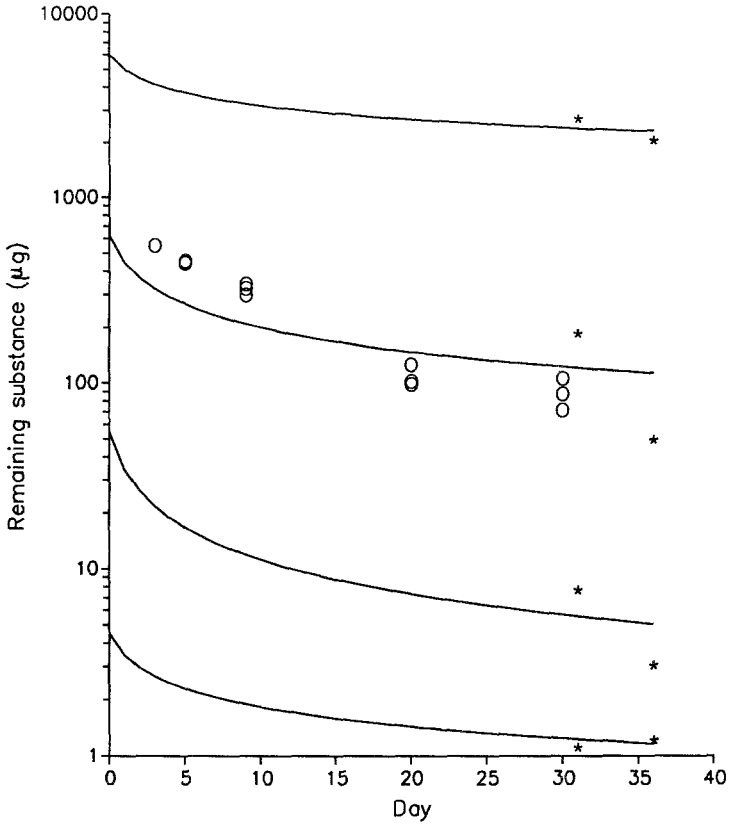


FIG. 4. Calculated remaining amounts of diprionyl acetate in dental rolls after different times in field. Curves are derived from the amounts extracted (*) after the experiments 88:1 and 2 were finished (Table 1) and from the shape of the regression curves in Figure 2a. The extracted amount of diprionyl propionate (600 µg applied) after different times in field is indicated by circles.

both acetate and propionate the catch was highest in the traps with strongest bait. For *trans*-perillenal most catches were not significantly larger than that of the unbaited trap (Table 2). When the three compounds were used in the same trap, no significant increase in catch compared to the acetate or propionate alone was noted.

However, in test 82:4 with release rates (measured) of (2*S*,3*S*,7*S*)-diprionyl acetate ranging from 0.01 to 300 µg/day, a clear dose–response relationship was found. Even for the lowest release rate, the average catch was significantly higher than in the unbaited trap (Figure 5).

The tests in 1988 (88:1 and 2) were designed to establish a dose–response

TABLE 2. TEST 81:1—CATCH OF MALE PINE SAWFLIES, *Neodiprion sertifer*, IN CROSS-BARRIER STICKY TRAPS BAITED WITH (2*S*,3*S*,7*S*)-DIPRIONYL ACETATE, (2*S*,3*S*,7*S*)-DIPRIONYL PROPIONATE OR *trans*-PERILLENAL[a]

Compound released (μg/day)			Catch, $\bar{X} \pm$ SD ($N = 21$)[b]
Acetate	Propionate	*trans*-Perillenal	
0.4			114 ± 83 abcde
0.9			128 ± 78 bcde
1.5			198 ± 279 def
3.3			125 ± 106 abcde
9.9			172 ± 115 def
11.2			386 ± 342 g
	0.4		120 ± 77 bcde
	0.9		114 ± 90 abcde
	1.5		164 ± 124 def
	3.3		86 ± 69 abc
	9.9		199 ± 121 ef
	11.2		265 ± 150 fg
		0.8	123 ± 91 abcde
		1.8	103 ± 72 abcd
		3.4	106 ± 156 ab
		6.6	87 ± 74 abc
		18.5	155 ± 116 cde
		19.8	118 ± 86 bcde
3.3	3.3	6.6	169 ± 137 cde
	blank		68 ± 59 a

[a] Three sets of traps were used and they were repositioned seven times, i.e., 21 replicates, September 1–9, 1981. Substances were released from capillaries with release rates estimated from the measurements done in tests 82:1, 3, and 6.

[b] Figures followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test after ln(catch + 1) transformation. ANOVA $F = 5.05$, $P < 0.001$.

relationship for the diprionyl acetate when released from dental rolls and to compare rolls with capillaries. The dental rolls were loaded with 0.06, 0.6, 6, 60, 600, or 6000 μg diprionyl acetate, and three capillary sizes (0.080, 0.28, and 0.546 mm diameter) were used. The release rates of the capillaries and of the four strongest dental rolls were measured. For the two weakest dental roll baits, the release was set to 1% and 10%, respectively, of the 6-μg bait, because of analytical limitations of the procedure described above. The catch increased with release rate up to approximately 10 (test 88:1) or 100 (test 88:2) μg/day (Figure 6A and B). The catches in traps with capillaries were in good agreement with those of traps with dental rolls of comparable release rates. Since capillaries can be expected to give a relatively steady release rate over time, whereas that

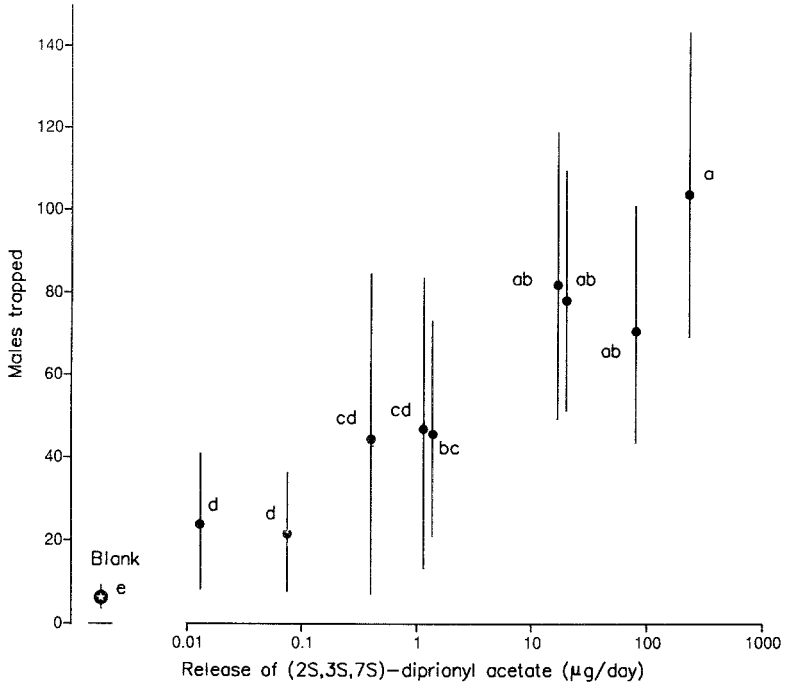


FIG. 5. Test 82:4. Average catch of male pine sawfly, *Neodiprion sertifer*, in horizontal Lund-I sticky traps releasing different amounts of (2*S*,3*S*,7*S*)-diprionyl acetate from capillaries. Three sets of traps were used, and they were repositioned five times, i.e., 15 replicates, August 31 to September 9, 1982. Bars indicate 95% CI. Means with the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test after ln (catch + 1) transformation. ANOVA $F = 10.5$, $P < 0.001$.

of dental rolls was shown to decline over time, one would expect that traps baited with dental rolls caught comparatively more sawflies in the beginning of an experiment and less at the end than traps with capillaries. However, no such tendency was found, which partly may be due to large differences between positions and replicates.

Tests of Synergism between Diprionyl Acetate and Propionate (81:2, 82:5). In test 81:2 one of the substances was released at the same rate (1.5 µg/day) from several traps, while the release rate of the other substance varied between traps (0, 0.4, 0.9, 1.5, 3.3, 11.2, 33.6, 112 µg/day). Average trap catches ($N = 12$) varied from 43 to 117 males in the 16 differently baited traps, and on average, 41 males were caught in the blank traps. Traps with the strongest baits (112 + 1.5 µg) caught the most, but there was no indication of synergism between the acetate and propionate.

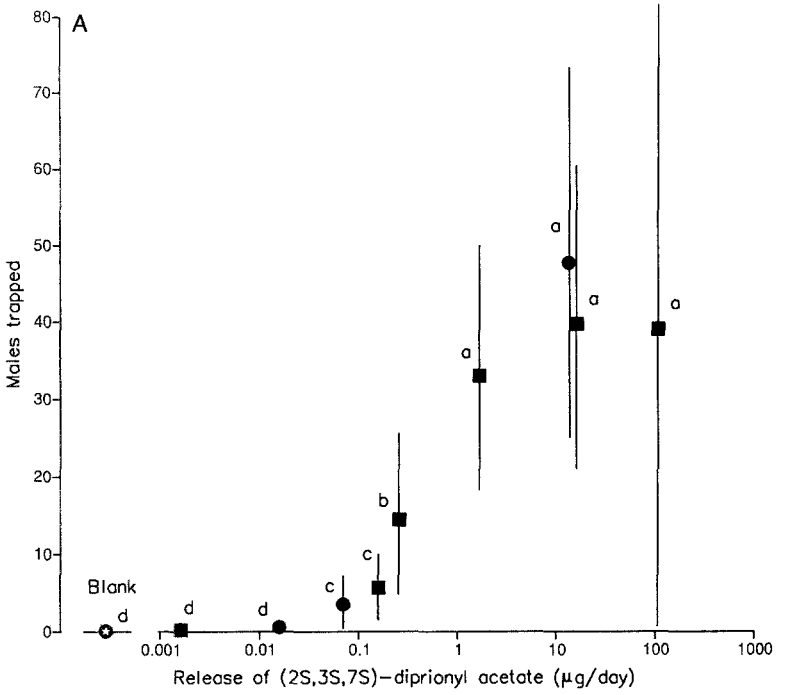


FIG. 6. Tests 88:1 (A) and 88:2 (B). Average catch of male pine sawfly, *Neodiprion sertifer*, in horizontal Lund-I sticky traps releasing different amounts of (2*S*,3*S*,7*S*)-diprionyl acetate from dental rolls (■) or capillaries (●). In 88:1, traps (one setup) were randomized 10 times, August 30 to September 30, 1988, and in 88:2, traps (one setup) were randomized 15 times, August 31 to October 6, 1988. Bars indicate 95% CI. Means with the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple-range test after ln (catch + 1) transformation. ANOVA $F = 33.2$, $P < 0.001$ in 88:1 and $F = 67.9$, $P < 0.001$ in 88:2.

In test 82:5 two release rates of each compound were used (1.8 or 3.5 µg/day, release rates estimated from the measurements done in tests 82:1 and 3). Traps were loaded with the acetate or propionate (four different baits) or with the lower release rate of both substances. No significant difference between the baits was noted. Average catches ($N = 6$) were between 66 and 82 for all five baits, and thus no synergistic effect was found in this experiment either. The two substances seem to be equally active attractants when used alone.

Tests of Synergistic Effects of trans-*Perillenal (81:3, 81:4, 82:6).* In 81:3 (2*S*,3*S*,7*S*)-diprionyl acetate and propionate were released together (3.3 µg/day, release rates estimated from the measurements done in tests 82:1, 3, and 6)

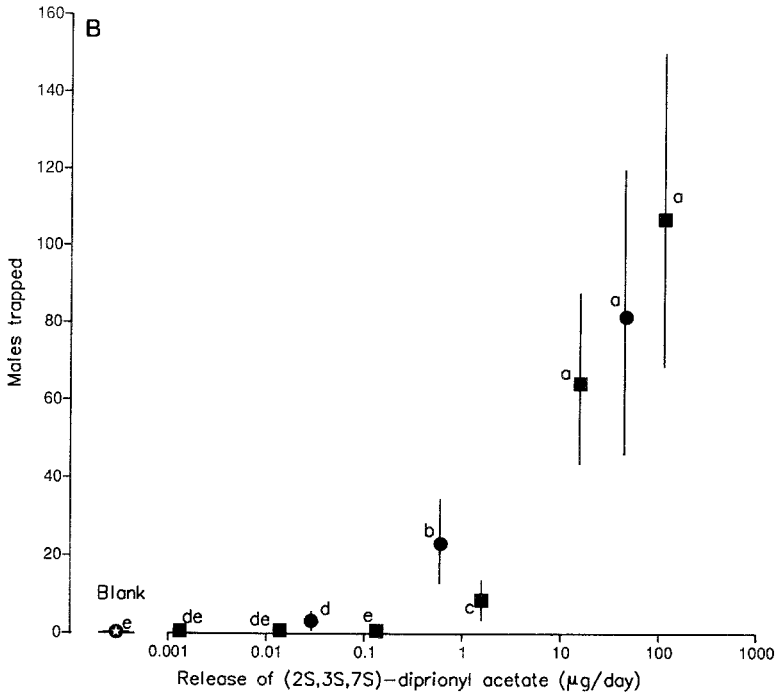


FIG. 6. Continued

with or without *trans*-perillenal (6.6 µg/day), at two localities. No significant differences in average trap catch ($N = 12$) between the two baits were detected in either locality (mean ± SD locality 1:212 ± 198 without *trans*-perillenal, 106 ± 91 with *trans*-perillenal; locality 2:180 ± 244 without, 204 ± 187 with). Also in 81:4 the acetate and propionate were released together. In each of two series, they were released at the same rate (11.2, series I) and (1.5, series II) µg/day each, respectively; the release rates were estimated from the measurements done in tests 82:1, 3, and 6) from all traps (except blank traps), while *trans*-perillenal was released at different rates (0, 0.8, 1.8, 3.4, 6.6, 18.5, 19.8 µg/day). In series I, the average catch ($N = 12$) of the unbaited traps was significantly lower (26) than the catch of the baited traps, but the catch of these did not differ from each other; averages ranged from 335 to 449. In series II, not even the average ($N = 9$) of the unbaited traps differed from the baited ones (averages from 36 to 62). The same approach was used in test 82:6, but here *trans*-perillenal was tested with the acetate only. However, no effect of *trans*-perillenal could be detected (Table 3).

TABLE 3. TEST 82:6—CATCH OF MALE PINE SAWFLIES, *Neodiprion sertifer*, IN HORIZONTAL LUND-I STICKY TRAPS BAITED (CAPILLARIES) WITH (2*S*,3*S*,7*S*)-DIPRIONYL ACETATE WITH ADDITION OF *trans*-PERILLENAL IN VARIOUS AMOUNTS [a]

Series I		Series II	
Release of *trans*-perillenal (μg/day)	Catch $\bar{X} \pm$ SD ($N = 12$)[b]	Release of *trans*-perillenal (μg/day)	Catch $\bar{X} \pm$ SD ($N = 18$)[b]
0 (blank)	2 ± 2 a	0 (blank)	2 ± 2 a
0	141 ± 74 b	0	111 ± 72 b
0.56	191 ± 65 b	0.0021	128 ± 87 b
1.80	188 ± 138 b	0.0054	91 ± 67 b
3.41	174 ± 97 b	0.022	130 ± 119 b
11.3	149 ± 94 b	0.078	103 ± 67 b
36.1	131 ± 137 b		
76.5	157 ± 108 b		

[a] In both series the average release of acetate was 1.63 μg/day, while the release of *trans*-perillenal varied. In series I three sets of traps were used and they were repositioned four times, i.e., 12 replicates, September 5–14, 1982, and in series II three sets of traps were repositioned six times, i.e., 18 replicates, September 13–30, 1982.

[b] Figures followed by the same letter are not significantly ($P = 0.05$) different according to Duncan's multiple range test after ln(catch + 1) transformation. ANOVA $F = 50.2$, $P < 0.001$ for series I and $F = 50.5$, $P < 0.001$ for series II.

DISCUSSION

In many studies of chemical communication, it is essential to know for each substance of a blend the amount released per unit of time. An ideal dispenser should have a constant and predictable release rate. However, few such dispensers exist (Byers, 1988). We have documented two fundamentally different release systems, and have found one of them, the dental cotton rolls, very suitable for releasing pine sawfly pheromones, with relatively low volatility. The release rate of diprionyl acetate is relatively constant over a long period, and, most importantly, the proportional release is independent of the load. This means that ratios between diprionyl acetate isomers persist for long time periods, although the absolute release rates decrease. Furthermore, there is a very small variation in release rate between individual cotton rolls. The only major drawback with the cotton rolls is that they require a rain shelter in order to avoid disintegration. Thus, although they are suitable for use in traps in monitoring programs, they are not appropriate if mating disruption for large-scale forest protection is attempted. The usefulness of cotton rolls as reference dispensers was recently demonstrated by Leonhardt et al. (1990), when testing various

commercial dispensers for the gypsy moth, *Lymantria dispar* (L.). They obtained a good correlation ($r^2 = 0.97$) between dose and release rate of (+)-disparlure.

The capillaries should, in theory, be ideal dispensers for diprionyl acetate and propionate, since the low volatility of these substances would make the length of the vapor-air column, and thus the release, nearly constant over time (Weatherston et al., 1985a). However, we did not find the glass capillaries to be practical. They are complicated to fill and show a large between-capillary variation in release rate. In part, this variation might be due to different influences of wind, depending on direction of the capillary. Another factor that may be important is the occasionally observed condensation of water inside the capillary, which reduces the cross-sectional area. When sealing one end to reduce the release rate (test 82:3, Figure 1A), one third of the capillaries did not release any substance at all, probably because of condensation of water. In this test, the capillary radius was small, 0.002–0.04 mm. Other factors introducing between-capillary variation have been suggested by Weatherston et al. (1985b), but were applicable mainly to nonglass capillaries with a fairly rough surface.

The field experiments describe the development not only of dispenser reliability, but also of trap design. Although the cross-barrier traps used in 1981 caught large numbers of sawflies, they were not very suitable for sophisticated studies of sawfly pheromones. The differences in catch between traps with baits of varying strengths were small, and in many cases unbaited traps caught nearly as many sawflies as did baited traps. These problems were solved with the introduction of the Lund-I trap (Anderbrant et al., 1989), which is very selective and gives very low blank-trap catches.

The field experiments showed that both diprionyl acetate and propionate are highly attractive to male *N. sertifer* in Sweden. This was earlier shown in North America (Kikukawa et al., 1983; Kraemer et al., 1983; Olaifa et al., 1987) and has recently been confirmed by electrophysiological studies as well (Hansson et al., 1991). This reveals a pattern that is different from pheromone specificity in moths. Usually such a structural variation as that between an acetate and a propionate analog causes a very large electrophysiological as well as behavioral response difference (Liljefors et al., 1984). However, diprionid sawfly males apparently have a different system for pheromone perception than moths (Hansson et al., 1991). A similar response to the acetate and propionate has been reported also from other *Neodiprion* species (Kraemer et al., 1983; Olaifa et al., 1984).

The function of *trans*-perillenal in the chemical communication of diprionids, if any, is still unknown. One possibility is that it is a precursor to diprionol or a by-product in the biosynthetic path (Wassgren et al., 1992). However, in contrast to the extensive knowledge about pheromone biosynthesis in moths, nothing is known about the site or chemical path of pheromone production in diprionid sawflies.

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EFRAPEPTIN PRODUCTION BY *Tolypocladium* FUNGI (DEUTEROMYCOTINA: HYPHOMYCETES): INTRA- AND INTERSPECIFIC VARIATION[1]

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Abstract—Production of the mitochondrial ATPase inhibitory peptides efrapeptins was evaluated by HPLC analysis in 44 strains of nine species of the fungal genus *Tolypocladium* (Deuteromycotina: Hyphomycetes). Efrapeptin identification was confirmed by mass spectral data for the first time in the species *T. cylindrosporum*. HPLC retention time data indicated that *T. nubicola* and *T. tundrense*, two species not previously known to produce efrapeptins, also produce the peptides. No efrapeptins were detected (<0.3 mg/100 ml broth) in single strains each of *T. balanoides*, *T. extinguens*, *T. parasiticum*, and *T. microsporum*. Five strains of *T. geodes* produced detectable amounts of efrapeptins and had compound F, or F and G, as the major component(s) in the mixture with the order of abundance being F > or ~ G > H ~ D ~ E > C. Eleven strains of *T. niveum* produced detectable amounts of efrapeptins and had efrapeptins D and E as the primary and secondary components, respectively, in the mixture with the order of abundance being D > E > F > C ~ G. A single *T. niveum* strain had an efrapeptin profile similar to that of the *T. geodes* strains. Ten strains of *T. cylindrosporum* had detectable amounts of efrapeptins. Of these, nine had F and one had G as the major component. *T. cylindrosporum* had higher ratios of E to D than did *T. geodes*. Efrapeptins were detected in one of two *T. nubicola* strains analyzed (F > G > H) and one of three *T. tundrense* strains (F > G > H > E). *T. niveum* strains could, in most cases, be identified to species on the basis of their efrapeptin profiles.

Key Words—Chemotaxonomy, fungus, *Tolypocladium*, fungal toxin, mycotoxin, efrapeptins.

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INTRODUCTION

The fungal genus *Tolypocladium* (Deuteromycotina: Hyphomycetes) includes species found in nature as pathogens of invertebrates as well as saprobic soil-inhabiting species, some of which are also capable of infecting arthropods in the laboratory (Bissett, 1983; Weiser, 1987). Because of their pathogenic characteristics, these fungi have attracted attention as potential biological control agents, especially for a number of mosquito species (Goettel, 1988 and references cited).

T. niveum and *T. geodes* produce efrapeptins, a microheterogeneous group of peptides rich in α-amino-isobutyric acid and other unusual amino acids, and having an unusual carboxy-terminal blocking group (Figure 1) that confers potent mitochondrial ATPase inhibitory activity on these compounds (Gupta et al., 1991, Gupta et al., 1992; Krasnoff and Gupta, 1991). Efrapeptins have insecticidal activity (Krasnoff et al., 1991), which may enhance the utility of these fungi as biological control agents.

Previously we reported that a strain of *T. geodes* was distinguished from a strain of *T. niveum* by the relative amounts of individual efrapeptins produced in the mixture (Krasnoff and Gupta, 1991). We hypothesized that efrapeptin profiles might be species-specific in this genus. To test this hypothesis, as well as to get a more complete picture of the production of efrapeptins in the genus as a whole, we carried out HPLC analyses of extracts of a variety of strains of these and other *Tolypocladium* species. In the process we isolated and identified efrepeptins from *T. cylindrosporum*, a species in which these compounds were previously unknown, and also found preliminary evidence of efrapeptin production by *T. nubicola* and *T. tundrense*. Herein we report these identifications,

C Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-AIB-X (FW=1606)
D Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-IVA-X (FW=1620)
E Ac-PIP-AIB-PIP-IVA-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-IVA-X (FW=1634)
F Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-ALA-LEU-IVA-X (FW=1634)
G Ac-PIP-AIB-PIP-IVA-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-ALA-LEU-IVA-X (FW=1648)

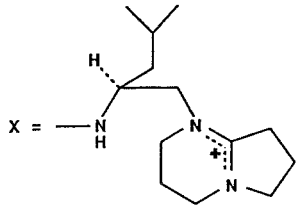

FIG. 1. Structure of efrapeptins C, D, E, F, and G. Amino acid residues are abbreviated as follows: alanine (ALA), glycine (GLY), leucine (LEU), α-aminoisobutyric acid (AIB), β-alanine (β-ALA), isovaline (IVA), and pipecolic acid (PIP). Ac = Acetyl, FW = formula weight.

describe variation in efrapeptin production in and among *Tolypocladium* species, and discuss the implications of this variation to the systematics of the group.

METHODS AND MATERIALS

Growth of Fungi. Fungal material from cultures maintained under liquid nitrogen in the USDA-ARS entomopathogenic fungi (ARSEF) collection was used to inoculate plates of Sabouraud dextrose agar + 1% yeast extract (SDAY) grown at 22 ± 1°C. For the study of interstrain variation in efrapeptin production, a small plug of this SDAY culture was used to inoculate 100-ml batches of Czapek-Dox (Difco) liquid medium supplemented with 0.5% bactopeptone (Difco) (CDB) in 250 Erlenmeyer flasks. All strains were grown in duplicate to afford duplicate extracts for HPLC analysis.

Conidia used as inoculum to grow *T. cylindrosporum* (ARSEF #962) for preparative HPLC (see below) were harvested in sterile 0.1% Tween-80 from a 20-day-old culture grown on SDAY slants at 22 ± 1°C. Concentrations of initial conidial suspensions were determined using an improved Neubauer hemacytometer. A suspension of ca. 3.4×10^6 conidia/ml was made by dilution of initial suspensions in sterile water and used to inoculate 100-ml batches of CDB in 250 Erlenmeyer flasks with 1×10^6 conidia. All liquid cultures were grown on a rotary shaker (160 rpm; 2.5 cm throw) for 11 days at 22 ± 1°C, exposed to natural light from a window.

Production of Extracts. Fungal extracts were produced as described previously (Krasnoff and Gupta, 1991). Briefly, fungal mycelium was separated from the culture broth by filtration. Following centrifugation of the broth, the pellet was discarded and the supernatant was extracted with dichloromethane. The organic layer was concentrated to dryness and weighed. Extracts were stored at −20°C. Prior to HPLC analysis samples were dissolved in methanol and filtered through a 0.45-μm membrane cartridge.

Isolation and Characterization of Efrapeptins. The efrapeptin fraction from the crude dichloromethane extract of culture broth of a single *T. cylindrosporum* strain (ARSEF #962) was prepared for fast atom bombardment mass spectrometry (FABMS) by repetitive semipreparative HPLC on a reverse-phase C8 column (0.9 × 50 cm) eluted with MeCN–12.5 mM $(NH_4)_2SO_4$ (75:25) at a flow rate of 5.5 ml/min. Detection was by monitoring UV absorption at 225 nm (0.5 aufs).

Efrapeptin profiles were analyzed by HPLC using a reverse-phase C8 analytical column (0.46 × 15 cm, particle size 5 μm, Supelcosil LC-8) eluted with MeCN–12.5 mM $(NH_4)_2SO_4$ (70:30) at a flow rate of 2 ml/min. Detection was by monitoring UV absorption at 225 nm (0.2 aufs). In samples from species for which the production of efrapeptins was confirmed by mass spectral data, indi-

vidual efrapeptin peaks were identified by comparison with HPLC retention times of a standard efrapeptin mixture of authentic efrapeptins from a *T. niveum* strain (ARSEF #616) partially purified by flash chromatography on silica gel and sephadex LH 20 (Gupta et al., 1992).

Quantification of efrapeptins was accomplished by comparing total efrapeptin peak area from an electronic integrator (Hewlitt Packard 3396B) with the peak area of a known amount of pure efrapeptin F. A calibration curve was generated to verify that the UV detector response to efrapeptin F was linear in the quantitative range analyzed. Total efrapeptin production for 100 ml of culture broth was calculated by dividing the estimated efrapeptin injected by the amount of crude extract injected and multiplying this factor by the total yield of crude extract.

RESULTS

Isolation and Identification of Efrapeptins. Preliminary HPLC analysis of a crude dichloromethane extract of *T. cylindrosporum* (ARSEF strain #962) indicated the presence of a mixture of efrapeptins. Peaks from the *T. cylindrosporum* extracts cochromatographed with authentic efrapeptins D, E, F, and G.

Identification of efrapeptins from *T. cylindrosporum* extracts was confirmed by FABMS analysis of an HPLC-purified mixture of efrapeptins, which showed molecular ions (M^+) at *m/z* 1606, 1620, 1634, 1648, and 1676. As noted previously (Gupta et al., 1992), the quaternary nitrogen in the C-terminal blocking group of the efrapeptins (Figure 1) produces unusually intense molecular ion peaks (M^+) instead of the usual $M+H^+$ under FABMS conditions. The spectrum obtained from *T. cylindrosporum* is consistent with the presence of efrapeptins C (1606), D (1620), E (1634), F (1634), G (1648), and a sixth compound of molecular weight 1676.

Retention time data and cochromatography with authentic efrapeptins indicated the presence of efrapeptins in strains of *T. nubicola* (ARSEF #3435), and *T. tundrense* (ARSEF #3401).

Efrapeptin profiles of 44 strains of nine *Tolypocladium* species were analyzed by HPLC (Table 1). In most cases, a 10-μl aliquot of a 5 mg/ml solution of crude dichloromethane extract of broth was sufficient to detect efrapeptins. In some cases it was necessary to inject a larger volume, concentrate the sample, or do both to detect efrapeptin peaks. Due to low signal-to-noise ratios in chromatograms of concentrated samples, efrapeptins were regarded as "undetected" in samples with an estimate of less than 0.3 mg (per 100 ml broth).

We analyzed six strains of *T. geodes*, four from Canada and two from Great Britain (Table 1). There were significant intraspecific differences in total

TABLE 1. *Tolypocladium* STRAINS ANALYZED FOR EFRAPEPTIN PRODUCTION[a]

Species	ARSEF #	DAOM #	Host	Site of origin
T. balanoides	2483	185098	nematode	Manitoba, Canada
T. cylindrosporum	705		*Aedes sierrensis*	California, USA
T. cylindrosporum	961	183952	sewage	Czechoslovakia
T. cylindrosporum	962	167325	soil	Mt. Allen, Alberta, Canada
T. cylindrosporum	963	167176	soil	Mt. Allen, Alberta, Canada
T. cylindrosporum	976		mosquito	New Zealand
T. cylindrosporum	1027		mosquito	New Zealand
T. cylindrosporum	1028		mosquito	New Zealand
T. cylindrosporum	1580		unknown	unknown (from GGS Soares Tc4c)
T. cylindrosporum	2777		noctuid	Denmark
T. cylindrosporum	2778		noctuid	Denmark
T. cylindrosporum	2911		mosquito	New Zealand
T. cylindrosporum	2912		lovebug (*Plecia nearctia*)	Florida, USA
T. cylindrosporum	2920		peat soil	England
T. cylindrosporum	2975		soil	Antarctica
T. cylindrosporum	3392	180479	soil	Nepal
T. cylindrosporum	3393	180480	soil	Nepal
T. extinguens	2921		*Arachnocampa luminosa*	New Zealand
T. geodes	3271	167321	soil	Mt. Allen, Alberta, Canada
T. geodes	3272	167323	soil	Mt. Allen, Alberta, Canada
T. geodes	3273	167326	soil	Mt. Allen, Alberta, Canada
T. geodes	3274	176470	soil	Sudbury, Ontario, Canada
T. geodes	3275	191333	soil	Gisburn Forest, UK

TABLE 1. CONTINUED

Species	ARSEF #	DAOM #	Host	Site of origin
T. geodes	3276	191336	soil	Pennines (Moorhourse), UK
T. microsporum	3431	191103	roots (*Cornus canadensis*)	Kirkland Lake, Ontario, Canada
T. niveum	3277	64352	soil	Obergurge, Oetstal, Austria
T. niveum	3278	160594	mite surface (*Mycobates* sp.)	Baffin Island, NW Terr., Canada
T. niveum	3279	167175	soil	Luscor, Alberta, Canada
T. niveum	3280	167322	soil	Mt. Allen, Alberta, Canada
T. niveum	3281	198395	tachinid puparium	Fredricton, New Brunswick, Canada
T. niveum	3320	63905	pulp mill slime	Corner Brook, Newfoundland, Canada
T. niveum	3321	199924	*Choristoneura fumiferana*	Acadia, New Brunswick, Canada
T. niveum	3394	183955	soil	Burt Lake, Ontario, Canada
T. niveum	3395	185687	lake sediment	Sudbury, Ontario, Canada
T. niveum	3396	191338	soil	Pennines, England
T. niveum	3398	210147	soil	White Mtn. Nat. For., New Hampshire, USA
T. niveum	3432	167132	soil	Mt. Allen, Alberta, Canada
T. niveum	3433	180481	soil	Nepal
T. nubicola	3434	167320	soil	Mt. Allen, Alberta, Canada
T. nubicola	3435	167324	soil	Mt. Allen, Alberta, Canada
T. parasiticum	3436	184880	bdelloid rotifers	Guelph, Ontario
T. tundrense	3399	167133	soil	Pennines, UK
T. tundrense	3400	167134	soil	Devon Island, NW Terr., Canada
T. tundrense	3401	191334	soil	Devon Island, NW Terr., Canada

[a]Abbreviations not in text: DAOM = Canadian National Collection of Fungus Cultures, Department of Agriculture, Ottawa; UK = United Kingdom, USA = United States of America.

efrapeptin yield (ANOVA significant at $P < 0.0001$ for two replicates each of six strains). Efrapeptins were detected in five strains (Table 2). The three Canadian strains, ARSEF #3271, #3272, and #3274, produced efrapeptins F and G in approximately equal amounts. The two British strains, ARSEF #3275 and #3276, had 1.5 and 2.6 times as much F as G, respectively. For all efrapeptin-producing strains of *T. geodes*, minor components were efrapeptins D, E, and H, present in approximately equal proportions. Efrapeptin C was present in only trace amounts or undetectable.

We analyzed 14 strains of *T. niveum*, originating from material collected in North America, Europe, and Asia (Table 1). There were significant intraspecific differences in total efrapeptin yield (ANOVA significant at $P < 0.001$ for two replicates each of 14 strains). Efrapeptins were detected in 11 strains (Table 3). Ten strains produced efrapeptin D and E as the primary component and secondary components, respectively, with the order of abundance of the components being D > E > F > G > C. One of 11 efrapeptin-producing strains (ARSEF #3321) had efrapeptins F and G as major components in approximately equal amounts, with minor components D > E > H.

We analyzed 16 strains of *T. cylindrosporum* (Table 1). There were significant intraspecific differences in total efrapeptin yield (ANOVA significant at $P < 0.0001$ for two replicates each of 14 strains; analyses for two strains that were not duplicated were not included in the ANOVA). Efrapeptins were detected in 10 strains (Table 4). F was the primary component in nine and the secondary component in one strain. G was the major component in one strain, the secondary component in seven strains, and the tertiary component in two strains.

Efrapeptin E was more prominent in the profiles of *T. cylindrosporum* than in those of *T. geodes* as reflected in the ratios of E to D, which ranged from 2.3 to 11.6 for 10 strains of *T. cylindrosporum* versus 0.8 to 1.4 for five strains

TABLE 2. EFRAPEPTIN PRODUCTION BY *Tolypocladium geodes*[a]

ARSEF # (origin)	Total efrapeptins (mg)	%C	%D	%E	%F	%G	%H
3271 (CAN)	8.1 (0.2)	0.7 (0.1)	6.7 (0.1)	9.3 (0.0)	34.9 (1.3)	36.6 (0.4)	11.8 (0.7)
3272 (CAN)	5.3 (0.1)	0.0 (0.0)	6.1 (0.3)	8.6 (0.1)	35.3 (0.6)	38.7 (0.1)	11.3 (0.3)
3274 (CAN)	9.7 (0.9)	0.0 (0.0)	7.0 (0.8)	8.1 (0.2)	38.2 (0.5)	37.1 (0.7)	9.5 (0.2)
3275 (UK)	8.6 (0.1)	0.6 (0.0)	6.1 (0.3)	4.9 (0.1)	50.2 (0.5)	33.6 (0.2)	4.5 (1.2)
3276 (UK)	29.0 (0.1)	1.2 (0.1)	8.8 (0.1)	7.3 (0.1)	59.3 (0.2)	21.3 (0.1)	2.2 (0.0)

[a] Shown are estimates for total efrapeptin production and percentage composition of each compound in the efrapeptin mixture. Numbers in parentheses are standard deviations for duplicate samples. Abbreviations: CAN = Canada, UK = United Kingdom.

TABLE 3. EFRAPEPTIN PRODUCTION BY *Tolypocladium niveum*[a]

ARSEF # (origin)	Total efrapeptins (mg)	%C	%D	%E	%F	%G	%H
3277 (AUS)	3.0 (0.5)	8.7 (1.4)	43.9 (1.3)	20.7 (0.9)	18.3 (1.1)	8.3 (0.7)	0.0 (0.0)
3278 (CAN)	3.1 (0.3)	8.5 (1.3)	48.6 (0.7)	24.5 (1.3)	13.1 (0.3)	5.3 (0.4)	0.0 (0.0)
3279 (CAN)	3.3 (0.0)	5.4 (0.1)	36.2 (1.2)	26.7 (0.7)	17.0 (0.3)	14.7 (0.7)	0.0 (0.0)
3280 (CAN)	3.0 (0.0)	9.3 (1.0)	43.7 (2.9)	22.9 (0.1)	15.5 (0.4)	8.5 (1.6)	0.0 (0.0)
3281 (CAN)	5.6 (0.5)	6.4 (0.3)	44.5 (2.3)	28.9 (0.4)	12.4 (0.5)	7.8 (1.0)	0.0 (0.0)
3321 (CAN)	14.4 (1.8)	0.6 (0.0)	9.7 (0.8)	9.1 (0.4)	37.9 (0.3)	35.8 (0.9)	6.9 (0.0)
3394 (CAN)	4.1 (0.6)	9.8 (2.6)	38.0 (1.0)	29.4 (2.2)	14.2 (1.3)	8.5 (0.7)	0.0 (0.0)
3395 (CAN)	5.8 (1.3)	6.8 (0.4)	36.1 (0.6)	25.8 (0.9)	18.7 (1.1)	11.0 (0.8)	1.5 (0.3)
3396 (UK)	2.8 (0.1)	3.0 (0.7)	40.1 (1.9)	24.6 (0.6)	21.6 (1.6)	10.6 (1.6)	0.0 (0.0)
3398 (USA)	1.9 (0.2)	10.3 (0.9)	42.8 (2.8)	22.4 (0.9)	16.9 (1.4)	7.5 (1.4)	0.0 (0.0)
3432 (CAN)	4.9 (0.1)	6.0 (1.1)	44.2 (0.2)	23.8 (0.4)	19.1 (0.0)	6.9 (0.5)	0.0 (0.0)

[a] Shown are estimates for total efrapeptin production and percentage composition of each compound in the efrapeptin mixture. Numbers in parentheses are standard deviations for duplicate samples. Abbreviations: AUS = Austria, CAN = Canada, UK = United Kingdom, USA = United States of America.

TABLE 4. EFRAPEPTIN PRODUCTION BY *Tolypocladium cylindrosporum*[a]

ARSEF # (origin)	Total efrapeptins (mg)	%C	%D	%E	%F	%G	%H
705 (USA)	1.7	0.0	3.6	11.3	48.0	37.0	0.0
961 (CZ)	1.6 (0.1)	0.0 (0.0)	3.4 (0.1)	11.8 (0.3)	62.2 (3.3)	22.6 (3.2)	0.0 (0.0)
962 (CAN)	8.3 (0.5)	0.0 (0.0)	3.4 (0.2)	11.4 (1.1)	54.3 (0.1)	31.0 (0.7)	0.0 (0.0)
963 (CAN)	0.5 (0.0)	0.0 (0.0)	0.3 (0.2)	1.0 (0.7)	22.4 (1.5)	48.2 (1.3)	28.1 (3.7)
976 (NZ)	0.9 (0.2)	0.0 (0.0)	0.0 (0.0)	16.7 (2.3)	43.2 (4.9)	23.0 (4.9)	17.1 (12.1)
1028 (NZ)	1.2	0.0 (0.0)	1.8 (1.3)	9.0 (0.2)	64.0 (0.1)	25.2 (1.4)	0.0 (0.0)
1580 (UNK)	2.1 (0.1)	0.0 (0.0)	4.9 (0.1)	11.4 (0.1)	58.3 (1.3)	25.4 (1.3)	0.0 (0.0)
2912 (USA)	0.3 (0.1)	0.0 (0.0)	4.5 (0.2)	15.9 (0.7)	67.3 (0.4)	12.3 (0.9)	0.0 (0.0)
3392 (NEP)	0.3 (0.1)	0.0 (0.0)	2.0 (1.4)	23.5 (3.3)	61.5 (1.0)	13.0 (0.9)	0.0 (0.0)
3393 (NEP)	0.3 (0.0)	0.0 (0.0)	0.0 (0.0)	15.2 (2.6)	66.1 (0.3)	18.7 (3.0)	0.0 (0.0)

[a] Shown are estimates for total efrapeptin production and percentage composition of each compound in the efrapeptin mixture. Numbers in parentheses are standard deviations for duplicate samples (if no parentheses no duplicate was analyzed). Abbreviations: CAN = Canada, CZ = Czechoslovakia, NEP = Nepal, NZ = New Zealand, UNK = unknown, USA = United States of America.

of *T. geodes*. E was the secondary component in two strains, the tertiary component in seven strains (E ~ H in one of these), and present as a minor component in trace amounts in one strain. No clear consensus order of minor components emerged but F > G > E was the order of the major components in six strains. Although no efrapeptin C was detected by HPLC in any of the *T. cylindrosporum* strains (Table 4), the more sensitive FABMS analysis of the HPLC-purified efrapeptin fraction from ARSEF #962 indicated that it is being produced by this species ($M^+ = 1606$; see above).

Strains of several other species of *Tolypocladium* were also analyzed by HPLC. Peaks with retention times corresponding to efrapeptins were detected in one of two strains of *T. nubicola* (ARSEF #3435). The estimate for total efrapeptin production of this strain was 0.8 ± 0.3 mg (±SD) with the profile: *F* (50.0 ± 4.3%) (±SD) > H (28.1 ± 2.9%) > G (21.9 ± 1.4%). No C, D, or E were detected. One of three strains of *T. tundrense* (ARSEF #3401) produced detectable amounts of efrapeptins (1.4 ± 0.0 mg) with the profile: F (40.8 ± 3.3%) ~ G (37.0 ± 1.7%) > H (19.2 ± 1.7%) > E (3.0 ± 0.2%); no C or D were detected.

No efrapeptins were detected in single strains each of *T. balanoides*, *T. extinguens*, *T. parasiticum*, and *T. microsporum*.

DISCUSSION

T. cylindrosporum is the third species in the genus for which production of efrapeptins has been confirmed. This species is frequently found in nature as a pathogen of mosquito larvae and consequently has attracted attention as a potential biological control agent (Soares, 1982; Gardner and Pillai; 1986, Goettel, 1987; Ravallec et al., 1989). We observed considerable variability in total efrapeptin production in this species. The possibility that interstrain variability in virulence (e.g., Soares et al., 1985) may correlate with efrapeptin production might be a fruitful area of future investigation.

Our HPLC data provide the first chemical evidence that efrapeptins are produced by *T. nubicola* and *T. tundrense*. Insecticidal effects reported for extracts of these species, and of *T. cylindrosporum*, *T. geodes*, and *T. inflatum* (=*niveum*), have been attributed to an unidentified material referred to as "tolypin" (Matha et al., 1988a,b; Weiser and Matha, 1988; Weiser, 1991). However, given the production of efrapeptins by these species shown here, and the demonstrated insecticidal activity of these compounds (Krasnoff et al., 1991), it is likely that efrapeptins are the agents responsible for these insecticidal effects.

Previously (Krasnoff and Gupta, 1991) we hypothesized that efrapeptin profiles might be useful characters for species level diagnostics and phylogenetic analysis in *Tolypocladium*. Although no inviolate species-specific chemotypes

emerged from this study, definite trends were discernible. These can be illustrated in bivariate scatter plots showing how strains group by percent composition of pairs of efrapeptins in the profile (Figure 2) and by a cluster analysis based on percent composition of efrapeptins D, E, F, and G (Figure 3).

T. geodes showed what may be two distinct subspecific chemotypes linked to geographic origin (Figures 2 and 3). The three Canadian strains produced F and G in approximately equal amounts while the two British strains produced a clear predominance of F over G, the latter being a secondary but still major component relative to the others (Table 2). The possibility that there are geographic chemotypes within this species, as there are, for example, in *Fusarium* spp. (Miller et al., 1991), should be pursued with more extensive sampling of strains from divergent sites of origin.

T. cylindrosporum showed less intraspecific consistency in efrapeptin profiles than either *T. niveum* or *T. geodes*, but generally resembled the latter more than the former (Figures 2 and 3; Tables 2–4), especially in the preeminence of compound F and/or G, and in the production of H as a secondary component in two strains. Another salient feature is the higher ratios of E to D relative to *T. geodes* and *T. niveum* (Figure 2b).

Of the three major species sampled, *T. niveum* showed the greatest consistency in efrapeptin profiles. However, as seen in Figures 2 and 3, and as mentioned above (see Results), a single strain (ARSEF #3321) from eastern Canada more closely resembled the Canadian *T. geodes* strains in efrapeptin content than it did the other 10 *T. niveum* strains (Figure 3). In fact, it more closely resembled the Canadian *T. geodes* strains than the latter resembled the British *T. geodes* strains (Figure 3). We grew this strain repeatedly and made several independent analyses, which confirmed that the original results were not due to experimental error.

The utility of efrapeptin profiles as a species level diagnostic character depends on the interpretation of this outlier. Consequently, we offer several possible explanations for this apparent anomaly: (1) *T. niveum* could be polymorphic for this character. If this is the case, then more extensive sampling ought to turn up similar *T. niveum* strains. (2) This strain could be the result of a rare mutation. *T. niveum* almost always produces a predominance of efrapeptins D and E, which both have glycine (Gly) at position 13 in the peptide chain, while the other four efrapeptin-producing species make a predominance of efrapeptins F and G, both of which have alanine (Ala) at position 13. As we pointed out in a previous study (Krasnoff and Gupta, 1991), this suggests that, at a particular step in biosynthesis, *T. niveum* incorporates Gly where the other species incorporate Ala (see above). Although their unusual amino acids and terminal blocking groups indicate that efrapeptins are not translation products, it is reasonable to assume that the choice of a Gly or an Ala at position 13 is mediated by an enzyme that is subject to mutation. Showing that mutagenic

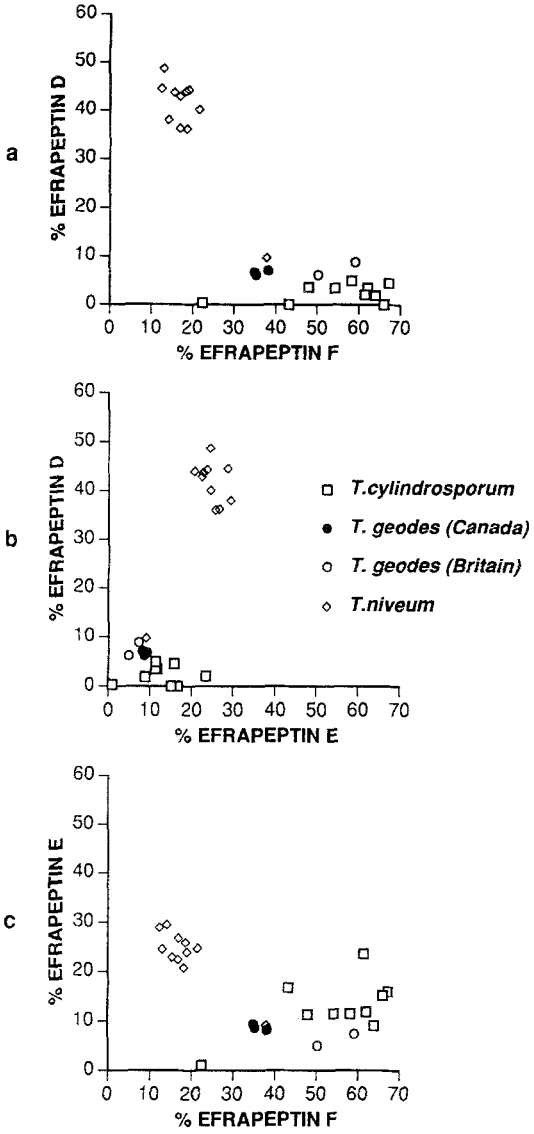

FIG. 2. Bivariate plots of percent efrapeptin composition for *Tolypocladium* species: (a) efrapeptin D vs. F, (b) efrapeptins D vs. E, (c) efrapeptins E vs. F.

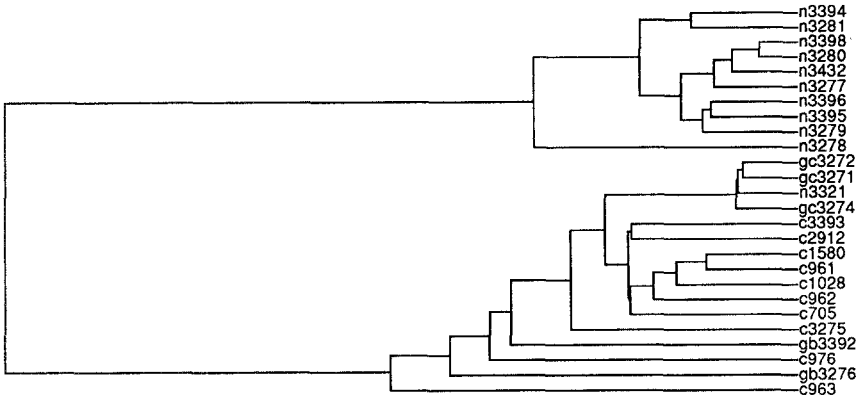

FIG. 3. Hierarchical single linkage euclidean distance cluster diagram (Anderberg, 1973) of *Tolypocladium* strains based on percent composition of efrapeptins D, E, F and G. Numbers at termini correspond to ARSEF # (Table 1) and are preceded by species designations: c = *T. cylindrosporum*, gc = *T. geodes* (Canadian strains), gb = *T. geodes* (British strains), n = *T. niveum*.

agents could change the efrapeptin profile of a strain from D-dominated to F-dominated would support this interpretation. (3) The strain might be a parasexual cross between *T. geodes* and *T. niveum*. Although liquid cultures of ARSEF #3321 did not have the characteristic earthy odor of liquid cultures of *T. geodes*, cultures of the strain on solid medium (SDAY) produced some phialides with swollen bases typical of *T. niveum* and some phialides more like those of *T. geodes*, which are less swollen basally with a more cylindrical shape. Thus the morphology of the phialides might be interpreted as intermediate between *T. geodes* and *T. niveum*. The collection of *T. cylindrosporum*, *T. geodes*, and *T. niveum* all from the same locality (albeit not the same locality from which ARSEF #3321 originated) establishes the possibility of contact among all three species (Table 1). Although we are unaware of any reports of *inter*specific parasexual crosses in the hyphomycetes, *intra*specific parasexual crosses have been produced in the laboratory with the entomopathogenic hyphomycetes, *Verticillium lecanii* (Jackson and Heale, 1987) and *Metarhizium anisopliae* (Messias and Azevedo, 1980).

In consideration of the chemically anomalous nature of ARSEF #3321, a *Tolypocladium* strain producing an efrapeptin profile dominated by F or G could not be diagnosed to species on the basis of the profile. However, of all the *Tolypocladia* sampled only *T. niveum* produced efrapeptin profiles dominated by compound D, so to this extent the character would still be useful in distinguishing most *T. niveum* from other species in the genus.

Gams (1971b) erected the genus *Tolypocladium* on the basis of defining

morphological features, phialides swollen basally, with a narrow, often bent neck, and small, one-celled conidia produced in "slimy heads" (Gams, 1971b; Bissett, 1983). Originally included in the genus were *T. cylindrosporum*, *T. geodes*, and *T. inflatum*. Barron described two new species, *T. parasiticum* (1980) and *T. trigonosporum* (1981). Bissett (1983) added two more new species, *T. nubicola* and *T. tundrense*, redisposed two others, *T. balanoides* and *T. microsporum*, which were previously described in other genera, and synonymized Gams' *T. inflatum* with *T. niveum*, proposing the latter as the name of the type species. Samson and Soares (1984) added *T. extinguens* to the genus. Finally, a new species, *T. terricola*, was proposed in an unrefereed forum (Weiser et al., 1989), so the species count for the genus stands at 10 officially with the possibility of an eleventh.

Tolypocladium taxonomy has several unresolved problems. The status of the genus was called into question by Von Arx (1986), who synonymized it with *Beauveria*, but this opinion has apparently not gained acceptance by others who have worked on the group (Gams, 1988). The placement of some species in the genus is also in dispute. There are countervailing opinions to Bissett's generic placement of *T. balanoides* and *T. microsporum*. The latter had been redisposed from *Verticillium* to *Sesquicillium* by Gams (1971a). Bissett (1983) moved it to *Tolypocladium* based on its production of spores in "minute" slimy heads rather than the "imbricate columns" indicative of *Sesquicillium*. *T. balanoides* was disposed to *Verticillium* by Dowsett et al. (1982), and this was supported by Gams (1988). Based on rRNA sequence data, Rakotonirainy et al. (1991) concluded that *T. extinguens* is more closely related to *Beauveria bassiana* than to *T. cylindrosporum* and, thus, that it does not belong in *Tolypocladium*.

We detected no efrapeptin production in *T. balanoides*, *T. extinguens*, and *T. microsporum*. While it is difficult to prove that a given strain or species does not produce a given metabolite, our negative evidence for these species may lend some support to those who argue for their placement in other genera. In view of the apparently widespread production of efrapeptins in the genus *Tolypocladium*, more extensive sampling needs to be done on species not represented in our sample, or represented here by only one or a few strains, before making any firm conclusions on generic disposition based on this chemical character.

Efrapeptin production is, so far, unknown outside of *Tolypocladium*. We have screened for it extensively in the genus *Beauveria* as well as in species of *Fusarium*, *Verticillium*, *Paecilomyces*, and *Hirsutella* (Krasnoff and Gupta, unpublished data). We suggest that efrapeptin production might be a defining synapomorphy for the genus, shared as it is among *T. geodes*, *T. niveum*, *T. cylindrosporum*, *T. tundrense*, and *T. nubicola*. Further taxonomic and chemical study may reveal it to be a less plastic and, therefore, more dependable character than some of the morphological features used to define the group.

In summary, we have reported the production of a mixture of efrapeptins by three new *Tolypocladium* species and have described a pattern of intra- and interspecific variation in efrapeptin profiles that supports their use in species determinations of *T. niveum*.

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ORIENTATION OF *Microplitis croceipes* (HYMENOPTERA: BRACONIDAE) TO GREEN LEAF VOLATILES: DOSE–RESPONSE CURVES

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Abstract—Female *Microplitis croceipes* wasps were tested in a wind tunnel for their ability to orient to various concentrations of eight different green leaf volatile (GLV) substances [hexanal, (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenyl acetate, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl propionate, and (*Z*)-3-hexenyl butyrate]. Overall, the esters elicited the greatest percentage of successful orientation flights, the alcohols elicited an intermediate response, and the aldehydes elicited a low response. The semilog dose–response curves were generally hill-shaped with high responses at medium release rates and low responses at high or low release rates. For the aldehydes, positive responses occurred at all GLV release rates between 0.01 and 100 nl/min. For some alcohols and esters, positive responses occurred at release rates as low as 1 pl/min and as high as 1 μl/min. These data show that *M. croceipes* wasps are strongly attracted to GLVs and are capable of orienting to GLV concentrations that would occur in nature when a caterpillar feeds on a green leaf. Hence, in nature, GLVs may be important clues, enabling *M. croceipes* to locate their hosts.

Key Words—Green leaf volatile, semiochemical, synomone, volatile attractant, *Microplitis croceipes*, Hymenoptera, Braconidae, *Heliothis zea*, Lepidoptera, Noctuidae, biological control, tritrophic, host location, parasitoid behavior.

INTRODUCTION

Many wasp parasitoids seek out and oviposit into plant-feeding caterpillars. Volatile semiochemicals originating from the caterpillars or their food plants

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serve as important cues for these wasps, enabling the wasps to find their hosts (Nordlund et al., 1988; Whitman, 1988a; Lewis and Martin, 1990; McAuslane et al., 1991; Turlings et al., 1991a,b). Among the volatile substances released from damaged plants are the six-carbon alcohols, aldehydes, and derivative esters that are collectively known as green leaf volatiles (GLVs) (Hedin et al., 1973; Saijo and Takeo, 1975; Wallbank and Wheatley, 1976; Visser et al., 1979; Hamilton-Kemp et al., 1988, 1989; Tollsten and Bergström, 1988; Hernandez et al., 1989; Lwande et al., 1989). Recently, we suggested that caterpillars feeding on green leaves cause plants to release GLVs and that parasitic wasps orient to these substances and hence to their caterpillar hosts (Whitman and Eller, 1990). In this tritrophic interaction, the GLVs would serve as plant-to-parasitoid synomones; their release would benefit both the plant and the parasitoid but would be detrimental to the caterpillar.

In this paper we examine the ability of female *Microplitis croceipes* (Cresson) wasps to orient to various concentrations of individual GLVs in a wind tunnel. *M. croceipes* is a braconid wasp that attacks the corn earworm caterpillar, *Helicoverpa* (= *Heliothis*) *zea* (Boddie) (Lepidoptera: Noctuidae). We show that the GLVs vary in attractiveness, that wasps can successfully orient to GLV concentrations ranging over five orders of magnitude, that there is variability in the low and high response thresholds of wasps to various GLVs, and that the dose–response curves for various GLVs differ more in magnitude and capacity than in shape. We also show that wasps can orient to release rates similar to those produced naturally when a caterpillar feeds on a green plant.

METHODS AND MATERIALS

Insects. Larval *M. croceipes* were reared on *Helicoverpa zea* at the Insect Biology and Population Management Research Laboratory at Tifton, Georgia. The *Heliothis* were cultured on artificial diet (Burton, 1969). Adult parasitoids were kept communally and allowed to mate in Plexiglas cages (30 × 30 × 17 cm) at 28°C, 50–70% relative humidity, a 16:8 light–dark photoperiod, and provided daily with fresh water and honey (Lewis and Burton, 1979). Adults were isolated from plant and host materials until tested.

Flight Tunnel. The ability of wasps to orient to GLVs was tested in a 75 × 75 × 200-cm laminar-flow Plexiglas wind tunnel (wind speed: 31 cm/sec; temperature: 26–27°C; relative humidity: 40–80%; wind tunnel light provided by four overhead 80-W fluorescent bulbs). An electric fan, placed outside the building, pulled air through the tunnel at a constant rate and vented it outside, eliminating contamination from recycled air.

Chemicals. Various GLVs were obtained from Sigma Chemical Co. and Aldrich Chemical Co. Following the tests, chemicals were assayed by GCMS; purity ranged from 93 to 99%.

Preparation of Water Extract of H. zea *Frass.* Ten grams of fresh moist *H. zea* frass (collected from caterpillars raised on cowpea seedlings) was thoroughly blended with 100 ml of distilled water. The resultant slurry was filtered through Whatman No. 1 filter paper, producing a green-colored filtrate. This filtrate was kept frozen and served as the stock extract throughout the tests. To produce the dried extract, approximately 1 ml of stock extract was unfrozen and shaken. Then 30 μl of extract was collected into a micropipet and spotted onto the center of a 110-cm disk of Whatman No. 1 filter paper. After the 1-cm diameter moist area dried ($\sim$7 min.), the filter paper was used to stimulate wasps.

Experimental Design. Three- to eight-day-old female *M. croceipes* wasps were tested for their ability to orient in a wind tunnel to various concentrations of eight different GLVs (see Figure 1 below). Eight to 10 different release rates were tested for each GLV, resulting in 73 different treatments. Thirty or more wasps were flown to each treatment; each wasp was tested only once. Eight control treatments were also employed: Sixty wasps were flown to a blank control consisting of a 1-cm^2 paper disk and 15–30 wasps were flown to each of seven different hexane release rates, which encompassed the full range of GLV release rates tested. Wasps were individually tested, allowing individual responses to be recorded. All trials were single choice tests and were balanced for compound, dose, day, and time of day. The odor source was placed in the flight chamber 13 cm above the chamber floor, along the longitudinal midline of the chamber, and 20 cm downwind from the up wind end of the chamber.

Immediately prior to testing, a wasp was stimulated for 30 sec by contact with dried water extract of *H. zea* frass. The wasp was then placed into a clean 4-ml shell vial directly 100 cm downwind from the odor source. Wasps usually walked upwards to the opening of the shell vial, encountered the lower portion of the odor plume, faced into the wind, and after 5 sec to 5 min took flight. Wasps that did not take flight in 5 min or wasps incapable of normal flight were discarded. Each wasp was given two successive chances to fly to the odor source and then was discarded. If a wasp flew toward and landed on the odor source or a paper target placed 3 mm from the odor source, a positive response was recorded and the trial was ended. If the wasp took flight, but failed to land on the odor source or target, a negative response was recorded.

In the above trials, wasps were released 100 cm downwind from the odor source. To examine the effects of distance, a second set of trials was run with wasps released only 15 cm downwind from the odor source.

Release and Calibration of Green Leaf Volatiles. Different methods were used to obtain low, medium, and high GLV release rates. For low release rates, one end of a 5-, 10-, 20-, 50-, or 100-μl-capacity glass micropipet (Fisher Scientific, Pittsburgh, Pennsylvania) was flame sealed, and a measured amount of GLV was placed into the open end. The pipet was then spun in a centrifuge

to pull the liquid down to the sealed end of the tube. The resulting tube contained a measured amount of GLV at the sealed end. After a 24-hr equilibration period, a single pipet was placed vertically in the wind tunnel with the open end up, providing a relatively constant release rate of GLV. By using various sized micropipets, various release rates were produced. A standardized release rate among same-sized pipets was assured by maintaining a standard distance of 110 mm between the GLV meniscus and the pipet opening.

For medium release rates, 0.5- or 1-μl-capacity glass micropipets (Drummond Scientific, Broomall, Pennsylvania) or 5-, 10-, 20-, 50-, or 100-μl-capacity micropipets (Fisher Scientific) were filled with 10 mm of a selected green leaf volatile substance. A filled pipet was suspended crosswise in the wind tunnel, but at a 45° angle from vertical. This arrangement assured that the lower meniscus of test liquid always remained just at the lower orifice of the pipet, producing a uniform evaporative surface. If the pipet was placed vertically or horizontally, uniform evaporation was not achieved; with a vertical pipet, a droplet of test liquid sometimes emerged from the tube, increasing volatilization. With a horizontal pipet, capillary action sometimes pulled the liquid into the center of the tube, decreasing volatilization.

Higher release rates were achieved by inserting an 8-mm^2 or 30-mm^2 triangular filter paper wick into the open end of a 100-μl micropipet containing 20 mm of GLV. Pipets were positioned at a 45 degree angle from vertical, with the tube and surface of the wick perpendicular to the air flow.

For all pipets, a 1-cm^2 disk of white filter paper served as a landing target. The disk was attached to the micropipet in a vertical position so that the perimeter of the disk was 3 mm from the releasing end of the tube. With this arrangement, a wasp orienting to volatiles emerging from the end of the micropipet usually landed on the vertical paper disk.

Medium and high release rates were determined directly by measuring the change in volume of the internal liquid when the pipets were kept in the wind tunnel for 24 hr.

The release rates from the low-release pipets were determined indirectly as follows: The eight synthetic GLVs were loaded individually into 10-, 20-, 50-, and 100-μl micropipets as described above. After equilibrating in a fume hood for 24 hr, the pipets were placed vertically in a volatile collection system for 24 hr. The volatile collection system consisted of a 20-cm × 2.2-cm-ID glass tube sealed on each end with a cork stopper. The bottom cork held a prefilter (7-cm × 4-mm-ID glass tube) packed with 6 mm of Super Q (80–100 mesh; Alltech Associates, Inc., Deerfield, Illinois) held between a 325-mesh stainless-steel screen and a glass-wool plug. The top cork held a similar filter with 4 mm of Super Q to collect volatiles. Air was drawn up through the tube at a flow rate of 130 ml/min. Collected volatiles were extracted from the downwind filter with 240 μl methylene chloride. Ten microliters of a 250 ng/μl

solution (i.e., 2500 ng) of 1-octen-3-ol was added to each filter extract as an internal standard to quantify collected volatiles. Volatile collections were analyzed using a Hewlett Packard 5890 Series II gas chromatograph (Hewlett Packard Co., Avondale, Pennsylvania) equipped with a fused silica HP-5 column (0.17 μm, 25 m $\times$ 0.32 mm ID) (Hewlett Packard), a flame ionization detector, and a Spectra-Physics SP4400 integrator (Spectra-Physics, Inc., San Jose, California). Injections were made in the splitless mode and after 0.60 min changed to the split mode. The temperature program was: 40°C for 3 min, then 10°C increase/min to 110°C. The injector and detector temperatures were 170°C and 250°C, respectively. Three collections were made of each compound–pipet combination.

RESULTS

Dose–response curves for female *M. croceipes* wasps orienting to various individual GLVs in a wind tunnel are shown in Figure 1. The overall response of wasps appeared to vary according to the functionality of the compound tested. The aldehydes [hexanal and (*E*)-2-hexenal] were not very attractive and elicited few successful orientations. The alcohols (*E*)-2-hexen-1-ol and (*Z*)-3-hexen-1-ol elicited moderate responses, and the esters (*E*)-2-hexenyl acetate, (*Z*)-3-hexenyl acetate, (*Z*)-3-hexenyl propionate, and (*Z*)-3-hexenyl butyrate were more attractive and elicited the greatest number of successful orientations. In contrast, no wasps oriented to the blank or hexane controls.

The dose–response curves for the eight tested compounds tended to be similar in shape when displayed on a semilog plot (Figure 1). The curves were generally hill-shaped, with high responses at medium release rates and low responses at either very high or very low release rates. Maximum responses generally fell at release rates between 0.1 and 10 nl/min.

The data show that the range of attractive doses varied among the different GLVs. With aldehydes, wasps responded to release rates that ranged across four orders of magnitude; above and below this active range, wasps failed to orient. For each of the alcohols and esters, positive responses were observed for over five orders of magnitude. Note, however, that for the alcohols and esters, we did not test above or below this range (it is difficult to reliably produce such extreme release rates). Hence, wasps may be able to orient over a greater range of release rates than shown here. For example, 30% of wasps successfully oriented to the lowest (*Z*)-3-hexenyl butyrate release rate tested (0.5 pl/min) and 32% of wasps oriented to the highest (*Z*)-3-hexen-1-ol release rate tested (1 μl/min).

Our data also indicated that the low threshold for response varied for different GLVs. For (*Z*)-3-hexenyl butyrate, some wasps oriented to release rates as low as 0.5 pl/min. For hexanal, however, this figure was only 10 pl/min.

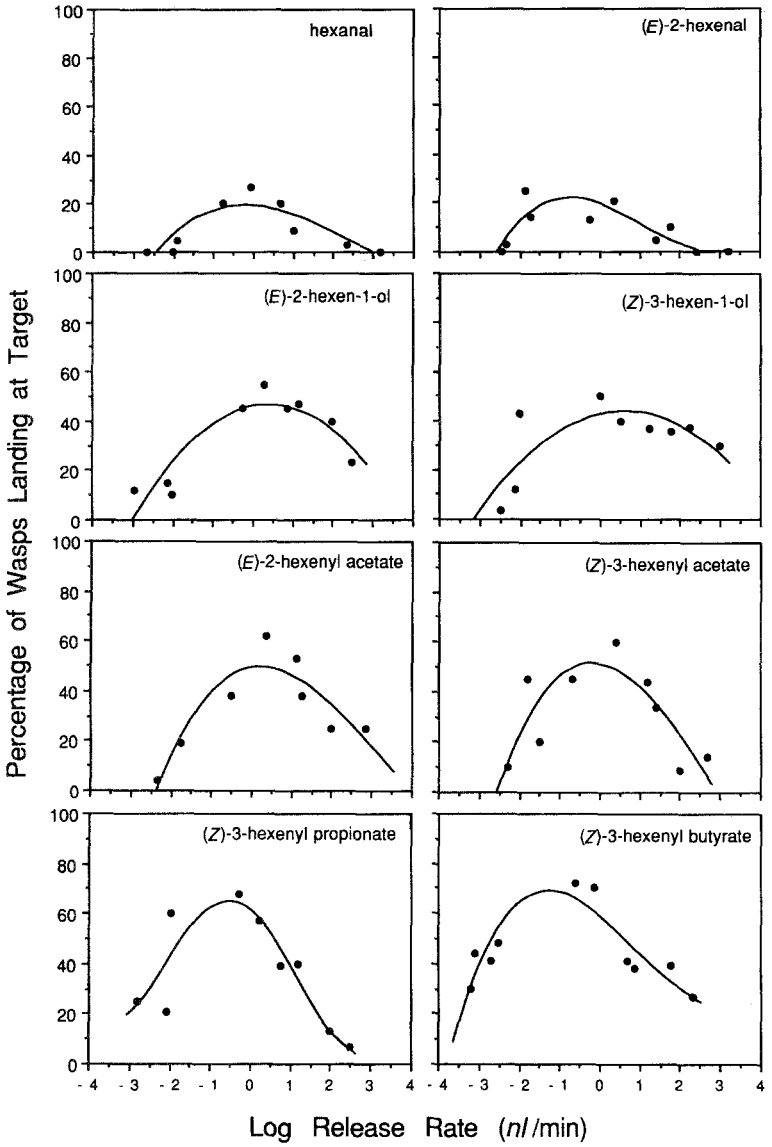

FIG. 1. Dose–response curves for female *Microplitis croceipes* wasps flying to various green leaf volatiles in a wind tunnel. Each point represents the results from 30 or more wasps. Curves generated and drawn by computer as second- or third-order polynomials. Each wasp was given two chances to successfully orient.

TABLE 1. ORIENTATION RESPONSE OF FEMALE *Microplitis croceipes* WASPS TO NEAR AND DISTANT SOURCES OF INDIVIDUAL GREEN LEAF VOLATILES

Volatile	Release rate (pl/min)	Percentage[a] of wasps successfully orienting to odor source from	
		100 cm[b]	15 cm[c]
(Z)-3-hexen-1-ol	3.2	3	80
(E)-2-hexenyl acetate	4.7	3	60
(Z)-3-hexenyl butyrate	1.9	40	80
(Z)-3-hexenyl butyrate	3.0	47	87

[a] Each wasp given two chances to orient.
[b] Thirty or more wasps tested to each volatile.
[c] Fifteen wasps tested to each volatile.

Wasps responded differently to high versus low release rates. At high release rates, wasps often oriented well when between 30 and 100 cm downwind from the odor source. However, when the wasps flew closer (between 5 and 20 cm from the odor source), they often turned sharply away and made no further attempts to enter the odor plume. In contrast, with low release rates wasps oriented poorly when 50–100 cm downwind from the odor source, but flew well when closer. When these wasps exited the odor plume, they often looped downwind or performed transverse zigzag flights until they reentered the plume. For example, when wasps were tested with (Z)-3-hexenyl propionate at a release rate of 10 pl/min, all 18 wasps that approached within 10 cm of the odor source landed, but at 13 nl/min, only 12 of the 22 wasps that approached within 10 cm landed. Likewise, at a release rate of 9 pl/min of (Z)-3-hexen-1-ol, all 13 wasps that approached within 10 cm of the odor source landed, but at 45 nl/min, only 12 of the 25 wasps that approached within 10 cm landed.

Table 1 shows that wasps were better able to orient to the low release rates of certain GLVs when the wasps were released close to the odor source.

DISCUSSION

Our results demonstrate that female *Microplitis croceipes* wasps can detect and orient to each of the eight GLVs tested. However, individual GLVs varied in attractiveness. Six-carbon aldehydes were the least attractive, with no responses above 30%. Six-carbon alcohols were moderately attractive, with some doses eliciting up to 55% successful flights. Eight- to 10-carbon esters were the most attractive, with 60–70% successful flights at some doses.

Our results also suggest that wasps can orient to extremely low doses of certain GLVs; release rates of approximately 2 pl/min for (*E*)-2-hexen-1-ol, (*Z*)-3-hexenyl propionate, and (*Z*)-3-hexenyl butyrate were attractive to some wasps. The fact that some wasps oriented to these low doses suggests that even lower release rates might be attractive.

The low release rates to which *M. croceipes* responded are comparable to GLV release rates that occur when a caterpillar feeds upon a green leaf. For example, 3–5 pl/min of GLVs are released when a *Heliothis zea* caterpillar feeds upon a cowpea leaf (Whitman and Eller, 1990), suggesting that *M. croceipes* are capable of responding to the levels of GLV that would be produced when a caterpillar feeds on a green leaf in nature. Undamaged plants release much lower levels of GLVs (Dicke et al., 1990a; Whitman and Eller, 1990; Turlings et al., 1991a,b). This difference in GLV release rates may allow *M. croceipes* to distinguish between undamaged and caterpillar-attacked plants; wasps might only orient to the higher release rates that occur when a caterpillar feeds on a leaf.

There is additional evidence to suggest that *M. croceipes* might orient to even lower GLV release rates than tested here: The cross-sectional area of an odor plume increases and the concentration of volatiles in that plume decreases with distance from the odor source. In our tests, *M. croceipes* were released 100 cm downwind, where the concentration of test volatile was relatively low. When wasps were released much closer to the odor source (15 cm distant), where odor concentrations were greater, wasps oriented much better (Table 1). This is an important point because in the field *M. croceipes* hunt by flying slowly 5–10 cm downwind from plants. At such a close range, the wasps should be able to detect very low levels of GLVs, perhaps lower than we have tested here.

In our tests, we used individual GLVs, but in nature, a caterpillar feeding on a plant would produce a complex blend of volatiles (Whitman and Eller, 1990; Turlings et al., 1990). A survey of previous literature (Drost et al., 1986, 1988; Eller et al., 1988; Prevost and Lewis, 1990) shows that when *M. croceipes* wasps were tested in wind tunnels to the full complement of plant and caterpillar odors, 45–90% of the wasps made successful orientation flights. This compares favorably with the highest responses (60–70%) recorded from our trials using individual compounds. Because both the sensitivities and behavioral responses of animals to odor blends may exceed the sum of component thresholds and responses (Laska et al., 1990), we suspect that combinations of two or more individual GLVs might elicit greater responses from the wasps.

Information about the sensory and neural mechanisms of the wasps' response to GLVs can be derived from the various GLV dose–response curves. All eight curves were fairly similar in shape, suggesting a similar stimulus–response mechanism. Generally, the height of the curve was proportional to its length;

compounds that elicited high responses were also active over a greater range. For each GLV, there was usually one concentration that produced a maximum flight response. Above or below that release rate, the orientation rate dropped. The decreased flight responses that were observed at low release rates probably occurred because GLV concentrations were insufficient to reach sensory or neural thresholds. Decreases in flight response at high release rates were possibly due to stimulus overload; as wasps approached the odor source, the GLV concentration increased to a point where it became repellent. This was shown by the fact that, when exposed to high GLV release rates, wasps often oriented well when far from the odor source, but turned sharply away when within 5–20 cm of the source. It should be noted, however, that such high release rates are artificial and would seldom occur in nature.

When the dose–response curves are plotted on numerical, instead of semi-log scales, the left-hand sides of the curves are very steep, and the maximum responses lie close to the low dose thresholds, suggesting that the maximum responses of the wasps have evolved to occur near their low sensory thresholds.

The dose–response curves reported here (Figure 1) clearly show that the response of *M. croceipes* wasps to GLVs varies with the dose. This underscores the great importance of always examining dose effects when investigating semiochemical interactions. Failure to consider dose effects when comparing semiochemicals could easily lead to erroneous conclusions about their absolute or relative activities.

Many authors have suggested that carnivorous natural enemies use plant chemicals to locate their herbivorous hosts (Whitman, 1988a,b; Ding et al., 1989; Navasero and Elzen, 1989; Sheehan and Shelton, 1989; Dicke et al., 1990a; Martin et al., 1990; McAuslane et al., 1991; Turlings et al., 1991a,b). Others have hypothesized that certain plant volatiles may have evolved to serve a tritrophic communicative function (i.e., plants release natural enemy attractive substances in direct response to herbivore attack) (Whitman 1988a,b; Dicke and Sabelis, 1989; Dicke et al., 1990a,b; Turlings et al., 1990, Whitman and Eller, 1990). The release of GLVs is undoubtedly advantageous for *M. croceipes* wasps because it allows the wasps to locate hosts. If it is shown that plants benefit when their attendant herbivores are destroyed by natural enemies that respond to these GLVs, then these chemicals should be regarded as plant-to-parasitoid synomones.

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INHIBITORY EFFECTS OF TERPENE ALCOHOLS AND ALDEHYDES ON GROWTH OF GREEN ALGA *Chlorella pyrenoidosa*

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Abstract—The growth of the green alga *Chlorella pyrenoidosa* was inhibited by terpene alcohols and the terpene aldehyde citral. The strongest activity was shown by citral. Nerol, geraniol, and citronellol also showed pronounced activity. Strong inhibition was linked to acyclic terpenes containing a primary alcohol or aldehyde function. Inhibition appeared to be taking place through the vapor phase rather than by diffusion through the agar medium from the terpene-treated paper disks used in the system. Inhibition through agar diffusion was shown by certain aged samples of terpene hydrocarbons but not by recently purchased samples.

Key Words—*Chlorella pyrenoidosa*, alga, terpenes, growth inhibition.

INTRODUCTION

Terpene hydrocarbons, alcohols, aldehydes, and ketones have been reported to have antibacterial and antifungal activity (Morris, 1972; Smirnoff, 1972; Uzdennikov, 1972; Andrews et al., 1980; Mathela, 1981), and it has been suggested that terpenes in the diet may be responsible for the gut of the larvae of the Douglas fir tussock moth *Orgyia pseudotsugata* being relatively free of microflora (Andrews et al., 1980). Therefore, terpenes may have an ecological significance in certain situations involving microorganisms. Studies involving the effects of terpenes on microalgae are more limited. The use of terpene alcohols as anti-blue-green algal agents has been described in the European patent literature (Juettner, 1983).

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In previous studies we have described the pronounced inhibitory effects of fungi-produced toxins (mycotoxins) on the growth of the green alga *Chlorella pyrenoidosa* (Ikawa et al., 1969, 1979, 1985; Sullivan and Ikawa, 1972). Many of the more active of these mycotoxins are terpenoids, and terpene intermediates are involved in their biosynthesis. This paper reports on an extension of this work to the simple terpenes themselves.

METHODS AND MATERIALS

Paper disks (6 mm, Difco Laboratories, Detroit, Michigan) were dipped into test solutions in ethanol, excess solution removed by touching the disks to the edge of the container (ca. 20 μl of solution was retained on the disk), and the disks placed on the surface of light yellow-green *Chlorella*-seeded agar plates (100-mm-diameter Petri plates, three disks per plate) prepared as previously described (Ikawa et al., 1985). The plates were placed under a fluorescent light at room temperature and zones of inhibition read after two days under constant illumination.

RESULTS AND DISCUSSION

The terpene hydrocarbons showed little or no inhibition at a disk concentration of 10 mg/ml (Table 1). However, it was found that samples of some of the terpenes that had been collected from various laboratories on campus and that presumably had been on the shelf for some time did show zones of growth inhibition (Table 1, footnote *d*), indicating that the older samples had probably undergone chemical changes to form inhibitory substances. These results recall those that had been observed with chlorellin, the antibacterial and autotoxic substance produced by *Chlorella*, where it was shown that chlorellin activity was due to photooxidation products of unsaturated fatty acids present in *Chlorella* (Spoehr et al., 1949).

Most of the terpene alcohols and aldehydes tested showed inhibition that was evident as a lightening or total wipe out of color in the light yellow-green *Chlorella* lawn (Table 1). That inhibition was taking place through the vapor phase rather than by diffusion through the agar was shown by observing the same results when the paper disks were placed on slightly larger Teflon disks and thus were not in direct physical contact with the agar surface. The greatest inhibition was shown by nerol, geraniol, citronellol, and citral, all of which possess the acyclic terpene structure with a primary alcohol or aldehyde function (Figure 1). Citral appeared to be the most active, showing complete plate color wipe out with three disks at 1 mg/ml. The cyclic terpene alcohols borneol and isoborneol and terpenes carrying the alcohol function at a nonterminal position

TABLE 1. INHIBITION OF *Chlorella* GROWTH BY TERPENES

Compound	Source[a]	(Net diameter of inhibition zone) (mm)[b]		
		10 mg/ml[c]	1 mg/ml[c]	0.1 mg/ml[c]
Terpene hydrocarbons				
(+)-Limonene[d]	Aldrich	2	0	
Myrcene	Aldrich	Tr		
(+)-α-Pinene[d]	Aldrich	0		
(−)-α-Pinene	Aldrich	0		
(−)-β-Pinene[d]	Aldrich	0		
α-Terpinene	Aldrich	0		
γ-Terpinene	Aldrich	0		
Terpene alcohols				
(−)-Borneol	K & K	*[e]	0	
Isoborneol	K & K	*	0	
Cineole	Aldrich	0		
Citronellol	Aldrich	**[e]	0	
Geraniol	Aldrich	**	*	0
Linalool	Aldrich	*		
Nerol	K & K	**	*	0
Terpinen-4-ol	Aldrich	*		
α-Terpineol[d]	Aldrich	0		
Farnesol (isomers)	Aldrich	6	Tr.	
Hexenols				
cis-2-Hexen-1-ol	Aldrich	0		
trans-2-Hexen-1-ol	Aldrich	0		
cis-3-Hexen-1-ol (leaf alcohol)	Aldrich	0		
trans-3-Hexen-1-ol	Aldrich	0		
trans-4-Hexen-1-ol	Aldrich	0		
5-Hexen-1-ol	Aldrich	0		
Terpene aldehydes and ketones				
(+)-Camphor		0		
Citral	Aldrich	**	**	0

[a] Sources: Aldrich Chemical Co., Milwaukee, Wisconsin; K & K Laboratories, Plainview, New York.

[b] Disk diameter (6 mm) subtracted from the total diameter of the inhibition zone. Figures represent the mean of all disks (three per plate) run on two or more separate occasions. Standard deviations generally ran $< 10\%$ of the mean for means > 5 mm.

[c] Concentration of test solution in ethanol.

[d] Older shelf samples of these compounds that were collected from various laboratories on campus showed inhibition at 10 mg/ml.

[e] * indicates platewise lightening of the lawn color as compared to control plates. ** indicates complete wipe out of the yellow-green lawn color with three disks per plate. Similar results were obtained when disks were prevented from direct contact with the agar surface by interposing larger diameter Teflon disks between the paper disks and the agar surface.

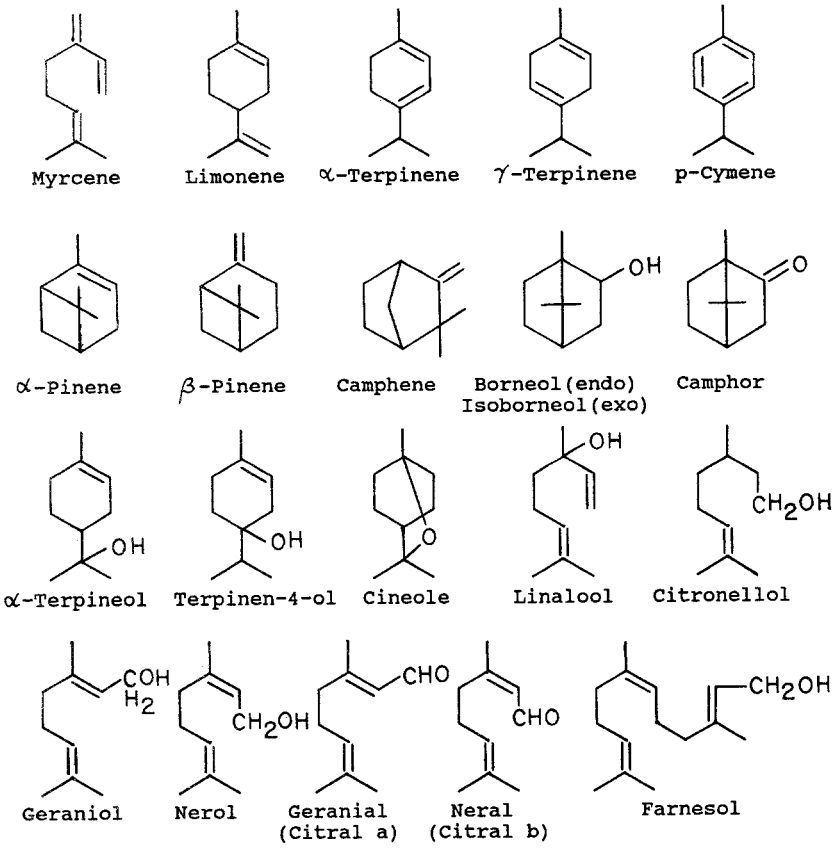

FIG. 1. Structures of terpenes tested against *Chlorella*.

(α-terpineol, terpinen-4-ol, linalool) showed less activity than the more active alcohols. The sesquiterpene alcohol farnesol showed some activity at 10 mg/ml through agar diffusion.

The leaf alcohol (*cis*-3-hexen-1-ol) and a series of related hexenols were also tested. As shown in Table 1, none of the compounds showed any activity at a concentration of 10 mg/ml.

In contrast to the activity of terpene hydrocarbons against bacteria and fungi, our results indicate that the hydrocarbons are not particularly toxic to the green alga *Chlorella*. Terpene alcohols showed activity, the highest activity being shown by acyclic compounds that carried the functional group on the terminal carbon atom. Acyclic terpene alcohols have been described as anti-blue-green algal agents, with farnesol being particularly active against *Synechococcus* (Juettner, 1983).

Lorber and Muller (1976) showed that when root tip cells of *Cucumis sativus* were exposed to the vapors of the volatile monoterpenes from the leaves of *Salvia leucophylla*, disruption of the membranes surrounding the nuclei, mitochondria, and dictyosomes took place. Disruption of the cytoplasmic membranes of *Bacillus thuringiensis* and *Saccharomyces cerevisiae* were also observed when these microorganisms were treated with α-pinene (Andrews et al., 1980). Lipophilicity appeared to be an important contributing factor in the inhibition of lettuce fruit germination by terpenes (Reynolds, 1987). In this case, the terpene alcohols, aldehydes, and ketones appeared to be active, while the more lipophilic hydrocarbons did not show inhibitory activity. In our case also, the inhibitory activity of the alcohols in contrast to the inactivity of the hydrocarbons indicated the need for a hydrophilic head and a lipophilic tail in a molecule for optimum activity. Although membrane effects may be the first actions of these compounds, these effects could, in turn, influence other cellular processes, such as respiration, photosynthesis, and protein synthesis. The trichothecenes, which are sesquiterpene alcohols, are strong inhibitors of protein synthesis (Sato and Ueno, 1977). Our previous results showed that there was good correlation between inhibition of protein synthesis and inhibition of *Chlorella* growth among the various trichothecenes (Ikawa et al., 1985). This suggests a possible link to processes beyond a primary disruption of membrane function.

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ANTENNAL OLFACTORY RESPONSIVENESS OF *Microplitis croceipes* (HYMENOPTERA: BRACONIDAE) TO COTTON PLANT VOLATILES

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Abstract—Antennal olfactory responses of the parasitoid, *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae), to 29 cotton volatile compounds were measured by electroantennogram (EAG) techniques. No significant sexual differences were found in EAGs of males and females to volatiles emanating from 100-μg stimulus loads of the 29 cotton compounds. Green leaf volatiles (saturated and monounsaturated six-carbon alcohols, aldehydes, and their acetate derivatives), heptanal, and the benzene derivatives, benzaldehyde and acetophenone, elicited the largest EAGs. Monoterpenes elicited moderate EAGs with β-ocimene being the most effective monoterpene tested. Among the sesquiterpenes tested, β-bisabolol was the most effective stimulus at the 100-μg dose. Dose–response curves constructed from EAGs of females revealed a low threshold for (*Z*)-3-hexenyl acetate, a compound previously shown to be an effective attractant in wind-tunnel bioassays. Comparison of relative volatilities of the various odorants indicated differential selectivity and sensitivity of *M. croceipes* antennal receptors to them. The roles of cotton plant volatiles in host habitat location of *M. croceipes* are discussed.

Key Words—*Microplitis croceipes*, Hymenoptera, Braconidae, chemoreceptors, cotton, electroantennogram, host habitat location, plant volatiles, parasitoid, EAG.

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INTRODUCTION

Microplitis croceipes (Cresson) (Hymenoptera: Braconidae) is a predominant parasitoid of two major cotton pests: the cotton bollworm [*Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae)] and the tobacco budworm [*Heliothis virescence* (F.)] in the United States (Powell and King, 1984). As an important biological control agent, *M. croceipes* has been studied extensively in recent years (Drost et al., 1986; Eller et al., 1988; Hopper and King, 1984; Lewis et al., 1988; Powell and King, 1984; Whitman and Eller, 1990). The success of the parasitic wasp in suppressing the pest population depends on its ability to locate hosts in a complex olfactory and visual environment. Understanding the mechanisms governing host-searching behavior by the wasp is critical to successful implementation of biological control programs.

Host-finding behavior in parasitoids is a hierarchy of behavioral events, which include host habitat location and host location (Vinson, 1981, 1984). Chemical cues are known to play a central role in each level of these behaviors. Electroantennograms (EAGs) to several plant volatiles by two parasitoids, *Campoletis sonorensis* (Ichneumonidae) (Baehrecke et al., 1989) and *Microplitis demolitor* (Ramachandran and Norris, 1991), have been previously reported. Numerous volatiles have been identified from the cotton plant (Chang et al., 1988; Hedin, 1976; Thompson et al., 1971). Some of these compounds play important roles in mediating host-searching behaviors in *Campoletis sonorensis* (Elzen et al., 1983, 1984; Baehrecke et al., 1989).

Behavioral responses of *M. croceipes* to volatiles from the plant–host complex have been studied by several workers (Drost et al., 1986; Eller et al., 1988; Whitman, 1988). Drost et al. (1986) showed that *M. croceipes* responded in a wind tunnel to odors by increasing the frequency of sustained and oriented flight. *M. croceipes* also showed a dose-dependent response to volatiles from its plant–host complex in an olfactometer (Eller et al., 1988). *M. croceipes* was also shown to orient in a flight tunnel to green leaf volatiles (Whitman, 1988; Whitman and Eller, 1990). No information is available on the nature of neurophysiological responses of *M. croceipes* to volatiles emanating from its plant–host complex.

The purpose of this study was to investigate neurophysiological responses of *M. croceipes* to cotton volatiles by means of electroantennograms (EAGs). Our results provide a basis for our future studies of single olfactory receptor neurons on the antenna.

METHODS AND MATERIALS

Insects. Microplitis croceipes used in this study were from a colony maintained at the USDA, ARS, Biological Control of Insects Research Lab, Columbia, Missouri. Pupae of the wasp were shipped overnight to the behavioral

physiology lab at the USDA, ARS, Boll Weevil Research Unit, Mississippi State, Mississippi, where they were kept in Petri dishes at room temperature (25 ± 1°C) until emergence. Newly emerged wasps were sexed and maintained in Petri dishes at room temperature and a light cycle of 16 hr light and 8 hr darkness. Adult wasps were provided water and honey until testing three to six days after emergence.

Odorous Stimuli. Compounds tested, their purities, and supply sources are listed in Table 1. These compounds were chosen based primarily on their presence in the cotton plant and/or in the green leaf volatile complex (Dickens, 1984). Compounds were diluted in spectrometric grade hexane, and tested for purity using a gas chromatograph (Tracor Instruments model 540, Austin, Texas). Serial dilutions of 0.001–100 $\mu g/\mu l$ were prepared for eight of the 29 compounds tested. All test solutions were kept at −4°C before and after each experiment. Solutions were allowed to reach room temperature before testing.

The odor delivery system and stimulation technique were the same as that described by Dickens (1984). Stimulus compounds diluted in hexane were delivered as 1-μl or 10-μl samples placed on No. 1 filter paper (7 × 16 mm) inserted into glass cartridges (80 mm long × 5 mm ID) and oriented toward the preparation from ca. 1.5 cm. Filtered and dried hydrocarbon-free air carried odor molecules evaporating from the filter paper over the preparation. Stimulus duration was 1.0 sec; air flow was 1 m/sec as measured by a thermistor. The atmosphere around the preparation was continuously exhausted.

Electrophysiological Recording Technique. Electroantennogram (=EAG) techniques used in this study were a modification of previous techniques and are described in detail elsewhere (Schneider, 1957; Dickens and Payne, 1977; Dickens, 1984). Ag–AgCl glass capillary microelectrodes were used. Following prepuncture with a sharpened tungsten needle, the recording electrode was inserted into the distal segment of the antenna. In the same manner, the indifferent electrode was inserted into the scape. The signal was amplified 10× by a Grass P-16 AC/DC microelectrode amplifier and viewed on a Tektronix 5111A storage oscilloscope. EAGs were recorded on graph paper by an Omniscribe recorder (Houston Instruments, Houston, Texas) for subsequent analyses and storage.

Experimental Procedure. Two series of experiments were conducted. In the first series of experiments, responses of the antennal receptors were measured by recording EAGs to 100-μg stimulus loads of each test odorant. In the second series of experiments, eight compounds were chosen based on results of the first series of experiments, and their function in host location of other parasitic wasps. Serial dilutions of each odorant were presented from the lowest to the highest dose. Because of the variation in volatility of the test compounds, only relative comparisons can be made between the stimuli.

In the first experimental series, EAGs were recorded from five wasps of

TABLE 1. SOURCE AND PURITY OF VOLATILE COMPOUNDS USED IN EAG STUDIES OF *M. croceipes*

Compound	Chemical purity (%)	Source of supply[a]
Aliphatic alcohols		
Propan-1-ol	>99	A
Butan-1-ol	>99	A
Pentan-1-ol	>99	B
Hexan-1-ol	98	C
(*E*)-2-Hexen-1-ol	97	C
(*Z*)-3-Hexen-1-ol	98	C
Heptan-1-ol	98	C
Octan-1-ol	>99	C
Nonan-1-ol	97	C
Decan-1-ol	>99	C
Aliphatic aldehydes		
Hexanal	99	C
(*E*)-2-Hexenal	99	C
Heptanal	95	C
Monoterpene hydrocarbons		
(−)-α-Pinene	98	C
(−)-β-Pinene	80–90	C
β-Ocimene	60	D
(−)-Limonene	97	C
Myrcene	85	C
Oxygenated monoterpenes		
Geraniol	>90	E
Nerol	>65	E
Linalool	99	C
Sesquiterpenes		
α-Bisabolol	92	F
β-Bisabolol	83	G
Caryophyllene oxide	97	C
β-Caryophyllene	90.2	F
Miscellaneous compounds		
(*Z*)-3-Hexenyl acetate	97	H
Acetophenone	99	C
Benzaldehyde	>98	C
Decane	>99	C
Undecane	99	C

[a] A, Fisher Scientific Co., Pittsburgh, Pennsylvania; B, J.T. Baker Chem. Co., Phillipsburg, New Jersey; C, Aldrich Chem. Co., Milwaukee, Wisconsin; D. Alfred Bader Lib. Rare Chem., Milwaukee, Wisconsin; E, Pfaltz & Bauer, Inc., Stamford, Connecticut; F, ICN-K&K Laboratories, Inc., Plainview, New York; G, P.A. Hedin, USDA, ARS, Mississippi State, Mississippi; H, Sigma Chemical Co., St. Louis, Missouri.

each sex for each test compound. Only female wasps were tested for the dose–response studies for each of eight chosen compounds. In both experiments, control stimulations (1 μl or 10 μl of hexane solvent on filter paper in a cartridge) were tested three to five times in each preparation. Hexan-1-ol (100 μg on filter paper) served as a standard and was tested following every two experimental stimuli. Response to the solvent control, if any, was deducted from all responses. Responses to the standard were used to normalize all responses so that the responses from different preparations could be compared. At least 3 min were allowed between each stimulation for recovery of the EAG.

EAGs to test compounds were evaluated by measuring the maximum amplitude of depolarization elicited by a stimulus, then subtracting the amplitude of the response to the preceding control. Millivolt responses to all stimuli were converted to percentage values of the mean responses to the two nearest standards (Dickens, 1984).

Threshold was defined as the stimulus load at which the mean EAG minus the standard error was greater than the mean EAG to the lowest stimulus load tested plus the standard error. Saturation was considered to be the stimulus load at which the mean EAG plus the standard error was greater than or equal to the mean EAG minus the standard error for the next highest dose tested.

Statistical Analyses. For the first series of experiments, EAGs to 100-μg doses of the various odorants were compared for significant differences using a general linear models procedure (SAS Institute, Cary, North Carolina). Dose–response curves were evaluated by analysis of variance. Where differences were noted, comparisons were made using Duncan's new multiple-range test.

RESULTS

Mean responses of the male [-3.53 mV (SE = ± 0.14 mV)] and female [-3.81 mV (SE = ± 0.13 mV)] *M. croceipes* to the 1-hexanol standard (100 μg) were not significantly different ($P < 0.05$).

Selectivity. Although no significant differences were found in responses of male and female wasps to the various odorants, EAGs to individual compounds did differ ($P < 0.05$) (Table 2). The most effective odorants were heptanal, benzenoids (i.e., benzaldehyde and acetophenone), and green leaf volatiles (GLVs). Responses of both sexes to primary alcohols with three to 10 carbons were maximal for the six-carbon alcohol (hexan-1-ol, a GLV), and declined as the carbon chain length increased or decreased (Figure 1A). All GLVs tested elicited strong responses in both male and female wasps, but EAGs for individual GLVs did not differ significantly ($P < 0.05$) (Figure 1B). Monoterpenes were less effective than GLVs and elicited moderate responses. Among the oxygenated monoterpenes tested, linalool elicited significantly higher responses

TABLE 2. MEAN EAGS OF FIVE MALE AND FIVE FEMALE *M. croceipes* TO 100-μg STIMULUS LOAD OF SELECTED COTTON VOLATILES AND OTHER COMPOUNDS[a]

Compound	Mean EAG[b]	I_k[c]
Heptanal	101.2 A	1040
Benzaldehyde	101.1 A	1172
Acetophenone	100.8 A	1270
(*E*)-2-Hexenal	97.0 A	965
Heptan-1-ol	94.8 A	[1080]
(*Z*)-3-Hexen-1-ol	86.0 A	990
(*E*)-2-Hexen-1-ol	85.6 AB	995
Hexanal	84.6 ABC	945
Octan-1-ol	71.2 BCD	[1180]
Pentan-1-ol	67.0 CDE	880
β-Bisabolol	62.3 DEF	1795
Decan-1-ol	57.2 DEFG	[1380]
β-Ocimene	56.3 DEFG	1210
Decane	55.8 DEFG	1000
Linalool	51.6 DEFGH	
Nonan-1-ol	48.5 EFGHI	[1280]
Undecane	47.2 EFGHI	[1100]
Butan-1-ol	44.9 FGHI	[780]
(−)-β-Pinene	44.0 FGHI	1105
Myrcene	41.9 GHI	1115
Geraniol	40.3 GHI	1425
(−)-Limonene	37.5 GHIJ	1168
Nerol	33.7 HIJ	1410
β-Caryophyllene	32.8 HIJ	1582
(−)-α-Pinene	30.3 IJ	1080
Propan-1-ol	18.9 J	[680]
α-Bisabolol	18.6 J	1765
Caryophyllene oxide	18.0 J	

[a] EAGs from males and females were combined since chemical * sex interaction was not significant. Numbers followed by different letters are significantly different ($P < 0.05$; Duncan's multiple-range test).
[b] EAGs are represented as percent response to the standard = hexan-1-ol (100 μg).
[c] Kovàts' indices on OV-17 column from Hedin et al. (1975). Values in brackets were extrapolated.

than either geraniol or nerol. β-Ocimene was the most effective monoterpene hydrocarbon with decreasing responses elicited by the remaining compounds of the same class: (−)-β-pinene > myrcene > (−)-limonene > (−)-α-pinene. With the exception of β-bisabolol, which elicited a moderate response, the sesquiterpenes were ineffective. Both of the alkanes tested, decane and undecane, elicited moderate responses that did not differ significantly.

Sensitivity. Dose–response curves were constructed from EAGs of female

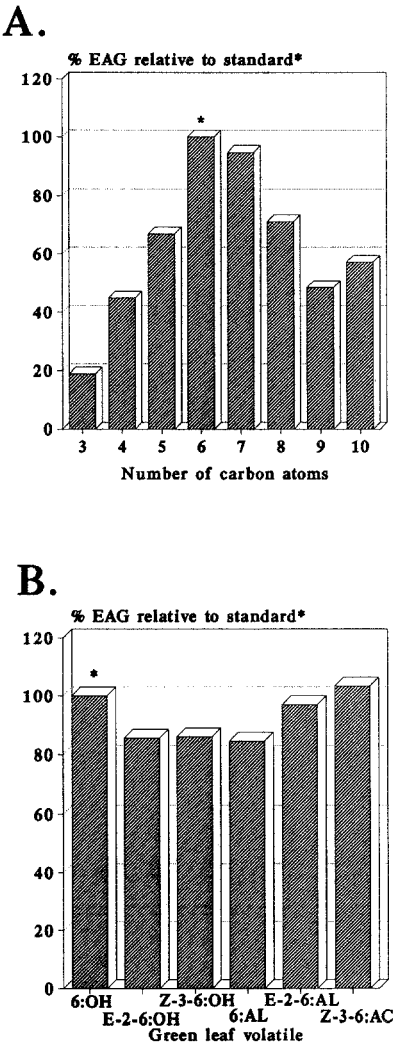

FIG. 1. Mean EAGs of *M. croceipes* (N = 10, five males and five females) to: (A) stimulus load of 100 μg of saturated primary alcohols with chain length from three to ten carbons; (B) stimulus load of 100 μg of green leaf volatile alcohols [hexan-1-ol = 6:OH, (*E*)-2-hexen-1-ol = *E*2-6:OH, (*Z*)-3-hexen-1-ol = *Z*3-6:OH), aldehydes (hexanal = 6:Al, (*E*)-2-hexenal = *E*2-6:Al], and acetate [(*Z*)-3-hexenyl acetate = *Z*3-6:Ac, N = 5 females for this compound]. See Table 2 for statistical analyses.

wasps to eight volatiles distributed among four chemical classes: monoterpenes, sesquiterpenes, green leaf volatiles, and benzene derivatives (Figure 2). Thresholds for compounds relative to those in the same class were: monoterpenes—β-ocimene (0.01 μg) > linalool (10 μg) > (−)-β-pinene (100 μg); sesquiterpenes—β-bisabolol (1 μg) = β-caryophyllene (1 μg); green leaf volatiles—(*Z*)-3-hexenyl acetate (0.01 μg) > (*E*)-2-hexenal (1 μg). Benzaldehyde had a threshold of 1.0 μg. Only β-caryophyllene clearly reached saturation at a stimulus load of 10 μg.

DISCUSSION

Characteristics of Electroantennograms. No significant sexual differences were found in EAGs to 100-μg stimulus loads among the 29 compounds tested. Similarity in antennal responses of male and female insects to plant odors was previously noted for several phytophagous pests (Fein et al., 1982; Dickens, 1984; Wellso et al., 1984; Light et al., 1988; Hansson et al., 1989). The explanation for such similarity was that males and females live in the same habitat and may share the same chemical cues to locate host plants on which to feed and mate. In contrast to phytophagous insects, female parasitic wasps use plant volatiles to locate host insects, while male wasps probably share some of the same chemical cues, possibly in conjunction with a sex pheromone (Powell and King, 1984), to locate females for mating. Differences in behavioral responses of male and female wasps to green leaf volatiles (Whitman, 1988; Whitman and Eller, 1990) may arise from sexual differences in higher-order processing of incoming peripheral olfactory information.

Both the differential EAGs recorded to 100-μg stimulus loads of the various odorants (Table 2) and the differing dose–response curves to selected odorants recorded from females (Figure 2) indicate differential selectivity and sensitivity of the antennal olfactory receptor system of *M. croceipes* for host habitat odors. Clearly the number of molecules emanating from the filter paper in the glass odor cartridge, and ultimately reaching the preparation, differs for the various odorants tested due to differing volatilities of the compounds and binding characteristics of the compounds to the filter paper. Thus, what is being compared is stimulus loading and not the number of molecules reaching the preparation. However, the observed differences in EAGs cannot be ascribed solely to differences in volatility of the various compounds. This point is illustrated by comparing Kovàts' retention indices (a measure of relative volatility) (Kovàts, 1961) for the various odorants with observed thresholds of neurons responsive to them (Table 2). Kovàts' indices previously calculated for the compounds, and threshold stimulus loads (μg) were: β-ocimene = 1210, 0.01; β-bisabolol = 1795, 1.0; β-caryophyllene = 1582, 1.0; (*E*)-2-hexenal = 965, 1.0; benzaldehyde =

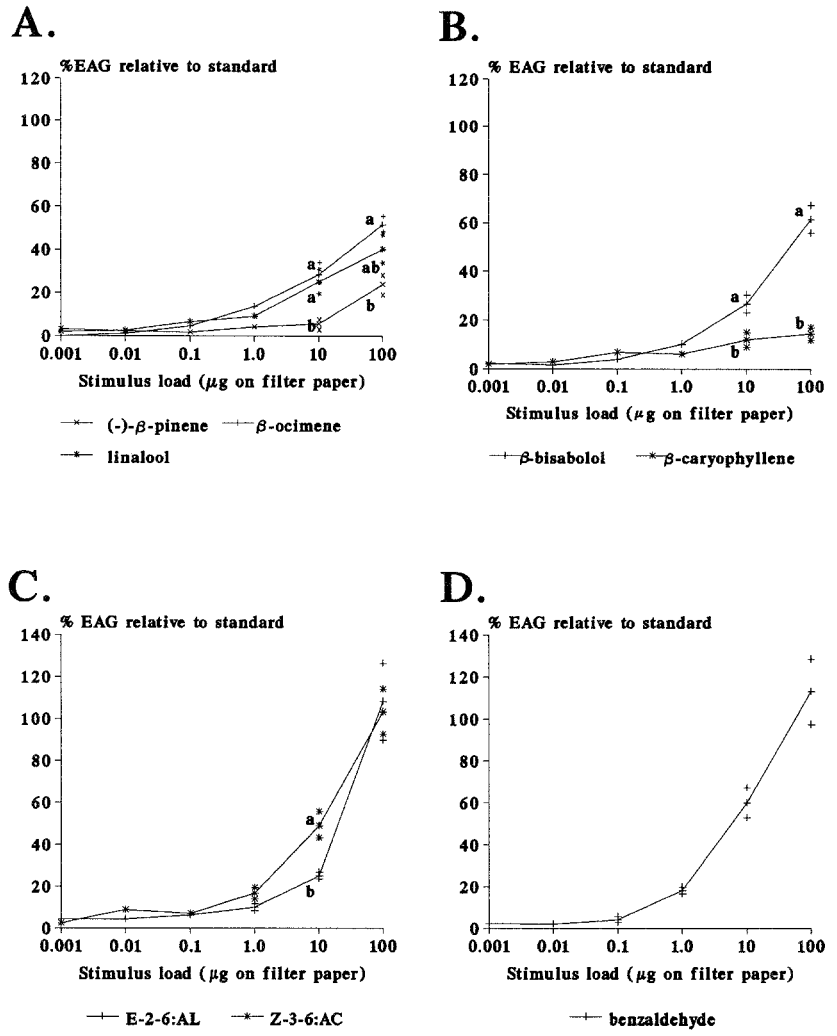

FIG. 2. Dose–response curves constructed from EAGs of female *M. croceipes* ($N = 5$) to serial stimulus loads of: (A) (−)-β-pinene, β-ocimene, and linalool; (B) β-bisabolol and β-caryophyllene; (C) (*E*)-2-hexenal and (*Z*)-3-hexenyl acetate; and (D) benzaldehyde. EAGs to different chemicals at the same dose in the same graph with different letters are significantly different ($P < 0.05$; Duncan's multiple-range test). Points above and below means represent standard errors.

1172, 1.0; and β-pinene = 1105, 100 (OV-17 column; Hedin et al., 1975). Kovàts' indices were unavailable for linalool (threshold = 0.1 μg), and (*Z*)-3-hexenyl acetate (threshold = 0.01 μg). However, geraniol, a monoterpene alcohol closely related to linalool, had a Kovàts' index = 1425 (Hedin et al., 1975). (*Z*)-3-Hexenyl acetate should be considerably less volatile than (*E*)-2-hexenal based on boiling points of compounds that are similarly related, for example, hexyl acetate (bp = 168°C) and hexanal (bp = 131°C) (boiling points from Windholz, 1983). EAGs of *M. croceipes* to alcohols and aldehydes reported here are similar to those reported previously for *M. demolitor* (Ramachandran and Norris, 1991).

Behavioral Correlations. Detection of host volatiles by phytophagous insects and their possible role in insect behavior have been the subjects of numerous studies. The most effective volatiles and the specific receptors involved in their detection have been identified in some insects by EAG and single-neuron recording techniques as well as behavioral studies (Dickens and Payne, 1977; Dickens, 1984, 1990a,b; Dickens et al., 1984; Dickens and Boldt, 1985; Guerin and Visser, 1980; Visser, 1979, 1986). In contrast, most work done on parasitic wasps is at the behavioral level (Whitman, 1988). Host location and acceptance by parasitic wasps were believed to be mediated by chemical stimuli emitting from the host and its plant (Vet et al., 1983; Vinson, 1984; Bouchard and Cloutier, 1985). For example, several sesquiterpenes, including β-bisabolol and β-caryophyllene, were identified as compounds released from cotton, which elicited behavioral responses from the parasitic ichneumonid, *Campoletis sonorensis* (Elzen et al., 1983, 1984). β-Bisabolol, which constitutes 50–60% of the volatiles emitted by maturing cotton plants (Thompson et al., 1971), elicited significantly larger EAGs in *M. croceipes* in our current study than any other sesquiterpene tested. Relatively large EAGs were generated by β-bisabolol in the boll weevil, a cotton-feeding insect, which has neurons selectively responsive to this sesquiterpene (Dickens, 1990a).

The importance of plant chemicals in host finding has been proven for several parasitic wasps including *M. croceipes* (Eller et al., 1988; Whitman, 1988; Ding et al., 1989; Navasero and Elzen, 1989). Large EAGs of *M. croceipes* females to green leaf volatiles correlate well with the previously reported behavioral studies indicating their attractiveness (Whitman, 1988; Whitman and Eller, 1990). (*Z*)-3-Hexenyl acetate was among the most effective odorants among the cotton volatile compounds tested in our current study. Acetate analogs of green leaf volatiles were among the most effective volatiles eliciting flight behavior in female *M. croceipes* (Whitman, 1988; Whitman and Eller, 1990). Insect predators and parasitoids are more attracted to damaged plants than undamaged plants (Sato, 1979; Elzen et al., 1983; Whitman, 1988; Dicke and Sabelis, 1989; Whitman and Eller, 1990). Corn seedlings release large amounts of terpenoid volatiles and (*Z*)-3-hexenyl acetate after being fed upon by cater-

pillars (Turlings et al., 1990). Both caterpillar-damaged and artificially damaged plants emit GLVs that are attractive to female *M. croceipes* (Whitman and Eller, 1990). Thus results of chemical analyses, behavioral studies, and our current neurophysiological study correlate well with reported female behavior and are indicative of chemical communication among the parasitoid, plant, and insect host. Green leaf volatiles also elicited large EAGs in *C. sonorensis*, another parasitoid associated with cotton insects (Baehrecke et al., 1989).

Similarity between parasitoids and host herbivores in responding to some host plant volatiles has been reported in several plant–herbivore–parasitoid systems (Salkeld, 1959; Camors and Payne, 1972; Guerin and Visser, 1980; Ramachandran and Norris, 1991). EAGs in response to cotton volatiles have not yet been reported for either *Heliothis virescens* or *Helicoverpa zea*, both hosts of *M. croceipes*. We suspect these hosts may use some of the same plant volatiles used by *M. croceipes* for locating host plants. In fact, one of us recently found that (Z)-3-hexenyl acetate, an attractant (Whitman, 1988; Whitman and Eller, 1990) for *M. croceipes*, and an effective EAG stimulus as reported herein, enhances the sex attractant pheromone of a host, *H. virescens* (Dickens et al., 1992).

While plant volatiles may be maladaptive in the sense of attracting herbivores, evolution of tritrophic chemical communication between plants and parasitoids is highly adaptive. More information about the olfactory responsiveness of *H. virescens* and *H. zea* is needed in order to better understand tritrophic communication among *M. croceipes*, its lepidopterous hosts, and host plant.

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NAIVE OPHIOPHAGUS LIZARDS RECOGNIZE AND AVOID VENOMOUS SNAKES USING CHEMICAL CUES

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Abstract—Monitor lizards prey on snakes. Conversely, venomous snakes prey on juvenile monitor lizards. Immediately after hatching, monitor lizards are naive to all prey items, thus correct assessment of snake prey is paramount for survival. Experiments were conducted to determine how hatchling monitor lizards (*Varanus albigularis*) with no previous exposure to snakes reacted to sympatric venomous and nonvenomous snakes. Hatchling lizards attacked harmless snakes, but avoided venomous species. Lizards readily accepted meat from skinned snakes, regardless of species. When invertebrate prey covered with skin segments from venomous snakes were restrained from moving, they were usually investigated by tongue-flicking and rejected. Unrestrained skin-covered prey, however, were generally attacked and eaten without prior evaluation by tongue-flicking. Attack was inhibited in trials in which unrestrained prey were tongue-flicked, suggesting that chemical cues contained in snake skins mediate avoidance of venomous snakes. Selection for the ability to perceive snake integumental chemicals may be especially strong in species that both consume and are consumed by snakes.

Key Words—Chemical cues, *Varanus*, snakes, lizards, monitor lizard.

INTRODUCTION

A variety of vertebrate prey react to the scent products of snakes (Weldon and Burghardt, 1979; Weldon, 1991), and certain prey use chemoreception to discriminate snakes that are their predators from those that are harmless (Weldon, 1982; Dial et al., 1989). The chemical senses also aid some predators in their

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identification of snake prey (Weldon and Schell, 1984). However, the responses by a species to chemical cues from organisms that are potentially either their predators or their prey have not been examined systematically.

As a genus, monitor lizards consume a variety of prey (King and Green, 1979; Losos and Greene, 1988) and chemoreception is extremely important in their foraging ecology (Auffenberg, 1978; Pianka, 1986; Cooper, 1989). Although invertebrates account for the majority of the natural diet, a substantial percentage of the annual caloric intake is obtained from vertebrate prey, including snakes (Shine, 1986; Branch, 1991). Conversely, as juveniles, many species of monitors are preyed upon by snakes (Shine, 1989; Shine and Slip, 1990).

The white-throated savanna monitor, *Varanus albigularis*, is a large (>5 kg) lizard that inhabits a variety of southern African habitats, including the arid savanna. In this environment, dramatic increases in vegetational growth and animal populations occur during the rainy season (Berry and Louw, 1982). As with most reptiles, hatching of snakes and monitor lizards occurs midway through the wet season (Branch, 1988). Growth of recently hatched snakes and lizards during this period is rapid, with most species amassing substantial fat deposits required for survival during the subsequent eight months of drought. Juvenile lizards and snakes are favored prey items of numerous species of birds and mammals, and lizards and snakes also prey upon one another (Branch, 1988).

Snakes that eat lizards and those that do not are likely to be visually similar at a distance, thus necessitating close-range investigation by lizards attempting to distinguish them. Because snakes are generally formidable predators, any misclassification of a predator species as prey is potentially very costly. In this study, we demonstrate the chemical discrimination of snake predators from snake prey by naive white-throated savanna monitor hatchlings.

METHODS AND MATERIALS

Five gravid female white-throated savanna monitors were caught and housed in a 50-m^2 outdoor enclosure at the Etosha Ecology Institute, Etosha National Park, in north-central Namibia. Oviposition occurred within two weeks of capture, and eggs were artificially incubated. The females were released at the site of their capture. Twenty-seven of 120 hatchlings (five or six hatchlings from each clutch) were retained in captivity; the remaining hatchlings were released in the area where their mother had been caught.

After the umbilical scar had completely sealed, the retained hatchlings were released in the outdoor enclosure. The hatchlings were exposed to human contact for 2 hr each day, and exhibited no discernible aggressive or defensive behavior to human presence in the enclosure. Hatchlings were offered daily a variety of invertebrates (grasshoppers, crickets, land snails, centipedes, scorpions) that are

known to be common prey of this species of monitor lizard (W.R. Branch, personal communication; J.A. Phillips, unpublished data). Prey items were offered with forceps individually or were placed in the enclosure en masse. Water was available ad libitum. Hatchlings were fed until satiated. All animal care was in compliance with the guidelines of the Zoological Society of San Diego Animal Care and Use Committee.

Experiment 1. At six weeks of age each hatchling (total length 220–250 mm) was offered unrestrained grasshoppers, and land snails, grasshoppers, and corn crickets restrained by forceps, on each of two occasions. Trials were begun between 1300 and 1400 hr, at which time the lizards had been actively searching for food for several hours. The sequence of tongue-flicking, aggressive and/or defensive postures, and attack and/or retreat were videotaped and analyzed for each prey item.

At seven weeks of age, hatchlings were assigned to one of three groups (nine hatchlings per group). Members of each group were individually exposed to either a whole fresh (less than 2 h old) road-killed sand snake (*Psammophis leightoni*), road-killed spitting cobra (*Naja nigricollis*), or road-killed horned adder (*Bitis caudalis*) at two-day intervals. The order of presentation of the three snakes was randomized among hatchling groups, and a total of nine different snake carcasses (three from each species) were used in the presentations. The behavioral responses of individual hatchling lizards to the road-killed snakes were videotaped and analyzed as above.

Experiment 2. At eight weeks of age, the hatchlings were assigned to one of five groups, with one to two hatchlings from each clutch represented in each group (numbers of hatchlings in each of the five groups were 5, 5, 5, 6, 6). Each member of the five groups of hatchlings was tested individually for its response to fresh road-killed sand snakes, spitting cobras, and horned adders under five treatment conditions: (I) whole snake, (II) 50-mm intact snake sections, (III) 50-mm sections of snake with the skin removed, (IV) live grasshopper covered with a coat of snake skin restrained from jumping by a pair of forceps (Figure 1), and (V) live unrestrained grasshopper covered with a coat of snake skin. The snakes used in the experiment were 700–930 mm total length. Treatments were presented to the five hatchling groups in a Latin-square design, thus the order of exposure to the five treatments differed for each group of hatchlings. Each of the three snake species was tested under each of the five treatment conditions in random order at 72- to 96-h intervals. Trials were begun between 1300 and 1400 hr.

Statistics. Repeated measures analysis of variance (ANOVA) was used to examine variation in the rate of tongue-flicking in response to invertebrate prey and venomous and nonvenomous snakes in experiment 1. In experiment 2, Latin-square ANOVA was used to compare the percentage (arcsine square-root transformed) of hatchlings in each of the five groups that attacked snakes under

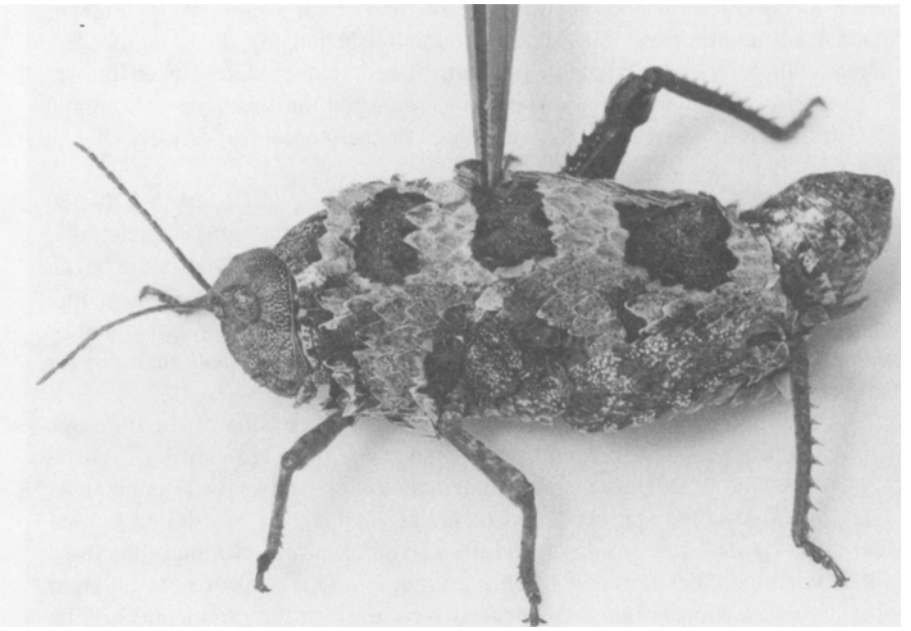

FIG. 1. Pamphagid grasshopper (approximately 50-mm body length) covered with a coat of skin from a horned adder.

the five treatment conditions. Differences in response to each of the three snake species were assessed using Fisher's protected least significant difference tests (Carmer and Swanson, 1973).

RESULTS AND DISCUSSION

In experiment 1, hatchlings exhibited tongue-flicking, a behavior that probably delivers odorants to the vomeronasal organ (Halpern, 1987), when exposed to invertebrate prey (Table 1). In most cases, two to four tongue-flicks were emitted before a prey item was attacked. However, most attacks on unrestrained grasshoppers occurred in the absence of tongue-flicking. Tongue-flicking rates were significantly lower in response to unrestrained moving grasshoppers than to the other invertebrate prey items presented ($F = 132.48$; $df = 3,53$; $P = 0.0001$), suggesting that visual cues may be more important than chemical cues in eliciting attack of moving prey. In no instance were aggressive or defensive behaviors observed in response to live invertebrate prey.

Sand snakes do not eat monitor lizards but are relatively common prey

TABLE 1. RESPONSES OF 27 HATCHLING SAVANNA MONITORS TO INVERTEBRATES AND WHOLE ROAD-KILLED SNAKES[a]

	Mean tongue flicks	Percent attack	Percent of hatchlings exhibiting defensive/ aggressive behaviors			
			Lateral	Hiss	Slap	Vent
Invertebrates						
Land snail (54)	4.33	100	0	0	0	0
Corn cricket (54)	4.35	100	0	0	0	0
Restrained grasshopper (54)	3.94	100	0	0	0	0
Unrestrained grasshopper (54)	0.18	100	0	0	0	0
Snakes						
Sand snake (27)	4.27	100	0	0	0	0
Spitting cobra (27)	4.84	22	100	93	52	100
Horned adder (27)	4.55	11	100	100	81	0

[a]Sample size in parentheses. Each invertebrate prey type was offered on two different occasions. Lateral compression of the body (lateral), hissing vocalizations (hiss), and tail slap (slap) are aggressive displays, whereas vent dragging (vent) is a territorial behavior.

items of adult savanna monitors. The stomach contents of 18 road-killed savanna monitors revealed two sand snakes, among other prey, accounting for about 10% of the total caloric value of the stomach contents (J.A. Phillips, personal observation). Sand snakes were universally attacked by hatchlings in a manner similar to invertebrate prey (Table 1). After initial attack, the hatchlings exhibited no hesitation in attempts to consume the sand snakes. No aggressive or defensive behaviors were noted in response to sand snakes.

The spitting cobra and the horned adder are highly venomous snakes that eat hatchling monitor lizards (Branch, 1988). Similar to other potential prey items, tongue-flicking by hatchling lizards occurred immediately after exposure to these two species. There was no difference in the rate of tongue-flicking in response to the three species of snakes ($F = 1.16$; $df = 2,78$; $P = 0.32$). However, in only nine of 54 cases was attack of venomous snakes observed (Table 1), and in no instance did the hatchlings attempt to consume venomous snake carcasses.

Regardless of whether snakes were attacked, all hatchlings exhibited one or more defensive and/or aggressive behaviors toward the venomous snakes (Table 1). The behavioral responses exhibited by hatchlings were specific to each of the two venomous snake species. In particular, posterior adpression (vent dragging), apparent in every encounter with the spitting cobra, was never elicited when hatchlings were exposed to horned adders. Hatchling savanna

monitors defecate at latrine sites, and when exiting these sites, often perform vent dragging (J.A. Phillips, personal observation). When other hatchlings investigate areas where vent dragging has occurred, aggressive posturing is frequently observed, suggesting that vent dragging may function in territorial marking.

In experiment 2, the hatchlings responded to whole bodies of the three snake species in a manner similar to that of experiment 1. Latin-square ANOVA revealed no effects of order of presentation ($F = 1.001$, $df = 4,12$, $P > 0.50$), no differences in response among the five hatchling groups ($F = 1.001$, $df = 4,12$, $P > 0.50$), and no differences in response across the five treatments ($F = 1.001$, $df = 4,12$, $P > 0.50$) for sand snakes (Figure 2). These results contrasted with those for the two venomous snake species. For spitting cobras, there were no effects of order of presentation ($F = 0.56$, $df = 4,12$, $P > 0.50$), no differences among hatchling groups ($F = 0.28$, $df = 4,12$, $P > 0.50$), but significant differences across treatments ($F = 6.84$, $df = 4,12$, $P < 0.01$). For horned adders, there were also no effects of order of presentation ($F = 1.62$, $df = 4,12$, $P > 0.20$), no differences among hatchling groups ($F = 0.78$ $df = 4, 12$, $P > 0.50$), but significant differences across treatments ($F = 48.10$, $df = 4,12$, $P < 0.001$).

For spitting cobras and horned adders, Fisher's protected least significant difference tests revealed that hatchlings were significantly more likely to attack skinned body sections and skin-covered unrestrained grasshoppers than whole snakes, sectioned snakes, or skin-covered restrained grasshoppers (Figure 2). Hatchlings avoided sectioned snakes and skin-covered restrained grasshoppers

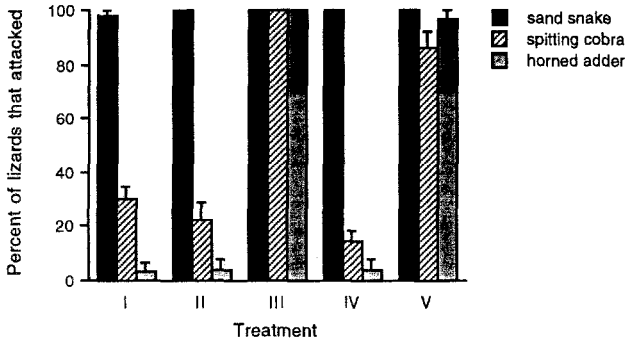

FIG. 2. Mean percentage (±SE) of hatchling savanna monitors in five groups that attacked (I) whole road-killed snakes, (II) snake sections, (III) skinned snake sections, (IV) a restrained grasshopper covered with a segment of snake skin, or (V) an unrestrained grasshopper covered with a segment of snake skin. Three snake species were presented, the sand snake, and two highly venomous species, the spitting cobra and the horned adder.

at a rate similar to that for whole snakes. Hatchlings attacked unrestrained grasshoppers covered with skin from either spitting cobras or horned adders in 49 of 54 trails. The five hatchlings that rejected skin-covered grasshoppers exhibited tongue-flicking, whereas all hatchlings that attacked grasshoppers did so without tongue-flicking. This indicates that chemical, rather than visual, cues associated with snake skins mediate avoidance of venomous snakes.

Past studies with scincid lizards have shown higher levels of tongue-flicking toward snakes that are their predators than those that are not (Cooper, 1990). Skinks are insectivorous, thus harmless snakes represent neither predator nor prey and are not expected to elicit a strong response. The lack of a difference in tongue-flicking rate to the three snake species in the present study probably reflects the fact that all of the snake species tested interact with savanna monitors. Although spitting cobras and horned adders are predators and sand snakes are prey, predator detection and foraging are both ecological contexts in which tongue-flicking occurs (Burghardt, 1970; Halpern and Kubie, 1983; Simon, 1983; Duvall et al., 1990).

Naive hatchling savanna monitors not only differentiated venomous from nonvenomous snakes in their propensity to attack them, they also reacted differently toward the two species of venomous snakes. Horned adders (Viperidae) and spitting cobras (Elapidae) possess divergent hunting strategies and chemically distinctive venoms (Visser, 1967). For these reasons, it is not unexpected that responses by their prey will differ. The tendency for some hatchlings to attack cobras and all to perform vent dragging behavior, which may be associated with aggression in savanna monitors, suggests that cobras may be less dangerous than adders, at least to these lizards. The long, stiff fangs of adders, capable of inflicting a deeper and more serious bite than those of cobras, may partially explain the differential treatment of the two predatory snake species.

The rejection of invertebrate prey bearing skins of venomous snakes and the indiscriminant acceptance of skinned snake meat suggest that savanna monitor hatchlings use skin-borne chemicals to identify snakes. That lizards having no previous experience with snakes exhibit these responses indicates that they are innate. Because snakes represent both predators and prey for savanna monitors, the ability to discriminate among them potentially functions in two different adaptive contexts. Foraging efficiency is increased when appropriate prey items are identified prior to attack, and survivorship is enhanced when potential predators are detected and avoided with minimal physical contact.

Previous experiments suggest that the movement of prey is important in the feeding behavior of reptiles (Drummond, 1985). Chemical cues also stimulate feeding behavior in snakes, and naive hatchlings recognize and attack their species-typical prey on the basis of chemical cues (Burghardt, 1966, 1968). Our results for monitor lizards indicate that visual cues associated with prey movement can override chemical cues. These visual responses may be learned, as

the tested lizards had been fed invertebrate prey since hatching. A hierarchy of cues involving multiple sensory modalities and both learned and unlearned behavior is probably used by lizards in the recognition of sympatric snake species and assessment of their relative suitability as prey.

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ALLELOPATHY OF *Sasa cernua*

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Abstract—Sasa (*Sasa cernua* Makino) is a very serious weed pest. Its allelopathy was studied using lettuce, wheat, timothy, and green amaranth as testing species. Cultured in the rhizosphere soil of *Sasa cernua*, the seedlings were inhibited by 42–80% compared with the controls cultured in normal soil and vermiculite. The phenolic fraction extracted with 1 M NaOH from the rhizosphere soil of *S. cernua* caused significant inhibitions on the seed germination and seedling growth of lettuce, timothy, green amaranth, and barnyard grass. *p*-Coumaric, ferulic, vanillic, and *p*-hydroxybenzoic acids and *p*-hydroxybenzaldehyde were identified as the main allelochemicals in sasa soil by HPLC and [^{1}H]NMR. Their contents in the rhizosphere soil were 5640, 1060, 860, 810 and 630 μg/100 g soil. The neutral fraction inhibited the seed germination and seedling growth of lettuce in the TLC direct bioassay. Volatile compounds released from sasa leaves also inhibited the growth of lettuce, wheat, timothy, and green amaranth grown under light, and the growth of etiolated seedlings of barley and wheat. These results confirm that *S. cernua* produces typical allelopathy through its rhizosphere soil and air space.

Key Words—Allelopathy, allelochemicals, growth inhibitors, rhizosphere soil, sasa, *Sasa cernua*, volatile compounds, phenolic compounds.

INTRODUCTION

Sasa plant is a very serious weed pest distributed widely in Japan and some other regions of Asia and Europe. In Japan, over 4300 kilohectares (kha) are

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covered with sasa. Many mountains, called "sasa mountains," are overgrown with sasa. In Hokkaido, sasa can be seen everywhere, and it covers over 3360 kha, constituting over 60% of the total forested area (Kawahara, 1979). The aggressiveness of sasa has led to serious problems in forestry and agriculture. When trees are cut, sasa grows rapidly and has made reforestation difficult.

Many studies have been made on the growth, distribution, and chemical components of sasa (Chuyen and Kato, 1982; Chuyen et al., 1982; Kawahara, 1979; Kazumori and Sakakibara, 1977; Ponomareva, 1971; Shibata et al., 1976; Suzuki et al., 1968). However, the allelopathic effects of the *Sasa* spp. have not been reported. Although in many places competing grasses grow poorly in the vicinity of a sasa community, they grow vigorously away from it, suggesting that sasa has allelopathic effects. Recently, there have been increasing reports on allelochemicals among plants (Chou and Waller, 1989; Putnam and Tang, 1986; Rice, 1984; Waller, 1987). Allelochemicals have numerous impacts on cultivated and natural ecosystems. In allelopathy between plants, allelochemicals must be transmitted from donor (emitting) plant to acceptor (affected) plant through either their rhizosphere soil or air space (volatile components).

Sasa cernua is one of the three main species of the *Sasa* genus in the Sapporo area. In this paper, the allelopathic effects of the rhizosphere soil and the volatile components of *S. cernua* were examined, the main allelochemicals in the rhizosphere soil were identified, and their possible roles discussed.

METHODS AND MATERIALS

Plant Culture Tests with Soil. Plant culture tests with soil were done using a plastic culture box set containing two 10 × 10 × 6 cm boxes. The lower box contained nutrient solution and the upper contained soil. A piece of gauze was used in each set to transport the nutrient solution in the lower box to the upper soil box by capillary force. This apparatus allowed easy replacement of the nutrient solution. Samples of rhizosphere soil (5- to 15-cm layer from soil surface) were collected in September 1990 under the pure stands of wild *S. cernua*. Two controls were used: control I was vermiculite and control II consisted of soils taken from the vicinity of the sasa community with no sasa growth or residue. Two-day-old etiolated seedlings of lettuce (*Lactuca scariola* L. var. *sativa* Bisch), wheat (*Triticum aestivum* cv. Norin No. 59), timothy (*Phleum pratense* L.), and green amaranth (*Amaranthus viridis* L.) were transplanted to the culture boxes and grown in a phytotron for 15 or 30 days, under a 14-hr light (white fluorescence, 3.8×10^3 ergs/cm^2/sec, 28°C) and 10-hr dark (20°C) cycle. Distilled water was used for irrigation during the first week and Hoagland's solution thereafter. Ten plants for each testing species were used. Each test was run in triplicate.

Volatile Components from Leaves of S. cernua. Ten germinated seedlings of each test species were planted in vermiculite in the culture boxes described above. They were placed into airtight glass containers (50 × 25 × 30 cm) with (treatment) or without (control) dried sasa leaves (dried in air at room temperature for two days, 500 g for each box). The containers were maintained in a greenhouse at 25°C with a photoperiod of 14 hr (2.0×10^3 ergs/cm^2/sec of white fluorescent light). The sasa leaves in the glass container were changed every three days. After one month, the fresh and dry weight of the plants were measured. For tests on etiolated seedlings, Petri dishes (33 mm diameter) with 15 seeds were put in the airtight glass box with (treatment) or without (control) sasa leaves for one day to allow air saturation with the volatile components, and then water was added to the dishes to let the seed germinate and grow in the dark at 25°C. The lengths of roots and aerial parts were measured after one week. Each test was repeated at least two times.

Extraction of Active Substances from Soil. The rhizosphere soil of *S. cernua* was dried in a desiccator at room temperature. The extraction procedure is shown in Figure 1.

Seed Germination Tests. Seed germination tests were done on Tokyo No. 1 filter paper in 33-mm-diameter Petri dishes with 1 ml test solution or control containing 100 ppm Tween 80. Thirty seeds were added to each dish and germinated in the dark at 25°C. Germinated seeds were counted after a certain number of hours according to plant species (see Table 1). Each test was repeated at least three times.

Seedling Growth Tests. For all the seedling growth tests, seeds were sterilized with 1% sodium hypochlorite for 5 min and washed with distilled water. Germination was carried out on filter paper moistened with distilled water in a plastic tray in dark at 25°C for 36 hr (lettuce), 48 hr (timothy, green amaranth), or 72 hr (barnyard grass, *Echinochloa crus-galli* Beauv var. *crus-galli*). Uniform seedlings with ca. 3-mm hypocotyls or coleoptiles and 5-mm roots were selected. All the growth tests were done in 33-mm-diameter Petri dishes with 1 ml test solution. For each test, five seedlings were used. The seedlings were cultured for 48 hr in the dark at 25°C. The lengths of roots and hypocotyls or coleoptiles were measured. Each test was repeated three times.

Direct Bioassay on TLC Plate. One 10-mg sample was applied on a TLC plate (6.6 × 20 cm with 0.25 mm thickness silica gel) and developed to 15 cm from the original with toluene–ethyl acetate–methanol, 5:3:2, v/v. The thickness of the TLC plate could be changed from 0.25 to 2.0 mm according to the amount of the sample size. After evaporation of the solvents in a desiccator overnight, a 3-mm-thick 0.8% agar layer was put on the TLC plate. The plates were kept in a culture box saturated with water for one night to allow the compounds to diffuse into the agar. Seeds were then sowed on the agar plate in lines and the plates placed in an airtight container and cultured in the greenhouse

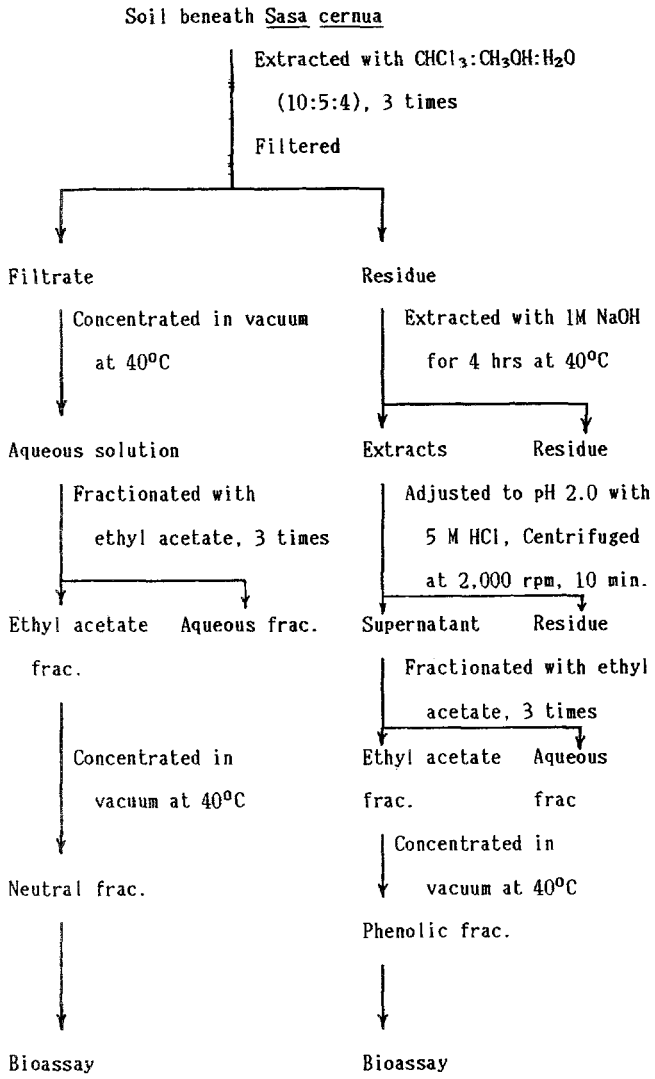

FIG. 1. Extraction procedure of allelochemicals from the rhizosphere soil of *Sasa cernua*.

at 25°C. Seed germination and seedling growth were recorded after two and five days, respectively.

Isolation and Identification of Allelochemicals in Rhizosphere Soil. The phenolic fraction was separated by HPLC (YMC-packed ODS column 10 × 300 mm) with UV detection at 254 nm. The sample was eluted with A (H_2O–

acetonitrile–acetic acid, 80:20:0.1, v/v/v) from 0 to 30 min, linear gradient to B (H_2O–acetonitrile, 9:1) from 30 to 60 min, and B from 60 to 70 min, at 2 ml/min. Purified compounds were identified by comparing their retention times and the [^{1}H]NMR spectra with those of the standard compounds.

RESULTS

Effects of Rhizosphere Soil of S. cernua *on Plant Growth.* Four testing plants (wheat, timothy, lettuce, and green amaranth) were cultured in a phytotron in the rhizosphere soil of *S. cernua* (treatment) with two controls (control I: in vermiculite; control II: in normal soil). Differences in growth could be seen after one week. The growth of wheat and timothy were inhibited by 80% and 45% after 15 days of culture, and the growth of lettuce and green amaranth were inhibited by 80% and 74% against control I after 30 days, respectively (Figure 2 and 3). All the treatments were significantly different from the two controls. In Figure 3, however, it can be seen that control II also showed a weak inhibition on lettuce, wheat, and timothy, but not on green amaranth, when compared to control I.

Effects of Extracts from Rhizosphere Soil of S. cernua *on Seed Germination and Seedling Growth.* The neutral and phenolic fractions were obtained from the rhizosphere soil of *S. cernua* (Figure 1). The phenolic fraction showed a strong inhibition on both seed germination and seedling growth of lettuce, green amaranth, timothy, and barnyard grass at 500 (pH 3.6) or 1000 (pH 3.3) ppm, although the magnitudes of the inhibitions differed according to plant species (Table 1). It had significant inhibition on both root elongation and the elongation of hypocotyl or coleoptile at 500 ppm. In the cases of timothy and barnyard grass, the inhibitions were stronger on root than on coleoptile. Seed germination was completely inhibited in lettuce and timothy, and inhibited by 90% in green amaranth at 500 ppm. Barnyard grass was less sensitive, its germination was inhibited by 30% and 45%, respectively, at 500 and 1000 ppm.

At 500 and 1000 ppm, the neutral fraction extracted with $CHCl_3$–CH_3OH–H_2O (10:5:4) did not show significant inhibition on either seed germination or seedling growth (data not shown). However, in the TLC direct bioassay, this fraction showed selective inhibition on different plant species, strongly inhibited germination and seedling growth of lettuce, but had little effect on timothy, green amaranth, and barnyard grass (Figure 4).

Identification of Main Allelochemicals in Rhizosphere Soil. The phenolic fraction was separated by HPLC. Five main inhibitory compounds, *p*-coumaric, ferulic, vanillic, and *p*-hydroxybenzoic acids and *p*-hydroxybenzaldehyde were identified. These phenolics are well-known allelochemicals (Chou and Lee, 1991; Chou and Waller, 1989; Putnam and Tang, 1986; Rice, 1984; Waller, 1987).

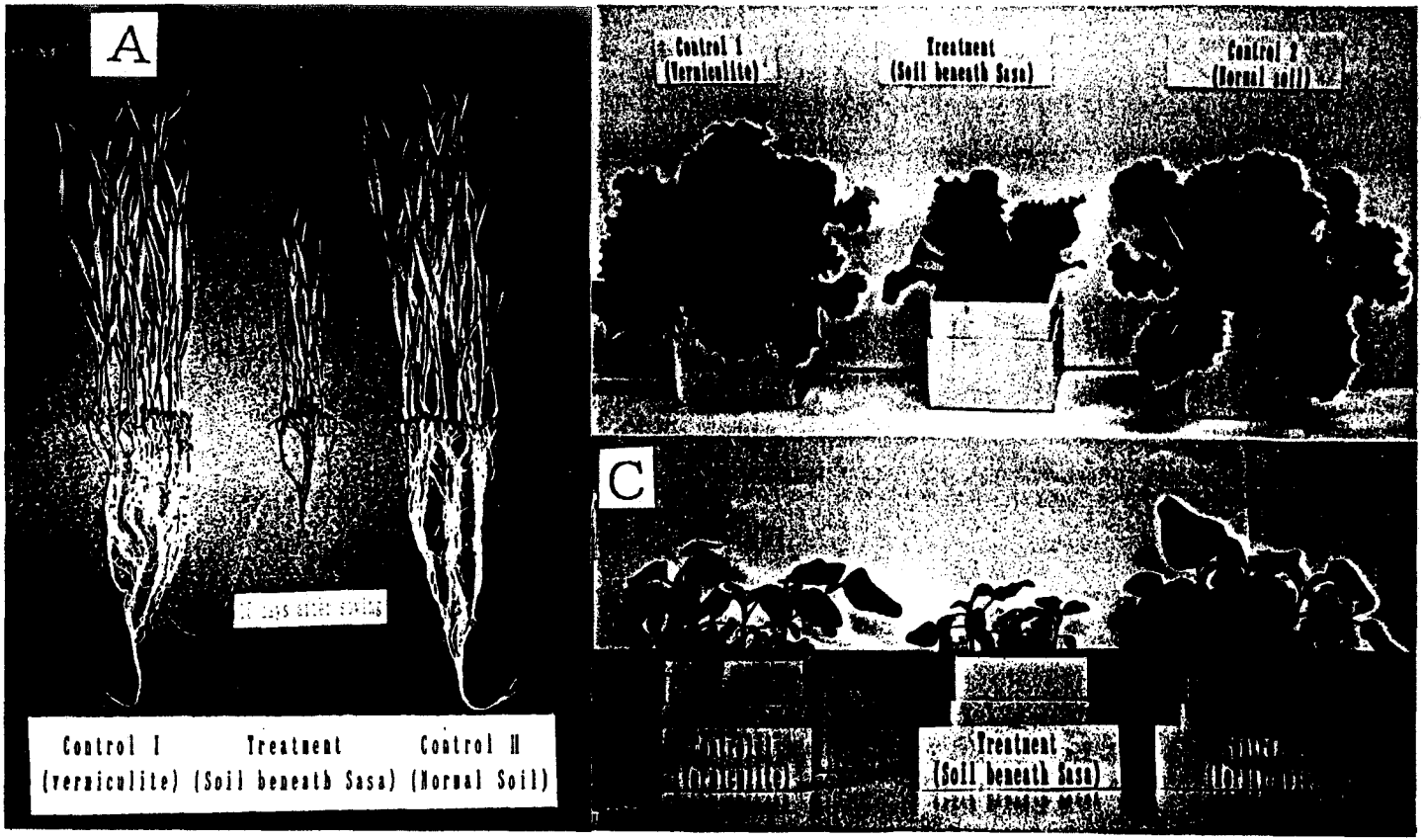

FIG. 2. Effects of rhizosphere soil of *Sasa cernua* on the growth of plants in soil assays. (A) wheat seedlings cultured for 15 days, (B) lettuce seedlings cultured for 30 days, (C) green amaranth seedlings cultured for 30 days.

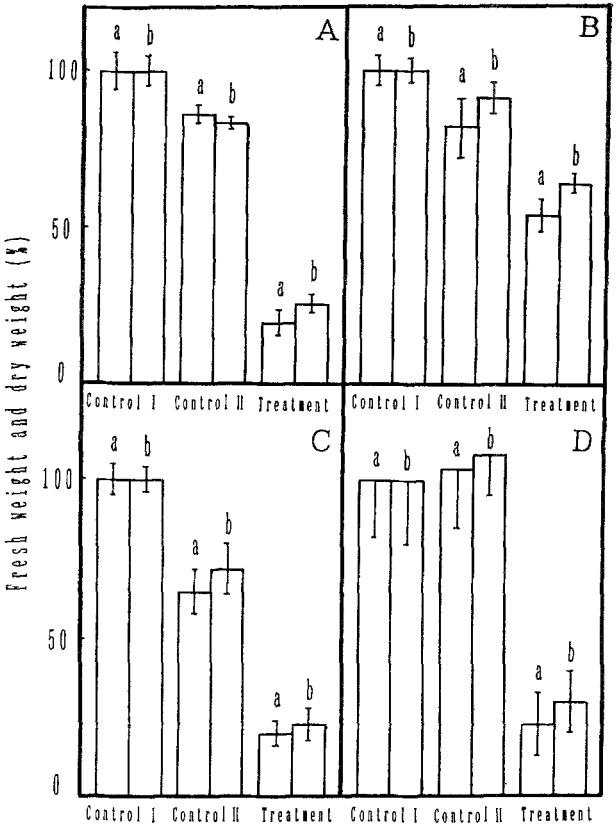

FIG. 3. Effects of rhizosphere soil of *Sasa cernua* on the fresh and dry weights of wheat (A, 15 days culture), timothy (B, 15 days culture), lettuce (C, 30 days culture), and green amaranth (D, 30 days culture). a: fresh weight, b: dry weight. Data were the means of 20 plants (two independent replicates each with 10 plants) ± SE (vertical bars).

They were reported to inhibit seed germination, root elongation, leaf expansion, photosynthesis, nutrient absorption, and the accumulation of dry matter in shoots and roots in plants (Blum and Rebbeck, 1989; Lyu et al., 1990; Rice, 1984). Contents of these five phenolic compounds in the rhizosphere soil were 5640 ± 500, 1060 ± 120, 860 ± 80, 810 ± 30 and 630 ± 90 μg/100 g soil, although the actual amounts in the soil might be higher than those detected. It was reported that phenolic allelochemicals in soil could be easily oxidized when the soil was removed from the natural status (Cast et al., 1990; Cheng, 1989).

The active substances from the neutral fraction were separated using column and thin-layer chromatography. The active substances, based on the seed

TABLE 1. EFFECTS OF PHENOLIC FRACTION OF EXTRACTS FROM RHIZOSPHERE SOIL OF *S. cernua* ON SEED GERMINATION AND SEEDLING GROWTH OF LETTUCE, GREEN AMARANTH, TIMOTHY, AND BARNYARD GRASS[a]

Plant species	Treatment (ppm)	Seed germination		Seedling growth (%)	
		Hours	%	Length of aerial part	Length of root
Lettuce	Control	24	87 ± 7	100 ± 5	100 ± 6
	500	24	0	44 ± 10	46 ± 5
	1000	24	0	35 ± 5	40 ± 5
Green amaranth	Control	48	72 ± 4	100 ± 9	100 ± 9
	500	48	8 ± 3	20 ± 5	45 ± 5
	1000	48	0	9 ± 3	35 ± 5
Timothy	Control	48	70 ± 5	100 ± 7	100 ± 10
	500	48	0	71 ± 5	15 ± 4
	1000	48	0	45 ± 5	9 ± 4
Barnyard grass	Control	72	53 ± 3	100 ± 9	100 ± 10
	500	72	38 ± 3	83 ± 4	61 ± 5
	1000	72	29 ± 6	60 ± 11	11 ± 3

[a]Data are the means of three independent replicates ± SE.

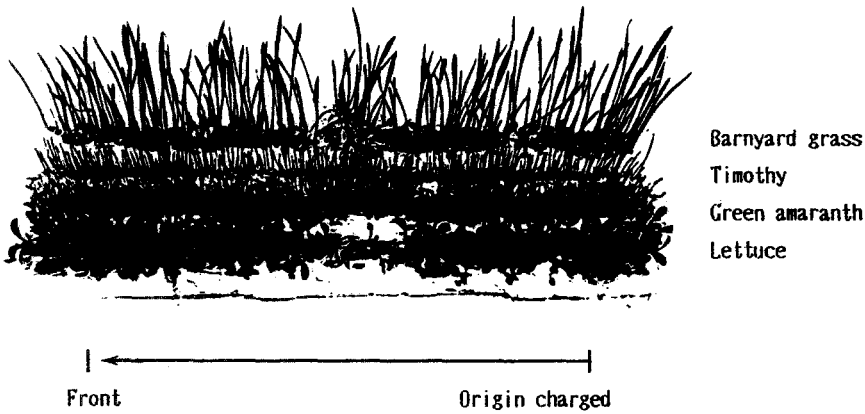

FIG. 4. Effects of the neutral fraction of extracts from the rhizosphere soil of *S. cernua* on TLC direct bioassay. TLC were developed with the solvents of toluene–ethyl acetate–CH_3OH (5:3:2). Photograph was taken six days after seeds were sowed.

germination test of lettuce, were unstable and remain unidentified. The [^{1}H]NMR spectrum of the unpurified active fraction showed that it might consist of a series of unsaturated long-chain esters of fatty acids.

Effects of Volatile Components from Leaves of S. cernua *on Plant Growth.* Wheat, timothy, lettuce, and green amaranth cultured in greenhouse were inhibited by 30, 44, 35, and 31%, respectively, in 20 days (Figure 5). Elongation of coleoptiles and roots were inhibited by 35% and 50% in etiolated seedlings of barley and by 46% and 35% in those of wheat after one week following germination. However, these inhibitions were weak on lettuce, green amaranth, and timothy (Table 2).

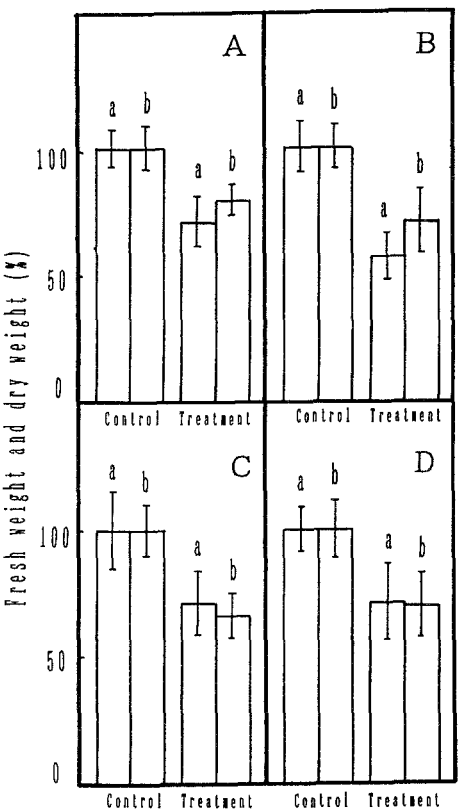

FIG. 5. Effect of volatile components released from the leaves of *S. cernua* on the fresh (a) and dry weights (b) of wheat (A), timothy (B), lettuce (C), and green amaranth (D) seedlings. Data are the means of 20 plants (two independent replicates each with 10 plants) ± SE (vertical bars).

TABLE 2. EFFECTS OF VOLATILE COMPONENTS FROM LEAVES OF *Sasa cernua* ON GROWTH OF ETIOLATED SEEDLINGS OF SEVERAL PLANT SPECIES[a]

Plant species		Length of aerial part		Length of root	
		cm	%	cm	%
Barley, *Hordeum distichum* cv. Delta	Control	12.5 ± 1.1	100 ± 9	12.3 ± 1.4	100 ± 11
	Treatment	8.1 ± 1.1	65 ± 9	6.0 ± 2.0	49 ± 16
Wheat, *Triticum aestivum* cv. Norin No. 59	Control	12.6 ± 2.0	100 ± 16	9.3 ± 2.0	100 ± 20
	Treatment	6.8 ± 2.1	54 ± 16	6.0 ± 1.1	65 ± 11
Timothy, *Phleum pratense* L.	Control	5.2 ± 0.4	100 ± 8	2.2 ± 0.1	100 ± 5
	Treatment	4.5 ± 0.4	87 ± 8	1.6 ± 0.3	73 ± 14
Green amaranth, *Amaranthus viridis* L.	Control	2.4 ± 0.2	100 ± 8	2.4 ± 0.2	100 ± 8
	Treatment	2.0 ± 0.1	83 ± 4	1.9 ± 0.3	79 ± 13
Lettuce, *Lactuca scariola* L. var. *sativa* Bisch	Control	2.6 ± 0.1	100 ± 4	4.6 ± 0.5	100 ± 11
	Treatment	2.4 ± 0.1	92 ± 4	4.1 ± 0.2	89 ± 4

[a] Lengths of aerial parts and roots were measured seven days after the start of treatment. Each value is the mean of 30 seedlings of three separate tests ± SE.

DISCUSSION

In the cultural tests with soil, it seems that suitable methods were important for the expression of allelopathic effect (Putnam and Tang, 1986). In the preliminary experiments, several traditional cultural methods were tested but did not yield uniform repeatable results. One problem seemed to arise from surface irrigation with water or nutrient solution. Usually, the small plants at the beginning of the experiments grew in the uppermost layer of the soil, and irrigation might dilute or wash away the allelochemicals (especially the water-soluble compounds). Nutrient conditions also affected the test results. It was reported that phenolic allelopathy may be more severe under low fertility, and raising the nutrient level can suppress some of the allelochemical effect (Einhellig, 1987). Lehmann et al. (1987) and Wang and Huang (1989) reported that inorganic ions in soil, such as Mn, Fe, Al, and Si, promoted the oxidation of phenolic allelochemicals. Inorganic irons might also affect the activities of acidic allelochemicals by salinization. Nutrient deficiencies or stresses might induce the ability for the root system to absorb substances including allelochemicals and inorganic irons from soil. In addition, nutrient deficiencies might have additive or even synergistic effects on allelopathy. In this paper, we used distilled water in the first week of the soil tests. To maintain sufficient nutrient levels for the test plants, especially for control I with vermiculite, nutrient solution

was added from the second week. The use of nutrient solution from the beginning of the tests resulted in lower inhibition (data not shown). The effects of nutrients on allelopathy should be studied further. The results on the soil tests for *S. cernua* (Figures 2 and 3) showed that this method was useful for detecting allelopathy in soil.

In the tests of the phenolic extracts from the rhizosphere soil of *S. cernua*, the low pH of the test solution might have some effect on seed germination and seedling growth. We tested the pH effect of the extracts from sasa soil and two kinds of phenolic acids (*p*-coumaric acid and *p*-hydroxybenzoic acid), which were identified in many plant soils and cultural solutions as allelochemicals (Chou and Lee, 1991; Chou and Waller, 1989; Putnam and Tang, 1986; Tang, 1986; Tang et al., 1989; Waller, 1987), on the growth of lettuce seedlings with pH 5.0 MES buffer and water solutions. The inhibitions of both the phenolic fraction from sasa soil and the phenolic acids on the growth of lettuce seedlings were lowered in the case of buffer solution compared to water solution. To obtain the same inhibition, two to five times higher concentrations were needed in the tests with the buffer solution than in those with water (data not shown). *S. cernua* might produce its allelopathy by releasing allelochemicals and lowering the pH of the soil.

In the tests for volatile components from the leaves of *S. cernua*, dried leaves were used in the airtight cultural boxes. At first, we used fresh leaves in the culture container with the petioles in cultural solution. It showed similar results (data not shown) as in the case of dried leaves. In the test with fresh leaves, however, the inhibition might be affected by the change of air components, which is difficult to control in an airtight container.

Chuyen and Kato (1982) identified 39 volatile flavor compounds in the headspace of *Sasa albo-marginata*; 1-penten-3-ol, *trans*-2-hexenal, acetaldehyde, *cis*-3-hexen-1-ol, hexanal α-terpineol etc. were the main components. Some of them had been demonstrated in residues of many plant species and shown to be toxic for plant growth as allelochemicals (Bradow and Connick 1990). Many other compounds were also toxic to plants, although their amounts were very low. Whether *S. cernua* produces the same volatile compounds is under investigation.

The dominant growth of sasa might result from many factors. The results above showed that sasa was allelopathic through its rhizosphere soil and air space. The significant allelopathic effects might be an important factor that contributes to the dominance of the landscape in Japan and some other countries.

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RESPONSIVENESS OF *Pseudaletia unipuncta* (LEPIDOPTERA: NOCTUIDAE) MALES, MAINTAINED AS ADULTS UNDER DIFFERENT TEMPERATURE AND PHOTOPERIODIC CONDITIONS, TO FEMALE SEX PHEROMONE

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Abstract—Different-aged *Pseudaletia unipuncta* (Haworth) males, maintained as adults at 10 and 25°C under 16:8 and 12:12 light–dark photoperiodic regimes, were flown to a 30-μg pheromone source in a wind tunnel at 20°C. While the responsiveness of individuals reared under both photoperiodic conditions at 25°C was similar by day 5 (65–75%), response levels of younger males showed that those reared at 16:8 light–dark matured more rapidly than those under 12:12 light–dark. A significant delay in the onset of responsiveness was observed for males reared under 10°C with less than 25% of individuals responding to the pheromone, even at 25 days old. At this temperature, photoperiodic conditions had no significant effect on male receptivity. When males are transferred from 10°C to 25°C 16:8 light–dark, their level of responsiveness after five days was similar to control 5-day-old males reared at 25°C, 16:8 light–dark. These results are discussed relative to the hypothesis that this species migrates in response to seasonal cues that indicate a predictable habitat deterioration.

Key Words—*Pseudaletia unipuncta*, true armyworm, Lepidoptera, Noctuidae, male responsiveness, temperature, photoperiod, female sex pheromone, seasonal migration.

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INTRODUCTION

Many environmental factors may influence daily and seasonal pheromone-mediated communication in moths: photoperiod (Fatzinger, 1973; Baker and Cardé, 1979), temperature (Cardé and Roelofs, 1973; Cardé et al., 1975; Comeau et al., 1976; Castrovillo and Cardé, 1979; Haynes and Birch, 1984), light intensity (Traynier, 1970; Kanno, 1981; Dreisig, 1986), relative humidity (Webster and Cardé, 1982), and wind velocity (Conner et al., 1985) are known to affect the time at which females produce and release the sex pheromone and/or the level of male responsiveness to it.

Saario et al. (1970) noted seasonal differences in the patterns of cabbage looper male captures in pheromone traps, with individuals being captured earlier in the day during cooler seasons than warmer ones. A rather different situation has been observed in the seasonal activity of the true armyworm *Pseudaletia unipuncta* (Haworth) (Lepidoptera: Noctuidae) where there are two distinct annual flights at Normandin, Quebec (McNeil, 1987). During the first, from May to July, the pattern of pheromone trap captures was similar to those of light traps, while in the fall flight, from August to October, very few males were attracted to pheromone traps even though they were caught in light traps.

Previous studies have shown that the age at which virgin *P. unipuncta* females initiated calling could be significantly influenced by abiotic conditions (Turgeon and McNeil, 1983; Delisle and McNeil, 1986, 1987). In this paper we examine how temperature and photoperiodic conditions during adult life influenced the responsiveness of virgin *P. unipuncta* males to a synthetic sex pheromone lure in a wind tunnel.

METHODS AND MATERIALS

Larvae of *P. unipuncta*, obtained from a laboratory colony established for less than 10 generations, were reared individually on a pinto bean diet modified after Shorey and Hale (1965). Pupae were sexed using the criteria described by Breeland (1958), and only males were kept. Both larvae and pupae were kept at 25 ± 1°C, 65 ± 5% relative humidity under a 16:8 light–dark photoperiod (750–1000 lux during photophase). Males were segregated by day of emergence in cages (20 × 20 × 30 cm) containing 8% sucrose solution and held under the appropriate temperature and photoperiodic conditions until tested.

To minimize the effect of handling close to the time of testing, males were placed in small individual wire mesh "release cages" 18–24 hr before flight tests. They were transferred to the standard test conditions of 20 ± 1°C and 65–90% relative humidity in the controlled environment chamber housing the Plexiglas wind tunnel (1 × 1 × 4 m) 1 hr before each trial. The logic behind testing males from different holding conditions at a standard condition in the

wind tunnel was to overcome potential problems associated with differences in thermoregulation and flight performance (Turgeon et al., 1983a), the release rates of the pheromone from the lure (Ioriatti et al., 1987), and dispersal of pheromone (Gaston et al., 1971) when experiments are carried out at different temperatures. The thoracic temperature of individuals transferred from 10°C conditions rapidly attained 20°C, the ambient temperature in the wind tunnel, and males were capable of flight within 2 min following the transfer. Thus, we believe that any differences observed in pheromone receptivity of males are due to major physiological changes and not to temperature shifts 1 hr prior to testing. A rubber septum loaded with 30 μg of (Z)-11-hexadecenyl acetate (Z11-16:Ac) and 0.15 μg of (Z)-11-hexadecenol (Z11-16:OH), the principal components of the female sex pheromone (Hill and Roelofs, 1980; McDonough et al., 1980), was placed on an insect pin 2.5 cm above a cork at the upwind end of the tunnel on a 30-cm-high galvanized steel stand. This concentration was chosen as it elicits a good male upwind flight under humidity conditions similar to those used in this experiment (Fitzpatrick et al., 1988). A release cage with an inactive male was placed on a 36-cm-high galvanized steel stand 230 cm downwind from the pheromone source. Once placed in the pheromone plume, the number of males exhibiting "locking on" behavior (initiation of directional flight towards the pheromone source) was scored, and for those initiating this behavior the distance flown towards the lure, the number touching the lure, and the flight speed calculated. Males not taking flight after 3 min, or those flying directly to either the walls or ceiling of the wind tunnel, were considered as nonresponsive to the pheromone. Males showing no activity during the 3-min period were nudged with a pencil to verify that each individual was capable of sustained flight. Less than 5% of males from any holding conditions were nonfliers, and these individuals were not included in the analyses. During each trial total light intensity was less than 0.17 lux, provided by diffused white light from above the tunnel and supplemented by a red point light source behind a white curtain on one side of the tunnel at the level of the release cage. Wind speed was 0.39 $\pm$ 0.02 m/sec. Males were only used once. Release cages were rinsed in acetone and dried after each test.

As the time at which females initiated calling behavior after lights out was affected by the length of the scotophase (Delisle and McNeil, 1986), an experiment was carried out to compare the receptivity of males held since emergence under 16:8 or 12:12 light–dark photoperiodic regimes at 25°C. Four- and 8-day-old males were tested in the second, fifth, and eighth hour of the 8-hr scotophase (16:8 light–dark) and in the second, fifth, eighth, and eleventh hour of the 12-hr scotophase (12:12 light–dark). Twenty males were tested at each period for each photoperiodic regime. No significant differences due to age or photoperiodic conditions were detected in the pattern of male response in the eighth hour of the scotophase, where responses ranged from 60 to 80% (χ^2 =

2.507, $df = 3$, $P = 0.4740$), so all subsequent experiments were carried out at that maximum response time.

A minimum of 20 males from each of the two photoperiodic regimes were tested at different ages: 2, 3, 4, and 5 days old at 25°C and 5, 10, 15, 20, and 25 days old at 10°C. To ensure that any differences in the response levels observed in males held at 10°C were not due to a shift in the time of maximum response (earlier in the scotophase), as noted for female calling behavior (Delisle and McNeil, 1987), 15-day-old males ($N = 9$) were also tested in the second and fifth hour of the scotophase but no response were observed at those times.

Turgeon et al. (1983a) demonstrated that the level of male response increased with an increase in pheromone concentration. Therefore, to ensure that the lack of responsiveness by males held at 10°C was not just a question of concentration, 5- and 10-day-old males, held from emergence at the two extreme conditions (10°C 12:12 light–dark or 25°C 16:8 light–dark), were tested using a 100-μg lure and compared with responses to a 30-μg concentration.

Males held at 10°C, 16:8 light–dark for 10 or 20 days were transferred to 25°C, 16:8 light–dark, held five days, and then tested to determine if males, like females (Delisle and McNeil, 1987), mature rapidly once placed under favorable conditions.

On every day that experiments were carried out, a number of 5- to 7-day-old males from the standard 25°C, 16:8 light–dark rearing conditions were also flown as a form of control to ensure that conditions on any given day were favorable for flight.

"Locking on" and touching behaviors were analyzed by contingency table (χ^2) techniques. Distance had a bimodal distribution, so a one-way nonparametric ANOVA for each effect was done (Kruskall-Wallis analysis). Speed, after transformation to its natural log, was normally distributed and had homogeneity of variance, but, since it was unbalanced, we used the GLM procedure with a type III SS (SAS Institute, 1987).

RESULTS

Short day conditions at 25°C induced a delay in the responsiveness of *P. unipuncta* male to the female sex pheromone on day 2 ($\chi^2 = 5.71$, $df = 1$, $P = 0.02$) and day 3 ($\chi^2 = 3.75$, $df = 1$, $P = 0.05$) compared with males held under long day conditions (Table 1). For example, 25% of males held under 16:8 light–dark responded on day 2 while none of the similar aged individuals at 12:12 light–dark did. Furthermore, response levels of day 3 males from 12:12 light–dark were similar to those observed for day 2 individuals held under long day conditions. However, by day 5 the responsiveness of males from both

photoperiodic conditions were comparable ($\chi^2 = 0.13$, $df = 1$, $P = 0.72$). Neither daylength ($\chi^2 = 0.04$, $df = 1$, $P = 0.84$; $F = 0.20$, $df = 1$, $P = 0.66$) nor age ($\chi^2 = 3.16$, $df = 3$, $P = 0.37$; $F = 0.67$, $df = 3$, $P = 0.58$) had any significant effect on either the distance flown or flight speed of males responding to the pheromone source (Table 1).

For males held at 10°C there was a slight age-related increase in the proportion of individuals locking on over the 25-day period (Figure 1), yet even 25-day-old males were significantly less responsive ($\chi^2 = 17.90$, $df = 1$, $P < 0.0001$) than 5- to 7-day-old males reared continuously at 25°C, 16:8 light–dark (Table 2). However, no significant photoperiodic effects ($P > 0.26$) were detected in males of any age reared at 10°C (Figure 1). As at 25°C, neither age ($\chi^2 = 3.11$, $df = 3$, $P = 0.37$; $F = 0.40$, $df = 3$, $P = 0.76$) nor photoperiodic conditions ($\chi^2 = 1.84$, $df = 1$, $P = 0.18$; $F = 0.56$, $df = 1$, $P = 0.46$) affected flight distance or speed (Table 2). An overall comparison of individuals held at 10 and 25°C, to a 100-μg cap, again demonstrated that males held at the lower temperature are significantly less responsive ($\chi^2 = 25.39$, $df = 1$, $P < 0.0001$) than those at the higher one (Table 3). As so few males held at 10°C responded, no meaningful comparisons could be made for distance flown or flight speed for individuals at the two temperatures. Furthermore, the responsiveness of males (both ages pooled) at 10°C did not increase with an increase in pheromone concentration ($\chi^2 = 2.57$, $df = 1$, $P = 0.11$) (Table 3). A similar trend was noted in the field: while an increase in pheromone concentration from 300 to 1000 μg increased trap efficiency during the summer flight (Turgeon et al., 1983b), this was not the case during the fall flight (McNeil, unpublished).

When males held at 10°C for 10 or 20 days were transferred to 25°C and

TABLE 1. EFFECT OF PHOTOPERIODIC CONDITIONS DURING ADULT LIFE ON RECEPTIVITY OF DIFFERENT-AGED *Pseudaletia unipuncta* VIRGIN MALES REARED AT 25°C AND TESTED IN A WIND TUNNEL AT 20°C USING A 30-μg PHEROMONE CAP

Photoperiod (light–dark, hr)	Age (days)	*N*	Locking on (%)	Touching (%)	Distance flown (cm; $X \pm$ SE)	Flight speed (cm/sec; $X \pm$ SE)
16:8	2	20	25	0	154.0 ± 74.2	14.3 ± 3.3
	3	20	55	20	209.5 ± 46.8	15.8 ± 7.0
	4	20	60	20	135.4 ± 81.3	13.5 ± 4.2
	5	20	70	45	188.9 ± 66.2	14.8 ± 6.2
12:12	2	20	0			
	3	20	25	10	169.0 ± 73.3	18.0 ± 6.0
	4	20	40	15	206.2 ± 45.0	14.6 ± 5.3
	5	20	75	40	166.3 ± 85.7	13.5 ± 4.2

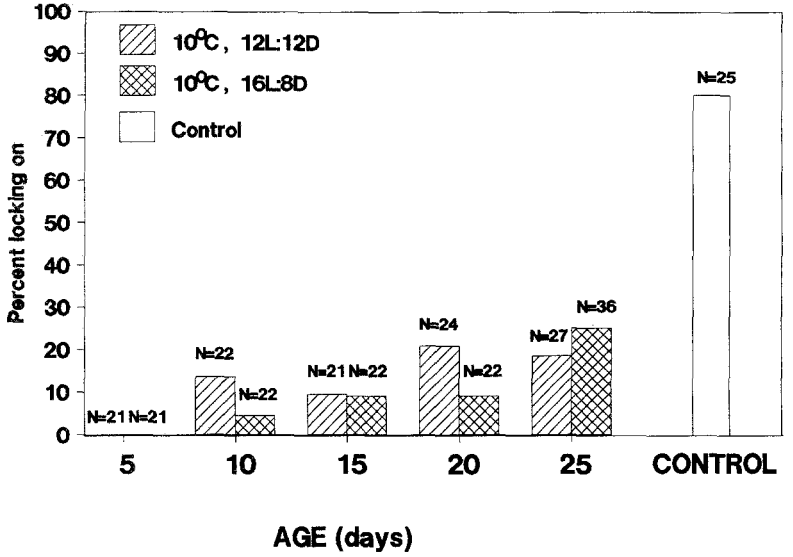

FIG. 1. Percent of *Pseudaletia unipuncta* males "locking on" to the pheromone plume as a function of age at 10°C/12L:12D and 10°C/16L:8D. The numbers of males tested are indicated directly above the histograms. Controls were 5- to 7-day-old males (N = 25) reared at 25°C/16L:8D.

TABLE 2. EFFECT OF PHOTOPERIODIC CONDITIONS DURING ADULT LIFE ON RECEPTIVITY OF DIFFERENT-AGED *Pseudaletia unipuncta* VIRGIN MALES HELD AT 10°C FROM EMERGENCE AND TESTED IN A WIND TUNNEL AT 20°C USING A 30-μg PHEROMONE CAP

Photoperiod (light–dark, hr)	Age (days)	N	Locking on (%)	Touching (%)	Distance flown (cm; $X \pm$ SE)	Flight speed (cm/sec; $X \pm$ SE)
16:8	5	21	0			
	10	22	4.6	0	30.0 ± 0.0	7.5 ± 0.0
	15	22	9.1	0	157.6 ± 9.5	13.5 ± 3.1
	20	22	9.1	0	37.5 ± 1.7	10.8 ± 0.6
	25	36	25.0	2.7	126.1 ± 71.6	9.9 ± 4.4
12:12	5	21	0			
	10	22	13.6	4.5	170.0 ± 38.4	9.7 ± 5.5
	15	21	9.5	0	220.0 ± 3.5	10.7 ± 1.7
	20	24	20.8	0	143.0 ± 61.4	7.2 ± 3.8
	25	27	18.5	2.8	118.0 ± 49.9	8.5 ± 5.3
Controls (25°C/16:8)	5–7	25	80.0	20.0	158.9 ± 88.4	10.9 ± 5.3

TABLE 3. EFFECT OF TEMPERATURE CONDITIONS DURING ADULT LIFE ON RECEPTIVITY OF 5- AND 10-DAY-OLD *Pseudaletia unipuncta* VIRGIN MALES TESTED IN A WIND TUNNEL AT 20°C USING 30- AND 100-μg PHEROMONE CAPS

Temp (°C)	Age (days)	Cap (μg)	*N*	Locking on (%)	Touching (%)	Distance flown (cm; $X \pm$ SE)	Flight speed (cm/sec; $X \pm$ SE)
25	5	30	12	83.3	58.3	228.5 ± 2.2	15.3 ± 7.1
	5	100	10	90.0	60.0	228.4 ± 2.0	15.4 ± 8.4
	10	30	20	60.0	20.0	225.4 ± 7.0	9.8 ± 3.9
	10	100	22	68.2	18.2	212.8 ± 26.2	10.5 ± 5.1
10	5	30	17	0.0			
	5	100	14	0.0			
	10	30	25	4.0	0	25.0 ± 0.0	2.3 ± 0.0
	10	100	21	14.3	0	80.0 ± 76.8	5.4 ± 3.8

tested five days later, their level of response was not significantly different ($\chi^2 = 0.25$, $df = 2$, $P = 0.88$) from 5- to 7-day-old control males (Figure 2).

DISCUSSION

There is very clear evidence that many moth species extend their geographic range each summer and establish temporary populations in regions where climatic conditions do not permit permanent occupation. However, the fate of these populations once weather conditions deteriorate in the fall is far from clear. Rabb and Stinner (1978) suggested that they die off, while Walker (1980) proposed the possibility of a return migration, a well-documented phenomenon in a number of butterfly species.

The true armyworm occurs each year throughout much of eastern Canada (Beirne, 1971), but does not have the ability to overwinter there (Fields and McNeil, 1984; Ayre, 1985), thus providing a working system to test the return migration hypothesis. All armyworm females reared under summer conditions (25°C, 16:8 light–dark) initiate calling behavior within nine days following emergence, while only 50% of those under fall conditions (10°C, 12:12 light–dark) do so within 35 days of emergence (Delisle and McNeil, 1987). Furthermore, those that called did so at a significantly older age ($X = 21.0 \pm 1.1$ days) than those under summer conditions ($X = 5.8 \pm 0.4$ days). More than 90% of all females captured in light traps during the summer flight were mated and have mature eggs in their ovaries, in sharp contrast to the fall flight where <2% of females were mated, and the vast majority of individuals captured had immature ovaries and large fat bodies (McNeil, 1987).

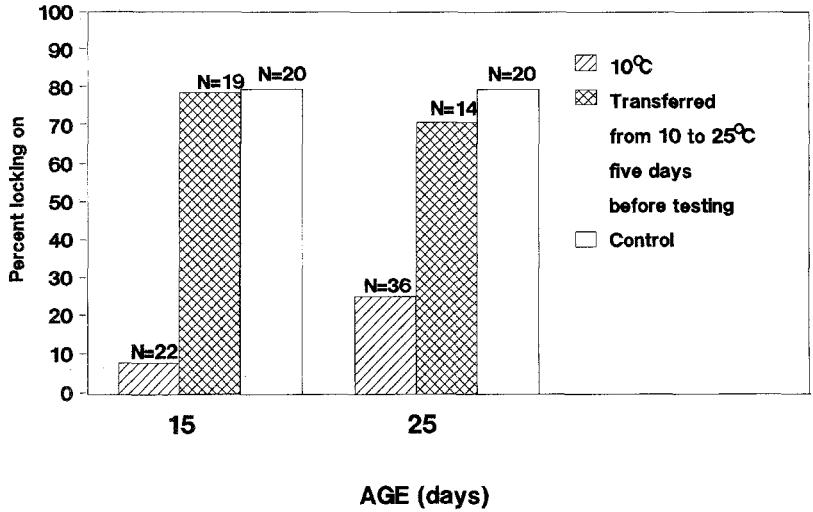

FIG. 2. Percent of *P. unipuncta males* "locking on" to the pheromone plume five days after being transferred to 25°C/16L:8D after being held for 10 or 20 days at 10°C/16L:8D. Controls were 5- to 7-day-old males reared continuously at 25°C/16L:8D. The numbers of males tested are indicated directly above the histograms.

Based on these results, McNeil (1987) hypothesized that the delay in reproduction induced by short-day, low-temperature conditions could be associated with a southward fall migration, as suggested by Johnson's "oogenesis–flight syndrome" (Johnson, 1969). Subsequently, Cusson and McNeil (1989a,b) demonstrated that the initiation of pheromone synthesis and calling behavior in the true armyworm are associated with the onset of ovarian development and that all three processes require the presence of juvenile hormone (JH). Rankin and Riddiford (1978) proposed a migration model for the milkweed bug: migration occurred when hemolymph levels of JH surpassed the "threshold for migratory flight" while remaining below the "threshold for ovarian development." Cusson et al. (1990) suggested a similar model may hold for the true armyworm as JH biosynthesis in females held for 35 days at 10°C, 12:12 light–dark was significantly lower than levels in 3-day-old individuals at 25°C, 16:8 light–dark. Furthermore, a marked increase in JH biosynthesis occurred within 24 hr of females being transferred to 25°C, 16:8 light–dark (Cusson et al., 1990).

In the summer flight, the pattern of male captures in pheromone-baited (300 μg) traps was similar to the pattern observed in light traps. However, in the fall flight, males are captured in the light traps but rarely attracted to pheromone lures (McNeil, 1987). These observations led to the suggestion that, like females, male maturation was delayed by short-day, low-temperature conditions

(McNeil, 1987). The results of the experiments reported here provide solid evidence that low temperatures do significantly delay the age at which males become responsive to pheromones and that, under certain conditions, day length may play a significant role.

True armyworm males produce JH acids (JHA) (Cusson et al., 1991), and the patterns of JHA biosynthesis parallel those of JH biosynthesis in females (Cusson et al., submitted). At 25°C, 16:8 light–dark there is a significant increase in JHA biosynthesis on the third day following emergence, coinciding with the age at which some males complete upwind flight to the lure (Table 1) and will mate with calling females (unpublish observations). However, JHA biosynthesis by males reared at 10°C, 12:12 light–dark remains low on day 5, but increases on day 10, the age at which males first show any level of response to pheromone lures (Table 2). As previously reported for females (Cusson et al., 1990), a switch from fall to summer conditions results in a significant and rapid increase in JHA biosynthesis (Cusson et al., submitted). In the present experiment, the response levels of such males are similar to controls (Figure 2).

Maturation of male accessory glands in the migratory grasshopper, *Melanoplus sanguinipes*, and the reduviid bug, *Panstrongylus megistus*, occurs following emergence and in both species is under the control of JH (Gillot and Friedel, 1976; Couche et al., 1985; Regis et al., 1985). In the armyworm, we dissected males and noted that accessory glands increase noticeably in volume over the five days following emergence at 25°C, 16:8 light–dark, while at 10°C, 12:12 light–dark there is little or no evidence of accessory gland development in the first 10 days of adult life. We therefore interpret the reduced responsiveness of males reared at 10°C in this study as a delay in sexual maturation. Thus, both sexes of the armyworm exhibit a hormonally mediated delay in the onset of mating in response to environmental cues that are reliable predictors of subsequent habitat deterioration, and this extended prereproductive period would provide a window during which long-distance migration could be undertaken.

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ANTIMICROBIAL TERPENES FROM OLEORESIN OF PONDEROSA PINE TREE *Pinus ponderosa:* A DEFENSE MECHANISM AGAINST MICROBIAL INVASION

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Abstract—The oleoresin of the ponderosa pine, *Pinus ponderosa* (Pinaceae) exhibited broad antimicrobial activity. In order to identify the active compounds, the oleoresin was steam distilled to give a distillate and residue. The distillate contained mainly monoterpenes and some sesquiterpenes, while the residue consisted chiefly of four structurally related diterpene acids. An antimicrobial assay with the pure compounds indicated that the monoterpenes were active primarily against fungi, but there was also some activity against gram-positive bacteria. The diterpene acids, in contrast, only exhibited activity against gram-positive bacteria. Although not all of the identified sesquiterpenes could be tested, longifolene showed activity only against gram-positive bacteria. Therefore, it appears that the oleoresin of *P. ponderosa* functions as a biochemical defense against microbial invasion.

Key Words—Antimicrobial activity, gram-positive bacteria, fungi, Pinus ponderosa, oleoresin, monoterpene, sesquiterpene, diterpene acid, defense mechanism.

INTRODUCTION

When pine trees are physically damaged or attacked by insects, they exude resins that apparently protect them from further damage. This damage is usually caused by microorganisms that may be transmitted by insects as well as by other

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means (Harris, 1948). Paradoxically, bark beetles are known to be attracted by pine oleoresins (Wood, 1982; Miller et al., 1986; Phillips et al., 1988). Thus, the response of bark beetles to attractants in oleoresins is a critical, initial step in the colonization of host tissues. However, as long as trees are healthy, the insects are probably expelled by resin flow before they can colonize the phloem tissue (Vité, 1961; Berryman, 1972; Wood, 1972). Often, the trees are damaged more by insect-transmitted microorganisms than by insect action itself (Raffa and Berryman, 1982; Cook and Hain, 1985; Pain and Stephen, 1987; Owen et al., 1987; Raffa and Smalley, 1988; Parmeter et al., 1989). Moreover, microorganisms may be transmitted by other means as well, since trees are continually exposed to microorganisms in the environment (Andrews et al., 1980). Therefore, we might expect that oleoresins have broad antimicrobial activity that protects the trees from these nonspecific microorganisms (Smirnoff, 1972; Morris, 1972).

This paper describes identification of the principal components in the distillate and residue of the ponderosa pine oleoresin, and their antimicrobial activities.

METHODS AND MATERIALS

General. All NMR spectra were run in $CDCl_3$ on a Nicolet spectrometer equipped with an Oxford superconducting magnet at 300 MHz for ^1H and 75 MHz for ^{13}C. Mass spectra were obtained with a Hitachi RMU 6-MG spectrometer in electron impact mode (EI-MS). UV spectra were recorded in ethanol on a Hitachi 100-80 spectrometer. IR spectra were recorded on a Perkin-Elmer 1310 spectrometer. Merck 35–70 mesh silica gel was used for column chromatography. The solvents used were of reagent or HPLC grade.

Chemicals. The xylem oleoresin was collected in vials screwed into holes bored into the stems of the ponderosa pine *P. ponderosa*, near Blodgett Research Forest (El Dorado County, California) in 1987. The oleoresin (850 g) was steam distilled for 2 hr to yield a distillate (126 g) and residue (690 g). The fractions were submitted to GC-MS analysis and R-HPLC separation, respectively. α-Pinene, β-pinene, 3-carene, and limonene were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). Terpinolene was purchased from Glidco Organics (Jacksonville, Florida), and abietic acid was purchased from Sigma Chemical Co. (St. Louis, Missouri). Longifolene was a gift from Takasago International Corporation (Tokyo, Japan). These compounds were all used for the assay without purification. *N,N*-Dimethylformamide (DMF) was purchased from EM Science (Gibbstown, New Jersey).

Microorganisms and Media. All microorganisms used for the antimicrobial assay were purchased from American Type Culture Collection (Rockville,

Maryland). They are *Bacillus subtilis* ATCC 9372, *Brevibacterium ammoniagenes* ATCC 6872, *Staphylococcus aureus* ATCC 12598 (a methicillin-susceptible strain), *Escherichia coli* ATCC 9637, *Pseudomonas aeruginosa* ATCC 10145, *Saccharomyces cerevisiae* ATCC 7754, *Candida utilis* ATCC 9226, *Penicillium chrysogenum* ATCC 10106, *Rhizopus stolonifer* ATCC 6227b, *Aspergillus niger* ATCC 16404, and *Mucor mucedo* ATCC 20094.

The culture media for bacteria consisted of 0.8% nutrient broth (BBL), 0.5% yeast extract (Difco) and 0.1% glucose. The culture media for fungi consisted of 2.5% malt extract broth (BBL).

Cultivation of Microorganisms. The freeze-dried microorganisms were reactivated as follows. *B. subtilis*, *S. cerevisiae*, *C. utilis*, *P. chrysogenum*, *R. stolonifer*, *A. niger*, and *M. mucedo* were cultured with shaking at 30°C. *B. ammoniagenes* was cultured stationarily at 30°C, and *S. aureus*, *E. coli*, and *P. aeruginosa* were cultured stationarily at 37°C.

Antimicrobial Assay. Preliminary routine antimicrobial assay was performed by a paper disk method. The test compound was dissolved in chloroform at a concentration of 8 mg/ml, and an aliquot of 0.1 ml of the sample solution was applied to a disk with a diameter of 8 mm (Toyo disk). This disk was placed onto the agar medium, which had been previously inoculated with a test microorganism. After incubation for two days at 37°C for bacteria and 30°C for fungi, the diameter of the inhibitory zone was measured with a caliper.

Since the *P. ponderosa* oleoresin was only slightly soluble in water, the paper disk method was an inadequate method because the water-insoluble oleoresin cannot disperse into the media. Therefore, throughout this report, the assay was performed by a broth dilution method (Taniguchi and Satomura, 1972) unless otherwise specified. For the broth dilution method, the test compound was dissolved in DMF and diluted in a twofold series with DMF, and 1% of sample solution was added to the broth medium. Microorganisms were cultured in a series of tubes with the different concentrations of the test compound in the broth medium. For the assay, the four common molds, *P. chrysogenum*, *R. stolonifer*, *A. niger*, and *M. mucedo*, were cultured with shaking, and other microorganisms were cultured stationarily. After two days (five days for molds), the growth was examined as a function of turbidity (OD at 660 nm) or visually in the case of molds. The lowest concentration of the test compound in which no growth occurred was defined as the minimal inhibitory concentration (MIC). It should be noted that the concentration of DMF in each test medium was always 1%. This did not affect the growth of any of the microorganisms tested.

GC and GC-MS Analysis. Analytical gas chromatography (GC) of the distillate was performed on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID) and fitted with a glass capillary column (0.25 mm ID × 30 m) coated with Carbowax bonded (20 m). The carrier gas was helium at 1.5 ml/min, and the oven temperature was programmed from 50 to 230°C at 4°C/min.

GC-MS analysis was carried out on a Hitachi M-808 double-focusing instrument equipped with a Hewlett-Packard 5890 gas chromatograph. The GC conditions were identical to the above analytical GC runs. Mass spectral data were acquired and processed by a built-in computer system (M-0101) (Takasago International Corporation, Tokyo, Japan). The distillate was composed of a mixture of almost exclusively mono- and sesquiterpene hydrocarbons. Components were identified on the basis of a comparison of the GC retention time and MS fragmentation with those of the authentic samples.

R-HPLC Analysis. Recycle HPLC was performed on a JAI LC-09 (Japan Analytical Industry, Tokyo, Japan) equipped with a JAI refractive index detector and a JAI UV detector operating 235 nm. An ODS column (25 cm × 25 mm ID) was employed. The residue (19 g) was first further purified by silica-gel chromatography with *n*-hexane–ethyl acetate eluant to obtain a major fraction (14.5 g), which showed a single spot on a TLC plate. This fraction (40 mg) was injected into the R-HPLC system. The mobile phase was methanol at a flow rate of 3 ml/min. R-HPLC was repeated until four known diterpene acids were separated (Figure 1). Their UV and [^{1}H]NMR were identical with those of the authentic samples.

RESULTS AND DISCUSSION

Preliminary antimicrobial assays of *P. ponderosa* oleoresin exhibited activity against the gram-positive bacteria, *B. subtilis* and *B. ammoniagenes*, but not against the gram-negative bacteria, *E. coli* and *P. aeruginosa* up to 500 μg/ml. For the fungi, the oleoresin showed activity against *C. utilis* but not against *S.*

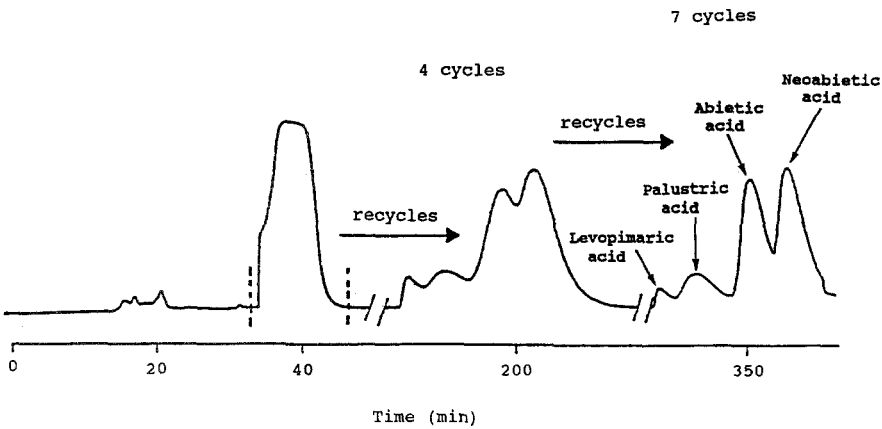

FIG. 1. Separation of diterpenoids by recycle HPLC.

cerevisiae at 10,000 ; gmg/ml (Table 1). As shown in Table 1, the distillate maintained almost the same activity as the oleoresin itself and showed additional activity against *S. cerevisiae*, but the residue lost activity against fungi. Thus, the distillate showed activity against fungi and gram-positive bacteria, while the residue exhibited activity only against gram-positive bacteria. Neither fraction showed any activity against gram-negative bacteria. It seems that the distillate and residue combine to yield the original antimicrobial activity of the oleoresin. It should be noted that none of these fractions showed any activity by the paper disk method. A possible reason for this is discussed later.

In order to identify the antimicrobial principles in both the distillate and residue, further analysis was performed. In the distillate, seven monoterpenes (α-pinene, β-pinene, 3-carene, myrcene, limonene, β-phellandrene and terpinolene), three sesquiterpenes (α-longipinene, longifolene, and germacrene D), and one phenolic compound (estragole) were identified by GC-MS analysis. This result is in general agreement with those reported earlier (Anderson et al., 1969; Smith, 1964).

On the other hand, the residue was found to be a mixture of primarily diterpene acids. The residue was first further fractionated by silica-gel column chromatography to remove the remaining volatile compounds resulting in a nondistillable fraction in 65% yield from the original oleoresin. Although this fraction showed a single spot on TLC plate, its NMR (^{1}H and ^{13}C) spectra indicated that it was still a mixture of abietic acid-type diterpenes. These structurally related diterpene acids were efficiently separated by R-HPLC (Kubo and Nakatsu, 1990). The mixture was injected into an R-HPLC system using an ODS column with the recycling carried out at intervals of about 50 min. By cycle 4 it was evident that this mixture contained four components as shown in

TABLE 1. ANTIMICROBIAL ACTIVITY OF OLEORESIN AND ITS DISTILLATE AND RESIDUE BY STEAM DISTILLATION

Microorganisms tested	Activity[a]		
	Oleoresin	Distillate	Residue
Bacillus subtilis ATCC 9372	+	+	+
Brevibacterium ammoniagenes ATCC 6872	+	+	+
Escherichia coli ATCC 9637	−	−	−
Pseudomonas aeruginosa ATCC 10145	−	−	−
Saccharomyces cerevisiae ATCC 7754	−[b]	+	−
Candida utilis ATCC 9226	+[b]	+	−

[a] +, Active at a concentration of 500 μg/ml; −, inactive at the concentration of 500 μg/ml.
[b] The concentration was 10,000 μg/ml.

Figure 1. Each fraction was collected, and R-HPLC was repeated under the same conditions to give four pure structurally similar diterpene acids; abietic acid, levopimaric acid, neoabietic acid, and palustric acid were isolated in natural form and identified by spectroscopic studies. These diterpene acids were previously identified in the same source by various methods (Anderson et al., 1969) including GC-MS analysis after methylation with diazomethane (Fujii and Zinkel, 1984).

Various biological activities of abietic acid-type diterpenes have been reported. For example, ingestion of the hexane extract of *P. ponderosa* needles, composed chiefly of eight diterpene acids, caused reproductive failure in mice during the early stages of gestation (Kubik and Jackson, 1981). The diterpene acids from various coniferous trees are also known as antifeedants against insects (Wagner et al., 1983; Schuh and Benjamin, 1984) and aphids (Rose et al., 1981).

Among the volatile hydrocarbons identified in the distillate, α-pinene, β-pinene, 3-carene, limonene, terpinolene, and longifolene were tested for antimicrobial activity. However, we were unable to test the antimicrobial activity of several compounds for the following reasons: (1) myrcene was barely soluble in DMF, so that we could not prepare the necessary sample solution for the assay; (2) germacrene D was unstable under the sample preparation conditions for the assay; and (3) some substances were minor and available only in small quantities. None of the volatile components tested showed activity against gram-negative bacteria up to 800 μg/ml (Table 2). Most monoterpenes showed activity against fungi, especially against *S. cerevisiae*, but almost no activity against gram-positive bacteria at concentrations up to 800 μg/ml. However, longifolene, a sesquiterpene hydrocarbon identified in the distillate showed no activity against fungi but exhibited activity against gram-positive bacteria, among

TABLE 2. ANTIMICROBIAL ACTIVITY OF PRINCIPAL VOLATILE IN DISTILLATE

	MICs against microorganisms[a] tested (μg/ml)					
Volatile	Bs	Ba	Ec	Pa	Sc	Cu
α-Pinene	>800	>800	>800	>800	50	800
β-Pinene	>800	>800	>800	>800	100	800
3-Carene	>800	>800	>800	>800	50	100
Limonene	800	>800	>800	>800	25	200
Terpinolene	800	800	>800	>800	50	100
Longifolene	3.13	200	>800	>800	>800	>800

[a]Bs, *B. subtilis*; Ba, *B. ammoniagenes*; Ec, *E. coli*; Pa, *P. aeruginosa*; Sc, *S. cerevisiae*; Cu, *C. utilis*.

which *B. subtilis* was much more sensitive than *B. ammoniagenes*. These results indicate that the original activity of the distillate against fungi was due to monoterpenes and the activity against gram-positive bacteria was due to sesquiterpenes. Therefore, even among the components of the distillate, monoterpenes and sesquiterpenes may share antimicrobial activity. Interestingly, none of these antimicrobial terpenes showed any activity by the paper disk method. There are two possible explanations for this: (1) Since these volatiles are not soluble in water they might not disperse into the media, and (2) they might have been partially if not entirely evaporated from the disk when the solvent was removed from the disk under reduced pressure. However, when these volatile monoterpenes were tested against insect-transmitted fungi in a closed system with the saturated vapor, all of them exhibited fungistatic activity (Cobb et al., 1968). Therefore, we retested the five monoterpenes against four common molds, namely *P. chrysogenum* (blue-green mold), *R. stolonifer* (black bread mold), *A. niger* (black mold), and *M. mucedo* in a broth media at 800 μg/ml. Results showed that only limonene exhibited activity against *M. mucedo* with a MIC of 400 μg/ml (Table 3).

In the case of the residue, the antimicrobial activity of abietic acid was extensively studied as a representative component, because the others were rather unstable and available only in minute quantities. Abietic acid exhibited strong activity against gram-positive bacteria (Table 4), comparable to the original activity of the residue itself. Since the three other diterpene acids are structurally similar to abietic acid, and the antimicrobial activity of pure abietic acid did not increase compared with the residue itself, they might be expected to show the same activity as abietic acid. Among gram-positive bacteria tested, *S. aureus* is an important human pathogenic bacterium. Based on this activity of abietic acid, the three other purified diterpene acids were also tested against this bacterium. All four diterpene acids exhibited the same antibacterial activity against *S. aureus* at MIC of 25 μg/ml.

TABLE 3. ANTIMICROBIAL ACTIVITY OF MONOTERPINES IN DISTILLATE AGAINST MOLDS

	MIC against molds[a] tested (μg/ml)			
Monoterpines	Pc	Rs	An	Mm
α-Pinene	>800	>800	>800	>800
β-Pinene	>800	>800	>800	>800
3-Carene	>800	>800	>800	>800
Limonene	>800	>800	>800	400
Terpinolene	>800	>800	>800	>800

[a]Pc, *P. chrysogenum*; Rs, *B. stolonifer*; An, *A. niger*; Mm, *M. mucedo*.

TABLE 4. ANTIMICROBIAL ACTIVITY OF RESIDUE AND ABIETIC ACID (COMMERCIAL)

Microorganisms tested	MIC (μg/ml)	
	Residue	Abietic acid
B. subtilis	12.5	12.5
B. ammoniagenes	25	12.5
S. aureus	25	25
E. coli	>800	800
P. aeruginosa	>800	>800
S. cerevisiae	>800	>800
C. utilis	>800	>800

The oleoresin of *P. ponderosa* may play an important role in protecting the tree from microbial invasion. Moreover, the viscous residue, comprised almost exclusively of diterpene acids, retains the volatile antifungal monoterpene hydrocarbons. Otherwise, these volatiles would rapidly escape into the environment and have only a short duration of antimicrobial activity. For our study, oleoresin was collected from the stems of *P. ponderosa* by boring through the bark into the xylem tissue. We speculate that the role of the oleoresin in defense against microbial invasion may not be only chemical but also biological. However, we were unable to determine if any of the substances identified in the oleoresin were a result of de novo synthesis. Furthermore, the relative amounts of these compounds in the outer and inner bark may effect microbial invasion of pines.

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A NEW METHOD FOR FAST ISOLATION OF INSECT ANTIFEEDANT COMPOUNDS FROM COMPLEX MIXTURES

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Abstract—Bioassay-guided isolation of bioactive natural substances requires monitoring of all fractionation and purification steps using a bioassay system. This is often a long and tedious process, especially with insect feeding bioassays. We report here a new method, based on the principle of bioautography, for a quick isolation of insect antifeedant compounds. TLC plates, after development, are coated with a thin layer of artificial diet and fed to *Spodoptera litura* larvae. The location of uneaten areas is then compared with the R_f values of the TLC spots, in order to determine rapidly the active fractions. This methods allows for a very fast determination of the most active antifeedant compounds in a complex mixture and considerably speeds up the isolation process. This new method was successfully applied in the study of antifeedant activity of several plant samples, and results are presented here for a model plant, *Skimmia japonica* (Rutaceae). Using this new method, the compounds responsible for the feeding-deterrent activity, three furanocoumarins (bergapten, xanthotoxin, and oxypeucedanin), were quickly and efficiently identified.

Key Words—Antifeedants, tobacco cutworm, *Spodoptera litura*, Lepidoptera, Noctuidae, bioautography, new bioassay, *Skimmia japonica*, Rutaceae, furanocoumarins.

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INTRODUCTION

In the past decade, the increased awareness of the potential dangers of synthetic pesticides, as well as a permanent increase in pest resistance, have prompted a search for new types of compounds with pest control properties. Among other leads, compounds affecting the feeding behavior of insects have been targeted as a promising group (Klocke, 1989; Jermy, 1990), and extensive searches for antifeedants of natural origin have been conducted. Plants have been the main focus of this search, since their resistance to herbivory by insects is often attributed to the presence of secondary compounds, which are thought to function primarily to deter herbivory through a variety of mechanisms (Feeny, 1976; Smith, 1989).

The screening of plant extracts for antiherbivore activity and the isolation and purification of active compounds are often long, expensive, and tedious processes. Numerous samples or chromatographic fractions are usually evaluated against a target insect, in a sequence of feeding bioassays. These bioassays require large numbers of insects, need to be replicated several times for statistical validation, are therefore generally laborious to conduct and evaluate, and have prompted a search for alternative methods (Alkofahi et al., 1989).

We propose here an alternative method to the classical leaf-disk choice bioassay (Lewis and Van Emden, 1986; Kubo, 1991), for the isolation of antifeedants against larvae of herbivorous insects. This new method is based on bioautography, a method widely used for the isolation of antifungal or antibacterial agents from complex mixtures (Hamburger and Cordell, 1987; Rios et al., 1988). In the general method, a solution containing candidate compounds is separated on a thin-layer chromatography (TLC) plate. The plate is coated with culture medium, inoculated with microorganisms, and incubated. Active components are quickly identified by comparing the location of growth inhibition zones with the R_f values of compounds on a control plate (Figure 1A).

We have successfully adapted the concept of bioautography to the isolation of insect antifeedant compounds, using feeding on TLC plates coated with an artificial diet as an indicator of activity. We present here details of this method as well as its application to the study of a Japanese plant, *Skimmia japonica*, which was previously found to possess insect feeding-deterrent properties in a broad screening of plants from Hokkaido (Escoubas et al., In press).

Although previous work has applied this concept to the study of insect (Metcalf et al., 1980; Ferguson et al., 1983) or marine gastropod (Sakata et al., 1984) feeding stimulants, to our knowledge, this is the first application of bioautography to the study of insect antifeedant compounds.

METHODS AND MATERIALS

Insect Rearing. Spodoptera litura (Noctuidae) were reared on an artificial diet (Insecta LF, Nihon Nosan Kogyo Co.) in a controlled environment chamber at 27°C and 70% relative humidity.

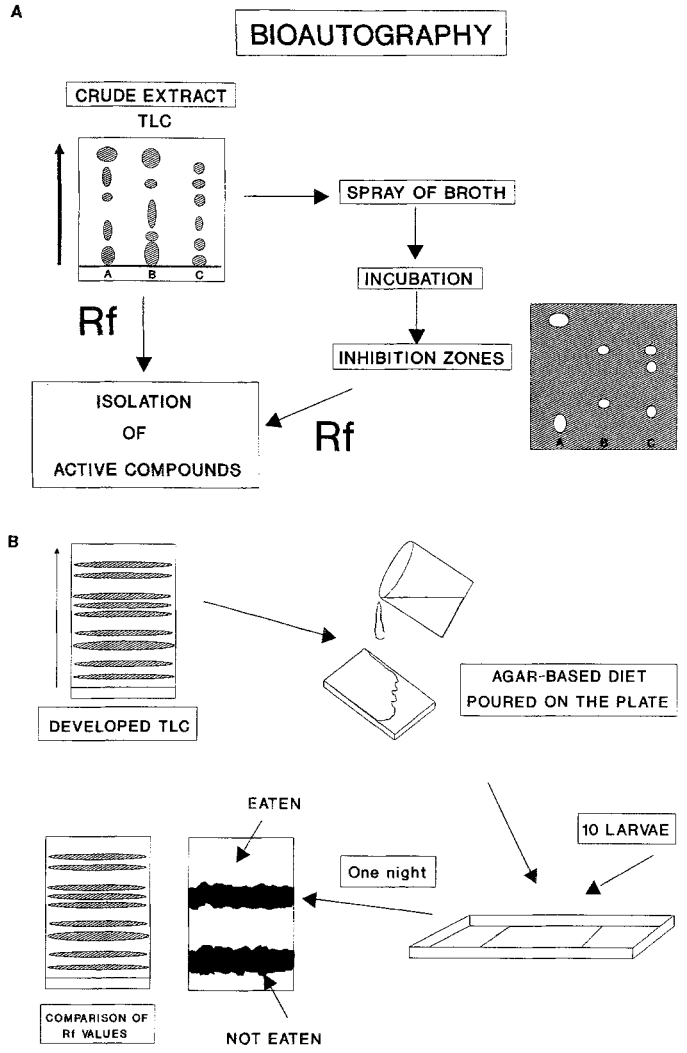


FIG. 1. (A) General principle of bioautography, as used for the identification of antibacterial or antifungal agents. (B) Application of bioautography to the identification of insect feeding-deterrent compounds.

Artificial Diet. The artificial diet used in the bioassay differs from the diet used for rearing the insects and is derived from a general-purpose insect diet (Singh, 1985). The composition is described in Table 1. The diet was prepared as follows: agar is boiled in 200 ml distilled water in a microwave oven (750 W, 1 min 30 sec). Other constituents except vitamins are added to 120 ml of distilled water and thoroughly mixed to avoid the formation of lumps. The

TABLE 1. COMPOSITION OF INSECT ARTIFICIAL DIET

Ingredients	Weight (g)
Agar	8.8
Wheat germ	12.8
Soy flour	14.8
Sucrose	12.8
Sorbic acid	0.4
α-Cellulose	1.9
Methyl *p*-hydroxybenzoate	1.9
Wesson salt mix	3.6
Vanderzant's insect vitamin mix	5.8
Distilled water	320 ml

two solutions are combined, and the vitamin mixture is added after the temperature has cooled to 55°C.

Diet Bioassay. TLC silica gel plates with fluorescent indicator (Merck Silica Gel 60 F_{254} 0.25 mm) were cut into 5 × 10-cm pieces. The sample containing candidate compounds (ca. 5 mg) is applied as a band, and the plate is developed in an appropriate solvent mixture in order to give the best possible separation. The solvent is then completely evaporated under reduced pressure in a glass desiccator. The plate is then coated with hot insect artificial diet and left to solidify for a few minutes. The diet is poured from a beaker onto a plate placed at a 45-degree angle in order to form a layer about 1–1.5 mm thick. The diet used quenches the fluorescence, making the coated plate appear dark under UV light (254 nm). For each experiment, a control plate with no sample is prepared. A second control plate developed under the same conditions, but not coated with diet, serves as reference for visualizing and measuring the R_f values of individual compounds.

Each plate is laid in a 10 × 15 × 1-cm plastic dish, lined with wet filter paper, with ten 10-day-old larvae of *Spodoptera litura* (end of fourth instar). The larvae are allowed to feed on the plate for 16–18 hr, at 27°C, in the dark, in order to avoid visual cues (Lewis and Van Emden, 1986). Under these conditions, the larvae usually consume most of the diet layer on the control plates, leaving only the underlying fluorescent silica gel. The plates are dried with a hair-dryer and then examined under UV light (254 nm) to visualize the uneaten areas. The location of these areas is compared with the R_f values of the compounds detected on a reference plate (Figure 1B). Compounds with feeding-deterrent properties produce a dark band or spot, due to quenching by the remaining diet. To evaluate the sensitivity of the method, known amounts of extracts

were applied as spots, at 2-cm intervals on TLC plates and submitted to the same protocol. The level of activity was estimated visually.

Leaf-Disk Bioassay. The dose–response activity of various pure compounds was determined. Ten sweet potato leaf disks (1 cm^2), treated with 10 μl of sample or solvent for control, were arranged alternately in 9-cm-diameter plastic Petri dishes, in three replicates. After allowing five 4-day old *Spodoptera litura* larvae to feed for one night in the dark at 27°C, leaf areas are measured using a video camera coupled with an Apple Macintosh computer. Calculation of an antifeedant index allowed for comparison of activities; an index of less than 20 indicated significant antifeedant activity (Alkofahi et al., 1989; Escoubas et al., In press) .

$$\text{Antifeedant index} = 100 \times \frac{\%T}{\%T + \%C}$$

$\%T$ = % of treated disks consumed, $\%C$ = % of control disks consumed. The index varies from 0 (total feeding-inhibition) to 100 (total feeding-stimulation).

Isolation of Skimmia japonica *Compounds. S. japonica* leaves were collected in the Nopporo Forest Park, Hokkaido, Japan, ground, and then extracted with 5% aqueous methanol for one week, at room temperature. This crude extract was subsequently partitioned between water and ethyl acetate (1 : 4). The aqueous extract was reextracted with butanol (1 : 1).

The first TLC separation of the ethyl acetate fraction was carried on silical gel TLC plates, using a chloroform–ethyl acetate mixture (8 : 2). Further separation of the active portion of the above chromatogram, obtained by preparative TLC, was achieved using a mixture of hexane–chloroform–ethyl acetate (7 : 2 : 1) and three successive developments. In all TLC separations, compounds were visualized under fluorescent light (365 nm).

Spots found to be active in the bioassays described were purified further by open-column chromatography using Silica Gel 60 (230–400 mesh). Compounds were eluted with a mixture of hexane–chloroform–ethyl acetate in various proportions (8 : 1 : 1, 7 : 2 : 1, and 6 : 3 : 1). Final purification of the compounds was done using preparative TLC, under the conditions described above. NMR data were obtained on a 270-MHz JEOL EX 270 spectrometer, and mass spectra were taken on a high-resolution JEOL JMS-DX 300 mass spectrometer.

RESULTS

Identification of antifeedants in Skimmia japonica. The first bioassay of the water, butanol, and ethyl acetate extracts of *S. japonica* was conducted without migration of the samples. When the three fractions were tested by bioautography, only the ethyl acetate extract showed activity (Figure 2A). When the bioas-

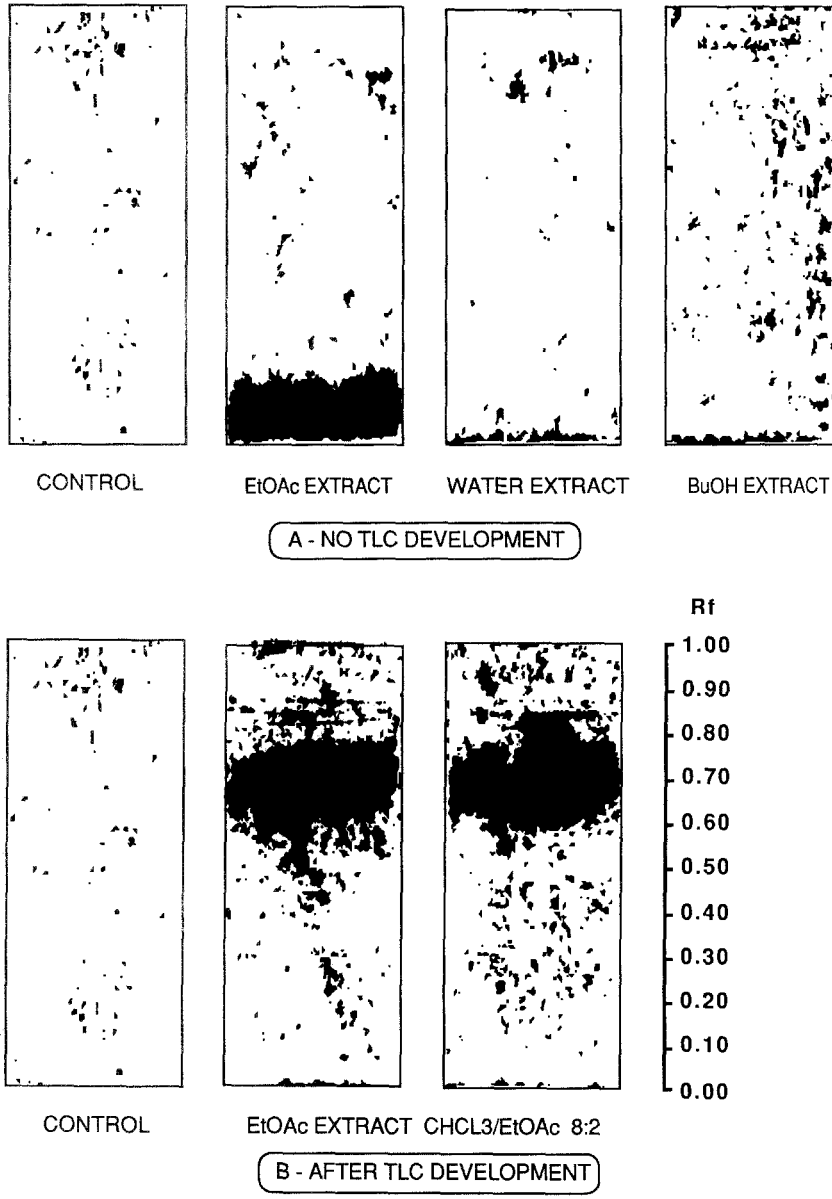


FIG. 2. Identification of *Skimmia japonica* active compounds. Actual pictures of experiments were digitized with a desktop computer scanner. White areas represent fluorescence at 254 nm, and black areas indicate quenching by the artificial diet. (A) Bioassay of the ethyl acetate, water, and butanol extracts, without development of the plates; (B) bioassay of the ethyl acetate fraction (two replicates) after development.

say of the ethyl acetate extract was repeated after development, the activity, visualized by the quenching, moved from the application point to the upper part of the plate, and was found at a R_f of 0.60–0.80. (Figure 2B). This result demonstrated that, after TLC separation, the activity of the total extract could be successfully localized in one portion of the chromatogram, with the diet being consumed in all other parts. When this band was subsequently isolated and bioassayed separately using the second solvent system, antifeedant activity appeared between R_f = 0.35 and 0.56. The active portion of the chromatogram contained four compounds (SKJ-1 to −4). No supplementary bioassay step was needed and the compounds were isolated and purified by column chromatography followed by preparative TLC.

Subsequent leaf-disk bioassay, at a concentration of 1 mg/ml (10 $\mu g/cm^2$), showed that only three of the purified compounds possessed antifeedant activity (Table 2). The structural analysis was therefore carried out only for these three compounds and showed them to be three furanocoumarins: bergapten (SKJ-1) xanthotoxin (SKJ-2), and oxypeucedanin (SKJ-3) (Figure 3). Evaluation of the dose–response activity in the leaf-disk bioassay, for the ethyl acetate fraction as well as for the pure compounds, showed that the most active antifeedant was bergapten, followed by xanthotoxin and oxypeucedanin (Figure 4).

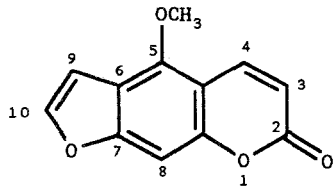
Spectral Data. The physicochemical and spectral data for bergapten and xanthotoxin were identical in all respects with those reported previously in the literature (Razdan et al., 1987).

Bergapten (M^+ 216.0386, calc. for $C_{12}H_8O_4$, 216.0608): Recrystallization from benzene gave colorless needles, mp 186–188°C. IR (KBr, cm^{-1}) 1725, 1607; UV (MeOH) max (nm), 222, 249, 260, 268, 310; [^{1}H]NMR ($CDCl_3$) 8.15 (d, 1H, $J_{4,3}$ = 10 Hz, H-4), 7.62 (d, 1H, $J_{10,9}$ = 2 Hz, H-10), 7.13 (s, 1H, H-8), 7.02 (d, 1H, $J_{9,10}$ = 2 Hz, H-9), 6.27 (d, 1H, $J_{3,4}$ = 10 Hz, H-3), 4.27 (s, 3H, CH_3O).

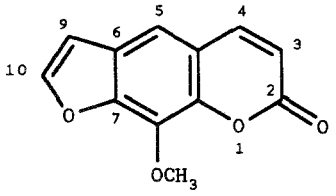
Xanthotoxin (M^+ 216.0396, calc. for $C_{12}H_8O_4$, 216.0608). Recrystalli-

TABLE 2. ANTIFEEDANT ACTIVITY OF ISOLATED CONSTITUENTS OF ACTIVE FRACTION, AT 1 mg/ml (10 μg/DISK) CONCENTRATION (MEAN ± SE)

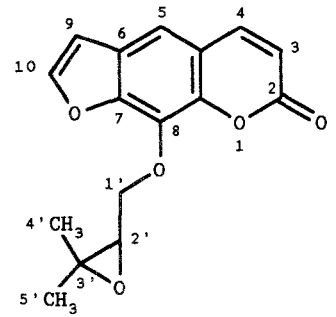
Compound	% treatment eaten	% control eaten	Index
Control	23.62 ± 9.64	17.02 ± 5.10	55.54 ± 14.49
SKJ-1	0.00 ± 0.00	50.66 ± 5.17	0.00 ± 0.00
SKJ-2	0.00 ± 0.00	65.97 ± 4.20	0.00 ± 0.00
SKJ-3	19.83 ± 6.91	32.97 ± 6.62	37.41 ± 12.67
SKJ-4	56.57 ± 6.17	18.24 ± 3.41	75.22 ± 3.29



SKJ-1
BERGAPTEN



SKJ-2
XANTHOTOXIN



SKJ-3
OXYPEUCEDANIN

FIG. 3. Antifeedant furanocoumarins of *Skimmia japonica*.

zation from chloroform gave colorless needles, mp 146–148°C, IR (KBr, cm^{-1}) 1710, 1610. UV (MeOH) max (nm) 215, 249, 264, 299, 315. [^{1}H]NMR ($CDCl_3$) 7.76 (d, 1H, $J_{4,3}$ = 10 Hz, H-4), 7.62 (d, 1H, $J_{10,9}$ = 2 Hz, H-10), 7.35 (s, 1H, H-5), 6.81 (d, 1H, $J_{9,10}$ = 2 Hz, H-9), 6.37 (d, 1H, $J_{3,4}$ = 10 Hz, H-3), 4.30 (s, 3H, CH_3O).

Oxypeucedanin gave identical physicochemical and spectral data with those previously published (Atkinson et al., 1974) (M^+ 286.0833, calc for $C_{16}H_{14}O_5$ 286.1078). Recrystallization from chloroform gave colorless needles, mp 102–104°C. IR (KBr, cm^{-1}) 1720, 1620. UV (MeOH) max (nm) 222, 247, 270, 306. [^{1}H]NMR ($CDCl_3$) 8.20 (d, 1H, $J_{4,3}$ = 12 Hz, H-4), 7.61 (d, 1H, $J_{10,9}$ = 2 Hz, H-10), 7.20 (s, 1H, H-5), 6.95 (d, 1H, $J_{9,10}$ = 2 Hz, H-9), 6.31 (d, 1H, $J_{3,4}$ = 12 Hz, H-3), 4.61 (dd, 1H, $J_{1a',1b'}$ = 11 Hz, $J_{1a',2'}$ = 4.5 Hz,

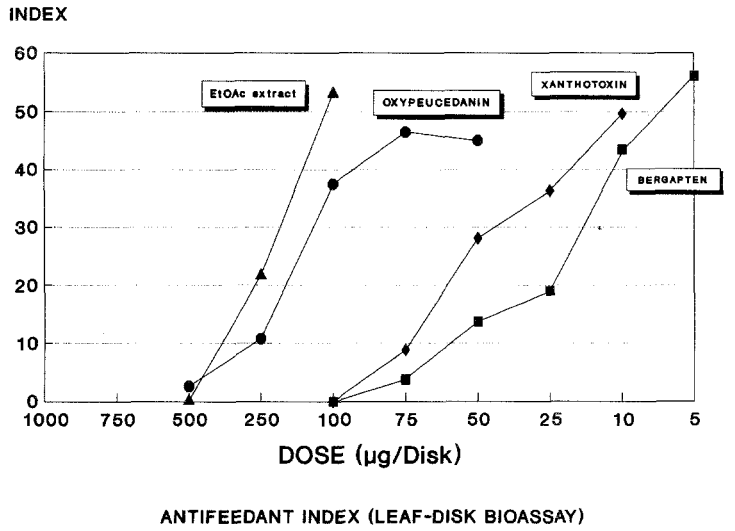


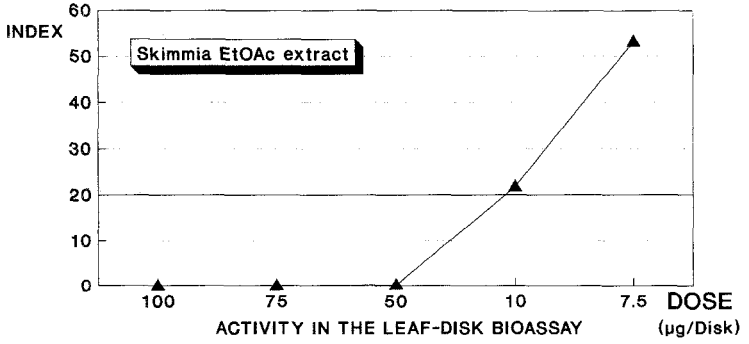
FIG. 4. Dose–response activity for the ethyl acetate fractions and the purified furanocoumarins.

H-1a′), 4.32 (dd, 1H, $J_{1b',1a'}$ = 11 Hz, $J_{1b',2'}$ = 4.5 Hz, H-1b′), 3.28 (dd, 1H, $J_{2',1a'}$ = 4.5 Hz, $J_{2',1b'}$ = 4.5 Hz, H-2′), 1.41 (s, 3H, CH_3), 1.33 (s, 3H, CH_3).

Evaluation of Sensitivity. Figure 5 summarizes the results of the comparison between the leaf-disk bioassay method and the bioautography method. The threshold of activity (index = 20) in the leaf-disk bioassay is approximately 10 $\mu g/cm^2$ (or 10 μg/disk). In the TLC bioassay, activity could be detected with a lower limit of 20 μg for spots and 50 μg for bands.

DISCUSSION

Our results show that bioautography is a quick and efficient method for the isolation of insect-active compounds from complex mixtures. In the case studied here, at least two fractionation steps and subsequent purification of the samples would have been necessary in order to isolate the active constituents using traditional leaf-disk methodology to monitor the activity. In terms of bioassays, this would have translated into several dozen individual experiments. In the example reported here, total time required to obtain the R_f values of the active compounds was only four days. This can be advantageously compared with the time usually required to monitor all fractionation steps with an insect bioassay. Hence, bioautography provides a substantial reduction in cost, time, and labor.



DOSE (µg)	500	250	100	50	20	10
SPOTS	+++	+++	+++	++	+	-
BANDS	+++	+++	++	+	-	-

DETECTION OF ACTIVITY ON THE TLC PLATE BIOASSAY

FIG. 5. Comparison of the sensitivity between the leaf-disk bioassay method and the TLC bioautography method. (+ indicates visually detectable quenching under UV light. SPOT/BAND indicates mode of initial sample application).

The sensitivity study shows that there is almost no loss of information when using this new method. Good sensitivity is important, especially in the final stages of the purification, when small amounts of material are available for testing, sometimes not compatible with the concentration or replication requirements of the classical bioassays. In any event, most problems with sensitivity can easily be overcome by increasing the amount of original material extracted. With plants, the amount of material is generally not a limiting factor.

One potential drawback of bioautography would be in the case of high synergistic effects between compounds otherwise possessing weak individual activity. Their elution and separation would then result in a loss of activity. Another potential problem is the possible degradation of proteinaceous substances or evaporation of volatile compounds when coming in contact with the hot diet. Although minimized with prior cooling of the diet, this problem should be considered when the presence of such compounds is suspected. Similarly, photoactivated compounds might not be detected unless a second set of experiments in the presence of UV irradiation is conducted. It should be noted that, although dark conditions were found preferable, *Spodoptera litura* larvae can feed in the presence of light.

The difference of sensitivity observed when applying the extract as spots or as bands can easily be explained by the concentration of the sample, higher

in the case of spots, therefore avoiding a "dilution" of the antifeedant effect during the bioassay. In the most favorable case, under the experimental conditions, the sensitivity of the TLC bioassay was only two times lower than the leaf-disk bioassay. Usually, application in bands was preferred since the response is clearer than with spots. A TLC analysis conducted with an amount of 1–5 mg of dry material would therefore represent a good compromise for starting an analysis. However, since the detection of activity depends on the sample, the nature of the active constituents, and their concentration in the extract, no absolute starting dose can be defined and a higher initial concentration can be used. However, personal experience has shown that an excessive amount of material can induce wide diffusion of the compounds in the medium, thus broadening the uneaten bands and reducing the precision of the method.

As for separation conditions, any type of TLC plate can be used, since the animals do not consume the chromatographic medium. However, a rigid support such as glass is preferable for easy manipulation and to avoid deformations of the diet layer.

The most important problem associated with this technique is the transfer rate of compounds from the silica plate into the agar-based diet. This rate is presumably dependent on the hydrophilic–lipophilic character of the compounds. In the examples we have studied so far, such as aristolochic acids, triterpenoids (Escoubas et al., unpublished data), or furanocoumarins, the transfer rate was satisfactory and allowed for a clear visualization of uneaten areas. This was probably due to the ability of these compounds to partially dissolve in aqueous solvents as well as to the partial lipophilic character of the diet, which contains a certain proportion of lipids, since these compounds are rather lipid-soluble ones (Harborne, 1984). In the case of very lipophilic compounds, migration into the diet could be a problem, preventing the successful identification of active compounds. New diet formulations, with an increased lipid content, might solve this problem. It should be noted, however, that excessive diffusion of compounds in the diet would defeat the purpose of the method, by extending the uneaten area and therefore inducing an error in determining the R_f values of the active components.

The three active furanocoumarins isolated have previously been identified in other representatives of the genus *Skimmia* (Weinstein and Craig, 1971; Razdan et al., 1987). Although furanocoumarins of *Skimmia japonica* have been previously investigated (Atkinson et al., 1974; Reisch and Achenbach, 1989), and ovicidal activity against acarians described (Tanaka et al., 1985), no mention was made of their role in the plant protection against herbivorous insects.

Under our experimental conditions, we found that bergapten was a more potent antifeedant against *S. litura* than oxypeucedanin or xanthotoxin. This result complements previous work (Yajima et al., 1977; Yajima and Munakata, 1979), and once again raises the question of whether or not a high antifeedant

activity is linked to the high phototoxic activity of xanthotoxin and bergapten, as noted previously (Klocke et al., 1989). Our bioassays were conducted in darkness, thus limiting the potential influence of phototoxicity on test results. According to our results, the substitution of a methoxy group in position 8, by a 2′,3′-epoxy-3′-methylbutyloxy group, substantially decreased the antifeedant activity against *S. litura*. However, response and activity levels vary with the test insects used, and structure–activity relationships can hardly be generalized.

S. japonica defense systems against herbivores and pathogens are complex and involve compounds with both feeding deterrent and growth-inhibitory activity (Ochi et al., 1988). The presence of limonoids and furanocoumarins in the leaves and fruits provides this plant with a very high defense potential against a wide array of natural enemies. Furthermore, both phototoxic and nonphototoxic metabolites are involved, making this plant an interesting model for the study of complex defense strategies. Furanocoumarins show phototoxic, chronic, and acute toxicity as well as phagodeterrent activities in members of the Rutaceae and Umbelliferae (Luthria et al., 1989; Nitao, 1990; Zobel et al., 1991) and undoubtedly represent an important element of the plant's defense system.

CONCLUSION

Isolation of bioactive compounds from complex plant extracts is usually a very long process. To fractionate and then subfractionate crude mixtures represents a laborious chemical endeavor. Assessing the activity of all fractions is equally time- and effort-consuming, considering that only a small percentage of that effort produces a significant output because active substances can comprise only a few out of a large number of constituents. Bioautography solves these problems by considerably speeding the isolation of active compounds. In the case of antifeedants, we demonstrate here that bioautography is faster than traditional leaf-disk bioassays, with no loss of information or sensitivity.

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ALLELOCHEMICALS FROM *Polygonum sachalinense* FR. SCHM. (POLYGONACEAE)

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Abstract—The root exudates from *Polygonum sachalinense* in a recirculating system significantly inhibited lettuce seedling growth. The rhizomes and roots of *P. sachalinense* were extracted with 80% acetone. Bioassay of the neutral–acidic fraction on the TLC agar plate showed the inhibitory activity corresponded to the two yellow pigment bands. Two orange needles were isolated and identified as anthraquinone compounds: emodin and physcion. Both compounds exhibited inhibitory activities against the seedling growth of several testing plant species. Glucosides were isolated from *P. sachalinense* and were identified as emodin-1-*O*-β-D-glucoside and physcion-1-*O*-β-D-glucoside, respectively. On plant growth bioassay, these glucosides showed no phytotoxic activity against lettuce seedlings. The concentrations of emodin, physcion, and their glucosides from rhizome with roots, aerial parts, fallen leaves, and soil were determined. The rhizome with roots and fallen leaves contained emodin and physcion at relatively high concentrations. Emodin also occurs in the soil of this plant community with effective concentrations in the fall. The results indicate that these anthraquinones are responsible for the observed interference and are potent allelopathic substances.

Key Words—*Polygonum sachalinense*, allelopathy, allelochemicals, anthraquinone, emodin, emodin-1-*O*-D-glucoside, physcion, physcion-1-*O*-D-glucoside, root exudates.

INTRODUCTION

Chemical interactions among plants have long been recognized. Many scientists have shown that certain plants produce secondary compounds that have the

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ability to inhibit growth and germination of other plants (Rice, 1984; Harborne, 1988). The genus *Polygonum* has about 300 species and is distributed all over the world. AlSaadawi and coworkers demonstrated that *Polygonum aviculare* was allelopathic, and several phenolic compounds and long-chain fatty acids appeared to be responsible for this activity (AlSaadawi and Rice, 1982a,b; AlSaadawi et al., 1983).

P. sachalinense (Sachaline giant knotweed), or "Ohitadori" in Japanese, is a perennial grass found in Honshu and Hokkaido. Its rhizome has been used as an herbal medicine for laxative and diuretic purposes. This grass forms colonies along roadsides and river banks in gravelly soils. Judging from its ability to rapidly colonize, we hypothesize that allelopathy contributes to its aggressiveness.

This paper deals with the isolation and identification of allelochemicals in *P. sachalinense* and the evaluation of these allelochemicals in the ecosystem.

METHODS AND MATERIALS

Root Exudate Recirculating Systems. The evaluation of the effect of the root exudates as allelochemicals was carried out according to the root exudate recirculating system of Stevens and Tang (1985). Lettuce seedlings were planted in the acceptor pot, while *P. sachalinense* was grown in the donor pot. Nutrient solution (Tadano and Tanaka, 1976) from the donor pot was lifted through 8-mm-diameter glass tubing to the acceptor pot using an air pump. Nutrient levels were maintained, and pH was kept 5.5 with H_2SO_4 or NaOH. Recirculating solution was not changed during the experiment except for replenishing water and nutrient solution. After 10–14 days, the growing condition of lettuce (*Lactuca sativa* L.) seedlings was compared with that of the control growing under the same conditions without the donor plant. The experiment was carried out in a greenhouse.

Plant Materials and Extraction. Rhizomes with roots, aerial parts, and fallen leaves of *P. sachalinense* were collected at several wild places near the campus of Hokkaido Tokai University. Excavated rhizomes were rinsed with tap water and brushed lightly to remove soil, and air-dried. The rhizome (5.1 kg) and aerial part (3.8 kg) were sliced and extracted in separate containers with 80% acetone for about two months at room temperature. The extracts were filtered and concentrated under reduced pressure. In another experiment, 100 g of fallen leaves were extracted with 300 ml of ether for 24 hr, and the residues were removed by filtration.

Fractionation. The acetone extract of rhizome with roots was divided into ether-soluble and water-soluble fractions. The ether-soluble fraction was further divided into neutral, acidic, and basic fractions in an usual manner. The water-

soluble fraction was evaporated to dryness for bioassay; it did not show any significant activity against the growth of lettuce seedlings.

TLC Agar Plate Bioassay. Thin-layer chromatography was carried out on a 20 × 7-cm silica gel glass plate (Merck, Kieselgel 60 F254) 0.25 mm thick using hexane–$CHCl_3$–MeOH (4 : 10 : 1 v/v/v) as developing solvents. The chromatogram was covered with a 0.5% agar layer (2 mm), and the seeds of green amaranth (*Amaranthus viridis* L.), timothy grass (*Phleum pratense* L.), crabgrass (*Digitaria adscendens* Henr.), and Chinese cabbage (*Brassica campestris* L.) were sown in a row perpendicular to the bands on the chromatogram. The seeds were incubated in a moisture saturated growth chamber at 24–26°C with 14 hr light and 10 hr dark. After four to seven days, seed germination and plant growth were examined.

Seedling Growth Bioassay. In a 25-mm-high × 40-mm-ID glass Petri dish 10 germinated seeds were placed on 0.5% agar gel with a known concentration of the test sample. Lettuce, green amaranth, and timothy grass were used for bioassay. The assay was done in duplicate. Incubation was for seven days in a growth chamber at 24–26°C with a 14-hr light and 10-hr dark cycle. The average length of hypocotyl or leaf sheath and the longest roots of the testing plants were measured and compared with that of the control growing on 0.5% agar gel only.

Isolation and Identification of Allelochemicals. The inhibitory substances against seedling growth were fractionated by SiO_2 column chromatography using a hexane–$CHCl_3$–MeOH gradient as an eluent, and the desired products were recrystallized from hexane–$CHCl_3$ to give two orange-needle crystals. The isolated compounds were identified from IR, UV, mass, and NMR spectra.

Emodin **(1):** IR (KBr) ν_{max} 3400 (H)(OH), 1630 (C=O) cm^{-1}; UV (MeOH) λ_{max} 221, 253, 265, 289, 437 nm; [^{1}H]NMR [$(CD_3)_2CO$] δ 2.46 (3H, s, Ar-CH_3), 6.65 (1H, d, J = 2.5 Hz, H-2), 7.12 (1 H, br s, H-7), 7.24 (1H, d, J = 2.5 Hz, H-4), 7.55 (1H, br s, H-5), 12.05 (1H, s, OH), 12.18 (1H, s, OH); HR-EI-MS m/z 270.0515 ($C_{15}H_{10}O_5$).

Physcion **(2):** IR (KBr); ν_{max} 3400 (OH), 1630 (C=O) cm^{-1}; UV (MeOH) λ_{max} 223, 254, 265, 287, 434 nm; [^{1}H]NMR ($CDCl_3$) δ 2.45 (3H, s, Ar-CH_3), 3.94 (3H, s, Ar-OCH_3), 6.68 (1H, d, J = 2.5 Hz, H-2), 7.08 (1H, br s, H-7), 7.36 (1H, d, J = 2.5 Hz, H-4), 7.63 (1H, br s, H-5), 12.12 (1H, s, OH), 12.31 (1H, s, OH); HR-EI-MS m/z 284.0704 ($C_{16}H_{12}O_5$).

Isolation and Identification of Anthraquinone Glycoside. The rhizomes with roots and the aerial part of *P. sachalinense* were collected in June 1991. Immediately after cutting, they were extracted in EtOH by refluxing for 5 hr and the EtOH solution concentrated to one third of the original volume. The aqueous concentrate was extracted with ether to remove free anthraquinones and other ether-soluble constituents. $Ba(OH)_2$ solution (5%) was added to precipitate the organic acids. After filtration, the filtrate was adjusted to pH 3 and was extracted

with *n*-BuOH. The *n*-BuOH layer was separated and evaporated in vacuo to give the crude glycoside fraction, which was further fractionated by SiO_2 column chromatography using a hexane—$CHCl_3$—MeOH as an eluent to obtain anthraquinone glycosides. Rechromatography and further recrystallization from MeOH gave two glycosides as orange-needle crystals. Identification was based on the comparison of the melting point, R_f values, and spectrometric data with those in Kang and Woo (1982) and Kato and Morita (1987).

Emodin-1-*O*-β-D-glucoside **(3):** mp 193°C; IR (KBr) ν_{max} 3400 (OH), 1630 (free C=O), 1600 (chelated C=O) cm^{-1}; UV (EtOH) λ_{max} 221, 253, 265, 286, 421 nm; [^{1}H]NMR (CD_3OD) δ 2.40 (3H, s, Ar-CH_3), 4.96 (1H, d, J = 7.5 Hz, anomeric H), 7.04 (1H, br s, H-7), 7.10 (1H, d, J = 2.5 Hz, H-2), 7.32 (1H, d, J = 2.5 Hz, H-4), 7.48 (1H, br s, H-5); FAB-MS (DMSO) *m/z* (NaCl addition) 455 $(M + Na)^+$, 478 $(M + 2Na)^+$.

Physcion-1-*O*-β-D-glucoside **(4):** mp 240°C; IR (KBr); ν_{max} 3400 (OH), 1640 (free C=O), 1600 (chelated C=O) cm^{-1}; UV (MeOH) λ_{max} 226, 273, 417 nm; [^{1}H]NMR (CD_3OD) δ 2.46 (3H, s, Ar-CH_3), 3.97 (3H, s, Ar-OCH_3), 4.96 (1H, d, J = 7.5 Hz, anomeric H), 7.11 (1H, br s, H-7), 7.30 (1H, d, J = 2.5 Hz, H-2), 7.57 (1H, d, J = 2.5 Hz, H-4), 7.60 (1H, br s, H-5).

Quantitation of Anthraquinones and Anthraquinone Glycosides. Plant samples were collected at several wild places near the campus of Hokkaido Tokai University. Fresh aerial parts, rhizome with roots, and fallen leaves were extracted with 80% acetone. After concentration, the aqueous residues were extracted with ether. The ether-soluble fractions were quantitatively analyzed by HPLC (JASCO System-800) and compared with standard emodin and physcion (purchased from Funakoshi Co. Ltd.). The analytical conditions are as follows: column—YMC-Pack ODS-AM (30 cm × 10 mm ID); solvent—MeOH/H_2O (80:20, v/v); flow rate—4 ml/min, wavelength of detection—245 nm.

Soil samples were collected in November 1990 from the center of a *P. sachalinense* community near the campus of Hokkaido Tokai University at the depth of about 10 cm. Litter was removed as completely as possible by sieving and the soil was dried at room temperature overnight. A 500-g soil sample was stirred in 2 liters of acetone–1 N HCl (2:1, v/v) for 1 hr at 50°C and further treated with ultrasonic energy for 1 min. After leaving overnight at room temperature, it was filtered. The filtrate was evaporated to remove acetone and extracted three times with ether. The ether extract was evaporated in vacuo and fractionated by SiO_2 column chromatography. The contents of emodin and physcion were determined by HPLC. The amounts of anthraquinone glycosides were determined by weighing the isolated crystals of emodin-1-*O*-β-D-glucoside and physcion-1-*O*-β-D-glucoside.

Spectrometers. IR spectra were recorded on a JASCO IR-810 as KBr disk for crystals. UV spectra were recorded on a Hitachi U-3210 spectrophotometer. NMR spectra were measured on a Bruker AM-400 (400 MHz) spectrometer

using TMS as an internal standard. Mass spectra were recorded using JEOL JMS-DX303HF GC-MS spectrometer.

RESULTS AND DISCUSSION

The root exudates from *P. sachalinense* showed an inhibition on lettuce seedling growth using the recirculating system. It is well known that organic compounds are exuded from healthy undamaged plant roots (Rovira, 1969), and we expect that allelochemicals or their precursors are present in the plant tissue and also released from the rhizome and roots.

From the results of the TLC agar plate bioassay, growth inhibitory activity was shown in the two bands of yellow pigments against green amaranth and timothy grass on the chromatogram of the neutral–acid fraction (Figure 1). Two corresponding orange needles were isolated and identified as anthraquinone compounds: emodin **(1)** and physcion **(2)** (Figure 2).

Emodin and physcion exhibited inhibitory activities against the seedling growth of several test species (Figure 3). Emodin inhibited root and hypocotyl or leaf sheath growth over 100 ppm (3.7×10^{-4} M). It severely inhibited the growth of lettuce seedlings at 50 ppm (1.85×10^{-4} M). Physcion was less active and inhibited root and hypocotyl growth at 200 ppm (7.0×10^{-4} M).

The presence of anthraquinone glucosides was expected since they are common for transportation and storage and are less toxic in the plant. As a result, two glucosides were isolated from both the rhizome and aerial part of *P.*

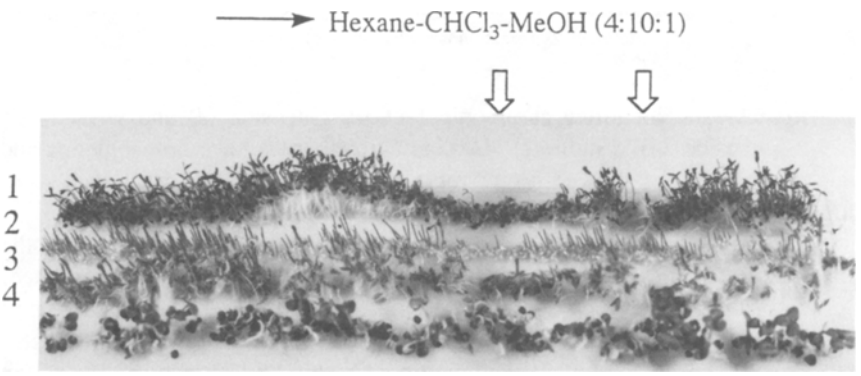
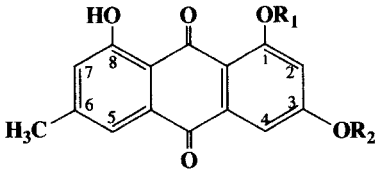


FIG. 1. Bioassay of neutral and acid substances from *Polygonum sachalinese* by TLC agar plate method (5 days). Arrows point to areas of inhibited plant activity. (1) green amaranth, (2) timothy grass, (3) crab grass, (4) Chinese cabbage.



(1) R_1=H, R_2=H Emodin
(2) R_1=H, R_2=CH_3 Physcion
(3) R_1=Glu, R_2=H Emodin-1-*O*-β-D-glucoside
(4) R_1=Glu, R_2=CH_3 Physcion-1-*O*-β-D-glucoside

FIG. 2. Chemical structures of allelochemical candidates from *Polygonum sachalinense*.

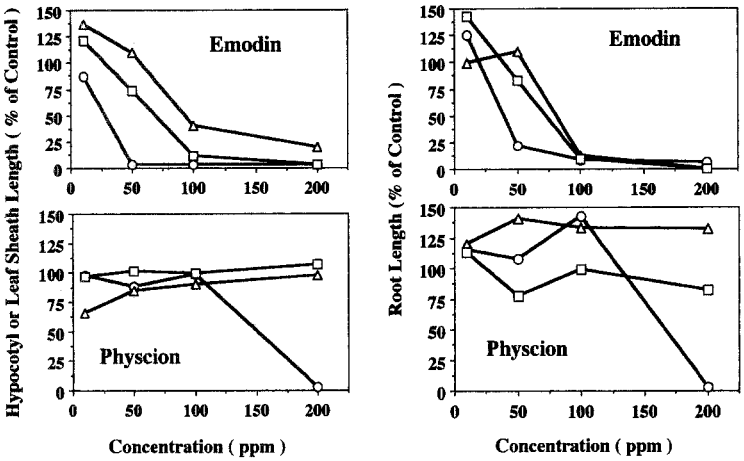


FIG. 3. Growth inhibitory activities of emodin and physcion against plant seedlings. ○: lettuce, △: green amaranth, □: timothy grass.

sachalinense and identified as emodin-1-*O*-β-D-glucoside **(3)** and physcion-1-*O*-β-D-glucoside **(4)** (Figure 2). In plant growth bioassay, both emodin and physcion glucosides showed no phytotoxicity at 200 ppm against lettuce seedlings.

The concentrations of emodin and physcion, and their glucosides in the rhizome, aerial part, fallen leaves, and soil were quantitatively determined. Contents of emodin and physcion in the fresh rhizome were ca. 158 and 32 mg/kg fresh weight, and in the aerial part, ca. 72 and 22 mg/kg fresh weight, respectively. Large amounts of anthraquinones were still detected in dry fallen leaves more than four months after defoliation: emodin, 213 mg/kg dry weight; physcion, 180 mg/kg dry weight. In addition, contents of emodin and physcion in the soil were 55 and 30 mg/kg dry weight, respectively; these concentrations

are enough to inhibit plant seedlings. These facts indicate that the anthraquinones are very stable in the ecosystem.

Allelopathic substances are released from plants in four ways (Tukey, 1969): (1) Litter of leaves and stems decomposes by physical or biological processes and the substance is released. (2) The active substance is released to the soil from the root directly by exudation or through the decay of dead roots. (3) Volatile material is vaporized from plants and acts on other plants through the air. (4) Rain and fog drips transfer toxic compounds from leaves to the soil.

We consider that the first two items above are associated with the allelopathy of *P. sachalinense*. It has anthraquinone glucosides in the plant tissue. We propose that either these glucosides or the aglycons are released from the rhizome. The glucosides are then decomposed to active forms—emodin and physcion—and subsequently exhibit plant growth inhibitory activities against other plant species. To confirm this hypothesis, isolating glucosides and aglycons from the root exudate solution is currently under investigation. Furthermore, anthraquinones exuded from the fallen leaves of *P. sachalinense* accumulate on the soil surface and affects early seedling growth of nearby plants of the community.

There are reports about antimicrobial activities of emodin and physcion (Podojil et al., 1978; Anke et al., 1980; Kitanaka and Takido, 1986). Some antimicrobial activity by emodin and physcion may directly or indirectly affect the growth of other plants. Rice (1964, 1984) and AlSaadawi et al. (1983) pointed out that some weeds produce allelopathic chemicals that inhibited soil bacteria and changed the soil environment to their own advantage. While these anthraquinone compounds are potent allelopathic substances, the question of the extent of their impact against the flora in *P. sachalinense* is yet to be fully evaluated.

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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

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Abstract—In an earlier study we documented attraction of the specialist parasitoid *Macrocentrus grandii* Goidanich to odors of corn, potato, and snap bean. In the present study the chemical bases of the parasitoid's attraction to these food plants of its European corn borer host were compared. Volatile compounds from corn leaves were isolated using Tenax and identified by capillary gas chromatographic–mass spectrometry. Twenty-one compounds including aldehydes, ketones, alcohols, esters, and sesquiterpenes were present in corn. These were separated into fractions by column chromatography on Florisil. Wind-tunnel bioassays of the fractions indicated that *M. grandii* was attracted to fractions containing nonpolar and slightly polar compounds including sesquiterpenes, aldehydes, a ketone, and esters. More polar compounds in corn, like alcohols, were not attractive. Attraction to potato odor was based on the presence of the same classes of compounds that were attractive in corn, but more polar compounds were involved in attraction to snap bean odor. This study indicated that only some compounds in each of the three odor complexes tested were attractive. It also documented that different compounds were involved in attraction of *M. grandii* to different plant odors.

Key Words—*Macrocentrus grandii*, Hymenoptera, Braconidae, European

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corn borer, *Ostrinia nubilalis*, Lepidoptera, Pyralidae, behavior, synomones, allelochemicals, column chromatography, gas chromatography, mass spectra.

INTRODUCTION

Parasitoids of phytophagous insects use olfactory cues for host location. In long-range orientation, volatile cues emanating from plants are used for locating habitats where hosts are likely to be present. Some parasitoids, however, are attracted to odors of only certain food plants of their hosts (Shahjahan, 1974; Elzen et al., 1983; Ding et al., 1989b; Navasero and Elzen, 1989; Martin et al., 1990). This is likely to affect the range of plants on which their hosts are parasitized. If the searching behavior of a parasitoid in the field can be increased by application of attractive plant odors, then this strategy can be incorporated in biological control programs for retaining a parasitoid in a habitat or introducing it to a less preferred habitat.

For their efficient use in biological control programs, attractive plant compounds need to be identified. Plant odors contain numerous compounds of which only some may be critical for parasitoid attraction. Plant compounds involved in interactions between plants and parasitoids are termed synomones (allelochemicals that mediate mutually beneficial interactions, Nordlund and Lewis, 1976). In several studies (Read et al., 1970; Camors and Payne, 1972; Elzen et al., 1984; Sugimoto et al., 1988; Turlings et al., 1991) plant synomones involved in habitat location behavior of parasitoids were identified. However, the chemical basis of a parasitoid's differential response to several food plants of its host has not been analyzed.

Macrocentrus grandii Goidanich (Hymenoptera: Braconidae) is a specialist parasitoid that was introduced into the United States for biological control of European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae) in corn (Parker, 1931). While *M. grandii* is established in several U.S. regions (Clausen, 1956; Peairs and Lilly, 1975; Lewis, 1982) parasitism levels in corn have been low. According to Parker (1931), the inefficiency of this parasitoid is due to the limited ability of the female to locate corn borers. The European corn borer is polyphagous but *M. grandii* locates it only in certain habitats. In an earlier study (Udayagiri, 1991) *M. grandii* exhibited different olfactory responses to food plants of its host. It was attracted to volatiles of corn, potato, and snap bean but not to those of pepper and soybean. It may be possible to increase searching behavior of female *M. grandii* by providing chemical stimuli attractive to the parasitoid. If specific volatile plant compounds critical for attraction are identified and synthesized, they might be used for increasing host-location efficiency of *M. grandii*. It may also be possible to use plant synomones for introducing *M. grandii* to less attractive habitats.

In the present study, we examined the chemical basis of habitat location

behavior in *M. grandii*. We were interested in (1) identifying constituents of corn odor that are responsible for long-range attraction by *M. grandii* and (2) determining whether attraction to other plant odors was based on the presence of similar or different volatile compounds than those found to be attractive in corn.

METHODS AND MATERIALS

Plant Material. Corn (Jubilee), potato (Reddale), and snap bean (Strike) were raised during the summer of 1990 at the Minnesota Agricultural Experimental Station, University of Minnesota, in St. Paul, Minnesota. Normal cultivation practices were used but no pesticides were applied.

Insects. European corn borer larvae were maintained on an artificial diet (Guthrie et al., 1971), and *M. grandii* were reared on European corn borer larvae as described by Ding et al. (1989a). Adult parasitoids were transferred on the day of emergence to Plexiglas/aluminum cages 30 × 30 × 45 cm with nylon netting on three sides, provided with honey and water, and maintained at 23°C and 70–80% relative humidity under a 18:6 light–dark photoperiod. Experiments were conducted 4–6 hr into the photophase. One-week-old, mated, naive (with no previous oviposition experience or exposure to larval frass or foliage) *M. grandii* females were used for bioassays.

Isolation of Plant Volatiles. Leaves were collected from corn, potato, and snap bean plants 40–50 days from planting date. In separate collections, volatile compounds were isolated from the leaves using the Tenax trapping method described by Buttery and Ling (1984). Whole leaves (500 g) were placed in a 20-liter flask connected to a Tenax trap. The trap consisted of a Pyrex tube (15 cm long and 1.5 cm diameter) packed with 2 g of Tenax. Collection of volatiles from the atmosphere around the leaves was continued for 12 hrs. Volatiles were eluted from the trap with 10 ml pentane. Each collection provided 600 g hr of volatiles [(500 g × 12 hr) ÷ 10 ml solvent where 1 g hr = volatiles from 1 g of plant material aerated for 1 hr and eluted with 1 ml solvent]. Plant extracts were stored at −70°C until used.

Identification of Plant Volatiles. Volatile compounds in corn extract were identified by gas chromatographic–mass spectral analysis. Identities were confirmed by comparison of gas chromatographic and mass spectral data with those of standards obtained from commercial sources.

Extracts of corn volatiles in pentane were separated with a J & W 30-m × 0.53-mm-ID DB-wax fused silica capillary column with 1 μm film thickness on a Hewlett-Packard 5890A gas chromatograph equipped with a flame ionization detector and coupled to a HP 3392A integrator. The gas chromatographic unit was temperature programmed from 40°C to 200°C at 5°C/min with a 2-min

initial delay. The carrier gas was helium at a linear velocity of 47 cm/sec at 150°C. For each extract, 1 μl of each sample (= 0.6 g hr) was injected in the splitless mode.

Equivalent chain length (ECL) (Miwa, 1963) for each corn compound was calculated by running internal and external standards. Two alkanes, dodecane (C-12) and eicosane (C-20), were added as internal standards to the extract of corn volatiles. A mixture of external standards was prepared by combining equal quantities of nine consecutive alkanes from dodecane to eicosane. The mixture of external standards and the corn extract containing the internal standards were chromatographed and the retention time (on the gas chromatograph column) of each compound was recorded. The chromatogram of corn extract with the internal standards was superimposed over the chromatogram of the external standard based on the two internal standard alkanes and ECLs were determined by comparison of the retention time of each corn compound with those of the alkanes.

Mass spectral data were obtained from a Kratos MS 25 gas chromatograph–mass spectrometer. Samples were injected in the splitless mode through the same J & W DB-wax column and the samples were fractionated using the temperature program listed above. The electron ionization energy was 70 eV. Mass spectral data were compared with mass spectra of standards obtained from commercial sources and with mass spectra published by McLafferty and Stauffer (1989).

Compounds in the corn extract were considered to be identified if their mass spectral and gas chromatographic retention data were consistent with those of standards obtained from commercial sources run on the same instruments. Standards used (purity given in parentheses) were: hexanal (99%), (*E*)-2-hexenal (99%), 2-heptanone (98%), hexanol (98%), and (Z)-3-hexen-1-ol (98%) from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin; cyclosativene (>99%) from Fluka Chemical Corp., Ronkonkoma, New York; β-caryophyllene (84%) from Pfaltz & Bauer, Inc., Waterbury, Connecticut; and (Z)-3-hexen-1-yl acetate (approximately 98%) from Sigma Chemical Company, St. Louis, Missouri.

Separation of Plant Volatiles. Compounds in volatile extracts of corn, potato, and snap bean were separated by column chromatography on Florisil. Florisil (2 g) was added to a Pyrex tube to create a 2 × 5-cm column. A small plug of glasswool was placed at the bottom to support Florisil and a narrow layer of anhydrous sodium sulfate (0.2 g) was placed above Florisil for absorption of water.

Volatile extracts from three collections (= 1800 g hr) were combined and concentrated to 0.5 ml under nitrogen. The concentrated material was loaded onto the Florisil column and the column was eluted consecutively with 3 ml (= one void volume of the column) of solvents of increasing polarity. The solvents used were pentane (P) + ether (E) in the following combinations: 100%

P, 97.5% P + 2.5% E, 95% P + 5% E, 92.5% P + 7.5% E, 90% P + 10% E, 80% P + 20% E, 50% P + 50% E and 100% E followed by acetone and methanol. Solvents used were of analytical quality. Ten fractions (each containing 3 ml of solvent) were collected and 1 μl of each fraction was injected on the gas chromatograph to determine evidence of plant compounds. Fractions that contained visible quantities (>1 ng) of plant compounds were retained and stored at $-70°C$.

Bioassay of Plant Volatiles after Separation. Fractions that contained plant compounds were bioassayed for behavioral activity by presenting them in separate tests in the wind tunnel olfactometer as described by Udayagiri (1991). Aliquots of each fraction (200 μl) were tested and the response of 10 naive *M. grandii* was determined. When a particular fraction was bioassayed, the solvent used for eluting the fraction was used as the control. Each bioassay was replicated five times.

Statistical Analysis. Categorical data from bioassays for each Florisil fraction were analyzed by constructing contingency tables and fitting log linear models (Fienberg, 1989). Goodness-of-fit for each model was determined by examining the log likelihood ratio chi-squared statistic [$= G^2 = 2\Sigma$ (observed No.) $\times$ log (observed No. $\div$ expected No.)] (Fienberg, 1989). Model selection was done as described by Sakamoto and Akaike (1978) using AIC (Akaike Information Criterion), where AIC $= G^2 - 2\,df +$ No. of cells in the contingency table. Models with low G^2 and large degrees of freedom that resulted in low AIC and $P > 0.05$ were considered to provide a good fit. The effect of a specific variable was determined by estimation of ΔG^2 values for nested models. High ΔG^2 values with low degrees of freedom and $P < 0.05$ were considered to be indicative of strong effects. The Bonferroni method (Devore and Peck, 1986) was adopted for multiple comparisons between treatments in each bioassay and P values were obtained by comparison of estimated values with values from a standard z table. Each Florisil fraction was assayed independently and hence comparisons were not made across fractions. G^2 and Bonferroni critical values were obtained using the 'S' package (Becker et al., 1988) on the UNIX system.

RESULTS

Identification of Plant Volatiles. Table 1 lists the 21 volatile compounds isolated from leaves of corn. These included aldehydes, ketones, alcohols, esters, and sesquiterpenes. Hexanal, (*E*)-2-hexenal, 2-heptanone, (*Z*)-3-hexen-1-yl acetate, hexanol, (*Z*)-3-hexen-1-ol, cyclosativene, and β-caryophyllene were identified by comparing the spectrum of the mass of each compound against that of a synthetic standard. ECLs of these compounds matched those of standards run on the same gas chromatographic column. Mass spectra of (*Z*)-3-

TABLE 1. VOLATILE COMPOUNDS ISOLATED FROM CORN LEAVES (JUBILEE) USING TENAX TRAPPING

Compound	Mass spectral ions[a]	ECL[b]	Rel %
Aldehydes			
Hexanal	44, 56, 72, 82	10.93	1.0
(Z)-3-Hexenal[c]	41, 69, 81, 55, 96	11.67	4.4
(E)-2-Hexenal	41, 55, 69, 83, 98	12.26	5.7
Ketones			
2-Heptanone	43, 58, 71, 114,	11.91	6.9
(E)-4-Hepten-2-one[c]	43, 67, 112, 53	12.60	2.9
Alcohols and esters			
2-Heptanol acetate[c]	43, 56, 87, 69, 98	12.74	2.9
(Z)-3-Hexen-1-yl acetate	43, 67, 82	13.28	2.3
(E)-4-Hepten-2-ol[c]	45, 55, 69, 83, 98	13.33	2.6
Hexanol	44, 56, 69	13.67	3.2
(Z)-3-Hexen-1-ol	41, 67, 55, 82, 69	13.97	12.4
Sesquiterpenes			
(+)-Cyclosativene	105, 41, 119, 93, 161, 204	14.96	18.0
Ylangene[c]	43, 119, 105, 161, 91	15.06	1.6
β-Caryophyllene	41, 69, 93, 79, 133, 55, 161	16.14	27.7
α-Humulene[c]	93, 43, 121, 105	16.86	1.3
δ-Cadinene[c]	161, 43, 91, 105, 119, 133	17.79	1.6
Unidentified sesquiterpene	43, 161, 120, 91, 69, 105	15.89	1.8
Unidentified sesquiterpene	43, 69, 91, 81, 119, 161	16.94	0.9
Unidentified sesquiterpene	43, 105, 161, 93, 69, 119	17.06	0.7
Unidentified sesquiterpene	43, 131, 161, 105	17.28	0.9
Unidentified sesquiterpene	43, 69, 105, 93, 161, 204	17.43	<0.3
Unidentified sesquiterpene	43, 105, 161, 91	18.20	0.9

[a] Major fragmentation ion values (*m/z*) over 40 in descending order of intensity.
[b] Equivalent chain length, which indicates the affinity of a compound on a DB-wax gas chromatographic column relative to that of standard alkanes.
[c] No authentic sample was available; identification based on published spectra (McLafferty and Stauffer, 1989).

hexenal, (*E*)-4-hepten-2-one, 2-heptanol acetate, (*E*)-4-hepten-2-ol, ylangene, α-humulene and δ-cadinene matched published spectra (McLafferty and Stauffer, 1989) of the same compounds, but commercial standards of these compounds were not available for confirmation of the identities. Mass spectral data indicated the presence of six additional sesquiterpenes.

Separation of Plant Volatiles. Gas chromatographic analysis indicated that visible quantities (>1 ng) of several compounds were present in each of the three plant extracts. During column chromatography 10 fractions were collected

for each plant extract. However, only fractions 1, 4, 5, and 7 contained visible quantities (>1 ng) of compounds based on gas chromatographic analysis.

The volatile profile of whole-corn extract prior to Florisil separation was compared with the profile of each Florisil fraction of corn on the gas chromatograph. Identities of compounds in each Florisil fraction are listed in Table 2.

Bioassay of Plant Volatiles after Separation. For analyzing results of the bioassays, 2 (No. of choices) $\times$ 5 (No. of replications) contingency tables with a total of 10 cells were set up. Model selection based on lowest AIC values indicated that, for all bioassays, the model that provided the best fit was the model that considered the effects of choice between the fraction containing plant odor and the control.

Corn. The effects of preference were significant for fraction 1 with $\Delta G^2 = 37.99$ at $df = 1$ and $P < 0.001$ and for fraction 4 with $\Delta G^2 = 13.55$ at $df = 1$ and $P < 0.001$. There were more landings on wicks containing fraction 1 and fraction 4 compared to control wicks (Figure 1). Based on the Bonferroni comparison approach, these differences in the number of landings were significant for fraction 1 with $z = 4.71$ and $P < 0.001$ and for fraction 4 with $z = 2.86$ and $P = 0.004$.

TABLE 2. SEPARATION OF COMPOUNDS IN VOLATILE EXTRACT OF CORN USING COLUMN CHROMATOGRAPHY ON FLORISIL

Fraction	Compounds eluted
1 (eluted with pentane)	(+)-cyclosativene
	ylangene
	β-caryophyllene
	α-humulene
	δ-cadinene
	6 unidentified sesquiterpenes
4 (eluted with 92.5% pentane + 7.5% ether)	hexanal
	(*Z*)-3-hexenal
	2-heptanol acetate
	(*Z*)-3-hexen-1-yl acetate[a]
5 (eluted with 90% pentane + 10% ether)	(*E*)-2-hexenal
	2-heptanone[b]
	(*E*)-4-hepten-2-one
7 (eluted with 50% pentane + 50% ether)	(*E*)-4-hepten-2-ol
	hexanol
	(*Z*)-3-hexen-1-ol

[a] Minor quantity eluted in fraction 5.
[b] Minor quantity eluted in fraction 4.

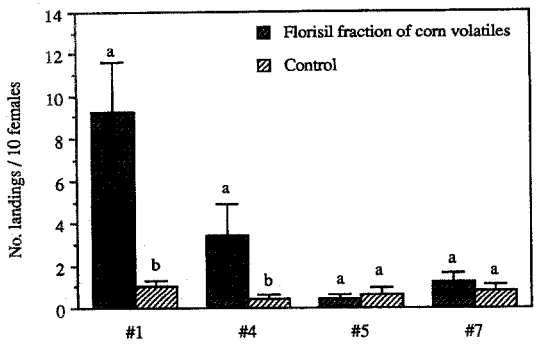

FIG. 1. Response of *Macrocentrus grandii* females to Florisil fractions of corn volatiles. Number of females tested in each assay was 50. Treatments with different letters within the same fraction are significantly different ($P < 0.05$). Comparisons cannot be made across fractions since each fraction was bioassayed in a separate test.

In bioassays that tested the activities of fractions 5 and 7, the effects of preference were not significant. For fraction 5 $\Delta G^2 = 0.2$ at $df = 1$ and $P = 0.65$ and for fraction 7 $\Delta G^2 = 0.4$ at $df = 1$ and $P = 0.53$. The difference between the number of landings on the wick containing fraction 5 and on the control wick was not statistically significant ($z = 0.44$; $P = 0.66$). Similarly, there was no significant difference between the number of landings on wicks containing fraction 7 and the control ($z = 0.63$; $P = 0.53$) (Figure 1).

Potato. The effects of preference were significant for fraction 1 where $\Delta G^2 = 19.81$ at $df = 1$ and $P < 0.001$ and for fraction 4 where $\Delta G^2 = 5.01$ at $df = 1$ and $P = 0.03$. Based on the Bonferroni comparison approach, these differences were significant for fraction 1 ($z = 3.72$; $P < 0.001$) and for fraction 4 ($z = 2.12$; $P = 0.03$) (Figure 2).

In assays that tested the effects of fractions 5 and 7, the effects of preference were not significant. For fraction 5 $\Delta G^2 = 2.32$ at $df = 1$ and $P = 0.13$ and for fraction 7 $\Delta G^2 = 2.84$ at $df = 1$ and $P = 0.09$. The difference between the number of landings between the wick containing fraction 5 and the control wick was not statistically significant ($z = 1.49$; $P = 0.14$) (Figure 2). There was also no significant difference between the number of landings on wicks containing fraction 7 and the control ($z = 1.64$; $P = 0.1$).

Snap Bean. The effects of preference were not significant for fraction 1 with $\Delta G^2 = 2.65$ at $df = 1$ and $P = 0.1$ and fraction 4 with $\Delta G^2 = 0.29$ at $df = 1$ and $P = 0.59$. There were no significant differences in the numbers of landings on control wicks and wicks containing fraction 1 ($z = 1.55$; $P = 0.12$) and fraction 4 ($z = 0.53$; $P = 0.59$) (Figure 3).

The effects of preference were significant for fraction 5 where $\Delta G^2 = 10.13$ at $df = 1$ and $P = 0.0014$ and fraction 7 where $\Delta G^2 = 10.13$ at $df =$

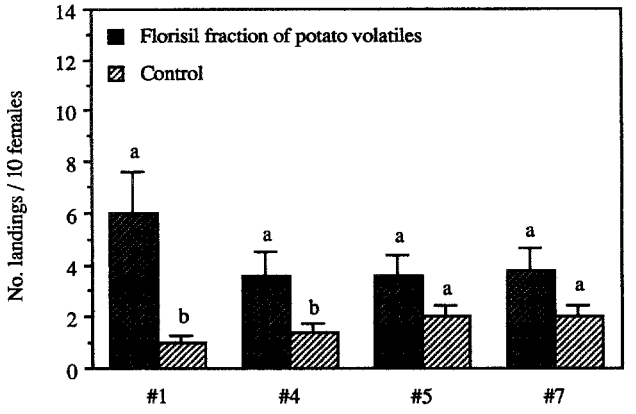

FIG. 2. Response of *Macrocentrus grandii* females to Florisil fractions of potato volatiles. Number of females tested in each assay was 50. Treatments with different letters within the same fraction are significantly different ($P < 0.05$). Comparisons cannot be made across fractions since each fraction was bioassayed in a separate test.

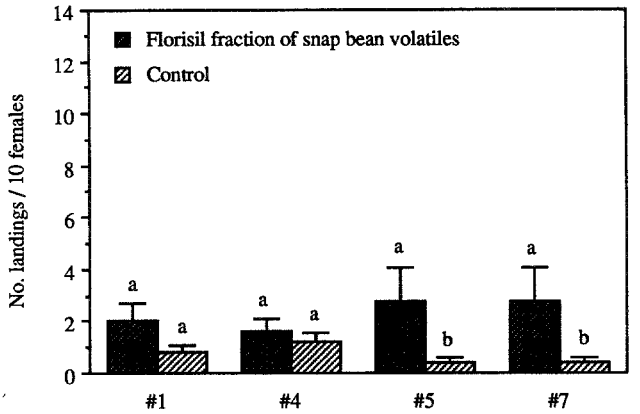

FIG. 3. Response of *Macrocentrus grandii* females to Florisil fractions of snap bean volatiles. Number of females tested in each assay was 50. Treatments with different letters within the same fraction are significantly different ($P < 0.05$). Comparisons cannot be made across fractions since each fraction was bioassayed in a separate test.

1 and $P = 0.0014$ (Figure 3). The difference between the landings on the wick containing fraction 5 with the landings on the control wick was significant ($z = 2.6$; $P = 0.009$). The difference in number of landings containing fraction 7 compared to the control was also significant ($z = 2.57$; $P = 0.01$).

DISCUSSION

Plant odors contain numerous compounds including many that are common among different plants and some that are unique to a plant. Common compounds include green-leaf volatiles, which are six-carbon alcohols, aldehydes, and esters present in varying ratios in different plant species (Visser et al., 1979; Dickens, 1989; Whitman and Eller, 1990). In earlier studies, compounds known to be present in plants were obtained from commercial sources and parasitoid attraction to these compounds was tested. Read et al. (1970) observed that the parasitoid *Diaretiella brassicae* was attracted to allyl isothiocyanate present in cruciferous plants, while Camors and Payne (1972) determined that parasitoids of bark beetles were attracted to α-pinene present in pines. However, a parasitoid could be attracted to more than one compound present in an attractive plant odor. Whitman and Eller (1990) and Ramachandran and Norris (1991) observed that parasitoids were attracted to several green-leaf volatiles obtained from commercial sources. They did not study attraction of these parasitoids to compounds that are unique to specific food plants of their hosts. For a parasitoid, a particular blend of commonly occurring and unique compounds may be critical for attraction. Hence analysis of plant synomones that play a role in plant–parasitoid interactions requires systematic isolation, identification, separation and bioassay of plant compounds.

In the present study, a systematic approach to analyzing plant synomones involved in habitat location behavior of *M. grandii* was attempted by initial isolation and identification of corn volatiles. The isolated volatile extract of corn appeared to contain at least 21 compounds that included six-and seven-carbon compounds and sesquiterpenes. Sesquiterpenes are 15-carbon compounds that are fragrant and present in the essential oils of several plants. Eleven sesquiterpenes were present in corn, of which the identities of five were established. The mass spectra of the remaining sesquiterpenes are very similar to spectra of several sesquiterpenes, but other sophisticated techniques will be required to establish their identities. Sesquiterpenes were present in minor quantities ($< 2\%$) except for β-caryophyllene (18%) and cyclosativene (27.7%). The six-carbon alcohols, esters, and aldehydes identified in corn are green-leaf volatiles common in plants. These compounds were present in varying amounts ranging from 1.0% to 12.4%. Ketones present in corn included 4-hepten-2-one (2.9%), which is unique to corn (Buttery and Ling, 1984), and 2-heptanone (6.9%).

Techniques used for collection and isolation of corn volatiles in the present study were similar to those used by Buttery and Ling (1984). They reported the presence of 24 compounds from leaves of Bonanza and Stylepak cultivars of corn that included 13 compounds that we obtained from Jubilee. In addition they obtained 2-methyl-2-pentanal, (*E*)-2-nonenal, myrcene, limonene, linalool, α- and β-copaene, (*E*)-β-farnesene, α-muurolene, methyl salicylate, and an

unidentified sesquiterpene. These compounds may have been present in Jubilee in low quantities. However, they did not find 2-heptanol acetate and α-humulene (also known as α-caryophyllene), which we obtained from Jubilee. We also obtained 11 sesquiterpenes, whereas they reported nine in their extracts. These differences in volatile profiles could be because different cultivars were used.

Our results differ from those of Turlings et al. (1991), who reported that significant quantities of volatiles were obtained only when corn plants were damaged by insects. They obtained (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*-3-hexen-1-ol, and (*Z*)-3-hexen-1-yl acetate from corn seedlings of Ioana cultivar only after the plants were damaged by beet armyworm larvae. However, we found these compounds in corn plants that were not damaged by insects. Concentrations of these compounds in the present study were similar to those reported by Turlings et al. (1991), except for (*Z*)-3-hexen-1-yl-acetate, of which they obtained 12–20%. (*E*)-Nerolidol, α-*trans*-bergamotene, (*E*)-β-farnesene, linalool, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene reported from Ioana were not observed in Jubilee in the present study. These compounds may be specific to the cultivar or age of plants used by Turlings et al. (1991). Alternatively, they may be insect-induced compounds. However, linalool and (*E*)-β-farnesene were also reported by Buttery and Ling (1984) from plants undamaged by insects.

Column separation on Florisil provided a simple method of separating compounds into groups based on their polarity. All 11 sesquiterpenes were collected in fraction 1, which was eluted with pentane. Esters were collected in fraction 4 eluted with 92.5% P + 7.5% E, aldehydes and ketones were eluted with 92.5% P + 7.5% E or 90% P + 10% E and collected in fractions 4 and 5, while alcohols were eluted with 50% P + 50% E and collected in fraction 7 (Table 2). Adequate separation was achieved for all compounds except (*Z*)-3-hexen-1-yl acetate and 2-heptanone. (*Z*)-3-Hexen-1-yl acetate was collected in fraction 4 but minor quantities of the ester were also present in fraction 5. The ketone 2-heptanone was eluted with 90% P + 10% E with minor quantities in the fraction eluted with 92.5% P + 7.5% E.

Wind-tunnel bioassays of the Florisil fractions made it was possible to identify components of corn odor that are critical for attraction of *M. grandii*. Fraction 1, which contained nonpolar compounds, elicited the greatest response. Few or all of the 11 sesquiterpenes present in this fraction may be critical for attraction of *M. grandii* to corn odor. Fraction 4 was also attractive while fractions 5 and 7 were not. Fraction 4 contained five compounds of which 2-heptanone and (*Z*)-3-hexen-1-yl acetate may not be attractive, since these were also present in fraction 5, which did not elicit positive responses in *M. grandii*. However, the concentration of a compound could be critical for attraction of a parasitoid, and these compounds may attract *M. grandii* at concentrations present in fraction 4. The compounds 2-hexenal and 4-hepten-2-one, which were present

in fraction 5 do not appear to be attractive to *M. grandii*. Alcohols present in fraction 7 were also not attractive.

More than one fraction was attractive, indicating that more than one class of compounds can elicit oriented flight behavior in *M. grandii*. The fraction containing sesquiterpenes was most attractive, which suggests that the compounds present in this fraction may play a major role in parasitoid attraction to corn plants. Further separation is required to determine whether all compounds in this fraction are attractive to *M. grandii*.

Turlings et al. (1991) used a similar approach and identified 11 compounds in corn damaged by beet armyworm. They documented that a blend of synthetic versions of these 11 compounds was attractive to the parasitoid *Cotesia marginiventris*. However, their study did not indicate whether all 11 compounds were essential for parasitoid attraction. In the present study, certain components of plant odor were not involved in parasitoid attraction. Elzen et al. (1984) observed that, in a Petri dish Y-tube bioassay, the parasitoid *Campoletis sonorensis* was attracted to five of six sesquiterpenes isolated from cotton. In a study by Sugimoto et al. (1988), ether-soluble components of volatiles of *Ranunculus glaber* were attractive to the parasitoid *Dapsilarthra rufiventris* in a Y-tube olfactometer. No information on flight responses to plant compounds were reported in these studies. The present study is the first to document positive flight responses of a parasitoid to isolated fractions of plant odor.

Parasitoids of polyphagous hosts are likely to be attracted to several food plants of their host. In a previous study, we documented that, besides corn, *M. grandii* was attracted to volatiles of potato and snap bean. Attraction to these plants could be based on the presence of compounds similar to those attractive in corn, or different compounds could be involved. The attraction to snap bean could be because the same attractive compounds were present in low concentrations, or attraction to snap bean could be based on different compounds that are less attractive to *M. grandii*.

In the present study, separation by column chromatography indicated that extracts of potato and snap bean contain volatiles that belong to the same four classes of compounds as those present in the volatile extract of corn. When potato fractions were bioassayed, *M. grandii* was attracted to fractions 1 and 4. Compounds in these fractions were not identified. However, based on column chromatography of corn, these fractions are likely to contain nonpolar and slightly polar compounds, respectively. These classes of compounds in corn were also attractive. This suggests that attraction to potato and corn are based on similar compounds. Fractions 5 and 7, which were not attractive in corn, were also not attractive in potato. When snap bean fractions were bioassayed, *M. grandii* responded positively to the more polar fractions 5 and 7 but not to fractions 1 and 4. Attraction to snap bean odor appears to be based on classes of compounds different from those responsible for attraction to corn or potato odor.

Preliminary gas chromatographic–mass spectral analysis (data not included) indicates the presence of several sesquiterpenes in potato extract. These are likely to have been eluted in fraction 1, which was attractive to *M. grandii*. This suggests that sesquiterpenes are responsible for attraction to potato odor. Snap bean extract did not appear to contain sesquiterpenes in significant amounts. This would explain lack of attraction to fraction 1 of snap bean. Detailed analysis of compounds in fractions 1 and 4 of potato and fractions 5 and 7 of snap bean are required to determine the identity of compounds in these plants responsible for attraction by *M. grandii*.

CONCLUSIONS

Parasitoids exhibit diverse responses to plants based on the chemical composition of plant odor. In an earlier study, we provided evidence that *M. grandii* exhibits differential flight response to odor of food plants of its European corn borer host. Whereas it exhibits positive responses to odors of corn, potato, and snap bean, its response to pepper is neutral and its response to soybean is slightly negative.

The present study documents that attractive components of corn, potato, and snap bean odors are extractable with pentane and can be isolated using Tenax. For each attractive extract, more than one fraction elicited positive responses, indicating that attraction is not based on a single attractive compound. An interesting result was that among attractive plants, different classes of compounds were responsible for eliciting positive responses in *M. grandii*. This indicates that location of food plants of its host is a complex process where foraging parasitoids select and respond to diverse classes of plant compounds. Plant odors thus influence attraction of a parasitoid to a particular plant where hosts are likely to be present.

Information on the chemical basis of a parasitoid's attraction to plant odors may be used for manipulation of its behavior toward increasing its efficiency in the field. While European corn borer is polyphagous, *M. grandii* has not been reported parasitizing it in diverse habitats. Absence of attractive compounds in certain plant odors could be the basis for lack of attraction to a plant despite the presence of hosts. Chemical cues for orientation of *M. grandii* are absent in pepper and soybean, and European corn borers are likely to escape parasitism when present in these plants. However, other plants that are not hosts of European corn borers may possess compounds that are critical for attraction of *M. grandii* from a distance. Intercropping these plants with pepper and soybean may increase parasitism of European corn borers in these habitats. Alternatively, attractive plant extracts may be useful in increasing parasitoid efficiency. In studies by Altieri et al. (1982) and Nordlund et al. (1985) application of plant

extracts in the field resulted in increased parasitism. By provision of attractive plant compounds, it may be possible to introduce *M. grandii* to less attractive habitats like those of pepper and soybean.

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INHIBITION OF SCOTS PINE SEEDLING ESTABLISHMENT BY *Empetrum hermaphroditum*

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Abstract—Poor establishment and reduced seedling growth of Scots pine (*Pinus silvestris* L.) in northern Sweden is related to an allelopathic inhibition by the dwarf shrub *Empetrum hermaphroditum* Hagerup. Indoor bioassays with green and brown leaves of *Empetrum* have strong negative effects on rooting ability, radicle elongation, and growth of Scots pine seedlings. Bioassays with soil samples show that phytotoxic substances leached from *Empetrum* foliage accumulate in the soil. Field experiments reveal that chemical inhibition by *Empetrum*, causing high mortality and slow growth of pine seedlings, can be reduced by adding activated carbon to the soil.

Key Words—Allelopathy, *Pinus silvestris*, *Empetrum hermaphroditum*, succession, regeneration failures, soil toxicity, activated carbon, forest fires.

INTRODUCTION

Empetrum hermaphroditum Hagerup is a 5- to 15-cm-high evergreen dwarf shrub forming extensive clones in postfire successions on acid mor humus soils with high C/N ratios in northern Scandinavia (Haapasaari, 1988; Odell and Drakenberg, 1991). Regeneration of Scots pine (*Pinus silvestris* L.) from seed trees and from planting operations on clear-cut areas dominated by *Empetrum* have often been unsuccessful (Arnborg, 1943; Malmström, 1949; Sarvas, 1950; Ebeling, 1979; Hagner, 1984). Traditional interpretations of these regeneration problems and slow growth have focused mostly on harsh climate, unfavorable soils, and dense plant cover. Recently, the possibility of chemical inhibition by *Empetrum* has been suggested (Zackrisson and Nilsson, 1992). *Empetrum* leaves

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were found to possess glands that are responsible for the production of water-soluble toxins easily leached out by rain. Chemical identification revealed that 3-methoxy-5,3′-dihydroxy, dihydrostilbene, or batatasin III (B-III) is one substance in water extracts of *Empetrum* that strongly inhibits seed germination under controlled indoor conditions (Odén et al., 1992). In this paper we have assessed the allelopathic potential of *Empetrum* on early seedling growth of Scots pine in both field experiments and indoor bioassays.

METHODS AND MATERIALS

Green leaves of living *Empetrum* were collected during late autumn at Rovågern (63°50′N), air-dried at 20°C, and stored at −18°C before use in the bioassays. A stock 5% solution of aqueous extract was prepared by soaking 50 g of dry green leaves in 1 liter of distilled water while slowly stirring for 48 hr. One leaf is about 3 × 7 mm and has a dry weight of 0.4 mg. The extract was filtered (Munktell No. 3 filter paper) to yield a clear, yellowish brown solution with a characteristic odor. The solution had an osmotic potential of 43 mmol/kg and a pH of 4.2. Scots pine seeds (Skaholma, 66°99′N, half-sib seeds with a viability of 92%) were used in all experiments, unless otherwise noted.

Analysis of Data

Variation among treatments was tested with analysis of variance or Student's t test. Tukey's multiple a posteriori range test and the least significant difference (LSD) (Sokal and Rohlf, 1981) were used to determine significant ($P \leq 0.05$) differences between treatments.

Experiment 1. Bioassays of Aqueous Leaf Extracts

Method 1. Empetrum extract (2 ml of 5% solution) was placed into 50-mm-diameter Petri dishes lined with a sheet of filter paper (Munktell No. 3). In seven replicate dishes for each treatment, seven seeds with a pregerminated radicle of 1 mm were exposed to the extract or to distilled water controls for 7, 19, 25, 48, 72, or 240 hr, and then rinsed in distilled water and transferred to Petri dishes lined with filter paper and containing 2 ml of distilled water. Petri dishes were maintained in a growth chamber under 20 hr illumination at 20°C. The length of the radicle and, later, total seedling length (root plus hypocotyl) were measured daily with a digital ruler.

Method 2. Scots pine seeds were germinated on moist, oven-sterilized (130°C, 12 hr) industrial quartz sand (Silversand 90) in the greenhouse. After 12 days, one seedling was transferred to each of 80 0.1-liter plastic pots containing sterile sand watered to field capacity, and placed in the greenhouse under

18 hr illumination and alternating temperatures of 20°C (day) and 15°C (night). All pots were watered every second day with a complete nutrient solution (5 mg NH_4-N/liter) recommended by Ingestad (1979). Twenty days after transfer, 4 ml of three concentrations (5%, 2.5%, and 1.25%) of aqueous extracts of *Empetrum* or distilled water was given twice a week. Seedling height, measured to the longest primary or secondary needle, and mortality were recorded at the start of the treatments and every fifth day thereafter. At harvest after 46 days of treatment, sand was rinsed off the roots, seedlings were dried at 70°C for 72 hr, dry weights of shoots and roots were determined separately, and root growth characteristics were recorded. All extracts were kept at 2°C during the experiment. Each treatment was replicated 20 times.

Experiment 2. Bioassays of Empetrum *Leaves*

Method 1. Scots pine seeds were germinated on industrial quartz sand watered to field capacity with distilled water in 10 90-mm-diameter 0.7-liter plastic pots. When seedlings reached 10 mm in height, they were thinned to 20 seedlings per pot. Then, 3.0 g of air-dried green leaves of *Empetrum* was added to five pots and 3.0 g of dry commercial greenhouse peat (unlimed, unfertilized and finely ground) was added to five pots as a control. Pots were misted several times a day with tapwater to maintain a high moisture level. On days 4, 7, 11, 14, and 17, seedling height and mortality were recorded. After 17 days of treatment, sand was rinsed off the roots, the seedlings were dried at 70°C for 72 hr, and the main root length and dry weights of shoots and roots were determined.

Method 2. Scots pine seeds were pregerminated in distilled water. When a radicle of 1 mm emerged, 10 seeds were carefully transferred, without changing their orientation, onto each of five replicate 0.7-liter pots containing moistened quartz sand and either 0.48, 0.12, 0.06 g or no dry green leaves and 3 g peat on the surface. Plastic bags allowing airflow were placed over the pots to maintain the moisture level. When the radicle penetrated the substrate and the hypocotyl grew above the substrate, the seedling was considered rooted.

Method 3. Green leaves and brown leaves, still connected to the three-year shoots, were removed and air-dried at 20°C. Ten Scots pine seeds, pregerminated in distilled water with a radicle of 1 mm length, were transferred to 60-mm-diameter 0.15-liter plastic pots half-filled with sterile quartz sand and 2 g of one of the following substrates on top: (1) green leaves, (2) brown leaves, (3) a control of soaked green leaves, (4) a control of soaked brown leaves and (5) peat. "Soaked" leaves were 25 g leaves in 1 liter of distilled water, repeatedly changed during eight days, to detoxify the leaves, yet maintain physical similarity in surface structure of the seed beds. As this experiment was intended to simulate leaves and litter exposed to natural wet conditions (heavy rainfall or

snow meltwater), the leaves and peat were soaked in 10 ml of distilled water for 48 hr before transfer to the pots. Ten replicates of each treatment were sealed with parafilm to maintain uniform moisture and placed in a climate chamber at 20°C and with 20 hr illumination. The numbers of rooted seedlings and seedling mortality were recorded daily for five days, after which the experiment was terminated and total length of seedlings (root plus hypocotyl) was measured.

Experiment 3. Bioassay of Freshly Collected Soil

To investigate the accumulation of phytotoxins in soils, six replicate soil samples were taken beneath a homogeneous clone of *Empetrum* at the Akkanålke field site (lat 65°40′N) in May, August, and October 1990, May and July 1991, and in May 1992. There were no trees, or ground layer of mosses or lichens at the sampling site. The sampling in May 1991 and May 1992 coincides with recent snow melt on frozen soil, but in May 1990 the frozen soil had thawed during unseasonably warm weather. Each sample included a complete humus soil profile plus the uppermost mineral soil (A00–A2) (Rieger, 1983). A similar fine-textured leached moraine soil (A2 horizon), where plant cover and humus had been removed three years earlier, was sampled nearby as a control. Soil samples were transferred to plastic bags and immediately transported at 2°C to the laboratory, where each sample was separated into A00 (litter), A1 (humus), and A2 (leached mineral soil) portions. The uppermost 0.5 cm of each layer was transferred to 50-mm-diameter Petri dishes. Industrial quartz sand was used as an additional control substrate in the bioassay. Fifty aspen (*Populus tremula* L.) seeds were sown on the soil in each dish because aspen germinates more quickly than pine, and a quick germination response is needed when diminishing levels of toxins are expected in samples brought indoors. The dishes were sealed with parafilm and placed in a growth chamber. Seed germination was recorded for seven days. Six replicates per treatment were used.

Experiment 4. Field Experiment

Three field sites on acid mor humus soils with cover dominated by *Empetrum* were selected at Akkanålke (65°40′N, 700 m asl), Kiutisvaara (67°30′N, 300 m asl), and Salmisjärvi (68°15′N, 430 m asl) in northern Sweden. At each site 40 level plots (0.75 × 0.75 m) were selected where *Empetrum* cover was about 80% and no mosses, lichens, or trees were present. On 20 randomly chosen plots, 280 g of fine-powdered (pro analysi) activated carbon (Labasco) was added (Zackrisson and Nilsson, 1992). In the central part of each plot, 100 seeds of Scots pine were sown in a 60 × 60-cm plastic exclosure with 10 mm mesh, at Akkanålke and Kiutisvaara in May 1988 and at Salmisjärvi in May 1989. Additional amounts of activated carbon were applied in the springs of 1988 (140 g/plot), 1989 (70 g/plot), and 1990 (45 g/plot). Germination, and

the survival and the stage of development of emerged seedlings were counted two to four times a year during 1988–1991. Seedling height to the apical bud was measured in September 1991.

Experiment 5. Bioassays of Activated Carbon

Method 1. To determine the effect of activated carbon on seed germination of Scots pine, 20 g of industrial quartz sand or 2.5 g of well-decomposed humus were added to 10 50-mm-diameter Petri dishes. One gram of fine-powdered activated carbon was added to five dishes, each filled with sand and humus. Each dish was watered to field capacity with distilled water and 50 Scots pine seeds were sown. When radicles reached seed length, they were counted as germinated and removed from the dishes daily until there was no further germination.

Method 2. Ten Scots pine seeds germinating on wet industrial quartz sand in the greenhouse were transferred when seedlings reached 10 mm length into each of 10 plastic pots filled with wet sand. Five grams of activated carbon (equivalent to field applications) was added to the sand surface of five pots. Shoot heights of the seedlings were measured every third day until termination at day 28, when shoot height and main root length were measured.

RESULTS

Experiment 1

Growth of germinating seedlings exposed to the 5% aqueous extract of *Empetrum* was significantly inhibited compared to controls exposed to distilled water (Figure 1). Degree of inhibition increased with time of exposure. Although exposed seedlings grew, inhibition after exposure was persistent and growth as a percent of control continued to decline. Seedlings continuously exposed or exposed 48 hr or 72 hr almost stopped growing—an indication of severe physiological damage. In addition, *Empetrum* aqueous extract significantly decreased growth of pine seedlings when application of extracts was initiated after depletion of the cotyledonary reserves (Table 1). As *Empetrum* extract concentration increased, mean dry weight of seedlings and elongation of roots decreased. In the 5% treatment, seedlings started to lose turgor after six days of treatment, and mortality reached 10% at day 22 of treatment, 40% at day 31, and 100% by day 46. Other treatments exhibited little mortality. Seedlings exposed to *Empetrum* extract had dark, discolored primary roots and strongly reduced side roots, chiefly occurring near the pot bottoms. The abnormalities appeared to be related to extract concentration.

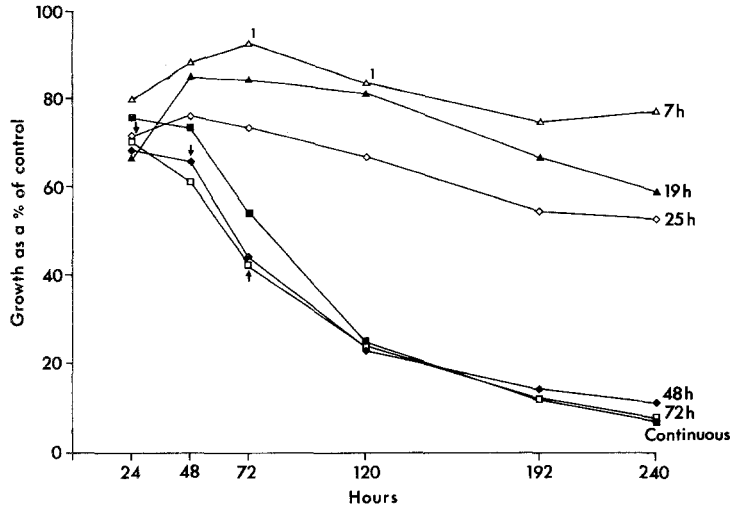

FIG. 1. Radicle growth as a percent of controls of Scots pine germinating seedlings exposed to 5% aqueous extract of *Empetrum* leaves for different times. All growth reductions are statistically different from controls except for the dots marked with "one." Arrows indicate when exposure to *Empetrum* ended.

TABLE 1. MEAN AND ± SE FOR SCOTS PINE SEEDLINGS AFTER 46 DAYS OF EXPOSURE TO DIFFERENT CONCENTRATIONS OF AQUEOUS LEAF EXTRACT OF *Empetrum* OR DISTILLED WATER CONTROLS[a]

	Empetrum treatment			
	1.25%	2.50%	5%	Control
Shoot height (mm)	44.4 ± 1.5a	41.1 ± 1.8a	41.8 ± 1.2a	46.4 ± 1.2a
Shoot weight (mg)	13.8 ± 0.8a	13.2 ± 0.7a	7.3 ± 0.4b	19.5 ± 1.0c
Root weight (mg)	15.6 ± 1.9a	10.0 ± 0.9b	2.6 ± 0.2c	20.1 ± 2.2d
Shoot-root ratio	1.2a	1.5a	3.2b	1.1a
Total weight (mg)	29.4 ± 2.4a	23.2 ± 1.4b	9.8 ± 0.4c	39.6 ± 3.1d
Main root length (mm)	110.8 ± 7.6a	102.9 ± 23.3a	56.6 ± 2.6b	121.2 ± 8.0a
Mortality (% of control)	5	0	100	0

[a] Values are means of 20 seedlings. Values in a row followed by the different letters are significantly different at $P \leq 0.05$ in Tukey's multiple range test.

TABLE 2. MEAN AND ± SE OF DRY WEIGHT OF SHOOTS AND ROOTS, SHOOT LENGTHS, ROOT LENGTHS, AND MORTALITY OF SCOTS PINE SEEDLINGS GROWN IN CONTACT WITH 3.0 GRAMS OF GREEN LEAVES OF *Empetrum* OR PEAT (CONTROL)[a]

	Treatment	
	Empetrum	Control
Shoot weight (mg)	129.6 ± 2.2[b]	229.8 ± 4.5
Root weight (mg)	30.4 ± 7.4[b]	72.0 ± 1.2
Shoot–root ratio	4.7 ± 0.5	3.2 ± 0.1
Shoot length (mm)	31.8 ± 0.7[b]	35.0 ± 0.5
Main root length (mm)	44.8 ± 2.9[b]	88.5 ± 1.8
Mortality (%)	57.6[b]	12.8

[a] Seedlings were harvested after 17 days of treatment. Values are means of 100 seedlings per treatment. Shoot and root dry weight values are means of five replicates.
[b] Significantly different from control at $P \leq 0.05$.

Experiment 2

In the first bioassay of *Empetrum* leaves, seedlings grown with air dried green *Empetrum* leaves had significantly higher mortality (58%) than the control (13%) (Table 2). Surviving seedlings in the *Empetrum* treatment had dry weights of shoots and roots and lengths of main roots about half those in the control (Table 2). Differences in shoot length were less exaggerated. A higher shoot–root ratio was also observed for *Empetrum* treated seedlings. Seedlings in the *Empetrum* treatment had dark-colored, dwarfed root systems whereas control seedlings were light-colored and healthy.

In the second bioassay of *Empetrum* leaves, rooting of pregerminated Scots pine seeds was inversely related to leaf weights in pots even after one day of treatment (Figure 2). In the 0.48-g treatment most radicles were stunted and died before rooting. Convergence of the treated seedlings over time is due to mortality. Variation in number of rooted seedlings is explained by mortality and delayed rooting.

In the third bioassay of *Empetrum* leaves, rooting of germinating Scots pine seedlings was shown to be severely affected by green leaves of *Empetrum* (Figure 3). Brown leaves also strongly inhibited the rooting. Soaked green and brown leaves revealed lower inhibition than the respective unsoaked leaves, although all treatments caused statistically significant growth reductions compared to the control. Mortality of germlings was 100% in the green leaf treatment, but they grew in the other treatments.

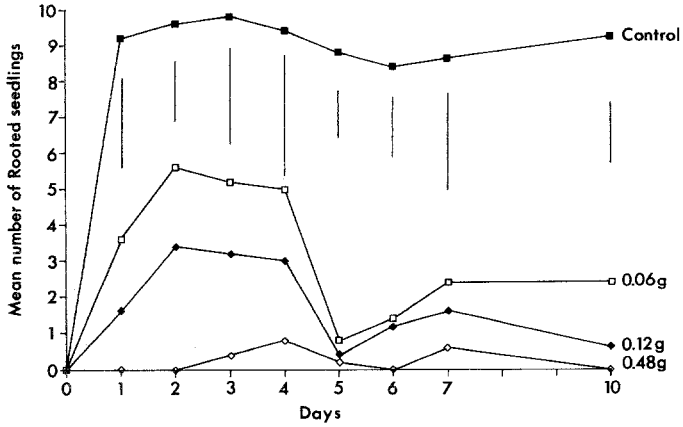

FIG. 2. Rooting ability of pregerminated Scots pine seeds grown in pots with different quantities of green leaves of *Empetrum* or peat (control). Vertical bars give the least significant differences (LSD) among treatments.

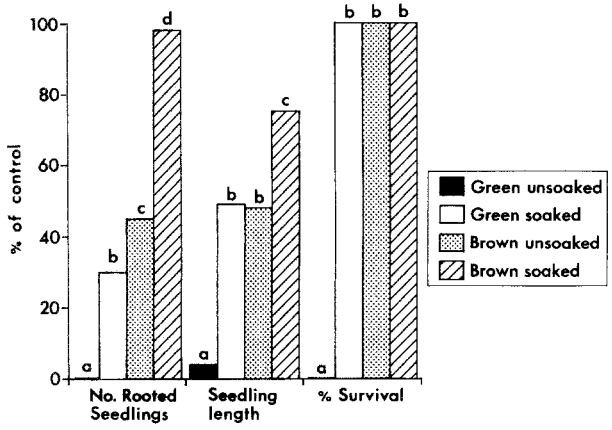

FIG. 3. Rooting ability and seedling length (root plus hypocotyl) of pregerminated Scots pine seeds grown in contact with soaked and unsoaked green and brown leaves of *Empetrum* during five days of treatment. Bars topped with the same letter do not differ significantly in Tukey's multiple-range test.

Experiment 3

Seed germination of aspen on different layers (A00, A1, A2) of *Empetrum* mor humus showed that inhibition was strongest during May 1991 and May 1992, and generally increased with depth in the soil profile from the litter layer

(A00) to the leached mineral soil (A2) (Figure 4). The mineral soil (A2) sampled from the soil uncovered with humus and the industrial quartz sand (data not shown) had no inhibitory effect on seed germination.

Experiment 4

A significantly smaller number of Scots pine seeds germinated and fewer seedlings became established in untreated *Empetrum* vegetation compared with the activated carbon-treated vegetation at all three field sites (Table 3). Ger-

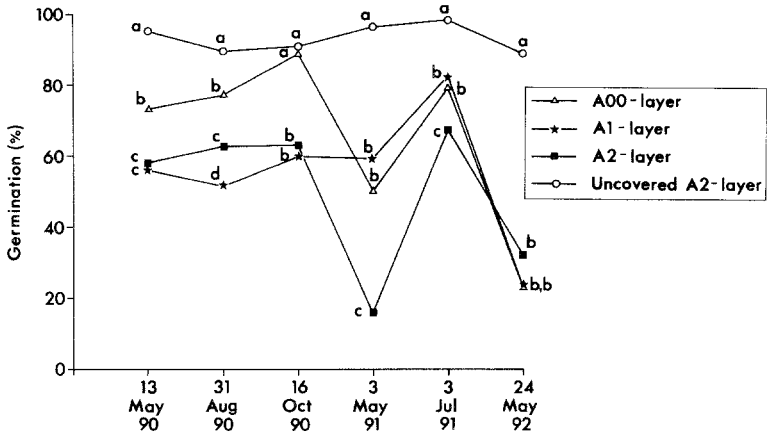

FIG. 4. Seed germination of aspen in soil collected at different depths and at different times in natural *Empetrum* vegetation. Means on the same date followed by the same letters do not differ in Tukey's multiple-range test.

TABLE 3. SEED GERMINATION, SURVIVAL, AND GROWTH OF 2000 SCOTS PINE PER PLOT IN UNTREATED AND ACTIVATED (A) CARBON-TREATED *Empetrum* VEGETATION AT THREE FIELD SITES IN INTERIOR NORTHERN SWEDEN, 1988–1991[a]

Site	Treatment	Germination observed (%)	Surviving in September 1991 (%)	Seedling height (mm) in September 1991 (Mean ± SE)
Akkanålke	Untreated	36.6[a]	11.4	39.7 ± 0.4[a]
	A carbon	52.2	15.0	45.7 ± 0.7
Kiutisvaara	Untreated	21.3[a]	3.1[a]	36.3 ± 1.0[a]
	A carbon	35.9	12.4	39.6 ± 0.7
Salmisjärvi	Untreated	11.0[a]	1.0[a]	43.9 ± 1.4
	A carbon	45.3	6.8	49.1 ± 1.0

[a]Significantly different from plots treated with activated carbon at $P \leq 0.05$ level.

mination was also delayed and seedlings grew more slowly in the untreated plots compared to the carbon-treated plots during the first season (data not shown). Seedling survival and seedling height were significantly higher in activated carbon-treated plots at the end of the experiment. Seedling density was compared to seedling height in order to check for interspecific competition in the plots, but no correlations were found.

Experiment 5

Activated carbon alone did not have any positive effect on seed germination. No differences in seed germination were evident between seeds grown in humus (96.8%), sand (96.8%), humus treated with carbon (99.2%), or sand treated with carbon (99.6%). There were also no differences in the rate of germination among the treatments (data not shown). For seedlings in pots, shoot height did not show any significant differences when grown in sand (39.1 $\pm$ 0.7 mm) or in sand treated with activated carbon (36.7 $\pm$ 0.7 mm). Main root lengths were 38.5 $\pm$ 1.4 mm and 39.2 $\pm$ 1.4 mm, respectively.

DISCUSSION

Indoor bioassays showed strongly increased mortality and reduced growth of Scots pine seedlings when grown in pots together with green or brown leaves of *Empetrum*, or when watered with different dosages of aqueous leaf extracts. Water-soluble phytotoxins may be leached from leaves of *Empetrum* and taken up by the developing radicle and primary roots, leading to stagnant growth and high mortality. The different results of growth and rooting ability between germinating seedlings grown in contact with soaked and unsoaked leaves could not be attributed to physical dissimilarities of the seed bed. Soaked leaves are probably less inhibitory than unsoaked leaves because allelochemicals were leached from the former during soaking. Soaking, however, did not totally reduce the inhibitory effect, indicating that all toxins are not easily washed away despite repeated soaking in large quantities of water. Compared to green leaves, brown leaves were also less inhibitory to rooting ability.

When a seed lands on the upper soil surface in an *Empetrum*-dominated microhabitat, it comes in direct contact with newly fallen toxic litter and is exposed to phytotoxins released from the green foliage of *Empetrum* by rain and snowmelt water. During the imbibition process, the seeds absorb water, and if the water contains toxins from *Empetrum*, the germination will be delayed or totally inhibited, depending on the toxin concentrations (Zackrisson and Nilsson, 1992). Before rooting occurs, the early radicle may be exposed in the same way. In this paper we have shown that very short exposure times of germinating seedlings to aqueous leaf extracts of *Empetrum* have a persistent inhibitory effect

on radicle growth. Osmotic pressure or pH in extracts are not responsible for the observed inhibition (unpublished data).

Our field data provide empirical evidence of a possible allelopathic inhibition by *Empetrum* on Scots pine. The treatments used in the field studies were designed to subject pine seeds and seedlings to the interference of *Empetrum*, without removing competitive influence, and to reveal possible allelopathic effects. Pine responded to this treatment by displaying significantly better establishment and growth when the phytotoxins were removed by activated carbon. The activated carbon will adsorb inhibiting substances produced by leaves and litter leached into the soil. The reduction in germination and lower seedling shoot growth is certainly due to chemical inhibition as the experimental design ruled out the possibilities that conditions of illumination, root competition, humidity, and nutritional conditions of the soil could differ between the two treatments. There is no reason to assume that seed or seedling predation would differ between the treatments. The activated carbon in itself does not contribute to any positive effects, as shown in our indoor bioassays.

Similarly, Eliasson (1959) showed that activated carbon, when present in a nutrient solution, did not stimulate the growth of wheat roots. Low amounts of activated carbon effectively detoxify aqueous extracts of *Empetrum* (Zackrisson and Nilsson, 1992). Smaller amounts than presently used in the field experiment are preferable in future studies, as an excess of carbon may retard growth of seedlings by blackening the photosynthetic tissues. Activated carbon can only be expected to detoxify substances originating from leaves and litter leaching into the soil. Toxins accumulated in the soil by *Empetrum* before the experiment started or through the decomposition process cannot be reduced by activated carbon on the surface. It was also found that germination and seedling growth were delayed in the untreated plots during the first growing season. This delay in growth probably also contributes to the lower total shoot height found after three (Salmisjärvi) and four growing periods (Akkanålke and Kiutisvaara). Indoor experiments with pine seeds also show that germination is delayed when exposed to low amounts of extracts (Zackrisson and Nilsson, 1992). Slow seedling growth in the first season (data not shown) may also cause a delay in the winter hardening process, giving rise to the increased mortality demonstrated in the untreated plots. Allelopathy could also cause seedlings to suffer from reduced mycorrhizal infection (Olsen et al., 1971; Brown and Mikola, 1974; Rose et al., 1983, Perry and Choquette, 1987; Coté and Thibault, 1988), which may inhibit the seedling's ability to compete for nutrients. No seedlings were harvested in our field experiments and conclusions could not be drawn about the below-ground effects of inhibition on pine root development. In the greenhouse experiment, however, we noted a greater negative impact of *Empetrum* on root growth than on shoot growth. Restricted root growth may result in mortality of seedlings due to desiccation. Desiccation interpreted in a wide sense is one of

the major causes of mortality registered during periods with spring and summer droughts.

The snowmelt water contains biologically inhibiting levels of toxins produced by *Empetrum* through the natural soaking of green vegetative parts and litter (Zackrisson and Nilsson, 1992). The accumulation of toxins in soil may be greatest during early spring when the snow has just melted, and ground ice still persists deep into the soil, preventing the snowmelt water from draining into the ground. The microbial detoxification in this situation is probably slow because of the low soil temperatures and anaerobic conditions. After the snow has melted away, the soil may remain frozen for weeks, as in May 1991 and 1992, when the toxicity level at all soil depths studied was high. In other years, such as May 1990, with hardly any frozen soil and rapid snowmelt (statistics from the Swedish Meteorological Institute), the water drained quickly into the soil and the threshold situation with persistently high toxin levels in the soil was probably very short. As pointed out by Jalal and Read (1983), Yoder-Williams and Parker (1987), Cast et al. (1990), and Molina et al. (1991), allelopathy could be operative in a specific environment only for a short period of the growing season or under specific environmental conditions. The most profound effects of pine seedling mortality are registered during the spring.

Allelopathic interference is one likely explanation of conifer seed regeneration failures found in *Empetrum*-dominated vegetation in northern Sweden. There are reasons to suspect that the substances leached from *Empetrum* could also affect more long-term growth of pine saplings. Martinsson (unpublished data) has shown that young coniferous forest stands established on *Empetrum*-dominated clear-cuts were less productive than nearby prescribed burned areas of the same age on sites previously influenced by *Empetrum*. *Empetrum* is fire sensitive and normally does not resprout vegetatively after burning. *Empetrum* is a slow seed recolonizer, and thus seldom reaches a dominance in the postfire forest floor vegetation before crown closure. After cutting without burning, *Empetrum* expands vegetatively and can form a dense field layer vegetation. In virgin pine stands, fire return intervals normally were short (Zackrisson, 1977, 1981), and *Empetrum* seldom reached the distribution and densities found today due to fire protection. Fire elimination will therefore indirectly contribute to the regeneration failures attributed to expansion of *Empetrum* vegetation.

Whether allelopathy is the major factor responsible for the regeneration problems described cannot be fully concluded from this study. However, regeneration failures and reduced growth under allelopathic influence of *Empetrum* may exacerbate an already stressful boreal environment. Although other environmental factors may contribute to regeneration failure, our study indicates allelopathy by *Empetrum* may be an important factor.

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EVOLUTION OF A CRUSTACEAN CHEMICAL COMMUNICATION CHANNEL: BEHAVIORAL AND ECOLOGICAL GENETIC EVIDENCE FOR A HABITAT-MODIFIED, RACE-SPECIFIC PHEROMONE

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Abstract—An earlier study of population genetics of an estuarine amphipod provided evidence from genomic DNA analysis for a habitat-specific race of amphipods within the species *Eogammarus confervicolus*. In some estuaries of the northeast Pacific, this race of amphipods exists sympatrically with other members of the species. Here we present evidence for a race-specific pheromone that appears to be the consequence of differential metabolism of the algae (*Fucus distichus* and *Pelvetia fastigiata*) characteristic of the habitat occupied by this race. The race-specific pheromone identified in this study is a subtle modification of an already existing communication system: females of the habitat-specific race produce the pheromone characteristic of the species as a whole but have an ability not shared by other females of the species to modify this pheromone when raised on the algal substrate characteristic of their habitat. Only males of this race make a distinction between the more specific pheromone and the species pheromone. The formation of hybrids (conceived and raised on the algal substrate) between members of the habitat-specific race and the other members of the species disrupted the ability to produce and distinguish the race-specific pheromone; hybrids still produced a pheromone, but it was indistinguishable from that produced by the species as a whole. Behavioral assays and the results of reciprocal, interpopulation crosses indicated pheromone response in males had evolved with production; males however, did not have to be raised on the algal substrate to respond to the alternate pheromone. No evidence for maternal effects or sex linkage were

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detected in the results of the crosses; more specific indications of the genetics underlying pheromone production were not evident.

Key Words—*Eogammarus confervicolus*, crustacean pheromone, mate choice, ecological genetics, amphipod, estuarine crustacean, sympatric population subdivision, race formation.

INTRODUCTION

Information on the origin of ethological isolating mechanisms is important to an understanding of the speciation process. Differences in sex pheromone communication systems between closely related species, or within a species, are an example of such an ethological isolating mechanism. The manner in which chemical communication channels differentiate is thus of intrinsic interest. Relatively little is known about the genetics and evolution of invertebrate sex pheromone communication systems. Most of the information that is available comes from insect species (e.g., tussock moth: Grant et al., 1975; *Drosophila melanogaster:* Averhoff and Richardson, 1976; budworm moths: Sanders et al., 1977; sulfur butterfly: Grula and Taylor, 1979; European corn borer: Klun and Maini, 1979; Klun and Huettel, 1988). Although crustaceans are known to produce sex pheromones, there is little or no information on genetics and evolution of pheromones in this class of arthropods (see Dunham, 1978, for review). Sex pheromones in insects are thought to be important reproductive isolating mechanisms (see Cardé, 1986, for a review), and there are cases where pheromone polymorphisms have been implicated in sympatric diversification or at least in the maintenance of sympatric subdivided population structure (e.g., Sturgeon and Mitton, 1986; Klun and Huettel, 1988). Population polymorphisms have not been demonstrated in crustacean pheromones but there are well-documented cases in certain insect species (e.g., European corn borer, *Ostrinia nubilalis:* Klun and Maini, 1979; Roelofs, et al., 1987; Klun and Huettel, 1988; Pena et al., 1988; Bengtsson and Lofstedt, 1990). Behavioral assays suggesting species-specific differences in amphipod sex pheromones have been reported (Borowsky, 1985). Although the role of pheromone communication systems in speciation remains largely speculative, there is little doubt that pheromone differences are effective premating isolating mechanisms in many insect species and, thus, the development of a new chemical communication pathway in any species clearly has the potential to contribute to the speciation process.

Stanhope and Levings (1985), in a study of population dynamics of a gammarid amphipod, *Eogammarus confervicolus*, found that populations occupying three distinct habitat types (characterized by three distinct substrate types) within the same estuary had size frequency distributions that suggested they were not experiencing any significant interhabitat immigration and emmigration.

Two of the populations were separated by only 200 m of intertidal mud flat, the third by a distance of about 1.5 km. Gammarid amphipods are highly vagile marine organisms (e.g., Fincham, 1972; Kolding, 1981; Miller, 1984), and thus these populations can satisfactorily be considered sympatric. The population dynamics data (Stanhope and Levings, 1985), concomitant with direct behavioral observations, indicate that animals mate and reproduce within their respective environment types (i.e., periods where most individuals are engaged in amplexus, or pair formation, are followed by high juvenile numbers in size frequency distributions). More recently, Stanhope (1989) has provided evidence from genomic DNA analysis that the amphipods comprising one of these populations are members of a distinct race within the species *E. confervicolus;* although this race has a clearly distinct genotype, it is not as divergent as the only other taxonomically recognized species in this genus (*Eogammarus oclari*). Interpopulation and interracial hybrids can be readily formed in the laboratory (Stanhope, 1989). As part of a larger study designed to determine the factors responsible for maintaining the distinct character of these sympatric groups, we have undertaken a behavioral and ecological genetic study of mate choice in this amphipod species. The results provide evidence for a race-specific pheromone that appears to be the consequence of differential metabolism of the algae characteristic of the habitat occupied by this race.

METHODS AND MATERIALS

Species Description. Eogammarus confervicolus is the most abundant and widely distributed gammarid amphipod on the North American Pacific coast (Bousfield, 1979). It occurs intertidally and in the shallow subtidal regions of estuaries and protected shores that have brackish waters, among sedge, under various species of algae, woody debris, or stones. Its salinity tolerance is 5–25 parts per thousand (ppt); optimum is 5–10 ppt (Sharp, 1980). Gammarid amphipods graze on epiphytic fungi, bacteria, diatoms, and various macrophytes (Hargrave, 1970; Kostalos and Seymour, 1976; Sutcliffe et al., 1981), and there are many examples of marine, estuarine, and lotic amphipods exhibiting substrate preferences (e.g., Fenchel and Kolding, 1979; Stoner, 1980; Kneib, 1982; Williams and Moore, 1986; DeWitt, 1987). Many of these studies suggest that the choice is based on food preference (e.g., Williams and Moore, 1986; DeWitt, 1987).

Populations and Their Habitats. Three habitat types were included in this investigation: woody debris, a particular type of *Fucus distichus* community, and embankments along the perimeter of *Carex lyngbyei* marshes.

Logs have been stored in estuaries in British Columbia (and throughout the northeast Pacific) for 75–100 years. In areas where log booms ground at low

tide, the result is a mud flat devoid of macrophytes with accumulations of bark fragments in depressions along the surface. *Eogammarus confervicolus* can be found among such wood debris. Such areas are commonly about 2.0 m above mean low water.

The second principal habitat type was a mixture of two brown algae: *Fucus distichus* and *Pelvetia fastigiata* (hereinafter termed *Fucus*) overlying soft dark black mud smelling of hydrogen sulfide. The amphipods were found within the algal mixture and at the mud surface. This algal community usually occurs as relatively small patches (rarely exceeding 1 ha) in estuaries or quiet, brackish water bays at about 3.5 m above mean low water.

Many estuaries of the northeast Pacific possess deltas covered predominately with *Carex lyngbyei*. The perimeter of such a marsh is an embankment over which hangs a mat composed of sand, clay, and *C. lyngbyei* rhizomes (hereinafter termed bank) at about 2.0 m above mean low water. *Eogammarus confervicolus* can be found on the underside of this mat, mixed with the rhizomes of the marsh plant.

One of each of these habitat types exists in the Squamish River estuary, British Columbia. (Figure 1). Wood debris and *Fucus* habitats in the Squamish estuary are separated by only 200 m of mud flat, whereas the distance between bank and wood debris habitats is approximately 1.5 km, with a variety of physical obstructions in the middle of the estuary. The area of the *Fucus* habitat in the Squamish estuary is approximately 5000 m^2; the wood debris habitat exists over an area approximately 10 times the size of the *Fucus* habitat. Bank habitat exists along the perimeter of the central and east deltas as well as along the sides of the many channels running through both deltas.

The *Fucus* population in the Squamish estuary is univoltine and the other two are bivoltine. The bivoltine populations are reproductively synchronous, and the adult members of all three populations are reproductively active in the spring and summer months (Stanhope and Levings, 1985).

In order to test whether the trends observed in mate choice involving the Squamish *Fucus* population reflected a unique situation in this estuary or was a characteristic more broadly representative of this race, we investigated pheromone production and response in several other geographically proximal and removed *Fucus* populations. Two additional populations were sampled: a population in Howe Sound (southern British Columbia coast), about 4 km south of Squamish (i.e., the Squamish estuary is at the north end of Howe Sound, see Figure 1) and a population in an estuary in the Queen Charlotte Islands, approximately 750 km north of Squamish.

Mate Choice. Mate choice experiments were performed using a Y-tube apparatus designed by Borowsky (1984) for choice tests in another amphipod species (diagramed in Figure 2). The base of the Y was connected to a 2-liter bottle (termed by Borowsky the departure bottle) by plastic tubing (2 cm ID);

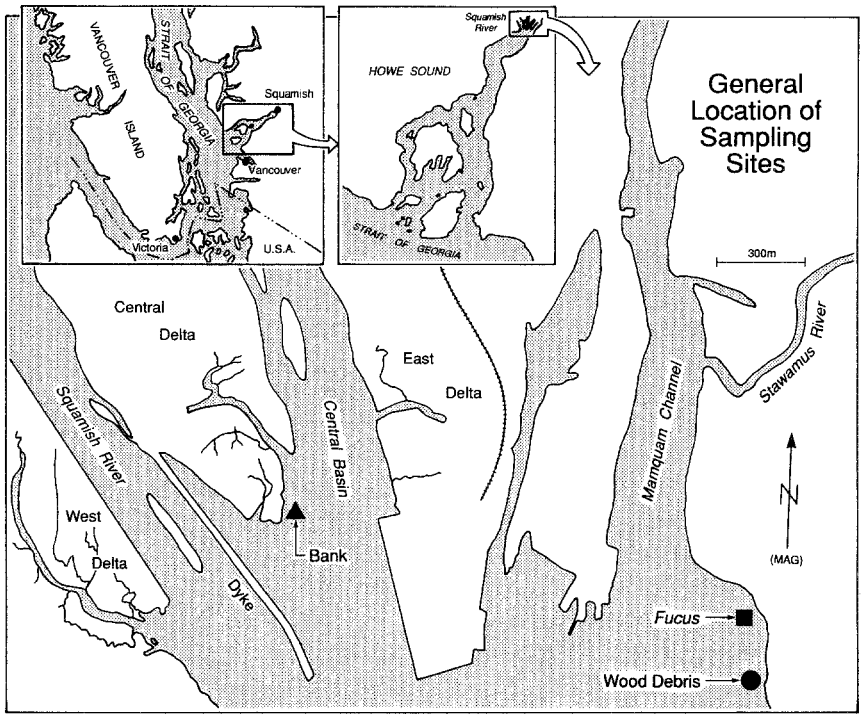

FIG. 1. Location and map of the Squamish estuary, showing the major physical features and the position of the three principal habitat types.

each of the arms of the Y were similarly connected to two additional 2-liter bottles (arrival bottles). The base and the arms of the Y were 25 cm long. Two further bottles (test chambers) were supported on a shelf slightly above the arrival bottles with plastic tubing stretching from an opening at their base into the mouths of the arrival bottles. Amphipods (30 individuals) whose secretions were to be tested were placed in these test chambers. The test chambers had fine mesh screen covering the opening at their base preventing amphipods from traveling through the tubing into the arrival bottles. Water at 10ppt and 10°C was passed into the test chambers "washing" the amphipods; their secretions thus traveled into the arrival bottles, on through the arms of the Y, into the base of the Y and finally into the departure bottle. A slight current was thus created in the direction of test chamber to departure bottle. Before each experiment, the appropriate individuals were placed in the test chambers and water was allowed to flow through the system for several hours. Water flow was then turned off, and the males concerned with a particular test were placed in the departure bottles. The top of this bottle was then covered with a fine mesh screen to

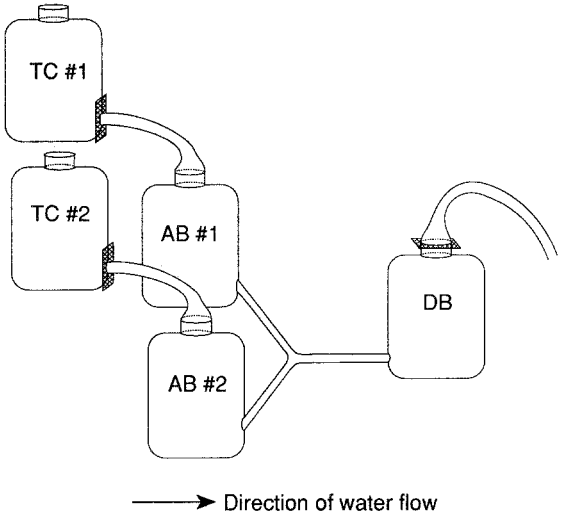

FIG. 2. Mate choice apparatus employed to test for differences in secretions of amphipods from the various populations, crosses, and manipulations. TC = test chamber; AB = arrival bottle; DB = departure bottle.

prevent males from escaping the system. A slight water current was then reapplied. Males could travel freely between the departure bottle and two arrival bottles. Five hours later, the number of males in each arrival bottle as well as those left in the departure bottle were noted. Thirty males were used for each replicate; the same individuals (male or female) were never used more than once. Chi-square was used in analysis and, since there was a single degree of freedom, Yates' correction for continuity was employed (i.e., absolute value of each deviation of observed frequency from expected frequency was reduced by 0.5) (Zar, 1974). To test whether individual replicates of particular experiments were homogeneous, we performed a heterogeneity chi-square analysis for each experiment (Zar, 1974). In those experiments where the replicates were judged homogeneous, the numbers were pooled in the final calculation of chi-square. The entire apparatus was disassembled and thoroughly washed between tests.

The first series of experiments were designed to test whether there was any preference of males for females from each of the three Squamish populations. The results of these tests led to some follow up experiments, designed to test whether the substrate on which the females were raised had any effect on male choice; in other words, could a particular pattern of mate choice be induced by environment? These reciprocal transplant experiments were performed in a series of laboratory 50-liter aquaria, each containing one of the three representative substrate types. The wood debris habitat simulation was simply a mixture of

wood chips overlying the sand–mud typical of that habitat. Bank substrate consisted of a layer of sand, several pieces of the rhizome mat, with additional bunches of entangled *Carex rhizomes*. *Fucus* substrate consisted of the algal mixture overlying a sand–mud mixture, without H_2S mud (i.e., H_2S mud eventually fouled the aquarium water). Aquarium water was kept well aerated and at 10°C and 10ppt. Water and substrate were changed regularly. Test animals for these transplant experiments were the offspring of wild-caught mating pairs. Amphipods in amplexus were collected from each of the respective locations and placed in the various laboratory transplant situations (including several control situations in which amphipods were raised in the laboratory simulation of their native habitat type); the offspring of these individuals, upon reaching reproductive maturity (first appearance in amplexus), were those used in the mate choice experiments.

Tests were then performed using amphipods from the other *Fucus* populations to see if the pattern observed for the *Fucus* Squamish population was unique to Squamish or was common to the race. Several tests were then performed on F_1s and F_2s of some select interpopulation hybrids. Parents for these crosses came from separating mating pairs in the respective estuarine habitats, or from laboratory maintained stocks. Stocks were maintained in a series of 50- or 200-liter aquaria, with the substrate type from which they arose. All crosses were established using approximately 50 pairs and were conducted in laboratory aquaria containing *Fucus* substrate (minus the H_2S mud). Females were held separately for a few weeks prior to crossing, in order to avoid any possible confusion due to an earlier fertilization. Hybrids were not only conceived but raised on *Fucus* substrate. Animals placed in test chambers were, for all the experiments, obtained by separating pairs joined in amplexus, whether they came from a laboratory culture or directly from the estuary. Most experiments had three replicates, each using different amphipods.

RESULTS

Sexually receptive females from all three Squamish populations produced some form of waterborne male attractant (Table 1); in controls without animals in the test chambers, most males never left the departure bottle (they were in the departure bottle at the end of the trial period and periodic checks of the apparatus indicated their presence throughout the test) and when only one chamber contained females, males were found in the arrival bottle fed from that chamber. Males did not produce an attractant.

Males from the Squamish bank and wood debris habitats were unable to make a distinction in the attractant produced by females arising from any of the three Squamish populations (Table 2, experiments 1–6). *Fucus* males, on the

TABLE 1. RESULTS OF VARIOUS MATE CHOICE CONTROL EXPERIMENTS USING Y-TUBE APPARATUS[a]

Exp.	Source of males	Contents of test chambers 1	Contents of test chambers 2	No. in each arrival bottle 1	No. in each arrival bottle 2	No. in departure bottle	Chi-square
1	BKSQ	BKSQ females	empty	69	9	12	44.63***
2	WDSQ	empty	WDSQ females	8	75	7	52.48***
3	FSQ	FSQ females	empty	77	3	10	66.61***
4	BKSQ	empty	empty		N/A		
5	WDSQ	empty	empty	4	5	81	130.06***
6	FSQ	empty	empty	9	5	76	106.06***
7	BKSQ	BKSQ males	empty		N/A		
8	WDSQ	WDSQ males	empty	5	5	50	67.50***
9	FSQ	FSQ males	empty	3	4	83	140.46***

[a]FSQ, BKSQ, and WDSQ refer to the Squamish *Fucus*, bank, and wood debris populations, respectively. Chi-square was calculated employing Yates' correction for continuity (because $df = 1$) except in those experiments where most amphipods were still in the departure bottle at the end of the trial period; in those particular situations the null hypothesis was: males were evenly distributed among the three possible chambers (i.e., two arrival bottles and the departure bottle) and thus $df = 2$. Replicates were judged homogeneous and thus pooled values are presented. N/A = not available; $***P < 0.0001$.

other hand, made distinct choices in favor of females from *Fucus* over any other females (Table 2, experiments 8 and 9) but would respond to females from the other two locations when not offered a choice (Table 2 experiments 7 and 10).

Males from the Squamish *Fucus* population showed no preference for Squamish *Fucus* females when the latter were raised on alternate substrates (Table 3, experiments 1, 2, 6, and 7); the females still produced an attractant, but the males did not distinguish it from that of the females native to the substrate upon which the amphipods had been raised (Table 3, experiments 1 and 2). *Fucus* males exercised a strong preference for native *Fucus* females over *Fucus* females raised on both bank and wood debris substrates (Table 3, experiments 6 and 7). These results suggest that the genotype of Squamish *Fucus* females alone is not solely responsible for the production of *Fucus* female pheromone. On the other hand, raising bank and wood debris females on *Fucus* substrate was not sufficient to induce their choice by *Fucus* males (Table 3, experiments 4 and 5). This, in turn, suggests that it is not simply the *Fucus* substrate that causes females to produce *Fucus* female pheromone. *Fucus* substrate alone did not appear to act as an attractant (Table 3, experiment 10), and *Fucus* females raised on wood debris substrate and then placed in the test chamber with *Fucus* substrate were indistinguishable from wood debris females (Table 3, experiment

TABLE 2. RESULTS OF MATE CHOICE TESTS INVOLVING AMPHIPODS FROM SQUAMISH POPULATIONS[a]

Exp.	Source of males	Source of females		No. in each arrival bottle		No. in departure bottle	Chi-square
		1	2	1	2		
1	BKSQ	BKSQ	WDSQ	20	10		2.70 ns
				10	17	3	1.33 ns
				9	16	5	1.44 ns
2	BKSQ	BKSQ	FSQ	50	33	7	3.08 ns
3	BKSQ	WDSQ	FSQ	13	10	2	0.17 ns
4	WDSQ	BKSQ	WDSQ	40	46	4	0.29 ns
5	WDSQ	BKSQ	FSQ	10	11	2	0.00 ns
6	WDSQ	WDSQ	FSQ	49	33	8	2.74 ns
7	FSQ	BKSQ	WDSQ	9	18	3	2.37 ns
				20	6	4	6.50*
				13	16	1	0.14 ns
8	FSQ	BKSQ	FSQ	15	71	4	35.17***
9	FSQ	WDSQ	FSQ	9	78	3	53.15***
10	FSQ	WDSQ	empty	40	9	11	18.37***

[a] FSQ, BKSQ and WDSQ refer to the Squamish *Fucus*, bank, and wood debris populations respectively. Replicates are listed individually when they were judged to be heterogeneous, otherwise pooled values are presented. *$0.01 < P < 0.05$, ***$P < 0.0001$, ns = not significant.

11). Males collected directly from the *Fucus* habitat in the Squamish estuary and offered a choice of females raised in the laboratory-simulated *Fucus* habitat and females collected directly from the estuary made no distinction (Table 3, experiment 3), suggesting the laboratory habitats were accurately simulating the factor(s) responsible for differences in mate choice. Thus, both the *Fucus* genotype and the *Fucus* environment combined are necessary to induce the production of *Fucus* female pheromone. The response of *Fucus* males to the pheromone produced by *Fucus* females was independent of the substrate upon which the males were raised (Table 3, experiments 8 and 9).

Mate choice tests involving *E. confervicolus* from other *Fucus* populations along the British Columbia coast suggested that populations occupying *Fucus* habitats were producing a common pheromone (Table 4, experiments 1–7). Like the Squamish *Fucus* population, the two other *Fucus* populations only produced this specific pheromone when raised on *Fucus* substrate (Table 4, experiments 8–11). When raised on wood debris substrate, they still produced a pheromone, but it was indistinguishable from that produced by wood debris females (Table 4, experiments 9 and 11).

The ability to produce and distinguish the *Fucus* pheromone was disrupted

TABLE 3. RESULTS OF MATE CHOICE EXPERIMENTS DESIGNED TO TEST WHETHER OBSERVED DIFFERENCES IN PHEROMONE PRODUCTION AND RESPONSE OF SQUAMISH POPULATIONS COULD BE INDUCED BY HABITAT TYPE[a]

Exp.	Source of males	Source of Females and/or contents of test chamber		No. in each arrival bottle		No. in departure bottle	Chi-square
		1	2	1	2		
1	FSQ	FSQ/Bk	BKSQ	42	45	3	0.05 ns
2	FSQ	FSQ/WD	WDSQ	50	38	2	1.37 ns
3	FSQ	FSQ/F	FSQ	36	50	4	1.96 ns
4	FSQ	BKSQ/F	BKSQ	42	43	5	0.00 ns
5	FSQ	WDSQ/F	WDSQ	47	37	6	0.96 ns
6	FSQ	FSQ	FSQ/Bk	23	5	2	10.32**
7	FSQ	FSQ	FSQ/WD	72	17	1	32.76***
8	FSQ/BK	FSQ	BKSQ	81	5	4	65.41***
9	FSQ/WD	FSQ	WDSQ	70	11	9	41.53***
10	FSQ	Fucus	empty	7	6	77	110.46***
11	FSQ	FSQ/WD + Fucus	WDSQ	42	39	9	0.05 ns

[a] FSQ, BKSQ, and WDSQ refer to the Squamish *Fucus*, bank, and wood debris populations, respectively. The following notations refer to situations in which amphipods were raised in laboratory simulated habitat types: /WD, /Bk, /F, i.e., raised in wood debris, bank, and *Fucus*, respectively. Replicates were judged homogeneous and thus pooled values are presented. $^{**}0.001 < P < 0.01$, $^{***}P < 0.0001$, ns = not significant.

in wood debris–*Fucus* interpopulation hybrids. F_1 and F_2 females of crosses between wood debris and *Fucus* (with the reminder that all such animals were raised on *Fucus* substrate) produced an attractant that *Fucus* males were not able to distinguish from that produced by wood debris females (Table 5, experiments 1–4). Wood debris males were attracted similarly to the substances produced by both groups of test individuals (Table 5, experiments 5 and 6). Hybrid males did not exercise any choice between wood debris or *Fucus* females (Table 5, experiments 7–10) as well as between hybrid and wood debris females (Table 5, experiments 11 and 12). In contrast, the formation of *Fucus–Fucus* interpopulation hybrids appeared to have no effect on either production or perception of the pheromone (Table 5, experiments 13–23). The frequency of choice was similar in all reciprocal crosses (contingency chi-square values were not significant for any pairwise comparison), indicating the differences in pheromone production were not due to maternal effects.

TABLE 4. RESULTS OF MATE CHOICE TESTS INVOLVING ADDITIONAL *Fucus* POPULATIONS[a]

Exp.	Source of males	Source of females		No. in each arrival bottle		No. in departure bottle	Chi-square
		1	2	1	2		
1	FSQ	FSQ	FQC	39	44	7	0.19 ns
2	FSQ	FHS	FSQ	31	45	14	2.22 ns
3	FQC	FSQ	FQC	20	9	1	3.45 ns
				14	15	1	0.00 ns
				7	18	5	4.00*
4	FHS	FHS	FSQ	12	17	1	0.55 ns
				21	9		4.03*
				11	16	3	0.59 ns
5	FSQ	FQC	WDSQ	77	12	11	46.02***
6	FQC	FSQ	WDSQ	83	4	3	69.93***
7	FQC	FQC	WDSQ	80	5	5	64.42***
8	FQC	FQC	FQC/WD	80	10		52.90***
9	FQC	FQC/WD	WDSQ	37	50	3	1.65 ns
10	FHS	FHS	FHS/WD	77	8	5	54.40***
11	FHS	FHS/WD	WDSQ	43	39	8	0.20 ns

[a] FQC: *Fucus* population from the Queen Charlotte Islands; FHS: Howe Sound *Fucus* population. /WD = amphipods raised in a laboratory simulation of the wood debris habitat. Replicates are listed individually when they were judged to be heterogeneous, otherwise pooled values are presented. *$0.01 < P < 0.05$, *** = $P < 0.0001$, ns = not significant.

DISCUSSION

A central topic in speciation theory is the study of the evolution of reproductive isolating mechanisms (White, 1978). An important and as yet unresolved question is whether the primary role in initiating speciation is a premating or postmating isolating mechanism. Results of our investigations of differentiated populations of *E. confervicolus* suggest that isolation in this gammaridean amphipod may result from premating mechanisms. Our array of choice test results indicates that the *Fucus* amphipods are a distinct race within the species *E. confervicolus* and have an ability to produce a modified pheromone (or alternate blend of pheromones) through the metabolism of *Fucus*, *Pelvetia*, or something associated with the algae. This ability is not shared by other non-*Fucus* populations and is disrupted when interpopulation hybrids are formed between *Fucus* and non-*Fucus* groups, indicating a genetic basis. *Fucus–Fucus* interpopulation hybrids did not disrupt *Fucus* pheromone production or response. F_1 and F_2 progeny in this study were produced by simply combining in the same aquaria

TABLE 5. RESULTS OF MATE CHOICE TESTS INVOLVING INTERPOPULATION HYBRIDS[a]

Exp.	Source of males	Source of females		No. in each arrival bottle		No. in departure bottle	Chi-square
		1	2	1	2		
1	FSQ	WDSQ-FSQ(F_1)	WDSQ	43	41	6	0.01 ns
2	FSQ	FSQ-WDSQ(F_1)	WDSQ	35	47	8	1.48 ns
3	FSQ	WDSQ-FSQ(F_2)	WDSQ	7	20	3	5.33*
				17	13		0.30 ns
				16	10	4	0.96 ns
4	FSQ	FSQ-WDSQ(F_2)	WDSQ	45	41	4	0.10 ns
5	WDSQ	FSQ-WDSQ(F_1)	WDSQ	31	42	17	1.37 ns
6	WDSQ	WDSQ-FSQ(F_1)	WDSQ	46	39	5	0.42 ns
7	FSQ-WDSQ(F_1)	FSQ	WDSQ	48	31	11	3.24 ns
8	WDSQ-FSQ(F_1)	FSQ	WDSQ	33	24	3	1.12 ns
9	FSQ-WDSQ(F_2)	FSQ	WDSQ	44	40	6	0.11 ns
10	WDSQ-FSQ(F_2)	FSQ	WDSQ	35	42	13	0.47 ns
11	WDSQ-FSQ(F_1)	WDSQ-FSQ(F_1)	WDSQ	34	47	9	1.78 ns
12	FSQ-WDSQ(F_1)	FSQ-WDSQ(F_1)	WDSQ	49	30	11	4.10*
13	FSQ	FSQ-FQC(F_1)	WDSQ	75	12	3	44.18***
14	FSQ	FQC-FSQ(F_1)	WDSQ	81	3	6	70.58***
15	FSQ	FSQ-FQC(F_1)	FSQ	30	23	7	0.68 ns
16	FSQ	FQC-FSQ(F_1)	FSQ	35	44	11	0.81 ns
17	FSQ-FQC(F_1)	FSQ	WDSQ	70	8	12	47.70***
18	FQC-FSQ(F_1)	FSQ	WDSQ	59	6	25	41.60***
19	FSQ-FQC(F_2)	FSQ	WDSQ	63	10	17	37.04***
20	FQC-FSQ(F_2)	FSQ	WDSQ	60	11	19	32.45***
21	FSQ-FQC(F_1)	FSQ	FQC	20	33	7	2.72 ns
22	FQC-FSQ(F_1)	FSQ	FQC	47	35	8	1.48 ns
23	FSQ-FQC(F_1)	FSQ-FQC(F_1)	FSQ	8	22		5.63*
				16	13	1	0.14 ns
				17	9	4	1.88 ns

[a]Males are listed first in each cross, i.e., WDSQ-FSQ(F_1) refers to the F_1 of a cross in which the male parent came from the wood debris Squamish population and the female parent from the *Fucus* Squamish population. FQC = the Queen Charlotte *Fucus* population. Replicates are listed individually when they were judged to be heterogeneous, otherwise pooled values are presented. *$0.01 < P < 0.05$, ***$P < 0.0001$, ns = not significant.

males and females from the respective populations. Stanhope (1989) found no evidence for differences in mating success (percentage of matings that produced offspring) in interpopulation crosses involving these same populations (both parental and F_1 crosses). Thus, a postmating isolating mechanism such as gametic incompatibility or zygote mortality does not appear to exist. Starch gel electrophoresis of 15 enzymes indicated that almost all loci were fixed for the same allele in the six sampled populations (BKSQ, WDSQ, FSQ, FHS, a wood debris

population on Vancouver Island, and a bank population in the Fraser River estuary near Vancouver). Mannose-6-phosphate isomerase and glucose-6-phosphate isomerase were the only detectable polymorphisms; however, *Mpi* and *Gpi* allele frequencies were not significantly heterogenous among all six sites (J.H. McDonald, Department of Ecology and Evolutionary Biology, Princeton University, Princeton, New Jersey 08544; Stanhope, unpublished data). In other words, starch gel electrophoresis of several enzymes did not reveal the fixed allelic differences between locations that would indicate hidden species were present. A more fine-tuned analysis, however, using genomic DNA RFLPs (restriction fragment length polymorphisms) of nine populations (three of each habitat type included in this study) and the only other species in this genus (*Eogammarus oclari*), has indicated that the three *Fucus* populations are a distinct phylogenetic group from the bank and wood debris populations (Stanhope, 1989). They are, however, much more similar to other *E. confervicolus* than to *E. oclari*. The fact that *Fucus–Fucus* interpopulation hybrids still produced the *Fucus* pheromone, and *Fucus*–wood debris hybrids did not, is further corroborating evidence that amphipods residing in *Fucus* substrate comprise a distinct genotype. In some additional tests of ours, two other clearly distinguishable and taxonomically recognized species of gammarid amphipod (*Lagunogammarus setosus* and *Rammellogammarus* sp.) both produced their own pheromone that was only recognized by males of that species (Stanhope, unpublished data), suggesting there is a distinct species specificity to pheromone production and response in gammarid amphipods.

Thus, it appears that *Fucus* amphipods are a distinct evolutionary line within the species *E. confervicolus* that have not developed any marked postmating isolating mechanisms but have evolved an unusual diet-modified sex pheromone. It appears that they produce the pheromone characteristic of the species as a whole but have an ability not shared by other members of the species to modify this pheromone when raised on *Fucus* substrate. We are aware of no other example of a diet-modified pheromone in crustaceans, but there are examples of insect chemical attractants that are blends of insect pheromone and host-tree volatiles (Bedard et al., 1969, 1980); the host-tree volatile is released when it is attacked. Our example differs from these, however, in that the "host" in our case provides an ingredient(s) that is transformed by the members of the race that occupy that habitat into a more specific pheromone. It is not simply a blend of algal chemical and amphipod pheromone, since a combination of amphipods (raised on wood debris substrate) and algae did not elicit the same response. The resulting surmise is that it is a metabolic by-product of the amphipods feeding on algal substrate (or possibly related epiphytes) that either functions as part of a blend or subtly alters the structure of the general pheromone.

Although no crustacean sex pheromone has been completely chemically characterized, Borowsky et al. (1987) present evidence that the amphipod *Micro-*

deutopus gryllotalpa produces a polar attractant that the authors hypothesize is an amino acid or charged organic conjugate. Perhaps the sex pheromone produced by *E. confervicolus* is a peptide that includes a prosthetic group which, in *Fucus* amphipods, either can be replaced or an additional one included, when the diet is changed. Borowsky et al. also found algae could be important in the chemical communication of their species; the intertidal alga *Ulva lactuca* inhibited the male response to receptive female secretions. Amphipods are known to make dramatically different food choices in response to structurally similar algal metabolic compounds (Hay et al., 1987). These findings emphasize the key role algae play in structuring marine intertidal communities through chemical communication channels.

Whatever the precise nature of the difference in pheromone between the *Fucus* race and the other populations, it is likely a contributing factor to reproductive isolation in this group. The close proximity of the wood debris and *Fucus* populations (separated by only 200 m of intertidal mud flat) means it is very likely that individuals from these two groups will encounter each other. At least some portion of the population enters the water column with the flooding tide; we know this from both personal observation and from data that indicate *E. confervicolus* is a major food organism for juvenile salmonids in the Squamish estuary (Goodman and Vroom, 1972; Levy and Levings, 1978). There is also information available indicating that gammarid amphipods are highly vagile aquatic invertebrates (Fincham, 1972; Kolding, 1981; Miller, 1984). Although our bioassay ran for 5 hr [(approximately the same time as in Borowsky's original experiment involving this choice test apparatus (Borowsky, 1984)], many of the amphipods made their choice within the first few minutes, with the rest of the individuals responding gradually over the course of the next several hours. Thus, there is reason to believe the *Fucus*-specific attractant will be, at least at high tide, a contributing factor towards assortative mating. Although it is not an aggregation pheromone per se, it may also have an effect as a "habitat homing" mechanism. As already mentioned, our DNA analysis (Stanhope, 1989) clearly indicates the three *Fucus* populations comprise a distinct race. This race has so far only been identified within *Fucus* habitats. These habitats are often represented by very small patches (FSQ and FHS, for example). The *Fucus*-specific pheromone may be an important factor in maintaining this habitat-based segregation by identifying (at least to males) the location of these relatively small environments.

Unfortunately, the results of the crosses do not provide much specific information on the genetics underlying pheromone production. They do indicate, however, that there was no evidence for maternal effects or sex linkage. Evidence for sex-linked pheromone production has been reported for several insect species (e.g., Sanders et al., 1977; Grula and Taylor, 1979). Pheromone response in *Fucus* males evolved with production; males responded preferentially to the

Fucus pheromone, and this ability was disrupted in interpopulation hybrids. The only apparent difference in production and response was that males did not have to be raised on *Fucus* substrate to respond to the *Fucus* pheromone. A more precise description of the genetic mechanisms underlying production and response may depend on chemical characterization of the different pheromones in order to score the phenotypes more accurately.

Borowsky and Borowsky (1987) have broken down the nature of reproductive stimuli in a gammaridean amphipod (*Gammarus palustris*) into several stages and suggest that a contact pheromone may be an important component in pair formation. We have no information to suggest whether a similar system exists in *E. confervicolus*. It raises, however, the intriguing question of whether different pheromone systems (contact vs. waterborne) would evolve together. Clearly, the situation in *E. confervicolus* is a subtle modification of an already existing communication system. Would we expect a similar modification in a contact pheromone and, if so, what would be the underlying genetic mechanism?

The modeling results of Diehl and Bush (1989) and Rice (1984) and the recent experimental work of Rice and Salt (1990) suggest that considerable sympatric population divergence (and in fact significant progress towards sympatric speciation) can occur if mate choice is closely coupled with habitat preference. Our case provides an unusual example of such a coupling, in that the production of the specific pheromone responsible for distinctions in mate choice is dependent on a key feature of the habitat.

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INHIBITION OF OXYGEN EVOLUTION BY ZALUZANIN C

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Abstract—The inhibition of ATP synthesis, proton uptake, and electron transport (basal, phosphorylating, and uncoupled) from water to methylviologen by zaluzanin C indicates that it acts as an electron transport inhibitor. Uncoupled photosystem I electron transport rate was not affected by zaluzanin C; however, uncoupled photosystem II electron flow from water to 2,3,5,6-tetramethyl phenylenediamine and from water to silicomolybdate was inhibited, but electron transport from diphenylcarbazide to silicomolybdate was not affected. We conclude that the site of inhibition by zaluzanin C is located at the oxygen evolution level.

Key Words—Zaluzanin C, *Zaluzania triloba*, chloroplast, oxygen evolution, inhibition, Hill reaction, photosystem I, photosystem II.

INTRODUCTION

Sesquiterpene lactones are characteristic constituents of Compositae, although investigations of other families (e.g., Magnoliacea and Umbelliferae) indicate that they occur sporadically (Dalvi et al., 1971, see reference herein). These secondary constituents are primarily classified on the basis of their hydrocarbon skeleton as germacranolides, guaianolides, eudesmanolides, pseudoguaianol-

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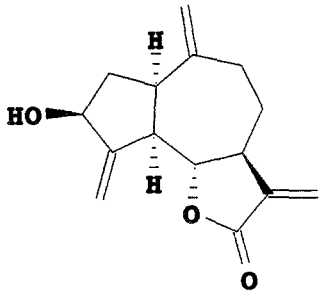
ides, eremophilanolides, and xanthanolides (Rodríguez, 1977 and Rodríguez et al., 1976).

Some sesquiterpene lactones have shown the following biological properties: they produce allergic contact dermatitis in humans; they have poisoning properties in livestock; they are insect feeding deterrents and microbial growth inhibitors; they have cytotoxic and tumor-inhibiting properties: they are plant growth inhibitors; and they have antibiotic properties (Dalvi et al., 1971; Garcidueñas et at., 1972; and see reference therein). It appears that the biological activity of sesquiterpene lactones depends on several structural characteristics: (1) lipophilicity; (2) exocyclic double bonds conjugated to γ lactone; (3) β unsubstituted cyclopentenone ring in pseudoguaianolides; (4) type of sesquiterpene lactone; (5) presence of functional groups, such as epoxide, hydroxyl, chlorohydrin; and (6) stereochemistry (Rodríguez, 1977 and Rodríguez et al., 1976 and see reference therein).

The sesquiterpene lactones with phytotoxic properties are vernolepin, an eudesmanolide that inhibits the extension growth of wheat coleoptile sections; parthenin, a pseudoguaianolide that inhibits the germination of *Phaseolus vulgaris* and affects the development of adventitious roots; and alantolactone and isoalantolactone, two eudesmanolides that greatly enhance the rate of respiration of *Chlorella pyrenoidosa* and inhibit the growth of the cell. Lactones isolate from the leaves of *Artemisia* (arbusalin, adiilin, desacetoxymatricarin, and viscidulin-B and -C) were found to inhibit the germination of three grasses (Kanchan, 1975; Lee et al., 1971). *Ambrosia cumanensis* was inhibited by several sesquiterpene lactones (Amo and Anaya, 1978). The ecological role in plants of zaluzanin C, a guaianolide, is not known, but it probably behaves as an allelochemical agent, interfering with the metabolism of other organisms. The purpose of this research is to study the effect of zaluzanin C (Figure 1) on energy metabolism in photosynthesis and to localize its target(s) of action. Zaluzanin



zaluzanin C

FIG. 1. Structure of zaluzanin C.

C was isolated from *Zaluzania triloba* from Hidalgo State, Mexico. The identification of zaluzanin C was made by comparison with its spectroscopic data reported in the literature (Romo de Vivar et al., 1967). Our results show that zaluzanin C inhibits photosystem II at the level of oxygen evolution. This fact suggests that zaluzanin C could interfere with the growth of photosynthetic organisms.

METHODS AND MATERIALS

Zaluzanin C was obtained from aerial parts of *Zaluzania triloba* and dried after successive chromatographic separations on silica-gel columns from the hexane and acetone extracts using eluent gradients of hexane-ethyl acetate as reported by Romo de Vivar et al. (1967). It was later dissolved in dimethylsulfoxide. Chloroplast thylakoids were isolated from marked spinach leaves (*Spinacea oleracea* L.) as described earlier (Lotina-Hennsen et al., 1987a; and suspended, unless indicated, in 100 mM sorbitol, 5 mM $MgCl_2$, 40 mM KCl and buffered with 30 mM Na^+ Tricine at pH 8.0; 0.05 mM methylviologen was added as an electron acceptor for the Hill reaction (water to methylviologen); KCN (1 mM) was added to inhibit catalase activity. Chlorophyll quantification (Strain et al., 1971), photosynthetic noncyclic electron transport from water to methylviologen (Lotina-Hennsen et al., 1991; Peña-Valdivia et al., 1991), proton uptake (Dilley, 1972), and ATP systhesis (Peña-Valdivia et al., 1991) were determined as described in the literature. Photosystem I and photosystem II (Lotina-Hennsen et al., 1991; Peña-Valdivia et al., 1991), electron transport from water to silicomolybdate (Giaquinta and Dilley, 1975), and diphenylcarbazide (DCP) to dichlorophenol-indophenol (DCIP) (Vernon and Shaw, 1969) were measured as reported. All reaction mixtures were illuminated with actinic light of a projector lamp (GAF 2660) and were passed through a 5-cm filter of a 1% $CuSO_4$ solution.

RESULTS AND DISCUSSIONS

Photosynthetic phosphorylation associated with the methylviologen reduction in spinach thylakoid was partially inhibited by zaluzanin C in concentrations up to 6.8 mM (Figure 2A), the I_{50} (concentration producing 50% inhibition) was 4.6 mM. The light-dependent proton uptake was 100% inhibited at 6.6 mM zaluzanin C concentration (Figure 2B, I_{50} was 4.2 mM); however, ATP synthesis was only 60% inhibited.

The light-dependent systhesis of ATP, phosphorylation, and proton uptake by illuminated thylakoids such as zaluzanin C may be inhibited in one of three ways:

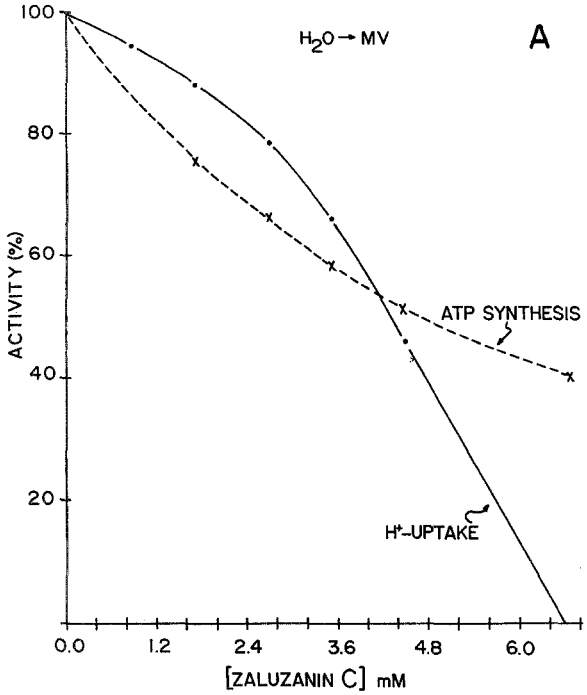


FIG. 2. (A) Effect of increasing concentrations of zaluzanin C on the inhibition of photophosphorylation and proton uptake from water to methylviologen. Proton uptake was measured as a pH rise between 8.0 and 8.1 as described by Dilley (1972) using a combination microelectrode connected to a Corning potentiomenter with expanded scale. The pH changes were recorded (Gilson recorder). After the illumination period H^+ ions produced were quantified by titration with a standard solution 0.01 N HCl. Control value for proton uptake (expressed in μeq H^+/hr/mg Chl) was 11.7. Photophosphorylation was measured as in proton uptake conditions in the presence of 1 mM ADP and 3 mM K^+ phosphate. Each cuvette contained 20 μg chlorophyll per milliliter in 100 mM Kcl, 5 mM $MgCl_2$, and 1 mM Na^+ tricine, pH 8.0; 0.05 mM methylviologen was added as electron acceptor; reaction time: 1 min; aerobic conditions; saturating white light. Control valve for ATP synthesis (expressed in μmol/hr/mg Chl) was 276. (B) Noncyclic electron transport from water to methylviologen (basal electron transport) as a function of zaluzanin C concentrations, was determined with an oxygraph YSI model 5300. Each cuvette contained 20 μg chlorophyll per milliliter in the reaction medium as in (A) except that Na^+ tricine concentration was changed to 15 mM; reaction time: 1 min, aerobic conditions; saturating white light. Other conditions are as described in Methods and Materials. Phosphorylating electron transport was measured as basal electron transport and 1 mM ADP and 3 mM KH_2PO_4 were added. Uncoupled electron transport was measured as basal electron transport and 3 mM NH_4Cl was added. Control values for basal, phosphorylating, and uncoupled electron transport rate (expressed in μeq/hr/mg Chl) were 217, 608, and 837 respectively.

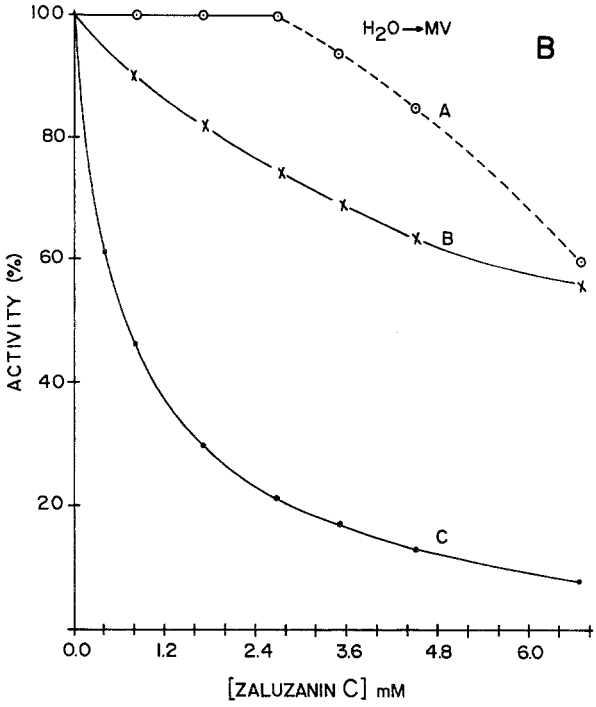


FIG. 2. Continued

TABLE 1. EFFECT OF ZALUZANIN C ON UNCOUPLED PHOTOSYSTEM I ELECTRON TRANSPORT FROM DAD TO MV[a]

Compound	Addition (mM)	H_2O to DAD (μeq/hr/mg Chl)	DPC to DCIP (μeq/hr/mg Chl)
Control	none	1200	118
Zaluzanin C	1.9	1185	117
	2.6	1207	120
	3.5	1201	119
	4.4	1193	118
	6.6	1203	118

[a] Reaction mixture as described in Figure 2A; concentrations—DAD 100 μM, $K_3[Fe(CN)_6]$ 200 mM, DCMU 10 μM, Na_4Cl 3 mM, chloroplast 20 μg Chl. Uncoupled electron transport from DPC to DCIP determined from Tris-treated chloroplasts, reaction mixture as for the photosystem I measurement except that $K_3[Fe(CN)_6]$ and DCMU were omitted and 200 μM DPC was added. Electron flow from DPC to DCIP was measured spectrophotometrically in the resuspended medium.

1. Blocking the electron transport. Reagents that block the electron transport also inhibit ATP systhesis because the generation of the transmembrane electrochemical gradients, the driving force for ATP synthesis, is dependent upon the electron flow.

2. Uncoupling ATP systhesis from the electron transport. Chemicals that increase the proton permeability of thylakoid membranes uncouple phosphorylation from electron flow. Uncoupling agents inhibit ATP synthesis by decreasing the proton gradient, but allow electron transport to occur at high rates.

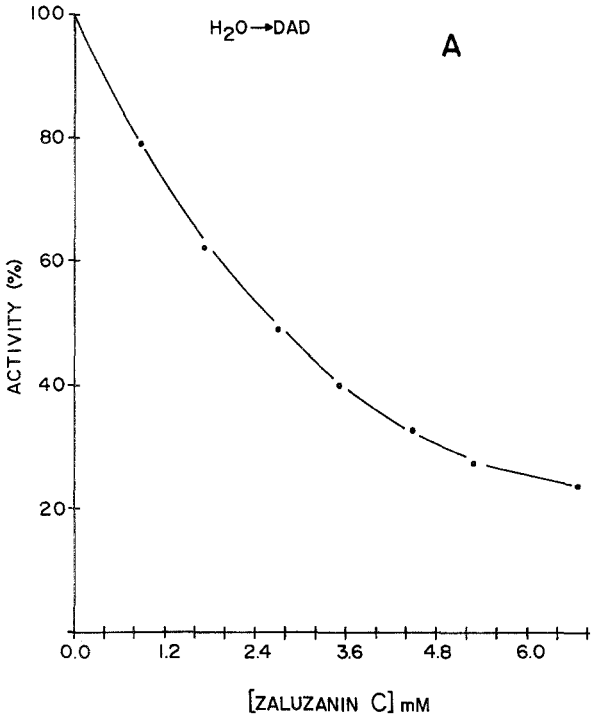


FIG. 3. (A) Effect of increasing concentrations of zaluzanin C on uncoupled photosystem II electron transport from water to DAD. The composition of the reaction mixture is similar to Figure 2B except that in photosystem II the following reagents were added: 0.1 mM DAD, 3 mM $K_3Fe(CN)_6$, and 1 mM DBMIB; methylviologen was omitted. Control valve for uncoupled photosystem II electron transport (expressed in μeq/hr/mg Chl) was 1305. (B) Effect of increasing concentrations of zaluzanin C on uncoupled electron transport from water to silicomolybdate. The composition of the reaction mixture is similar to Figure 2B, except that the following reagents were added: 200 μM Na^+ silicomolybdate and 10 μM DCMU; MV was omitted. Control valve for electron transport rate from water to silicomilybdate (expressed in μeq/hr/mg Chl) was 112.

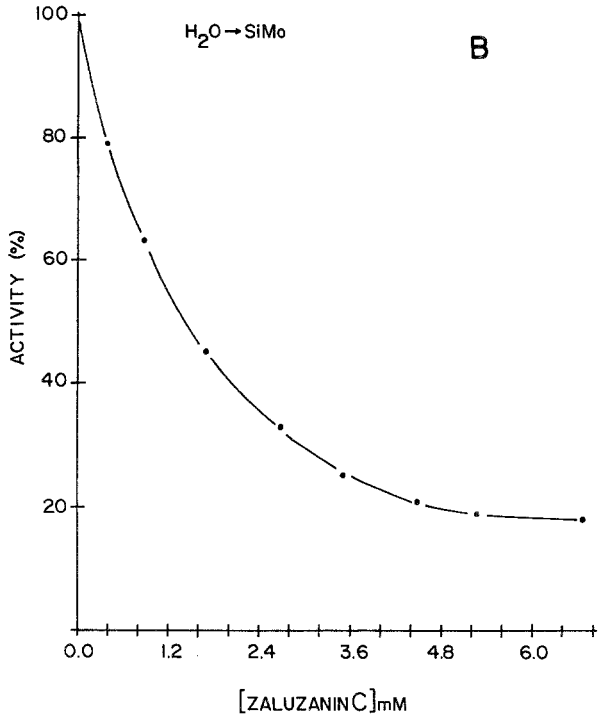


FIG. 3. Continued

3. Blocking the phosphorylation reaction itself (energy transfer inhibitors). In contrast to the other mechanisms, direct inhibitors of photophosphorylation block phosphorylation and the portion of electron transport that is a consequence of proton efflux linked to phosphorylation.

In order to obtain further information, we studied the effect of zaluzanin C on photosynthetic electron transport. Figure 2B shows that basal, phosphorylating, and uncoupled electron transport rates from water to methylviologen were inhibited as zaluzanin C concentrations increased up to concentrations similar to those affecting ATP synthesis and proton uptake (compare Figure 2B with 2A). These results indicate that zaluzanin C behaves as a typical electron transport inhibitor (process 1), since it inhibited uncoupled electron transport and phosphorylation, as well as proton uptake. Taking into account that uncoupled electron transport (I_{50} was 0.7 mM) was severely inhibited compared to the basal and phosphorylating electron rate, these data suggest that zaluzanin C binds to the target of an enzyme that suffers conformational changes when the membrane is energized and shields the zaluzanin's site.

In order to localize the inhibition site of zaluzanin C, photosystems I and

TABLE 2. EFFECT OF DIFFERENT COMPOUNDS IN REACTION OF WATER OXIDATION ENZYMES COMPLEX AS REPORTED IN LITERATURE[a]

Compound	Concentration (mM)	Oxygen evolution inhibition (%)
Ammonia (1)[a]	10	100
Methylamine (1)	100	100
Hydroxylamine (2)	1	100
Tris-washing (3)	800	60–80
DCMU (4)	0.2	80
Cu^{2+}, Cd^{2+}, Zn^{2+} (5)	0.005	80–100
Cacalol (6)	0.06	100
Diamines (7)	0.01–10	100
Zaluzanin C (8)	0.8	50
ANT2p (9)	0.0005	80
p-Diazonium benzenesulfonate (10)	1.5	50
Z^{2+} (11)	4.0	100

[a](1) Izawa and Good, 1972; (2) Joliot, 1966; (3) Yamashita and Butler, 1968; (4) Renger, 1973; (5) Singh and Singh, 1987; (6) Lotina-Hennsen et al., 1991; (7) Lotina-Hennsen et al., 1987b; (8) data from this work; (9) ANT2p = (2-(3-chloro-4-trifluoromethyl)amilino-3,5-dinitrothiophene); Packham and Robert, 1986; (10) Giaquinta et al., 1974; (11) Tripathy and Mohanty, 1980.

II were measured in the presence of this chemical. The activity of photosystem I from 2,3,5,6-tetramethyl-p-phenylandiamine (DAD) was not affected by this compound (Table 1). However, zaluzanin C inhibited coupled photosystem II electron transport from H_2O to DAD; the I_{50} was 2.7 mM (Figure 3A). Electron transport from water to silicomolybdate was inhibited by zaluzanin C with I_{50} 1.4 mM (Figure 3B). The concentration of zaluzanin C is low because silimolybdate destroys the coupling capacity of PSII (Giaquinta et al., 1974).

To determine the site of electron transport inhibition between water and Q_A by zaluzanin C, electron flows were measured from DPC to DCIP. The results show that this span of electron transport was not affected by zaluzanin C (Table 1). Therefore, this result indicates that zaluzanin C inhibits the Hill reaction by acting between H_2O to P680.

The water-splitting enzyme system is a key event in photosynthesis. It is known to be far more labile (with ammonia, methylamine, hydroxylamine, etc.) than the photosystem II trapping center; therefore, various inhibitors and treatments are effective in selectively blocking or destroying the water oxidation step (Izawa and Good, 1972; see references therein). Table 2 shows that the concentration of each compound needed to inhibit water oxidation varies widely. The structure of these compounds also varies greatly. Thus, these data indicate that these compounds inhibit the enzyme at different sites and with different

mechanisms of action. It is important to note that the only natural product that inhibits oxygen evolution as zaluzanin C does is cacalol (Lotina-Hennsen et al., 1991). The I_{50} of zaluzanin C (0.8 mm) is in the average range of various inhibitors at the same target.

As far as we know, there are eudesmanolides that greatly enhance the rate of respiration of *Chlorella pyrenoidosa*, but the effect of sesquiterpertene lactones on photosynthesis is unknown. This is the first time that zaluzanin C is reported as an inhibitor at this target (at the level of oxygen evolution). This result is similar to that found for cacalol (Lotina-Hennsen et al., 1991). How significant the effect of zaluzanin C is in the normal growth pattern of the plant has yet to be determined.

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MULTICOMPONENT SEX PHEROMONE IN *Macrocentrus grandii* GOIDANICH (HYMENOPTERA: BRACONIDAE)

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Abstract—A multicomponent sex pheromone in *Macrocentrus grandii* Goidanich (Hymenoptera: Braconidae) was demonstrated using wind-tunnel and field bioassays. In wind-tunnel bioassays, three Florisil fractions from female wasps (hexane, 5%, and 50% ether in hexane) and one from male wasps (50% ether in hexane) were attractive to males. The hexane and 5% ether in hexane fractions each elicited similar male behavioral responses. These included upwind anemotaxis, casting, landing on the source, wing fanning, and mating attempts between males. The 50% ether in hexane fraction, whether male- or female-derived, initiated a strong flight response by males but few landings on the source. In addition, the 50% ether in hexane fraction was found to synergize strongly the hexane fraction. All females tested in the wind tunnel gave no responses to male- or female-derived extracts. Field tests generally supported the wind-tunnel results, although no field attraction was observed for the 5% ether in hexane fraction.

Key Words—Parasitoid, pheromone, bioassay, wind-tunnel, Hymenoptera, Braconidae, *Macrocentrus grandii*, Lepidoptera, Pyralidae, *Ostrinia nubilalis*.

INTRODUCTION

The integration of natural enemies with other means of control in pest management programs is receiving increased attention (Chiang and Palmer, 1978; Lewis, 1982; Greany et al., 1984; Tumlinson, 1988). An understanding of the chemical ecology of these natural enemies is an important part of the integration and enhancement of beneficial insects in these programs. This may include the iden-

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tification and evaluation of parasitoid pheromones, which could provide a means for assessing parasitoid activity and density and for predicting rates of host parasitism (Lewis et al., 1971; Robacker et al., 1976; Greany et al., 1984; Morse and Kulman, 1985).

Eller et al. (1984) reviewed the contributions citing sex pheromones in parasitic Hymenoptera. Additional investigations have been made in the families Braconidae (Weseloh, 1977, 1980; Vinson, 1978; Tagawa and Kitano, 1981; Tagawa, 1983; Powell and King, 1984; Jones, 1989), Pteromalidae (Assem et al., 1981), Ichneumonidae (Rotheray, 1981) Eulophidae (Takahashi and Sugai, 1982; Qui et al. 1984; Gonzalez et al., 1985), Trichogrammatidae (Pintureau and Toonders, 1983), and Aphidiidae (Singh and Sinha, 1982; Powell and Zhang, 1983). These works present behavioral evidence for the existence of a sex pheromone in over 35 species and in 10 families of parasitic Hymenoptera by using live wasps, body parts or fluids, and/or solvent extracts of these elements as sources of attraction.

The isolation, identification, and field bioassay of a parasitoid's sex pheromone has been described for only one species. Eller et al. (1984) identified and field tested synthetic ethyl (Z)-9-hexadecenoate, a sex pheromone from *Syndipnus rubiginosus* Walley (Ichneumonidae). Later, Morse and Kulman (1985), using sex pheromone traps, studied the relationship between catches of *S. rubiginosus* and its host *Pikonema alaskensis* (Rohwer), the yellowheaded spruce sawfly, and subsequent sawfly-caused defoliation. They found a significant relationship between defoliation and trap catches (host and parasitoid) and suggested such information could provide a pest manager with information on the natural control and the growth phase of a pest population.

Macrocentrus grandii Goidanich is an introduced larval parasitoid of the European corn borer, *Ostrinia nubilalis* (Hübner). *M. grandii* was imported from France and the Orient as early as 1926, and today, throughout much of its range, it is in good synchrony with the peak abundance of its favored larval host stages (Winnie and Chiang, 1984). Despite this synchrony, rates of parasitism in many areas have generally been poor ($<1\%$) except for periodic and localized high parasitism rates (9–56%) (Andreadis, 1980, 1982; Burbutis et al., 1981; Lewis, 1982). Why these fluctuations in parasitism rates have occurred is unclear, but factors such as pesticide usage, susceptibility to pathogens, and low host density have been proposed (Andreadis, 1982; Chiang, 1978; Lewis, 1982).

An understanding of *M. grandii's* courtship behavior and knowledge of its sex pheromone could lead to enhanced efficacy of this beneficial parasitoid. The purpose of this investigation was to document and define the role of pheromones in the sexual behavior of *M. grandii*. The study was prompted by observations in the laboratory that the presence of female-associated chemicals elicited attraction and wing fanning by males.

METHODS AND MATERIALS

Insect Material. M. grandii and *O. nubilalis* were reared according to Ding et al. (1989) and Guthrie et al. (1971), respectively. Voucher specimens have been placed in the University of Minnesota insect museum.

Pheromone Collection and Purification. To collect pheromone(s) associated with courtship behavior, whole-body extracts of male and female virgin wasps (one to three days after pupal emergence) were made by soaking wasps (sexes separate) in hexane for several days. After soaking, the hexane extract was drawn off, reduced under nitrogen, and fractionated by column chromatography on Florisil (2.5% water by weight). The column was eluted consecutively with aliquots of hexane, then 2.5, 5.0, 7.5, 10.0, and 50.0% ether in hexane (hereafter labeled 2.5E, 5E, 7.5E, 10E, and 50E), ether, acetone, and methanol. The volume of each eluant was about twice the void volume of the column. Fractions were capped under nitrogen and stored at -70°C until used for bioassay.

Laboratory Bioassay. Pheromone bioassays were conducted indoors in a rectangular fish tank (31 × 42 × 92 cm) modified to function as a wind-tunnel. The wind-tunnel was screened on each end and was all glass elsewhere. Air was circulated through the wind-tunnel by a fan at the downwind end that pulled air through the wind-tunnel at ca. 0.25 m/sec. The bioassay arena was held in a walk-in environmental chamber in which 16:8 hr, light–dark, 25°C constant temperature, and 80–90% relative humidity were maintained. Air, drawn by the fan through the tunnel, was subsequently exhausted out of the environmental chamber. A bank of four 40-W fluorescent lights, 5 cm above the wind tunnel, ran parallel with the length of the tank. About 150–200 male or female wasps were used for a bioassay. Chemical extract preparations were placed on 4-cm watch-glass plates. After the solvent had evaporated, each plate was attached to a Boston clip and each clip with its plate was then slipped onto a wire hook. The hooks were positioned in the upwind end of the tunnel 21 cm or 10.5 cm apart (distance apart depended on whether we were bioassaying two or three treatments) and 15 cm above the wind-tunnel floor. All chemical preparations were tested at the rate of 1/3 of a male or female equivalent (ME or FE). All chemical preparations were used for only one test, with the exception of the experiment testing the activity of three Florisil fractions over 24 hr. In this experiment, the initial chemical treatments were the same treatments bioassayed at 15 min, 1 hr, and 24 hr, and with the same male wasps. Live virgin wasps (male or female), when bioassayed, were placed in 4×2-cm screen cylinders and positioned likewise in the upwind end of the tunnel.

Observed behavior included upwind flight, casting, landing on the source, wing fanning, and mating attempts between males. Visits at the source were usually brief, and the bioassay was quantified by recording the total number of

wasps landing on each plate during a 1-min period. Since the wasps were not trapped, the same wasp could land more than once and/or land on more than one treatment. All replicates were run usually with untested male or female wasps in the wind-tunnel. For pairwise comparisons, data were analyzed by a one-sided paired t test. Chemical positions were exchanged for each replicate. Three-way comparisons were considered a randomized complete block design with each bioassay labeled a block. Chemical preparations were positioned randomly for each bioassay. Data were transformed ($\sqrt{x + 1}$) to stabilize variance and tested for homogeneity with Levene's test. An analysis of variance (ANOVA) was then applied (degrees of freedom for error = 4) with mean separations by Fisher's (protected) least significant difference (PLSD), $\alpha \leq 0.05$ (Steel and Torrie, 1980).

Field Bioassay. Field bioassays were conducted within a University of Minnesota test plot of hybrid field corn. An area of no less than 52 rows × 75 hills was chosen and all experiments were run in a randomized complete block design with rows of corn as blocks. Treatments were 10 hills apart, blocks 10 rows apart, and traps (Pherocon 1C) were secured to the corn stalk 1.0–1.5m above ground. Block rows were always a minimum of 10 rows from the field border. Chemical extract preparations were placed on 4-cm watch-glass plates centered inside the trap bottom. Live virgin wasps (male or female) were placed with honey and water in 4 × 2-cm screen cylinders that were centered under the top of each trap. However, even with the provision of honey and water, many of the wasps within the cylinders were dead by the end of the experiment (24 hr).

Because of low and variable natural populations of *M. grandii*, laboratory-reared males were released into the test plots to ensure adequate densities. Males were used for all field bioassays, and they were released from emergence cups into the plots immediately after extracts were placed into the traps. They were released along each fifth row in between blocks, and the number of cups per row and their distance apart varied and was dependent upon male availability. In three separate field tests, ca. 1100, 2000, or 3000 males were released. Males were also released five rows outside the experimental area. All field experiments were begun in the early afternoon. Field data was transformed to $\sqrt{x + 1}$ to stabilize variance and tested for homogeneity with Levene's test. Treatments in which all replicates had zero captures were not included in the analysis. An ANOVA was applied with mean separations by PLSD, $\alpha \leq 0.05$.

RESULTS

Laboratory Bioassay. Evaluation of male *M. grandii* response to female-derived Florisil fractions indicated that the hexane, 5E, and 50E fractions contained attractive chemicals as measured by landing at the source (Table 1,

TABLE 1. LANDING RESPONSES OF MALE *M. grandii*[a] TO VIRGIN FEMALE OR MALE AND TO EXTRACT AND FLORISIL FRACTIONS OF VIRGIN FEMALES OR MALES IN LABORATORY WIND-TUNNEL

	Means[c]	
Treatment[b]	Female/Control	Male/Control
Virgin wasp (1 wasp)	**46.5/0.0	0.0/0.0
Hexane extract (1/3 WE)	**28.2/0.0	*10.0/0.2
Florisil fractions (1/3 WE)		
Hexane	**40.5/0.2	*3.5/0.0
2.5E	4.0/0.0	0.0/0.0
5E	**30.8/0.0	0.0/0.0
7.5E	2.7/0.2	0.0/0.0
10E	0.3/0.0	0.0/0.0
50E	*6.0/0.0	2.0/0.0
Ether	0.5/0.0	0.0/0.0
Acetone	0.2/0.2	0.0/0.0
Methanol	1.2/0.0	0.0/0.0

[a] 150–200 males 1–5 days old were tested.
[b] Each treatment plate was paired with a control. The female treatments utilize female wasps and extracts, the male treatments utilize male wasps and extracts. (WE) implies wasp equivalent.
[c] Six replications with each replicate using a new group of untested males; counts represent the mean number of male wasps landing on each plate during a 1-min interval; * and ** imply significant differences from control at the $\alpha \leq 0.05$ and $\alpha \leq 0.01$ levels (one-sided paired t test).

Female/Control). The hexane and 5E fractions elicited similar quantitative activity as the virgin female or to its hexane extract. The hexane and 5E fractions, the female hexane extract, and the virgin female also elicited qualitatively similar male behaviors in the wind-tunnel. These responses included flight initiation, upwind anemotaxis, casting, landing on the source, walking, wing fanning, and mating attempts between males.

The 50E fraction elicited a very different response. Although there was a very strong upwind anemotaxis, landings on the watch-glass were few. Males also were observed to land on the floor of the wind-tunnel directly under the 50E treatment watch-glass; this was a behavior not observed frequently with the other Florisil fractions.

In the evaluation of male wasp response to male-derived Florisil fractions, significant landing responses were observed with the hexane extract and the Florisil hexane fraction (Table 1, Male/Control). Again no significant landing response was observed with the 50E fraction, but it elicited a very strong upwind anemotaxis response, as observed by males responding to the female-derived 50E fraction. In addition, the male hexane extract gave similar flight responses

and landings under the watch-glass as the male- or female-derived 50E fractions. The male-derived hexane Florisil fraction was active, but there was no strong flight response. However, the few males attracted to the plate exhibited landing and wing-fanning behaviors. Whether this attraction to the male-derived hexane fraction is significant in nature seems doubtful because it is very weak compared to the male response to female-derived material.

Evaluation of female wasp response in the wind-tunnel to male- or female-derived extracts or Florisil fractions of these extracts ($N = 6$; identical to the Florisil fractions in Table 1) elicited no observable behavioral response.

To test for synergism, combinations of the fractions were presented to males. A significant increase in landing response was observed when the female-derived hexane and male- or female-derived 50E fractions were combined (Table 2; tests 1 and 2). No other fraction or combination of male- or female-derived fractions, when compared to these fractions, was as attractive (Table 2; tests 8–12). In fact, the previously "very attractive" female-derived hexane or 5E fractions gave a minimal response when tested along with the hexane plus 50E combination (Tables 1 and 2; tests 1, 2, and 8). The combined female-derived hexane and male- or female-derived 50E fractions generally "dominated" the bioassay arena at 1/3 male or female equivalent. In contrast, combining the 50E fraction (male- or female-derived) with the female-derived 5E fraction appeared to reduce attraction to the 5E fraction (Table 2; test 5 and 6). Combining the female-derived hexane and 5E fractions also provided a significantly more attractive source than either of the fractions alone (Table 2; test 7). However, combining the female-derived hexane and 5E fractions was not as attractive as a combination of the hexane and 50E (Table 2; test 9).

The three active female-derived Florisil fractions—hexane, 5E, and 50E—were tested for their activity over time (Table 3). Only the hexane fraction and hexane plus 50E fractions elicited male attractancy behavior after the initial bioassay. After 15 min, the 5E fraction was nearly inactive and the 50E fraction no longer elicited upwind anemotaxis. After the initial bioassay, the hexane plus 50E fraction's activity also dropped to a level comparable to the hexane fraction only. The activity of the hexane fraction remained quite active even 24 hr later.

Field Bioassay. When female-derived Florisil fractions were individually field tested, only the hexane fraction was highly attractive (Table 4). This activity was similar to that elicited by female-derived hexane extract and virgin females (Tables 4–6).

The 5E fraction (which in the wind-tunnel was highly attractive) was unattractive in the field bioassay (Tables 1 and 4). Results of the wind-tunnel bioassay had indicated that attraction to the 5E fraction was short-lived (Table 3; test 1). Therefore, attempts were made to increase the attraction time of the 5E fraction by increasing its concentration and/or combining it with other fractions or release-rate retardants. However, all efforts to demonstrate field activity with the 5E fraction were unsuccessful.

TABLE 2. LANDING RESPONSES OF MALE *M. grandii* TO COMBINATIONS OF FLORISIL FRACTIONS OF VIRGIN FEMALES OR MALES (1/3 FE OR ME PER PLATE) IN LABORATORY WIND-TUNNEL

Treatment[a]	Mean[b]	Treatment	Mean
Test 1		Test 2	
Hexane + 50E	68.7a	Hexane + 50E (M)	39.0a
Hexane	7.6b	Hexane	10.3b
50E	1.0c	50E (M)	0.0c
Test 3		Test 4	
Hexane (M) + 50E (M)	0.7a	50E (M)	0.3a
Hexane (M)	0.0a	50E	4.3a
50E (M)	1.0a	Control	0.0a
Test 5		Test 6	
5E + 50E	20.3a	5E + 50E (M)	12.3
5E	31.3a	5E	36.0b
50E	0.3c	50E (M)	0.0c
Test 7		Test 8	
Hexane + 5E	41.7a	Hexane + 50E + 5E	18.0a
Hexane	16.7b	Hexane + 50E	40.0b
5E	13.7b	5E	2.7c
Test 9		Test 10	
Hexane + 50E	57.0a	Hexane + 50E	63.7a
Hexane + 5E	23.7b	Hexane + 2.5E + 7.5E + 10E	15.3b
5E + 50E	1.3c	Hexane + ether + acetone + methanol	6.7b
Test 11		Test 12	
Hexane + 50E	66.7a	Hexane + 50E	60.3a
5E + 2.5E + 7.5E + 10E	9.7b	50E + 2.5E + 7.5E + 10E	7.0b
5E + ether + acetone + methanol	6.0b	50E + ether + acetone + methanol	3.3b

[a] Unless noted with the letter M, which implies male-derived, Florisil fractions are female-derived.
[b] Three replications with each replicate using a new group of 150–200 untested males 1–5 days old; counts represent the mean number of males landing on each plate during a 1-min test. In each test, column means followed by different letters are significantly different (PLSD, $\alpha \leq 0.05$).

Field testing of the 50E fraction demonstrated very low trap catches (Table 4). This was not surprising since the wind-tunnel bioassays also had demonstrated infrequent landing behavior and short-lived activity for the 50E fraction (Tables 1 and 3). To increase the attraction period of the 50E fraction, its concentration was increased to two or three times the dosage of the other fractions in subsequent tests. The change in concentration did appear to increase the 50E trap catch. In addition, when the 50E fraction was combined with the

TABLE 3. LANDING RESPONSES OF MALE *M. grandii*[a] TO FLORISIL FRACTIONS OF VIRGIN FEMALES OVER TIME IN WIND-TUNNEL

	Mean			
Treatment[b]	Initial[c]	15 min	1 hr	24 hr
Test 1				
Hexane	65.3a	38.7a	34.3a	24.7a
5E	29.3b	0.7b	1.7b	0.3b
50E	2.3c	0.3b	0.3b	0.0b
Test 2				
Hexane	33.0a	20.7a	11.3a	19.7a
Hexane + 50E	74.7b	19.0a	15.0a	18.7a
Control	0.0c	0.0b	0.0b	0.0b

[a] 150–200 males 1–5 days old were tested.
[b] All treatments 1/3 FE per plate.
[c] Three replications with each replicate using a new group of untested males. Counts represent the mean mumber of males landing on each plate during a 1-min test. In each test, column means followed by different letters are significantly different (PLSD, $\alpha \leq 0.05$).

TABLE 4. MEAN CATCH OF MALE *M. grandii*[a] TO VIRGIN FEMALES AND TO EXTRACT AND FLORISIL FRACTIONS OF VIRGIN FEMALES IN CORN FIELD

Treatment	Mean[b] ($N = 4$)
Control (empty trap)	0
Virgin female (4 females per trap)	4.8b
Hexane extract (15 FE per trap)	22.0a
Florisil fractions (15 FE per trap)	
Hexane	13.8ab
2.5E	0
5E	0.5b
7.5E	0
10E	0.2b
50E	1.2b
Ether	0
Acetone	0
Methanol	0.5b

[a] About 1100 laboratory-reared males were released for this field bioassay.
[b] Column means followed by different letters are significantly different (PLSD, $\alpha \leq 0.05$).

TABLE 5. MEAN CATCH OF MALE *M. grandii*[a] TO VIRGIN FEMALES AND TO EXTRACT AND FLORSIL FRACTIONS OF VIRGIN FEMALES IN CORN FIELD

Treatment	Mean[b] ($N = 4$)
Control (empty trap)	0
Virgin females (4 per trap)	7.8a
Hexane extract (10 FE per trap)	9.0a
Florisil fractions[c]	
Hexane	12.2a
50E	5.5a
Hexane + 5E	2.2a
Hexane + 50E	37.8b
Hexane + ether + acetone + methanol	5.5a
Hexane + 2.5E + 7.5E + 10E	2.2a

[a] About 3000 laboratory-reared males were released for this field bioassay.
[b] Column means followed by different letters are significantly different (PLSD, $\alpha \leq 0.05$).
[c] All Florisil fractions 10 FE per trap except the 50E fraction which was 30 FE per trap.

TABLE 6. MEAN CATCH OF MALE *M. grandii*[a] TO VIRGIN FEMALES, VIRGIN MALES, AND FLORISIL FRACTIONS OF VIRGIN MALES AND FEMALES IN CORN FIELD

Treatment	Mean[b] ($N = 4$)
Control (empty trap)	0
Virgin female (5 per trap)	4.5a
Virgin male (5 per trap)	3.2a
Virgin male and female (5 of each per trap)	5.0a
Florisil fractions[c]	
Hexane	3.8a
50E (M)	3.5a
Hexane + 50E (M)	18.0b
Hexane + virgin males (5 per trap)	3.2a

[a] About 2000 laboratory-reared males were released for this field bioassay.
[b] Column means followed by different letters are significantly different (PLSD, $\alpha \leq 0.05$).
[c] Florisil hexane fractions are 10 female wasp equivalents, 50E fractions are 20 male wasp equivalents.

hexane Florisil fraction, catches increased synergistically (Table 5). This observation was similar to that observed in the wind-tunnel. All other fractions bioassayed individually or in conjunction with other fractions were not significantly active in the field.

When the male-derived 50E fraction was field tested with the female-derived

hexane fraction, activity analogous to that found with a mix of the female-derived hexane and 50E fractions was observed (Table 6). This result was comparable to that observed in the wind-tunnel. Tests also showed that virgin males were attractive to males (Table 6). This attractancy was similar to that of virgin females or a mix of the males and females. The attractancy of traps baited with virgin males plus the female-derived hexane fraction was not significantly different from the female-derived hexane fraction only. However, the death of many of the live wasps in the live bait treatments leaves these results unclear.

DISCUSSION

Both the wind-tunnel and field studies support the view that *M. grandii* has a multicomponent sex pheromone. The combination of the Florisil hexane fraction with the male- or female-derived 50E fraction was the most attractive source. The 50E fraction elicits a strong flight response in male wasps, and the hexane fraction elicits flight, landing, and mating behaviors. Together these components appear to comprise a potent sex pheromone with long-range attraction, landing, and mating behavior.

The function of the 5E fraction in this scenario is unexplained. It appeared very attractive in wind-tunnel bioassays, whether bioassayed alone on in conjunction with the female-derived Florisil hexane fraction. However, the attractiveness of the 5E fraction decreased when combined with the 50E fraction and the 5E fraction was not attractive in the field. This latter result may be due to the field procedures, since release of males into the test site took several minutes after the extracts were applied, and ephemeral extracts could lose much of their attractiveness during this time.

The Florisil separation indicated the likelihood of at least three different chemicals contributing to pheromone activity. The hexane fraction would contain nonpolar compounds (i.e., hydrocarbons), the 5E fraction would contain moderately polar compounds (i.e., ketones, aldehydes, or esters), and the 50E fraction would contain chemicals similar in polarity to the primary alcohol. In addition, the fact that the hexane fraction was attractive 24 hr later indicated the chemical(s) responsible for its attractiveness were either quite abundant, slow to vaporize, and/or a product of a continually occurring chemical reaction (e.g., photo oxidation). The active component(s) of the 5E and 50E fractions were very volatile and/or unstable because their activity diminished significantly in 15 min.

The wind-tunnel and field data also demonstrated that males, with the male-derived 50E fraction eliciting qualitatively identical male responses as the female-derived 50E fraction, may be "chemically" active in the mate finding scenario. Lewis et al. (1971) observed the behavior of males attracting males with *Car-*

diochiles nigriceps and postulated male flight aggregation with subsequent response to females that fly into the area. They had observed groups of males flying through the field. Although we have not observed *M. grandii* flying freely in the field, these data indicate that male aggregation may play a role in the behavior of *M. grandii*.

Identification and clarification of the roles of these behavioral components for *M. grandii* is the subject of subsequent papers.

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(Z)-4-TRIDECENAL, A PHEROMONALLY ACTIVE AIR OXIDATION PRODUCT FROM A SERIES OF (Z,Z)-9,13 DIENES IN *Macrocentrus grandii* GOIDANICH (HYMENOPTERA: BRACONIDAE)

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Abstract—(*Z*)-4-Tridecenal was identified as a sex pheromone component of *Macrocentrus grandii*, a larval parasitoid of the European corn borer. The aldehyde was found to be a common air oxidation product from a series of (*Z,Z*)-9,13-dienes of 27–33, 35, 37, 39, and 41 carbon atoms in female wasps. Synthetic (*Z*)-4-tridecenal and (*Z,Z*)-9,13-heptacosdiene each elicited flight initiation, upwind anemotaxis, casting, landing on the source, walking, wing fanning, and copulatory attempts by male wasps in a wind-tunnel. Field studies demonstrated that both compounds are synergized by a more polar component. Synthetic (*E*)-4-tridecenal was not attractive.

Key Words—Pheromone, hydrocarbon, diene, (*Z*)-4-tridecenal, oxidation, Hymenoptera, Braconidae, *Macrocentrus grandii*, parasitoid.

INTRODUCTION

Macrocentrus grandii Goidanich is a larval parasitoid of the European corn borer, *Ostrinia nubilalis* (Hübner). In a previous report we described evidence of a female-produced multicomponent sex pheromone for *M. grandii* (Swedenborg and Jones, 1992). These components occurred in the hexane, 5% ether in hexane (5E), and 50% ether in hexane (50E) fractions eluted from Florisil. In this paper we report the isolation, identification, and synthesis of two pheromone components originating from the hexane and 5E fractions. In addition,

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we report evidence for a second pheromone component occurring in the 5E fraction.

METHODS AND MATERIALS

Wasps, Extracts, and Florisil Fractions. *M. grandii* and *O. nubilalis* were reared according to Ding et al. (1989) and Guthrie et al. (1971), respectively. Handling, extraction, and initial purification of the extracts using Florisil were as described by Swedenborg and Jones (1992). Voucher specimens have been placed in the University of Minnesota insect museum.

Chromatography. Open-column argentation chromatography was conducted using a 9-in. Pasteur capillary pipet packed with 5 cm of 20% silver nitrate on silica gel (60/200 mesh). The column (void volume ca. 1 ml) was eluted first with 2 ml hexane, followed by 2 ml of 10% ether in hexane. The sample was the original Florisil hexane fraction (Swedenborg and Jones, 1992). Similar chromatography was conducted using Florisil (2.5% water by weight). This column was eluted consecutively with aliquots of hexane then 2.5, 5.0, 7.5, 10.0, and 50.0% ether in hexane, ether, acetone, and methanol. The samples were the products of diene oxidation and chemical syntheses. All fractions were capped under nitrogen and stored at −70°C until used for bioassay or analysis.

High-pressure liquid chromatography (HPLC) was done isocratically with a Waters Associates M6000A pump and a R401 differential refractometer detector. Columns used were a Serva 250-mm × 4.6-mm-ID Servachrome silica gel column coated in situ with 20% silver nitrate, as described by Heath and Sonnet (1980), and an Altex 300-mm × 8-mm-ID μ-Spherogel 50 Å size-exclusion column. Samples on the Serva Column were eluted with 25% toluene in hexane (hydrocarbon analysis) or 100% toluene (aldehyde analysis) at 2.0 ml/min. Samples on the Altex column (aldehyde analysis) were eluted with 25% ether in hexane at 1.0 ml/min.

Gas chromatography (GC) was performed with Hewlett Packard 5830A and 5890A instruments, both equipped with flame ionization detectors. The 5830A was modified to contain an effluent splitter and thermal gradient collector (similar to that described by Brownlee and Silverstein, 1968). Columns used were: glass columns, 1.9 m × 2 mm or 4 mm ID packed with 3% Dexsil 300 on 80/100 Gas Chrom Q for hydrocarbon and aldehyde analysis, collection, and hydrocarbon equivalent chain length (ECL) determination; a bonded fused silica column, Supelco Supelcowax 10, 30 m × 0.53 mm ID, for aldehyde analysis and collection; a bonded fused silica capillary column, J & W DB-1, 30 m × 0.32 mm ID, for hydrocarbon and aldehyde analysis; and a fused silica capillary column, Quadrex SLP-Silar-10C 50 m × 0.25 mm ID, for aldehyde double bond configuration. Key GC temperature programs are noted with results.

Spectroscopy. Coupled gas chromatography–mass spectroscopy (GC-MS) was carried out with either an LKB-9000 instrument using a 0.6-m × 2-mm-ID 3% Dexsil 300 column and electron ionization energy of 20 eV or 70 eV, or a Kratos MS25 instrument using a J & W DB-1 15-m × 0.53-mm-ID column.

The Fourier-transform infrared (FT-IR) spectrum was taken with a Nicolet 170SX spectrometer. The IR spectrum was run with ca. 2 mg of the HPLC-purified diene material held between two KBr 25-mm × 4-mm disks. Bands were reported in wave numbers (cm^{-1}).

Chemical Tests. Ozonolysis was done as described by Beroza and Bierl (1967). Samples (0.1–2 μg of diene material) were ozonized in 20 μl of carbon disulfide for 2 min at −70°C. The ozonides were then reduced with triphenylphosphine to aldehydes. Subsequently, ozonized samples were run on the GC or GC-MS to identify reaction products.

For molecular sieve tests, samples (1–2 μg of diene material) were evaporated under nitrogen then resolubilized in isooctane containing activated 5 Å molecular sieve pellets to separate branched from straight-chain compounds (Hutchins and Martin, 1968). Control samples included the dienes but no molecular sieve pellets. Twenty-four hours later the sample solutions were analyzed by GC for changes in diene concentration.

For diene oxidation tests, six samples (20 μg each) of natural and synthetic (Z,Z)-9,13-heptacosadiene, after purification by Florisil, HPLC, and GC, were solubilized in hexane and placed on 6-cm watch-glass plates. Three replicates of each source were placed on the lab bench under fluorescent ceiling lights and three were placed in an ultra-low freezer −70°C. After 24 h, the watch-glass plates were each rinsed with 1 ml hexane. Each replicate was then chromatographed through a Florisil column (to separate dienes from diene oxidation products) followed by GC or GC-MS to look at reaction products and bioassayed for activity. In addition, a similar oxidation was performed with 200 μg of the entire diene series.

Synthesis of (Z,Z)-*9,13-Heptacosadiene.* Diene synthesis was guided by the procedure of Sonnet (1974) utilizing a Wittig condensation with the cosolvent, dimethyl sulfoxide. A procedural change involved the preparation of the alkyltriphenylphosphonium salt from 1,4-dibromobutane, which, upon simultaneous addition of nonanal and tetradecanal, provided for double-bond formation on each end of the four-carbon precursor (Bartelt et al., 1986). Following synthesis, the dienes were separated from polar by-products on Florisil and 9,13-heptacosadiene was separated from the nonpolar symmetrical by-products of 9,13-docosadiene and 14,18-dotriacontadiene using gas chromatography. HPLC, using a silver nitrate-coated silica gel column, eluted with 25% toluene in hexane, was used to provide geometrical purity of the synthetic dienes (Bartelt et al., 1982). All starting chemicals were obtained from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, and used without further purification.

Synthesis of (Z)-*4-Tridecenal and* (E)-*4-Tridecenal.* (Z)-4-Tridecenyl acetate and (*E*)-4-tridecenyl acetate were purchased from Bedoukian Research, Inc., Danbury, Connecticut. The isomers were base hydrolyzed to their respective alcohols using KOH in 95% aqueous ethanol. Following hydrolysis, the ethanol was distilled off, and the sample was diluted with methylene chloride and dried over 4 Å molecular sieves. Without further purification, the alcohols were oxidized with chromium trioxide and pyridinium chloride to provide (*Z*)-4-tridecenal and (*E*)-4-tridecenal (Svirskaya et al., 1980). Following synthesis, the aldehydes were purified using Florisil, HPLC, and GC.

Laboratory and Field Bioassay. Bioassays were conducted as described by Swedenborg and Jones (1992). For the laboratory bioassay a wind-tunnel was utilized with treatments on 4-cm watch-glass plates at the upwind end of the tunnel. About 150–200 males were used for a bioassay. Male behaviors in the bioassay included flight initiation, upwind anemotaxis, casting, landing on the source, walking, wing fanning, and mating attempts between males. Visits at the source were usually brief, and the bioassay was quantified by recording the total number of males landing on each plate during a 1-min period. Open column silver nitrate fractions were tested at 1/3 female equivalent (FE), HPLC fractions were tested at 5 FE, and GC fractions were tested at 1/3 FE (HPLC purified dienes) or 5 FE (diene oxidation products and HPLC purified 5E). All synthetic and female-derived dienes were tested at a 100-ng dosage (the total diene material extracted from one female using hexane is ca. 9 μg), and all sources of 4-tridecenal were tested at a 17-pg dosage [the total (Z)-4-tridecenal extracted from one female using hexane is ca. 50 pg]. For pairwise comparisons, data were analyzed by a one-sided paired *t* test. Chemical positions were exchanged for each replicate. Three-way comparisons were considered a randomized complete block design with each bioassay being a block. Data were transformed ($\sqrt{x + 1}$) to stabilize variance and tested for homogeneity with Levene's test. An analysis of variance (ANOVA) was then applied (degrees of freedom for error = 4) with mean separations by Fisher's (protected) least significant difference (PLSD), $\alpha \leq 0.05$ (Steel and Torrie, 1980).

In two separate field tests ca. 1200 and 800 laboratory-reared males were released into a test plot of hybrid field corn. Traps (Pherocon 1C) were secured to the corn stalk 1.0–1.5 m above ground. Chemical extract preparations were placed on 4-cm watch-glass plates centered inside the trap bottom. A Hercon polymer-bonded slow-release wafer formulation (0.15 mg isomer/cm^2) of (Z)-4-tridecenal and (*E*)-4-tridecenal was used at target release dosages of 50 ng/hr each for test 1 (release rates not verified). The 50E fraction was applied at 15 FE for test 1 and 20 FE for test 2. Synthetic (Z,Z)-9,13-heptacosadiene was applied at 20 FE or 180 μg diene for test 2. Each test was initiated near midday and run for 24 hr. Wasps were released immediately after the treatments were applied. Field data were transformed ($\sqrt{x + 1}$) to stabilize variance and tested

for homogeneity with Levene's test. An ANOVA was applied with mean separations by PLSD, $\alpha \leq 0.05$.

RESULTS

Female-Derived Florisil Hexane Fraction. Bioassays of the Florisil hexane fraction were consistently active in laboratory and field studies (Swedenborg and Jones, 1992). This fraction (475 FE) was concentrated to 100 μl and chromatographed on a pipet column of 20% silver nitrate on silica gel to separate paraffins from olefins. The active fraction was the 10% ether in hexane fraction, which contained the olefins (Table 1; test 1). The olefin fraction was comparable in activity to the original Florisil hexane fraction (Table 1; test 2), and activity was not improved by addition of the hexane- or paraffin-containing fraction (Table 1; test 3).

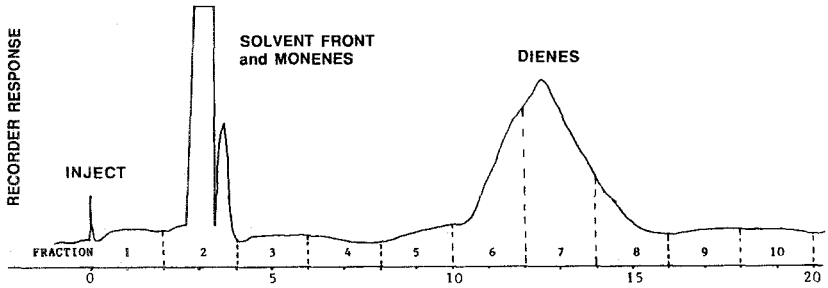
Subsequently, the olefin fraction was chromatographed using HPLC on a silver nitrate-coated silica gel column eluted with 25% toluene in hexane to separate monoenes from dienes (Figure 1). Bioassay activity coincided with fractions 5–9. GC and/or GC-MS analysis of the HPLC fractions identified the

TABLE 1. LANDING RESPONSES OF MALE *M. grandii* TO "SILVER NITRATE" OPEN COLUMN FRACTIONS OF VIRGIN FEMALES IN LABORATORY WIND-TUNNEL

Treatment[a]	Mean[b]
Test 1	
10% ether in hexane	35.0a
Hexane	0.3b
Control	0.3b
Test 2	
10% ether in hexane	35.0a
Original Florisil hexane	29.3a
Control	0.0b
Test 3	
10% ether in hexane	35.3a
Hexane + 10% ether in hexane	39.3a
Control	0.0b

[a] The paraffin and olefin portions of the original Florisil hexane fraction are represented by the hexane and 10% ether in hexane treatments, respectively.

[b] Three replications with each replicate using a new group of 150–200 untested males 1–5 days old; counts represent the mean number of males landing on each plate during a 1-min test; all fractions were bioassayed at 1/3 FE; in each test column means followed by different letters are significantly different (PLSD, $\alpha \leq 0.05$).



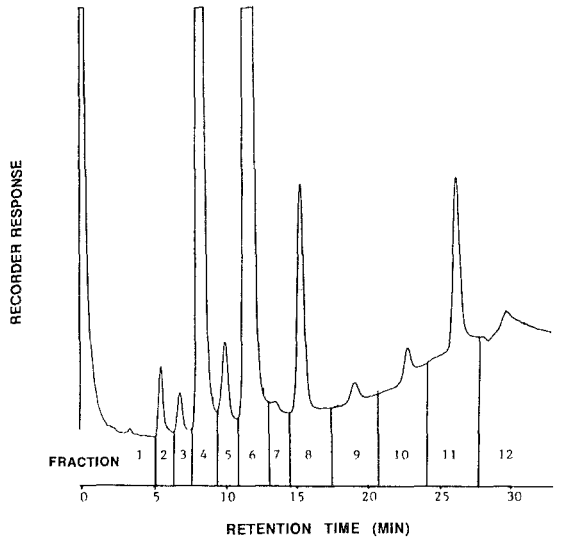
DATA:

FRACTION	1	2	3	4	5	6	7	8	9	10
RESPONSE	0.0	0.0	0.0	0.5	18.4**	31.3**	16.5**	19.7**	4.6**	1.2

FIG. 1. HPLC chromatogram using a Serva ServaChrom silica gel column coated in situ with silver nitrate. Sample represents 425 FE from the olefin fraction. Fractions were collected every 2 ml. The data represent the mean landing response of male *M. grandii* to each HPLC fraction in a laboratory wind-tunnel during a 1-min interval. Each treatment plate was paired with a solvent-only control; control plates paired with HPLC fractions 1 and 5–10 hand zero landings, plates 2–4 had 0.2 mean landings. 150–200 males 1–5 days old were tested; five replications with each replicate representing a new group of untested males; all HPLC fractions were bioassayed at 5 FE; * and ** imply significant differences from controls at the $\alpha \leq 0.05$ and $\alpha \leq 0.01$ levels (one-sided paired t test).

presence of monoenes in fractions 2 and 3 and dienes in fractions 5–9. The analysis demonstrated that dienes of longer chain length eluted slightly ahead of shorter-chain dienes on the silver nitrate-coated column. Therefore, each fraction did not contain the same mixture of dienes as the original olefin fraction. An identical analysis using male hydrocarbons demonstrated a virtual absence of dienes in male wasps with monoenes representing over 99% of the olefin component, whereas monoenes represented 39% of the olefin component in female wasps. In both male and female wasps saturated hydrocarbons represented ca. 20% of the total hydrocarbon component. Fraction 7 contained the bulk of the active HPLC peak. GC analysis showed fraction 7 over three times more concentrated with diene material than fractions 6 or 8. The reduced behavioral response to fraction 7 may have been due to an imbalance in the diene mixture and/or to pheromone overload in this fraction.

The active HPLC fractions (5–9) were combined and chromatographed on a 3% Dexsil 300 column (Figure 2). The peaks were collected separately and bioassayed at 1/3 FE. GC fractions 2–12 were significantly attractive. Male activity increased with greater peak area. The ECLs for the active diene peaks were 0.41–0.50 units less than the corresponding *n*-alkane (Table 2). The ECL



DATA:

FRACTION	1	2	3	4	5	6	7	8	9	10	11	12
RESPONSE	1.4	34.5*	42.5*	106.0**	35.1*	78.2**	16.6*	43.9*	14.1**	9.1*	26.8*	10.9*

FIG. 2. GC chromatogram of the HPLC purified dienes using a 3% Dexsil 300 1.9-m × 4-mm column. The GC temperature program used was an initial temperature of 240°C for 1.0 min followed by a 3°C/min increase to 325°C and held at 325°C for 10 min. The carrier was nitrogen at 70 ml/min. The data represent the mean landing response of male *M. grandii* to each GC fraction in a laboratory wind-tunnel during a 1-min interval. Each treatment plate was paired with a solvent-only control; all control plates paired with the GC fractions had zero landings. 150–200 males 1–5 days old were tested; four replications with each replicate representing a new group of untested males; all GC fractions were bioassayed at 1/3 FE; * and ** imply significant differences from controls at the $\alpha \leq 0.05$ and $\alpha \leq 0.01$ levels (one-sided paired t test).

fractional part increased with increasing chain length as predicted by the model set forth by Bartelt et al. (1986).

Mass spectra of the GC peaks were similar in appearance with a discernible molecular ion and an absence of the intense odd mass peaks indicative of branching. Experiments with 5 Å molecular sieve pellets also indicated an absence of branching. Molecular ions were consistent with hydrocarbons of 27–33, 35, 37, 39, and 41 carbon atoms; each with two degrees of unsaturation (Table 2). The mass spectrum for the peak in GC fraction 6 is shown in Figure 3A.

Double-bond positions were determined by ozonolysis of the dienes. GC retention times or GC-MS of the aldehydes (products of diene ozonolysis) were compared to those of standard aldehydes. For each unsaturated hydrocarbon,

TABLE 2. PHYSICAL CHARACTERISTICS OF DIENE PEAKS

Fraction[a]	ECL[b]	Molecular weight[c]	Carbon number[d]	Ozonolysis fragments[e]	Double-bond positions[f]	Peak amount[g]
2	26.50	376	27	9:Al, 14:Al	9,13	2.3
3	27.50	390	28	9:Al, 15:Al	9,13	1.3
4	28.50	404	29	9:Al, 16:Al	9,13	44.4
5	29.51	418	30	9:Al, 17:Al	9,13	1.9
6	30.53	432	31	9:Al, 18:Al	9,13	44.3
7	31.52	446	32	9:Al, 19:Al	9,13	0.1
8	32.54	460	33	9:Al, 20:Al	9,13	2.5
9	34.57	488	35	9:Al, 22:Al	9,13	0.2
10	36.59	516	37	9:Al, 24:Al	9,13	0.8
11	38.59	544	39	9:Al, 26:Al	9,13	1.9
12	40.59	572	41	9:Al, 28:Al	9,13	0.6

[a] Fractions are those collected by GC and bioassayed (Figure 2).
[b] Peak equivalent chain length (ECL) based on a coinjection of *n*-alkane standards *n*-24–40. The column used was a glass column 1.9 × 2 mm ID packed with 3% Dexsil 300. The GC temperature program was 180°C for 1 min followed by a 3°C min increase to 325°C and held at 325°C for 10 min. The carrier was nitrogen at 20 ml/min.
[c] GC-MS molecular weight determinations.
[d] Carbon number based on molecular weight and/or ECL determinations.
[e] Ozonolysis fragments were determined by GC retention times based on aldehyde standards or GC-MS.
[f] Double-bond positions were hypothesized from the ozonolysis fragments.
[g] Peak amount is the percent of total 9,13-dienes.

nonanal was found, and tetradecanal–eicosanal, docosanal, tetracosanal, hexacosanal, and octacosanal were found for dienes of 27–33, 35, 37, 39, and 41 carbon atoms respectively (Table 2). Butanedial (possibly unstable) was not found, but the double-bond positions could be hypothesized from the data as 9,13 for all dienes.

To determine the configurations of the double bonds, an FT-IR spectrum was obtained for HPLC purified diene material. Typical hydrocarbon bands were found at 2924, 2855, 1466, 1380, and 723 cm^{-1}. A peak at 3007 cm^{-1} (olefinic C—H stretch) indicated the presence of double bonds. Peaks at 1655 cm^{-1} (C=C stretch) and 1400 cm^{-1} (olefinic C—H in-plane bending) indicated the *Z* configuration. The lack of a peak at ca. 970 cm^{-1} (olefinic C—H out-of-plane bending) indicated the absence of the *E* configuration. Therefore, the geometrical configuration for the diene material was determined to be *Z,Z* and the tentative structure determination was complete as a series of (Z,Z)-9,13-dienes.

(Z,Z)-9,13-Heptacosadiene was synthesized and purified by Florisil, GC, and HPLC. Mass spectra and FT-IR spectra of the synthetic were identical to the female-derived (Z,Z)-9,13-heptacosadiene. Synthetic and female-derived

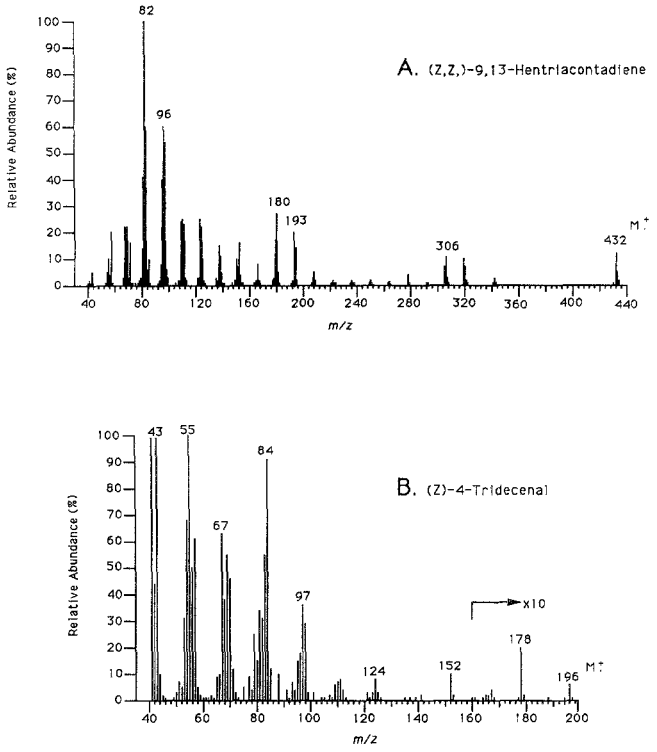


FIG. 3. (A) Electron impact (70 eV) mass spectrum of the GC peak in fraction 6, Figure 2, (Z,Z)-9,13-hentriacontadiene. A member of the homologous series of (Z,Z)-9,13-dienes in female *M. grandii*. (B) Electron impact (70 eV) mass spectrum of (Z)-4-tridecenal. An oxidation product of any member of the female-derived (Z,Z)-9,13-diene series.

(Z,Z)-9,13-heptacosadiene were bioassayed on equal weight bases, 100 ng, and the two were found comparable (Table 3; test 1). In addition, 100 ng of 27-carbon synthetic and 100 ng (1/30 FE) of HPLC-purified female-derived dienes, which contained the entire diene series in the female, were bioassayed (Table 3; test 2). These results were equivalent, demonstrating that the dienes may substitute for one another. A by-product of the synthesis was (Z,Z)-9,13-docosadiene. This symmetrical diene was not found in *M. grandii*; however, since it was a member of the homologous series of 9,13 dienes, it was bioassayed and found to be as attractive as the female-derived dienes (Table 3; test 3).

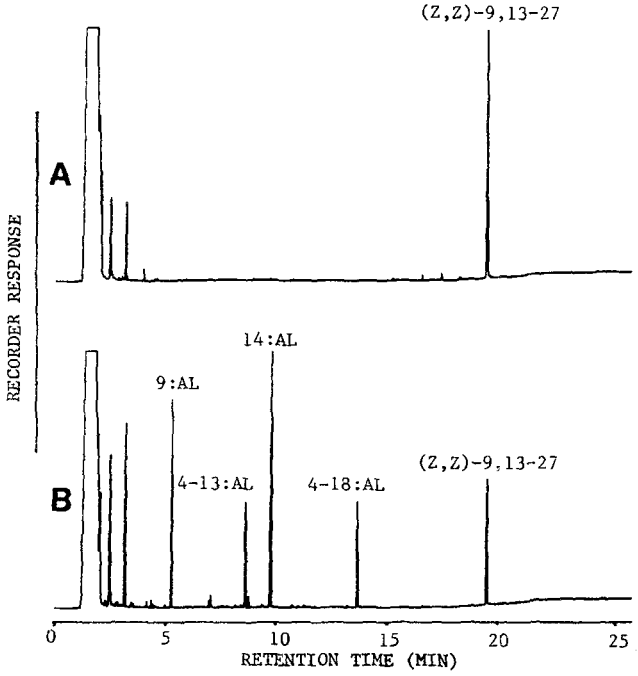
Female-Derived Florisil 5E Fraction. This fraction produced a similar qualitative response in bioassays as the original Florisil hexane fraction; however, the length of time the 5E fraction remained active was shorter (less than 15 min

TABLE 3. LANDING RESPONSES OF MALE *M. grandii* TO FEMALE-DERIVED AND SYNTHETIC (Z,Z)-9,13-HEPTACOSADIENE, AND SYNTHETIC (Z,Z)-9,13-DOCOSADIENE IN LABORATORY WIND-TUNNEL

Treatment	Mean[a]
Test 1	
Synthetic (Z,Z)-9,13-heptacosadiene	40.7a
Female-derived (Z,Z)-9,13-heptacosadiene	45.0a
Control	0.0b
Test 2	
Synthetic (Z,Z)-9,13-heptacosadiene	31.7a
Female-derived HPLC dienes	33.0a
Control	0.0b
Test 3	
Synthetic (Z,Z)-9,13-docosadiene	21.3a
Female-derived HPLC dienes	19.7a
Control	0.0b

[a] 150–200 males 1–5 days old were tested. Three replications with each replicate using a new group of untested males; counts represent the mean number of wasps landing on each plate during a 1-min interval; all diene fractions contain 100 ng diene material; in each test column means followed by different letters are significantly different (PLSD, $\alpha \leq 0.05$).

versus at least 24 hr for the hydrocarbon fraction) and the activity of the 5E fraction was generally reduced when combined with the 50E fraction (Swedenborg and Jones, 1992). Our initial effort in identifying the active component(s) in the 5E fraction was to look at oxidation products of the (Z,Z)-9,13-dienes. Previously, in this laboratory, cuticular dienes of *Pikonema alaskensis* (Rohwer), the yellowheaded spruce sawfly were demonstrated to oxidatively decompose to yield a pheromonally active aldehyde (Bartelt and Jones, 1983). An aldehyde would elute in the 5E fraction using the ether in hexane elution gradient on Florisil. During oxidative degradation, due to their homologous nature, each (Z,Z)-9,13-diene would be expected to produce nonanal, 4-tridecenal, butanedial, and two unique aldehydes. The analogous bioassay activity of all the dienes and their continuous activity over time may be due to a common oxidation product. To verify this hypothesis, we exposed synthetic and female-derived (Z,Z)-9,13-heptacosadiene to the fluorescent ceiling lights above our lab bench at room temperature. As a control, a like number were placed in the dark inside an ultra-low temperature freezer. After 24 hr, the samples were resolubilized in hexane and chromatographed on the GC. Four major oxidation products were found for the synthetic and female-derived "benchtop" (Z,Z)-9,13-heptacosadiene samples (Figure 4B). In addition, over 70% of the original diene material



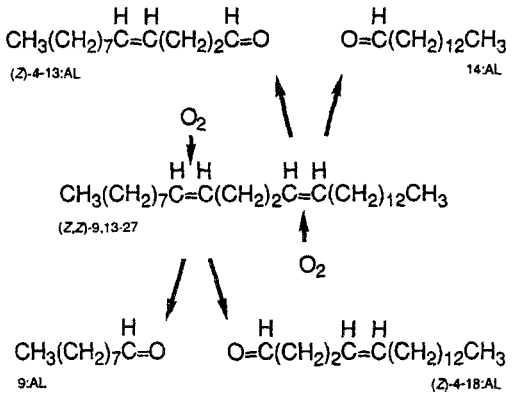


FIG. 4. Top: GC chromatograms using a DB-1 column of (Z,Z)-9,13-heptacosadiene after a 24-hr period in the freezer (A) or exposed on the benchtop (B). The four major oxidation products were nonanal (9:Al), (Z)-4-tridecenal (4-13:Al), tetradecenal (14:Al), and (Z)-4-octadecenal (4-18:Al). The GC temperature program used was at an initial temperature of 40°C for 0.5 min followed by a 20°C/min increase to 150°C, a subsequent programming of 10°C/min to 300°C and held at 300°C for 5 min. The carrier was helium at a linear velocity of 35 cm/sec (150°C). Below, the expected aldehyde cleavage fragments from double-bond oxidation of (Z,Z)-9,13-heptocosadiene.

(20 μg) was gone from these watch-glass plates. However, there was no evidence of oxidation for the samples placed in the −70°C freezer, and the diene material recovered from the freezer plates was greater than 95% (Figure 4A).

The diene oxidation products from the benchtop samples were separated from the remaining dienes by Florisil and the four major products eluted in the 5E fraction. They were identified by GC-MS as a saturated 9-carbon aldehyde, a monounsaturated 13-carbon aldehyde, a saturated 14-carbon aldehyde, and a monounsaturated 18-carbon aldehyde (Figure 4B). Each of the four oxidation products was GC collected and bioassayed. Only the 13-carbon peak was active. The mass spectrum of the active 13-carbon peak verified it as a monounsaturated aldehyde with a discernible molecular ion at 196 and a significant M−18 (loss of water) (Figure 3B). Ozonolysis revealed the double bond to be in the 4 position.

The diene oxidation products obtained from oxidation of 200 μg of the complete diene series were similar to that of (*Z*,*Z*)-9,13-heptacosadiene except additional aldehydes were found due to the increased chain length of the other dienes. Bioassays of the diene oxidation products demonstrated that only the 13-carbon peak (Table 4, fraction 7) was active.

Configuration of the female-derived active oxidation product 4-tridecenal was determined to be 100% *Z*, based on its identical retention time to that of synthetic (*Z*)-4-tridecenal on a Silar-10C capillary column. There was no chromatographic evidence of the *E* isomer as a product of oxidation.

In laboratory bioassay, all sources of 4-tridecenal were bioassayed using 17 pg, the amount of *Z* isomer determined to exist in 1/3 FE of the 5E fraction. Bioassays determined female-derived (*Z*)-4-tridecenal (collected via watch-glass diene oxidation) and synthetic (*Z*)-4-tridecenal elicited comparable activity (Table 5; test 1). Laboratory bioassays of the synthetic *Z* and *E* isomers also demonstrated the *E* isomer was inactive and, when the *E* isomer was added to the *Z* isomer, there was no inhibition or synergism (Table 5; tests 2 and 3). Additionally, the synthetic *Z* isomer and the 5E fraction bioassayed equivalent (Table 5; test 5).

A chemical analysis of the 5E fraction was performed to further verify the oxidation hypothesis and to check for additional biologically active components in the 5E fraction. HPLC analysis was conducted with 200 FE of the 5E fraction on Servachrome (silver nitrate coated) and Spherogel columns (Figures 5 and 6). A broad biologically active region was demonstrated on both columns; however, specific peaks could not be associated with the activities. An HPLC analysis of standard (*Z*)-4-tridecenal on each column using identical chromatographic conditions demonstrated (*Z*)-4-tridecenal would elute predominantly in the two most attractive fractions of either column. A subsequent GC collection using Supelcowax 10 and 3% Dexsil 300 columns was conducted with 100 FE of the combined active HPLC fractions from either column and compared with the

TABLE 4. LANDING RESPONSES OF MALE *M. grandii*[a] TO GC-PURIFIED COLLECTIONS OF DIENE OXIDATION PRODUCTS, ACTIVE HPLC SPHEROGEL FRACTIONS (5–9), AND ACTIVE HPLC SILVER NITRATE ON SILICA GEL FRACTIONS (9–13) OF VIRGIN FEMALES IN LABORATORY WIND-TUNNEL

	SOURCE[c]		
GC fraction[b]	Diene oxidation product[d]	Spherogel[e]	$AgNO_3$[e]
1–6	0.0	0.3	0.0
7	25.5**	29.8**	23.2**
8	4.3**	4.8*	0.0
9	2.7**	1.7*	0.0
10	0.3	0.8	0.2
11–13	0.0	0.3	0.2
14	0.0	37.0**	52.8**
15	0.0	2.0**	0.3
16	0.0	6.5**	0.0
17–18	0.2	4.3**	0.0
19–20	0.3	2.5*	0.3
21–26	0.3	0.3	0.2

[a] 150–200 males 1–5 days old were tested.

[b] Each source was collected on the same GC column (Supelcowax 10, 15 m × 0.53 mm ID) and under the same GC conditions. The GC temperature program was an initial temperature of 100°C for 1 min followed by a 10°C min increase to 250°C and held at 250°C for 10 min. Fractions were collected at minute intervals.

[c] Each treatment plate was paired with a solvent-only control; Six replications with each replicate generally using a new group of untested males; counts represent the mean number of wasps landing on each plate during a 1-min interval; all GC fractions were bioassayed at 5 FE except the diene oxidation product source, which was based on the amount of (Z)-4-tridecenal in fraction 7 ($AgNO_3$ source) as determined by GC and normalized to this concentration; * and ** imply significant differences from controls at the $\alpha \leq 0.05$ and $\alpha \leq 0.01$ levels (one-sided paired *t* test). All control plates averaged less than one landing, most were zero.

[d] Diene oxidation products were the products of diene oxidation from the complete series of *M. grandii* female dienes (200 μg) previously purified utilizing argentation chromatography. Diene oxidation products were separated from the dienes using open column chromatography with Florisil support. The Florisil 5E fraction represented the oxidation products source utilized for GC collection.

[e] The Spherogel and $AgNO_3$ sources (their source was the original 5E fraction) represent the active HPLC fractions from these respective columns. The HPLC active fractions were combined, concentrated, and injected (100 FE) on the GC for collection.

diene oxidation products from the complete female diene series. The data from the Supelcowax 10 column is shown in Table 4. Fraction 7 and, where significant, fractions 8 and 9 were active due to the presence of (Z)-4-tridecenal in all three sources. Using capillary GC retention times, the presence of (Z)-4-tridecenal was demonstrated in these fractions. In addition, fraction 14 demonstrated biological activity for both HPLC sources and additional active frac-

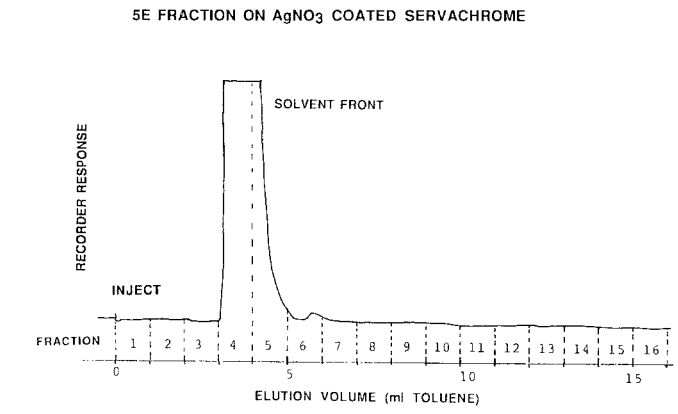
TABLE 5. LANDING RESPONSES OF MALE *M. Grandii* TO FEMALE-DERIVED (*Z*)-4-TRIDECENAL AND SYNTHETIC (E)-AND (*Z*)-4-TRIDECENAL IN LABORATORY WIND-TUNNEL

Treatment	Mean[a]
Test 1	
Synthetic (*Z*)-4-tridecenal	34.7a
Diene oxidation product (*Z*)-4-tridecenal	28.0a
Control	0.0b
Test 2	
Synthetic (*Z*)-4-tridecenal	30.6a
Synthetic (*E*)-4-tridecenal	0.2b
Control	0.0b
Test 3	
Synthetic (*Z*)-4-tridecenal	32.8a
Synthetic (*Z*) + (*E*)-4-tridecenal	34.5a
Control	0.0b
Test 4	
Synthetic (*Z*)-4-tridecenal	32.8a
Female-derived 5E fraction (1/3 FE)	32.2a
Control	0.0b

[a] 150–200 males 1–5 days old were tested. Three replications with each replicate using a new group of untested males; counts represent the mean number of wasps landing on each plate during a 1-min interval; all sources of the *E* and *Z* isomers were tested at 17-pg dosages, the amount of *Z* isomer found in 1/3 FE of the 5E fraction; in each test column means followed by different letters are significantly different (PLSD, $\alpha \leq 0.05$).

tions (15–20) were observed for the Spherogel source. Fraction 14 eluted with the retention of a 26- to 27-carbon hydrocarbon or a 20- to 21-carbon aldehyde on the Supelcowax 10 column. Qualitatively, the active fractions 14–20 demonstrated identical biological activity as (*Z*)-4-tridecenal. This included upwind anemotaxis, casting, landing, wing fanning, and mating attempts between males. Similar findings of activity, additional to (*Z*)-4-tridecenal, were observed for the Dexsil column. The source and nature of this component(s) is unknown. However, we believe it is not a hydrocarbon oxidation product produced by the method that yields (*Z*)-4-tridecenal. There was no evidence of biological activity in fraction 14 in the diene oxidation products collection. Additionally, female monoenes were previously demonstrated as inactive.

In field bioassays (*Z*)-4-tridecenal or (*Z,Z*)-9,13-heptacosadiene, when combined with the 50E fraction, were the most attractive treatments (Table 6, tests 1 and 2). No other fraction or combination of fractions was as attractive as these two combinations. In addition, (*E*)-4-tridecenal demonstrated no activity alone or in combination with the 50E fraction. Trap catch observations were



DATA:

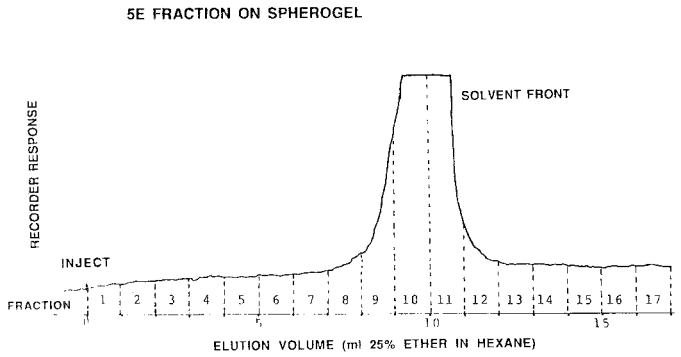
FRACTION	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
RESPONSE	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.7**	37.2**	27.8**	7.5**	0.0	0.0	0.0	0.0

FIG. 5. HPLC chromatogram using a Serva ServaChrom silica gel column coated in situ with silver nitrate. Sample represents 200 FE from the original 5E fraction. Fractions were collected every 1 ml. The data represent the mean landing response of male *M. grandii* to each HPLC fraction in a laboratory wind-tunnel during a 1-min interval. Each treatment plate was paired with a solvent only control; all control plates paired with the HPLC fractions had zero landings. 150-200 males 1–5 days old were tested; six replications with each replicate representing a new group of untested males; all HPLC fractions were bioassayed at 5 FE; ** implies significant differences from controls at the $\alpha \leq 0.01$ levels (one-sided paired t test).

made for both tests. During the first ca. 30 min after each test began, many male wasps were observed actively searching near the traps treated with the combined attractive fractions [(Z)-4-tridecenal or (Z,Z)-9,13-heptacosadiene with the 50E fraction], while few if any were observed around any of the other treatments. It also appeared that many more wasps were searching near the attractive traps than were eventually captured. However, following this initial period, few wasps were observed near even the most attractive traps. Perhaps the ephemeral activity of the 50E component (Swedenborg and Jones, 1992) (Table 3) reduced the synergistic attraction to a very short period after which these treatments were no more attractive than the controlled release treatments of the aldehyde or diene.

DISCUSSION

Large cuticular hydrocarbons that elicit sex pheromone behaviors have been described for a number of species. The works by Howard and Blomquist (1982), Blomquist and Dillwith (1985), and Lockey (1988) provide reviews. Most infor-



DATA:

FRACTION	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
RESPONSE	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.2**	2.6**	10.1**	19.6**	6.3**	0.0	0.2	0.2	0.2

FIG. 6. HPLC chromatogram using an Altex Spherogel 50 Å size exclusion column. Sample represents 200 FE from the original 5E fraction. Fractions were collected every 1 ml. The data represent the mean landing response of male *M. grandii* to each HPLC fraction in a laboratory wind-tunnel during a 1-min interval. Each treatment plate was paired with a solvent only control; all control plates paired with the HPLC fractions had zero landings. 150–200 males 1–5 days old were tested; six replications with each replicate representing a new group of untested males; all HPLC fractions were bioassayed at 5 FE; ** implies significant differences from controls at the $\alpha \leq 0.01$ levels (one-sided paired t test).

mation concerns the Diptera, where cuticular olefins and methylalkanes have been demonstrated to be short-range attractants or contact pheromones (e.g., Carlson et al., 1971; Sonnet et al., 1979; Uebel et al., 1978). Long-range attraction due to large cuticular hydrocarbons (greater than 25 carbons) has not been demonstrated clearly. In the Hymenoptera, a series of (Z,Z)-9,19-dienes (28–37 carbon atoms) for *Pikonema alaskensis* (Rohwer), the yellow-headed spruce sawfly, were found to elicit long-range attraction in laboratory wind tunnel and field studies (Bartelt et al., 1982). However, Bartelt and Jones (1983) later proposed and documented an alternative hypothesis that the (Z,Z)-9,19-dienes were oxidizing and a common oxidation product, (Z)-10-nonadecenal, was responsible for the pheromonal activity of the dienes. This work also demonstrated that oxidation of the dienes and consequent production of active (Z)-10-nonadecenal occurred in the absence of the insect.

We propose that (Z)-4-tridecenal is a primary sex pheromone of *M. grandii* and, like the sawfly, a homologous series of dienes are apparently precursors to this component. Diene oxidation occurs on the cuticle or isolated from the cuticle to produce the pheromone aldehydes. It is not known whether these insects have a controlling mechanism for this oxidation. There is evidence that

TABLE 6. RESPONSE OF MALE *Macrocentrus grandii* TO *E* AND *Z* ISOMERS OF 4-TRIDECENAL AND FEMALE-DERIVED 50% ETHER IN HEXANE FRACTION IN CORN FIELD

Treatment	Mean[a] ($N = 4$)
Test 1[b]	
Control	0.0
Virgin females (3)	1.2ab
50E fraction	0.8a
(*E*)-4-Tridecenal	0.5a
(*E*)-4-Tridecenal + 50E fraction	0.5a
(*Z*)-4-Tridecenal	2.8ab
(*Z*)-4-Tridecenal + 50E fraction	8.2c
(*E*)-4-Tridecenal + (*Z*)-4-tridecenal	0.5a
(*E*)-4-Tridecenal + (*Z*)-4-tridecenal + 50E fraction	5.0bc
Test 2[c]	
Control	0.0
Virgin females (3)	1.0a
50E fraction	1.5a
(Z,Z)-9,13-Heptacosadiene	6.25ab
(Z,Z)-9,13-Heptacosadiene + 50E fraction	11.25b

[a] In each test column means followed by different letters are significantly different (PLSD, $\alpha \leq 0.05$).

[b] (*E*)-4-Tridecenal and (*Z*)-4-tridecenal were released via a Hercon polymer-bonded show-release wafer at a target dosage of 50 ng/hr. The 50E fraction was released at 15 FE per trap. Approximately 1200 laboratory-reared males were released for this field bioassay.

[c] Synthetic (Z,Z)-9,13-heptacosadiene was applied at 180 μg/trap. The 50E fraction was released at 20 FE/trap. Approximately 800 laboratory-reared males were released for this field bioassay.

in certain insects olefin oxidation is inhibited. Atkinson et al. (1973) demonstrated that (Z,Z)-6,9-heptacosdiene (the major hydrocarbon component of *Periplaneta americana* L.) was stable when undisturbed on the cuticle. However, the (Z,Z)-6,9-diene oxidatively decomposed very readily when isolated from the cuticle. In our diene oxidation studies, the (Z,Z)-9,13-dienes decomposed rapidly when isolated from the wasp [greater than 70% of 20 μg of (Z,Z)-9,13-heptacosadiene were decomposed or evaporated within 24 hr]. Inhibition of diene oxidation by other cuticular chemicals may occur in *M. grandii* and *P. alaskensis*. Atkinson et al. (1973) reported that polyhydric phenols, extractable from cuticle, would inhibit diene oxidation when isolated. However, inhibition does not necessarily mean control of pheromone release. In the case of *M. grandii* we have observed females to mate only once; however, they remain attractive to males long after mating. This indicates a pheromone is still being released.

If the production of (Z)-4-tridecenal from cuticular olefins is an uncontrolled event as it appears to be, it is possible that this system represents an early to intermediate stage for sex pheromone evolution. Once any sexual dimorphism in olefin quality occurs, for whatever reason, the stage is set for males to become rapidly selected for their ability to detect any odor differences. Subsequently, those males that best detect this difference and those females that produce more of the unique odor will be favored for selection. Over time and continuous selection, some part of the female integument would specialize in the production of the subject chemical, thus producing a pheromone gland. It is possible that the elaborate system seen in Lepidoptera began with males taking advantage of a serendipitous event in female cuticle composition.

M. grandii (Braconidae) and *P. alaskensis* (Tenthredinidae) are phylogenetically very distant. However, both species have apparently evolved a very similar mechanism for release of sex pheromone. With the ubiquitous nature of hydrocarbons in insects and with the frequent occurrence of the olefin component, this mechanism may be repeated often.

In addition to the (Z,Z)-9,13-dienes and (Z)-4-tridecenal, the pheromone blend for *M. grandii* includes the previously demonstrated 50E fraction and at least one additional component in the 5E fraction. The 50E fraction was previously found to synergize the Florisil hexane fraction (Swedenborg and Jones, 1992) and in this study synergized (Z)-4-tridecenal and (Z,Z)-9,13-heptacosdiene in field bioassays. The identification of the 50E component is the subject of a future paper. The unidentified component of the 5E fraction elicited similar bioassays as the (Z,Z)-9,13-dienes and (Z)-4-tridecenal. Whether it is related to these compounds and/or responsible for the reduced attraction of the 5E fraction when combined with the 50E fraction (Swedenborg and Jones, 1992) or additional behaviors remains to be clarified.

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ASSESSMENT OF ALLELOPATHIC POTENTIAL IN *Artemisia princeps* var. *orientalis* RESIDUES

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Abstract—Field and laboratory studies were conducted to examine the differential phytotoxicity of residues of *Artemisia princeps* var. *orientalis* (wormwood) using various plants as test species. Seedling elongation and dry weights of receptor plants were inversely proportional to the concentration and incubation time of dry leaves of *A. princeps* var. *orientalis* in vermiculite. In seedling growth tests with abandoned field soils (control) and soil underneath wormwood plants (test), the elongation, dry weight, and caloric content of seedlings grown in the soil from under wormwood plants were severely inhibited, thereby suggesting that certain growth inhibitors were released from wormwood and the inhibitor remained in the soil.

Key Words—*Artemisia princeps* var. *orientalis*, allelopathy, growth inhibition, wormwood residues.

INTRODUCTION

Allelopathic chemicals are released from plant tissue in a variety of ways, including exudation of volatile chemicals from living plant parts (Muller et al., 1964; Heisey and Delwiche, 1984); exudation of water-soluble toxins from belowground parts (AlSaadawi et al., 1986); leaching of water-soluble chemicals from aboveground parts in response to the action of rain, fog, or dew (Al-Naib and Rice, 1971; Kumari and Kohli, 1987); and release of toxic chemicals from nonliving plant parts through leaching of toxins from litter, sloughed root cells, or tissues decomposed by microorganisms (Patrick and Koch, 1958; Goel et al., 1989).

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Virtually all parts of plants have been bioassayed, and the inhibitors were found in their organs (Horsley, 1977). Rice (1984) recognized that low concentrations of allelopathic substances may be stimulatory or nontoxic, but higher concentrations of the same substances are quite inhibitory.

Many plant residues have been reported to inhibit growth of plants (Rice, 1984). The degree of inhibition appears to depend on such factors as the test plants; quantity, placement, and weathering of residues; nutrient status of the soil; microbial activity and other soil parameters (Mason-Sedum et al., 1986). The objective of this research was to determine the allelopathic potential of *Artemisia princeps* var. *orientalis* residues.

METHODS AND MATERIALS.

Donor and Receptor Plants. For the study of allelopathic potential of *Artemisia princeps* var. *orientalis* (Pampan.) Hara (wormwood), the donor plant, ten receptor plants were selected. The receptor species included; *Lactuca sativa* L., *Achyranthes japonica* (Miq.) Nakai, *Artemisia princeps* var. *orientalis*, *Oenothera odorata* Jacq., *Plantago asiatica* L., *Zoysia japonica* Steud., *Echinochloa crus-galli* (L.) Beauv., *Hordeum vulgare* var. *hexastichon* Aschers., *Chrysanthemum boreale* Makino, and *Artemisia rubripes* Nakai.

Plant Residues Test. Powdered air-dried fresh leaves were added to vermiculite at various concentrations, and the mixture was put in plastic pots (12 cm). Vermiculite without the powder was used as the control pot. Seeds of receptor plants were sown separately in these pots and, six weeks after sowing, receptor plants were harvested and their lengths and dry weights measured.

Incubation Treatment of Vermiculite and Powder Mixture. Powdered air-dried fresh leaves (5 g) were added to vermiculite (100 g) and then 100 ml of Hoagland's solution (Hoagland and Arnon, 1950) was added to the mixture. These final mixtures were allowed to decompose at 30°C (in the dark) for three, six, and nine days, respectively. The treated vermiculite mixture was put into plastic pots, and then seeds of test plants were sown. Vermiculite without powdered leaves was used in control pots. Hoagland's solution (30 ml) was supplied to each pot every two days. Four weeks after sowing, all plants were harvested and elongation and dry weights determined. This procedure was a slight modification of the method of de Jong and Klinkhamer (1985) and Mason-Sedum and Jessop (1988).

Field Soil Test. To determine whether allelopathic compounds were released from wormwood plants into soil, soil samples were collected from an abandoned field in which wormwood plants were growing. Soil for the control set was collected from a field without wormwood. The soil was screened with a sieve (mesh, 2.0 mm), and seeds of receptor plants were sown in plastic pots con-

taining the soils. When germination was completed and small plants were thinned out, the largest 10 seedlings were grown for four to five weeks.

The results were taken by measuring the total length of each seedling and the dry weight. After the samples were oven-dried at 80°C and reached constant weight, the dry weight of the seedlings was measured and the caloric content was determined by using a calorimeter (Shimadzu, CA-4). All bioassays consisted of four replications, and each experiment was repeated four times. Relative elongation ratio and relative calorie ratio were calculated as suggested by Rho and Kil (1986).

$$\text{Relative elongation ratio (RER)} = \frac{\text{Mean length of tested plant}}{\text{Mean length of control}} \times 100$$

$$\text{Relative calorie ratio (RCR)} = \frac{\text{Calorie of tested plant}}{\text{Calorie of control}} \times 100$$

RESULTS

Effect of Residues on Seedling Growth. Elongation and increase in dry weight of *Achyranthes japonica* were inhibited by addition of powdered wormwood leaves to vermiculite (Table 1). The dry weight was more strongly inhibited than was elongation. The dry weight was only 43.7% of the control in the 10-g powder treatment.

Effect of Incubation Time on Phytotoxicity of Residues. It is evident from Figure 1 that there were significant changes in length of the receptor plants treated with incubated wormwood leaf. The growth of all receptor plants was significantly decreased in accordance with increased incubation time (three, six, and nine days).

TABLE 1. THE DECREASE OF ELONGATION AND DRY WEIGHT OF *Achyranthes japonica* BY ADDITION OF DIFFERENT AMOUNTS OF WORMWOOD LEAF TO VERMICULITE[a]

Amount of dry leaf (g/100 g vermiculite)	Plant growth	
	Elongation (mm)	Dry weight (mg)
0.0	345.5a	327.0a
0.2	308.9ab	232.0b
1.0	276.0bc	182.0b
5.0	246.9c	182.0b
10.0	241.5c	143.0b

[a]Values in a column having the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

The dry weight and caloric content of receptor plants were also strongly decreased by the decomposed wormwood leaf. The order of the inhibitory effect by chemical substances from the wormwood residues was caloric content > dry weight > elongation. Among the receptor plants *A. princeps* var. *orientalis* itself was inhibited most severely (Table 2, Figure 2).

Seedling Growth of Receptor Species in Wormwood Field Soil. Seedling length of receptor plants in abandoned field soil with wormwood plants is graphically shown in Figure 3. *L. sativa* was inhibited severely to 36.8% of RER. *A. japonica*, *O. odorata*, *P. asiatica*, and *A. princeps* var. *orientalis* grew comparatively well in wormwood field soil. This difference may be attributed to differences in species sensitivity to wormwood residues in field soil.

In contrast to seedling lengths, the dry weights of receptor plants, except *P. asiatica* and *A. princeps* var. *orientalis*, were found to be reduced. RWR of

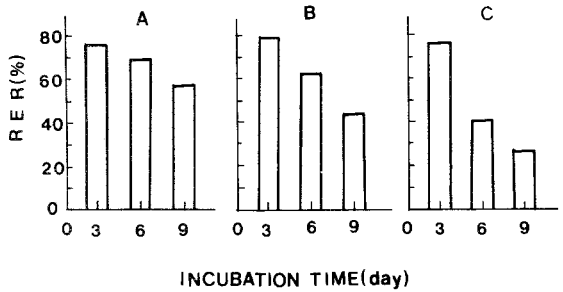

FIG. 1. Relative elongation ratio (RER) of receptor plants grown in pots with different incubation time of wormwood leaf. (A) *Lactuca sativa*; (B) *Achyranthes japonica*; (C) *Artemisia princeps* var. *orientalis*.

TABLE 2. SEEDLING DRY WEIGHT (MG) OF THREE RECEPTOR PLANTS GROWN IN POTS WITH DIFFERENT INCUBATION TIMES OF WORMWOOD LEAF

	Incubation time(day)[a]			
Plant species	0	3	6	9
Lactuca sativa	230.0a	124.0b	54.0c	41.0c
Achyranthes japonica	222.0a	116.0b	47.0b	45.0b
Artemisia princeps var. *orientalis*	227.0a	151.0a	31.0b	9.0b

[a] Values in a row having the same letters are not significantly different at the 5% level by Duncan's multiple-range test.

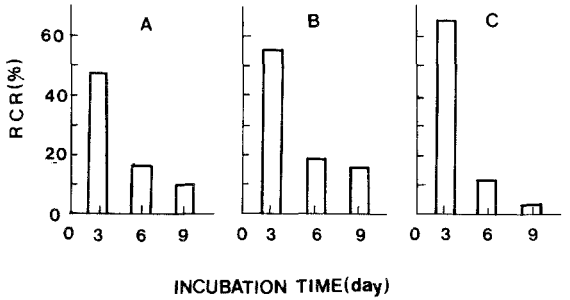

FIG. 2. Relative calorie ratio (RCR) of receptor plants grown in pots with different incubation time of wormwood leaf. (A) *Lactuca sativa*; (B) *Achyranthes japonica*; (C) *Artemisia princeps* var. *orientalis*.

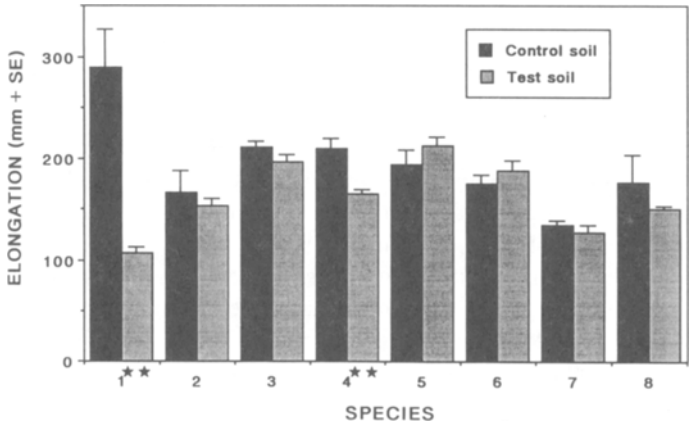

FIG. 3. Seedling length of receptor plants tested in soils from underneath wormwood plants. Keys to species: 1, *Lactuca sativa*; 2, *Plantago asiatica*; 3, *Artemisia princeps* var. *orientalis*; 4, *Chrysanthemum boreale*; 5, *Artemisia rubripes*; 6, *Zoysia japonica*; 7, *Achyranthes japonica*; 8, *Oenothera odorata*. Bar is standard error. Student's *t* test: *significantly different, $P \leq 0.05$; **$P \leq 0.01$.

A. rubripes and *Z. japonica* were less than 43.5% and 68.7% of control, respectively (Figure 4).

DISCUSSION

Incorporating wormwood leaf powder into vermiculite in which test plants were planted showed that elongation and dry weight were progressively decreased with increasing concentrations of wormwood leaf powder. Irons and Burnside

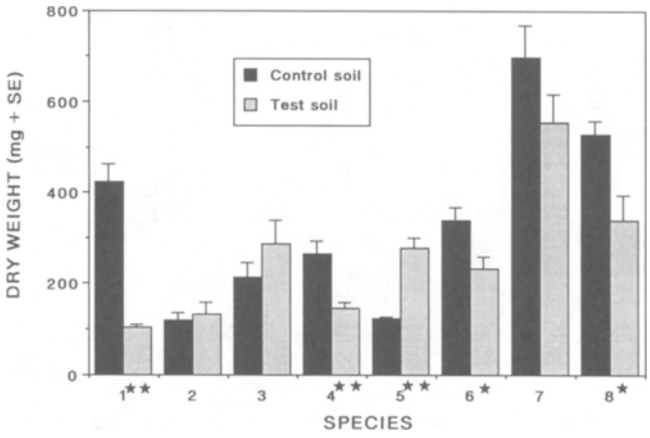

FIG. 4. Seedling dry weight of receptor plants tested in soils from underneath wormwood plants. Keys to species as in Figure 3. Bar is standard error. Student's t test: *significantly different, $P \leq 0.05$; **$P \leq 0.01$.

(1982) reported that 2% or greater amounts of ground, mature sunflower leaves mixed into the soil significantly reduced emergence, height, fresh weight, and dry weight of the tested plants. It was reported that incorporation of dried shoot or root residues of lantana into the soil significantly reduced milkweedvine growth compared with growth in control soil (Achhireddy and Singh, 1984). White et al. (1989) reported that cotton and pitted morning glory emergence and dry weight were decreased approximately 60–80% when these plants were grown under greenhouse conditions in the presence of increasing amounts of field-grown crimson clover or hairy vetch debris incorporated into the soil medium, while corn dry weight was increased 20–75% when legume debris was placed on the soil surface.

It was found that increasing the incubation time of wormwood leaf decreased elongation, dry weight, and caloric content of receptor plants. Patrick and Koch (1958) noted that substances possessing phytotoxic properties may be formed during the decomposition of plant residues in the soil; Al-Naib and Rice (1971) found that decaying sycamore leaves significantly reduced seed germination and seedling growth of all test species. Kuo et al. (1981) pointed out that the aqueous extraction of decomposing Chinese cabbage leaves completely inhibited mungbean seed emergence. Phytotoxicity of Chinese cabbage was caused by substances produced by decomposing Chinese cabbage leaves or microorganisms associated with the decomposition rather than by microorganisms becoming pathogenic agents for the following mungbean crop. The fact that the increase in growth inhibition of tested plants with incubation time may have been due to a release of inhibitory substances from decomposing plant tissue or to the

formation of toxic substances by microorganisms associated with the decomposing leaves was discussed by Barnes and Putnam (1983) and Harrison and Peterson (1986). Thus, it is likely that the allelopathic effect of the wormwood plants was partly due to this interaction between residues and microorganisms.

Field soil collected under the wormwood plants significantly reduced or somewhat promoted growth of the test plants. These results are in agreement or disagreement with following results: soil collected from some plant fields exhibited phytotoxity by reducing growth (Al-Naib and Rice, 1971; AlSaadawi and AlRubeaa, 1985; Inam et al., 1989) and soil from below some test plants did not inhibit germination and growth of the test species (Fadayomi and Oyebade, 1984; Goel and Sareen, 1986).

The current study demonstrates phytotoxicity of wormwood residues in soil.

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ISOTHIOCYANATES AS ALLEOPATHIC COMPOUNDS FROM *Rorippa indica* Hiern. (Cruciferae) ROOTS

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Abstract—The ethyl acetate extracts of *Rorippa indica* Hiern. contained hirsutin, arabin, camelinin, and three novel ω-methylsulfonylalkyl isothiocyanates (n = 8, 9, and 10). These compounds severely inhibited lettuce (*Lactuca sativa*) hypocotyl and root growth at 0.1 mM or above. The precursor glucosinolates of hirsutin, arabin, and camelinin were isolated. Presence of the three ω-methylsulfonylalkylglucosinolates, along with other glucosinolates in the roots were verified by the isolation and identification of their desulfo-derivatives. Using the continuous root exudate trapping apparatus and GC-MS, hirsutin and the three ω-methlylsulfonylalkyl isothiocyanates were detected in the root exudates of *R. indica*, suggesting that these isothiocyanates are the primary candidate of allelopathic compounds contributing to the aggressiveness of this cruciferous weed.

Key Words—Allelopathy, root exudates, hirsutin, glucohirsutin, arabin, glucoarabin, camelinin, glucocamelinin, ω-methylsulfonylalkyl isothiocyanates, *Rorippa indica*, cruciferae.

INTRODUCTION

Rorippa indica Hiern. (Inugarashi in Japanese, Cruciferae) is a common perennial weed in the fields and pastures of Japan. The weed has the ability to propagate through its vegetative organs such as roots or stems (Ito et al., 1989). As the weed exists in very dense and practically pure stands, this suggests that the species may produce substances that are allelopathic to other surrounding plants.

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Recently, we reported that *Rorippa sylvestris* contained 8-methyl sulfinyloctyl isothiocyanate (hirsutin) as a major inhibitory principle in the roots and that the compound inhibited the growth of lettuce (Kawabata et al., 1989). The content of glucohirsutin and deoxyglucohirsutin in the root increased during both the vegetative and the reproductive growing stages. Isothiocyanates in cruciferous plants are thought to play an important role as inhibitors of plant growth, in addition to their antimicrobial activity (Harborne, 1988). These sulfur-containing compounds are usually found in macerated tissues owing to the mixing of the precursor glucosinolates with the hydrolytic enzyme thioglucosidase (Olsen and Sørensen, 1981). The most frequent fate of the unstable aglycones generated by initial hydrolysis is to undergo spontaneous rearrangement via either a proton-independent Lossen rearrangement concerted with a loss of sulfate to yield an isothiocyanate, or a competing proton-dependent desulfation to yield a nitrile (McGregor et al., 1983).

Tang and Takenaka (1983) reported that for 2-month-old trees (*Carica papaya* L.), the rate of benzyl isothiocyanate released from the root system was 2–3 μg/tree/day in the continuous root exudate trapping system. Interestingly, Tang (1971) pointed out that herbicidal secondary metabolites are released from the undisturbed plant root system.

As many studies of allelopathic interactions in germination and growth have only dealt with plant extracts or leachate from soils, it is usually difficult to assign the active principle in plant–plant interactions. For this reason, we applied the continuous root exudate trapping system according to Tang and Young (1982) to the exploration and identification of the constituents in the root exudates of *R. indica* which inhibit the growth of lettuce.

METHODS AND MATERIALS

Plant Collection and Extraction. Roots (2.4 kg, fresh weight) of *R. indica* were collected on the campus of Hokkaido University in July 1989 and were extracted with EtOAc for one month at room temperature. The extract was evaporated under reduced pressure, and the aqueous concentrate was fractionated into acidic and neutral fractions in the usual way.

Bioassay of Inhibitory Activities against Lettuce. We examined the activity of each extract, chromatographic fractions, and isolated compounds by a lettuce seed germination bioassay. An appropriate amount of the sample was dissolved in 0.5 ml of acetone, and the solution was poured into a 6-cm-diam. Petri dish with two sheets of filter paper (No. 3, 5.5 cm, Toyo Roshi Co., Ltd.) placed at the bottom. The acetone was evaporated, and 2 ml of Tween-80 aqueous solution (100 ppm) was poured into each Petri dish. The dishes were covered and kept in the dark for 24 hr at room temp. Lettuce seeds (30 seeds per dish)

were placed on the filter paper and then incubated at 23°C in the dark. Germination percentages were recorded after 24 hr. A control containing only the Tween-80 aqueous solution was included in each experiment. All experiments were duplicated.

For those isolated compounds with significant biological activities, a lettuce hypocotyl and radicle growth bioassay was also used. A sample was dissolved in 150 μl acetone and poured into the 15-ml hot 0.35% agar solution in a Petri dish. The agar solution was cooled, and 20 lettuce seedlings (24 hr after germination) were placed in each dish. The dishes were covered and kept in a growth chamber for one week at 23°C under a 12-hr light–dark cycle. The hypocotyl and radicle lengths for each seedling were measured to the nearest millimeter. The mean length was determined for 20 seedlings (two replications). A control without the sample was included in each experiment.

Fractionation of EtOAc Extract. The neutral fraction inhibited the lettuce seed germination by 90% at 500 ppm and was subjected to silica gel column chromatography using $CHCl_3$–MeOH as eluents. The $CHCl_3$–MeOH (99.5:0.5) and the $CHCl_3$–MeOH (99:1) eluates inhibited lettuce seed germination by 97% and 100% at 500 ppm, respectively. Both fractions were further purified by HPLC using a Megapak Sil C18T, 5 μm, 10 × 250 mm (Gaskuro Kogyo). The UV detector measured absorbance at 254 nm. The eluent system was MeOH–H_2O–CH_3CN (60:25:15) at a flow rate of 4 ml/min. By HPLC, the former eluate was divided into three fractions, and from each fraction, compounds 1, 2, and 3 (37.8, 868, and 44.1 mg) were isolated, respectively. From the latter eluate, three compounds, i.e., compounds 4 (0.2 mg), 5 (42.5 mg), and 6 (6.6 mg) were isolated.

Preparation and Isolation of Desulfoglucosinolates. Fresh roots of *R. indica* were collected on the campus of Hokkaido University in June 1989. The roots (55.7 g) were added slowly to 100 ml of boiling 80% ethanol, and boiled further for 15 min at boiling point. After having been cooled and then homogenized, the resulting suspension was filtered through filter paper. The ethanol was removed by rotary evaporation at 35°C. After transferring the aqueous solution to an Erlenmeyer flask, 5 ml of 0.3 M barium and lead acetate solution were added. The solution was centrifuged (5×10^3 rpm, 5°C, 10 min) and the supernatant was augmented to 50 ml, applied to DEAE-Sephadex A-25, and desulfated with aryl sulfatase (Sigma, H-1), as described by Ishii et al. (1988). Desulfoglucosinolates were obtained after an overnight reaction with sulfatase at ambient temperature (Figure 1). Nine desulfoglucosinolates (7–15) were separated on an HPLC column (Unisil Q C_{18}, 7.6 × 300 mm) (Figure 2). The column was eluted (2.5 ml/min, UV detection; 227.5 nm) with H_2O–CH_3CN (3:2).

Detection of Allelopathic Compounds in R. indica *Root Exudates.* Whole *R. indica* plants were collected on the campus of Hokkaido University and were

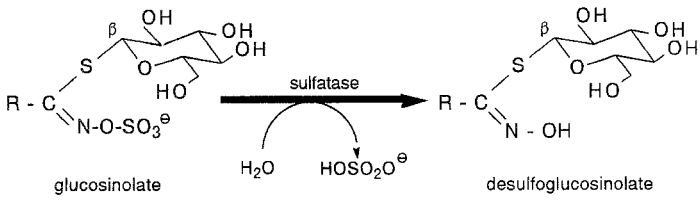

FIG. 1. Preparation to desulfoglucosinolate from glucosinolate.

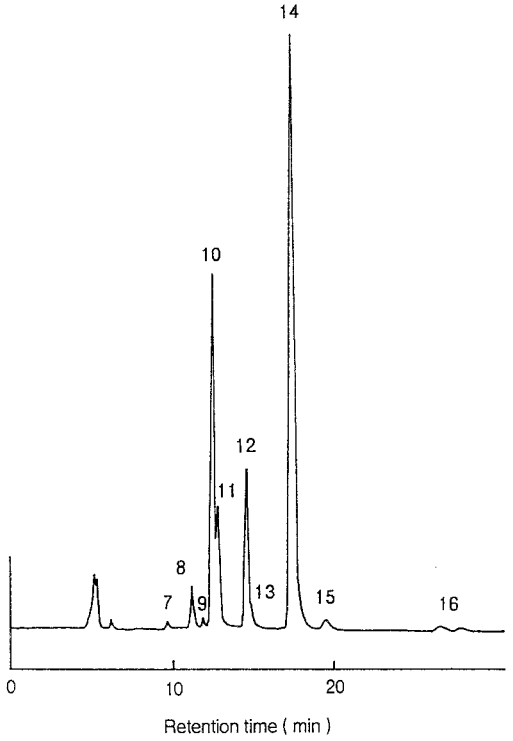

FIG. 2. HPLC separation of desulfoglucosinolates from *R. indica* root. Column: Unisil Q C_{18}, 7.6 × 300 mm, 5 μm; mobile phase: H_2O–CH_3CN (3:2), 2.5 ml/min; UV detection: 227.5 nm

then transplanted into the continuous root exudate trapping system similar to that devised by Tang and Young (1982). The pots were irrigated with a circulating nutrient solution (Tadano and Tanaka, 1976) at a rate of ca. 1 liter/hr. It was necessary to replace the nutrient solution every five days to obtain the best growth.

The plants were ca. 150 mm tall with healthy and proliferous root systems, and collection of root exudates commenced 10 days after transplanting. Prior to the collection, the pots were washed by leaching with 2 liters of distilled water to rinse off accumulated residues, after which a column containing 15 ml of cleaned XAD-4 resin was attached to the pot, fresh nutrient solution was circulated at 1 liter/hr for 48 hr. The charged column was detached from the pot, and then washed with 100 ml of distilled water followed by elution with 100 ml of acetone. The eluate was concentrated to ca. 10 ml at 40°C using a rotary evaporator, and the resultant solution was extracted twice with 10 ml of ether. The combined organic extracts were dried with anhydrous Na_2SO_4 and concentrated to ca. 500 μl for GC analysis.

Chemical Analysis. GC analysis was performed on a Hitachi Gas Chromatograph 263-30, with an OV-1 capillary column (25 m × 0.35 mm ID). The column temperature was programmed from 100°C to 300°C at 4°C/min. GC-mass spectrometry was conducted using a JEOL DX-300 GC-MS system with a 25 m OV-1 column. EI-MS and FD (FI)-MS determinations were carried out, respectively, on a JEOL DX-300 and JEOL JMS-01SG-2. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a BRUKER AM-500 instrument at 500 MHz and 125 MHz, respectively, with TMS as an internal standard. Infrared spectra were obtained using a Hitachi model 285, and UV spectra were recorded on a Hitachi model U-3210 spectrophotometer.

RESULTS

Isothiocyanates from EtOAc Extracts. The bioassay results revealed that the neutral fraction of the EtOAc extracts remarkably inhibited lettuce seed germination. Silica gel column chromatography, preparative TLC, and subsequent HPLC of these fractions, based on the lettuce seed germination bioassay, resulted in the isolation of inhibitors **1–6**.

The MS of compound **1** [FD-MS *m/z* (%): 249 (M^+, 100)] suggested a hirsutin-related structure. The presence of an isothiocyanate group in **1** was confirmed by the IR spectrum (ν −N=C=S at 2170 and 2100 cm^{-1}), and the absorption at 1290, 1270, 1250, and 1130 cm^{-1} suggested the presence of a sulfonyl moiety. The ^{1}H-NMR spectrum indicated an ω-methylsulfonylalkyl isothiocyanate. From the physiochemical data shown in Table 1, compound **1** was assigned to the structure of 8-methylsulfonyloctyl isothiocyanate (Figure 3).

The MS of **2** [FD-MS *m/z* (%): 263 (M^+, 100)] and **3** [FD-MS *m/z* (%): 277 (M^+, 100)] suggested homologs of 1. From the physicochemical data shown in Table 1, compounds **2** and **3** were assigned to the structures of 9-methylsulfonylnonyl isothiocyanate and 10-methylsulfonyldecyl isothiocyanate, respectively (Figure 3).

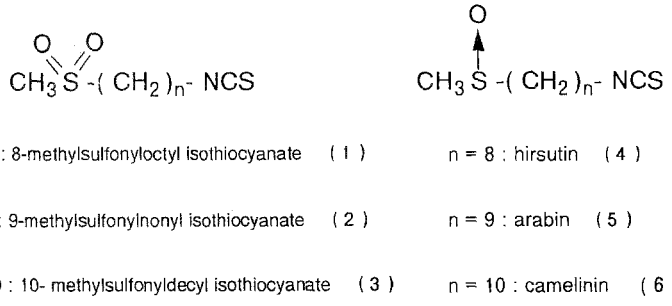

FIG. 3. Chemical structures of isothiocyanates; the allelochemical candidates referred to the text.

The MS of **4** (buff-colored oil) suggested hirsutin that we had isolated from the root of yellow fieldcress (*R. sylvestris*, Kirehainugarashi in Japanese) (Kawabata et al., 1989), and its optical rotation was $[\alpha]_D^{23} = -50°$ ($c = 0.145$, MeOH). The ^{1}H-NMR, IR, and UV spectra of **4** were identical with those of hirsutin (Figure 3).

The MS of **5** and **6** suggested homologs of **4**. Compound **5** has optical rotation $[\alpha]_D^{23} = -51°$ ($c = 0.153$, MeOH), and the ^{1}H-NMR, IR, and UV spectra of compound **5** were assigned to the structure of 9-methylsulfinylnonyl isothiocyanate (arabin) (Figure 3). Compound **6** has optical rotation $[\alpha]_D^{23} = -55°$ ($c = 0.003$, MeOH) and its ^{1}H-NMR, IR, and UV spectra were assigned to the structure of 10-methylsulfinyldecyl isothiocyanate (camelinin) (Figure 3).

Inhibitory Activities against Lettuce. Hirsutin (Kawabata et al., 1989), arabin, camelinin and ω-methylsulfonylalkyl isothiocyanates ($n = 8$ and 9) inhibited seed germination at 0.5 mM by 100, 88, 9, 100, and 58%, respectively. On the other hand, 10-methylsulfonyldecyl isothiocyanate had no effect upon seed germination (Table 2). All compounds inhibited root and hypocotyl elongations of lettuce seedling at 0.4 mM (Table 3). In particular, 9-methylsulfonylnonyl isothiocyanate showed a remarkable inhibitory activity and reduced the hypocotyl and radicle elongations of lettuce seedling at 0.2 mM by 63 and 76%, respectively. Even at 0.1 mM (26 ppm), it inhibited the hypocotyl elongation of lettuce seedlings by more than 30%. As a reference compound to clarify the activity of 9-methylsulfonylnonyl isothiocyanate, juglone was used. Although the tendencies of inhibition for lettuce seedling growth differed for the compounds, it could be concluded that the inhibitory activity of 9-methylsulfonylnonyl isothiocyanate was as half strong as that of juglone.

Physicochemical Properties of Isolated Desulfoglucosinolates (DS-GSLs). The main DS-GSLs extracted from the *R. indica* root were further purified on HPLC, and nine DS-GSLs were isolated (**7–15**) (Figure 2). All the DS-GSLs were identified by FD-MS and ^{1}H-NMR spectra as follows.

TABLE 1. PHYSIOCHEMICAL DATA OF COMPOUNDS

No.	Name	mp (°C)	Appearance	IR	UV	^{1}H-NMR	^{13}C-NMR	EI-MS m/z (%)
1	8-methyl-sulflonyloctyl isothiocyanate	49.5–50.5	colorless needles	2170, 2100, 1290, 1270, 1250, 1130, 950	242 (MeOH)	3.52, 3.01, 2.91, 1.85, 1.70, 1.32–1.52, $CDCl_3$		FD: 249 216(17), 170(78), 115(56), 109(17), 101(13), 86(11), 81(31), 72(28), 69,(83), 67(25)
2	9-methyl-sulfonylnonyl isothiocyante	52.5–53.5	colorless needles	2170, 2100, 1290, 1270, 1250, 1130, 950	242 (MeOH)	3.51, 3.01, 2.90, 1.86, 1.70, 1.30–1.50 $CDCl_3$		FD: 263 230(31), 184(36), 123(13), 115(71), 101(13), 96(10), 86(10), 83(23), 81(31), 72(24), 69(67), 55(100), 41(78)
3	10-methyl-sulfonyldecyl isothiocyanate	56.2–58.2	colorless needles	2170, 2100, 1290, 1270 1250, 1130, 950	242 (MeOH)	3.51, 3.01, 2.90, 1.85, 1.70, 1.27–1.50 $CDCl_3$	129.6, 54.9, 45.1, 40.5, 29.9, 29.2, 29.1, 29.0, 28.7, 28.4, 26.5, 22.4	FD: 277 244(36), 198(20), 115(63), 81(28), 69(48), 55(100), 41(85)
4	hirsutin		buff colored oil					233(8), 216(47), 170(25), 72(12), 69(48), 55(100), 41(85)

TABLE 1. CONTINUED

No.	Name	mp (°C)	Appearance	IR	UV	^{1}H NMR	^{13}C NMR	EI-MS m/z (%)
5	arabin		buff colored oil	2170, 2090, 1020	244.5 (MeOH)	3.51, 2.70, 2.57, 1.77, 1.70, 1.30–1.50 $CDCl_3$		247(1.6), 230(64), 184(25), 83(30), 69(49), 55(100), 41(53)
6	camelinin		buff colored oil					261(1.0), 244(52), 198(26), 83(30), 69(49), 55(100), 41(53)
7	8-methyl-sulfinyloctyl-desulfogluco-sinolate		colorless oil			4.78, 2.80, 2.63, 2.57, 1.60–1.80, 1.35–1.55 CD_3OD		FD: 413
8	9-methyl-sulfinylnonyl-desulfogluco-sinolate		colorless oil			4.69, 2.80, 2.63, 2.56, 1.60–1.80, 1.35–1.55 CD_3OD		FD: 427
9	8-methyl-sulfonyloctyl-desulfogluco-sinolate		colorless needles			4.78, 3.1, 2.94, 2.57, 1.80, 1.68, 1.30–1.50 CD_3OD		FD: 429
10	desulfogluco-brassicin		colorless needles			7.64, 7.33, 713, 7.10, 7.01, 4.69, 4.27, 3.93 CD_3OD		FD: 368

11	4-methoxy-desulfoglucobrassicin	colorless needles	6.99, 6.93, 6.91, 6.47, 4.71, 4.48, 4.07, 3.90 CD_3OD	FD: 398
12	9-methylsulfonylnonyl-desulfoglucosinolate	colorless needles	4.78, 310, 2.94, 2.56, 1.80, 1.67, 1.30–1.50 CD_3OD	FD: 443
13	DS-glucocarnelinin	colorless oil		FD: 441
14	1-methoxy-desulfoglucobrassicin	buff colored needles	7.66, 7.39, 7.30, 7.21, 7.07, 4.68, 4.22, 4.05, 3.93 CD_3OD	FD: 398
15	10-methylsulfonyldecyl-desulfoglucosinolate	colorless needles	4.78, 3.10, 2.94, 2.56, 1.80, 1.67, 1.30–1.50 CD_3OD	

TABLE 2. EFFECTS OF ISOTHIOCYANATES ISOLATED FROM *R. indica* ROOT ON GERMINATION OF LETTUCE

Treatment	Germination (%)[a]			
	0.1 mM	0.2 mM	0.5 mM	1.0 mM
$CH_3SO(CH_2)_n$-NCS				
$n = 8$	68.3	5.0	0	0
$n = 9$	100.0	66.7	11.7	0
$n = 10$	100.0	100.0	91.1	20.0
$CH_3SO_2(CH_2)_n$-NCS				
$n = 8$	81.1	7.8	0	0
$n = 9$	93.3	71.1	42.2	32.2
$n = 10$	100.0	100.0	100.0	100.0

[a]Each Petri dish contained 30 seeds and was replicated three times. As percent of controls (24 hr).

TABLE 3. RELATIVE ACTIVITIES OF ISOTHIOCYANATES ISOLATED FROM *R. indica* ROOT ON LETTUCE HYPOCOTYL AND ROOT GROWTH

Compound	Hypocotyl elongation (%)[a]			Radicle elongation (%)[a]		
	0.1 mM	0.2 mM	0.4 mM	0.1 mM	0.2 mM	0.4 mM
$CH_3SO(CH_2)_n$-NCS						
$n = 8$	71d	58d	10e	111b	63c	14d
$n = 9$	80bc	66c	24d	93e	66c	25c
$n = 10$	81ab	72b	44b	95de	77b	61b
$CH_3SO_2(CH_2)_n$-NCS						
$n = 8$	78c	61d	32c	99d	66c	13d
$n = 9$	68e	37e	31c	106c	24d	13d
$n = 10$	83a	78a	70a	141a	105a	90a

[a]Elongations (percent of control) for a particular compound. Means within column followed by the same letters are not significantly different at the 0.05 level according to Duncan's multiple-range test.

Compound **7** (0.2 mg): 8-methylsulfinyloctyldesulfoglucosinolate (DS-glucohirsutin); compound **8** (1.1 mg): 9-methylsulfinylnonyldesulfoglucosinolate (DS-glucoarabin); compound **9** (0.5 mg): 8-methylsulfonyloctyldesulfoglucosinolate; compound **10** (2.4 mg): desulfoglucobrassicin, compound **11** (0.8 mg): 4-methoxydesulfoglucobrassicin; compound **12** (5.6 mg): 9-methylsulfonylnonyldesulfoglucosinolate; compound **13** (0.1 mg): 10-methylsulfinyldecyldesulfoglucosinolate (DS-glucocamelinin); compound **14** (5.4 mg): 1-me-

thoxydesulfoglucobrassicin; and compound **15** (0.7 mg): 10-methylsulfonyldecyldesulfoglucosinolate.

The anomeric proton doublets of **7–15** (H-1′) were observed in the ^{1}H-NMR, and these signals indicated a β-linked glucoside structure, by comparison with prepared desulfosinigrin (J = 9.8 Hz).

The FD-MS of the lowest polar fraction **16** (colorless oil, 0.2 mg) indicated a mixture of three homologous compounds (M^+ 397, M^+ 411, and M^+ 425) and suggested the structures of 8-methylthiooctyldesulfoglucosinolate, 9-methylthiononyldesulfoglucosinolate, and 10-methylthiodecyldesulfoglucosinolate, respectively.

Candidates of Allelopathic Compounds in R. indica *Root Exudates.* The hydrophobic substances in the recirculating solution inhibited lettuce seed germination by 85% at 200 ppm and also reduced the hypocotyl and root elongations of lettuce seedling at 100 ppm by 35% and 7%, respectively. Even at 10 ppm, it affected the growth of lettuce seedling hypocotyl.

The chromatogram of root exudates obtained from GC equipped with FID showed more than 50 peaks, and the retention time of the isolated standard compounds and GC-MS revealed the presence of **1** (t_R 17 min 24 sec), **2** (t_R 18 min 39 sec), **3** (t_R 19 min 42 sec), and **4** (t_R 15 min 48 sec). 8-Methylsulfonyloctyl isothiocyanate (**1**), MS *m/z* (%): 249(M^+, 0.1), 216 (8), 170 (50), 115 (36), 81 (17), 69 (79), 55 (100); 9-methylsulfonylnonyl isothiocyanate (**2**), MS *m/z* (%): 263 (M^+, 2), 230 (16), 184 (5), 115 (13), 81 (26), 69 (51), 55 (100); 10-methylsulfonyldecyl isothiocyanate (**3**), MS *m/z* (%): 277 (M^+, 0.2), 244 (15), 198 (3), 115 (78), 69 (35), 55 (100); 8-methylsulfinyloctyl isothiocyanate (**4**), MS *m/z* (%): 233 (M^+, 1), 216 (11), 170 (23), 69 (53), 55 (90). The other phytotoxic isothiocyanates that we isolated may exist in the root exudates at lower concentrations.

DISCUSSION

The isolation of compounds from *R. indica* root extracts revealed not only the presence of phytotoxic isothiocyanates, hirsutin, arabin, and camelinin that had been reported previously for the essential oil (Kjaer and Christensen, 1958; Kjaer and Gmelin, 1956; Kjaer et al., 1956) but also novel ω-methylsulfonylalkyl isothiocyanates (n = 8, 9, and 10). This is the first report of their occurrence as major phytotoxic isothiocyanates in *R. indica*.

The data in Table 2 clearly demonstrate a close relationship between chemical structure and inhibitory activity. The inhibition rate of lettuce seed germination by ω-methyl-sulfinyl (and sulfonyl) alkyl isothiocyanates (n = 8, 9, and 10) was gradually decreased as the values of n increased. However, this hypothesis was not applicable for the inhibition of lettuce seedling growth by ω-meth-

ylsulfonylalkyl isothiocyanate (n = 8, 9, and 10) (Table 3). The difference may be attributed to the differences in the mode of action between the inhibition of lettuce seed germination and the inhibition of lettuce seedling growth (Einhellig, 1986).

In order to elucidate how these isothiocyanates are generated, we needed to isolate their precursor glucosinolates. Kjaer et al. reported that they have isolated glucohirsutin, glucoarabin, and glucocamelinin in addition to an extremely small amount of deoxyglucohirsutin, deoxyglucoarabin, and deoxyglucocamelinin as desulfoglucosinolate (Kjaer et al., 1956; Kjaer and Christensen, 1957, 1958; Kjaer and Gmelin, 1956), but no one has previously shown that these compounds are also present in *R. indica*. We have found, for the first time, that ω-methylsulfonylalkylglucosinolates (n = 8, 9, and 10) isolated for desulfoglucosinolate are naturally occurring glucosinolates. Although the indole glucosinolates glucobrassicin, 4-methoxyglucobrassicin, and 1-methoxyglucobrassicin represent a significant proportion of the total glucosinolate content of cruciferous vegetables (Fenwick et al., 1983; Truscott et al., 1982), while Virtanen (1965) has demonstrated that, following the rupture of the cells of plant material, indole glucosinolate is hydrolyzed by the endogenous enzyme myrosinase to a number of compounds, including indolyl nitrile in acidic condition or thiocyanate ion and indolyl alcohol in neutral condition, it is still necessary to investigate the mechanism of these chemodynamics and the role of those compounds in *R. indica*.

Chisholm (1972) has reported that in *Cheiranthus kewensis* DL-methionine-2-^{14}C and DL-homomethionine-2-^{14}C were incorporated into 3-methylthiopropylglucosinolate and 3-methylsulfinylpropylglucosinolate; these results suggested the following sequence of biochemical reactions: methionine → homomethionine → 3-methylthiopropylglucosinolate → 3-methylsulfinylpropylglucosinolate. This result suggests that, in *R. indica*, the oxidation of ω-methylthioalkylglucosinolate into ω-methylsulfinylalkylglucosinolate and of ω-methylsulfinylalkylglucosinolate to ω-methylsulfonylalkylglucosinolate may also be performed by enzymatic oxidation, since isothiocyanates are usually found in macerated tissues owing to the mixing of glucosinolates and the hydrolytic enzyme thioglucosidase (Olsen and Sørensen, 1981). We therefore consider that ω-methyl-sulfonyl (and sulfinyl) alkylglucosinolates (n = 8, 9, and 10) are the precursors of phytotoxic ω-methyl-sulfonyl (and sulfinyl) alkyl isothiocyanates (n = 8, 9, and 10).

Because many studies of allelopathic interactions on plant seed germination and growth have only dealt with crude plant extracts or leached compounds from soils, it is difficult to assign the active principle in plant–plant interactions. For this reason we used the continuous root exudate trapping method. Tang and Takenaka (1982) reported that in 2-month-old trees of *Carica papaya* L., benzyl isothiocyanate was released from the undisturbed plant root system. Our study

has revealed that the inhibitory activity of hydrophobic exudates from *R. indica* roots upon lettuce seed germination and lettuce seedling growth may be caused by phytotoxic isothiocyanates including ω-methylsulfonylalkyl isothiocyanates (n = 8, 9, and 10) and hirsutin. Einhellig (1987) reported that allelopathic inhibition of germination and plant growth typically occurred as a result of the joint action of several allelochemicals. In the same way, isothiocyanates may interact in an additive or synergistic manner. Owing to their low water solubility, ω-methyl-sulfonyl (and sulfinyl) alkyl isothiocyanates released from the root surface will diffuse slowly into the soil solution, resulting in a concentration gradient at the root–soil interface. The continuous release of ω-methyl-sulfonyl (and sulfinyl) alkyl isothiocyanate from normal growing *R. indica* roots would suggest that this metabolite plays an important active role in the rhizospheric ecosystem.

Our investigation clearly shows that several isothiocyanates are potent phytotoxins to lettuce seed and lettuce seedling. Furthermore, these isothiocyanates appear to be allelochemical compounds in plant–plant interactions.

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DETRIMENTAL EFFECTS OF *Cinchona* LEAF ALKALOIDS ON LARVAE OF THE POLYPHAGOUS INSECT *Spodoptera exigua*

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Abstract—Young *Cinchona ledgeriana* plants contain two types of alkaloid: indole alkaloids in the leaves and quinoline alkaloids in the root. From *Cinchona* leaves, a crude alkaloid extract was made, containing the cinchophylline type of indole alkaloids and a small amount of 5-methoxytryptamine. The leaf alkaloid extract exerted a strong detrimental effect on the growth of larvae of the polyphagous beet armyworm, *Spodoptera exigua* (Lepidoptera). Feeding of larvae on an artificial diet containing the leaf alkaloids at the same concentrations as those found in the plant resulted in significant growth reduction, retardation in development, and mortality of the larvae. Cinchophyllines are composed of 5-methoxytryptamine coupled to a corynantheal unit. When incorporated into the artificial diet, 5-methoxytryptamine alone had no effect on the *S. exigua* larvae. Corynantheal, however, had a strong detrimental effect on growth of the larvae, its effect being comparable to that of the leaf alkaloid extract. In contrast to the indole-type leaf alkaloids, the quinoline-type *Cinchona* root alkaloids did not affect growth and development of the larvae. These results suggest that the indole-type alkaloids, which in *Cinchona* plants are present at the highest concentrations in the young, vulnerable leaflets, are involved in the chemical defense of the plant against herbivorous insects.

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Key Words—Chemical defense, *Cinchona*, indole alkaloids, leaf alkaloids, Lepidoptera, Noctuidae, quinoline alkaloids, root alkaloids, *Spodoptera exigua*.

INTRODUCTION

Alkaloids are present in an enormous range of chemical structures in about 20% of the higher plant families. These secondary plant compounds often have a feeding-inhibitory or toxic activity towards herbivores (Janzen et al., 1977; Devitt and Philogène, 1980; Clay and Cheplick, 1989; Harborne, 1989). *Cinchona ledgeriana* Moens (fam. Rubiaceae) is indigenous to South America, and this species produces a variety of alkaloids, including the well-known antimalarial agent quinine (McHale, 1986). In young *C. ledgeriana* plants the various types of alkaloids are present in different organs: indole cinchophylline alkaloids in the aerial parts, and quinoline alkaloids in the roots (Aerts et al., 1991a).

All *Cinchona* alkaloids are ultimately derived from the common precursor alkaloid, strictosidine (Nagakura et al., 1979). Strictosidine is formed in a reaction catalyzed by the enzyme strictosidine synthase (Scott et al., 1977; Stöckigt and Zenk, 1977). In a previous study it was shown that in 6-month-old *C. ledgeriana* plants, the highest strictosidine synthase activity of the plant is present in the young, expanding leaflets (Aerts et al., 1991a). Indeed, the highest alkaloid content of the plant is also found in the young leaflets, in which the cinchophylline concentration is about 15 mM (15 nmol/mg fresh weight). In old leaves the cinchophylline concentration is five times lower than in young leaflets (Aerts et al., 1991a). Much lower strictosidine synthase activity than in the young leaflets is found in the roots of young *C. ledgeriana* plants, where quinoline alkaloids are present in concentrations of about 6 mM (Aerts et al., 1991a).

The high content of cinchophyllines in the young, nutrient-rich leaflets of *Cinchona* plants, and the striking difference in content between young leaflets and old leaves, may point to a protective role of the cinchophylline alkaloids against herbivores. In a number of cases, plants have been shown to express an explicit defense strategy in the timing and localization of alkaloid accumulation. The alkaloids produced by *Coffea arabica* L., for instance, have a deterrent or lethal effect on insect larvae (Janzen et al., 1977; Usher et al., 1988). Furthermore, during leaf development in coffee plants, the highest alkaloid content is present in the young, soft leaflets at the moment they emerge from their protective stipules (Frischknecht et al., 1986). Likewise, the alkaloid halostachine deters feeding by *Locusta*. In rye grass (*Lolium perenne* L.), a high concentration of halostachine is present in young, vulnerable seedlings just after germination, and the concentration decreases as the plant matures. Accordingly, the

meal size taken by *Locusta* nymphs from rye grass seedlings is much smaller than from mature leaves of the same species (Bernays and Chapman, 1976).

Here, we set out to investigate the possible protective role of *Cinchona* cinchophylline alkaloids. To this end, the alkaloids were incorporated into an artificial diet, and the effects were monitored on growth of larvae of the polyphagous beet armyworm *Spodoptera exigua* (Hübner) (Lepidoptera, Noctuidae).

METHODS AND MATERIALS

Extraction and Quantification of Leaf Alkaloids. Twenty-one of the youngest leaves (22.8 g) were collected from several *Cinchona ledgeriana* Moens plants. The leaves were cut in pieces and frozen in liquid nitrogen. The plant material was ground in a mortar and extracted twice with methanol. After evaporation of the methanol, the alkaloids were taken up in 2% H_2SO_4 and, after alkalinization with NH_4OH, were extracted three times in chloroform. The procedure was repeated once more: the chloroform extract was extracted three times with 2% H_2SO_4, and, after alkalinization of the aqueous extract, the alkaloids were again extracted three times in chloroform. The chloroform extract was filtered, dried over Na_2SO_4, and taken to dryness.

Identification and quantification of the alkaloids was by means of reference compounds and a HPLC system coupled to a photodiode array detector as described previously (Aerts et al., 1991a). In addition, identification was also by TLC as described previously (Aerts et al., 1991a,b). The crude extract contained the 3α,17β-, 3α,17α-, and 3β,17α-cinchophyllines (cf. Mulder-Krieger et al., 1982; Keene et al., 1983), as well as 7% 5-methoxytryptamine.

Other Alkaloids Used. The other alkaloids used were from commercial sources or gifts: 5-methoxytryptamine (Sigma); cinchonine, cinchonidine, quinine, and quinidine (a kind gift of ACF Chemiefarma); corynantheal (a kind gift of Dr. G. Massiot, Reims); emetine (gift).

Feeding Studies. An artificial diet was prepared as follows: 3 g agar was dissolved in 88.5 ml water and, after boiling, 14.5 g cornmeal, 3.5 g ground wheat germ, and 4 g brewer's yeast were added and mixed for 2 min. When the mixture had cooled to 60°C, vitamin C (0.5 g), benzoic acid (0.16 g), methyl *p*-hydroxybenzoate (0.13 g) and Aureomycin (8 mg) were added, and the complete diet was mixed for 3 min and further cooled. The alkaloids, dissolved either in water or in 96% ethanol, were thoroughly mixed into the artificial diet. Controls were provided by diets treated with solvent, i.e., water or 96% ethanol, only. After evaporation of the ethanol, the diets were offered to newly hatched *Spodoptera exigua* (Hübner) larvae. For each condition, 30–45 larvae, divided over four to six separate dishes, were used and were kept on the diet in the dark at 26–27°C. Every two to three days, the larvae received freshly

prepared artificial diet with alkaloid added. After six days, the mortality and average weight of the surviving larvae were determined. The average weight of the larvae was expressed as a percentage of the average weight of larvae kept on control diet (= 0 nM alkaloid, 100% growth).

To check for sensitivity of the larvae towards changes in ionic strength, larvae were also kept on artificial diet containing 15 mM NaCl. The NaCl had no effect on the growth of the larvae. The HPLC pattern and absorption spectra of the leaf alkaloids were unchanged after four days in the artificial diet, indicating no breakdown of the alkaloids.

Injection Studies. Fifth-instar larvae of *S. exigua* were mildly anesthetized with CO_2 and injected into the hemolymph with 1-μl volumes of solutions of the leaf alkaloids in 96% ethanol. Injections were performed by means of micropipets made with a Narishige Micropipette Puller. For each dose, 17 larvae were injected; controls were injected with 96% ethanol only. After injection with the highest dose (80 μg), the yellowish colored alkaloid solution could be clearly observed inside the larvae. The larvae were subsequently kept individually on artificial diet (see Feeding Studies) in the dark at 26–27°C. Seven days after injection, the pupae were transferred to containers with sawdust at the bottom. The pupae were kept in a 12 : 12 hr light–dark regime at 25–26°C. After emergence, the adults were offered a 10% honey solution.

RESULTS AND DISCUSSION

An extract of *Cinchona* leaf alkaloids was incorporated into an artificial diet in a concentration range similar to that found in *Cinchona* plants. The modified diet was offered to newly hatched *Spodoptera exigua* larvae, and the effects on growth of the larvae were monitored (see Methods and Materials). The leaf alkaloids exerted a strong detrimental effect on growth of the larvae (Figure 1, circles). At concentrations similar to those present in young leaflets of *Cinchona* plants (15 mM; in Figure 1 indicated with an arrow), the growth of the larvae was reduced to about 10% of that of controls. At a concentration of 5 mM, the growth of the larvae was approximately reduced by half. Concomitant with growth reduction, retardation in development was observed. At the highest concentration of leaf alkaloids used (15 mM), most of the larvae were still in their first instar after six days, whereas controls were already in their second or third instar.

Besides effects on growth and development of the larvae, the leaf alkaloids also caused high mortality: 41% of the larvae did not survive a six-day period on a diet with a concentration of alkaloid such as was present in young *Cinchona* leaflets (Table 1). In preliminary experiments, *Cinchona* leaf alkaloids were found to have comparable detrimental effects on larvae of *Biston betularia* L. and *Trichoplusia ni* (Hübner) as on the *Spodoptera exigua* larvae (not shown).

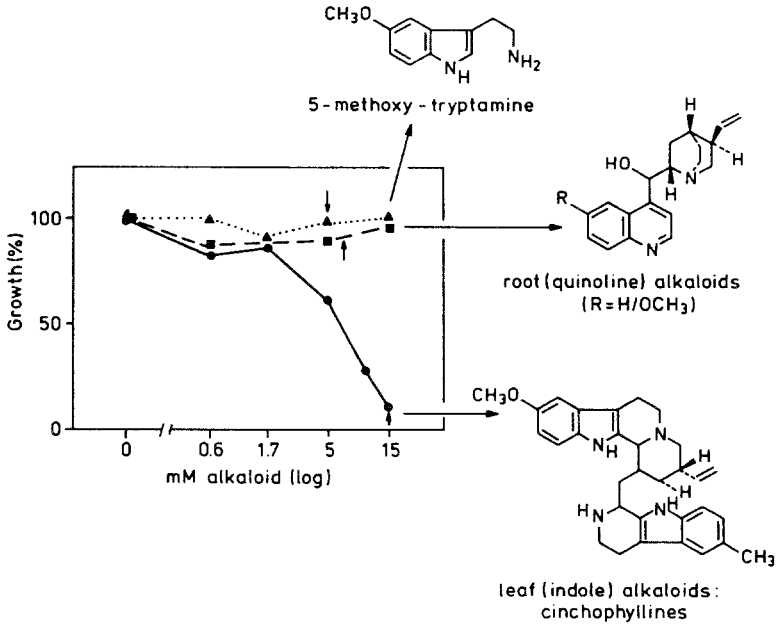

FIG. 1. Effects of the various alkaloids present in young *Cinchona ledgeriana* plants on the growth of larvae of the polyphagous insect *Spodoptera exigua* (Noctuidae, Lepidoptera). Newly hatched larvae were offered an artificial diet with the alkaloids added at several concentrations. After six days, the average weight of the surviving larvae was determined. Growth (=weight) of the larvae is expressed as a percentage of that of control larvae (= 0 mM alkaloid; see Methods and Materials). Arrows indicate the highest concentrations of the alkaloids such as found in 6-month-old *C. ledgeriana* plants.

TABLE 1. PERCENT MORTALITY OF NEWLY HATCHED *Spodoptera exigua* LARVAE AFTER 6 DAYS ON DIET PROVIDED WITH 15 mM ALKALOID

Alkaloid added	Mortality (%)
None	2
5-Methoxytryptamine	2
Root (quinoline) alkaloids[a]	3
Leaf (indole) alkaloids	41
Corynantheal	22
Emetine	97

[a]Cinchonine, cinchonidine, quinine, and quinidine (1:1:1:1)

To distinguish between effects of cinchophylline alkaloids and 5-methoxytryptamine, which were both present in the leaf extract (see Methods and Materials), the effect of 5-methoxytryptamine alone on growth and mortality of *Spodoptera exigua* larvae was investigated (Figure 1, triangles; Table 1). 5-Methoxytryptamine alone had no effect on the larvae, either at the concentration such as present in *Cinchona* plants (in Figure 1 indicated with an arrow) or at higher concentrations. This indicates that the effect of the leaf-alkaloid extract was due to the cinchophylline alkaloids.

In young *Cinchona* plants, 5-methoxytryptamine is present in plant parts that are rich in cinchophyllines (Aerts et al., 1991a) and these may very well be involved in the synthesis of cinchophyllines, since 5-methoxytryptamine constitutes part of the cinchophylline molecule (Figure 1, structures). The other part of the cinchophylline molecule consists of a corynantheal unit (Figures 1 and 2, structures). Because 5-methoxytryptamine alone did not influence growth of the larvae, the effect of corynantheal on the larvae was investigated and was shown to have a detrimental effect on growth of the larvae, in a manner similar to the leaf alkaloids (Figure 2, circles; cf. Figure 1). At the highest concentration used (15 mM), corynantheal reduced growth of the larvae to about 15% of that untreated controls, and at a concentration of 5 mM, growth was reduced by approximately half. Again, concomitant with growth reduction, both retardation in molting and mortality of the larvae were observed (Table 1).

These results may indicate that in cinchophylline molecules the corynan-

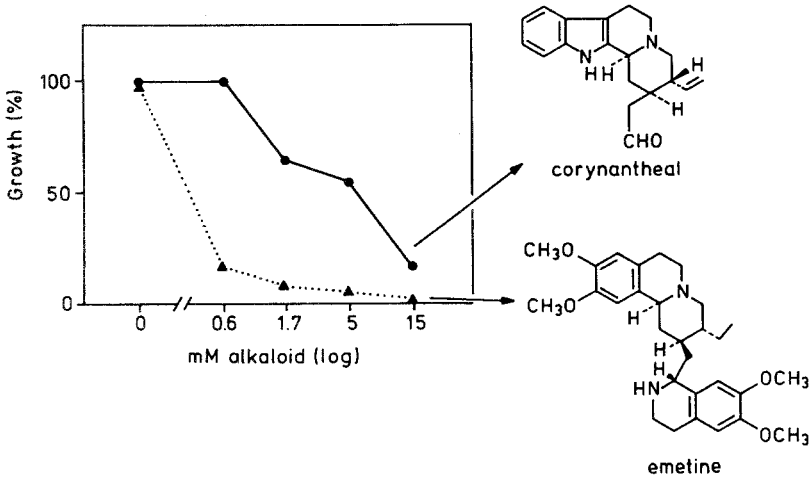

FIG. 2. Effects on the growth of *S. exigua* larvae of the corynantheal moiety of cinchophylline alkaloids from *Cinchona* and the alkaloid emetine from *Cephaëlis*. For experimental details, see legend to Figure 1.

theal moiety is the active site exerting the detrimental effects on the larvae. Alternatively, the detrimental effect of corynantheal may be related to the aldehyde group present in this molecule (Figure 2). This aldehydic function may also explain the coupling of corynantheal to 5-methoxytryptamine in the plant.

The quinoline-type alkaloids found in the underground parts of young *C. ledgeriana* plants were also tested for their effect on growth of *S. exigua* larvae. Intriguingly, in contrast to the indole-type leaf alkaloids, the quinoline-type root alkaloids did not affect growth of the larvae (Figure 1, Table 1).

Emetine is an alkaloid from *Cephaëlis ipecacuanha* and, like *Cinchona* is a member of the Rubiaceae family and has medicinal and amebicidal properties (Hughes and Genest, 1973; Keene et al., 1986). Because the mode of biosynthesis of emetine is closely related to that of cinchophylline alkaloids (Hegnauer, 1973; Verpoorte et al., 1985), and the chemical structures of emetine and cinchophylline alkaloids have features in common (Figure 1 and 2, structures), the effect of emetine was tested, and it turned out to have strong detrimental effects on the growth of the larvae (Figure 2, Table 1).

In general, the protective effect of secondary compounds in plant tissues can be due to their toxicity, to their feeding deterrence, or to a combination of the two. To study the possible toxicity of the *Cinchona* leaf alkaloids directly, known amounts of the alkaloids were injected into the hemolymph of final-instar *S. exigua* larvae (see Methods and Materials). Figure 3 shows that seven days after injection, the percentage of larvae that became pupae was unchanged at all amounts injected, as compared to controls injected with carrier only. The

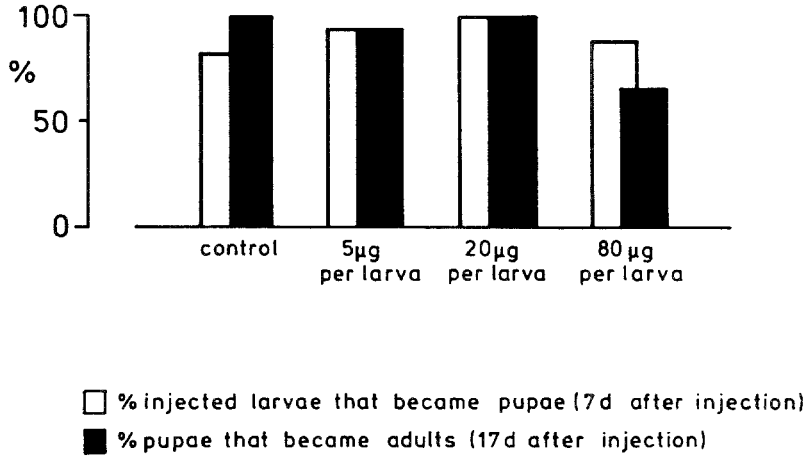

FIG. 3. Development of *S. exigua* final-instar larvae after injection into the hemolymph of increasing doses of *Cinchona* leaf alkaloids. For each condition, 17 larvae were injected. Controls were injected with carrier only.

percentage of pupae that subsequently became adults 17 days after injection declined only at the highest amount injected (80 μg) (Figure 3). Under all conditions, the adults produced eggs from which larvae hatched. Thus, acute, pronounced toxic effects of the *Cinchona* leaf alkaloids were not observed at the amounts injected.

The largest amount injected (80 μg) corresponds to a dose of about 0.4–0.8 mg/g of body weight. For comparison, in leaves of *Encelia asperifolia* and *E. actoni*, the toxic sesquiterpene lactone encelin is present in concentrations roughly corresponding to those of alkaloids in *Cinchona* leaves (Srivastava et al., 1990). However, encelin caused 60% mortality within two days when final-instar *Spodoptera littoralis* larvae were injected into the hemolymph with a dose of only about 0.1 mg/g of body weight (Srivastava et al., 1990). Likewise, when fifth-instar *Locusta migratoria* and *Schistocerca gregaria* larvae were injected into the hemolymph with the triterpenoid azadirachtin or the alkaloids nicotine or tomatine, the doses at which 50% mortality occurred within 24 hr were estimated at 0.08–0.16 mg/g for azadirachtin, 0.6–0.8 mg/g for nicotine, and 0.1–0.7 mg/g for tomatine (Cottee et al., 1988). Compared to these natural compounds, which are present in plants in comparable (nicotine, tomatine) or lower (azadirachtin) concentrations as the *Cinchona* leaf alkaloids, the latter show very low toxicity. How, exactly, the leaf alkaloids exert their detrimental effects on the larvae is at present unknown.

We have shown that *Cinchona* leaf alkaloids, when introduced into an artificial diet at concentrations such as present in the plant, exert detrimental and lethal effects on larvae of *S. exigua*. As has been stated before (Harborne, 1989), if secondary compounds play a protective role in the plant, then the timing and localization of accumulation of these compounds can be expected to reflect a defense strategy. Indeed, the highest concentration of leaf alkaloids in *Cinchona* plants has been shown to be present in the vulnerable, young expanding leaflets (Aerts et al., 1991a). These results suggest that the leaf alkaloids play a role in the chemical defense of the plant against phytophagous insects.

Evidence suggests that the *Cinchona* leaf alkaloids have, in fact, multiple defensive functions, since the alkaloids were shown to have amebicidal and antimicrobial activities as well (Keene et al., 1986; Caron et al., 1988). More secondary compounds synthesized by *Cinchona* may be involved in the chemical defense of the plant, since the anthraquinones produced by *Cinchona* also have antimicrobial activity, and a role of the anthraquinones as phytoalexins has been suggested (Verpoorte et al., 1985; Wijnsma et al., 1985). Finally, with respect to the root alkaloids of young *Cinchona* plants, which did not affect the *S. exigua* larvae, it is interesting to note that these alkaloids have been shown to have antigastropodan activity (Aerts et al., 1991b).

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FOLIAR CHEMICALS OF WHEAT AND RELATED GRASSES INFLUENCING OVIPOSITION BY HESSIAN FLY, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae)

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Abstract—More than twice the number of mated female Hessian flies, *Mayetiola destructor* (Say) entered a zone within 1 cm of a paper strip treated with one plant equivalent (PE) of a chloroform extract of wheat foliar waxes compared to a strip treated with solvent only; females also stayed six times longer and laid 10 times more eggs on the strip treated with the wheat extract. Column chromatographic fractionation of the wheat extract and application of these fractions onto filter paper strips showed four fractions elicited significant numbers of eggs to be laid. Single, binary, and tertiary combinations of three of these fractions (two of the four fractions apparently contained similar compounds) were tested. The greatest numbers of eggs were laid on strips treated with the tertiary combination or the binary combination containing the two most active fractions (3 and 6); three times the number of eggs were laid on strips treated with this binary combination than the sum of eggs laid on strips treated with these two fractions separately. A comparison of grasses and their extracts showed female Hessian flies laid greater numbers of eggs on wheat or rye than on barley or oat. Fractionated barley and oat extracts were tested for activity as for wheat, and a similar pattern was observed, i.e., the greatest numbers of eggs were laid on fractions 3 and 6. Dose–response tests, using these two fractions of wheat, barley, or oat showed the same threshold of activity for fraction 3 for all three extracts, i.e., 2 PE. In contrast, fraction 6 of wheat was active at the lowest dosage tested, 0.25 PE, while the same fraction of either barley or oat was not active until tested at a dosage of 2 PE. It appears that (at least) two chemicals in the foliar

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waxes of these grasses influence ovipositional behavior of female Hessian flies. Furthermore, given the similar foliar chemistry of these grasses and the strong synergistic interaction between fractions 3 and 6 shown for wheat extract, it is likely that the ovipositional preferences exhibited by female Hessian flies towards these grasses may be explained by quantitative differences in the amount(s) of the active chemical(s) in their respective fraction 6 (most polar fraction tested).

Key Words—Hessian fly, *Mayetiola destructor*, Diptera, Cecidomyiidae, oviposition stimulants, wheat, rye, barley, oat.

INTRODUCTION

The Hessian fly, *Mayetiola destructor* (Say), is one of the most destructive insect pests of wheat, *Triticum aestivum* L. The Hessian fly is found in most of the major wheat-growing areas in the Northern Hemisphere, as well as in New Zealand in the Southern hemisphere (Hatchett, 1986). In the United States, it is found in all the major wheat-growing areas from the Atlantic coast to the Great Plains and in the coastal areas of California, Oregon, and Washington (Gallun et al., 1975; Morrill and Nelson, 1976; Pike and Antonelli, 1981).

Although wheat is the best known host of the Hessian fly, Hessian fly larvae feed and survive on other grasses in the tribe Triticeae, such as barley (*Hordeum vulgare* L.) and rye (*Secale cereale* L.) (McColloch, 1923; Jones, 1938; Stokes, 1957). In contrast, oat, *Avena sativa* L. (tribe Aveneae), does not support growth of Hessian fly larvae (Morrill, 1982). The mobility of Hessian fly larvae is, however, limited to movement down the leaf from the site where the egg is laid to feeding sites near the crown of the plant. Consequently it is the adult female, by ovipositing, that determines which plant the larva feeds upon.

Recently some of the factors influencing host-plant selection by the female Hessian fly have been studied. Using a surrogate wheat leaf, Harris and Rose (1990) found that female Hessian flies were strongly influenced by chemical, visual, and tactile cues in their choice of an oviposition site. Chemicals extracted from the surface of wheat foliage were found to stimulate female oviposition such that significant increases in the number of eggs were laid on surrogate leaves when treated with levels as low as 0.01 plant equivalents (PE).

We have further investigated the role of foliar chemicals that elicit female Hessian flies to oviposit. We had four objectives in this work: (1) to determine the effects of wheat foliar chemicals on behaviors of mated female Hessian flies preceding and succeeding landing, (2) to determine whether there was more than one chemical in the wheat extract that influenced oviposition by female Hessian flies, (3) to determine whether female Hessian flies exhibited ovipositional preferences towards different grasses, and (4) if females exhibited preferences

towards the various grasses, to determine whether these preferences were due to differences in foliar chemicals of the grasses, and if so, whether these differences were qualitative or quantitative.

METHODS AND MATERIALS

Insects. Hessian flies were from a laboratory culture originally collected from wheat fields in Ellis County, Kansas, in 1986. Although the Great Plains biotype is predominant in Ellis County, other biotypes are present (biotypes A and B, Dr. J. Hatchett, personal communication) and consequently may have been used in experiments. The laboratory colony was reared on the susceptible wheat cultivar Newton in the two- to three-leaf stage. Single, mated females were placed in cages (10 cm diam. × 30 cm height) containing 20 plants and allowed to oviposit; adults emerged from the wheat plants approximately 30 days later and were harvested for use in the experiments reported here.

Mated females for the experiments were obtained from a cage containing 20–100 each of newly emerged males and females. Virgin females could be easily distinguished from mated females by their distended ovipositors; females were usually mated within 15 min of being placed with males.

Grasses and Their Foliar Extracts. Plants of wheat (cultivar Newton), oat (cultivar Don 6), barley (cultivar Kanby) and rye were grown outdoors in September 1989 either in pots (10 cm diam.) or in field plots at Tuttle Creek Research Farm (Manhattan, Kansas). Plants were grown to the three- to four-leaf stage and then either used intact or the leaves were cut at the base of the plant and extracts of their foliar waxes prepared by dipping them in cold (0°C) chloroform for 60 sec according to the method of Bianchi and Figini (1986). Crude extracts were concentrated to 1 PE/30 μl by rotary evaporation at 40°C and stored in glass vials at −15°C.

The concentrated, foliar extract of 500 plants of each grass (each extract containing ca. 30–100 mg of wax) was fractionated on a column (24 cm length × 1.2 cm diam.) containing 12 g of silica gel G (Woelm Pharma, Eschwege, Germany), using mixtures of hexane and diethyl ether according to Kate (1972, p. 404): 45 ml hexane (yielding fraction 1), 95 ml hexane–diethyl ether (99 : 1 v/v) (fraction 2), 60 ml hexane–diethyl ether (95 : 5) (fraction 3), 75 ml hexane–diethyl ether (92 : 8) (fraction 4), 115 ml hexane–diethyl ether (85 : 15) (fraction 5) and 45 ml diethyl ether (fraction 6). Each of the fractions was concentrated, using a rotary evaporator, to 1 PE/30 μl as for the mother solution.

Thin-Layer Chromatography. Extracts and fractions were analyzed by thin-layer chromatography (TLC) using 10 cm (width) × 20 cm (length), 250-μm silica gel-coated glass plates (Fisher Scientific). The various extracts and fractions were applied to the origin of the plates and the plates developed using a

mixture of chloroform–1% ethanol (Tulloch and Hoffman, 1971). After the plates were developed, they were sprayed with rhodamine G (Sigma Chemicals, St. Louis, Missouri). The spots were visualized under 254 nm ultraviolet light. The R_f values of the various spots from the extracts and fractions were compared with R_f values of mixtures of C_{22}–C_{32} alkanes and alcohols, hentriacontane-14,16-dione, 8-hydroxyhentriacontane-14,16-dione, and a mixture of C_{44}–C_{52} esters run on plates under the same conditions.

Behaviors Preceding and Succeeding Landing. At 0800 hr, virgin females ($N = 20$) that had eclosed between 0600 and 0700 hr were placed in cages with males ($N = 20$) and allowed 10 min to mate. At 1100 hr, the mated females were transferred to an arena (18 cm height × 10 cm diam. transparent acrylic walls, a screen top, and moist soil on the floor) containing two filter paper strips (0.5 × 10 cm), one yellow (treated with a vegetable dye, McCormick and Co., Baltimore, Maryland) and treated with 1 PE (in 100 μl) of a chloroform extract of wheat leaves, and the other yellow and treated with 100 μl of chloroform. The strips were placed 4 cm apart and 2 cm away from the arena walls and were held in position by metal clips placed in the soil.

The observer recorded for 20 min: (1) the number of females entering the 1-cm zone surrounding each strip, and (2) the number of females landing on the strips. A landing was scored when all six legs of a female contacted a strip, and the female's wings were folded over her abdomen. At the end of the observation period the numbers of eggs laid on each strip were counted. The test was replicated 10 times with 10 different groups of females.

In tests quantifying the behaviors of female Hessian flies after landing (see Harris and Rose, 1989, for detailed descriptions of behaviors), the observer waited for the first female to land on either of the two treatments (yellow filter paper with or without wheat extract) and then recorded (using a tape recorder) the following events or behaviors for that female: (1) time from landing to flight; (2) the frequency of antennation (antennae brought into contact with or within ca. 1 mm of the filter paper), arching (abdomen arched so that ovipositor touches filter paper), and sitting (abdomen straightened and held parallel to the filter paper); (3) percent of total time on the filter paper that was spent in the arching posture; and (4) the numbers of eggs laid by the female before flying off the strip. After this first observation, the same data were recorded for the next females landing on the other strip, using the same group of mated females. These paired observations were made using 12 different groups of females so that, in all, 12 females were observed both on chloroform-treated and wheat extract-treated yellow strips. Since a randomized complete block design was used in both sets of observations, the data were analyzed by a two-way ANOVA.

These tests were conducted in a climate-controlled room (22.5 ± 0.5°C, and 60–70% relative humidity) under a light intensity of 290 μW/cm^2 [generated using six full-spectrum fluorescent tubes, (Verilux Inc., Greenwich, Connecti-

cut) and measured using a United Detector Technology Model 61 Radiometer (Hawthorne, California)].

Bioassays. Egg-laying responses to the grasses, wheat, rye, barley, and oat [three- to four-leaf stage, 10 plants of each, transplanted into pots (37 cm diam.) with a cage top (18 cm height × 37 cm diam.) transparent acrylic walls, a screened top, and moist soil on the floor] were compared. At 1000 hr, 20–30 mated females were introduced into the cage. Females were removed 6 hr later, and the numbers of eggs laid on the four different grass species were recorded.

For the experiments comparing egg-laying responses to plant foliar extracts and fractions of extracts, extracts or chloroform were applied in appropriate volumes to yellow filter paper strips (10 × 0.5 cm), and the solvent was left to evaporate for 60 min before the paper strips were placed in cages (cages consisted of a transparent acrylic cylinder, 10 cm diam. × 10 cm height, with a screened top, placed over a 10 cm diam. pot containing moist soil). Filter paper strips were held upright (long axis) by small metal clips placed in soil and were placed to maximize the distance between adjacent strips and also the cage walls. Five to 15 mated females were introduced into each pot at 1000 hr. The pots were arranged randomly in an illuminated Percival model E-54B plant growth chamber (22 ± 1°C, 60–80% relative humidity). At 1400 hr, the number of eggs laid on each of the strips was recorded. In experiments with more than six treatments, the same procedures were used except the strips were presented to females in a larger cage (18 cm diam. × 21 cm height).

The experimental design for each of these experiments was a randomized complete block. Data were subjected to Bartlett's test for homogeneity of variances, transformed (square root or log) if heteroscedastic, and analyzed by a two-way ANOVA. Means of treatments were compared using Fisher's protected least significant difference tests. Unless otherwise stated, all statistical differences are reported to $P < 0.01$. In the experiment using the factorial design, the interactions between the different fractions were tested using a general linear models procedure (SAS Institute, 1988).

RESULTS

Behaviors Preceding and Succeeding Landing. Over twice as many females entered the 1-cm zone around the strip treated with wheat extract as entered the 1-cm zone around the chloroform-treated (blank) strip (significantly greater, $P < 0.001$). Upon entering the 1-cm zones, females typically changed to a hovering type of flight in front of the filter paper strip before they landed or flew out of the zone. Of these females, 33% and 77% went on to land on filter papers treated with chloroform or a chloroform extract of wheat, respectively. Wheat extract continued to influence female behavior after landing. Females that landed

on the wheat extract-treated filter paper strip laid twice as many eggs as females that landed on the blank strip (Table 1).

The influence of wheat extract on behaviors after landing on a surface was examined in greater detail by recording the behaviors of individual females on wheat extract and blank yellow filter paper strips (Table 2). Females spent a larger portion of their time arching on wheat extract-treated strips but exhibited higher frequencies of antennation and sitting on blank strips. Overall, females spent ca. 6.8 times longer on wheat extract-treated strips than on the blank strips. The greater mean time and percentage of time spent arching on the strips treated with wheat extract corresponded with a significantly higher ($P < 0.001$) mean number of eggs laid on these strips compared to the blank strips.

Activity of Fractions of Wheat Extract. Using the egg-count bioassay, the six fractions of the wheat extract (each at 4 PE) were tested for their ability to

TABLE 1. COMPARISON OF BEHAVIORS PRECEDING AND SUCCEEDING LANDING OF MATED FEMALE HESSIAN FLIES TO FILTER PAPER STRIPS TREATED WITH SOLVENT (CHLOROFORM) OR 1 PLANT EQUIVALENT (PE) OF WHEAT EXTRACT[a]

Treatment	Total encounters	Landed (%)	Eggs (*N*/landing)
Solvent blank	63.2	33	0.6
Wheat extract (1PE)	134.1	77	1.2
F value	12.91	80.52	7.99
Significance	$P < 0.001$	$P < 0.001$	$P < 0.05$

[a] Total encounters refers to the number of females flying into a 1 cm zone (2 cm diam) surrounding filter paper strips. Landed (%) refers to the number of females flying into the 1-cm zone that went on to land on the filter paper strip. Data derived from 10 replicates; statistical comparison was made by a two-way ANOVA.

TABLE 2. COMPARISON OF BEHAVIORS EXHIBITED BY MATED FEMALE HESSIAN FLIES ON FILTER PAPER STRIPS TREATED WITH SOLVENT OR 1 PLANT EQUIVALENT OF WHEAT EXTRACT[a]

		Frequency (events/10 sec)				
Treatment	Time arch (%)	Anten	Arch	Sit	Total time	Eggs (*N*)
Solvent blank	64	18.4	2.47	0.89	8.9	0.3
Wheat extract	87	4.5	0.28	0.19	60.9	3.6
F value	5.34	17.75	4.9	5.38	35.48	48.1
Significance	$P < 0.05$	$P < 0.001$	NS	$P < 0.05$	$P < 0.001$	$P < 0.001$

[a] For a description of the behaviors arching (Arch), antennation (Anten), and sitting (Sit), see text. Data derived from observations of 10 females each for blank and wheat extract.

stimulate oviposition by female Hessian flies. Females laid significantly more eggs on strips treated with fractions 3, 5, and 6, than on the blank strips (Figure 1). Females also laid more than twice as many eggs on fraction 1 (mean of 40.7/strip) than on the blank strips (mean of 15.7/strip; significantly different at $P < 0.05$). In a separate experiment testing only fraction 1 (at 4 PE) versus the blank, the mean number of eggs laid on strips treated with fraction 1 (103.2/strip) was significantly greater ($P < 0.001$) than the mean number of eggs laid on the blank strips (40.6/strip).

Silica gel TLC of the four active fractions of the wheat extract showed that these fractions contained compounds with R_f values corresponding to the following standards: hydrocarbons (fraction 1), esters (fraction 3), and alcohols (fractions 5 and 6).

Single Fractions versus Blends of Fractions of Wheat Extract. Using a factorial design, the relative activities of single, binary, and tertiary combinations of fractions 1, 3, and 6, at dosages of 1 PE, were determined (Figure 2). At this lower dosage, only fractions 3 and 6 (of the single fractions) exhibited significant activity relative to the blank. Strips treated with the binary combinations containing fraction 1 did not have significantly greater numbers of eggs laid on them than the corresponding unitary fractions, 3 or 6. However, strips treated with the binary combination of fractions 3 and 6 had over three times the number of eggs laid on them (mean of 198) than the combined sum of eggs laid on fraction 3 (mean of 23) and fraction 6 (mean of 42). The greatest number of eggs was laid on strips treated with the tertiary combination, although this was not significantly different from the number laid on the binary combination

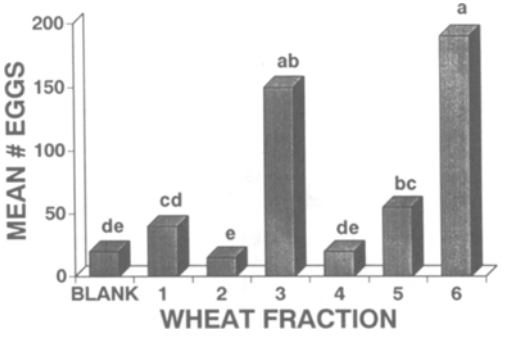

FIG. 1. Mean number of eggs laid by mated female Hessian flies on six different fractions (each at a dosage of 4 PE on filter paper strips) of wheat foliar extract; filter paper strips treated with chloroform were used as the blank. See text for details of silica gel column fractionation. Means with different letters are statistically different at $P < 0.01$ (ANOVA and Fisher's protected least significant difference test). The means were derived from three replicates.

of fractions 3 and 6. Analysis of the data showed a significant interaction between fractions 3 and 6 ($F = 22.3$, $P < 0.0001$), and no other significant interactions.

A Comparison of Egg-Laying Responses to Various Grasses and Their Extracts. The relative number of eggs laid on plants of wheat, rye, barley, and oat by female Hessian flies was determined (Figure 3). There were no significant differences in numbers of eggs laid on wheat and rye, but significantly fewer eggs were laid on both barley and oat.

Chloroform extracts of wheat, rye, barley, and oat plants on filter paper strips were similarly compared. The pattern of egg-laying on the filter paper strips treated with the different extracts showed a similar, but slightly different,

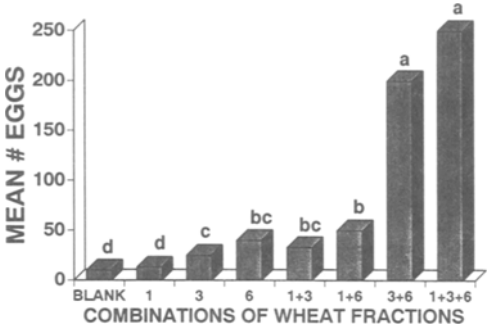

FIG. 2. Mean number of eggs laid by mated female Hessian flies on unitary, binary, and tertiary mixtures (each at a dosage of 4 PE) of three fractions of wheat extract; filter paper strips treated with chloroform were used as the blank. See text for details of silica gel column fractionation. Means with different letters are statistically different at $P < 0.01$ (ANOVA and Fisher's protected least significant difference test). The means were derived from three replicates.

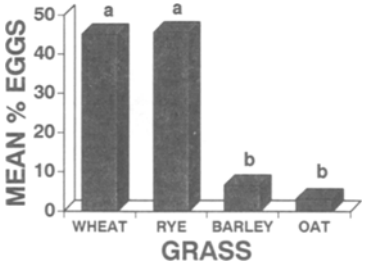

FIG. 3. A comparison of the percentage of eggs laid by mated females Hessian flies on plants, in the two- to three-leaf stage, of wheat, rye, barley, and oat. Means with different letters are statistically different at $P < 0.01$ (ANOVA and Fisher's protected least significant difference test). The means were derived from three replicates.

pattern to that on the grasses themselves; there was no significant difference between the numbers of eggs laid on rye and barley, and there was a significant difference between the numbers of eggs laid on barley and on oat (Figure 4). All the filter paper strips treated with extracts from the various grasses had significantly greater numbers of eggs laid on them than the blank strips.

Comparative Activity of Fractions of Extracts of Different Grasses. The chloroform extracts of barley and oat were fractionated by silica gel G column chromatography into six fractions, as for wheat, and each of the resulting fractions tested at 4 PE. A pattern of activity similar to that for wheat was observed for the six fractions of both barley and oat (Figure 5), with females laying the greatest number of eggs on strips treated with fractions 3 and 6. In the case of barley, strips treated with fraction 1 also had a significantly greater number of eggs laid on them than the blank strips.

Silica gel G TLC analysis of fractions 1, 3, and 6 of both barley and oat showed compounds with the same R_f values as for wheat, i.e., hydrocarbons (fraction 1), esters (fraction 3), and alcohols (fraction 6).

Dose–Response Relationships of Active Fractions of Extracts of Grasses. In order to determine whether the extracts of wheat, barley, and oat had different activities for eliciting oviposition in their respective fractions 3 and 6, different dosages of these fractions were applied to the filter paper strips and presented along with a blank in separate bioassays. The threshold of activity for fraction 3 was the same for all of the extracts (Figure 6); only at a dosage of 2 PE did females lay significantly more eggs on these strips than on the blank strips. However, for strips treated with fraction 6, the dose-response pattern for wheat differed from those for barley or oat (Figure 7). Females laid significantly more eggs on strips treated with fraction 6 of wheat extract, even at the lowest dosage

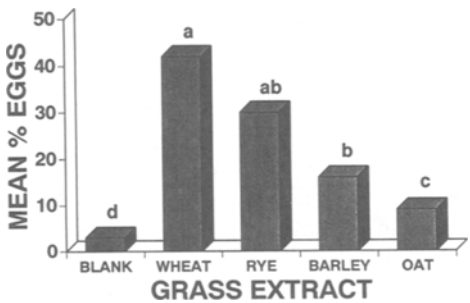

FIG. 4. A comparison of the percentage of eggs laid by mated female Hessian flies on filter paper strips treated with foliar extracts of wheat, rye, barley, and oat; filter papers strips treated with chloroform were used as the blank. Means with different letters are statistically different at $P < 0.01$ (ANOVA and Fisher's protected least significant difference test). The means were derived from three replicates.

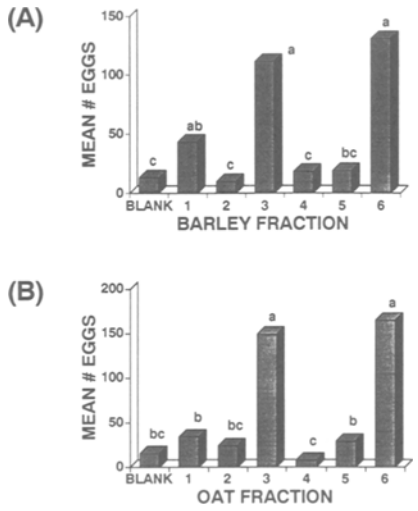

FIG. 5. Mean number of eggs laid by mated female Hessian flies on six different fractions (each at a dosage of four plant equivalents) of (A) barley and (B) oat foliar extract; filter paper strips treated with chloroform were used as the blank. See text for details of silica gel column fractionation. Means with different letters are statistically different at $P < 0.01$ (ANOVA and Fisher's protected least significant difference test). The means were derived from three replicates.

tested of 0.25 PE, than on the blank strips. In contrast, for fraction 6 of both barley and oat, females did not lay significantly greater numbers of eggs on strips treated with dosages up to 1 PE, compared to the blank strips. Only at a dosage of 2 PE did strips treated with fraction 6 of barley and oat have significantly more eggs laid on them than the blank strips.

DISCUSSION

Previously it had been shown (Harris and Rose, 1990) that at least three sensory inputs influence host finding and acceptance by female Hessian flies, namely, visual, tactile, and chemical. In this study we have investigated the chemical inputs in greater detail.

Chemicals present in the chloroform extract of the foliar waxes of wheat leaves clearly exert a major influence on the behaviors exhibited by mated female Hessian flies before and after landing. Over twice as many females entered the 1-cm zone surrounding the wheat extract-treated filter paper strip, suggesting that female Hessian flies are able to perceive, presumably through the olfactory modality, the wheat extract, at least from distances of 1 cm. Foliar chemicals

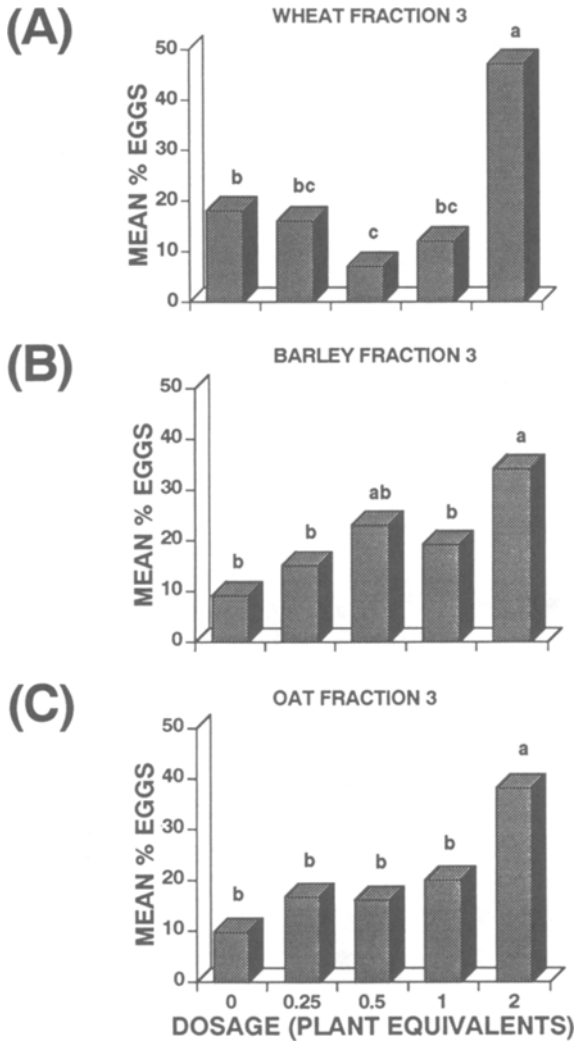

FIG. 6. Dose-response tests of the number of eggs laid by mated female Hessian flies on filter paper strips treated with different dosages of fraction 3 (see text for details of silica gel fractionation) of foliar extract of (A) wheat, (B) barley, and (C) oat; filter paper strips treated with chloroform were used as the blank. Means with different letters are statistically different at $P < 0.01$ (ANOVA and Fisher's protected least significant difference test). The means were derived from three replicates.

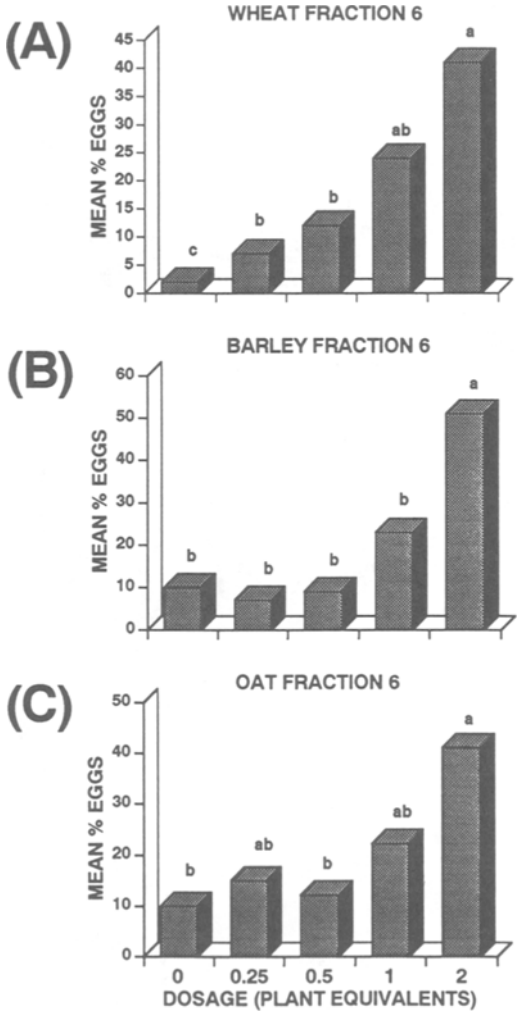

FIG. 7. Dose–response tests of the number of eggs laid by mated female Hessian flies on filter paper strips treated with different dosages of fraction 6 (see text for details of silica gel fractionation) of foliar extract of (A) wheat, (B) barley, and (C) oat; filter paper strips treated with chloroform were used as the blank. Means with different letters are statistically different at $P < 0.01$ (ANOVA and Fisher's protected least significant difference test). The means were derived from three replicates.

also influenced females after they had landed on the filter paper strips, with females staying six times longer and laying 10 times more eggs on strips treated with wheat extract as opposed to only solvent.

Subsequent experiments testing various fractions of the wheat extract (see below) indicate that there is more than one chemical that exerts an influence on the number of eggs laid on wheat. We do not know whether different chemicals in the extract elicit the distinct behaviors preceding and succeeding landing, or whether these behaviors are influenced by the same chemical(s). However, clearly at least one chemical in the wheat extract must be volatile enough to elicit orientation of females in flight, while the same or another chemical(s) must be relatively unvolatile, such that females may perceive it (them) on the surface of the plant. It has been suggested that volatile chemicals in wheat leaves, including low-molecular-weight alcohols, aldehydes, and esters, as well as some sesquiterpenes, may be attractants for some insects (Buttery et al., 1985). It is worth noting that our method for concentrating the extract would largely remove the more volatile chemicals present in the initial extract of the wheat foliage.

The stimulatory chemicals in the wheat extract were investigated by fractionating the extract and presenting single fractions to mated females. Of the six fractions, four, when placed on filter paper strips, showed some ability to stimulate oviposition by females. However, one of these fractions (fraction 1) only elicited a significant level of oviposition when presented at a relatively high dosage (4 PE) and did not significantly increase the number of eggs laid when added to the other active fractions (see below), while another of these fractions (fraction 5) appeared to contain the same class of compounds as the subsequent fraction (fraction 6). Thus, conservatively, we can state that there are two compounds in wheat foliar waxes that, at natural levels, appear to stimulate oviposition by female Hessian flies. However, it is possible that there may be more than two compounds that stimulate oviposition by female Hessian flies. For example, the two active fractions may each contain more than one active compound or fraction 5 may contain an additional, unique active compound.

While the compounds in the two (most) active fractions of wheat extract, when presented by themselves, elicited oviposition by females, it appears that, in their natural context, their effect on female Hessian flies is synergistic. That is, when presented together they elicit a significantly greater ovipositional response from females than the sum of the responses they elicit when presented separately. Such a synergistic effect on different chemicals on stimulating oviposition has been noted in other insects. For example, the addition of up to five other compounds synergizes the effects of the most stimulatory chemical (i.e., that with the lowest activity threshold), a polyacetylene falcarindiol, on oviposition by female carrot flies (Städler and Buser, 1984).

While Hessian flies are best know as pests of wheat, females also oviposit

on other grass species (McColloch, 1923; Jones, 1938; Stokes, 1957). In our experiments, females showed an order of preference to the various grasses of wheat $\approx$ rye $>$ barley $\approx$ oat. This order of preference appears to be mediated for the most part by the foliar chemistry of the grasses; when filter paper strips were treated with extracts of each grass, the order of preference was similar to that of the grasses themselves, i.e., wheat $\geq$ rye $\geq$ barley $>$ oat. The inclusion of a chloroform control in this experiment also revealed that all the grasses contained behaviorally significant amounts of stimulatory chemicals in their foliar waxes.

Column chromatographic fractionation of the foliar extracts of barley and oat separated each of these extracts into six fractions, at least two of which, when applied to filter paper strips, elicited female Hessian flies to lay significant numbers of eggs. Extracts of wheat, barley, and oat appear to have these same active fractions in common. To explain the greater activity of the wheat extract over those of barley and oat, we surmised that the wheat extract must contain greater activity in either or both of these two fractions. The dose–response experiment showed that fraction 3 elicited significant oviposition by female Hessian flies at a threshold of 2 PE for all three grass extracts. In contrast, fraction 6 of wheat elicited oviposition by female Hessian flies at a dosage as low as 0.25 PE, while for the extracts of barley and oat, fraction 6 did not elicit significant oviposition until at a dosage of 2 PE. Therefore, the greater activity of wheat extract (and to a large extent that of the grass itself) to elicit oviposition by female Hessian flies, over those of the other grasses, is due to the greater activity of the compound(s) in its fraction 6.

There are two possible explanations for this observation. First, the grasses have qualitative differences in their respective fraction 6, i.e., wheat contains a different and more powerful ovipositional stimulant than does barley or oat. The second explanation is that there are quantitative differences in the chemicals in their respective fraction 6, i.e., wheat (and probably rye) contains more of a particular ovipositional stimulant(s) than does barley or oat. We believe, given the similarities between the epicuticular wax chemistry of the grasses (see below), the dose–response results of fraction 6, and the powerful synergistic interaction between fractions 3 and 6 shown in wheat extract, that the latter explanation is more likely. In further support of this is the activity of fraction 5 in wheat, this fraction apparently containing more of the same or similar types of chemicals as fraction 6. Clearly, only identification of the oviposition-stimulating chemicals and their quantification in the different grasses will equivocally establish (or disestablish) this postulate.

The chemistry of the epicuticular waxes of a number of grasses in the tribe Triticeae (family Poaceae) has been studied extensively (Tulloch et al., 1980; Bianchi and Figini, 1986). The principal classes of compounds include long-chain hydrocarbons, alcohols, free fatty acids, esters, aldehydes, and β-diketones

and hydroxy-β-diketones. Most of the compounds within the classes are homologous, differing in series of two carbons, with the exceptions of the β-diketones and hydroxy-β-diketones, which are usually hentriacontane-14,16-dione and 8-, 9-, or 25-hydroxyhentriacontane-14,16-dione, respectively. The observed differences in epicuticular wax content between the different species or varieties of grasses are usually in the relative proportions of the classes or homologs or in the presence or absence of different isomers of a particular chemical. For example, spring wheat, *Triticum aestivum* L., varieties Selkirk and Manitou, differ in their relative proportions of unsaturated esters, free fatty acids, and β-diketones, while the hydroxy-β-diketone fraction in these varieties consists of a mixture of the 8- and 9-isomers, as opposed to the 25-isomer found in Durum wheat, *Triticum durum* Destf. (Tulloch and Hoffman, 1973). The identity of the chemicals that elicit oviposition by female Hessian flies is as yet unknown. TLC data indicated that fractions 3 and 6 contained large quantities of esters and alcohols, respectively. While compounds of these classes may be the actual oviposition stimulants, the crudeness of the techniques employed here does not preclude other classes of chemicals that either coelute with these classes or exist as trace (and undetected) components within these fractions as the actual oviposition stimulants.

Identification and quantification of the chemicals in the various grasses that elicit the behaviors and behavioral differences outlined in this paper will allow us greater insight into the chemical mechanisms involved in host selection be female Hessian flies.

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EFFECT OF FERULIC ACID ON GROWTH AND HYDROLYTIC ENZYME ACTIVITIES OF GERMINATING MAIZE SEEDS

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Abstract—Ferulic acid was tested for its effect on the growth of root and shoot, fresh and dry weights, and hydrolytic enzyme activities of germinating maize seeds. The results showed that root growth was inhibited more than shoot growth. A significant reduction in the activities of hydrolytic enzymes was also observed, which reflects a mechanism of action for the natural growth inhibitors, which may include other phenolic compounds.

Key Words—Allelopathy, ferulic acid, amylase, maltase, invertase, acid protease, acid phosphatase, *Zea mays*, growth, root, shoot.

INTRODUCTION

Allelopathy in agriculture is receiving considerable attention because of the allelochemicals that reduce the yields of crop plants due to phytotoxicity. This phenomenon is explored in the biological control of pests, diseases, and as a source of natural chemicals to solve other problems that arise in conventional agricultural crops. A number of potential growth inhibitory organic compounds are released into the environment in appreciable quantities via root exudates, leaf leachates, roots and other degrading plant residues, which include a wide range of phenolic acids such as *p*-hydroxybenzoic, *p*-hydroxycinnamic, ferulic, vanillic, and salicylic acids; alkaloids; and terpenoids (Glass, 1975a; Rice, 1987). Among the phenolic acids identified as allelochemicals, ferulic acid is a common one in leaf leachates and other degrading plant parts (Glass, 1975a; Rice, 1984).

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Several phenolic acids are known to modify growth and development of plants, including germination and early seedling growth (Al-Quadan et al., 1981; Boya et al., 1990; Chen and Klessig, 1991; Glass, 1973, 1975a,b; Holappa and Blum, 1991; Hoover et al., 1977; Kabi et al., 1981; Lyu and Blum, 1990; Lyu et al., 1990; McClure et al., 1977; Sharma et al., 1990; Sharma and Singh, 1987; Tayal and Sharma, 1985). However, the details of the biochemical mechanism through which a particular compound exerts a toxic effect on the growth of the plants are not known. One of the early events of seedling growth involves the mobilization of reserve food material, which supplies the required nutrients for the developing embryo and various other physiological processes.

Therefore, the aim of this work was to study the effect of ferulic acid on the early growth of maize seedlings by analyzing the activities of hydrolytic enzymes, viz., amylase, maltase, invertase, acid protease, and acid phosphatase. Any inhibition or promotion of activity of these enzymes will have a concomitantly major role in the early phase of the seedling growth. Ferulic acid was chosen as an allelochemical for our study because of its wide distribution and its toxicity to a variety of plant species.

METHODS AND MATERIALS

Maize seeds (*Zea mays* cv. Ganga-5) were purchased from the local market, surface sterilized with 5% sodium hypochlorite solution for 15 min, and washed with distilled water three times. The sterile seeds were soaked in water for 12 hr and placed on filter paper disks (9 × 9 cm) in polypropylene boxes (9 × 9 cm, inner dimensions). The seeds were treated with 10-ml solutions of 1–5 mM ferulic acid (Sigma Chemical Co., St. Louis, Missouri) for eight days using distilled water as the control. The seedlings grown in the light were used to determine the root and shoot length and fresh and dry weight.

Ferulic acid-treated seeds and distilled water controls grown in the dark (to exclude any light and photosynthetic effects on enzymes) were used for enzyme assays. On the specified days, the shoot and root portions of the germinated seedlings were removed, leaving behind the seed proper. Seed tissue (1 g), washed with water, was homogenized at 4°C in 5 ml of extraction buffer, viz., 50 mM sodium acetate buffer containing 20 mM calcium chloride, pH 4.8, for amylase; 50 mM sodium acetate buffer, pH 5.0, for maltase and invertase; 100 mM sodium acetate buffer, pH 5.0, containing 20 mM magnesium chloride for acid phosphatase; and 0.1 M acetate buffer containing 10 mM mercaptoethonol for proteases. The extract was centrifuged at 10,000*g* for 20 min, and the supernatant was used to determine enzyme activity. For day zero the 12-hr soaked seeds ground in the homogenization buffer. All the experiments were repeated six times.

Enzyme Assays. Amylase (E.C. 3.2.1.1) activity was determined by a modified method (Anonymous, 1979). Incubation of 1 ml of the reaction mixture containing 2 mg of amylopectin and 25 μl of enzyme extract in 50 mM acetate buffer with 20 mM calcium chloride, pH 4.8, in test tubes was carried out at 37°C for 10 min. One milliliter of dinitrosalicylic acid (Miller, 1959) was added to terminate the reaction and then placed in a boiling water bath for 5 min. The tubes were then cooled to room temperature, 2 ml of double-distilled water added, and absorbance was measured at 540 nm. Maltose was used as the standard (50–500 μg) reducing sugar. One unit of amylase activity was defined as the quantity of protein that liberates 1 μmol of reducing sugar.

Maltase (E.C. 3.2.1.20) activity was measured by the method of Yamasaki and Suzuki (1980). The reaction mixture, containing 0.1 ml of 0.1% maltose, 0.35 ml of 50 mM acetate buffer, pH 5.0, and 50 μl of enzyme extract in a final volume of 0.5 ml, was incubated at 37°C for 1 hr. The reaction was stopped by keeping the tubes in a boiling water bath for 5 min. The amount of glucose formed was determined using the arsenomolybdate reagent (Nelson et al., 1944). Maltase activity was represented as micromoles of glucose equivalents released per milligram of protein per hour.

Invertase (E.C. 3.2.1.26) activity was measured by the method of Billet et al. (1977): 1 ml of reaction mixture containing 100 mM sucrose, 50 μl enzyme extract in 100 mM acetate buffer, pH 5.0, was incubated at 35°C for 1 hr. The reaction was terminated by keeping the tubes for 5 min in a boiling water bath and the released glucose was measured using arsenomolybdate reagent (Nelson, 1944). Invertase activity was expressed as micromoles of glucose equivalents released per milligram of protein per hour.

Acid protease (E.C. 3.4.11.2) activity in the seeds was measured by the method of Mathur et al. (1988): 2 ml of assay mixture contained 100 μl of enzyme extract, 0.5 ml of 1% casein, and 0.1 M acetate buffer containing 10 mM mercaptoethonol, pH 5. The mixture was incubated for 1 hr at 40°C, and the reaction was stopped by adding 2 ml of 10% trichloroacetic acid, and the precipitated protein was removed by centrifugation. The supernatant was used for the estimation of amino acids by the method of Rosen (1957) using ninhydrin. Protease activity is expressed in micromoles of amino acids formed per hour per gram of fresh weight of tissue, using a standard curve for L-leucine.

Acid phosphatase (E.C. 3.1.3.2) activity in the seeds was measured by the method of Shinishi and Kato (1979). The reaction mixture, containing 0.1 M acetate buffer, pH 5, 20 mM magnesium chloride, 50 μl of enzyme extract, and 0.5 mM *p*-nitrophenylphosphate in a total volume of 1 ml, was incubated for 10 min at 30°C. The reaction was terminated by adding 2 ml of 1 N sodium hydroxide. The absorbance was measured at 400 nm. One unit of enzyme activity was defined as the amount of protein that liberates 1 μmol of *p*-nitrophenol per minute. A molar extinction coefficient of 18,000 is used in the calculations.

RESULTS

The germination bioassays indicate that ferulic acid (FA) suppressed the length of shoot (30–75%) and root (50–80%) (Figure 1A). Reduction in fresh weight of roots (38–78%) and shoots (35–60%) was also observed (Figure 1B). The dry weight was also decreased (roots 15–75%, and shoots 35–60%) (Figure 1B). The length, fresh weight, and dry weight of shoots and roots drastically decreased up to 2 mM FA treatment; thereafter, the reduction was not very steep with 3, 4, and 5 mM FA treatment.

Effect of Ferulic Acid on Activities of Hydrolytic Enzymes. Amylase activity increases gradually and reaches a maximum on the sixth day in control seeds. The 2 mM FA treatment had slightly inhibited the amylase activity on day 2 (4.5%), which was not significant. However, in other treatments, FA stimulated the activity up to 28%. FA after four days of treatment inhibited the activity by 10–22%. Significant reduction in the amylase activity was observed on days six and eight after FA treatment. Amylase activity was decreased by 30–50% and 18–45% on days six and eight, respectively (Table 1).

In the control seeds, maltase activity showed a biphasic increase on days 2 and 6. The maltase activity increased with 2 mM and 3 mM FA two days

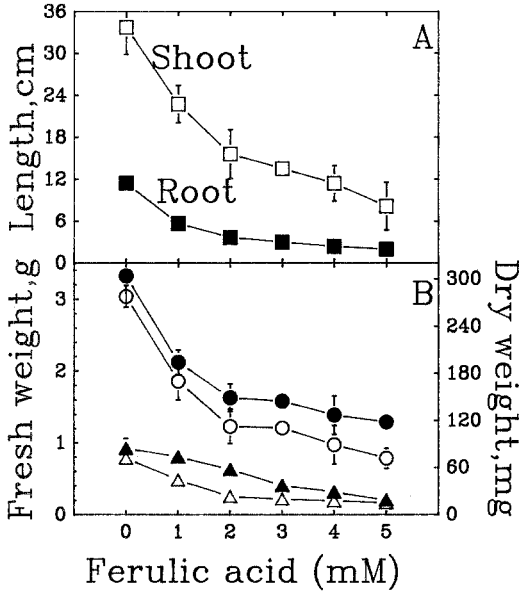

FIG. 1. (A) Effect of ferulic acid on the root and shoot length of *Zea mays*. (B) The fresh weight and dry weight of root and shoot. Circles = shoot, triangles = root. Hollow symbols = fresh weight, solid symbols = dry weight.

TABLE 1. EFFECT OF FERULIC ACID ON AMYLASE ACTIVITY IN MAIZE SEEDS

Ferulic acid treatment	Maltose (μmol/mg protein/min)			
	2 days	4 days	6 days	8 days
Control	1.54 ± 0.032	2.02 ± 0.04	2.68 ± 0.014	1.65 ± 0.065
1 mM	1.86 ± 0.053 (+20.77)[a]	1.72 ± 0.02 (−14.85)	1.89 ± 0.009 (−29.47)	1.23 ± 0.032 (−25.45)
2 mM	1.47 ± 0.045 (−4.57)	1.80 ± 0.02 (−10.89)	1.57 ± 0.017 (−41.41)	1.23 ± 0.016 (−25.45)
3 mM	1.97 ± 0.059 (+27.92)	1.58 ± 0.01 (−21.78)	1.43 ± 0.035 (−46.64)	1.35 ± 0.003 (−18.18)
4 mM	1.73 ± 0.048 (+12.33)	1.82 ± 0.03 (−9.90)	1.45 ± 0.054 (−45.89)	0.92 ± 0.028 (−44.24)
5 mM	1.66 ± 0.048 (+7.79)	1.71 ± 0.02 (−15.34)	1.36 ± 0.029 (−49.25)	1.05 ± 0.016 (−36.36)

[a]Figures in parentheses are percentages of increase (+) and decrease (−) over control.

after treatment by 6–8%. Its activity was further stimulated by 3 mM and 4 mM FA treatment to 16–33% on the fourth day. However, 5 mM FA inhibited activity by 58% on the fourth day. All concentrations of FA treatment inhibited activity on the sixth day (15–53%) and the eighth day (35–42%) (Table 2).

In untreated seeds, invertase activity was constant, with a slight rise in activity on the fourth day of germination that was not significant. FA at 1 mM and 2 mM two days after treatment increased activity by about 18%, and 5 mM FA decreased the activity by 33%. On the fourth day, 2 mM and 3 mM FA-treated seeds showed stimulation in invertase activity of 28% and 16%, respectively. However, 4 mM and 5 mM FA-treated seeds did not show significant change in activity compared to the control. By the sixth day, inhibition in invertase activity was prominent (16–42%), and its activity was suppressed by 19–47% with all FA treatments on the eighth day (Table 3).

In the control seeds, protease activity reached its peak on the sixth day. On treatment with FA (1–5 mM), 9–35% stimulation in activity was observed on day 2. However, there was a significant reduction in activity in FA-treated seeds at four, six and eight days. FA treatment after four days resulted in significant reduction in the activity by 19–57%. Similarly, after six and eight days, drastic inhibition in the activity (40–70% and 38–61%, respectively) was observed in FA-treated seeds (Table 4).

Phosphatase activity in untreated seeds was maximal (85 units) on the sixth day. On the second day only 1 mM and 2 mM FA treatments slightly stimulated

TABLE 2. EFFECT OF FERULIC ACID ON MALTASE ACTIVITY IN MAIZE SEEDS

Ferulic acid treatment	Glucose (μmol/mg protein/hr)			
	2 days	4 days	6 days	8 days
Control	9.72 ± 0.35	5.49 ± 0.39	7.53 ± 0.23	5.56 ± 0.30
1 mM	9.67 ± 0.70 (−0.51)[a]	6.77 ± 0.18 (+23.31)	4.96 ± 0.48 (−34.13)	3.53 ± 0.38 (−36.15)
2 mM	10.46 ± 0.74 (+7.61)	5.42 ± 0.16 (−1.27)	3.75 ± 0.36 (−50.19)	3.61 ± 0.17 (−35.07)
3 mM	10.34 ± 0.29 (+6.37)	7.32 ± 0.16 (+33.33)	6.37 ± 0.25 (−15.40)	3.24 ± 0.28 (−41.72)
4 mM	8.70 ± 0.20 (−10.49)	6.39 ± 0.33 (+16.39)	3.60 ± 0.31 (−52.19)	3.24 ± 0.11 (−41.72)
5 mM	8.53 ± 0.25 (−12.24)	8.71 ± 0.11 (−58.65)	3.57 ± 0.20 (−52.58)	2.52 ± 0.22 (−54.67)

[a]Figures in parentheses are percentages of increase (+) and decrease (−) over control.

TABLE 3. EFFECT OF FERULIC ACID ON INVERTASE ACTIVITY IN MAIZE SEEDS

Ferulic acid treatment	Glucose (μmol/mg protein/hr)			
	2 days	4 days	6 days	8 days
Control	5.34 ± 0.30	5.67 ± 0.31	5.53 ± 0.16	4.56 ± 0.19
1 mM	6.32 ± 0.19 (+18.35)[a]	5.59 ± 0.15 (−1.41)	4.65 ± 0.26 (−15.91)	2.83 ± 0.13 (−37.93)
2 mM	6.33 ± 0.29 (+18.53)	7.28 ± 0.32 (+28.39)	4.66 ± 0.23 (−15.73)	2.43 ± 0.17 (−46.71)
3 mM	5.33 ± 0.31 (−0.18)	6.55 ± 0.27 (+15.52)	4.29 ± 0.28 (−22.42)	3.70 ± 0.22 (−18.85)
4 mM	5.41 ± 0.27 (+1.31)	5.33 ± 0.30 (−5.99)	4.62 ± 0.39 (−16.45)	3.23 ± 0.19 (−29.16)
5 mM	3.56 ± 0.40 (−33.33)	5.74 ± 0.16 (−1.23)	3.22 ± 0.15 (−41.77)	2.55 ± 0.30 (−44.07)

[a]Figures in parentheses are percentages of increase (+) and decrease (−) over control.

TABLE 4. EFFECT OF FERULIC ACID ON PROTEASE ACTIVITY IN MAIZE SEEDS

Ferulic acid treatment	Amino acids (μmol/g fresh weight/hr)			
	2 days	4 days	6 days	8 days
Control	7.19 ± 0.11	11.96 ± 0.25	15.58 ± 0.21	9.74 ± 0.88
1 mM	7.22 ± 0.29 (+0.38)[a]	7.74 ± 0.18 (−35.28)	9.31 ± 0.12 (−40.24)	6.01 ± 0.45 (−38.29)
2 mM	8.96 ± 0.13 (+24.58)	7.70 ± 0.29 (−35.61)	8.69 ± 0.77 (−44.22)	5.77 ± 0.13 (−40.75)
3 mM	7.89 ± 0.27 (+9.70)	5.60 ± 0.13 (−53.17)	5.49 ± 0.12 (−64.76)	4.72 ± 0.16 (−51.54)
4 mM	6.68 ± 0.10 (−7.11)	9.69 ± 0.91 (−18.97)	4.64 ± 0.10 (−70.21)	3.54 ± 0.70 (−63.65)
5 mM	9.72 ± 0.38 (+35.55)	5.12 ± 0.15 (−57.19)	4.64 ± 0.81 (−70.21)	3.75 ± 0.59 (−61.49)

[a] Figures in parentheses are percentages of increase (+) and decrease (−) over control.

its activity (4–15%) and 3–5 mM FA inhibited activity (10–27%). On the fourth day, except in 1 mM FA-treated seeds, there was a linear inhibition in the activity of acid phosphatase with 2–5 mM FA (13–30%). Drastic inhibition in the activity was observed with all the FA treatments on days six and eight (17–44% and 23–48%, respectively) (Table 5).

DISCUSSION

The results on the growth of the FA-treated maize seedlings show a considerable reduction in the growth, which conformed with the previous reports (Blum et al., 1984; Blum and Dalton, 1985; Einhellig and Rasmussen, 1979). In mature maize seeds, starch is the important reserve food material. The degradation of this to glucose via the amylolytic pathway involves three principal enzymes, i.e., amylase, maltase, and invertase (Nomura et al., 1969).

As can be seen in Table 1, the reduction in amylase activity by the action of FA will lead to a reduction in the production of maltose. As maltose is the substrate for maltase, the reduction in maltose levels may lead to alteration in the levels of maltase during germination. FA treatment resulted in the inhibition of both amylase and maltase, which would result in a reduced supply of glucose from the starch via the amylolytic pathway, which may influence several physiological processes, including the respiration, growth, and development of the

TABLE 5. EFFECT OF FERULIC ACID ON ACID PHOSPHATASE ACTIVITY IN MAIZE SEEDS

Ferulic acid treatment	*p*-Nitrophenol (μmol/mg protein/min)			
	2 days	4 days	6 days	8 days
Control	57.78 ± 2.46	67.99 ± 4.52	85.21 ± 3.08	74.84 ± 11.8
1 mM	60.30 ± 5.96 (+4.36)[a]	68.89 ± 7.67 (+1.32)	70.85 ± 4.89 (−16.85)	57.81 ± 5.65 (−22.75)
2 mM	66.46 ± 8.75 (+15.02)	58.86 ± 8.32 (−13.42)	65.71 ± 5.95 (−22.88)	58.35 ± 2.58 (−22.03)
3 mM	51.59 ± 6.74 (−10.71)	54.77 ± 2.04 (−19.44)	55.59 ± 7.43 (−34.76)	46.60 ± 5.80 (−37.73)
4 mM	42.08 ± 5.12 (−27.17)	52.81 ± 3.55 (−22.32)	55.84 ± 1.06 (−34.46)	39.9 ± 1.86 (−46.67)
5 mM	57.10 ± 4.32 (−1.17)	46.92 ± 4.92 (−30.97)	47.62 ± 4.79 (−44.11)	38.97 ± 2.40 (−47.92)

[a]Figures in parentheses are percentages of increase (+) and decrease (−) over control.

seedling. Similar changes in amylase activity were observed with other phenolic compounds such as coumarin and tannic acid (Jacobson and Corcoran, 1977; Kabi et al., 1981). The initial rise in maltase activity in FA-treated seeds might be due to the supply of the maltose produced in the initial stages of germination.

Sucrose is the main transportable sugar from endosperm to the embryonic tissue. The breakdown of sucrose into glucose and fructose is mediated by invertase. Suppression in the activity of invertase by FA treatment only at later stages of germination suggests further interference of FA with carbohydrate metabolism.

At the early stages of seed germination, the supply of smaller peptides and amino acids that are essential for a variety of metabolic processes are produced by the action of proteases on the reserve proteins. FA inhibited the protease activity, and the effect will obviously reflect on the growth and development of seedlings. Compared to carbohydrate metabolism, the protein degradation is conspicuously affected at the early stages of germination (Table 4).

The reserve phosphates in the seeds are mobilized through phosphatases. Interference in phosphate metabolism due to FA may further bring about deleterious changes in the growth of the plants, as phosphorus is involved in various energy-dependent and energy-mediated processes, such as ATPases, and cell components such as phospholipid and nucleotide synthesis, respiration, etc. (Beweley and Black, 1985).

The results obtained in the present study support the hypothesis that FA,

through its deleterious effect on the activities of amylase, maltase, protease, and phosphatase, might interfere with carbohydrate, protein, and phosphate mobilization during germination, exerting a direct or indirect influence on various physiological and biochemical processes such as respiration and nitrogen and carbohydrate metabolism, etc. The synergistic effects of the inhibition of these hydrolytic enzymes might account for the reduction in growth of the maize seedlings that has been observed with FA treatment in this investigation.

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DEFENSIVE ROLE OF *Allium* SULFUR COMPOUNDS FOR LEEK MOTH *Acrolepiopsis assectella* Z. (LEPIDOPTERA) AGAINST GENERALIST PREDATORS

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Abstract—It has been shown previously that sulfur volatiles produced by *Allium* plants affect the behavior of their specialist phytophages and of their specialist entomophages. The action of these compounds in protecting the leek moth *Acrolepiopsis assectella* against generalist entomophages was studied in comparison to the proposed original defensive role of these compounds against generalist herbivorous insects. Two ants species, *Formica selysi* and *F. fusca*, were used as generalist predators. Six behavioral criteria of the predatory behavior of the ants were studied in presence of the last-instar caterpillars (C_5). C_5 reared on artificial diets with or without leek components were tested, as well as C_5 soaked in frass of leek-reared caterpillars or disulfide solutions. In addition, the response of the ants to pure chemicals found in leek was studied using honey solutions with or without sulfur compounds. The sulfur allelochemicals of *Allium* plants have a negative action on predatory ants. Interestingly, the nonvolatile precursors of sulfur volatiles of *Allium* plants seem to have a protective role for their phytophagous insects against generalist entomophages.

Key Words—Ants, defense, phytophage, predation, sulfur allelochemicals, *Allium*, *Formica fusca*, *Formica selysi*, Hymenoptera, Formicidae, *Acrolepiopsis assectella*, Lepidoptera, Acrolepiidae.

INTRODUCTION

Plants of the genus *Allium* produce sulfur amino acids, *S*-alk(en)yl-L-cysteine sulfoxides, precursors of thiosulfinates and volatile alkyl disulfides (Auger et

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al., 1989a), which are given off when the leaves are broken (Virtanen, 1965). These volatile compounds are perceived by the specialist phytophagous insect *Acrolepiopsis assectella* (Hyponomeutoïdea), attracting adults and caterpillars, while precursors of these volatiles are phagostimulant for them (Lecomte et al., 1987; Al Rouz and Thibout, 1988, 1989). The disulfides are very abundant in the frass and are also perceived by the specialist parasitoid *Diadromus pulchellus* (Ichneumonidae), where they modify locomotor behavior and favor the search for host chrysalids (Lecomte and Thibout, 1984; Thibout et al., 1988; Auger et al., 1989b). The primary defensive role of allelochemical compounds put forth in certain hypotheses (Fraenkel, 1959; Rosenthal, 1988) has thus been diverted by some specialist insects to their benefit, regardless of the trophic level. Even though one of the raisons d'être of these allelochemicals is a protective role, particularly against insects, the phytophagous insect that has successfully diverted them should, in turn, also be able to be protected from generalist entomophagous species. This has, in fact, been described in several cases (Aplin et al., 1975; Pasteels et al., 1988; Belofsky et al., 1989; Nishida and Fukami, 1989), but it is perhaps general in phytophagous insects feeding on allelochemical-rich plants.

We thus sought a repulsive or potentially deterrent action of *Allium* allelochemicals, more particularly those of the leek *A. porrum*, on the predatory activity of ants when encountering leek moth caterpillars. These hymenopterans were chosen because a large number of species are generalist predators (Finnegan, 1974), whose role in maintaining insect populations is well known (Sterling et al., 1979). In addition, they had been used previously to test various allelochemicals (Eisner et al., 1980; Godzinska, 1986; Bowers and Larin, 1989). Furthermore, one of us had witnessed the attack on *A. assectella* chrysalids by ants, of which several species are often observed on leeks during the summer.

METHODS AND MATERIALS

Phytophagous Insect. Caterpillars from the last instar (C_5) were obtained from three sources. The first was on the host plant, leeks (C_5 L). Photoperiod was 18:6 hr light–dark with a synchronous 25°C/18°C thermoperiod, with relative humidity varying from 50% to 70%. The second was on artificial medium, according to the technique of Arnault (1979) in 14:10 hr light–dark at constant 25°C and 70% relative humidity. The medium contained about 10% (dry weight) powdered leek leaves (C_5 M+L). The third source was identical to the second except that the medium contained no constituent from the host plant (C_5 M). Strain L was renewed every summer from caterpillars gathered in leek fields. Strain M+L has been raised in the laboratory for the past 15 years at about one generation per month. Strain M is formed from M+L neonatal caterpillars whenever necessary.

Predators. Formica selysi (Bondroit) and *F. fusca* (L.) (Formicidae) were used. Colonies were maintained in the same conditions as C_5 L in artificial ant nests composed of a cylindrical tube (5 cm × 8 cm in diameter) in darkness at high relative humidity. An 80-cm-long, 0.5-cm-diameter tube connected the nest to a 12 × 18-cm foraging area, open to air and containing water. During experimental periods, the colonies were given honey water and cut *Tenebrio molitor* larvae once a week. Colonies were composed of 300–400 ants.

Preliminary Experiments. Preliminary experiments were carried out with ten *F. fusca* or *F. selysi* removed from the foraging area of the colonies. They were placed in a 7-cm-diameter Petri dish containing one *A. assectella* C_5 L (10 replicates) and one C_5 of *Ephestia kuehniella* (10 replicates) or *Plodia interpunctella* (five replicates). The latter two Lepidoptera were raised on wheat flour and on corn flour + glycerol, respectively, in 18:6 hr light–dark and 25°C/18°C. These two species were chosen because they have nearly the same size as *A. assectella* (C_5 length = 1 cm) and because they do not eat leek. All observations were made in photophase at 25°C and lasted for 1 hr. The last observation was made 24 hr later. Results obtained with this type of experiment led to a modification in the procedure.

Behavior of Ants in Presence of A. assectella *C_5 of Different Food Origins.* One leek moth C_5 was deposited in the foraging area of an ant colony, and the behavior of the ants present in the area was observed for 30 min. Thirty minutes after the end of the observation period, a second C_5 of a different food origin was deposited and also observed for 30 min. The following behavioral criteria were noted: (1) time before the first attack, (2) time during which C_5 remained in the foraging area, (3) time required to transport the C_5 into the tube leading to the nest, (4) number of C_5 brought into the nest, (5) number of ants in contact with the C_5 every 30 sec, and (6) relative frequency of antennal cleaning among the ants. Three categories were distinguished for the last criterion: 0 = no cleaning, + = several cleanings, ++ = numerous and frequent cleanings.

With *F. selysi*, an initial experiment compared their behavior in the presence of C_5 M and C_5 M+L and a second in the presence of C_5 L and C_5 M+L. In the case of *F. fusca*, three groups of C_5 M were compared, immersed 10 min before the experiments in: (1) an extract of frass (C_5 F, 0.25 g/ml of 10% methanol), (2) 0.1% dipropyl disulfide (DSP_2) in 10% methanol (C_5 D), and (3) in 10% methanol alone. In the morning and afternoon, two or three observations were made, alternating the C_5 of different origins.

Behavior of Ants in Presence of Honey with Sulfur Chemicals. The last type of experiment was used to test the repulsive or deterrent action of precursor amino acids and volatile disulfides towards the ants. A control drop of honey (5% in water) was deposited in the foraging area of the *F. fusca* colony. A second drop of honey was diluted in either an aqueous solution of DSP_2 at 10^{-3} (solution D, five replicates) or an aqueous solution of *S*-propyl cysteine sulfoxide

(PCSO) at 10^{-3} (solution P, eight replicates) and placed at 2 cm from the control. The latter compound is a sulfur amino acid produced by leeks and the precursor of DSP_2. The quantity of precursor in one fresh *Allium* is about 10^{-3} (Lancaster et al., 1984). The number of ants in contact with each drop of honey was noted every 30 sec.

Statistical analyses were carried out with χ^2 or the t test for comparing means two by two.

RESULTS

Preliminary Experiments. The preliminary experiments provided data on the number of attacks on the C_5 during the hour of observation and the number of C_5 eaten by the ants 24 hr after the beginning of the experiment (Table 1). Despite the small number of data, the C_5 of *A. assectella* raised on leeks were attacked less and eaten less frequently by both species of the ants used than the C_5 of the other two Lepidoptera (Table 1). The eaten C_5 distribution of the three moth species is not statistically homogeneous whatever the predatory ant, *F. fusca* ($\chi^2 = 14.856$; $df = 4$) or *F. selysi* ($\chi^2 = 10.059$; $df = 4$). An analogous result was also obtained comparing the *F. fusca* attacks on the three moth C^5 ($\chi^2 = 8.944$; $df = 2$), but the differences are not significant for *F. selysi* attacks ($\chi^2 = 3.860$; $df = 2$).

Owing to the behavioral differences in the C_5 of the different prey species—more rapid movements by *E. kuehniella*, agility in climbing the vertical walls by *P. interpunctella*—and, above all, the hard integument of these two species, the results of these experiments were used to define experimental conditions and to choose the behavioral criteria used in subsequent experiments.

TABLE 1. PRELIMINARY OBSERVATIONS ON PREDATION OF THREE MOTH SPECIES BY *F. fusca* AND *F. selysi*[a]

			Attack		Eaten C_5 (%)		
		C_5 (N)	Weak	High	0	0–50	>50
F. fusca	*A. assectella*	10	8	2	4	5	1
	E. kuehniella	10	4	6	0	1	9
	P. interpunctella	5	0	5	0	2	3
F. selysi	*A. assectella*	10	5	5	2	6	2
	E. kuehniella	10	3	7	1	2	7
	P. interpunctella	5	0	5	0	0	5

[a] Caterpillars can be weakly or highly attacked and can be highly (% > 50), partly (0 < % < 50) or not (% = 0) eaten.

Behavior of F. selysi *in Presence of C_5 M or C_5 M+L.* Both types of *A. assectella* C_5 were successively placed in the foraging area 1 hr apart. The analysis had to take the presentation order into account, since it could have modified the behavior of the ants due to experience. This order was systematically inverted in each replicate. One C_5 M was added first (C_5 M1) in one experiment, followed by a C_5 M+L (C_5 M+L2), while in the following series a C_5 M+L was added first (C_5 M+L1), followed by a C_5 M2.

Comparing the results with C_5 M1 and M2 showed no difference, regardless of the behavioral criterion chosen (Table 2), and the data were thus pooled. The same was true for C_5 M+L1 and M+L2, where only the mean number of ants in contact with the C_5 per 30 sec was higher with C_5 M+L1 than with C_5 M+L2 (Table 2).

Once the data were pooled, the comparison of ants with C_5 M or C_5 M+L showed significant differences for all the criteria observed, except for the time required by the ants to attack the C_5. The times spent by C_5 in the foraging area or that of transport of C_5 to the tube were all longer with C_5 M+L. Whereas all the C_5 M were taken into the nest, only a small number of C_5 M+L was taken in. In this context, we often observed a forager trying to advance to the nest with a C_5 M+L, while another ant recently emerged from the nest was pulling it back to the foraging area. The number of ants paying attention to the C_5 M+L in the area was lower than that of ants interested in C_5 M. Finally, antennal cleanings in ants with C_5 M were infrequent and were manifested by only a few insects, while they were very frequent in a large number of ants when they were in presence of C_5 M+L (Table 2).

Behavior of F. selysi *in the Presence of C_5 M+L or C_5 L.* As above, there was no difference between the C_5 placed in the foraging area first and those placed second. Both types of C_5 were thus similar (Table 3). With the exception of the time taken for the ants to attack the C_5 being higher with C_5 L than with C_5 M+L, there was no significant difference in the behavior of the ants in the presence of C_5 L and that of the ants in the presence of C_5 M+L, regardless of the criterion used (Table 3).

In addition, data obtained with C_5 M+L in the previous experiment (Table 2) are very similar to those obtained here with the C_5 M+L (Table 3). Even though statistical comparison was impossible because both experiments were not done at the same time, it is clearly seen (Table 3) that only the number of C_5 M+L brought into the nest varied from one experiment to the other.

Behavior of F. fusca *in the Presence of C_5 M, C_5 F, or C_5 D.* In contrast to the prior two experiments, the order of C_5 presentation was not taken into account, since the number of combinations was excessive. There was a very high similarity between the behavior of the ants in presence of C_5 F and of C_5 D (Table 4). In contrast, the ants behaved very differently with C_5 M, and the data (Table 4) are reminiscent of those in Table 2. Only the mean number of ants in contact with the C_5 every 30 sec was the same in the three cases.

TABLE 2. COMPARISON OF BEHAVIORAL CRITERIA OF *F. selysi* PREDATION ON *A. assectella* CATERPILLARS REARED ON ARTIFICIAL MEDIUM WITH (C_5 M+L) OR WITHOUT (C_5 M) LEEK POWDER[a]

	C_5 (*N*)	Time before attack	Time in area	Transport time	C_5 at nest (%)	Ants contacting C_5/30 sec	Antennal cleaning (%)		
							0	+	++
C_5 M1	22	0.33 ± 0.07a	8.13 ± 1.20a	3.22 ± 0.68a	100a	1.8 ± 0.2a	77.3	22.7	0
C_5 M2	19	0.73 ± 0.43a	8.53 ± 1.13a	4.45 ± 1.10a	100a	1.5 ± 0.1ba	78.9	21.1	0
C_5 M+L1	19	1.03 ± 0.28b	23.07 ± 2.08b	11.77 ± 2.00b	15.8b	1.3 ± 0.1b	0	26.3	73.7
C_5 M+L2	22	0.97 ± 0.33ab	17.70 ± 2.13b	15.92 ± 2.27b	9.1b	0.9 ± 0.1c	4.6	27.2	68.2
C_5 M	41	0.53 ± 0.20a	8.32 ± 0.82a	3.47 ± 0.63a	100a	1.6 ± 0.1a	78.0	21.9	0.0
C_5 M+L	41	1.00 ± 0.22a	19.95 ± 1.55b	13.75 ± 1.43b	12.2b	1.1 ± 0.1b	2.4	26.8	70.7

[a]Times given in minutes. Data followed by the same letter are not statistically different at $P = 0.05$. Means are given with their SEM.

TABLE 3. COMPARISON OF BEHAVIORAL CRITERIA OF *F. selysi* PREDATION ON *A. assectella* CATERPILLARS REARED ON LEEK PLANT (C_5 L) OR ON ARTIFICIAL MEDIUM WITH LEEK POWDER (C_5 M+L)[a]

	C_5 (*N*)	Time before attack	Time in area	Transport time	C_5 at nest (%)	Ants contacting C_5/30 sec	Antennal cleaning (%)		
							0	+	++
C_5 M+L1	15	1.42 ± 0.17ba	11.53 ± 2.57a	12.87 ± 2.02a	66.7a	1.0 ± 0.1a	0	13.3	86.7
C_5 M+L2	15	1.17 ± 0.17a	17.05 ± 2.22a	9.20 ± 1.70a	66.7a	1.1 ± 0.05a	0	26.7	73.3
C_5 L1	15	1.92 ± 0.32b	14.52 ± 2.07a	11.48 ± 2.13a	60.0a	1.2 ± 0.1a	0	20.0	80.0
C_5 L2	15	1.93 ± 0.30b	16.50 ± 2.18a	10.38 ± 1.80a	66.7a	1.3 ± 0.1a	0	20.0	80.0
C_5 M+L	30	1.28 + 0.12a	14.28 ± 1.73a	10.97 ± 1.33a	66.7a	1.0 ± 0.05a	0	20.0	80.0
C_5 L	30	1.93 ± 0.22b	15.52 ± 1.48a	10.73 ± 1.42a	63.3a	1.2 ± 0.1a	0	20.0	80.0

[a] For further information refer to Table 2.

TABLE 4. COMPARISON OF BEHAVIORAL CRITERIA OF *F. fusca* PREDATION ON *A. assectella* CATERPILLARS REARED ON ARTIFICAL MEDIUM WITHOUT LEEK POWDER SOAKED IN FRASS (C_5 F) SOLUTION OR DISULFIDE (C_5 D) SOLUTION[a]

	C_5 (*N*)	Time before attack	Time in area	Transport time	C_5 at nest (%)	Ants contacting C_5/30 sec	Antennal cleaning (%)		
							0	+	++
C_5 M	30	0.32 ± 0.13a	5.27 ± 0.43a	1.97 ± 0.22a	100a	1.4 ± 0.1a	96.7	3.3	0
C_5 F	30	0.85 ± 0.15b	12.28 ± 1.03b	5.07 ± 1.03b	96.7ab	1.2 ± 0.1a	0	26.7	73.3
C_5 D	30	0.73 ± 0.13b	14.37 ± 1.42b	5.67 ± 0.90b	83.3b	1.1 ± 0.05a	0	20.0	80.0

[a]For further information refer to Table 2.

Although the data in Tables 3 and 4 cannot be compared statistically, there are certain similarities and occasionally differences in the behavior of the ants in the presence of C_5 L or C_5 M+L and those in the presence of C_5 F or C_5 D. The time before the first attack, the time spent in the area, the mean number of ants in contact with the C_5, or the frequency of antennal cleanings are similar with the four types of C_5. The transport times in the tube, however, were much lower with C_5 F and C_5 D, while the numbers of C_5 brought into the nest were lower with C_5 L and C_5 M+L. The effects of frass and of DSP_2 are apparently less persistent.

Behavior of F. fusca *in Presence of Honey with* DSP_2 *or PCSO.* A honey solution became less attractive and phagostimulating for the ants when DSP_2 was added (Figure 1). In five experiments done at irregular time intervals, more ants were counted on pure honey than on honey with DSP_2 in four cases. Among a total of 1369 ants observed eating, 61.8% were on pure honey in 30 min ($P < 0.05$), and this percentage was even higher in 15 min at 65.1% for 764 ants ($P < 0.001$).

When PCSO was added to the honey, the results were comparable to those obtained with DSP_2. Among 610 ants observed during eating, 68.0% were on pure honey ($P < 0.001$). The observations in this experiment were carried out at more regular intervals than in the preceding one, and so changes in responses were analyzed as a function of time (Figure 2). Initial observations strongly suggested that *F. fusca* did not initially differentiate between the two food sources, but they gradually "learned" to recognize PCSO and avoid it, particularly if two observations were separated only by a few hours.

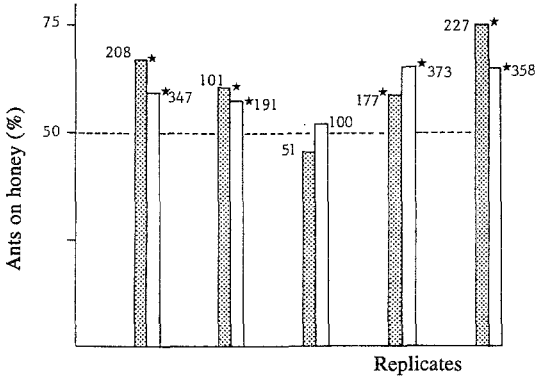

FIG. 1. Percentage of *F. fusca* feeding on pure honey in comparison to the total number of ants feeding (pure honey + honey + DSP_2). The asterisks indicate that the percentage is 50% different in $P = 0.05$ (χ^2). The numbers indicate the total number of ants. Gray columns: observations during 15 min, open columns: observations during 30 min.

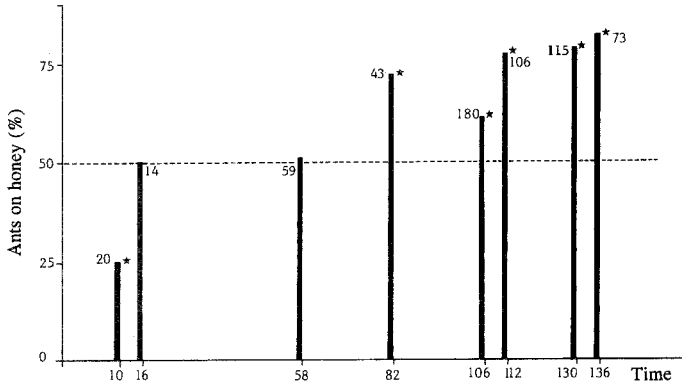

FIG. 2. Percentage according to time (in hours) of *F. fusca* feeding on pure honey in comparison to the total number of ants feeding (pure honey + honey + PCSO). See further explanations in Figure 1 legend.

DISCUSSION

A comparison between the two predatory species used would seem to indicate that *F. selysi* and *F. fusca* show a very similar predatory behavior, because the data in presence of C_5 M are analogous (Tables 2 and 4). On the other hand, in the experiments with honey, *F. fusca* changes its behavior over time, whereas *F. selysi* does not. As a large number of publications have shown, ants can modify their behavior as a function of acquired experience (Alloway, 1972; Bowers and Larin, 1989; Godzinska and Lenoir, 1990; Hölldobler and Wilson, 1990). When replicates are conducted at relatively short times, *F. fusca* seems to "learn" to avoid PCSO.

As shown previously, the predatory behavior of ants differs in the presence of C_5 M and C_5 M+L or C_5 L. Compounds arising from the host plant, the leek in this case, are thus perceived by the ants and lower their predatory efficiency by increasing the times of attack and decreasing the number of C_5 brought into the nest.

In preliminary experiments, the ants manifested a preference for caterpillars that had not been exposed to *Allium* compounds. Comparison with results obtained with C_5 D or C_5 F and C_5 M+L or C_5 L indicates that the values of behavioral criteria observed at the beginning of the experiment are relatively similar—time before the first attack, time spent in the hunting area—while the values of behavioral criteria observed at the end of the experiment are different—transport time in the tube, number of C_5 brought into the nest. Predatory behavior is thus more intense at the end of the experiment in the presence of C_5 F and D, while it is lower in the presence of C_5 L and M+L. The DSP_2 present

on the C_5 F and C_5 D could be responsible for this behavioral difference. This compound is volatile and its concentration on the surface of the C_5 automatically decreases with time. The volatility of leek constituents that disturb ant attacks must thus be low or nonexistent, and they may be precursor sulfur amino acids. This hypothesis is apparently confirmed by the results obtained when studying the feeding behavior of ants in the presence of honey. The phagostimulating power of honey was decreased by the presence of nonvolatile PCSO and also by DSP_2, but in this case the effect was more pronounced in the first quarter hour.

If PCSO is indeed the (or one of the) compound(s) in the leek that disturbs predation exerted by the two ant species, it would be interesting to elucidate the metabolism of these precursors in *A. assectella* caterpillars and determine if they are present in the cuticle, hemolymph, fat body, or only in the digestive tube. Are there sequestration phenomena involved (Duffey, 1980) or is there just a simple contamination by contact?

This example of the protection of a phytophagous insect against predation, even if in the present case it is a phytophagous species already specialized, tends to reinforce the hypothesis of Bernays and coworkers, according to which generalist predators would cause a selection pressure tending to limit the diversity of plant hosts among their phytophagous prey (Bernays, 1988, 1989; Bernays and Cornelius, 1989). Individuals eating plants that are the richest in allelochemical compounds would be those undergoing the lowest selection pressure by the generalist predators. This phenomenon would be all the more interesting in *A. assectella* since it was relatively unpredictable. Caterpillars are well protected physically since they develop in mines they dig in the parenchyma of host plant leaves.

Chemical protection related to the host plant towards generalist predators in this phytophagous species could nevertheless have an effect during the search for a metamorphosing site and while the cocoon is being spun.

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SEX PHEROMONES OF MALE YELLOWFIN BAIKAL SCULPIN (*Cottocomephorus grewingki*): ISOLATION AND CHEMICAL STUDIES

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Abstract—Three components of the male yellowfin Baikal sculpin pheromonal signal have been isolated from urine by diethyl ether extraction, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC). Using mass spectrometry, we have identified two of them as testosterone (T) and 11β-hydroxytestosterone (11HT). These steroids are synthesized in testes during full spermatogenesis, and they are excreted in male urine with milt. The third component is not a steroid. It is more likely to be a polyene alcohol (farnesol). 2*Z*,6*E*-Farnesol possesses behavioral activity.

Key Words—Teleost fish, yellowfin Baikal sculpin, Cottocomephorus grewingki, sex pheromones, testosterone, 11β-hydroxytestosterone, farnesol.

INTRODUCTION

Identification (or close characterization) of male pheromone has been reported for three teleost species: black goby (Colombo et al., 1989), zebrafish (Van den Hurk et al., 1987), and African catfish (Resink et al., 1989). In all cases the proposed pheromones are steroid glucuronides from the testes or seminal vesicles. Nonsteroids were not studied.

The yellowfin Baikal sculpin has a developed system of chemical communication that determines a complex of reproductive behaviors. It includes the

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preparation of a "nest" by the male, territory guarding, attracting partners, stimulation of maturation, and care of the young. The source of the male sex pheromones is a spawning male's urine (U). Introduction of aqueous urine solution (1×10^{-8}, vol. %) into a Y-maze aquarium with spawning females elicited the whole complex of stereotype elements of sexual behavior (Dmitrieva and Ostroumov, 1986).

Thus the presence of a male pheromonal signal, which determines female sexual behavior, sexual dimorphism, and organ source of the male sex pheromones make the yellowfin Baikal sculpin an ideal model for identification of sex pheromones. Our strategy to identify the pheromones was based on fractionation of the mixture containing signal substances; isolation of fractions preserving behavioral activity, with subsequent substance identification.

METHODS AND MATERIALS

Spawning fish were caught in the South Baikal and brought to the Irkutsk University Biostation (Bay Bolshie Koty, Lake Baikal). Fish were put into 600-liter pools with a constant inflow of running lake water. After a two-day acclimatization period, the urinary bladder was isolated from males and its contents were collected in ampules and frozen. The urine (0.1–1 ml) was taken from each individual and a total of 430 ml was collected from fish.

Separation. Silica gel plates (5 × 20 cm) with a 0.25-mm layer of sorbent (Merck) were used for TLC. HPLC was carried out on a microanalytical chromatograph, Milichrom (USSR), with 62 × 2-mm column (Nucleosil C_{-18}, 5 μm, Macherey Nagel). Detection was carried out at 220, 250, and 280 nm. All solvents and chemicals were of analytical grade.

Isolation of C_{19} Steroids from Testes. The spawning male testes (16 g) were homogenized on ice with (3 × 50 ml) of ethanol–diethyl ether mixture (3:1, v/v). The combined homogenates were centrifuged. Supernatant was evaporated in vacuum at 30°C. The residue was dissolved in 30 ml of water. Steroids from the water–ethanol solution were extracted by ethyl acetate (3 × 60 ml). The combined extracts were washed with 50 ml of 0.1 N NaOH and 50 ml of water during 2 min and evaporated. Then the residue was dissolved in 10 ml of 70% methanol. The purification from lipids was carried out by cold precipitation at −20°C during 24 hr. Methylene chloride (CH_2Cl_2) was then added (4 × 50 ml) to the extract of the free steroids from the water–methanol solution. The combined extracts were evaporated and subjected to HPLC.

Mass Spectrometry. Finnigan MAT-4615 mass spectrometer with a data processing system (Incos-2000) was used. The ionization potential was 70 eV. The reagent with chemical ionization was NH_3. Input port temperature was 40°C and was increased 1.5 min after input to 250°C for the 2.5.6 component and

to 500°C for 2.7.3 and 2.7.4 components. The temperature of the ionization chamber was 150°C.

Bioassays. The behavioral activity of the fractions was studied using spawning females during the light period after two days of adaptation. A two-choice preference test tank (40 liter; rate of flow: 2–3 liters/min) was used. Lake water was used in the tanks, and the temperature corresponded to the current lake temperature. The aqueous fractions (100 ml, 1×10^{-8} mg/liter) were added to one of the two compartments of tanks, and the behavior was observed during 30 min. This included the whole complex of stereotypical elements of female sexual behavior: female "testing motion" (periodic wide opening of mouth, rotating movements of thoracic fins and turning towards a stimuli source); attracting towards a stimuli compartment; "courting dance" (snakelike body movements in the opposite direction, head bending towards to bottom); movements imitating entering a "nest" (head bending towards to bottom and short fast movements forward with short standstills); imitation of a pose of laying oocytes (female attempt to turn upside down). We put these elements in the scale of criteria of sexual behavior of females (Dmitrieva et al., 1988). The procedure and results of behavioral testing were described in detail in our previous articles (Dmitrieva and Ostroumov, 1986; Dmitrieva et al., 1988, 1991; Katsel, 1990).

RESULTS AND DISCUSSION

We carried out the progressive isolation of the pheromonal signal from the urine of the male yellowfin Baikal sculpin using the procedures outlined in Figure 1. The first stage included urine fractionation by consecutive extraction with a series of organic solvents: hexane, diethyl ether, chloroform, and ethyl acetate. The extracts were dried in vacuum, diluted in water, and tested for behavioral activity. The pheromonal signal was present in fractions 2 and 3 (Figure 1). In the second stage, active fractions were studied by TLC using benzene–chloroform (1:2, v/v). Sulfuric acid, 10% solution of phosphomolybdic acid in ethanol, saturated $SbCl_3$ solution in chloroform, iodine vapor, and UV light (250 nm) were used for detection. We found that fractions 2 and 3 contained multiple components. To find out which component possessed behavioral activity, chromatograms were divided into zones as described in an earlier paper (Dmitrieva et al., 1988). The substances were eluted from the plates using methanol, dried, diluted in the water to the same concentration as in the previous fractionation stage, and bioassayed. It was established that zones 2.5 and 2.7 in fraction 2 and the same zones in fraction 3, containing substances with $R_f = 0.35$ and 0.04, as well as starting components possessed behavioral activity (Dmitrieva et al., 1988). In the third stage, the isolated zones 2.5 and

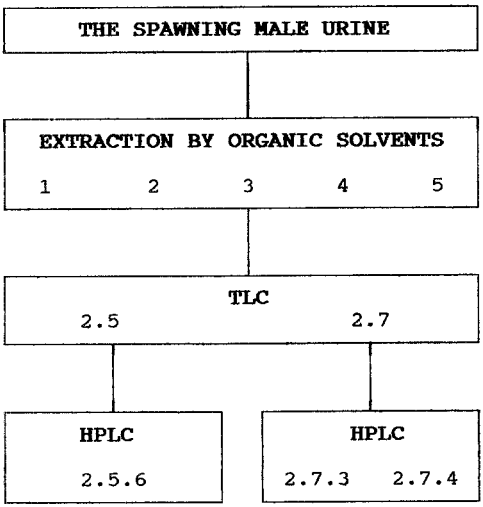

FIG. 1. Diagram of isolation of male sex pheromones. Fractions: 1, hexane; 2, diethyl ether; 3, chloroform; 4, ethyl acetate; 5, water residue.

2.7 were analyzed by HPLC. Zone 2.5 was fractionated in a linear gradient of methanol in water (40–100%); time of gradient formation = 12 min, elution rate = 100 μl/min. Eight components were discovered, which were bioassayed as in previous stages. We found that component 2.5.6 (retention time: 13 min 45 sec) had pheromonal activity (Katsel, 1990). This component has also been found in fraction 3, but in much lower concentration. Zone 2.7 was fractionated in a linear gradient of acetonitrile in water (30–100%); time of gradient formation = 14 min; elution rate = 100 μl/min. As a result, seven components were separated and bioassayed. Two components, 2.7.3 and 2.7.4 had pheromonal activity (retention times: 6 min 08 sec and 6 min 50 sec, respectively) (Katsel), 1990).

It should be pointed out that female behavioral reactions to the three isolated components were different. After introduction of the 2.5.6 component, the latent period of reaction did not exceed 5 min. The latent periods of 2.7.3 and 2.7.4 components were longer (more than 10 min); the elements of a courting dance and the imitation of entering the nest were observed. At the end of these reactions, a considerable increase in female activity was noted. Only females that had ovulated showed these reactions. Females that had not ovulated did not demonstrate the elements of sexual behavior in response to components 2.7.3 and 2.7.4. We observed the same female behavioral reactions in response to some Δ^4-3-keto C_{19} steroids (Dmitrieva et al., 1991). The effect of the 2.5.6 component consisted mostly of attracting females to the compartment with a

stimulus. The same bioassay results were obtained with both ovulating and unovulating females. We also studied the effect of isolated components on ovary maturation (Katsel, 1990; Dmitrieva et al., 1991). We found that the 2.7.3 component induced almost 100% maturation of the females.

In the process of isolating components 2.7.3 and 2.7.4, we supposed that these substances might be products of spermatogenesis, since they were isolated only from the urine of males with mature testes. During this period there was a precipitate in the urine that contained spermatogenic cells (Dmitrieva et al., 1988). Further, one can assume that these are nonconjugated steroids since they were extracted by a nonpolar solvent—diethyl ether. The fraction of steroid conjugates (glucuronides and sulfates) that were isolated from urine did not possess behavioral activity in our bioassay (Katsel, 1990). The results of fish sex pheromone studies indicated that steroid hormones and their metabolites can function as sex pheromones (review: Stacey et al., 1987). HPLC analysis in the presence of C_{19} and C_{21} steroids indicated that 2.7.3 retention time coincides with 11HT and 2.7.4 coincides with T. We registered mass spectra of isolated

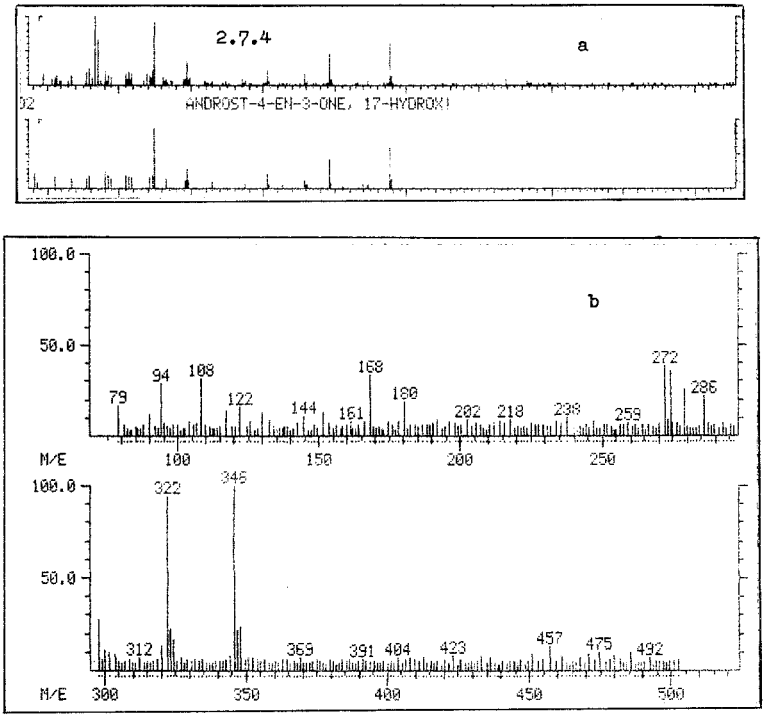

FIG. 2. Mass spectra of electron ionization of 2.7.4 and T (a), and chemical ionization of 2.7.3 (b).

compounds. The mass spectrum of electron ionization of 2.7.4 corresponded to T (Figure 2a). In the chemical ionization mass spectrum of 2.7.3, the ammonium adduct ion $(M+NH_3)^+$ m/z 322 was discovered, which indicates a molecular weight of 304 (Figure 2b). 11HT has the same mass. There is also a peak at m/z 346. The reconstruction of the curve of change of total ion current according to the given ions indicated that these are different compounds. At present, a study of the chemical structure of a component that has ion m/z 346 is being carried out.

It is possible that these steroids get into the urine with the seminal liquid. The aqueous extracts of mature testes homogenates elicited sexual behavior in females that had ovulated (Dmitrieva and Ostroumov, 1986). We isolated C_{19} steroids from the testes of the males during the spawning period. As a result, it was found that 11HT and T were contained in the testes during the final stage of spermatogenesis.

The calculation of identified steroids concentration in male urine according to mole response showed that 11HT concentration is approximately 9×10^{-8} M and T is 1×10^{-9} M. These data correspond to the data on T concentration in sea lamprey urine (Adams et al., 1987).

As for component 2.5.6, we surmised that this compound is not a steroid. In terms of fragmentation as a whole the electron ionization mäss spectrum of 2.5.6 (Figure 3) may be attributed to the spectrum of olefins. However the m/z 256 and 284 ions which are in the spectrum and may be molecular ions, do not correlate with masses of olefins. Calculation of mass numbers suggests that it

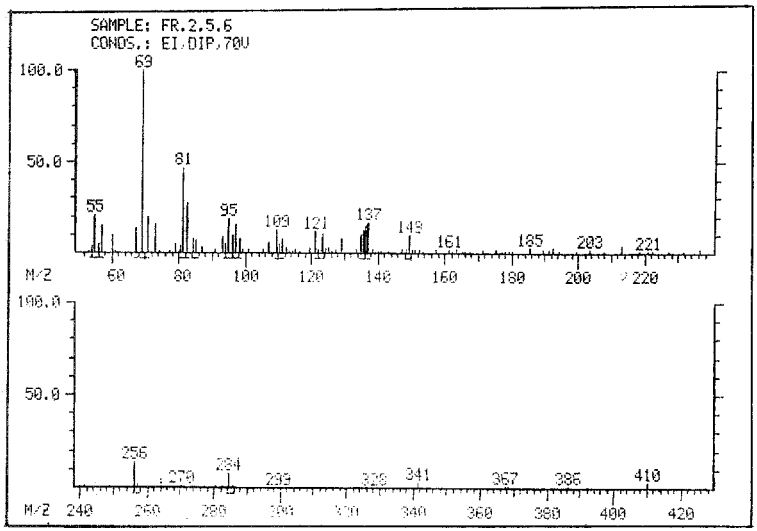

FIG. 3. Mass spectrum of electron ionization of 2.5.6.

is an oxygen-containing compound. Palmitic and stearic acids were found in fraction 2.5.6 using gas chromatography–mass spectrometry. These acids have masses corresponding to ions *m/z* 256 and 284. Solutions of these acids did not possess pheromonal activity (Katsel, 1990). The library search of the spectrum of Figure 3 indicates that it may be a polyene alcohol—farnesol (2*Z*,6*E*), M^+ 222. The molecular ion $(M+H)^+$ 223 demonstrated here using chemical ionization, superimposes on the known molecular ion of farnesol (Katsel, 1990). Farnesol and its derivatives have been reported as pheromones in insects (Regev and Cone, 1973) and mammals (Wheeler et al., 1982; Novotny et al., 1990). It is likely that farnesol is a key compound in the synthesis of steroid hormones. We conducted behavioral testing of steroisomers of farnesol (Katsel, 1990). Only 2*Z*, 6*E*-farnesol possessed pheromonal activity at 1×10^{-8} M. The identification of farnesol in the male urine is presently proceeding.

We found 2.5.6 in male urine at the beginning of the spawning period when steroid pheromones were absent. Excreting polyene alcohol with urine may be a signal to females that have not ovulated of the presence of males. This pheromone provokes the attraction of females that have not ovulated to nests where males are located. The attracted females do not enter nests. They remain on the periphery. Probably at that moment primer pheromones of the partners provoke a synchronization of their maturation. We have not yet identified the female sex pheromone. However, it was discovered that a primer pheromone—11HT excreted by the male at the final stage of spermatogenesis—initiated the maturation of oocytes (Dmitrieva et al., 1991). In addition, 11HT and T elicited sexual behavior of females that had ovulated, which we characterize as the phase of "sexual activation" (Katsel, 1990). The female's behavioral reaction are completed on entering the nests and spawning.

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DIFFERENCES AMONG SIBLING SPECIES *Rhagoletis mendax* AND *R. pomonella* (DIPTERA: TEPHRITIDAE) IN THEIR ANTENNAL SENSITIVITY TO HOST FRUIT COMPOUNDS

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Abstract—Prior electrophoretic and morphological studies have identified two closely related, economically important tephritid flies, *R. mendax* (Curran) and *R. pomonella* (Walsh), which infest the fruits of ericaceous and rosaceous plants, respectively. Further studies also have shown consistent differences among these species in their ovipositional preferences for apples and highbush blueberries and have determined that their ovipositional behavior is elicited by extracts obtained from these fruits. In this paper we report the results of an experiment that tested whether these species show distinct electroantennogram (EAG) responses to a large array of compounds present in gas chromatograph-fractionated pentane extracts of apples and highbush blueberries. *R. mendax* and *R. pomonella* flies were found to have significant differences in their antennal sensitivity to 11 blueberry and nine apple extract peaks, which correspond to 24.4% of all blueberry and 25.0% of all apple peaks that elicited a measurable EAG response from either species. Interspecific differences in peripheral sensitivity were more pronounced for blueberry than apple extract; *R. pomonella* flies were most sensitive to blueberry compounds with low retention times, whereas *R. mendax* flies responded to blueberry compounds with a broader range of retention times. Both species were most sensitive to apple peaks with high retention times. The retention times of most apple and blueberry peaks that elicited EAG responses from *R. mendax* and *R. pomonella* flies were different from the retention times of seven attractant fruit esters that were previously identified by Fein et al. (1982). The identification of these unknown apple and blueberry compounds could lead to the

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discovery of new chemical cues that mediate the host-plant preferences of these sibling species.

Key Words—Electroantennogram, GC splitter, *Rhagoletis pomonella*, *Rhagoletis mendax*, Diptera, Tephritidae, sibling species, antennal sensitivity differences, host odor compounds, host-plant recognition, speciation.

INTRODUCTION

Many phytophagous insects utilize odor perception to find and recognize host plants that are suitable for feeding, mating, and oviposition (e.g., see Guerin and Städler, 1982; Miller and Strickler, 1984; Visser, 1986). Prior studies have investigated this aspect of host-plant selection for several tephritid fruit flies and oftentimes found that volatile compounds that emanate from the fruits of their host plants are effective attractants that can also stimulate ovipositional behavior (e.g., Pritchard, 1969; Prokopy et al., 1973; Fein et al., 1982; Reissig et al., 1982; Levinson and Haisch, 1983; Metcalf et al., 1983; Averill et al., 1988; Bierbaum and Bush, 1990b). Although prior studies have shown that fruit volatiles influence the host selection behavior of several tephritid species, there are few studies that have examined whether closely related species have evolved distinct behavioral and chemosensory responses to host-related chemical cues. Recent comparative studies have, however, begun to test whether two closely related, economically important species, *R. mendax* (Curran) and *R. pomonella* (Walsh), have evolved divergent chemosensory and ovipositional responses to host-associated fruit chemical cues (Diehl and Prokopy, 1986; Bierbaum and Bush, 1990b; Frey and Bush, 1990).

These sibling species occur sympatrically over much of eastern and central North America where *R. pomonella* infests rosaceous plants (primarily apples, *Malus pumila* Miller = *Pyrus malus* L., and hawthorns, *Crataegus* spp.), and *R. mendax* infests ericaceous plants (primarily highbush, *Vaccinium corymbosum* L., and lowbush blueberries, e.g., *V. angustifolium* Ait.). In addition to species-specific alleles at several electrophoretic loci (Berlocher, 1980; Feder et al., 1989), they can be distinguished by differences in adult and pupal size (Bush, 1966; Bierbaum and Bush, 1990a). Prior field and laboratory studies have also found that *R. mendax* and *R. pomonella* flies show consistent differences in their ovipositional responses to apples and highbush blueberries (Diehl and Prokopy, 1986; Bierbaum and Bush, 1987, 1990a), as well as apple-treated versus blueberry-treated artificial fruits [see the results of the experiment reported by Bierbaum and Bush (1990b) in which artificial fruits were treated with pentane-extracted apple and blueberry compounds, and the experiment reported by Diehl and Prokopy (1986) in which compounds were rubbed off the surface of apple and blueberry fruits with a cotton swab, then rubbed onto the surface of

an artificial fruit]. The results of both studies indicate that the ovipositional responses of *R. mendax* and *R. pomonella* flies to apple-treated and blueberry-treated artificial fruits parallel their ovipositional responses to apples and blueberries. Furthermore, Bierbaum and Bush (1990b) found that the addition of apple esters to waxy artificial fruits increased the number of eggs that were laid in the fruits by *R. pomonella* flies. In summary, prior studies have shown that fruit compounds are one of the plant cues that mediate the ovipositional preferences of *R. mendax* and *R. pomonella* flies.

Prior laboratory and field studies have also shown that volatile apple esters are effective attractants for *R. pomonella* flies (Fein et al., 1982; Reissig et al., 1982; Averill et al., 1988). Fein et al. (1982) initially identified a blend of seven apple esters that increased the number of *R. pomonella* flies that alighted on odor sources at the upwind end of a wind tunnel. Moreover, they observed that the electroantennogram (EAG) responses of *R. pomonella* flies to specific GC fractions of apple extracts were correlated with the "attractiveness" of the extract fractions in their wind-tunnel bioassays (i.e., the extract fractions that attracted the most flies to the upwind end of a wind tunnel also elicited the largest EAG responses from olfactory chemoreceptors on their antennae). Subsequently, Frey and Bush (1990) found differences among *R. mendax* and *R. pomonella* flies in their EAG responses to crude, unfractionated, pentane extracts of apples and highbush blueberries, as well as a few identified fruit volatiles. The results of their experiment indicate that the antennal sensitivity of both species is selectively adapted to odors that are associated with the fruits of their respective host plants.

Additional experiments using purified, fractionated fruit extracts and identified fruit compounds could help pinpoint plant chemical cues that mediate the distinct host-plant preferences of these sibling species and also test whether their olfactory chemoreceptors show a high degree of specificity in their responses to host-related volatiles. To address this latter objective, we measured the EAG responses of *R. mendax* and *R. pomonella* flies to a large array of compounds present in GC-fractionated pentane extracts of apples and blueberries. Specifically, we analyzed the antennal sensitivity of both species to compounds present in apple and blueberry extracts by injecting the extracts onto a capillary GC column that had its outlet split between a flame ionization detector and an electroantennogram apparatus, which recorded the EAG responses of individual flies to each peak in the fractionated fruit extract. The following questions were addressed in our study: (1) What is the antennal sensitivity of each species in response to the full spectrum of compounds found in the GC-fractionated host and nonhost fruit extracts? (2) Are there differences among *R. mendax* and *R. pomonella* flies in their EAG responses to individual apple or blueberry peaks, and, if so, which extract peaks elicit the largest interspecific response differences? (3) Do any of the extract peaks that elicit significant EAG responses

from *R. mendax* and *R. pomonella* flies have retention times that differ from the retention times of the esters identified by Fein et al. (1982)?

METHODS AND MATERIAL

Mature (5- to 11-day-old) *R. mendax* and *R. pomonella* females were used in the electroantennogram bioassays. Recordings were made from seven *R. pomonella* and eight *R. mendax* flies exposed to apple extract, and six *R. pomonella* and five *R. mendax* flies exposed to blueberry extract. *R. pomonella* flies were reared from pupae that were obtained from an abandoned apple orchard near Hart, Michigan, and *R. mendax* flies were reared from pupae that were obtained from an abandoned highbush blueberry orchard near Sawyer, Michigan. After the pupae were collected, they were kept in moist vermiculite at 4°C for five months, then incubated in a 25°C chamber with 16:8 hr light–dark photoperiod until the adults emerged.

Whole-fruit extracts were obtained from Bluehaven blueberries and McIntosh apples by the methods described in detail in Bierbaum and Bush (1990b). The following paragraphs describe additional extract purification procedures and gas chromatography–electroantennographic detector (GC-EAD) methods that were used in the present study. In our initial fruit extractions, methanol, methylene chloride, and pentane were used as solvents. However, methanol and methylene chloride removed less extract material from the fruits than pentane, and the cuticular wax residues that were obtained by submerging fruits in pentane had a stronger fruity odor than those obtained using methanol and methylene chloride. Therefore, reagent-grade *n*-pentane (98% minimum purity, Columbus Chemical Industries, Columbus, Wisconsin) was chosen as the solvent for the fruit extractions. To increase its purity prior to the extractions, the pentane was distilled on a fractional distillation column at 36°C.

Several weeks prior to the start of the extractions, we used nylon-mesh bags to cover fruit-bearing branches of McIntosh apple trees and Bluehaven blueberry bushes in an abandoned orchard in East Lansing, Michigan. These protected the fruits from insect infestation without using pesticides. After the fruits had ripened, they were harvested and brought into the laboratory approximately 40 min before the start of the extractions. Thirty-two McIntosh apples and 785 Bluehaven blueberries were than placed in separate glass beakers and submerged in pentane (apples: 1940 ml; blueberries; 320 ml) for 60 min. The fruits were removed from the beakers and the remaining apple and blueberry extracts were each concentrated to a volume of 25 ml by distilling off excess pentane under a vacuum at 30°C. The concentrated fruit extracts were then stored at −70°C until the GC-EAD recordings.

Cuticular waxes and other undissolved solids in the extracts were removed

by centrifugation in a microcentrifuge for 15 min at 12,400 rpm. This produced a bilayer consisting of a lower layer of undissolved solids and an upper layer of pentane extract. This upper layer was centrifuged for another 15 min to remove the remaining undissolved solids. This produced the clear upper layer of pentane extract used in the experiment.

At the start of each recording session, 1 μl of either blueberry or apple extract solution was injected into a model 3700 Varian gas chromatograph equipped with a 25-m × 0.32-mm-ID OV-1 capillary column (methyl silicone, film thickness 1.05 μm; Quadrex Corp., New Haven, Connecticut). This volume of extract contained either ca. 3% of the total dissolved extract from a single blueberry or ca. 0.1% of the extract from a single apple. Helium was used as the carrier gas at a linear velocity of 24 cm/sec (at 100°C) and the GC oven underwent the following temperature-programmed changes after each sample injection: 40°C for the first 5 min, followed by an increase of 5°C/min up to a maximum of 205°C.

The end of the capillary column was divided into two pieces by a TEE-splitter (Valco Instruments Co., Houston, Texas), which enabled 50% of the effluent extract material to pass into the flame ionization detector (FID) and 50% to pass to an electroantennographic detector (EAD) mounted on top of the GC. The EAD apparatus consisted of a 10-cm-long × 1-mm-ID piece of L-shaped glass tubing that carried the column effluent to an antennal preparation, which was mounted as described in Frey and Bush (1990). The separated fruit compounds were delivered to the fly's antennae and the FID by adding purified compressed air through valves mounted after the TEE-splitter. This compressed air swept the separated fruit compounds off the end of the capillary column, into the L-shaped glass delivery tube and over the fly's antennae at a rate of 300 ml/min. Although there may have been some band-broadening of the eluting compounds due to the use of an unheated glass delivery tube, the use of the compressed air minimized this problem. The outputs from the EAD and FID were stored on a Zenith microcomputer. During the recordings, we observed small time delays between a given FID peak and a fly's corresponding EAG response, which were adjusted for by using the fly's response to the solvent peak (pentane).

In preliminary runs using the above-described conditions, we assessed the retention time (R_t) of several reference esters: hexyl acetate (15.8 min), hexyl propanoate (18.9 min), hexyl butanoate (21.5 min), and hexyl octanoate (31.6 min). From these data, the R_t of the hydrocarbons missing in this series can be interpolated: hexyl pentanoate (23.8 min), hexyl hexanoate (26.4 min), and hexyl heptanoate (29.1 min). Hexyl acetate, hexyl propanoate, and hexyl butanoate are three of the seven Fein blend esters. In addition to these, we also ran the other Fein blend esters: (*E*)-2-hexen-1-yl acetate had the same R_t as hexyl acetate (15.9 min) and butyl hexanoate the same as hexyl butanoate (21.6 min).

Butyl-2-methyl butanoate ran off at 16.8 min and propyl hexanoate at 18.6 min. Finally, we also ran three esters with shorter R_t's: methyl pentanoate (9.0 min), ethyl pentanoate (10.3 min), butyl butanoate (15.5 min).

Since we did not detect clear EAG responses in the second half of our 40-min GC runs, we used the first 20 min of each run for our data analysis. The EAG responses to the GC effluent were quantified as follows: For the EAG responses to the apple extract, the amplitudes of each individual fly were measured, averaged within each species, and then standardized relative to the EAG responses to the extract peak with an R_t of 6.62 min, which elicited the largest average amplitude EAG response from both species [1.06 $\pm$ 0.49 mV (average $\pm$ standard deviation) for *R. mendax* flies and 1.31 $\pm$ 0.53 mV for *R. pomonella* flies]. The same procedure was used for the EAG responses to the blueberry extract where the extract peak with an R_t of 6.55 min was used for standardization of the other peaks. This peak elicited the largest average EAG response from *R. pomonella* flies (1.46 $\pm$ 0.80 mV) and the second largest mean response from *R. mendax* flies (0.96 $\pm$ 0.56 mV). The EAG responses of both species to this blueberry peak were similar to the 6.62-min apple peak; however, it is not known whether these apple and blueberry peaks contained the same compound(s). Differences among species in their EAG responses to single extract fractions were analyzed by Mann-Whitney U tests.

RESULTS

The Fein blend esters elicited clear EAG signals from the flies' antennae with a signal-to-noise ratio of between 10 and 18 for all compounds. Specifically, we recorded a response of 2.00 mV to propyl hexanoate at 10^{-3} dilution (v/v) in pentane, the same compound at 10^{-4} gave a response of 1.44 mV, butyl hexanoate at 10^{-4} gave a response of 1.28 mV, hexyl propanoate at 10^{-4} one of 0.83 mV, butyl-2-methyl butanoate at 10^{-4} one of 0.76 mV, and hexyl acetate at 10^{-3} one of 0.66 mV and at 10^{-4} one of 0.28 mV. Our data allowed estimating the content of hexyl acetate in the apple crude extract to be about 4 $\times$ 10^{-4} μl/μl injected crude extract.

We detected distinct differences in the number of apple versus blueberry FID peaks within two separate ranges of retention times. Blueberry extract contained more large-amplitude peaks than apple extract from 1 to 5 min R_t, and vice versa from 5 to 10 min R_t (Figure 1A, B). Both extracts contained few large-amplitude peaks between 11 and 15 min R_t, and both contained an abundance of peaks with retention times greater than 15 min. In summary, our results show that the composition of apple and blueberry extracts differed most for peaks with retention times between 1 and 10 min. However, these differences among the extracts may be largely due to differences in the quantity of specific

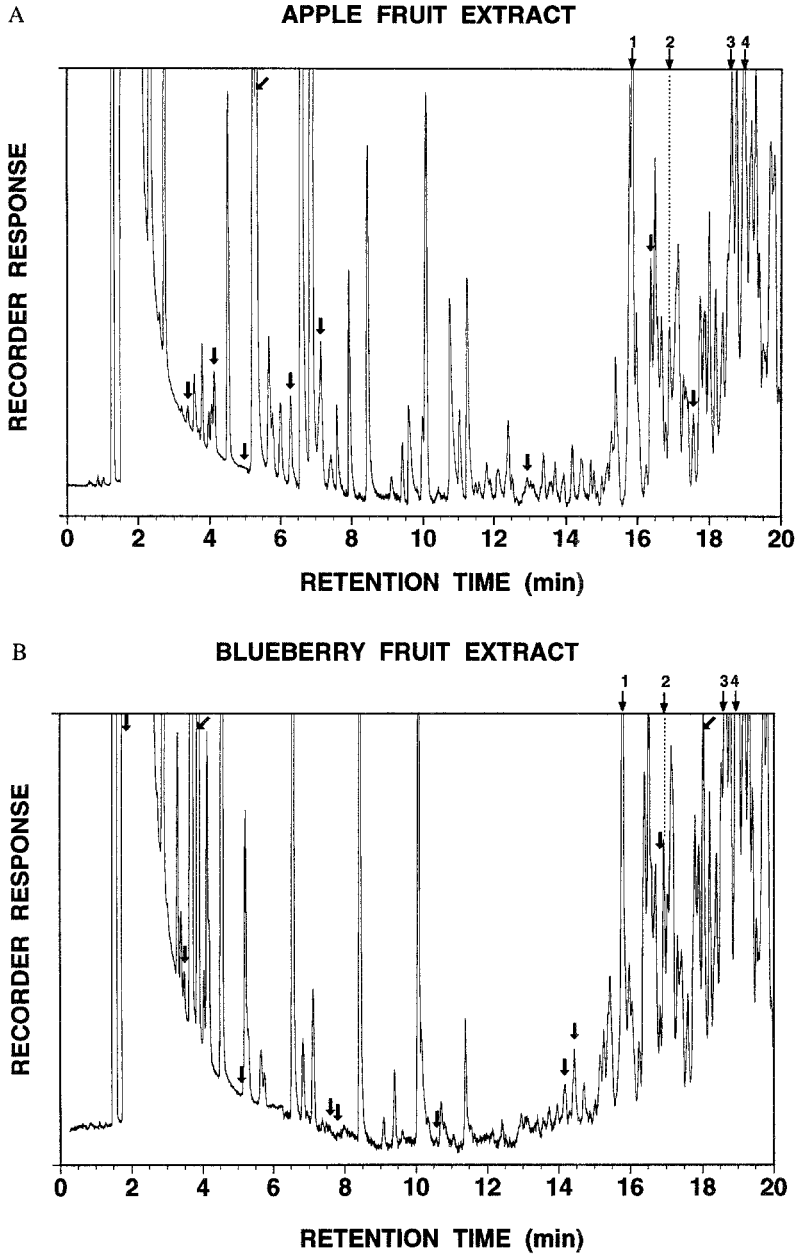

FIG. 1. Gas chromatogram of (A) apple fruit extract and (B) blueberry fruit extract. Compounds eliciting EAG responses with significant interspecific indifferences are marked by an arrow. Peaks marked by numbered arrows correspond to the retention times of the Fein blend esters: (1) hexyl acetate and (*E*)-2-hexen-1-yl acetate, (2) butyl-2-methyl butanoate, (3) propyl hexanoate, (4) hexyl propanoate.

compounds, rather than the complete presence or absence of compounds, since traces of almost every major peak found in one extract were also found in the other.

The results of our electroantennogram recordings indicate that blueberry fruit extract elicited 43 EAG response peaks above threshold level from *R. pomonella* flies and 45 EAG peaks from *R. mendax* flies, whereas apple fruit extract elicited 30 EAG peaks from *R. pomonella* flies and 33 EAG peaks from *R. mendax* flies (Table 1 and 2). Interspecific differences in the relative amplitude of these EAG responses were significant at the $P < 0.05$ level for 11 responses to blueberry extract and nine responses to apple extract (Figures 2A, B). The data in Tables 1 and 2 also indicate that differences in the peripheral sensitivity of *R. mendax* and *R. pomonella* flies were more pronounced for blueberry extract than apple extract. This is indicated by the approximately twofold greater sum of EAG amplitude differences among *R. mendax* and *R. pomonella* flies responding to blueberry extract compared to apple extract (see summations at the bottom of Tables 1 and 2). Furthermore, the 10 largest-amplitude EAG responses of *R. mendax* and *R. pomonella* flies were more similar for apple extract than blueberry extract; eight of their 10 largest responses corresponded to the same apple extract peaks (Table 1), whereas only four of their 10 largest responses corresponded to the same blueberry peaks (Table 2).

Several differences were found among *R. mendax* and *R. pomonella* flies in their EAG responses to blueberry compounds with retention times less than 6 min. *R. pomonella* flies were very sensitive to these compounds (seven of their 10 largest EAG responses corresponded to blueberry peaks with retention times less than 6 min, whereas only one of these responses corresponded to a blueberry peak with a retention time greater than 8 min; see Table 2). In contrast, the 10 largest-amplitude EAG responses of *R. mendax* flies corresponded to blueberry peaks with a broader range of retention times. Only three of these responses corresponded to blueberry peaks with retention times less than 6 min, whereas four of them corresponded to blueberry peaks with retention times greater than 8 min (Table 2). Lastly, both *R. mendax* and *R. pomonella* flies had more large-amplitude responses to apple peaks than blueberry peaks with high retention times (i.e., peaks with retention times greater than 6 min; see Tables 1 and 2).

DISCUSSION

Our results indicate that there are significant interspecific differences in the antennal sensitivity of *R. mendax* and *R. pomonella* flies to 11 blueberry and nine apple extract peaks. Currently, we do not know the structure of these compounds, but their identification is in progress.

TABLE 1. AVERAGE RELATIVE PEAK AMPLITUDES (IN %) OF ELECTROANTENNOGRAM RESPONSES TO APPLE FRUIT EXTRACT[a]

GC peak retention time (min)	*R. pomonella* ($N = 7$)		*R. mendax* ($N = 8$)		Absolute difference in mean responses
	Mean	SE	Mean	SE	
1.72	31.5	8.6	23.2	2.8	8.3
1.92	54.1 [4]	12.1	57.0 [3]	6.2	2.9
2.73	33.9	6.1	27.3	3.8	6.6
3.07	36.0	10.4	21.2	3.5	14.8
3.39	8.6	1.1	21.4	4.0	12.7*
4.04	19.3	3.9	19.5	3.5	0.1
4.13	25.9	4.1	15.1	2.3	10.8*
4.45	24.9	5.6	17.0	2.4	7.9
4.57	21.6	4.1	17.7	2.4	3.9
4.97	8.6	1.1	25.6	4.1	17.0*
5.32	48.7 [5]	8.8	19.8	2.8	28.9*
5.43	28.7	6.5	17.1	4.7	11.6
5.65	23.4	7.1	20.5	6.2	3.0
6.28	8.6	1.1	25.1	3.4	16.5*
6.62	100.0 [1]	0.0	100.0 [1]	0.0	0.0
6.88	44.7 [7]	8.8	51.0 [5]	6.1	6.4
7.13	22.7	6.1	8.6	1.5	14.1*
7.38	28.5	9.3	25.5	5.8	3.0
7.58	58.0 [3]	9.6	55.7 [4]	14.2	2.3
7.98	37.4	8.7	31.5 [10]	6.7	5.9
8.43	47.8 [6]	10.1	39.3 [9]	6.8	8.5
9.69	8.6	1.1	28.3	6.8	19.7
11.03	42.3 [9]	13.1	39.7 [8]	6.7	2.7
11.91	31.7	8.5	22.1	5.3	9.6
12.43	30.5	6.4	23.3	4.1	7.2
12.93	7.4	1.7	22.4	5.1	15.0*
14.10	18.2	3.3	21.7	4.4	3.5
14.47	16.6	4.4	18.8	2.6	2.3
14.65	32.5	5.9	26.1	4.3	6.4
14.90	8.6	1.1	16.3	4.9	7.7
15.38	96.0 [2]	17.3	71.1 [2]	15.3	24.9
15.76	31.4	5.2	30.2	8.5	1.1
15.97	44.2 [8]	10.1	49.2 [6]	8.9	4.9
16.36	27.8	5.3	8.6	1.5	19.3*
17.56	42.2 [10]	8.1	8.6	1.5	33.6*
18.95	41.1	10.4	45.5 [7]	10.5	4.4
Sum of interspecific difference in average relative amplitude for statistically significant comparisons					167.9

[a]SE = standard error of the mean; N = number of female flies; *$P < 0.05$. Background noise threshold level: 8.6; values in brackets indicate the 10 largest-amplitude responses of each species.

TABLE 2. AVERAGE RELATIVE PEAK AMPLITUDES (IN %) OF ELECTROANTENNOGRAM RESPONSES TO BLUEBERRY FRUIT EXTRACT[a]

GC peak retention time (min)	*R. pomonella* ($N = 6$)		*R. mendax* ($N = 5$)		Absolute difference in mean responses
	Mean	SE	Mean	SE	
1.87	43.9 [10]	12.7	13.3	4.5	30.6*
1.99	47.0 [8]	9.9	34.6	8.4	12.4
2.10	98.7 [2]	17.6	101.9 [1]	14.6	3.1
2.14	33.2	1.8	39.1	8.9	5.9
2.51	47.2 [7]	9.4	43.5	9.8	3.7
2.89	66.9 [4]	5.6	70.5 [6]	7.6	3.5
3.32	34.2	6.7	35.4	14.5	1.2
3.49	44.5 [9]	11.6	13.3	4.5	31.2*
3.87	25.7	4.0	57.9 [7]	12.3	32.2*
5.11	41.6	8.4	13.3	4.5	28.3*
5.28	32.3	9.5	33.3	9.3	1.0
5.49	54.1 [6]	8.1	36.7	10.3	17.3
6.55	100.0 [1]	0.0	100.0 [2]	0.0	0.0
6.80	34.0	6.7	32.1	8.6	1.9
7.16	36.1	9.6	34.8	8.3	1.4
7.24	37.6	10.6	32.6	8.2	5.0
7.59	30.9	6.8	86.1 [3]	16.5	55.2*
7.61	19.3	3.5	30.5	7.2	11.2
7.68	29.3	5.1	27.7	6.7	1.6
7.80	35.7	10.3	13.3	4.5	22.4*
7.99	19.6	4.1	23.1	6.1	3.5
8.42	77.5 [3]	9.9	72.2 [4]	15.8	5.3
8.85	25.4	5.3	24.7	5.3	0.7
9.10	34.2	13.0	23.7	4.8	10.5
9.73	19.9	3.6	31.6	4.3	11.7
10.20	33.1	6.6	33.2	9.3	0.1
10.28	42.2	8.7	34.6	11.2	7.7
10.57	36.9	8.0	13.3	4.5	23.6*
10.90	16.4	2.1	13.3	4.5	3.1
11.38	34.4	4.5	70.6 [5]	16.3	36.2
14.17	7.3	1.1	43.2	5.7	35.8*
14.44	7.3	1.1	33.0	8.5	25.7*
15.08	41.4	8.9	44.7 [10]	7.9	3.7
15.44	57.9 [5]	19.8	37.3	8.7	20.6
15.97	35.6	7.2	48.3 [8]	15.6	12.6
16.17	36.8	6.8	41.6	12.0	4.9
16.61	26.9	4.8	35.2	9.1	8.3
16.83	30.8	5.5	13.3	4.5	17.5*
17.32	25.5	3.9	27.2	5.7	1.7
17.61	29.8	3.1	28.6	5.8	1.2
17.89	33.1	7.1	36.9	11.3	3.8
18.02	24.8	7.2	45.4 [9]	7.0	20.6*
18.70	34.6	6.3	38.4	8.9	3.8
19.30	37.6	4.1	52.5	8.5	14.9
19.63	24.4	4.7	33.0	9.4	8.5
Sum of interspecific difference in average relative amplitude for statistically significant comparisons					323.1

[a]SE = standard error of the mean; N = number of female flies; *$P < 0.05$. Background noise threshold level: 7.3; values in brackets indicate the 10 largest-amplitude responses of each species.

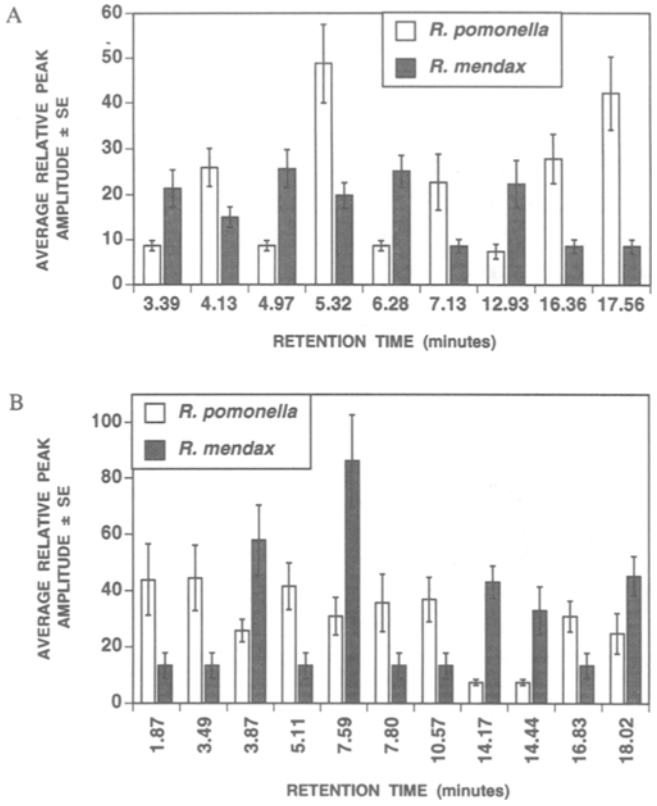

FIG. 2. Significant interspecific differences in antennal sensitivity to (A) apple extract compounds and (B) blueberry extract compounds ($P < 0.05$).

Several of the above threshold EAG responses were elicited by compounds with the same retention time as some Fein blend compounds, which, under the GC conditions used in our study, showed retention times between 15.8 and 21.5 min (e.g., in the apple extract hexyl acetate and hexyl propanoate and in the blueberry extract hexyl acetate, butyl-2-methyl butanoate, and propyl hexanoate; see Tables 1 and 2). Some of the GC peaks corresponding to the retention times of the Fein blend esters did not elicit EAG responses, probably because of low concentration in the extracts. However, there are cases where the GC trace showed a peak at a position where one of these esters was expected to elute but no EAG response was recorded. This may be caused by two reasons: (1) there may be two different compounds eluting at the same R_t, the concentration of the ester expected to elicit an EAG response being too low, or (2) the fly's

antenna may still be in the recovery period of a response to a compound running off just before the relevant ester and may therefore not be able to respond.

The results of our study indicate, however, that 87% of the apple peaks and 77% of the blueberry peaks that elicited measurable EAG responses from either *R. mendax* or *R. pomonella* flies have retention times that are smaller than those of the seven esters identified by Fein et al. (1982). Similarly, 94% of the apple peaks and 78% of the blueberry peaks that elicited EAG responses from *R. mendax* flies had smaller retention times than any of the Fein blend esters. Moreover, the majority of the largest-amplitude EAG responses of both species were to apple and blueberry peaks with retention times less than those of the Fein blend compounds (see Tables 1 and 2). The identification of these unknown apple and blueberry compounds could lead to the discovery of new chemical cues that mediate the host selection behavior of *R. mendax* and *R. pomonella* flies. For example, the addition of identified compounds to artificial fruits with suitable visual and size characteristics (Prokopy and Bush, 1973; Papaj and Prokopy, 1986; Bierbaum and Bush, 1990b) could lead to the discovery of new chemical cues that either elicit or deter oviposition.

In designing such bioassays, it is important to realize that the actual differences in host odor recognition between *R. mendax* and *R. pomonella* flies may be even more pronounced than indicated by the results of our EAG measurements. The transmission of peripheral information to the central nervous system (CNS) can be divided into two different modes (Visser and De Jong, 1988): (1) a large number of relatively unspecific generalist receptors may transmit their signals across many fibers of olfactory neurons to the CNS (i.e., across-fiber patterning), and (2) a low number of specialist receptors, each of which responds exclusively to a single compound in the blend, may transmit their signals via labeled lines directly to the CNS. If a small number of highly specific receptor cells project their signals via labeled lines into the CNS, a significant behavioral response may be elicited although no EAG response can be detected. Therefore, future attempts to identify plant compounds that elicit behavioral responses from *R. mendax* and *R. pomonella* flies should also include fruit volatiles that do not evoke measurable EAG responses from these flies.

Lastly, the results in Tables 1 and 2 indicate that there were significant differences among 25.0% of all *R. mendax* and *R. pomonella* responses to apple extract peaks and 24.4% of their EAG responses to blueberry peaks. Therefore, approximately 75% of their EAG responses to either apple or blueberry peaks were not measurably different. This result is consistent with the findings of van der Pers (1981), who recorded the EAG responses of seven closely related ermine moth species to 57 plant volatiles. Six of these species are associated with distinct principal host plants, and olfactory cues appear to play an important role in their selection of host plants. He observed that all of the compounds elicited a measurable EAG response from all of the species and that the EAG

response spectra of each of the seven species were very similar (i.e., less than 10% of the plant compounds elicited significantly different EAG responses from different species). The results of van der Pers (1981), as well as our results for *R. mendax* and *R. pomonella* flies, suggest that the olfactory receptors of some closely related oligophagous and monophagous insects are specifically tuned to only a small percentage of the plant compounds they detect. A similar pattern of responses was found among olfaction-deficient mutants of *Drosophila* whose antennal sensitivity is specifically affected by some odor compounds, but not others (Venard and Pichon, 1984). Furthermore, several studies on *Drosophila* flies indicate that genetic changes at only one or a few genes can affect their peripheral sensitivity to odor compounds (e.g., Falk and Atidia, 1975; Venard and Pichon, 1984; Arora et al., 1987; Lilly and Carlson, 1989). These results suggest that genetic changes at relatively few loci could influence an insect's peripheral sensitivity to key plant compounds, which may be sufficient to trigger changes in host preference. In host-specific parasitic organisms that mate on their host, such changes may result in a genetically based shift onto a new host when there are very low levels of gene flow among the host-associated populations. Thus, genetically based changes in the perception of plant odors may have played a significant role in mediating the host shifts of *Rhagoletis* fruit flies (Bush, 1975).

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(1*S*,2*R*,4*S*,5*S*)-ANGELICOIDENOL-2-*O*-β-D-GLUCOPYRANOSIDE—A MOOSE DETERRENT COMPOUND IN SCOTS PINE (*Pinus sylvestris* L.)

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Abstract—Hydrophilic extracts of Scots pine (*Pinus sylvestris* L.) twigs have been investigated for palatability to moose in feeding experiments. The predominant repellent effect was observed from the 2-*O*-β-D-glucopyranoside of the monoterpene (1*S*,2*R*,4*S*,5*S*)-angelicoidenol. Of other isolated and tested substances, only taxifolin-3′-*O*-β-D-glucopyranoside had a deterring effect. One fraction—containing mainly aliphatic compounds—also had a strong effect, but the effect was lost after further fractionation. Tannin-containing fractions had no effect. Angelicoidenol glucoside has not previously been reported from *P. sylvestris*. The results are discussed in view of phenols as defense compounds.

Key Words—Phenols, monoterpenes, glycosides, chemical defense, feeding experiments, moose browsing, *Alces alces*, Scots pine, *Pinus sylvestris* L., angelicoidenol glucoside, taxifolin glucoside.

INTRODUCTION

Phenols are considered to act as defense substances in plants against herbivory (Palo, 1984; Harborne, 1985; Haslam 1988). On this basis, many investigations have been made to elucidate the relations between food choice and phenols. One of the earlier studies was made by Feeny (1970), who studied caterpillar feeding behavior in relation to tannin content in oak leaves and found that leaves rich in tannins were consumed less. Tannins—quantified by protein-precipitating ability of plant extracts—had various effects on protein availability to moose (Robbins et al., 1987). Another study by Häggman and Rousi (1985) showed

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that the total phenolic content in Scots pine was negatively correlated to browsing by mountain hare (*Lepus timidus* L.). None of these investigations included isolation of single substances, so the compound(s) causing the effect is(are) still unknown. However, Bryant et al. (1983) and Clausen et al. (1986) have shown that the phenolic compound pinosylvin and its monomethyl ether—both isolated from green alder (*Alnus crispa*)—are highly repellent to snow-shoe hare (*L. americanus*).

In a previous study (Sunnerheim et al., 1988), the effect of birch twig extractives on digestibility in vitro in goat rumen liquor was studied. It was shown that platyphylloside [5-hydroxy-1,7-bis-(4-hydroxyphenyl)-heptan-3-one-5-*O*-β-D-glucoside] was the most effective compound for reducing digestibility, while other fractions—including those containing tannins—showed minor effects.

A few investigations of terpenes have also been performed for effect on food selection by moose. For instance, Danell et al. (1990) found that there is a negative correlation between the presence of the diterpene pinifolic acid and moose browsing of Scots pine. However, Löyttiniemi and Hiltonen (1978) as well as Löyttiniemi (1981) and Niemelä and Danell (1988) found no correlation between the total amount of ether extractives of Scots pine and moose browsing.

Moose (*Alces alces* L.) feed to a great extent on Scots pine (*P. sylvestris*), (Ahlén, 1968; 1975; Cederlund et al., 1980). However, some individual pine trees are preferred over other, apparently equal, pines in the same clump (Haukioja, 1983; Danell et al., 1990). This fact indicates that pines are provided with a chemical defense varying in strength between individuals.

The aim of the present work was to investigate the chemical defense in pine by testing extracts and isolated substances for their palatability to moose in feeding experiments.

In a parallel study, the palatability of pine individuals as a function of phenolic composition is studied by means of multivariate data analysis (Sunnerheim-Sjöberg and Hämäläinen, 1992).

METHODS AND MATERIALS

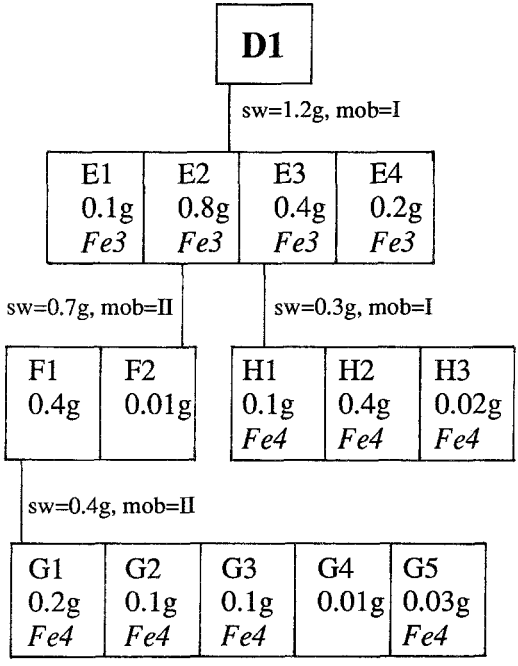
Plant Material. Twigs from unbrowsed Scots pines (*P. sylvestris*) were collected in winter outside Umeå in coastal northern Sweden. The twigs were stored at −20°C or used directly. Twigs from birch (*Betula pendula*) were collected in winter in Uppsala, Sweden. The birch twigs were used without storage.

Extraction. Frozen pine twigs (including needles) <5 mm diameter (5.6 kg) and cut in pieces were extracted in a homogenizer with a total of 10 liters 95% ethanol followed by 10 liters 80% aqueous ethanol. The extracts were filtered, and the ethanol was evaporated. The remaining aqueous phase was

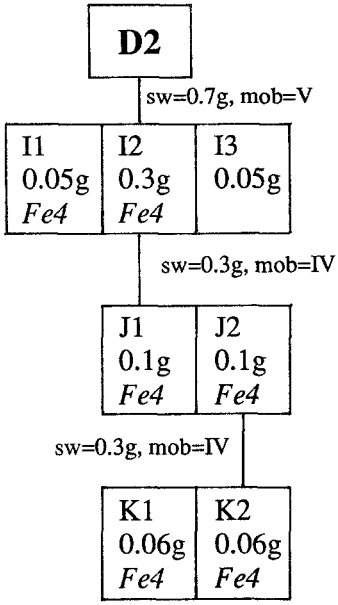
diluted with water to 2.0 liters and extracted with 3 × 2.0 liters petroleum ether (bp 40–60°C) (P) followed by 3 × 2.0 liters ethyl acetate (E). The organic phases were dried over anhydrous sodium sulfate and evaporated. Evaporations were performed at reduced pressure below 40°C. The remaining water extract (W) was lyophilized. The three extracts (W, 203 g; E, 112g; P, 142 g) were tested in feeding experiment 1 (Fe1).

Fresh pine twigs (3.0 kg fresh weight) were extracted as above for further fractionations.

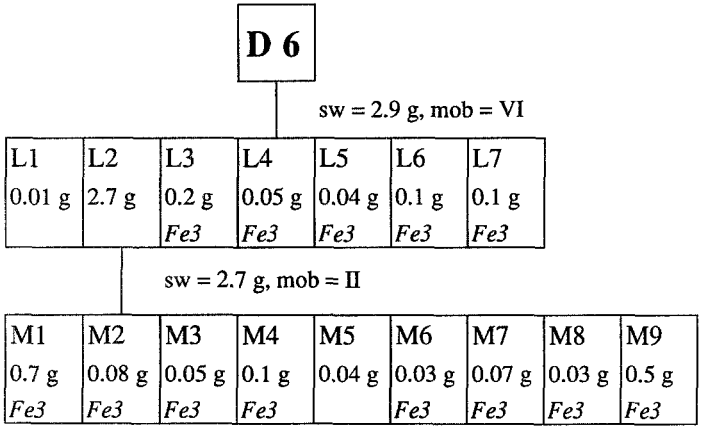
Fractionation of Extracts. The ethyl acetate extract (30 g) was chromatographed on a Sephadex LH-20 column (9 × 30 cm). Water, 30%, 60% and 95% aqueous ethanol, and finally acetone (3 liters of each) were used as eluents. The fractions were examined by TLC. Nine fractions were collected: (D1) 2.73 g, (D2) 1.46 g, (D3) 4.04 g, (D4) 3.02 g, (D5) 3.27 g, (D6) 3.73 g, (D7) 2.68 g, (D8) 1.38 g, (D9) 4.10 g. Parts of D1–D9 were tested in feeding experiment 2 (Fe2). The remaining parts of fractions D1, D2, and D6 were rechromatographed on silica gel columns to E–H, I–K, and L–M, respectively according to Schemes 1–3. Weights of extracts and fractions might include solvent residues.



SCHEME 1. Subfractions of D1. sw = sample weight, mob = mobile phase, *Fen* = fraction tested in feeding experiment *n*.



SCHEME 2. Subfractions of D2. sw = sample weight, mob = mobile phase, *Fe4* = fraction tested in feeding experiment 4.



SCHEME 3. Subfractions of D6. sw = sample weight, mob = mobile phase, *Fe3* = fraction tested in feeding experiment 3.

Subfractionations. Liquid chromatography (LC) was performed on a silica gel (Riedel-de-Haen, 0.032–0.064 mm) column (2 × 50 cm). Thin-layer chromatography (TLC) was performed on Merck HF-254 silica gel plates. The following eluents were used for TLC and for LC (silica gel): (I) chloroform–butanone–methanol–acetic acid (10:7:4:2, v/v); (II) chloroform–methanol–water (80:15:1, v/v); (III) chloroform–methanol–water (120:15:1, v/v); (IV) chloroform–methanol–water (40:15:1, v/v); (V) ethyl acetate–95% ethanol–acetic acid (9:2:1, v/v); and (VI) ethyl acetate–95% ethanol–acetic acid (18:2:1, v/v).

The TLC plates were inspected under UV light at 254 nm. Spray reagents used were: (i) diazotized sulfanilic acid (Fluka AG, CH 9470) in 10% aqueous sodium carbonate, followed by 50% sulfuric acid (v/v) (phenols); (ii) vanillin in 18% hydrochloric acid (catechin derivatives); (iii) 50% sulfuric acid, followed by heating at 100°C for about 1 min (organic compounds in general).

High-performance liquid chromatography (HPLC) was performed using an analytical column (Chrompack CP-TM-spher C18, 4.6 × 250 mm). Mobile phases were linear gradients of water with increasing amounts of methanol with constant concentration of 1% formic acid. Detection was made with a UV detector at 280 nm.

Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz and 101 MHz, respectively. A Varian VXR-400 spectrometer was used. Chemical shifts (δ) are given relative to tetramethyl-silane (TMS) as internal reference. Mass spectra were recorded at 70 eV with a Finnigan 4000 spectrometer using a direct insertion probe. Chemical ionization was performed using NH_3 as the reactant gas.

Reference Substances. Shikimic acid was commercially available. Taxifolin-3′-*O*-β-D-glucopyranoside was available at our laboratory (Lundgren, 1987; Lundgren and Theander, 1988).

Isolated Substances. (1*S*,2*R*,4*S*,5*S*)-(−)-Angelicoidenol-2-*O*-β-D-glucopyranoside (A, Figure 1) was isolated from fraction K2, 63 mg (Scheme 2).

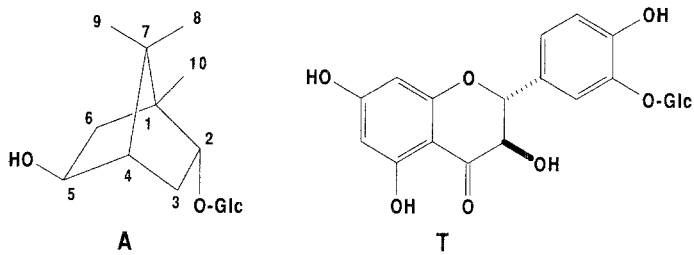


FIG. 1. Isolated substances with deterrent effect. (1*S*,2*R*,4*S*,5*S*)-(−)-Angelicoidenol-2*O*-β-D-glucopyranoside (A) and taxifolin-3′-*O*-β-D-glucopyranoside (T).

TLC; Eluent I: R_f = 0.34, color with spray (iii): greyish violet. ^{1}H NMR and ^{13}C NMR data—both in pyridine-d_5—were in accordance with those published by Inoshiri et al. (1988). ^{1}H NMR (CD_3OD) (δ): 0.85 (3H, s, CH_3-8), 0.92 (3H, s, CH_3-10), 1.01 (1H, dd, $J_{2\beta}$ = 3.0; $J_{3\beta}$ = 13.4 Hz, H-3α), 1.08 (3H, s, CH_3-9), 1.31 (1H, d (broad), $J_{6\alpha}$ = 13.2 Hz, H-6β), 1.69 (1H, d, $J_{3\beta}$ = 5.4 Hz, H-4α), 2.18 (1H, ddd, $J_{2\beta}$ = 9.3 Hz, H-3β), 2.44 (1H, dd, $J_{5\alpha}$ = 8.0 Hz, H-6α), 3.14 (1H, t, $J_{1'}$ = 7.9 Hz, H-2′), 3.18–3.26 [1H, m (broad), H-5′], 3.27–3.36 (2H, hidden, H-3′ and H-4′), 3.66 (1H, dd, $J_{5'}$ = 11.7; $J_{6'}$ = 5.6 Hz, H-6′), 3.85 (2H, m, H-6′ and H-5α), 4.05 (1H, d, H-2β), 4.21 (1H, d, H-1′). 2D NMR was run with standard methods. The structure was verified with COSY, long-range COSY, C,H-COSY and NOESY. $[\alpha]_D$ = −23.8° ± 3° (c = 0.65 in MeOH); ($[\alpha]_D$ = −26.3° [c 1.04 in MeOH], Inoshiri, 1988). EI-MS m/z (relative intensity): 170(4.3), 153(26.8), 152(20.4), 137(28.1), 135(19.4), 108(100), CI-MS: 350 $[M+NH_4]^+$.

Angelicoidenol and glucose were obtained by enzymatic hydrolysis: A (10 mg) was incubated in 2 ml of water with 1 mg of pectinase (Sigma Chemical Co. No. P4625). After 20 hr at room temperature in the dark, the solution was extracted with 2 × 2 ml ethyl acetate. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was identified as angelicoidenol by ^{1}H NMR spectroscopy in pyridine-d_5, the spectrum being in accordance with literature data, (Mahmood et al., 1983; Gunawardana and Cordella, 1988). ^{1}H NMR (CD_3OD) (δ): 0.77 (1H, dd, $J_{2\beta}$ = 3.2 Hz, $J_{3\beta}$ = 13.7 Hz, H-3α), 0.85 (3H, s, CH_3-8), 0.87 (3H, s, CH_3-10), 1.08 (3H, s, CH_3-9), 1.32 (1H, d(broad), $J_{6\alpha}$ = 13.3 Hz, H-6β), 1.67 (1H, d, $J_{3\beta}$ = 5.2 Hz, H-4α), 2.25 (1H, ddd, $J_{2\beta}$ = 9.8 Hz, H-3β), 2,32 (1H, dd, $J_{5\alpha}$ = 7.9 Hz, H-6α), 3.80 (1H, dd, $J_{6\beta}$ = 3.4 Hz, H-5α), 3.84 (1H, ddd, $J_{6\beta}$ = 2.0 Hz, H-2β). NMR and TLC data were in accordance with a synthetic sample prepared according to Marquet and Gabard (1967).

Glucose was identified by gas chromatography as trimethylsilyl derivative (Sweeley et al., 1963).

Taxifolin-3′-*O*-β-D-glucopyranoside (T, Figure 1), was isolated from fraction M9, 540 mg (Scheme 3) and was identified by ^{1}H NMR spectroscopy and co-TLC and co-HPLC with an authentic sample.

Feeding Experiments. Birch twigs were treated with pine twig extractives dissolved in 95% ethanol. The birch twigs were placed in a beaker, and the extract was evenly distributed over the twigs with a Pasteur pipet. To n g dry matter (DM) of birch twigs, extract from approximately 3.5 × n g (DM) of pine twigs was added. The control birch twigs (C) were treated with 95% ethanol.

In feeding experiments 1–4 (Fe1–Fe4), artificial trees were constructed from nine treated birch twigs and a rod with nine holes for the twigs. The trees—one tree per extract in each test—were planted in the snow in a pasture for moose

in a zoological park in Lycksele, Sweden. After about 1-hr the amount consumed was determined.

In feeding experiment 5 (Fe5), the test animal was fed by hand—one bunch of treated twigs and one control were offered simultaneously.

The number of test animals was: Fe1, varied between three and five in each test; Fe2, test 2 : 1—two adult bulls, two cows and three calves, Test 2 : 2—two adult bulls, one cow and three calves; Fe3, one adult cow in both tests; Fe4, one adult bull; and Fe5, one adult bull.

Quantification of consumption was as follows: normalized consumption = r_i/r_c, where relative consumption (r) = biomass removed/biomass available; r_i = relative consumption of sample i = $(b_i - a_i)/b_i$, where b_i = weight of birch twigs of artificial tree i before test, a_i = weight of birch twigs of artificial tree i after test, r_c = relative consumption of the control is defined analogously.

RESULTS AND DISCUSSION

Water, Ethyl Acetate, and Petroleum Ether Extracts. The three pine extracts (water, ethyl acetate, and petroleum ether) and one control were used in Fe1. Eleven tests were performed with four artificial trees in each test (Figure 2).

The water extract did not markedly affect the palatability of the twigs. The twigs were browsed more than 80% in 10 of the 11 experiments. The water phase contained mainly carbohydrates and hydrophilic glycosides. Glucose, fructose, and sucrose together constitute about 7% of the dry matter of pine needles (Popoff and Theander, 1976).

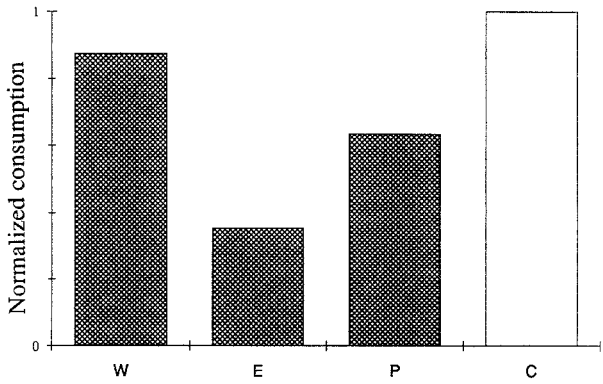


FIG. 2. Results from feeding experiment 1 (Fe1). Normalized consumption (means, $N = 11$) for test twigs treated with water (W), ethyl acetate (E), or petroleum ether (P) extract. Standard deviations: W, 0.29; E, 0.35; P, 0.29. Untreated twigs were used as control (C).

Browsing was least on twigs treated with ethyl acetate extract. In eight of the 11 experiments the sample twigs were consumed less than 50%. Ethyl acetate extract of Scots pine have been shown to contain compounds of intermediate hydrophilicity, such as glycosides—both phenolic and non phenolic—as well as aglycones (Popoff and Theander, 1975, 1976, 1977; Lundgren et al., 1982; Andersson and Lundgren 1988; Lundgren and Theander, 1988). To a lesser extent hydrophilic compounds, such as shikimic acid, are also extracted by ethyl acetate.

The petroleum ether phase of pine needles contains the most lipophilic compounds, such as chlorophyll and terpenoids. The content of the diterpene pinifolic acid is 1–2% of DM in pine needles (Enzell and Theander, 1962; Danell et al., 1990). As the concentration of pinifolic acid has been shown to be negatively correlated to moose browsing (Danell et al., 1990), the presence of this compound might be part of the explanation to the moderately but clear deterrent effect of the petroleum ether extract.

The most active fraction, the ethyl acetate extract, was further fractionated to D1–D9 as described above.

Fractions from Ethyl Acetate Extract. The nine fractions, D1–D9 (originating from Sephadex LH-20 chromatography of the ethyl acetate phase), and one control (C) were tested in each of two tests (Fe2). Figure 3 shows the mean value of results from tests 2:1 and 2:2. Fractions D1 and D2 were most deterrent in both tests. According to ^{1}H NMR spectroscopy and TLC, fraction D1 contained mostly sugars, several other aliphatic and small amounts of phenolic compounds. Fraction D2 was dominated by sugars and two other aliphatic compounds.

According to Popoff and Theander (1977) fractions D3–D6 were expected to contain several phenolic glycosides. This was confirmed by ^{1}H NMR spectroscopy and TLC. D6 was slightly deterrent in both tests, and D4 in one test. Twigs treated with fractions D3 and D5 showed no repellent activity (Figure 3).

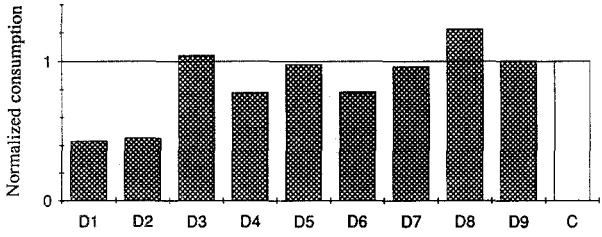


FIG. 3. Results from feeding experiment 2 (Fe2). Normalized consumption for test twigs treated with fractions D1–D9. Untreated twigs were used as control (C). Mean of two tests.

Fractions D7–D9 contained several phenolic compounds, such as catechin and probably also oligomers of catechin, i.e., condensed tannins (Proanthocyanidins) (Haslam, 1988). It has also been stated by Gupta and Haslam (1981) that needles of Scots pine are rich sources of proanthocyanidins. Catechin derivatives are easily detected with vanillin/hydrochloric acid and are assumed to have the ability to complex with proteins (including enzymes), leading to astringent taste and digestibility-reducing properties (Haslam, 1988). However, these fractions containing condensed tannins were not repellent to moose.

Fractions D1, D2, and D6 were further fractionated on silica gel columns as described as above.

Subfractions from D1. The strong effect of fraction D1 was lost after two fractionations (Scheme 1). Apart from the uncertainties in the feeding experiments there might be several explanations to this: (1) Synergistic effects might be caused by two or more compounds, which after a chromatographic procedure occur in different subfractions. (2) Active substance(s) might be divided into several fractions leading to a decrease in concentration. (3) Chemical reactions can occur, e.g., oxidation, during the work-up procedures and tests. (4) Active compounds might also be completely retained on the silica gel column.

Subfractions from D2: Isolation and Test of (1S,2R,4S,5S)-(−)-Angelicoidenol-2-O-β-D-glucopyranoside. The two main compounds of the deterrent fraction D2 were obtained after three subfractionations (Scheme 2). K1 was pure shikimic acid, identified with ^{1}H NMR spectroscopy. K2 was angelicoidenol glucoside A.

Fractions I1, I3, J1, K1, and K2 were tested in a feeding experiment (Fe4) in each of two tests. Unfortunately, the moose was not interested in feeding trials in the second test. Figure 4 shows results from test 1.

(−)-Angelicoidenol-2-*O*-β-D-glucopyranoside was the most deterrent sub-

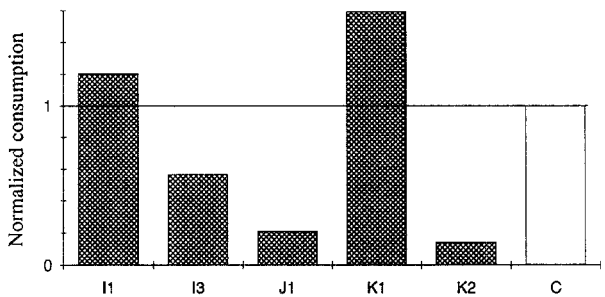


FIG. 4. Results from feeding experiment 4 (Fe4). Normalized consumption for test twigs treated with fractions I1, I3, J1, K1, and K2 (A). Untreated twigs were used as control (C).

stance tested. In test 1, the birch twigs treated with A were eaten only 17% compared to the control.

The palatable fraction I1 lacked A, while fraction I3 and J1, both eaten less than the control in the first test, contained A among other substances. Since shikimic acid (fraction K1) was the most palatable substance tested, this compound could be excluded as a cause of the deterrent effects of the extracts containing both compounds. Thus, angelicoidenol glucoside might be responsible for the repellent effect.

Even the water extract, tested in feeding experiment 1 (Figure 2), contained A in small amounts, but the taste might have been masked by the high sugar content.

Many phenols, glycosidically bound as well as free, have been isolated from pine (Norin, 1972; Popoff and Theander, 1975; 1976; 1977; Niemann, 1979; Lundgren et al., 1982; Niemann and Van Genderen, 1980; Theander, 1982; Theander and Lundgren, 1989). Terpenes also have been intensively studied, and a great number have been isolated (Norin, 1972; Enzell and Theander, 1962; Gref, 1981, 1987; Dev, 1989). However, glycosides of terpenes seem not to occur so frequently in pine species.

(1*S*,2*R*,4*S*,5*S*)-(−)-Angelicoidenol-2-*O*-β-D-glucoside has, to my knowledge, never been isolated from pine and is reported only once in the literature (Inoshiri et al., 1988). The aglycone angelicoidenol has four chiral centers, but, due to the bridgehead, can only occur in eight isomeric forms. However, only (1*S*,2*R*,4*S*,5*S*)-(−)-angelicoidenol and its enantiomer—glycosidically bound or as the free aglycone—have, to my knowledge, been found in nature. The 2-*O*-β-D-glucopyranosides of the two enantiomeric angelicoidenols were isolated from *Berchemia racemosa* by Inoshiri et al. (1988). [They call the two compounds (+)- and (−)-angelicoidenol-2-*O*-β-D-glucopyranoside, respectively, although both isomers have negative specific rotation.] The 2-*O*-β-D-glucoside of (1*R*,2*S*,4*R*,5*R*)-(+)-angelicoidenol (amomumoside) isolated from ginger (*Amomum xanthoides*) by Fujioka et al. (1988) was found to have sedative properties.

(1*S*,2*R*,4*S*,5*S*)-(−)-Angelicoidenol has been named by Mahmood et al. (1983), who isolated it from the Himalayan herb *Pleurospermeum angelicoides*. Gunawardana and Cordell (1988) found the same compound, here called (−)-5-*exo*-hydroxyborneol, in the Australian plant *Hedycarya angustifolia*.

This compound has also been formed by synthetic (Marquet and Gabard, 1967; Allen et al., 1979) and microbiological hydroxylation of borneol (Allen et al., 1979).

Based upon comparison with ^{1}H and ^{13}C NMR spectroscopy and optical rotation data from previous studies (Mahmood et al., 1983; Fujioka et al., 1988; Inoshiri et al. 1988), A was deduced to have the absolute configuration 1*S*,2*R*,4*S*,5*S*.

Subfractions from D6: Isolation and Test of Taxifolin-3'-O-β-D-Glucopyranoside. Subfractions—all containing phenols—from the slightly deterrent D6 were tested in feeding experiment 3 (Fe3). No strong deterrent effects were shown.

Naringenin glycosides are frequently occurring in the plant kingdom. Naringin (naringenin-7-*O*-β-neohesperidose) is responsible for the bitter taste of grapefruit, but, e.g., naringenin-7-[β-D-glucosyl (1→2) β-D-galactoside] is tasteless to man (Kamiya et al., 1974). Therefore it was of interest to test fraction M7 in a feeding experiment. ^{1}H NMR spectroscopy of this fraction showed that the main component was a 7-*O*-glucoside (probably the β-D-glucoside) of naringenin. Identification was made by comparison with the ^{1}H NMR spectrum of naringenin-7-*O*-β-D-glucopyranoside isolated from spruce needles (Lundgren, personal communication). Glucose was identified as described above. This fraction was only slightly deterrent, so the naringenin-7-*O*-glucoside is probably not too unpalatable to moose.

Fraction M9 was pure taxifolin-3'-*O*-β-D-glucopyranoside (T), which was shown to be slightly deterrent (Fe3). Twigs prepared with T were eaten 80% compared to the control. Taxifolin glucoside is a well-known flavonoid in conifers (Lundgren and Theander, 1988; Strack et al., 1989). In some clones of Scots pine, T is the main phenolic compound with a concentration of about 3% of needle DM, while it is absent in other clones (Sunnerheim-Sjöberg et al., 1992). The facts that (1) the concentration of T was shown to be correlated to moose browsing in the models in the multivariate study (Sunnerheim-Sjöberg and Hämäläinen, 1992), (2) T is the main phenolic compound in some of the pine individuals, and (3) T is shown to be genetically controlled (Sunnerheim-Sjöberg et al., 1992), made it of special interest to investigate the palatability of this compound.

Test of Angelicoidenol and Taxifolin Glucosides. Different concentrations of (−)-angelicoidenol-2-*O*-β-D-glucopyranoside and taxifolin-3'-*O*-β-D-glucopyranoside (both compounds obtained from additional isolations) were tested in simple two-choice feeding trials (sample and control, Fe5).

Three concentrations (A1–A3) of A were tested in Fe5 (Figure 5): A1; two samples, conc. 0.05% of DM; A2; one sample, conc. 0.15% of DM; and A3; two samples, conc. 0.25% of DM.

Even at the lowest concentration of A, the moose ate less from twigs treated with A compared to control twigs. The moose ate 29% and 79%, respectively, in the two tests and expectorated some. Higher concentrations caused complete aversion of the prepared twigs.

In Fe3 T was tested in a concentration of about 0.1% of dry matter. In this experiment (Fe5) three concentrations (T1–T3) of T were tested (Figure 5). T1; two samples, conc. 0.14% of DM; T2; two samples, conc. 2.8% of DM; and T3; one sample, conc. 5.5% of DM.

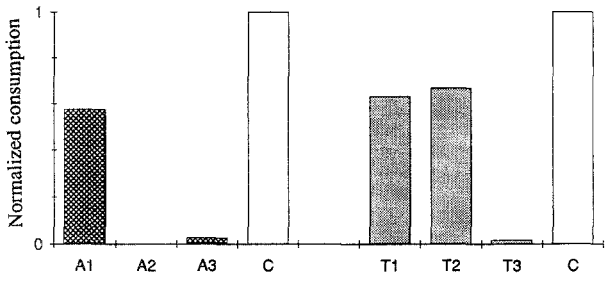


FIG. 5. Results from feeding experiment 5 (Fe5). Normalized consumption of twigs treated with different concentrations of A and T.

T was only slightly deterrent. In the lowest concentration, the twigs treated with T were eaten 55–100% compared to the control. At medium concentration (T2), the consumption was 44–100%. This concentration corresponds to the natural concentration in pines of high-T-chemotype (3% DM). However, if the concentration of T was doubled (T3), the moose expectorated the twigs after tasting.

The deterrent fraction D1 discussed earlier did not contain angelicoidenol glucoside or taxifolin glucoside.

Advantages and Disadvantages with Feeding Experiments. The large amount of sample required for a feeding experiment sets limits to the number of experiments. Here, some of the extracts were tested in only two trials, which, of course, can not be regarded as statistically significant. In some cases the moose had no interest in the artificial trees, so the test was of no value. However, the deterrent effect of angelicoidenol glucoside seems to be unequivocal: All the fractions from the ethyl acetate phase that contained A were avoided by the moose in the feeding experiments. These extracts were tested in 21 tests, and in 16 of these, the twigs were eaten less than 50% as compared to the control. Concerning food choice among the samples: in 18 of these 21 tests, the moose ate least from twigs prepared with extracts containing A, and in the remaining three tests, these twigs were chosen second to last. Only one extract containing A, the water extract (W) in Fe1 was not deterrent. However, this fraction also contained substantial amounts of sugars.

One advantage of feeding experiments in studying chemical defense is that food choice is a more direct measurement compared to quantities such as digestibility effects or toxicity. However, the normal diet of captured moose is not the same as for wild-living ones, which might influence the food choice.

Phenols as Chemical Defense Substances? Phenols—particularly tannins—have long been considered to be involved in the chemical defense of plants against herbivores (Feeny, 1970, 1976; Haslam, 1988). This work shows, how-

ever, that many of the phenol-containing fractions were not repellent. Moreover, the only fractions that might have contained condensed tannins, the vanillin-positive, were not deterrent. On the contrary, the most predominant effect was connected with a monoterpene glycoside and with fractions that almost lacked phenols. In another study (Sunnerheim-Sjöberg and Hämäläinen, 1992) 55 compounds absorbing at 280 nm, i.e., including phenols but not angelicoidenol glucoside, were analyzed. None of these compounds alone could explain the moose food choice among individual pines. However, there were significant correlations between the phenol pattern in Scots pine needles and moose food choice. Taxifolin glucoside was shown to be an important substance for food choice, although a high concentration of T was not sufficient for unpalatability of a pine individual. In view of the many observations of phenols as herbivore-deterring factors and the results above, one can conclude that, if there is a chemical defense in pine against moose browsing based upon phenols, it is of a complex nature. However, twigs prepared with extracts containing the monoterpene angelicoidenol glucoside were unpalatable to the moose tested. Thus, A might be an important part of the chemical defense of pine.

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SEMIOCHEMICAL BASIS OF INFESTATION OF HONEY BEE BROOD BY *Varroa jacobsoni*

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Abstract—Capping of worker *Apis mellifera* cells is elicited by four fatty acid methyl esters (Methyl palmitate, methyl oleate, methyl linoleate, and methyl linolenate) that are present on the surface of the worker and drone larvae only a few hours before the cell is closed. The amount of the pheromone reaches its maximum value when the cell has just been capped, at 8.5 and at 10.25 days of age, respectively, for worker and drone larvae. Thereafter, the amount of the pheromone decreases to its initial level. These data suggest that the esters also have a role in the capping of the drone cells, the temporal signal allowing the worker bees to recognize the age of the larvae and then to do the appropriate behavior. Two pheromonal components, methyl palmitate and methyl linolenate, and the inactive ethyl palmitate are kairomones attractive to *Varroa* females. Their secretion by the larvae follows the same pattern of development as the pheromonal signal. The longer and greater kairomonal signal in drone larvae, compared to worker secretion, could explain the preference of *Varroa* towards drone brood.

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Key Words—*Apis mellifera*, brood pheromone, brood recognition, capping behavior, fatty acid ethyl ester, fatty acid methyl ester, kairomone, *Varroa jacobsoni*.

INTRODUCTION

Honey bee (*Apis mellifera*) brood is reared in wax cells throughout its development. After hatching, the larva is fed by adult bees, which thereafter close the cell with a wax cap, a process called capping behavior. The behavior differs between worker and drone brood, because cap shape and cell size is different for these two kinds of larvae. Pupation occurs under shelter of the cap, and at about 21 and 24 days after egg laying, respectively, in workers and drones, the adult bee emerges from its cell.

Four fatty acid methyl esters [methyl palmitate (MP), methyl oleate (MO), methyl linoleate (ML), and methyl linolenate (MLN)] trigger the capping of the worker cells by the adult bees (Le Conte et al., 1990). These compounds are secreted by the worker larvae a few hours before the cell is closed and are present in great amounts on the larval surface during the capping period. They disappear during the following days (Trouiller et al., 1991). The temporal secretion of the brood pheromones seems to allow the adult workers to perform the capping behavior at the appropriate period. The six other methyl and ethyl esters present in the worker larvae [methyl stearate (MS), ethyl palmitate (EP), ethyl stearate (ES), ethyl oleate (EO), ethyl linoleate (EL), and ethyl linolenate (ELN)] do not induce capping behavior.

Two of the pheromonal compounds, MP and MLN, and the inactive EP, are kairomones attractive to *Varroa jacobsoni* (Le Conte et al., 1989). *Varroa* is responsible for a virulent, almost worldwide, parasitism of *Apis mellifera* (Ritter, 1981; Arnold, 1990). Adult *Varroa* feed on the hemolymph of the adult and immature bees, and their development is associated with bee brood. To reproduce, the *Varroa* female enters a cell containing a larva before it is capped (Grobov, 1977). After the closing of the cell, the *Varroa* females lays eggs and its offspring grow at the expense of the bee pupa. Later on, the mature acarians emerge from the cell with the adult bee. *Varroa* females prefer to infest drone brood rather than worker brood, when they are both present in the colony (Grobov, 1977; Sulimanovic, 1982; Tewarson, 1983; Schulz, 1984; Fuchs, 1990). As with the pheromonal compounds, the worker *Apis mellifera* brood only secrete the kairomonal compounds a few hours before the cell is closed (Trouiller et al., 1991). It is only during this period that *Varroa* enters the cell (Ifantidis, 1988; Boot et al., 1990; Wieting and Ferenz, 1991).

In this paper, we investigated the quantitative and qualitative variations in the secretion of the esters by drone larvae in order to attempt to explain the

differences in *Varroa* infestation and in the capping behavior of drone and worker brood. The data obtained from the drone brood were compared with those obtained in a previous study in worker brood (Trouiller et al., 1991).

METHODS AND MATERIALS

Biological Material

Rate of Development of Drone Brood. A queen (*Apis mellifera ligustica*) was allowed to lay during a short period (3 hr) on a comb set up with drone cells, isolated from the colony with a queen excluder (May 1991). Thereafter, the queen was excluded from the frame, which was left in the colony. From the sixth day, 20 larvae, chosen randomly, were removed every 12 or 24 hrs, the mean weight of the individuals and the form of the larvae and of their cells were noted. The size of the young larvae (between 7 and 8.5 days) was estimated in the cell. For larvae at their maximum development (between 9.5 and 10.25 days), the main criterion was the formation of the wax cap (no cap, capping in progress, and end of the capping). At subsequent ages (between 11.5 and 15 days), we noted the stage of the insect (traces of the larval cocoon, prepupa, and white eyed pupa). Using these data, we chose a series of stages of development that were easily distinguishable, and we assigned to them a mean age obtained from the growth curves and the other criteria (form of the cap and stage of the insect).

Comparison between Drone and Worker Brood. In order to compare the chemical data from the drone brood with that obtained from worker brood in 1990 (Trouiller et al., 1991), we also established a growth curve for the worker brood. To get this curve, we used the same protocol as described above: we placed a comb with worker cells in the same colony during the period that the drones were being reared.

Sample of Drone Brood. We took larvae from the same colony and reared during the same period. A queen (*Apis mellifera ligustica*) was allowed to lay for 24 hr on a comb set up with drone cells according to the same protocol described before. Seven days later, we took brood samples according to the criteria we had established from the growth curves. We used one or two samples of each stage, each sample consisting of 15–30 g of brood.

Chemical Analysis

The insects were taken intact from their cells and their cuticle extracted twice with *n*-pentane and thereafter once with dichloromethane. Each extract was shaken for 15 min. We used 2 ml of solvent per gram of insects for each of the three extractions of each sample; the three extracts were combined and

concentrated under a purified nitrogen flow. Then the residue was solubilized in 1 ml of pentane.

Each sample was fractionated on a silica gel column (Merck, 230–400 mesh) of 0.5 cm I.D. and 20 cm length. The silica gel was first washed with dichloromethane and dried at 60°C under vacuum. The first fraction, containing cuticular hydrocarbons (characterization by GC-MS), was obtained by passing 6 ml of pentane through the column; thereafter a second fraction containing the esters was obtained using 6 ml of dichloromethane.

Because of the presence of great amounts of di- and triglycerides, the second fraction was purified with HPLC. We used a Waters 6000 pump equipped with a Waters U6K injector and a refractometer detector (Erma ERC 7510). A mixture of *n*-heptane and ethyl acetate (95:5), with a flow of 1 ml/min was passed through a silica column (Interchrom 250, diameter 4.6 mm, intersphere 5 μm). The samples were recovered between 190 and 420 sec, dried under a nitrogen flow, and solubilized in *n*-hexane.

The esters were quantified by GC (Carlo Erba 6000 equipped with a on-column injector and a flame ionization detector) with a Carbowax capillary column (SGE, length 50 m, internal diameter 0.32 mm, film thickness 0.5 μm). We used hydrogen as carrier gas at a pressure of 40 kPa. The oven temperature was programmed from 55°C to 230°C at 7°C/min and held at 230°C for 20 min.

The amounts of total esters (TE) were determined by the use of 10 standard curves obtained by injection of quantities of external standard esters ranging from 0.01 to 1 μg for each of the 10 esters quantified: methyl palmitate (MP), ethyl palmitate (EP), methyl stearate (MS), ethyl stearate (ES), methyl oleate (MO), ethyl oleate (EO), methyl linoleate (ML), ethyl linoleate (EL), methyl linolenate (MLN), and ethyl linolenate (ELN).

Compounds were identified according to their retention times and molecular fragmentation by GC-MS, and we compared the mass spectra of the compounds present in the extract with those of reference esters (Sigma). The GC apparatus was a Varian 3400 equipped with a split–splitless injector. We used the same column and temperature program. The MS was an Incos 50 (Finnigan). Analysis was performed with positive ionization, at a 0.015 torr pressure and the electromultiplier was set at 70 eV.

RESULTS

Rate of Development of Drone and Worker Larvae. Between 6 and 8 days of age, drone and worker larvae had the same rate of growth. The worker cells were capped between 8 and 9.25 days; half of the cells were capped at 8.5 days (mean capping age). The mean capping age for workers, observed during this

experiment, differed to some extent from that observed by us in the summer of 1990. Accordingly, we reduced by 12 hr the mean age of capping assigned to the 1990 worker larvae.

When capped, the worker larvae stopped growing and reached a mean weight of 161 mg. The growth of drone larvae continued after 8 days until the cells were capped, which occurred between 9.5 and 11 days of age; the mean capping age was 10.25 days. At this time, the drone larvae weighed 410 mg, 2.5 times heavier than workers. The mean ages of spinning larvae, prepupae, and white-eyed pupae were, respectively 11.5, 13, and 15 days for drones, and 9.5, 11, and 12.5 days for workers.

Amounts of Esters in Drone Larvae. At 7 and 8.5 days of age, total esters (TE) were present at 10 and 60 ng per drone larva, respectively (Figure 1). When the cell is capped (10.25 days), there are 1850 ng of TE per larva; thereafter, the amount of TE decreases until only 40 ng are present in the white-eyed pupae (15 days).

The esters attractive to *Varroa* (VE: MP, EP, and MLN) and those eliciting the capping of the worker cells (CEW: MP, MO, ML, and MLN) follow the same quantitative pattern (Figure 1), with a peak of 955 ng for VE and 845 ng for CEW (10.25 days).

Between 8.5 and 10.25 days, the secretion of the esters by drone brood does not have the same amplitude for each ester (Figure 2). The amounts of the methyl and ethyl esters of the palmitic and linolenic acids increase strongly, and the proportions of the total esters also increase. The amounts of the esters

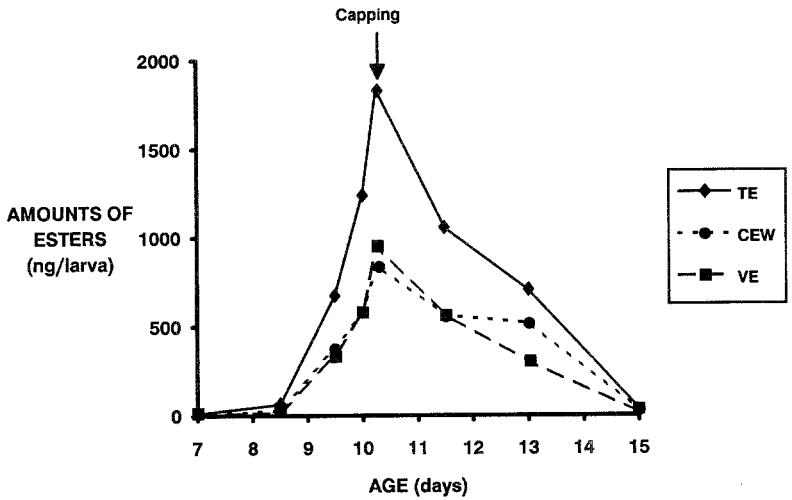

FIG. 1. Amounts of esters in drone brood of different ages. TE: total ester; CEW: esters eliciting the capping of worker cells, VE: esters attractive to the *Varroa* mite.

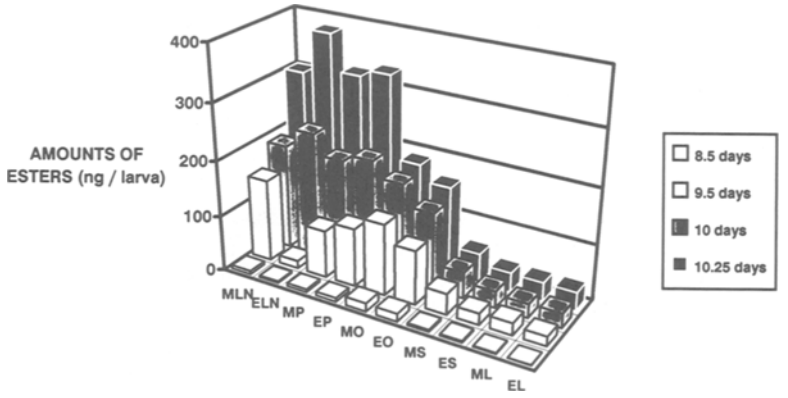

FIG. 2. Ester secretion in drone larvae 8.5 to 10.25 days of age during the capping period.

of oleic acid increase, but their relative amounts decrease; those of the stearic and linoleic acid esters show little increase.

To ensure that the ester secretion increase was not due simply to the increase of larval weight, we expressed the amounts of secretion per gram of larvae. The amounts per gram follow a similar pattern of change to that shown in Figure 1. Between 8.5 and 10.25 days, VE increased from 90 to 2600 ng/g of larva, and CEW from 140 to 2300 ng/g, an increase of 29 and 16 times, respectively.

Comparison between Drone and Worker Secretions. The quantification of esters in workers has been published (Trouiller et al., 1991), and the ages of the worker brood observed in the summer of 1990 have been modified to reflect the growth curve obtained in 1991.

The pheromonal signal of drone and worker broods has many similarities (Figure 3). As in worker brood, the ester secretion changes during the development of the insects. In both sexes, the amounts of esters reached their maximum just after the cells were capped and then decreased. On the other hand, the duration of the chemical signal is shorter in the worker brood; it began after 7.5 days of age and reached its maximum at 8.5 days, while in the drone brood the signal only appeared after 8.5 days of age and reached its maximum 40 hr later, at 10.25 days.

In both sexes, the amounts of esters were not directly proportional to the weight of the larvae. This is clear for 8.5-day-old larvae; in workers (151 mg larvae) there were 280 ng of CEW, while in drones (200 mg larvae) there were only 28 ng. The amounts of esters per gram of larvae reach a maximum value during capping. This signal is due to an intensified secretion of esters during the period before the capping.

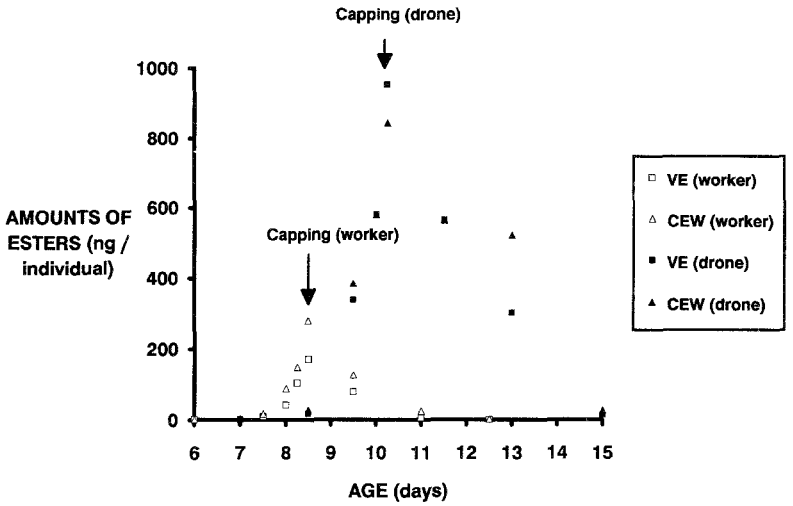

FIG. 3. Drone and worker secretion as a function of age. VE: esters attractive to the *Varroa* mite; CEW: esters eliciting the capping of worker cells.

The drone's pheromonal signal is more abundant than the worker's. During capping, there were 950 ng VE per drone larva while there were 170 ng in workers, 5.6 times more. At a maximum, there were 840 ng of brood pheromones per drone larva, whereas workers contained only one third as much (280 ng). Thus, the difference between the two sexes is much greater for the kairomones attractive to *Varroa*.

The day before capping, the increase in the secretion of esters was different between drone and worker larvae. In drones, the greatest increase of secretion was that of methyl and ethyl esters of palmitic and linolenic acids (MP, EP, MLN, and ELN, Figure 2). In the worker brood, the methyl esters were emphasized (MP, MS, MO, and MLN) (Trouiller et al., 1991).

During capping, the relative amounts of the esters are different between drone and worker broods (Figure 4). In drones, for each fatty acid, the methyl and ethyl esters were present in equal amounts, while there was about twice as much of the methyl esters than ethyl esters in workers. The most abundant compounds in drones were the esters of palmitic, linolenic, and oleic acids. In workers, MLN and MO were also major compounds, the corresponding ethyl esters being present in smaller amounts; MS was also a major ester, while in drones it was present in low amounts. Some major compounds, like EP, EO, and ELN in drones, and MS in workers, were inactive in eliciting capping behavior.

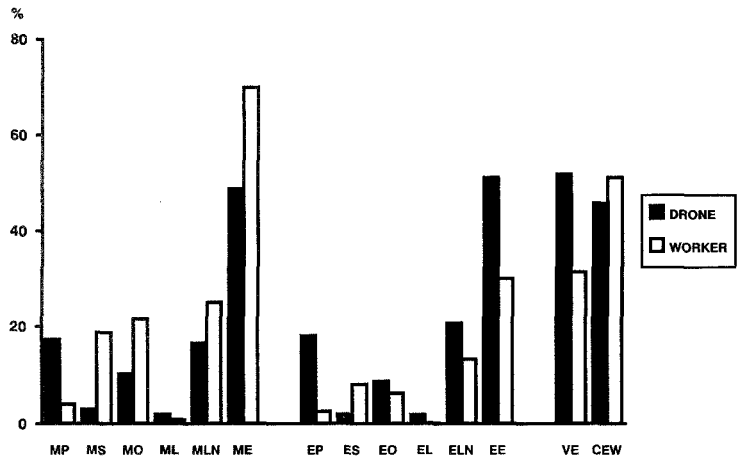

FIG. 4. Comparative percentage of esters in relation to total esters in worker and drone larvae at the end of capping: ME: methyl esters; EE: ethyl esters; VE: esters attractive to the *Varroa* mite; CEW: esters eliciting the capping of worker cells.

DISCUSSION

Kairomonal Secretion and Varroa jacobsoni *Infestation.* To reproduce, the female mites leave the adult bees and enter cells containing bee larvae. Several studies have shown that larvae, even when isolated from the colony, are attractive to *Varroa* females (Rosenkranz et al., 1984; Issa et al., 1986; Fuchs and Müller, 1988; Otten and Fuchs, 1988; Le Conte et al., 1989; Fuchs, 1990). It has been shown using a behavioral bioassay, that, in the experimental conditions used, the three kairomonal compounds completely mimic the attractive signal emitted by the larvae, because the esters are as attractive as living larvae (Le Conte, 1990).

In a colony, the mites invade only the cells that contain last-instar larvae, when these larvae occupy almost all the cell width (Grobov, 1977). After the *Varroa* has entered a cell, it becomes immersed in the brood food at the bottom of the cell and stays there until the food is totally eaten by the larva a few hours after capping. The invasion of the brood (L5) has two advantages for *Varroa*, it allows it to limit the immobilization period in the larval food and the size of the larva protects the acarian from adult bees, which cannot remove the mite at this stage.

The age-dependent attractiveness of the larvae has been analyzed with different bioassays. The young 5- to 6-day larvae (L3–L4 stages) and the white-eyed pupae are much less attractive than L5 larvae, both in the worker and drone broods (Rosenkranz et al., 1984; Fuchs and Müller, 1988; Le Conte,

1990). When comparing these observations with our data, it seems that a correlation exists between the amounts of kairomones present on the larvae and the attractiveness of the brood. The L5 worker and drone larvae, which are the most attractive towards *Varroa*, contain the greatest amounts of kairomones. However, the greater attraction of pupae to young larvae (Fuchs and Müller, 1988) is not correlated with the ester concentration, because the kairomones are present in the same amounts in the two stages. We can make the assumption that other chemical compounds could be present in pupae and could allow the acarians to be directed towards the bee pupa, after the invasion of the cell, to ensure its feeding.

The *Varroa* infestation rate of the brood has been studied inside the colony. Ifantidis (1988) observed that the infestation rate was a function of the larval weight; he showed that the invasion begins when worker larvae weight 100–125 mg and drone larvae 200–250 mg. According to our data, these larval weights correspond to the releasing of the kairomonal secretion at eight days in workers and at nine days in drones.

The time of the infestation has also been precisely determined relative to capping time. The infestation of the worker brood begins 20–27 hr before their cells are capped and 36–40 hr before in drones (Boot et al., 1990, Wieting and Ferenz, 1991). The beginning of brood infestation is correlated with the period at which the release of esters in larvae begins to increase strongly. The data concerning the entrance rate of the mite in relation to the age of the brood is variable (Boot et al., 1990; Wieting and Ferenz, 1991), and hence it is not possible to compare entrance rate with the amount of kairomones.

When both worker and drone broods are present, the drones are more infested than workers by the mite. In different races of *Apis mellifera*, the infestation rate of drone brood is five to nine times higher than in workers (Sulimanovic et al., 1982; Schulz, 1984; Issa et al., 1986; Fuchs, 1990). The drone preference is advantageous to *Varroa* because the rate of reproduction is greater in drone brood; this is related to the longer postcapping period (about 12 days in worker versus 15 days in drones), and higher fecundity (Schulz, 1984). Behavioral studies showed that L5 drone larvae were more attractive than worker L5 larvae (Rosenkranz et al., 1984; Issa et al., 1986; Otten and Fuchs, 1988; Le Conte, 1990). These observations are supported by our present data. During the capping, the amounts of attractive esters are 5.6 times higher in drone larvae. Moreover, the chemical signal, before the cell is capped, lasts longer in drone larvae (two days versus one day in workers) (Figure 3). The lesser attractiveness of worker brood could also be due to the fact that the most attractive compounds, MP and EP, are present in low amounts, whereas the less attractive MLN is the major kairomonal compound (Le Conte, 1990). In drone brood, MP and EP constitute the most abundant kairomones.

Compounds of low volatility, still unidentified, could also be active in the

recognition of the larva. It has been shown for another acarian bee parasite, *Acarapis woodi*, that cuticular hydrocarbons may be involved in host recognition (Phelan et al., 1991).

If chemical stimuli, according to the bioassays, seem to explain the recognition of the larva by *Varroa*, in the colony other factors could also play an important role in the location and choice of its host. Several experiments have demonstrated the importance of the different size and shape of the cells in the mite attraction (Message and Gonçalves, 1983; Camazine, 1986; De Ruijter, 1986).

According to our data, it seems that the kairomonal esters are used by *Varroa* to locate bee larvae of both sexes. The use of the temporal pheromonal secretion by *Varroa* seems to be a specific adaptation to the strategy of infestation by the mite, which allows it to choose an appropriate host and to invade it at the right time.

Brood Pheromone Secretion. To allow for normal development of the brood, the nurse bees perform behaviors adapted to the age, sex, and caste of the brood: nutrition (Townsend and Shuel, 1962; Haydak, 1970; Dietz, 1972; Rembold et al., 1974), capping behavior, sometimes removal of a part of the brood (Woyke, 1977; Free and Winder, 1983), and incubation behavior (Koeniger, 1978). Among the factors that elicit these behaviors, the chemical stimuli secreted by the brood should be important and allow for the recognition of the sex, age, and caste of the larvae. Free (1987) put forward the hypothesis that "a relatively involatile pheromone present on the body surface of brood is the primary brood recognition signal" and that, in addition, other pheromones like a capping pheromone could exist. The first bee brood pheromone, which elicits the incubation of the pupae, has been identified as the glyceryl-1,2-dioleate-3-palmitate (Koeniger and Veith, 1983). This pheromone is present in greater amounts in drone and queen larvae than in workers (Koeniger and Veith, 1984). This can be associated with the fact that, by using a bioassay, the larvae of the first two castes are preferentially incubated compared to worker larvae (Koeniger, 1978). However, no correlation between the ages of the larvae, the amounts of the pheromone, and the different incubation behaviors of the nurse bees has been demonstrated.

The wax cap that covers the cell that contains a drone larva is raised and domelike, whereas it is flat when the cell contains a worker larva. This aspect of the cap seems to be determined by the sex of the larva, because in dequeened colonies, when the laying workers deposit drone eggs in worker cells, the cap is domelike. The cells are also different between the two sexes, the diameter and the length of the drone cells are greater than those of worker cells. The caps are built by adult workers at each reproductive cycle, and are destroyed by the emerging adult bees, whereas the cells are used numerous times.

The recognition of the sex of the drone larva by the adult bees could be

mediated by a chemical factor. It seems, however, that the drone pheromonal signal is not the same as the worker signal (CEW), because when drone larvae are 9.5 days old, one day before the capping of the cell, the amounts of CEW are 1.5 times higher than the maximum pheromonal amounts that elicit capping of worker larvae cells. Qualitative and quantitative differences in the pheromonal signal composition could exist between the two sexes. The existence of the intensive secretion of esters before capping suggests that some of them could play a role in eliciting the capping of drone cells; for example three ethyl esters (EP, EO, and ELN) are inactive for the capping of the worker cells (Le Conte et al., 1990). Other semiochemicals than the 10 esters analyzed could be involved in the capping of the cells containing a drone larva.

The importance of mechanical stimuli is demonstrated by the fact that when pure esters are placed on the bottom of a cell, capping is not elicited (Le Conte and Trouiller, unpublished data). Both the presence of the mass of a lure and the chemical factor are necessary for eliciting capping behavior. Furthermore, the importance of both mechanical and chemical stimuli has been shown in the incubation behavior of the brood by adult bees (Koeniger, 1978; Koeniger and Veith, 1983). Mechanical stimuli linked to the larvae, such as the distance from the larva to the surface of the cell, could be of importance in eliciting the domed cap in drone cells (Buchner, 1953; Gontarski, 1954).

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DEFENSIVE SECRETION OF FIRST-INSTAR LARVAE OF ROOTSTALK BORER WEEVIL, *Diaprepes abbreviatus* L. (Coleoptera: Curculionidae), TO THE FIRE-ANT *Solenopsis geminata* (F.) (Hymenoptera: Formicidae)

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Abstract—Since several species of predatory ants show some kind of repulsion towards the first-instar larvae (FIL) of *Diaprepes abbreviatus* L., the predatory behavior of *Solenopsis geminata* (F.), a common ant in the citrus groves in Guadeloupe, was studied. Different extracts of larvae were disposed on egg masses of *D. abbreviatus* and presented as prey to the ants, both in the field and in the laboratory. The ants are repelled by the FIL extracts. The allelochemicals involved are produced in large amounts, from 5 to 20 ng per larva. Physiochemical analyses have led to the identification of two sesquiterpenes of molecular weight 218 and 234, secreted in the respective proportions of 65 and 35%.

Key Words—*Diaprepes abbreviatus*, Coleoptera, Curculionidae, defensive secretion, *Solenopsis geminata*, Hymenoptera, Formicidae, sesquiterpenes, 5-methyl-8-isopropenyl-3, 4,4a,5,6,7,8,8a- octahydro-2-naphthalene carboxy aldehyde.

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INTRODUCTION

Diaprepes abbreviatus, commonly known as the rootstalk borer weevil, is an important pest of citrus and sugarcane in the Caribbean basin. This species has a broad host range, including fruit, vegetables, and ornamentals (Martorell, 1945; Wolcott, 1948; Woodruff, 1968) but the main problem concerns citrus groves and nurseries, especially in Florida (Schroeder and Beavers, 1977), Puerto Rico (Woodruff, 1964), and the French West Indies (Mauléon and Mademba-Sy, 1988).

The adult weevils feed on the young foliage and deposit their eggs in masses between mature leaves, which are held together with an adhesive secretion. After hatching, first-instar larvae drop and burrow into the soil where they spend all their larval stages. Larvae feed on roots, causing severe damage. After pupation, the adults emerge from the soil.

Among the natural enemies, several species of predatory ants can play a role. Egg mass predation in the trees by *Monomorium floricola* Jerdon, *M. ebinium* Forel, *Pheidolle fallax* Forel, and *Ectatomma ruidum* Roger is reported (Buren and Whitcomb, 1977).

In Florida and Puerto Rico, ants are the dominant predators on first-instar larvae (Richman et al., 1983); the major ant species involved are *Tetramorium similum* Roger, *Pheidolle dentata* Mayr, and *P. floridana* Emery in Florida and *P. fallax* and *Tetramorium bicarinatum* (Nylander) in Puerto Rico. In a qualitative evaluation of ants as predators on *Diaprepes* species in the French West Indies, Jaffé et al. (1991) report predation of egg masses, larvae, and adults by several species of ants, but in most cases, ants showed some kind of repulsion towards the first-instar larvae. In the literature, there is no report of such a phenomenon. In this study, we tested this repellency, using first-instar larvae or egg masses treated with first-instar larvae extracts and presenting it as prey to a common ant of Guadeloupe: *Solenopsis geminata* (F.). This species is an important predator in citrus groves (Jaffé et al., 1991). The aim of the present work is to determine whether there is any chemical basis for repellency. Gas chromatography, mass spectrometry, nuclear magnetic resonance, and infrared spectrometry were used for the detection and identification of the chemicals involved.

METHODS AND MATERIAL

Insects. Fragments of ant colonies were collected in the field by digging up a nest and placing the soil containing large numbers of ants into boxes whose sides were coated with Fluon. The ants were separated from the soil by allowing water to slowly drip into the box and providing them with several wire bridges over which they could escape into a second Fluon-coated box containing the

nest. The nests consisted of plaster of Paris in standard Petri dishes, with a centrally positioned tube to permit the nest to be artificially moistened, and then covered with red plastic to simulate darkness. The ants were fed on larvae of the greater wax moth, *Galleria mellonella*.

The weevil species *Diaprepes abbreviatus* was collected in Guadeloupe and maintained in the laboratory at 26 ± 3°C, in large plastic cages. Adults were fed on young citrus foliage, and females oviposited readily between pieces of sulfurized paper stapled to a leaf, as well as between leaves. Egg masses were collected daily and placed in eclosion boxes. On hatching, the neonates drop through the gauze into a vial. They were collected daily, and some were placed in plastic vials with medium (supplied by Dr. W. J. Schroeder, USDA, Orlando, Florida) for rearing while others were utilized for extracts. The larvae used for extracts were kept in clean plastic vials with a piece of moistened paper towel to prevent dehydration and removed when required.

Preparation of Extracts. These were made by washing with hexane. For eggs, the number in a mass on sulfurized paper was counted and the paper cut to the smallest size possible. Larvae were counted and collected in a Pasteur pipet. Extracts were made from first-instar larvae up to 7 days old and also second instar larvae up to 30 days old. The eggs or larvae were placed in a Pasteur pipet that had a small plug of glass wool inserted. A volume of 500 μl was allowed to pass through the pipet and collected in a glass vial. After concentration under argon, extracts were stored at −18°C.

Field Bioassay. Before developing a laboratory bioassay, we simply observed the behavioral response of the ants near their nest on the soil of a citrus grove in Guadeloupe. Egg masses of *D. abbreviatus* were available to ants on the soil. Test and control stimuli consisted of 10 μl of first-instar larvae (FIL) extract at 1 L1 equivalent/μl (1 L1E/μl), 10 μl of second instar extract (1 L2E/μl), and 10 μl of hexane, respectively, applied to masses of approximately 10 eggs. Each stimulus was tested with 30 ants. The assays were conducted between 9:00 and 10:00 AM. When an ant came in contact with an egg mass, its behavior was recorded.

Laboratory Bioassay. A box containing the artificial nest of *S. geminata* was connected to a smaller box, containing the usual food of the ants, by three glass tubes (Figure 1). The stimulus (egg mass or larva) was placed in the central tube and when an ant entered this tube, the entrances were closed, using pieces of wrapped Parafilm as plugs. The ant was observed for a period of 5 min, and its behavioral response when it came in contact with the larva or egg mass noted. After 5 min, the plugs were removed and the ant was allowed to leave. This was repeated 20 times for each nest with the same test stimulus, and the glass tube and the stimulus were replaced each five replicates. An egg mass of 10 eggs or one larva was used for each test. For the tests that utilized extracts, 10 μl of a solution (concentration: 1 larva equivalent per microliter) were placed

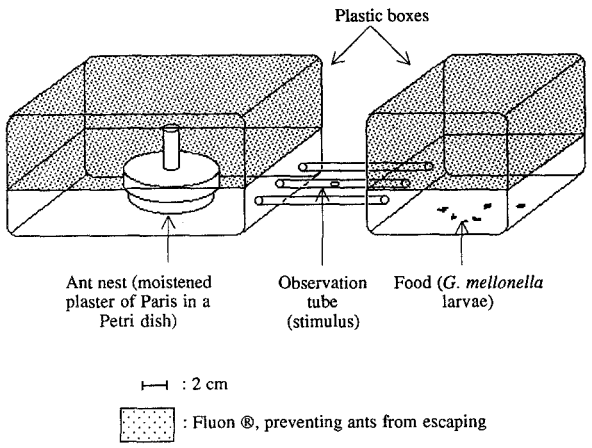


FIG. 1. Laboratory bioassay.

on the egg mass. Controls using 10 μl of pure hexane were also included. The following stimuli were tested: 5-day-old eggs, 5-day-old eggs treated with hexane, 5-day-old eggs treated with either a 0.5- or 5-day-old FIL extract and FIL of 0.5, 1, 2, 3, 4, and 5 days old. For statistical analysis, chi-square tests were carried out using the system STATITCF computer package for microcomputers.

Another laboratory bioassay was developed to test the activity of the two compounds (A1 and A2) isolated from the FIL extracts. One microliter of the tested solution was disposed on a piece of Bristol paper (1 × 1 cm), washed with a Soxhlet system. The exact place of the stimulus was delimited by a spot made with a pen. After a mechanical excitation of the ant nest, the piece of paper was placed on the Petri dish and the number of ants coming in contact with the source was recorded during 90 sec. The following stimulations were applied to four ant nests: compound A1 at 10, 100, 500 ng; compound A2 at 10, 100, 500 ng; a mixture of A1 (10 ng) and A2 (10 ng); and a mixture of A1 (100 ng) and A2 (100 ng) and a 12-hr-old FIL extract (10 L1E). The control consisted of 1 μl of *n*-hexane, and seven repetitions were made for each stimulus. For statistical analysis, the non-parametric U test of Mann and Whitney was used.

Chemical Analyses. Gas chromatography (GC) was performed with a HP 5890 Hewlett Packard chromatograph equipped with a Ross injector system fitted with a 25-m × 0.32-mm-ID CPSil8CB, WCOT; column temperature: 180°C (carrier gas: helium at 1 kg/cm^2). Gas chromatography–mass spectrometry (GC-MS) was carried out with a Nermag R-10-10C quadrupole mass spectrometer (electron impact at 70 eV) coupled with a Girdel 32 gas chromatograph equipped with a Ross injector. The system was fitted with a fused

silica capillary column (25 m WCOT CPSil5CB, 0.32 mm ID, Chrompack, Middleburg, The Netherlands). The column temperature was 200°C, using helium as carrier gas at 1 kg/cm^2. Chemical ionization–mass spectrometric data were obtained using ammonia as reactant gas at 92.5 eV.

To determine the number of unsaturations in the molecules, catalytic microhydrogenation was done, using PtO_2 in methanol.

Micropreparative capillary gas chromatography was performed on a Girdel 300 (Malosse, 1990) fitted with a wide-bore fused-silica capillary column (5 m WCOT HP-1, 0.53 mm ID, 160°C, Hewlett-Packard). The carrier gas was helium at 0.3 kg/cm^2. The split system was a Y glass tube (Chrompack) with capillaries giving a split ratio of 85:15. The different fractions were collected in 18-cm × 0.8-mm-ID glass capillaries and cooled with a mixture of acetone and Dry Ice.

Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker 600 MHz spectrometer. Samples separated by micropreparative GC were rinsed from collection tubes with C_6D_6 (99.93% CEA, France). After analysis, the different fractions were used to test their activity on ants.

Gas chromatography-infrared (GC-FTIR) was carried out with a IFS-85 Bruker coupled to a HRGC 5160 Carlo Erba chromatograph (on-column injector) fitted with a fused-silica capillary column 30 m WCOT DB-5, 0.32 mm ID, heated from 30 to 170°C (10°C/min) and from 170 to 220°C (5°C/min), with helium as carrier gas. The light pipe was heated at 220°C.

RESULTS

Field Bioassay. Three kinds of behavior were observed when ants came in contact with the egg mass. First, predation of eggs: the ant stops, antennates the egg mass, and picks off an egg with its mandibles with a few seconds and carries it away. Secondly, repulsion: the ant avoids the egg mass or, after a short period of antennation (less than 2 sec), it quickly moves away from the egg mass, then stops and cleans its antennae with its forelegs. At last, indifferent behavior: the ant arrives close to the eggs but continues without stopping or antennating.

Table 1 shows that in most cases, ants refused the egg mass treated with FIL extract (1 L1E/egg). In contrast, when exposed to solvent control or to the 1 L2E/egg extract, ants always accepted eggs as prey after antennation. The proportion of indifferent behavior is less significant for the treated eggs. That means that a close contact is not necessary, indicating a relative volatility of the chemicals involved in this host–prey relationship.

Laboratory Bioassay. Figure 2 shows clearly the significant results of the tests. Eggs treated with FIL extracts (FIL of ages less than 12 hrs or 5 days)

TABLE 1. PREDATORY RESPONSE OF *Solenopsis geminata* TO LARVAL EXTRACTS OF *Diaprepes abbreviatus* DISPOSED ON EGG MASSES, AT PHYSIOLOGICAL DOSES

Treatment[a]	Acceptance of eggs	Indifferent behavior	Repulsion
Solvent (a)	15	13	2
L1 extract (b) (1 L1E/egg[b])	2	5	23
L2 extract (a) (1 L2E/egg)	14	15	1

[a] Treatments followed by the same letter do not differ significantly (χ^2 test, $P < 0.05$).
[b] One first-instar larva equivalent per egg.

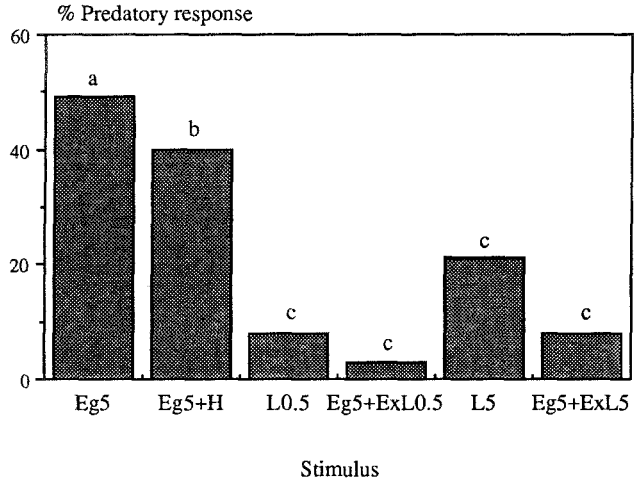


FIG. 2. Degree of predation by *Solenopsis geminata* upon *Diaprepes abbreviatus*. Results based upon the means of tests with 20 ants per nest, with three different nests per stimulus. Eg5: 5-day-old eggs; Eg5+H: 5-day-old eggs + hexane; L0.5: 0.5-day-old first-instar larvae; Eg5+ExL0.5: 5-day-old eggs + extract of larvae 0.5 days old; L5: first-instar larvae 5 days old; Eg5+ExL5: 5-day-old eggs + extract of larvae 5 days old. Histogram bars with the same letter are not significantly different (χ^2 test, $P < 0.05$).

are less preyed upon than untreated eggs or eggs with hexane. Similarly, 0.5-day-old and 5-day-old first-instar larvae are preyed upon less than eggs. This confirms that first-instar larvae produce a chemical repellent that is active immediately after hatching.

There is a significant difference between the responses of ants to 5-day-old eggs and 5-day-old eggs treated with hexane. Hexane appears to make eggs less attractive. In the majority of the tests, predation seems slightly less on eggs +

larval extracts than on larvae of the corresponding age. It is possible that hexane dissolves phagostimulant compounds present at the surface of the chorions.

Figure 3 shows the evolution of the predatory response with the age of the larvae used for the tests or for the extracts. In both cases, we can see that direct predation is low (less than 30%) at any age. Repulsion varied from 30% to 70%. Indifferent behavior decreases with the age of the larvae. It can tentatively be stated that predation increases with larval age, but there is no clear pattern for repulsive behavior.

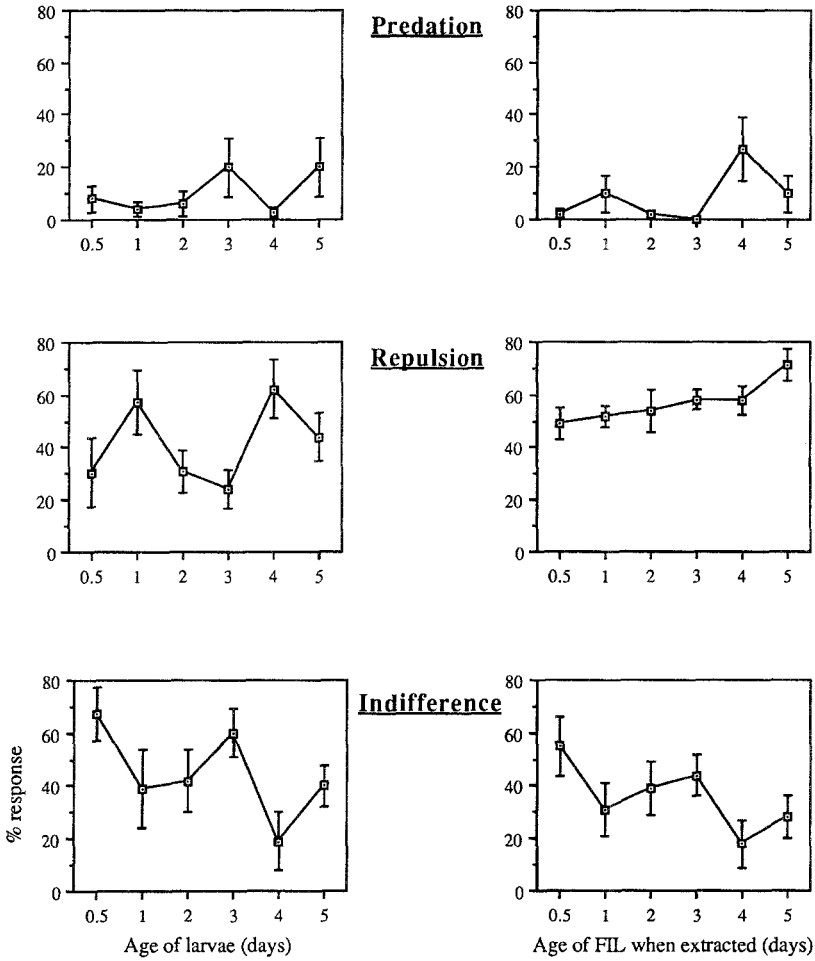


FIG. 3. Mean (±SEM) behavioral response of *S. geminata* towards *D. abbreviatus* first-instar larvae of different ages and egg masses treated with extracts of first instar larvae of different ages. An average of 170 ants were observed for each test.

The results with isolated compounds A1 and A2 are represented in Figure 4. A1 and A2 had no repellent activity at the dose of 10 ng. However, at 100 and 500 ng, their respective activities were significantly different from the control at the 5% level. The repellent activity of the whole extract was not different from the activities of each compound. The mixture of the two compounds was no more active than the compounds alone.

Chemical Analyses. Chromatograms of the *D. abbreviatus* first-instar larvae extracts showed the existence of two main peaks: A1 (65%) and A2 (35%) (Figure 5), which are nearly absent in the second-instar larvae and egg extract chromatograms. In addition to these specific compounds present in FIL extracts, hydrocarbons and saturated and unsaturated hexadecanoic acids were detected in all extracts.

According to the quantitative GC results, concentration of the repellent appears to decrease with the time, being at its highest in neonates and apparently absent from larvae after about four days (Figure 6). The results of the tests with *S. geminata* show some consistency with this result.

Micropreparative capillary GC led to the isolation of ca. 150 μg of compound A1 and 50 μg of compound A2 from 5000 first-instar larvae. The low resolution GC-EI-MS spectrum of A1 shows ions at *m/z* (% of relative abundance): 218 (M+, 38), 203 (29), 200 (5), 187 (52), 175 (21), 161 (13), 148

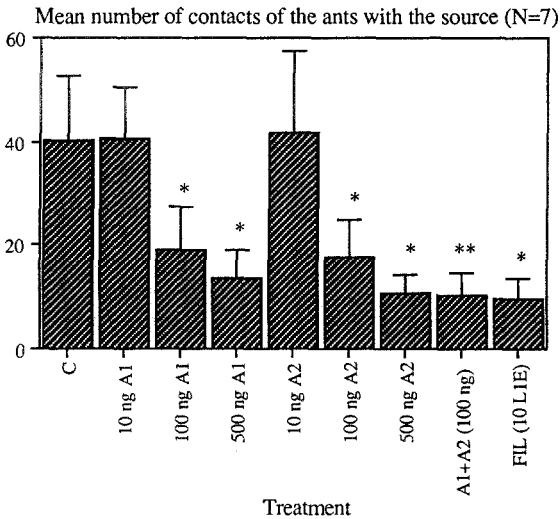


FIG. 4. Repellent activity of diaprepal A1 and A2 on *Solenopsis geminata*. C: control (1 μl of hexane); FIL (10 L1E): 1 μl of 12-hr-old FIL extract, corresponding to 10 FIL. Bars with asterisk are significantly different from the control, Mann and Whitney test (*$P < 0.05$, **$P < 0.01$).

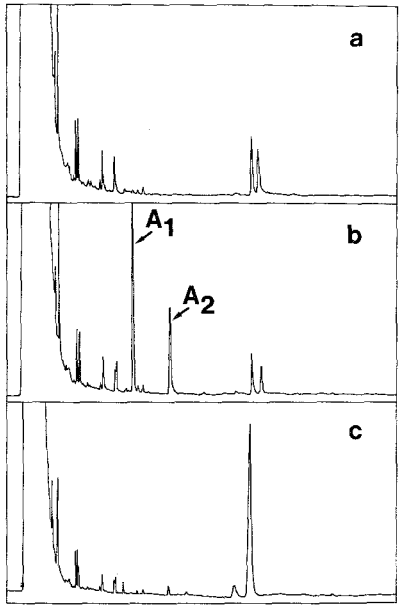


FIG. 5. Chromatograms of *Diaprepes abbreviatus* extracts: (a) egg extract; (b) first-instar larvae extract; (c) second-instar larvae extract. (HP 5890 chromatograph equipped with a Ross injector, system fitted with a 25-m × 0.32-mm-ID (CPSil8CB); column temperature: 180°C; carrier gas: helium at 1 kg/cm^2).

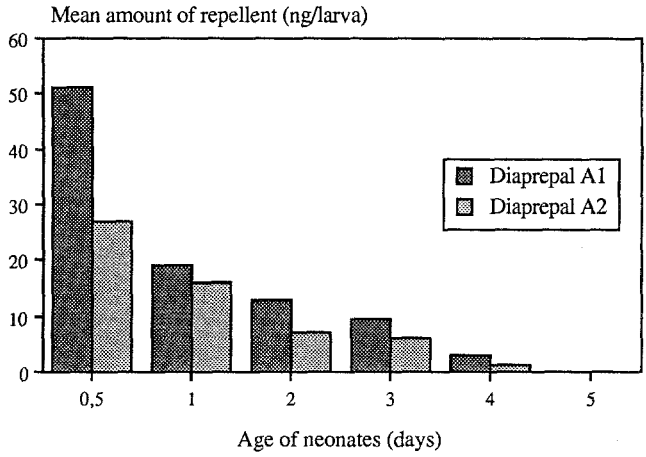


FIG. 6. Mean amounts of repellent extracted from *Diaprepes abbreviatus* first-instar larvae, at different ages.

(18), 147 (18), 133 (11), 119 (13), 107 (39), 91 (46), 79 (100), 67 (93), 55 (56), and 41 (73). GC (NH_4) CI-MS spectrum giving ions at *m/z* 236 ($(M+NH_4)+$, 31), 219 (MH+, 100), 201 ($(MH-H_2O)+$, 5), and 187 (4) confirmed the molecular formula as $C_{15}H_{22}O$ (HRMS calculated for $C_{15}H_{22}O$: 218,1658, found: 218,1658).

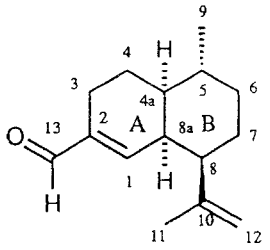
Catalytic microhydrogenation gave ions at *m/z* 222 (M+, 2), 204 (24), 191 (2), 179 (80), 161 (100), 119 (11), 109 (11), 105 (22), 95 (15), 81 (49), 69 (54), 55 (48), and 41 (56), indicating the occurrence of two unsaturations. The oxygen function was assigned to an aldehyde group regarding data from GC-FTIR showing absorption at 2800, 2708, 1705, and 1381 cm^{-1}. GC-EI-MS (loss of 43 amu) and GC-FTIR (absorption at 897 cm^{-1}) also confirmed the presence of an isoprenyl group. We proposed for the structure of compound A1, called diaprepal A1, a monounsaturated naphthane skeleton with three substituents: an aldehyde group, a methyl group, and an isoprenyl group. The final structure has been determined by ^1H NMR spectrometry (600 MHz). One-dimensional (1D) extensive decoupling and 2D COSY experiments led us to propose the structure represented on Figure 7.

Stereochemistry of the *cis* decalinic system is surmised from the observation of a small coupling constant between H-4a and H-8a (J = 3 Hz). Determination of the equatorial position on the B ring of the methyl and of the isoprenyl group is provided by measurements of characteristic *trans* diaxial coupling constants between H-5 and H-6α, H-5 and H-4a, and between H-7β and H-8. (9, 11, and 11.5 Hz, respectively). Proton H-8a is in an equatorial position in ring B, according to the coupling constant between H-8 and H-8a (J = 2 Hz). Location of the double bond (α to the ring junction) is obtained by observation of two ^5J coupling constants characteristic of the cyclohexene ring between H-8a and both H-3 (J = 6 and 3 Hz), the latter being in an allylic position according to their geminal coupling constant (J = 21 Hz) and coupled with both H-4. No coupling constant is detected between H-8a and H-1 owing to a dihedral angle of 90° between the two bonds H-8a—C-8a and H-1—C-1 (Figure 8a).

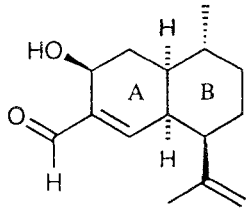
^1H chemical shifts (δ ppm): 1.12 (m, H-5); 1.2 (m, H-6α); 1.68 (m, H-6β); 1.69 (m, H-7β); 1.00 (bt, H-7α); 1.27 (dt, H-4a); 2.07 (bd, H-8); 2.65 (bs, H-8a); 2.22 (tm, H-3α); 2.57 (bdd, H-3β); 2.00 (dm, H-4α); 1.4 (tdd, H-4β); 6.55 (bs, H-1); 5.22 (s), 4.95 (s) (2H, H-12); 9.55 (s, H-13); 0.95 (d, 3H, H-9); 1.85 (bs, 3H, H-11).

Coupling constants were: $J_{4a,5}$ = 11 Hz; $J_{5,6\alpha}$ = 9 Hz; $J_{6\alpha,6\beta}$ = 10.5 Hz; $J_{6\alpha,7\beta}$ = 10 Hz; $J_{7\alpha,7\beta}$ = 12 Hz; $J_{7\beta,8}$ = 11.5 Hz; $J_{8,8a}$ = 2Hz; $J_{8a,3\alpha}$ = 3Hz; $J_{8a,3\beta}$ = 6 Hz; $J_{3\alpha,3\beta}$ = 21 Hz; $J_{3\alpha,4\beta}$ = 11 Hz; $J_{3\beta,4\beta}$ = 6 Hz; $J_{4a,4\beta}$ = 2 Hz; $J_{4a,4\alpha}$ = 3Hz; $J_{4\alpha,4\beta}$ = 15 Hz.

The GC-EI-MS spectrum of the minor compound (A2) gives ions at *m/z* 234 (M+. 14), 219 (3), 216 (55), 205 (14), 201 (25), 191 (14), 187 (43), 173 (27), 159 (25), 146 (38), 131 (26), 119 (14), 107 (39), 91 (62), 79 (87), 68



Diaprepal A_1



Diaprepal A_2

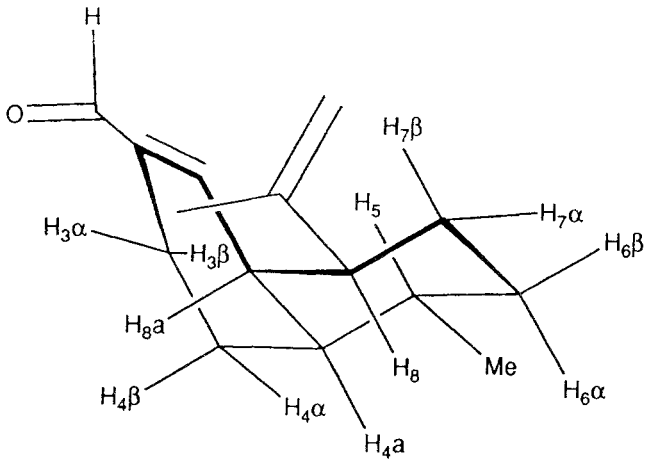


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FIG. 7. Diaprepal A1 and A2 from first instar-larvae of *Diaprepes abbreviatus* (*spatial representation of A1).

(90), 67 (100), 55 (88), 41 (84), and GC (NH_4) CI-MS, ions at *m/z* 252 [(M+NH_4+), 27], 235 (MH+, 27), 234 (M+, 46), 217 [(MH−H_2O)+, 100], 199{[(MH−H_2O)−H_2O]+,4} and 187 (4).

In agreement with these data, compound A2 has $C_{15}H_{22}O_2$ as its molecular formula. The second oxygen function was assigned as a hydroxy group. The GC-FTIR spectrum exhibiting absorptions at 3595 cm^{-1} and 1026 cm^{-1}. Lowering of absorption at 3595 cm^{-1} and also at 1695 cm^{-1} (compared to 1705 cm^{-1} for compound A1) indicates an intramolecular hydrogen bond between the hydroxy and the carbonyl group.

Close spectral NMR data of A1 and A2 led us to propose for compound A2 the same structure as diaprepal A1 with an additional hydroxy group at C-3 as represented in Figure 7. Owing to the fact that H-8a is in an equatorial position in the B ring and that H-3 on the carbon bearing the hydroxy group H-bonded to the aldehyde function has a coupling constant with only one proton H-4, the β position of the hydroxy group seems to be the more suitable (Figure 8b).

^{1}H chemical shifts (400 MHz, C_6D_6) δ (ppm) were as follows: 9.15 (s, H-13); 6.37 (s, H-1); 5.03 (s), 4.76 (s) (H-12); 4.48 (d, H-3); 2.3 (dd, H-4β); 2.27 (m, H-8a); 1.61 (s, H-11); 1.12 (s, H-9); and 0.82–2.1 (m, 8H, H-6, H-7, H-5, H-4α, H-4β).

Coupling constants were: $J_{3,4\alpha}$ = 5.8 Hz; $J_{4\alpha,4\beta}$ = 11 Hz; $J_{4\beta,4a}$ = 3 Hz.

DISCUSSION

In this study, we report for the first time the presence of a defensive secretion produced by a curculionid larva: the first-instar larvae of a citrus root weevil, *Diaprepes abbreviatus*, produce semiochemicals that have a repellent effect upon an important predator in citrus groves, *Solenopsis geminata*. The repellency reduces predation by about 40%. These allomonal compounds are not detected

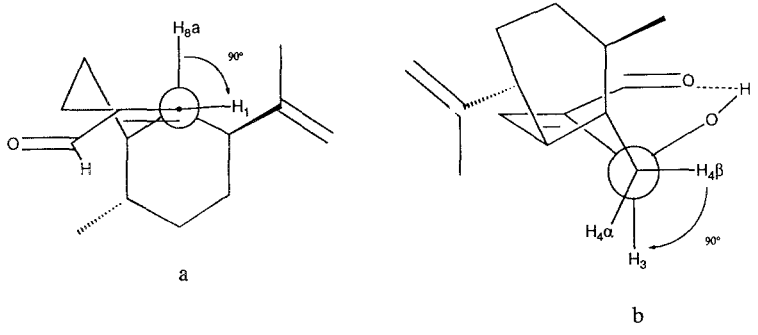

FIG. 8. Spatial representation of diaprepal A1 (a) and A2 (b).

in the second instars, nor in the egg masses. In the citrus trees, egg masses are stuck between two leaves, and they are not easily preyed upon by ants. When they drop on the soil, the neonates are very vulnerable, and their secretions allow them to be protected against their main predators.

For *D. abbreviatus*, the secretion has been identified as a mixture of two bicyclic sesquiterpenes aldehydes, in the proportion 65% and 35%. They have not been identified previously and we call them diaprepal A1 and A2. Sesquiterpenes are very frequent in plants and insects, and they can have different functions, such as alarm pheromones in aphids (Blum, 1969) or sex pheromones in Pentatomidae (Baker et al., 1987). Moreover, caryophyllene and caryophyllene epoxyde (terpenoids produced by neotropical plants) are known to be deterrent to leafcutter ants (Howard et al., 1989). Tricyclic sesquiterpenes (zizanal and epizizanal) (Jain et al., 1982) and sesquiterpene aldehydes (cadinenal, dehydroxycadinenal) (Plavkan, 1985, in Davidson et al., 1990), present in the essential oil of *Vetiveria zizanoides*, have potent topical irritant activity on cockroaches and flies.

It would be interesting to extend the investigation by testing for the same or related repellents in other *Diaprepes* species or in other curculionids. The repellent appears to be effective against the predator as long as necessary under natural conditions, because, as already mentioned, the neonates generally remain on the soil surface and are subject to predation by ant species for less than 3 hr. In these species, all living stages are relatively protected against predators, except the young emerging adults on which natural regulation by predatory ants seem to be more effective than on neonates.

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BEHAVIORAL RESPONSES TO OWN AND OTHER SPECIES' SCENT MARKS IN *Lemur fulvus* AND *Lemur macaco*

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Abstract—Behavioral responses (duration and frequencies of sniffing, scent-marking activity) were studied in one captive group each of *L. fulvus* and *L. macaco* when confronted with their own and other species' scent marks. Both species showed less interest in neutral objects or objects carrying their own group's odor than odors from an unfamiliar group of the same species or from another lemur species. The persistence of scent marks was also investigated. *L. fulvus* scent appeared to possess greater longevity than *L. macaco* scent. Olfactory long-term memory was analyzed in *L. fulvus*. The results showed that familiar rejected group members could be discriminated from an unfamiliar conspecific through odor cues 10 months after their eviction from the group.

Key Words—Olfactory discrimination, scent marking, Primates, *Lemur fulvus*, *Lemur macaco*, olfactory memory, persistence of marks.

INTRODUCTION

Lemurs are particularly important for the understanding of the evolution of communicatory systems in primates and more generally in mammals. They have conserved a number of primitive behavioral characteristics, one of which is their ability to communicate through olfactory cues, especially scent marks (Jolly, 1966; Tattersall, 1982). *Lemur macaco* and *Lemur fulvus* live in multimale, multifemale groups (number of individuals per group: 6–18; Petter et al., 1977;

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Tattersall, 1977). They have well developed visual and acoustic systems for communicating, but scent marking has retained an important role: in both species, adult individuals of both sexes perform anogenital marking, allomarking being only performed by males.

Surprisingly, only a few studies have addressed the information content of scent marks in *Lemur* species (Schilling, 1979). In *L. fulvus*, it has been shown that adults can discriminate individuals by scents (Harrington, 1976) and that males can discriminate males' scent marks from those of females (Harrington, 1974, 1977). However, the same studies failed to show evidence of subspecific discrimination or of male discrimination of estrous vs. anestrous females by scent only. To our knowledge, no such studies have been carried out in *Lemur macaco*. In *Lemur fulvus*, the studies were done using individual odors. In both species, several adult individuals often deposit their secretions on the same places in the environment. It seems likely therefore that under natural conditions, the animals are confronted with a mixture of individual scent marks.

In a long-term correlational study in these two species we did not find a significant change in scent-marking frequencies during the breeding season, or a direct relation between scent marking and emitted aggression (Fornasieri and Roeder, 1993). The results suggested that qualitative rather than quantitative changes in scent might play a role in social and reproductive processes, by transmitting information about the emitter. In order to test this hypothesis, we assessed the informative content of scent marks by recording behavioral responses towards various scent marks. The specific aims of this comparative study were to investigate discrimination between conspecific and heterospecific group odors in *L. macaco* and in *L. fulvus*, to study the persistence of scent marks in the two species, and to investigate the possibility of olfactory long-term memory in *L. fulvus*.

METHODS AND MATERIALS

The subjects were two groups of *L. macaco macaco* and *L. fulvus mayottensis* formed from unrelated wild-born adults and their offspring born in captivity. The *L. macaco* group contained one adult male (Q), two adult females (P, R), and their offspring (three males: pe, ro, and ram, which were 24, 18 and 7 months old, respectively, at the start of the experiments). The *L. fulvus* group contained two adult males (B, M), five adult females (H, R, V, P, F), one young male (fo), and one young female (he), 12 and 24 months old, respectively. The animals were individually identified using differences in coat color and condition and by distinctive features such as tail shape or facial scars. Each group was housed at the Centre de Primatologie de l'Université Louis Pasteur (Strasbourg, France) in a spacious indoor–outdoor cage (indoors: 4.90 × 2.10

× 2.60 m, outdoors: 3.00 × 2.90 × 2.60 m) furnished with three trunks providing aerial walkways, and two wooden boxes attached to the walls of the indoor area for use as resting sites. Food (commercial primate pellets) and water were available ad libitum, and fresh fruits and vegetables were provided twice per week.

During the study, two *L. fulvus* females (P, F) were removed from the group following persistent serious targeting aggression (see Vick and Pereira, 1980); they were housed temporarily in individual cages under the same conditions. Another captive group of each species, housed under similar conditions at the Laboratoire d'Embryologie de la Faculté de Médecine de Strasbourg was used as a scent donor. The *L. macaco macaco* donor group consisted of one adult male and two adult females, and the *L. fulvus mayottensis* donor group contained one adult male and one adult female. A conspecific was considered "unfamiliar" for the receiver group if it had never had any direct olfactory contact with the group before the experiment.

Experiment 1: Conspecific and Heterospecific Discrimination. In both groups, the reaction of each individual to conspecific and heterospecific scent marks was observed. Olfactory stimuli were collected on plastic boxes (10 × 10 × 10 cm) placed in the donors' cage 8–15 days before the test session. Concurrent observations were done to confirm that the donors scent marked the boxes. A mark mainly consisted of secretion of the anogenital area (skin glands), but urine and feces could also come in contact with the boxes. After removal from the donor's cage, the latency until scent marks were presented to the subjects never exceeded eight days. In each group, daily sessions were given on 20 successive days. During each session, each group was presented successively with four types of olfactory stimuli in random order: control unscented boxes, familiar conspecific scent (the group's own scent), unfamiliar conspecific scent (the scent of an unfamiliar conspecific group) and heterospecific scent (*L. macaco* scent for *L. fulvus* and vice versa). Two successive presentations during the same session were separated by a 5-min interval. During each test, the box was placed on the cage floor for a 15-min period, previous observations having shown that the animals were no longer interested after this period. Between each session, new boxes were used or previously used boxes were cleaned with alcohol and always served for collecting the same odors. All sessions occurred outside the females' estrous period.

For each individual, the frequency and duration of sniffing the box and frequency of marking on the box were recorded. The occurrence of scent marking elsewhere in the cage was also noted. The durations of sniffing and marking frequencies in the different conditions were compared using a Mann-Whitney U test. Sniffing duration and frequency were always strongly positively correlated, therefore analysis only used the duration measure.

In the *L. fulvus* group, the two subordinate females, P and F, were not

included in the analysis due to their infrequent response to the stimulus boxes, which did not allow statistical analysis. Similarly, young individuals were included in statistical analysis only when their response rates were sufficiently high, as they generally showed reduced interest towards the stimulus boxes compared to adults. During the overall experiment period, some juvenile individuals began to perform scent marking and therefore are or are not represented on the figures.

Experiment 2: Persistence of Scent Marks. The persistence of scent marks was investigated by recording the discriminatory responses to scent marks of differing ages. Each individual's responses towards an unfamiliar group's scent marks were compared to those directed towards the group's own scent marks of the same age. Scent marks were collected according to the usual procedure (boxes staying eight days in the donors' cage) and were stored in contact with air until the test. A series of 10 trials was conducted for each age. A trial consisted of the simultaneous presentation of the two stimuli (own group marks/ unfamiliar group marks). The same behavioral measures as above were recorded. In the *L. fulvus* group, a single series of tests was conducted using 3-month-old scent marks. In the *L. macaco* group, three series were conducted using 3-month, 5-week, and 15-day-old marks, respectively. All the series were done outside the breeding season. For statistical analyses we used the Wilcoxon *T* test.

Experiment 3: Olfactory Long-Term Memory. In this experiment, the possibility of olfactory memorization of familiar rejected groups members' scent marks was investigated in *L. fulvus*. Two females (P, F), which had been members of the group since its formation (four years earlier), were removed from it due to persistent and violent targeting aggression and served as scent donors. Two different females were used as donors for unfamiliar scent marks. All the donor females were isolated and housed under the same conditions, receiving the same diet. Scent marks were collected on plastic boxes introduced in the donors' cage several days before the test and removed just before the beginning of a test. Discrimination between fresh scent marks of one of the previously rejected females and scent marks from an unknown female was tested in the group. Four series of 20 tests were conducted with the scent from each of the rejected females. Each test consisted of the simultaneous presentation of the two stimuli (familiar vs. unfamiliar). These four series were carried out during three testing periods: 1–6 months, 8–10 months and 10–12 months from the date of the donors' removal from her group. The measures recorded were the same as in the preceding experiments. The second testing period was the only one during which females were receptive. No test was conducted on days when copulation was observed. Statistical analysis used the Wilcoxon *T* test.

RESULTS

Experiment 1. In both species, sniffing durations were significantly higher when investigating scents from unfamiliar conspecifics and from a heterospecific group than when investigating the control stimulus (Figure 1a and 2a). The only

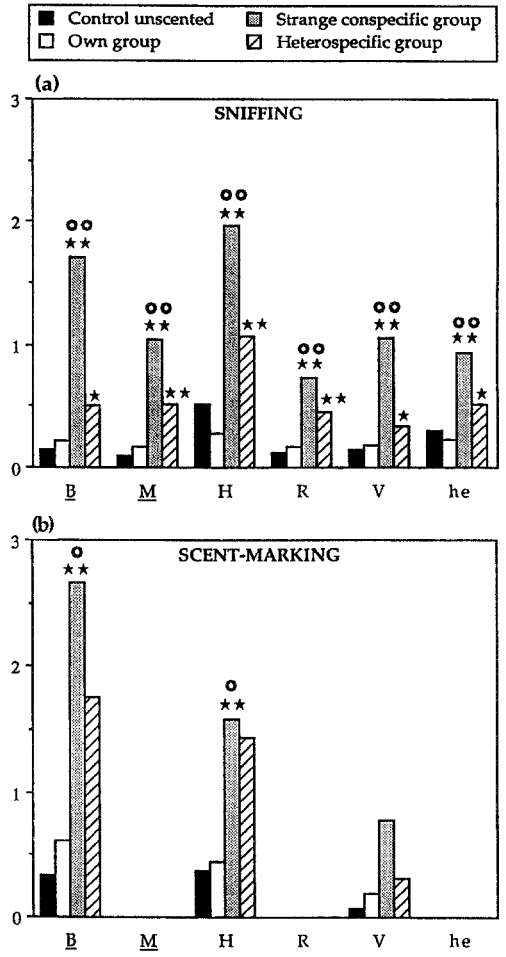

FIG. 1. Olfactory discrimination by *L. fulvus* individuals of various stimuli of conspecific and heterospecific origin: (a) mean sniffing duration (in minutes) and (b) mean scent-marking frequency (Mann-Whitney U test: comparisons with control ★:$P < 0.05$, ★★: $P < 0.01$, comparison familiar conspecific/strange conspecific: ✪: $P < 0.05$, ✪✪: $P < 0.01$). Male subjects are underlined.

exception was the male *L. macaco* (Q), who did not show more interest towards heterospecific scent marks compared to the control. In all cases, the duration of sniffing the group's own odor was not statistically different from that sniffing the control. Both species showed significantly more interest in unfamiliar conspecific group odors than in their own group odors. The comparison between unfamiliar conspecific and heterospecific scent marks showed that in *L. fulvus*, three individuals were significantly more interested in the former (Mann-Whit-

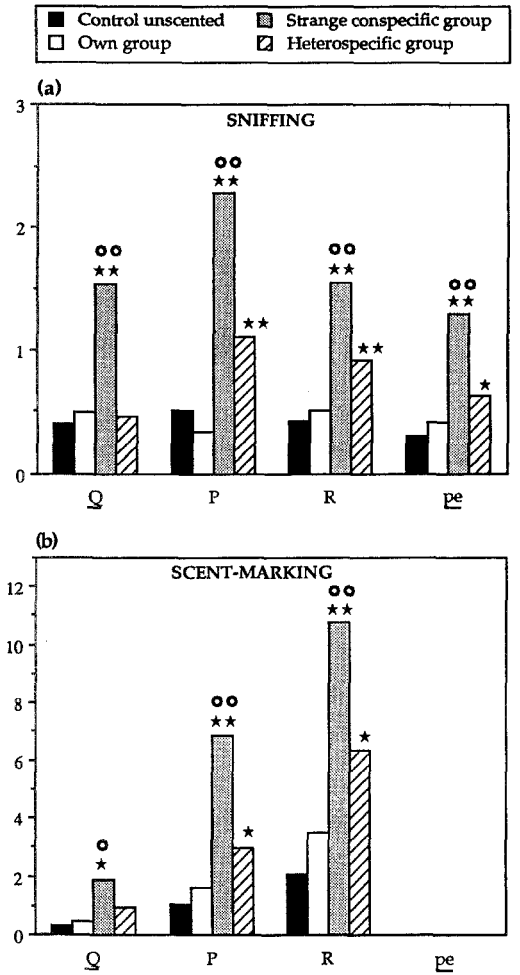

FIG. 2. Olfactory discrimination by *L. macaco* individuals of various stimuli of conspecific and heterospecific origin: (a) mean sniffing duration (in minutes) and (b) mean scent-marking frequency (Mann-Whitney U test: comparisons with control ★:$P < 0.05$, ★★: $P < 0.01$, comparison familiar conspecific/strange conspecific: ⭘: $P < 0.05$, ⭘⭘: $P < 0.01$). Male subjects are underlined.

ney B: n1 = 18, n2 = 17, U = 38; H: n1 = 19, n2 = 18, U = 74.5; V: n1 = 18, n2 = 13, U = 35; $P < 0.01$ in all cases). In *L. macaco*, this difference was statistically significant for all adult individuals (P: n1 = 17, n2 = 20, U = 65, $P < 0.01$; Q: n1 = 16, n2 = 17, U = 21, $P < 0.01$; R: n1 = 19, n2 = 17, U = 88, $P < 0.05$).

Marking responses were less clear, with important individual differences (Figures 1b and 2b). Two of the five adult *L. fulvus*, one male (M) and one female (R), never marked the boxes during the tests (the juvenile female did not yet perform anogenital marking at this test period). When scent marking did occur, it paralleled the sniffing response. Thus, the boxes most frequently marked were, in decreasing order, the unfamiliar conspecific stimulus then the heterospecific stimulus with no difference between the group's odor condition and the control. Three *L. fulvus* performed more scent marking on the box scented by unfamiliar conspecifics compared to all other conditions. This difference was statistically significant for two of them compared to control and to their own group's odor (Figure 1b). The difference was not statistically significant when comparing scent marking with unfamiliar conspecifics and heterospecific scents (B: n1 = 18, n2 = 16, U = 103; H: n1 = 19, n2 = 18, U = 152; V: n1 = 18, n2 = 13, U = 88; ns in all cases).

All *L. macaco* adults performed scent marking on the boxes. In this group, marking responses also paralleled sniffing responses (Figure 2b). A comparison between unfamiliar conspecific and heterospecific scents revealed a significant difference in only one female (R: n1 = 19, n2 = 17, U = 88, $P < 0.05$). No change in marking activity in the cage was observed in either species during the tests compared to their scent marking rates determined from regular observations done outside the test periods.

Experiment 2. In the *L. fulvus* group, the 3-month-old scent of the unfamiliar conspecific group still elicited more sniffing and marking than the group's own scent, although responses were weaker than with fresh marks. The difference in sniffing responses towards the two stimuli was statistically significant for each adult subject (Figure 3a,b). Scent marking responses gave a less clear picture, a significant difference emerging only in one individual (female H, Figure 3b).

In the *L. macaco* group, sniffing duration in response to the group's own 3-month-old scent marks did not differ from that obtained with unfamiliar conspecific scent marks. Five-week-old scent marks elicited no olfactory discrimination, sniffing responses appearing inconsistent (Figure 4c). Finally, only the third test series using 15-day-old scent marks resulted in significant discriminatory responses by some individuals: in three animals (Q, P, R), the unfamiliar group's scent marks were sniffed for significantly longer than the group's own marks; one individual (a juvenile male) showed more interest towards his group's scent marks than towards those of the unfamiliar group (Figure 4b). Such differences were not apparent when considering the mean marking frequencies.

Experiment 3. Surprisingly, the most important sniffing response was elicited by the familiar female's scent marks. During the first test period, all individual responses showed the same tendency. The difference for the whole group was statistically significant and persisted over the second period (Table 1). After

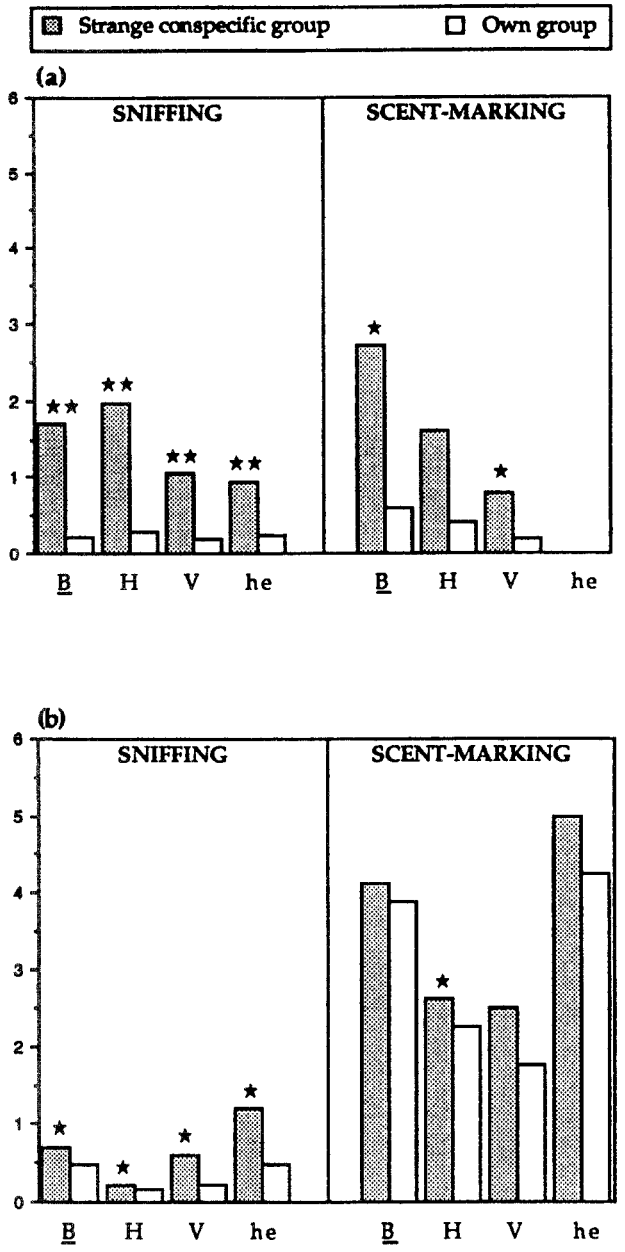

FIG. 3. Persistence of scent marks in *L. fulvus*: individual responses (mean sniffing duration in min and mean marking frequency) to (a) fresh and (b) 3-month-old scent marks (Wilcoxon T test: ★: $P < 0.05$, ★★: $P < 0.01$). Males subjects are underlined.

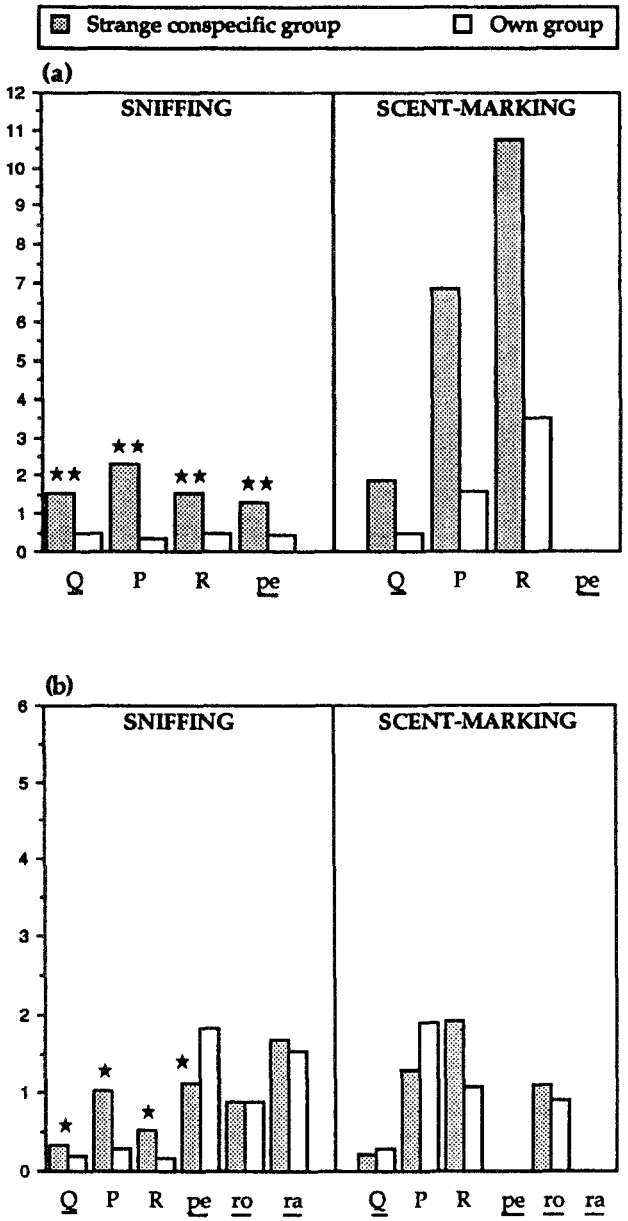

FIG. 4. Persistence of scent marks in *L. macaco*: individual responses (mean sniffing duration in min and mean marking frequency) to (a) fresh and (b) 15-day-old, and (c) 5-week-old scent marks (Wilcoxon *T* test: ★: $P < 0.05$, ★★: $P < 0.01$). Males subjects are underlined.

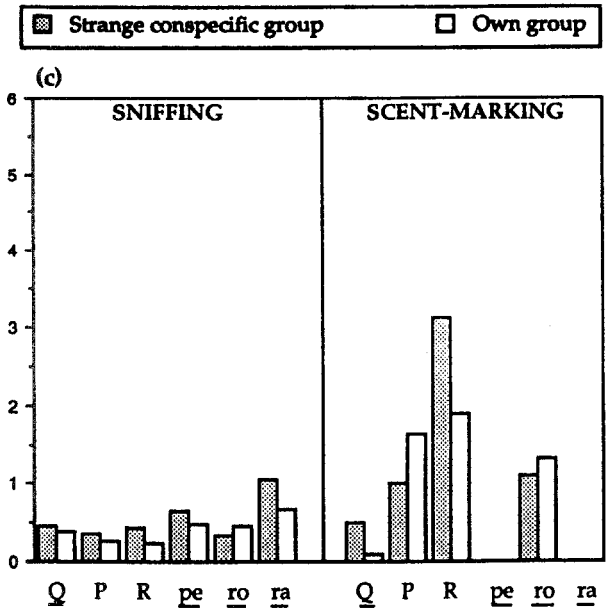

FIG. 4. Continued

TABLE 1. MEAN SNIFFING DURATION IN MINUTES (SD) OF FAMILIAR VS. CONTROL BOXES FOR THREE SUCCESSIVE TESTING PERIODS (1 = 1–6 MONTHS, 2 = 8–10 MONTHS, 3 = 12–14 MONTHS)

		Period 1				Period 2				Period 3			
		Familiar		Control		Familiar		Control		Familiar		Control	
Males	B	0.6	(0.4)	0.5	(0.4)	0.5	(0.3)	0.3	(0.3)	0.2	(0.2)	0.2	(0.1)
	M	0.4	(0.4)	0.3	(0.3)	0.4	(0.4)	0.3	(0.3)	0.2	(0.1)	0.3	(0.3)
	f	0.4	(0.4)	0.3	(0.3)	0.1	(0.1)	0.1	(0.1)	0.02	(0.02)	0.04	(0.03)
Females	H	1.2	(0.6)	0.8	(0.5)	0.8	(0.4)	0.5	(0.4)	0.5	(0.3)	0.5	(0.4)
	R	0.5	(0.4)	0.3	(0.3)	0.4	(0.2)	0.2	(0.1)				
	V	0.8	(0.5)	0.5	(0.4)	0.5	(0.4)	0.3	(0.4)	0.4	(0.3)	0.2	(0.2)
	h	0.9	(0.5)	0.6	(0.3)	0.6	(0.4)	0.3	(0.3)	0.3	(0.2)	0.2	(0.2)
Wilcoxon		$P < 0.001$				$P < 0.001$				NS ($T = 8$)			

one year, the difference in sniffing duration between the familiar and unfamiliar stimulus was no longer statistically significant for the group.

In general, the time course of marking responses paralleled that of sniffing responses: the familiar stimulus was scent marked significantly more frequently than the control stimulus up to a period of 10 months (first and second test period), but not after this time (due to low response rates, the juvenile male was dropped from the statistical analysis) (Table 2). Marking responses showed large individual variations. In particular, two adult females scent marked frequently during some test series but did not scent mark at all during others (at 6 and 10 months), although they sniffed the boxes. This temporary absence of scent marking extended to the entire cage environment. One adult male showed consistently low scent-marking rates on the boxes and at other marking sites, in parallel with a generally low response level. The juvenile male (fo) showed particularly variable sniffing and marking responses during these tests: he did not contact the boxes in more than 50% of tests, and what responses he did show were weaker than in adults. No significant differences were found for fo.

DISCUSSION

In both species, several individuals deposited their scent marks at the same location. Therefore, a receiver must analyze not only an individual mark but a mixture of different individual odoriferous substances.

Our results clearly demonstrate the possibility of conspecific and heterospecific discrimination based on odor cues in *L macaco* and *L. fulvus*. All

Table 2. MEAN SCENT-MARKING FREQUENCY (SD) ON FAMILIAR VS. CONTROL BOXES FOR THREE SUCCESSIVE TESTING PERIODS (1 = 1-6 MONTHS, 2 = 8-10 MONTHS, 3 = 12-14 MONTHS)

		Period 1				Period 2				Period 3			
		Familiar		Control		Familiar		Control		Familiar		Control	
Males	B	1.7	(2.2)	1.8	(1.5)	1.5	(1.9)	0.9	(1.1)	1.8	(1.3)	2.1	(1.7)
	M	0.6	(1.0)	0.1	(0.3)	0.9	(1.0)	0.7	(0.7)	0.9	(1.2)	0.8	(1.1)
	fo	0.03	(0.1)	0.1	(0.4)	0.1	(0.4)	0.05	(0.1)	0		0	
Females	H	2.1	(1.9)	1.9	(1.9)	0.8	(0.8)	0.6	(0.6)	2.5	(2.1)	2.3	(2.4)
	R	0.3	(0.5)	0.2	(0.5)	0		0					
	V	2.6	(2.4)	1.1	(0.9)	1.7	(2.1)	0.9	(1.3)	0.9	(1.1)	0.8	(1.1)
	he	2.2	(2.4)	1.3	(1.3)	2.7	(2.2)	1.5	(1.4)	3.0	(2.1)	2.3	(1.5)
Wilcoxon		$P < 0.05$ (without f)				$P < 0.001$				NS ($T = 4$)			

individuals in the two groups were able to differentiate not only between the scent of the other species but also that of an unfamiliar group of their own species, and their own group's odor. Over marking does not appear to have any effect on the behavioral response of the observed subjects as animals do not react differently to control objects and objects carrying their own group's odors. These results suggest that both species can recognize scent marks as being those of their own group, as the animals responded less to these than to marks of any other origin. It seems likely that in *L. macaco*, as demonstrated in *L. fulvus* and *L. catta* (Mertl, 1975; Harrington, 1976, 1977; Dugmore et al., 1984), other individual scent marks (e.g., a familiar vs. an unfamiliar conspecific) could be discriminated. Such studies remain to be conducted. Similar discriminatory abilities have been demonstrated in several other primate species (Epple, 1985; Epple and Smith, 1985), suggesting that the information content of scent marks or urine does not differ greatly between lemurs and other species.

In the present experiments, discrimination might have been related to differences in quantities of scent produced by the different groups. However, under natural conditions animals are not confronted with neatly quantified samples of scent. Instead, they must obtain specific information from a mixture of scents. It would be interesting to investigate whether species that are largely dependent on olfactory cues are able to discriminate one particular scent mark combined with others.

The low sniffing and marking rates observed towards control objects and a group's own scent marks suggest that scent marks may have a role in familiarizing the animals with their physical environment (Eisenberg and Kleiman, 1972). This interpretation is reinforced by the finding that striking increases in scent marking occur in unfamiliar environments (Fornasieri and Roeder, 1993). However, the function of scent marking in the maintenance of intra- and intergroup social relationships has not been well studied in the species used here. How such discriminatory abilities act under natural conditions to maintain intergroup spatial distribution and perhaps prevent hybridization remains to be studied.

Our results showed differences between *L. fulvus* and *L. macaco* in the persistence of scent marks. In *L. fulvus*, olfactory discrimination of 3-month-old unfamiliar conspecific vs. familiar scent marks occurred. In *L. macaco*, discrimination persisted until only 15 days after the collection of scent marks. The diminished olfactory response in *L. macaco* may be related to the lesser development of the anogenital glandular field (Rumpler and Oddou, 1970) and less frequent anogenital marking compared to *L. fulvus* (Koenders, 1990). Personal observations over a two-year period have also shown that the mean marking rate in *L. macaco* is low compared to that of *L. fulvus* except over some restricted and occasional periods (Fornasieri and Roeder, 1992). Our results are

in accordance with Koender's hypothesis (1990) that *L. macaco* should show a greater tendency towards the development of visual communication at the expense of olfactory cues than *L. fulvus*. The scent mark stimuli were stored indoors. It is not known whether persistence would be the same for scent marks deposited in the natural environment and therefore exposed to climatic conditions. However, the biological significance of such differences in scent mark persistence in free-living populations remains to be investigated.

In contrast to the results of the above-described experiments, the animals showed more interest towards the marks of previously known conspecifics than towards totally unknown conspecifics. They discriminated the scent marks of a rejected group member from those of an unfamiliar conspecific up to 10 months after the stimulus animal's removal from the group. Our results do not demonstrate that this discriminative ability was lost after this time, but the recorded behavioral responses no longer made this ability apparent. In comparison, similar studies have shown that such "long-term memory" lasts eight days in some rodents (Cheal et al., 1982) and 17 weeks in some carnivores (Roeder, 1983). Our results also argue for the possibility of individual recognition in *L. fulvus* through olfactory cues and that such cues may play an important role in the maintenance of their social organization, which appears to be characterized by frequent migration from one social group to another (Tattersall, 1977).

In general, scent marking responses were less consistent than sniffing responses, and showed larger intra- and interindividual variability. Some individual differences were related to social status. For example, the consistently low-marking adult male *L. fulvus* was subordinate to the other adult male and showed relatively low scent-marking rates at all times (Fornasieri and Roeder, 1993). Although the females usually showed quite high marking rates, the absence of marking responses during some experimental sessions occurred in parallel with a virtual absence of scent marking during the regular behavioral observations at the same time.

In olfaction-dependent species, the informative content of scent marks is traditionally assessed through discrimination tests. It is another problem to know how the animals use the information transmitted by olfactory cues. Are they able to discriminate one particular scent mixed with several others? Do they memorize (and if so, for how long) individual scent marks belonging to conspecifics with which they have had previous social experience? Responding to such questions will increase our understanding of the animals' olfactory language.

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DIVERSITY IN STEROIDOGENESIS OF SYMBIOTIC MICROORGANISMS FROM PLANTHOPPERS

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Abstract—Ergosta-5,7,24(28)-trien-3β-ol (trienol) **6** was isolated from the intracellular symbiotes (symbiotic microorganisms) of the planthoppers, *Nilaparvata lugens* and *Laodelphax striatellus*. The steroidogenic end product of the symbiotes was found to depend on the environmental conditions, i.e., although trienol **6** was produced under symbiotic conditions, ergosterol **4** was formed in aerobic culture. When the normal diet was replaced by a steroid-deficient artificial one, the ratio of 24-methylenecholesterol **5** to total insect sterols was significantly increased. The above study offers further corroborating evidence for our assumption that the host insects require 24-methylenecholesterol **5** as an alternate source for cholesterol **1** under certain environmental conditions. In our previous biotransformation experiments, 24-methylenecholesterol **5** was shown to be an immediate precursor of cholesterol **1.** However, the complexity of the insects' vital functions hindered all subsequent attempted transformations into cholesterol **1** of sitosterol **2,** trienol **6,** and ergosterol **4,** under injection conditions.

Key Words—Ergosta-5,7,24(28)-trien-3β-ol, trienol, intracellular symbiotes, steroidogenesis, planthoppers, *Nilaparvata lugens*, *Laodelphax striatellus*, Homoptera, Fulgoridae, steroid-deficient diet.

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INTRODUCTION

Insects cannot biosynthesize steroids (Clayton, 1964), which are essential membrane components and the precursors of molting hormone ecdysteroids. Therefore insects' steroid requirement is usually met by dietary sterols, such as cholesterol **(1)** and/or phytosterols. The senita cactus fly *Drosophila pachea* (Heed and Kirscher, 1965) and ambrosia beetle *Xyleborus ferrugineus* (Chu et al., 1970) are exceptional in requiring sterols with a 7-ene or a 5,7-diene system for metamorphosis to adults. Phytophagous insects are generally capable of dealkylating the side chains of phytosterols (Svoboda and Thompson, 1985; Ikekawa, 1985), such as sitosterol **(2),** stigmasterol, and campesterol to produce cholesterol via desmosterol **(3).** On the other hand, some homopterous insects, exemplified by planthoppers, may rely on their obligatory intracellular symbiotes in addition to their diet as a steroidal source. For example, the brown planthopper (*Nilaparvata lugens* Stål) and the small brown planthopper (*Laodelphax striatellus* Fallén), which host yeastlike symbiotes (Nasu, 1965), can live on an artificial diet without sterols. In these insects, the symbiotes are transmitted from the mycetocytes in the host's fat body through the ovary to the next host generation (Noda, 1977).

Previously, we reported that the yeastlike symbiotes (*Candida* sp.) Nl-1 and Nl-2 from *N. lugens*, and Ls-1 and Ls-2 from *L. striatellus* synthesize sterols to meet the host's need (Eya et al., 1989). The yeastlike symbiotes produced ergosterol **(4)** as the sole product when cultured. A comparative study of the steroids present in the cultured symbiotes, the normal and aposymbiotic insects, and dietary rice plants (*Oriza sativa* L. Koshihikari) led to the hypothesis that ergosterol is the key steroid provided to the host insects and is converted by the host via 24-methylenecholesterol **(5)** to cholesterol **1.** The biotransformation of injected 24-methylenecholesterol-(23,23,25-d_3, **5**-d_3) into cholesterol-d_3 by *N. lugens* supported this hypothesis (Eya et al., 1989).

We report here subsequent findings to further clarify previous results. Ergosta-5,7,24(28)-trien-3β-ol (trienol) **(6)** has now been isolated and identified as the major sterol component of the symbiotes, which were separated from the host insects by Percoll density gradient centrifugation; this also showed that our previous identification of ergosterol **4** in the host insects required revision. Namely, careful reinvestigation of mass spectra with the aid of capillary GC-MS revealed that the compound previously identified as ergosterol **4** in the host insects was in fact trienol **6,** a precursor of ergosterol in fungi, including *Saccharomyces cerevisiae* (Barton et al., 1972) and *Phycomyces blakesleeanus* (Goulston and Mercer, 1969). As yet we have not been able to secure direct evidence for the conversion of trienol **6** into cholesterol **1** via 24-methylenecholesterol **5**; all attempts to transform trienol **6** (symbiote origin), ergosterol **4** (feasible symbiote origin), and sitosterol **2** (plant origin) into cholesterol **1** by

injection were hindered by the complexity of the insects' vital functions. However, 24-methylenecholesterol **5** was shown to be a readily acceptable intermediate in cholesterol **1** synthesis (Eya et al., 1989).

Incubation of the symbiotes, separated from their host by centrifugation, with the cell culture medium MGM-448 without fetal bovine serum (Mitsuhashi, 1984) at 28°C for 4 hr, did not change trienol content and produced no additional sterols. In addition, sterols will not be released into the medium in measurable quantities. Incubation of insect homogenate, at 28°C for 3 hr, with culture medium containing trienol **6**-d_7 did not induce deuterium incorporation into any other sterols. Thus, the requirement for symbiote sterol by *N. lugens* was reinvestigated by administration of a steroid-deficient artificial diet, as an easier alternative to ingestion of labeled phytosterol by hard-to-obtain aposymbiotic insects. When third-instar *N. lugens* were switched from a normal diet to a steroid-deficient artificial one, growth and molting were slightly disrupted, and the ratio of 24-methylenecholesterol **5** to total sterols was significantly increased. The present study offers further corroborating evidence for our assumption that the host insects require 24-methylenecholesterol as an alternate source for cholesterol under certain environmental conditions.

METHODS AND MATERIALS

Chemicals and Supplies. 24-Methylenecholesterol was a gift from Professors John Blunt and Murrary H.G. Munro, University of Canterbury, Christchurch, New Zealand. Other chemicals were obtained from the following commercial suppliers: 2-bromopropane-(d_7), MSD Isotopes; cholesterol, Nakarai Chemicals; desmosterol, Steraloids; Emulgen 913, Kao Co.; ergosterol, Wako Pure Chemical Industries; lanosterol, Nacalai Tesque; Percoll, Pharmacia LKB, Sweden; β-sitosterol and 3β-hydroxy-Δ^5-cholenic acid, Tokyo Kasei Kogyo. Other reagents were obtained from Nacalai Tesque or Wako Pure Chemical Industries. Thin-layer chromatography R_f values refer to elution of Merck silica gel 60 F254 plates of 0.25-mm layer thickness with ether–hexane 1 : 1. Flash chromatography was performed with Merck 230–400 mesh silica gel. Cell culture medium MGM-448 without fetal bovine serum was prepared according to the procedure outlined previously by Mitsuhashi (1984).

Insects and Dietary Plants. The insects *N. lugens* and *L. striatellus* were collected at Shimane Prefecture in Japan. The insects were reared on seedlings of their dietary plant, *Oriza sativa* L. in plastic cages at 25°C under photoperiodic conditions of 16 : 8 hr light–dark for successive generations.

Steroid-Deficient Artificial Diet. N. lugens were reared on their dietary plant up to the third instar and then switched over a steroid-deficient artificial diet (Mitsuhashi and Koyama, 1971) for one week. Within one week, the insects

reared on rice seedlings reached the fifth instar; however, growth of the experimental insects was restricted to the fourth instar.

Symbiotes. The yeastlike symbiotes were separated from the host insects according to the method of Noda and Omura (1991). Adults and nymphs (8 g) were homogenized with 80 ml of 0.85% brine, and the insect debris was removed by filtration through cotton cloth. The pellet obtained after centrifugation (5 min at 100*g*) was purified by repetition of the suspension and centrifugation steps. The suspension of the final pellet in 0.85% brine was treated with four times its volume of Percoll. The mixture was ultracentrifuged at 85,000*g* for 40 min on a Beckman Type 70 Ti angle rotor. The upper insect lipid and the lower symbiote fractions were lyophilized.

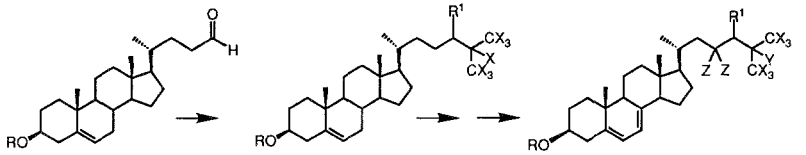
Fractionation of Sterols. Throughout the experiments involving the insects *N. lugens* and *L. striatellus* and their yeastlike symbiotes, exposure to light was minimized by use of aluminum foil. The procedure for sterol fractionation of insect and symbiote samples was essentially the same as that reported previously (Eya et al., 1989). In a typical experiment, ca. 200 insects were ground with a pestle and then homogenized by a Teflon homogenizer with chloroform–methanol (2 : 1, 5 ml) under ice cooling. After sonication for 30 min, the homogenate was centrifuged at 3000 rpm for 5 min. The supernatant was removed, and the solid residue was extracted in the same fashion again with fresh solvent (5 ml). The combined supernatants were concentrated to dryness. The residue was stirred magnetically with 2 M aq. potassium hydroxide (1 ml), methanol (1 ml), and tetrahydrofuran (1 ml) at reflux under a nitrogen atmosphere. The mixture was acidified with 0.2 M aq. hydrogen chloride (10 ml), and extracted with three portions of methylene chloride. The usual treatment of the combined organic extracts afforded 26.3 mg of light brown semisolid residue, which contained free sterols and fatty acids. After esterification of fatty acids with diazomethane–ether, the sterol fraction (0.1 mg) was separated by flash chromatography (10 × 150-mm column, ether–hexane 6 : 4) and chromatographed on HPLC [Cosmosil $5C_{18}$ column (Nacalai Tesque), 4.6 × 250 mm, methanol–water 95 : 5, flow rate 1 ml/min].

GC-MS of Sterols. The sterol fraction (0.1 mg) was treated with hexamethyldisilazane (100 μl) and chlorotrimethylsilane (50 μl) in pyridine (250 μl) for 30 min at room temperature under nitrogen. The reaction mixture was cooled to 0°C, diluted with pentane, and water was added. The aqueous layer was extracted twice with pentane to yield TMS-derivatized sterols, which were analyzed by GC-MS (Hewlett-Packard 5710 chromatograph connected to a Hitachi M-80 mass spectrometer). A 50-m Hewlett-Packard HP-5 cross-linked 5% phenyl methyl silicone gum phase column (0.2 mm ID) was employed. Other GC settings were as follows: column flow rate 0.4 ml/min, split ratio between 10 : 1 and 30 : 1, injector and auxiliary heater 250°C, column temperature program 200–290°C at 2°C/min. Mass spectrometer settings for sterol identification

were as follows: ionization voltage 70 eV, source slit 500 μm, collector slit 150 μm, scanning rate m/z 60–600 in 1.08 sec, rescan duration 1 sec, sampling time 25 μsec, multiplier 1500 V. The following mass spectrometer settings were employed for measurement of isotopic abundance ratios (MID mode): ionization voltage 70 eV, source slit 600 μm, collector slit 300 μm, scanning rate m/z 0–2000 in 128 sec, rescan duration 10 sec, sampling time 100 μsec, multiplier 1500 V. TMS sterols (R = TMS in **1, 2** and **4–6**) were identified from their mass spectra and by coinjection whenever authentic samples were available.

Preparation of Authentic Ergosta-5,7,24(28)-trien-3β-ol **6.** A $\Delta^{7,8}$ double bond was introduced into 24-methylenecholesterol: (1) benzoyl chloride, pyridine; (2) *N*-bromosuccinimide, azoisobutyronitrile (AIBN), CCl_4, reflux; (3) $(MeO)_3P$, CH_3CN; (4) 2% aq. KOH, MeOH, THF.

Synthesis of Labeled Sterols. Scheme 1 outlines the synthesis of ergosta-5,7,24(28)-trien-3β-ol-(25,26,27,d_7) (**6**-d_7, upper sequence) together with 23,23,25-d_3 (**6**-d_3, lower sequence). Grignard coupling of tetrahydropyranyl aldehyde (I) (Eya et al., 1989) with heptadeutero isopropyl magnesium bromide [from 2-bromopropane-(d_7)] in ether gave (II) (81%), which was oxidized to ketosteroid (III) (PCC, CH_2Cl_2, 81%). The THP protecting group was replaced by benzoyl (1. 1 N HCl-MeOH; 2. benzoyl chloride in pyridine) to afford (IV). Introduction of the Δ_7 double bond and the 24-methylene group of **6**-d_7 was accomplished according to the procedures that Fryberg et al. (1973) reported for the nondeuterated material. Compound **6**-d_7 was identified by the usual spectroscopic methods. Compound **6**-d_3 was obtained by deuteration of the intermediate (iv, X = Y = Z:H) four times in a solution of methanol-d_1 and 1% K_2CO_3 in D_2O for 20 min at 70°C. The deuterium incorporation was greater than 99% based on mass spectroscopy [overall yield, 4% from (i)].



(I) R: THP

(II) R: THP, R^1: OH, X: D
(III) R: THP, R^1: =O, X: D

(IV) R: Bz, R^1: =O, X=Y: D, Z: H
(V) R: Bz, R^1: $=CH_2$, X=Y: D, Z: H
6-d_7 R: H, R^1: $=CH_2$, X=Y: D, Z:H

(i) R: TBDMS

(ii) R: TBDMS, R^1: OH, X: H
(iii) R: TBDMS, R^1: =O, X: H

(iv) R: Bz, R^1: =O, X=Y=Z: H
(v) R: H, R^1: =O, X: H, Y=Z: D
6-d_3 R: H, R^1: $=CH_2$, X: H, Y=Z: D

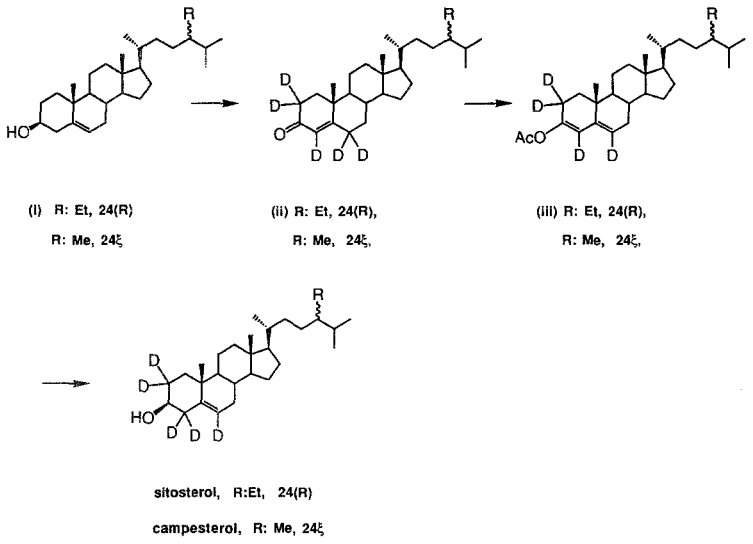
THP: tetrahydropyranyl
TBDMS: t-butyldimethylsilyl
Bz: benzoyl

SCHEME 1. Synthesis of ergosta-5,7,24(28)-triene-3β-ol-d_7 and -d_3 (**6**-d_7 and **6**-d_3).

For the preparation of sitosterol-(2,4,6-d_5, **2**-d_5) in Scheme 2, we adapted and improved upon Greunke and Craig's (1979) synthesis of cholesterol-(2,4,6-d_5) (**1**-d_5) to give the side-chain alkylated variant in greater isotopic purity and yield (> 90% d_5, 38% yield from sitost-4-en-3-one vs. 68% d_5, 18% yield for **1**-d_5 from cholest-4-en-3-one). A mixture of 4-en-3-ones (R = Et and Me), obtained in one step (Dow et al., 1983) from commercially available sitosterol (i: R = Et (R) 61%, R = Me (ξ) 39%), was exchanged with five portions of MeOD at reflux in the presence of NaOMe (Dufourc et al., 1984) to afford the corresponding mixture of (ii: R = Et and Me) in 84% yield and > 90% isotopic purity. Lithiation with Ph_3CLi (from PhLi and Ph_3CH, DME, r.t.), followed by injection of acetic anhydride, gave the thermodynamically favored heteroannular 3,5-dien-3-ol acetates (iii: R = Et and Me) in 70% yield. Sodium borohydride reduction (NaOD, D_2O, dioxane, 33°C; Barton et al., 1971), followed by reverse-phase HPLC separation gave pure sitosterol **2**-d_5 in 39% yield (63% based on pure iii-d_4, R = Et). Campesterol-(2,4,6-d_5) was also isolated in 27% yield (69% based on pure iii-d_4, R = Me). Structures were confirmed by spectroscopic and HPLC comparison with authentic, nondeuterated samples. Mass spectroscopic measurements showed that **2**-d_5 was > 90% isotopically pure.

RESULTS

Ergosta-5,7,24(28)-trien-3β-ol **6**. From the symbiotes separated from 8 g of *N. lugens* (see above), the sterol fraction (2.1 mg) was trimethylsilylated and analyzed by GC-MS as described above. After desilylation (2 M aq. KOH-



SCHEME 2. Synthesis of sitosterol-d_5 and campesterol-d_5.

MeOH-THF, each 1 ml), the major sterol (1 mg, 85% of the total sterol content) was purified by HPLC. Its ^{1}H NMR spectrum indicated the structure to be ergosta-5,7,24(28)-trien-3β-ol **6,** which was confirmed by comparison with an authentic sample prepared as described above. ^{1}H NMR ($CDCl_3$, 360 MHz) δ: 0.63 (s, 3 H, 18-H), 0.94 (s, 3 H, 19-H), 0.98 (d, J = 6.3, 3 H, 21-H), 1.03 (d, J = 7, 6 H, 26-H and 27-H), 1.10–2.53 (m, 23 H, CH_2 × 9 and CH × 5), 3.64 (m, $W_{1/2}$ = 22.7 Hz, 1 H, 3-H), 4.67 (br s, 1 H, 28-H_a), 4.72 (br s, 1 H, 28-H_b), 5.39 (m, $W_{1/2}$ = 11.2 Hz, 1 H, 7-H), 5.58 (m, $W_{1/2}$ = 10.8 Hz, 1 H, 6-H); ^{13}C NMR ($CDCl_3$, 75.5 MHz) δ: 11.83 (C-18), 16.31 (C-19), 18.85 (C-21), 21.13 (C-11), 21.88 and 22.01 (C-26 and C-27), 23.05 (C-15), 28.09 (C-16), 31.04 (C-23), 32.02 (C-2), 33.83 (C-25), 34.63 (C-22), 36.12 (C-20), 37.03 (C-10), 38.39 (C-1), 39.21 (C-12), 40.81 (C-4), 42.97 (C-13), 46.25 (C-9), 54.50 (C-14), 55.76 (C-17), 70.48 (C-3), 105.98 (C-28), 116.31 (C-7), 119.60 (C-6), 139.78 (C-5), 141.35 (C-8), 156.82 (C-24); EI-MS *m/z* (relative intensity) 396 (M^+, 100, $C_{28}H_{44}O$, calculated for 396.3392, found 396.3417), 381 (M − CH_3, 5), 378 (M − H_2O, 7), 363 (M − CH_3 − H_2O, 72), 337 (M − C_3H_7O, 32), 312 (M − C_6H_{12}, 4), 294 (M − C_6H_{12} − H_2O, 5), 279 (M − C_6H_{12} − H_2O − CH_3, 4), 271 (M − side chain, 7), 269 (M − side chain − 2H, 5), 253 (M − side chain − H_2O, 12), 251 (M − side chain − 2H − H_2O, 8).

Sterols in Whole Insects Reared on Rice Plant. The three major sterols, cholesterol **1,** 24-methylenecholesterol **5,** and trienol **6,** were found in different relative amounts in both *N. lugens* and *L. striatellus*. Neither species contained more than a trace of ergosterol **4,** save one exceptional sample of *N. lugens*. Small amounts of sitosterol **2** and desmosterol **3** were found in some *N. lugens* samples. Episterol **(7)** was tentatively identified in both insect species, on the basis of the mass spectrum of the TMS ether derivative.

Sterols in Symbiote and Insect Lipid Fractions. The symbiote and insect lipid fractions were separated from whole *N. lugens* or *L. striatellus* by density gradient centrifugation. The lipid fraction from each insect species displayed approximately the same sterol composition as the corresponding whole insects. In each symbiote fraction, trienol **6** occurred as the only major sterol together with small amounts of cholesterol **1,** 24-methylenecholesterol **5,** and lanosterol **(8).** Trace amounts of ergosterol **4** were tentatively identified in some samples. Tentative identifications were made for episterol **7,** 4α-methyl-5α-ergosta-8,24(28)-dien-3β-ol **(9),** 14α-methyl-5α-ergosta-8,24(28)-dien-3β-ol **(10),** 24-methylene-24,25-dihydrolanosterol **(11),** and 4,4-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol **(12)** on the basis of mass spectroscopic evidence. All sterol constituents of symbiotes from the two insect species were essentially the same.

Sterols in Whole Insects Reared on Artificial Diet. N. lugens insects that had been administered a steroid-deficient diet for one week contained the same major steroid constituents as control insects, i.e., cholesterol **1,** 24-methylene-

cholesterol **5**, and trienol **6**, according to GC-MS measurements of the TMS ether derivatives. However, the artificially fed insects contained more than three times the amount of **5**, relative to total sterols, than the control insects.

DISCUSSION

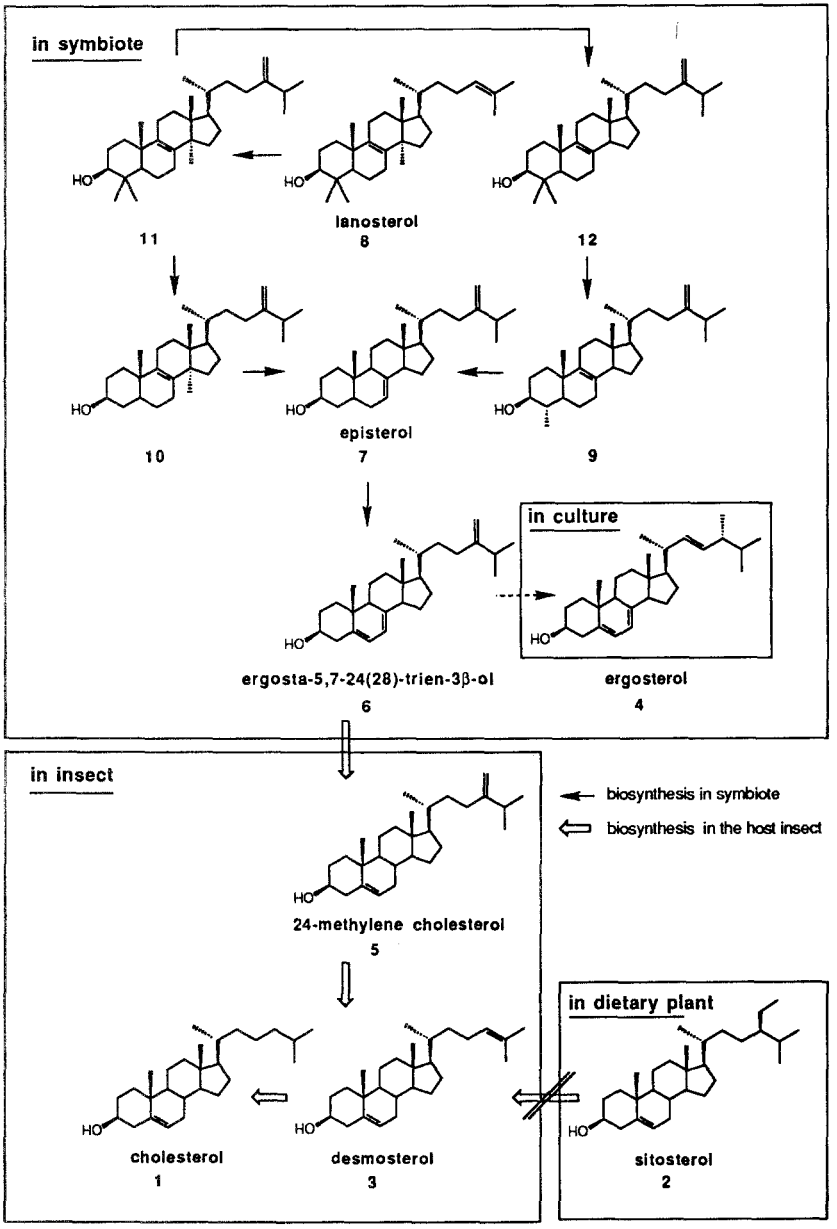
Isolation and identification of trienol **6** from the symbiotes separated by centrifugation prompted us to reinvestigate our previous results (Eya et al., 1989). Our present experiments revealed that trienol **6** was incorrectly assigned as ergosterol **4** in our previous report, although the characterization of ergosterol in cultures of Nl-1, Nl-2, Ls-1, and Ls-2 was correct. Ergosterol **4** was the single major sterol in the culture, but trienol **6** was the only sterol found in substantial quantities in both the symbiote and insect fractions separated by density gradient centrifugation. It has now become clear that the similarity in the mass spectra of the two compounds led us to erroneously conclude that the two were identical. The EI mass spectra of TMS-trienol (TMS-**6**) and TMS-ergosterol (TMS-**4**) were almost indistinguishable except for the generation of low-intensity *m/z* 294 and *m/z* 279 peaks by the former, but not by the latter. The *m/z* peak was attributed to loss of C_6H_{12} from the side chain in a McLafferty fragmentation and loss of Me_3SiOH. Loss of an additional CH_3 radical from *m/z* 294 provided *m/z* 279. Now, trienol appears to be the key compound supplied by the symbiote to the host insect. This hypothesis is supported by our previous heat-treatment experiment, in which removal of the yeastlike symbiotes from *N. lugens* led to the complete disappearance of **6** (not **4**), as well as to a 10-fold reduction in total sterol content, due to restriction of the insect growth (Eya et al., 1989). A dramatic decrease in 24-methylenecholesterol **5** as well as trienol **6** in aposymbiotic insects suggested that these compounds are essential to the development of host insects. The occurrence of a large amount of 24-methylenecholesterol **5** and a small amount of desmosterol **3** in the normal insect lipid fraction indicated that consumption of **5** is the rate-determining steroidogenic step in the host insects.

The co-occurrence of sterols **6–12** in the yeastlike symbiotes strongly suggests that the symbiotes in the host insects methylenate primarily at the stage of lanosterol **8.** In this respect, the yeastlike symbiotes are similar to *Phycomyces blakesleeanus*, *Agaricus campestris* (Goulston et al., 1975), *Mucor pusillus* (Mercer and Carrier, 1976), and *Neurospora crassa* (Renaud et al., 1978), among others, but different from *S. cerevisiae* (Barton et al., 1972). In the latter, elimination of the *gem*-dimethyl groups at C-4 occurs before introduction of the methylene group at C-24. In *Candida albicans*, preliminary evidence suggests that C-24 methylation can occur at either the demethyl sterol or the lanosterol stage (Shimokawa et al., 1986; Cannon and Kerridge, 1988).

Interestingly, the symbiotes Nl-1, Nl-2, Ls-1 and Ls-2 produced only ergosterol as their end product under aerobic culture conditions (Eya et al., 1989). In contrast, reduction of the $\Delta^{24(28)}$ double bond and introduction of the Δ^{22} double bond are both inhibited in the symbiotic environment of these microorganisms. The disabling of these two conversions in the symbiotic environment could result from active inhibition by the host insect, or from changes in the concentrations of cofactors required for these conversions (e.g., oxygen, see Osumi et al., 1978). It is also possible that the symbiotes isolated from the eggs of *N. lugens* and *L. striatellus* underwent mutation under the artificial culture conditions to produce ergosterol.

Ergosterol **4** has been suggested to be a growth factor for *S. cerevisiae* (Pinto et al., 1983). If **4** serves the same function in these yeastlike symbiotes, severe curtailment by the host insect of **4** biosynthesis in the symbiotes may allow the insect to control the growth rate of its guest, to the mutual benefit of host and guest. The relatively high concentrations of 24-methylenecholesterol **5** in *N. lugens* and *L. striatellus* are peculiar among insects, as noted previously (Noda et al., 1979). A need by the host insects for an extremely efficient Δ^7-reductase to allow successful competition with the symbiotes for limited supplies of trienol **6** could at least partially account for the unusual buildup of **5** in the insect tissues.

In order to clarify the origin of cholesterol via 24-methylenecholesterol in the host insects, biotransformations of the deuterated precursors (**6, 4, 2**) were examined. The carrier for injection was selected according to the solubility of the compound to be injected. Insects were injected with **6**-d_7 suspended in 20% aqueous glycerol containing 0.2% Emulgen 913 as the carrier, or in olive oil containing 0.2% Emulgen 913, in which **6**-d_7 had slightly greater solubility. The insects that were injected with the latter suspension were maintained for 28 hr and 96 hr before sacrifice. The sterol fraction was separated, TMS-derivatized, and analyzed by capillary GC-MS in multiple ion detection (MID) mode (selected ion monitoring). No deuterated 24-methylenecholesterol and/or cholesterol was detected. Another deutero compound, **6**-d_3, which had improved solubility, was prepared and injected in a similar manner. The result was the same as before. Insects injected similarly with **4**-d_2 and retained for 25 hr and 70 hr also afforded no conversion. However, the observation of a dramatic reduction of trienol **6** and 24-methylenecholesterol **5** in the aposymbiotic insects, and an increase of **5** in the insects reared on a steroid-deficient diet, strongly suggested the involvement of symbiote-derived **6** in cholesterol biosynthesis of the symbiotic insects. The ability of the insects to biotransform the major sterol of their dietary plant was also tested. Insects injected with sitosterol-2,4,6-d_5 (**2**-d_5) dissolved in either olive oil or 0.2% Emulgen 913 in 20% aqueous glycerol were allowed to live for three days before sterol extraction and analysis. Biotransformation of sitosterol into cholesterol and/or desmosterol was not observed under these conditions.



SCHEME 3. Proposed steroidogenesis in *N. lugens* hosting symbiotes.

In conclusion, all injection experiments with deuterated trienol **6**, ergosterol **4**, and sitosterol **2** failed to yield deuterated cholesterol **1**; only 24-methylenecholesterol **5** was biotransformed into cholesterol **1**. The present study may suggest that there are specific mechanisms in situ for transportation of the essential compounds; sitosterol **2** can be transformed by an ingestive route, whereupon cholesterol was the only excreted sterol. Administration of a steroid-deficient diet provided an alternate proof that 24-methylenecholesterol **5** originates from the symbiotes. Thus, Scheme 3 illustrates the proposed steroidogenesis in the symbiotic insects.

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INHIBITION OF *Schizachyrium scoparium* (POACEAE) BY THE ALLELOCHEMICAL HYDROCINNAMIC ACID

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Abstract—Bare zones around shrubs in the Florida scrub indicate the possibility of allelopathy by shrubs controlling the distribution of grasses invading from adjacent sandhills. The allelochemical, hydrocinnamic acid, has been identified as a breakdown product of ceratiolin, which is released from the shrub *Ceratiola ericoides*. Here, hydrocinnamic acid (HCA) was shown to have a strongly inhibitory effect on shoot and root biomass of the grass *Schizachyrium scoparium* in greenhouse bioassays lasting 4.5 months. Linear increases in the concentration of HCA from 0 to 200 ppm, applied biweekly, resulted in exponential decreases in root and shoot biomass at harvest. Plants grown at 200 ppm HCA had root and shoot biomasses 13% and 17% of controls, respectively. Concurrent investigation of reduced nutrient levels indicated greater inhibition by HCA in a reduced nitrogen (N) treatment and in a reduced potassium (K) treatment relative to HCA inhibition in the full nutrient treatment. The negative slopes of the regressions of log of biomass on HCA concentration were steepest in the reduced N and reduced K treatments. Root and shoot biomasses in reduced N treatments were 20–43% and 24–34% less than the respective biomasses in the full nutrient treatment. Comparable reductions in the reduced K treatment were as much as 19% and 10% for root and shoot biomasses, respectively. The effects of HCA in a reduced phosphorus (P) treatment and in a reduced P and K treatment were not significantly different from the effects of HCA in the full nutrient treatment. Extraction of the soils at harvest indicated no buildup of HCA at the end of the experiment. The sensitivity of *Schizachyrium scoparium* to HCA

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in general and increased sensitivity under low N and low K solutions may be important in the Florida scrub community where levels of N and K are known to be low.

Key Words—Allelopathy, *Schizachyrium scoparium*, *Ceratiola ericoides*, hydrocinnamic acid, nitrogen, phosphorus, potassium, fire, sand pine scrub, sandhill.

INTRODUCTION

Two distinct vegetative communities coexist on the upland dry sandy soils of Florida, the sandhill and the sand pine scrub. The sandhill is an open woodland with longleaf pine (*Pinus palustris* Mill.) and slash pine (*Pinus elliotti* Engelm.) towering over a savannah dominated by little bluestem [*Schizachyrium scoparium* (Michx.) Nash] and wire grass (*Aristida stricta* Michx.). The scrub is dominated by sand pine [*Pinus clausa* (Chapm. ex Engelm.) Vassey ex Sarg.] with an understory of rosemary (*Ceratiola ericoides* Michx.) (Empetraceae) and scrub oaks, but little herbaceous ground cover. One notable difference in the selective regimes of the two communities is the frequency of lightning-originated fires. Surface fires are common in the sandhill, typically occurring every three to eight years (Christensen, 1988). The sand pine scrub has more intense crown fires every 20–50 years (Richardson and Williamson, 1988; Williamson, 1990). Webber (1935) called the scrub a "fire fighting machine," as fires sweeping through the sandhills are often extinguished at the scrub ecotone due to changes in fuel availability. In the absence of fire in the sandhills, scrub species will invade; thus, different fire regimes are necessary for the existence of the two communities (Veno, 1976).

An examination of soils in both regions has revealed few differences in physical and chemical soil characteristics to a depth of 5 m, indicating a uniform substrate across vegetative boundaries (Kalisz and Stone, 1984). The differences in soil color or organic matter that are sometimes observed may be induced by the vegetation types. There is little evidence to support the early hypothesis that the abrupt boundary is caused by differences in soils, with the scrub community being characterized by nutrient-poor soils relative to the sandhill (Laessle, 1958; Christensen, 1981).

In addition to fire regime, allelopathy may have a large role in establishing and maintaining the boundaries (Richardson and Williamson, 1988; Weidenhamer and Romeo, 1989; Williamson et al., 1991). The scrub shrubs release chemicals that may inhibit the growth of the sandhill grasses and pines that provide fuel for fires that would kill the shrubs. *Ceratiola ericoides* is one shrub that has been implicated in allelopathic interactions (Fischer et al., 1988). Ceratiolin, a novel dihydrochalcone, is washed from the leaves and decaying litter by rainwater (Tanrisever et al., 1987). Although allelopathically inactive, the

compound decomposes in aqueous solution to form hydrocinnamic acid (HCA), which is highly phytotoxic in Petri dish bioassays to native sandhill grasses, including little blustem, *Schizachyrium scoparium* (Michx.) Nash (Fischer et al., 1988; Williamson et al., 1991).

We present here an investigation of the effects of HCA on growth of *S. scoparium* in greenhouse bioassays conducted in pots with sand as a substrate and under natural light regimes. These studies differ from earlier bioassays of leaf washes and mists and of pure HCA on seeds and seedlings in Petri dishes in the dark (Tanrisever et al., 1987; Fischer et al., 1988). In addition, reduced nutrient levels were used to test for interactions between nutrient reduction and inhibition by HCA. Allelopathy involving phenolic compounds has been suggested to be more important in soils of lower fertility, and in some cases reduced nutrient levels have increased allelopathic effects (Glass, 1976; Stowe and Osborn, 1980). Adsorption of potassium and phosphate is decreased by many phenolic acids identified as allelopathic inhibitors (Glass, 1973, 1974; Harper and Balke, 1981). Scrub soils are known to be low in some soil nutrients, notably potassium, calcium, and nitrogen (Kalisz and Stone, 1984; Richardson, 1958), so toxic effects of phenolic allelochemicals may be intensified. We applied HCA with nutrient solutions at both low concentrations and higher concentrations designed to overcome any nutrient limitation present.

METHODS AND MATERIALS

Schizachyrium scoparium was used as the test species for bioassays of both the phytotoxicity of hydrocinnamic acid in aqueous concentrations of 0 to 200 ppm and the influences of nutrient reduction. Thirty seeds were planted at a depth of 1 cm in sand in plastic pots 10 cm^2 × 7.5 cm high. Each pot was filled 5 cm deep with Easy Blast blasting sand, a river sand washed extensively to remove organic matter and free nutrients. The bottom of each pot was lined with a fiberglass screen covered with 1 cm of pea gravel to allow free drainage without soil loss.

The pots were placed in a completely randomized block design in a shaded greenhouse receiving 55% of full sunlight as measured with a Li-Cor model LI-185B Quantum/Radiometer/Photometer. Initially, pots were watered twice a week with 90 ml of tapwater. To deter fungal growth, Captan, 2.5 ml/liter of solution, was added to the water when the seeds were planted and again after five weeks. After two weeks, seedlings were thinned to one plant per pot. Two weeks later the sand surface was covered with a 1-cm layer of black gravel to deter surface algal growth, and the nutrient–HCA treatments were initiated. On Mondays and Fridays pots received 90 ml of one of five levels of HCA and one of five nutrient treatments. Ten replicates of each combination were used.

To preclude the accumulation of toxic levels of nutrient salts, pots were flushed on Wednesday with tapwater from a hose until water ran both through and over the pot top.

HCA was applied at concentrations of 0, 25, 50, 100, and 200 ppm. Five nutrient solutions, prepared according to Stowe and Osborn (1980), were used: full nutrient solution (F), reduced nitrogen (N), reduced potassium (K), reduced phosphorus (P), and reduced potassium and phosphorus (KP). The full nutrient solution contained 81 ppm N, 9 ppm P, and 78 ppm K. Other nutrient concentrations were held constant, as in Bradshaw et al. (1964). The reduced concentration of a nutrient was 1/9 the concentration in the full solution. In the reduced KP solution, both nutrients were reduced to 1/9 the full concentration. As concentrations of sulfate in the reduced K and reduced KP solutions decreased, sodium sulfate was added proportionally to maintain a constant sulfate concentration. Solutions were prepared with deionized water and were adjusted to pH 5.5 using NaOH and HCl.

Plants were harvested 4.5 months after planting. The pots were overturned and the plants gently rinsed in tap water until soil particles were freed from the roots. Each plant was divided into shoot and root components, the tissues were oven dried for 48 hr at 60°C, and dry weights were obtained. Shoot weights included living biomass and total biomass, the latter including dead leaves attached to the plant at the time of harvest. Data were then $\log_{10}$ transformed and analyzed by regression analysis using SAS (SAS Institute, 1985) to determine the effect of HCA and nutrient treatments.

Soil samples were taken from each full nutrient solution–HCA combination in order to determine the concentration of HCA at the time of harvest. The soil was extracted into water and analyzed using HPLC according to the methods of Jordan (1990). Identity of HCA was confirmed by comparing UV spectra and retention times to standards. A Hewlett Packard 1090 Liquid Chromatograph equipped with a diode array detector and auto injector was used with detection at 257 nm, and a C-18 reverse-phase column, 10 μm Carbosphere (250 mm $\times$ 4.6 mm ID, Phenomenex, Palo Alto, California). A two-solvent system of (A) methanol and acetic acid (99.5:0.5) and (B) double-distilled deionized water and acetic acid (99.5:0.5) was used, with a gradient of 50% (A) to 100% (A) in 17 min.

RESULTS

As the HCA concentration increased from 25 to 200 ppm, the overall mean shoot and root dry weights of *S. scoparium* decreased (Figure 1). Biomass reduction, clearly visible at harvest, was greater in the roots than in the shoots (Figure 2). In 200 ppm HCA, root biomass was reduced to 13% of the control,

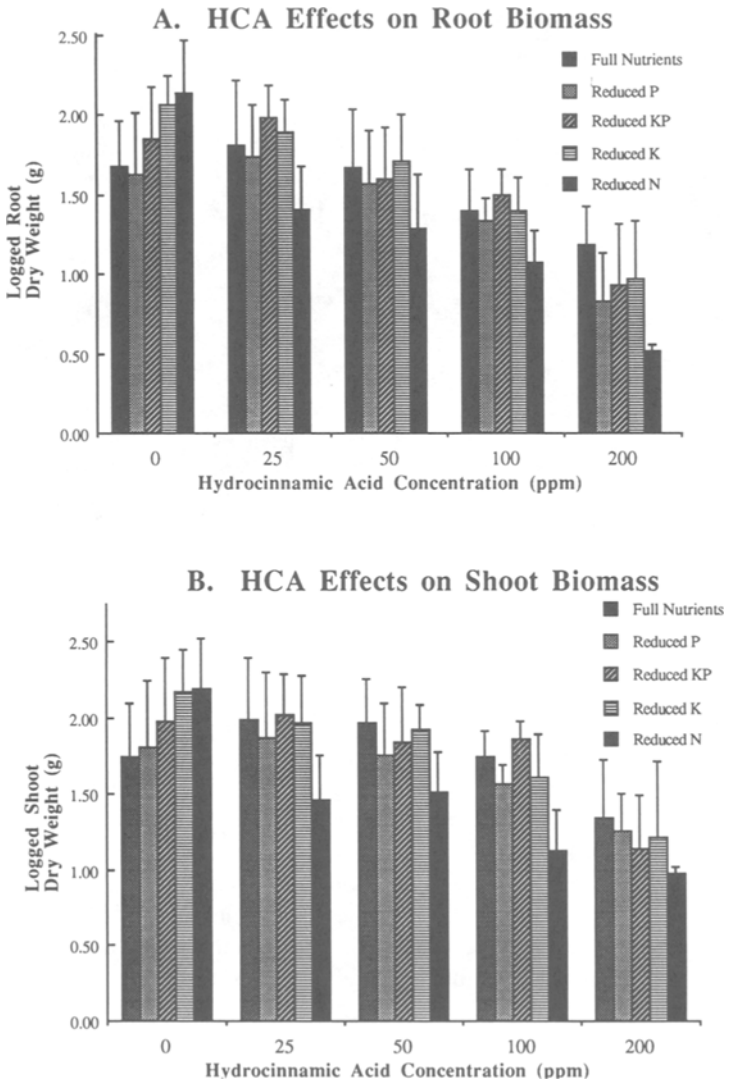

FIG. 1. Dry biomass of roots (A) and living shoots (B) at harvest of *Schizachyrium scoparium* after 4.5 months of growth exposed to 0, 25, 50, 100, and 200 ppm HCA in full nutrient (F), reduced phosphorus (P), reduced phosphorus and potassium (KP), reduced potassium (K), and reduced nitrogen (N). Data are means $\pm$ 1 SD.

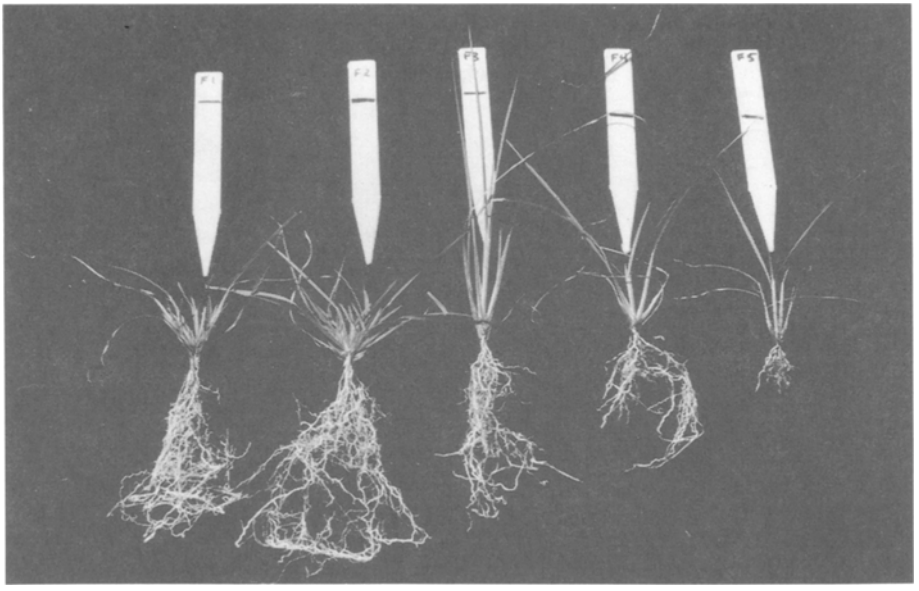

FIG. 2. Representative *Schizachyrium scoparium* plants at harvest, from the full nutrient treatment and HCA concentrations of 0, 25, 50, 100, and 200 ppm, labeled F1–F5, left to right.

living shoot biomass to 17% of the control, and total shoot biomass to 24% of the control. Linear regressions of the log of root, living shoot, and total shoot biomasses on HCA concentration were statistically significant for all nutrient treatments ($P \leq 0.05$) (Table 1). Therefore, as HCA concentration increased linearly, biomass at harvest decreased exponentially.

Reduced nutrient treatments had variable effects (Figure 1). A covariance model tested for differences in slope of the dependent variable, biomass, on the covariate, HCA concentration, with nutrient treatment as the class variable and a nutrient–HCA interaction term, where significance of the latter indicated different slopes. The slopes for both reduced N and reduced K were significantly different from the slope for the full nutrient solution for both root and living shoot biomasses (Table 1). For HCA concentrations from 25 to 200 ppm, reduced N produced root biomass from 20% to 43% of the control, living shoot biomass from 24% to 34% of the control, and total shoot biomass from 33% to 46% of the control. However, at 0 ppm HCA, reduced N exhibited an increase over the control of 27%, 26%, and 19%, for root, living shoot, and total shoot biomasses, respectively. Effects of reduced K were less pronounced than reduced

TABLE 1. RESULTS OF REGRESSIONS OF ROOT AND LIVING SHOOT BIOMASS OF *Schizachyrium scoparium* ON HCA CONCENTRATION INDIVIDUALLY FOR FIVE NUTRIENT TREATMENTS: FULL NUTRIENT (F), REDUCED PHOSPHORUS (P), REDUCED PHOSPHORUS AND POTASSIUM (KP), REDUCED POTASSIUM (K), AND REDUCED NITROGEN (N)[a]

	Slope	Intercept	R^2	P
Root biomass				
Full nutrients	−0.136	1.94	0.27	0.002
Reduced P	−0.183	1.97	0.36	0.0001
Reduced KP	−0.245	2.31	0.52	0.0001
Reduced K	−0.267*	2.40	0.72	0.0002
Reduced N	−0.326**	2.26	0.62	0.0001
Living shoot biomass				
Full nutrients	−0.09	2.02	0.10	0.05
Reduced P	−0.132	2.05	0.21	0.005
Reduced KP	−0.198	2.37	0.35	0.0007
Reduced K	−0.228*	2.43	0.51	0.0001
Reduced N	−0.269*	2.22	0.50	0.001

[a] Regression parameters are slope and intercept with the associated R^2 and probability of significance. Slopes significantly different from the full nutrient treatment are indicated by * if $p \leq 0.05$ and by ** if $p \leq 0.01$.

N and resulted in root and living shoot biomasses ranging from 82% to 123%, and 90% to 125%, respectively, of the full nutrient treatments (Figure 1).

The slope of the reduced KP treatment approached a statistically significant difference from the slope of the full nutrient treatment for root biomass ($P = 0.08$), living shoot biomass ($P = 0.13$), and total shoot biomass ($P = 0.16$) (Table 1). The slope of reduced P treatment was not different from the slope of the full nutrient treatment for root biomass ($P = 0.43$), living shoot biomass ($P = 0.54$), and total shoot biomass ($P = 0.63$). As the slopes of the reduced KP and reduced P treatments were not different from the slope of the full nutrient treatment, a second test for differences in intercepts (at HCA = 0 ppm) was performed, but it also yielded no significant differences ($P \geq 0.05$). Finally, comparisons between reduced KP versus reduced K and reduced KP versus reduced P treatment were also conducted to compare the double nutrient reduction with the single nutrient reductions, but no differences were found in slopes or intercepts.

Analysis of soil samples taken from pots at time of harvest revealed only trace concentrations of HCA in some samples, and no acetophenone. The concentrations of HCA found were several orders of magnitude lower than concentrations applied, and followed no apparent trend relative to application dosage.

DISCUSSION

Past studies of hydrocinnamic acid in Petri dish bioassays showed inhibition of germination and radicle elongation of *Schizachyrium scoparium* at levels of 63 ppm (Fischer et al., 1988). Leaf and litter washes of *Ceratiola* were also inhibitory to germination and radicle growth of *Schizachyrium scoparium* and other grasses in Petri dish bioassays at concentrations lower than 60 ppm (Tanrisever 1987, Williamson et al., 1991). The effect produced from both fresh foliage and litter was subject to seasonal variation (Richardson and Williamson, 1988). Studies of whole plants were deemed necessary to investigate the effects on growth over longer periods of time.

In this study the whole plants subjected to HCA treatments showed exceptionally strong inhibitory effects (Figure 1). At 200 ppm, roots were reduced to 13% of controls and live shoots were 17% of controls. At 25 ppm, the lowest concentration applied, roots were reduced to 82% of controls and live shoots to 78% of controls. These effects were achieved under greenhouse conditions that simulated natural conditions. The plants were grown in sand and the treatments were applied twice weekly. These pulses of application simulate the way natural doses are carried into the soil by sporadic rainfall leaching ceratiolin from leaves and litter of *Ceratiola*. HCA has been found in concentrations of 15–418 ppm in soil near *Ceratiola* (Jordan, 1990).

Previous studies have shown that the rate of formation of HCA from *Ceratiola* is greater than the rate of degradation of HCA into acetophenone (Jordan, 1990). This allows HCA to persist in the soil despite rapid decomposition when continuously resupplied. When nonsterile soil is spiked with HCA only once, recovery is 36% after 30 hr (Jordan, 1990). However, when HCA is spiked every other day and extracted 48 hr after the fifth addition, the amount extracted is 75% greater than an individual spike (Jordan, 1990). HCA in soil is known to degrade microbially into acetophenone (Jordan, 1990), a compound that also has allelopathic activity in Petri dish bioassays (Obee, unpublished data). The microbial degradation is rapid and stimulated by higher temperatures. Leaf washes of *Ceratiola* showed the most activity during summer months (Richardson and Williamson, 1988). This is likely due to both increased degradation during the hotter summer months and increased production caused by higher metabolic rates.

Soil samples were taken at the time of harvest from the HCA–nutrient experiment to determine whether HCA had accumulated in our pots at levels greater than those applied. Results showed that the samples contained only trace amounts of HCA in some pots and no acetophenone, a toxic degradation product of HCA. Since significant accumulation was not detected, the three- to four-day period between the biweekly applications may have been sufficient time for the breakdown of HCA. As the soil HCA was monitored only at the end of the

experiment, it is still possible that sporadic accumulation occurred at other times during the 4.5 months.

Reduced nutrient solutions were applied to test for nutrient stress and for interactive effects between nutrient stress and HCA. In each nutrient treatment increasing concentrations of HCA from 0 to 200 ppm caused decreases in plant biomass (Figure 1). Regression analysis showed that the effects of HCA did vary consistently according to nutrient treatments (Table 1): HCA had the strongest effect with reduced N, and successively milder effects with reduced K, reduced KP, reduced P, and finally the full nutrient treatment. The reduced N and reduced K treatments resulted in decreases in plant biomass that were significantly different from the HCA effect in the full nutrient treatment, whereas HCA effects in reduced P and reduced KP treatments were not significantly different from HCA effects in the full nutrient treatment. The Florida soils where *Schizachyrium scoparium* proliferates are considered nutrient poor (Christensen, 1988) and have low levels of K and N (Kalisz and Stone, 1984; Richardson, 1985), but an adequate level of P (Kalisz and Stone, 1984).

Since *Schizachyrium* grows on nutrient poor soils, it may be expected to be tolerant of reduced nutrient conditions. Here the inhibition of growth due to reduced nutrients was less severe, although significant for nitrogen and potassium, than the inhibition due to HCA. With reduced nitrogen or potassium, the allelopathic effects of HCA on *Schizachyrium* are intensified. This effect is similar to results of Stowe and Osborn (1980), who found that low nutrients combined with phenolics caused significant inhibition of barley not seen with phenolics alone. Einhellig (1989) found no evidence that more favorable phosphorus levels would overcome the allelopathic effects of ferulic acid on grain sorghum. In this case of phosphorus reductions the grain sorghum had the same insensitivity as the native *Schizachyrium*. Although here a native and agricultural species are shown to have similar reactions, comparisons should always be made with caution, as agricultural species are often more susceptible to nutrient limitation than native species.

Higher concentrations of HCA had a significant effect on mean dry weights (Figure 1). This effect, intensified by nutrient reduction, may be important where grasses grow near the shrub *Ceratiola*, the source of ceratiolin and its degradation product, HCA. In nutrient poor soils, grasses may be unable to colonize near the shrub where HCA concentrations reach inhibitory or toxic levels in the soil. This interaction between HCA, low nutrients, and the grasses may account for bare zones in the *Ceratiola* "balds," near pure stands of the shrub, and at some ecotones between sandhill and scrub where the latter is dominated by *Ceratiola*. Other environmental factors, notably high temperature and low soil moisture, may also increase the phytotoxicity of HCA (Einhellig and Eckrich, 1984; Einhellig, 1989). The well-drained sands of the Southeastern Coastal Plain have soil surface temperatures in excess of 50°C on sunny days (Richardson,

1985), and moisture stress is a significant factor in the dry season and during pauses in summer rains. The low plant densities in the scrub may also contribute to the enhanced effectiveness of allelochemicals such as HCA. The density of seeds and germinating seedlings has been shown to have a major impact on the magnitude of phytotoxic effects (Weidenhamer et al., 1987). Herbivory, which has been suggested as an explanation for bare zones around shrubs in the California chaparral (Bartholomew, 1970), may be a factor in the scrub. However, individual *Ceratiola* plants that are too small to harbor rodents also exhibit "halos" when colonizing disturbed scrub areas (Williamson, 1990). Where colonizing shrubs are sufficiently aggregated or grow sufficiently large to harbor rodents, both herbivory and allelopathy may contribute to a lack of plant growth around shrubs.

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AMINO ACID PROFILES AND LIPOSOMES: THEIR ROLE AS CHEMOSENSORY INFORMATION CARRIERS IN THE MARINE ENVIRONMENT

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Abstract—Yellowfin tuna enhance their hunting success in the vast pelagic environment by using their sense of smell to detect intact (uninjured) prey that are beyond visual range. However, the olfactory cues that tuna use would normally face huge and rapid dilution in the open ocean. We demonstrate that these prey odors are complexed within biologically derived lipid structures that probably delay the dilution of the amino acids to subthreshold concentrations and provide persistent arousal and search cues for the tuna. This may be the first demonstration of an extracorporeal biological function for liposomes. Tuna may also form "chemical search images" to maximize feeding efficiency. We demonstrate that the amino acid profiles of various prey species are consistent over time and between schools, which makes the formation of search images feasible.

Key Words—yellowfin tuna, *Thunnus albacares*, amino acid profiles, liposomes, olfaction, search image, lipid complexes.

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INTRODUCTION

Yellowfin tuna, *Thunnus albacares*, feed primarily on schooling prey that are patchily distributed in a huge ecosystem and that are consequently difficult to detect even in clear pelagic waters. Captive tuna respond to the odors of intact prey organisms (Atema et al., 1980), and, in the wild, arousal to food beyond visual range is probably mediated by olfactory detection of excretory and mucal slough-off compounds produced by the schools of prey. Aqueous rinses of the surfaces of tuna prey organisms contain complexes of lipids and amino acids, the latter being chemosensory feeding stimuli for a wide range of fish species including tuna (Bardach and Villars, 1974; Atema, 1980; Atema et al., 1980; Hara, 1982; Olsén et al., 1986).

Maximum energetic advantage (i.e., energy expended in hunting versus caloric value of the food consumed) may be obtained by use of a "chemical search image" shaped by recent feeding success on locally or seasonally abundant food types, and there is evidence that tuna respond preferentially to the odors of recently consumed prey species (Atema et al., 1980). However, for a chemical search image to be effective, the amino acid profile of a particular prey type must remain constant over time and be qualitatively similar between different schools of the same species. Furthermore, to provide arousal and, possibly, chemotactic cues, the prey odors must stay above detection thresholds even in the face of the immense dilutions possible in the open ocean. Detection thresholds for yellowfin tuna are around 5×10^{-6} g/liter for squid rinse (Yost, 1988) and estimated as 1×10^{-11} M for single amino acids (Atema et al., 1980). However, this estimate was extrapolated from minimum detectable amino acid concentrations in complex prey stimuli and is in the range of background concentrations found in ocean water (Carr and Derby, 1986).

There were two principal objectives of the current study: first, determine whether the amino acid profiles of tuna prey species were consistent over time and between schools, and second, because our previous attempts to separate prey rinses into discrete molecular weight fractions were consistently confounded by the presence of small-molecular-weight compounds in the supposedly pure large-molecular-weight fractions, investigate the possibility that amino acids were being entrained in the lipid components of the prey rinses.

METHODS AND MATERIALS

Rinses of the following prey were analyzed: (1) Hawaiian mackerel—"opelu" (*Decapturus macarellus*), (2) Hawaiian anchovy—"nehu" (*Stolephorus purpureus*), (3) bigeye scad—"akule" (*Selar crumenopthalmus*), and squid (*Loligo sp.*). Specimens were collected live from fishermen, from the wild, or from a local wholesale fish auction. In all cases, contact with possible contam-

inants was nonexistent or minimal. For the fish species, rinses were obtained through gentle washing of fresh dead whole prey in an agitating bath of either fresh- or saltwater for about 20 min. Washing was always terminated before any rupturing of the gills occurred. Squid rinses were obtained in the same way from previously frozen specimens. The rinses were filtered, and analyses were performed on both the fresh and lyophilized product.

Amino acid content of the rinses was determined by ion-exchange chromatography using a Beckman autoanalyzer with ninhydrin postcolumn derivatization. The lipophilic components of the prey rinses were Soxhlet-extracted using dry methylene chloride or chloroform. These extracts were then analyzed with high-performance thin-layer chromatography (HPTLC). The quantities of the various lypophilic compounds were ascertained using enzymatic methods of analysis (Anon., 1987).

Opelu rinses were prepared for electron and fluorescence microscopy by filtering the prey rinse through a Whatman No. 1 paper, followed by centrifugation at 5000 rpm for 20 min. For fluorescence microscopy, 2-ml aliquots of rinse were mixed with equal volumes of the derivitizing agent *o*-phthalaldehyde (Pickering Labs., Mountain View, California), which is specific for primary amine-bearing compounds (Chen et al., 1979) and which was prepared by dissolving 80 mg in 10 ml of 100 mM borate buffer, pH 9.4, and adding 100 μl of β-mercaptoethanol. One drop of derivatized prey rinse was viewed under UV illumination (340 nm) and observed through a 4-nm narrow band-pass filter. The image was captured with a video attachment interfaced with an IBM XT computer. Samples of opelu rinse for electron microscopy were stained with either phosphotungstic acid or uranyl acetate, both 2% in seawater. To ensure that the lipid vesicles were not an artifact of the centrifugation procedure, a sample of crude, filtered opelu rinse was scanned for the presence of liposomes.

Fluorescence spectroscopy was conducted on fresh opelu rinse diluted 1:20 with filtered seawater and excited with UV light at 280 nm. The intrinsic fluorescent properties of tryptophan (Trp) allowed us to utilize this amino acid as a probe to determine the location of amino acids occurring in the prey rinses. Fluorescence-quenching studies were conducted by adding increasing concentrations of potassium iodide to the prey rinse and measuring the degree of quenching with a multifrequency phase fluorometer (Alcala et al., 1985). Prey rinses were also analyzed with ^{1}H NMR techniques in which the emission spectra of Trp in opelu rinse was compared with the spectra of free Trp in deuterated seawater.

Behavioral bioassays, which quantify the responses of captive tuna to the presentation of dilute chemical stimuli, were conducted as described by Atema et al. (1980). A small school of tuna (three to five animals) was tested daily and their responses quantified by an observer who did not know the nature of the stimulus. Responses were ranked on a scale of 0 to 9, with 0 being no

response and 9 a very strong behavioral response. The fish were fed to satiation on the day before testing to minimize variations in response intensity due to hunger motivation. The stimuli were presented in a quasirandomized sequence with trials separated by about 1 hr. Responses to the whole squid rinse served as a measure of the tuna's responsiveness on each day and allowed evaluation of the relative efficacy of the test stimuli.

Four types of stimuli were used: (1) pure lipids produced from soybean lecithin (0.9 g/500 ml starting concentration); (2) reconstituted squid amino acid profile (0.5 g/500 ml); (3) reconstituted amino acid profile (0.5 g) plus soybean liposomes (0.9 g/500 ml); and (4) a control stimulus of lyophilized whole squid rinse (0.5 g/500 ml). The lipids and lipid–amino acid mixtures were sonicated for approx. 15 min to promote the formation of lipid complexes in the form of liposomes (Naoi et al., 1977; Klein et al., 1971).

RESULTS

The amino acid profiles proved to be species specific and consistent across replicate samples of each species taken over a period in excess of two years (Figure 1). In addition to these physiological amino acids (i.e., those occurring naturally in proteins), taurine was also found in the rinses of all prey species and was incorporated into the artificial amino acid mixtures. Microscopic examination using ultraviolet (UV) illumination of fresh rinses combined with the derivatizing reagent *o*-phthalaldehyde revealed that fluorescence was localized within the lipid structures and absent from the background medium, thereby verifying that amino acids were sequestered in the lipid domain.

The emission spectra of prey rinses excited at 280 nm demonstrated a Trp fluorescence that was blue-shifted by up to 30 nm compared with the emission maximum of 355 nm observed for Trp in pure seawater, indicating that Trp was complexed within the lipid structures. Quenching of this Trp fluorescence with potassium iodide resulted in Stern-Volmer plots (Lehrer, 1971), which indicate that the Trp associated with the liposomes was substantially shielded relative to Trp free in solution (Figure 2). The fluorescence lifetime of Trp in liposomes was also found to be heterogeneous and lengthened (average = 3.6 nsec) compared to Trp free in solution (average = 2.9 nsec), which supports the conclusion that Trp is associated with lipid matrices in the prey rinses.

Further indication of lipid–amino acid complexation was acquired from ^{1}H NMR examination of the rinses. Spectra of Trp in the presence of liposomes formed both with lipid extracts from the prey fish rinses and from standard lipid mixtures showed chemical shift and line broadening when compared with spectra of free Trp in deuterated seawater.

Examination of rinses under the electron microscope revealed classic mul-

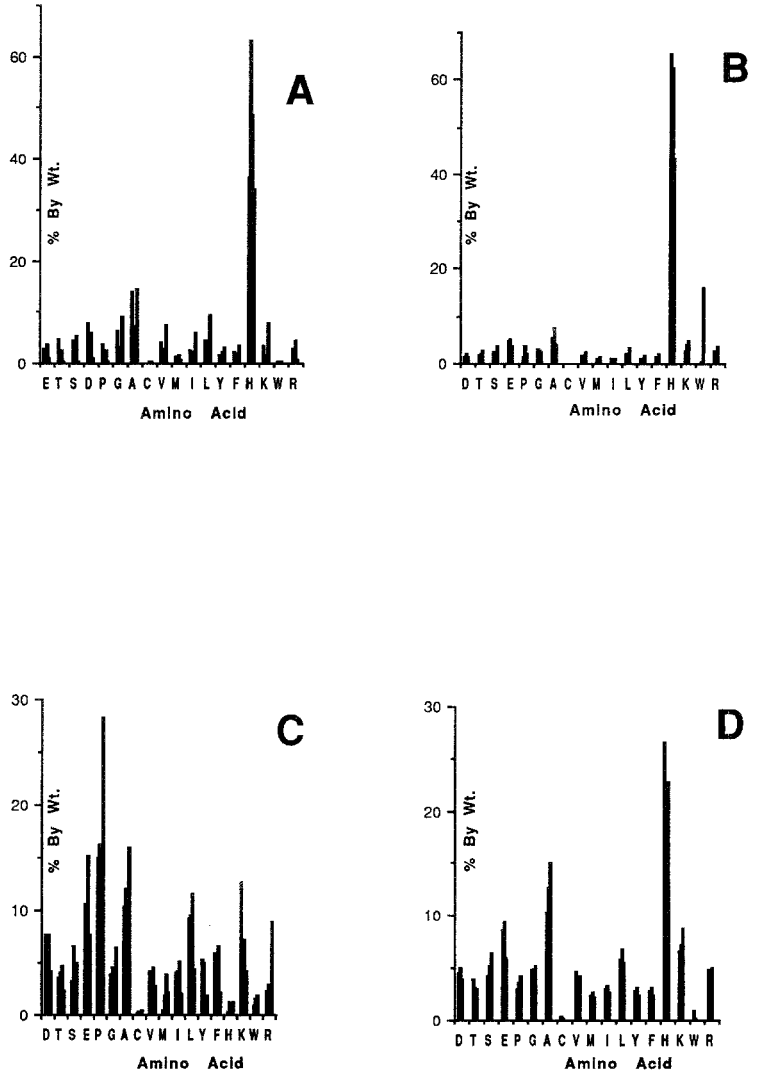

FIG. 1. Amino acid profiles of the aqueous rinses of four tuna prey species. (A) Opelu: profiles of rinses from four schools acquired over a period of two years. (B) Akule: profiles of rinses from three samples collected over two years. (C) Squid: four profiles of rinses sampled over two years. (D) Nehu: profiles of samples from three separate schools of fish taken from Kaneohe Bay, Oahu, Hawaii, over a period of approximately three years. Amino acids are as follows: A, alanine; C, cysteine; D, apartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; P, proline; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

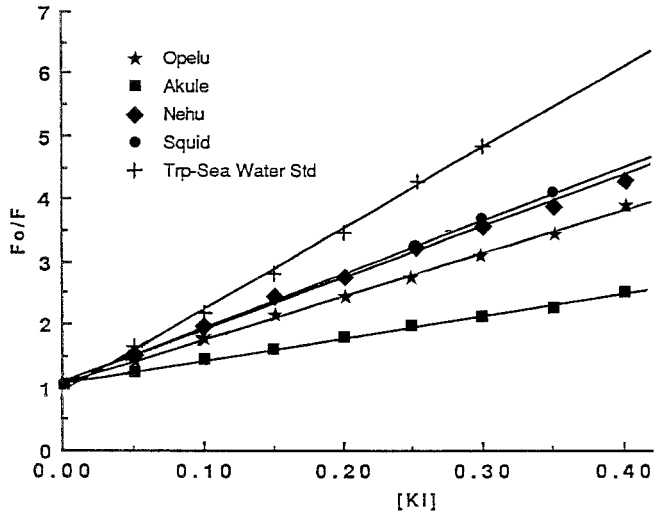

FIG. 2. Stern-Volmer iodide quenching plots of the rinses of four tuna prey species and plain sea water. F_0 = fluorescence intensity of Trp in the rinse in the absence of quencher; F = Trp fluorescence intensity with quencher. The flatter slope of F_0/F for the prey rinses relative to Trp free in solution indicates that Trp is occluded to varying degrees within lipid matrices in the rinses of each species.

tilamellar onion-skin structures, which are usually associated with the formation of liposomes (Figure 3). These structures were observed in both the centrifuged and crude opelu rinses.

HPTLC analysis of the extracts revealed the presence, in all species, of cholesteryl esters, cholesterol, free fatty acids, glycero-fatty acid triesters, and phospholipids.

The behavioral response intensities of captive tuna to the various stimuli are shown in Table 1. Kolmogorov-Smirnov chi-square approximation analysis of the behavioral data indicated that response intensities to the reconstituted squid amino acid profile and to the same profile complexed with soybean lipids were not significantly different. They were both significantly better stimuli than the lipids alone, but evoked significantly weaker responses than the squid odor control. The average response to the amino acid–liposome mixture was 62% as strong as responses to the unaltered squid odor.

DISCUSSION

The specificity and constancy of the amino acid profiles among schools of the same species, in some cases over periods in excess of two years, supports the concept of chemical search images as a viable foraging technique for tuna.

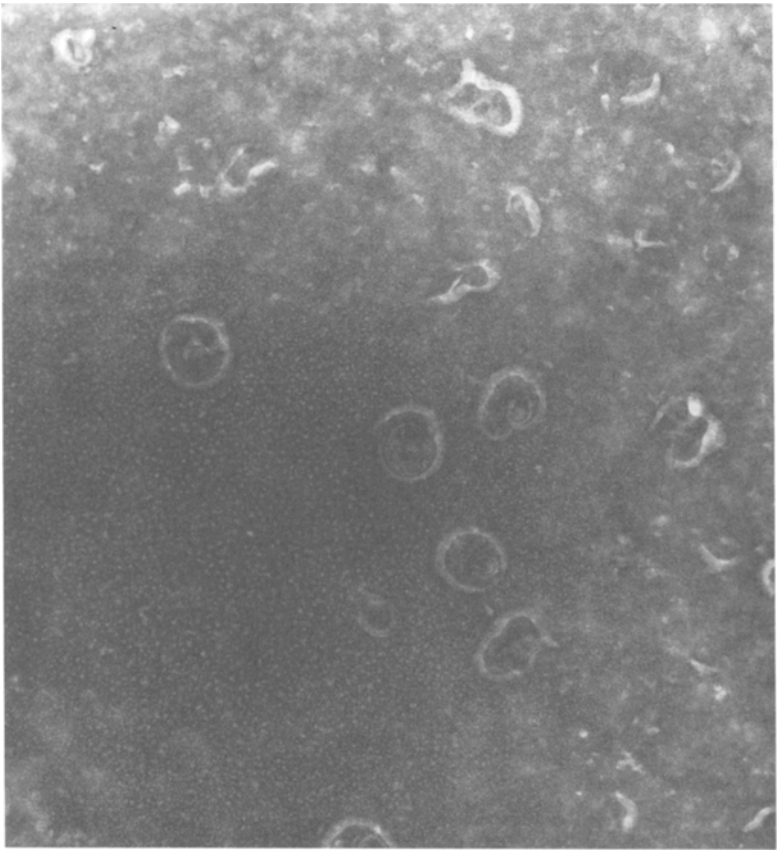

FIG. 3. Transmission electron micrograph of opelu seawater rinse at 127,500×. Lipid vesicles display the classic onion-skin structure of liposomes.

TABLE 1. BEHAVIORAL RESPONSE INTENSITIES TO OLFACTORY STIMULI

Stimulus type	Average response intensity (SD)	Number of trials
Soy bean liposomes	0.8 (0.8)	9
Amino acid mixture	3.0 (1.6)	7
Soy liposomes plus amino acid mixture	4.4 (0.5)	9
Whole squid (control)	7.1 (1.0)	11

These chemical search images may heighten appetitive feeding behavior in response to the odors of previously consumed prey (Atema et al., 1980) and thereby improve foraging efficiency. Even without a learned response to a particular prey species, the predictable nature of the prey odors could facilitate innate responses to specific food types. The qualitatively similar profiles of opelu and akule perhaps reflect the fact that they are both in the family Carangidae.

Amino acids appear to be routinely bound into a variety of biogenic lipid structures. The presence of tryptophan fluorescence in the liposomes, and its absence in the background medium, suggests that a large portion of amino acids emanating from prey are incorporated into lipid complexes. The HPTLC results indicate that lipid compounds suitable for liposome formation are present in the prey rinses. The rate of efflux of a particular amino acid from lipid vesicles is determined by the size and complexity of the lipid structure, the type of lipids from which the liposome is constructed, pH, and temperature (Klein et al., 1971; Naoi et al., 1977). Therefore, the time course of amino acid elution from liposomes in the open ocean will be variable, but it is probable that the lipid complexation significantly delays the dilution of prey odors to subthreshold levels.

The similar behavioral response intensities to the free amino acids and to the amino acid–liposome mixture indicate that lipid complexation does not significantly alter the biological potency of prey amino acid profiles. The reconstituted amino acid–lipid mixtures were not as effective as the whole rinse, but this is consistent with results from tuna and other species, which indicate that, although amino acids are important components of prey odors, there are other compounds present in the natural stimuli that contribute to their total effectiveness (Carr and Derby, 1986; Atema et al., 1980; Holland, 1978; Carr and Chaney, 1976). Diminution of maximum stimulus strength is probably less important to a passing school of tuna than the prolonged suprathreshold dwell time induced by lipid complexation of the amino acids. This would improve the chances of the tuna detecting the presence of food beyond visual range and possibly provide orientation cues for subsequent food search behavior.

Even though the amino acid profiles were temporally and spatially consistent and may permit olfactory recognition of the type of prey, lipid complexation may modify the profile detected by the predator because different amino acids elute from liposomes at different rates (Klein et al., 1971). Thus, the perceived prey profiles may well be constant, but somewhat modified, from those determined by our methods of analysis. This could explain why amino acid profiles constructed from ninhydrin-based analyses do not match the biological potency of the original natural stimulus. We believe this is the first demonstration of biogenic liposomes mediating interspecific interactions and that lipid structures may have a widespread role in the semiochemistry of aquatic chemoreception.

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METABOLISM AND EXUDATION OF CANAVANINE DURING DEVELOPMENT OF ALFALFA (*Medicago sativa* L. cv. VERKO)

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Abstract—The structural analog of amino acid L-arginine, L-canavanine (2-amino-4-guanidinooxybutyric acid), is found in 26 cultivars of alfalfa. Its concentration ranges from 6 to 16 mg/g of dry seeds. Canavanine represented more than 70% of the total soluble nitrogen in seeds. Practically all of the canavanine was stored in the cotyledons. Comparison is made of the canavanine content in the cultivars Verko and Europa harvested in different years. During sprouting, 29% of the guanidinooxy compound was translocated into the hypocotyl and radicle in 24 hr. In this early stage of seedling development, the level of the nonprotein amino acid, canavanine, increased threefold whereas the protein amino acid, arginine, as well as asparagine increased 11- and 35-fold, respectively. Two-day-old seedlings are capable of synthesizing canavanine derived from canaline up to 25%. Contrary to this finding in seedlings grown in the time range of 24 days, the guanidino compounds canavanine and arginine were metabolized rapidly, whereas asparagine increased. Furthermore, the toxic canavanine got into the environment of swelled seeds or into the rhizosphere of young seedlings and increased in the milieu to concentrations at 3–57 μM. In a biotest, this inhibited the growth of a tomato cell suspension culture as well as the growth of cabbage radicle.

Key Words—Canavanine, arginine, asparagine, amino acids, root exudates, growth inhibition, alfalfa, *Medicago sativa*.

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INTRODUCTION

The nonprotein amino acid canavanine (2-amino-4-guanidinooxybutyric acid) is a structural analog of L-arginine (2-amino-5-guanidinovaleric acid) and its metabolic antagonist (Rosenthal, 1977a, 1991; Natelson, 1985a; Lavin, 1986). This toxic compound is stored in the seeds of many leguminous plants (Rosenthal, 1977a; Lavin, 1986). During the imbibition and germination of seeds the nitrogen-rich canavanine is mobilized and degraded, resulting in the formation of aminooxy acid canaline and urea (Töpfer et al., 1970; Bell et al., 1978; Downum et al., 1983; Rosenthal, 1990). Contrary to this finding, canavanine increased in the first days of cultivation of the sword bean, *Canavalia gladiata* (Jaqu.) DC (Fujihara et al., 1986) and alfalfa, *Medicago sativa* L. (Kasai and Sakamura, 1986). In seedlings of *Medicago sativa* L. (Miersch et al., 1988) and *Canavalia ensiformis* (L.) DC (Rosenthal, 1972) the toxic amino acid canavanine was localized in roots, leaves, and stems. Obviously, distinct canavanine-free (or very low) stages of growth periods can be detected in the life cycle of *Medicago* (Miersch et al., 1988), whereas *Canavalia* is a canavanine-containing legume during the whole growing time (Rosenthal, 1977b; Fujihara et al., 1986).

In this paper, some experiments are carried out to clear up the following points: (1) the distribution of canavanine in seeds as well as in the developing seedlings of alfalfa, and (2) the secretion of canavanine into root space and the growth inhibition of receptor plants.

METHODS AND MATERIALS

Seed Material and Cultivation of Alfalfa. Seeds of alfalfa (*Medicago sativa* L.) were gifts of various institutions (see Table 1). The seeds were germinated and the seedlings were cultivated under sterile as well as nonsterile conditions according to Gorski et al. (1991).

Biotests. The influence of canavanine was measured on cell growth of tomato suspension culture (*Lycopersicon esculentum* MILL., cv. Lukullus) described earlier (Grancharov et al., 1985). The experiment was started by inoculation of cells and application of filter-sterilized solution of canavanine at concentrations of 10^{-3}–10^{-6} M.

The inhibition of radicle growth of cabbage (*Brassica oleracea* L.) was estimated during a period of seven days at 20°C and 6000 lux. For example, 40 seeds of cabbage (control) and 40 seeds of cabbage and 40 or 80 seeds of alfalfa were placed on a paper disk in Petri dishes of 8 cm diameter saturated with 5 ml of water. After seven days, the radicle length of the cabbage was measured.

Statistical Analysis. The data were analyzed statistically according to Weber

TABLE 1. CANAVANINE IN SEEDS OF SOME EUROPEAN CULTIVARS OF ALFALFA (*Medicago sativa* L.)

Cultivars	Collection year	Origin	Canavanine		
			mg/g dry mass	μg/10 seeds	%
Hybrida Milfenil(3)[a]	1986	France	5.7	113	0.6
Tomashskaja(1)	1984	USSR	5.7	104	0.6
Verko(1)	1987	GDR	5.9	110	0.6
H 27/55(3)	1986	Rouman	6.5	134	0.7
Triesdorfet(3)	1986	FRG	6.6	153	0.7
S.z.U.V.(3)	1986	H	6.6	146	0.7
Dunowka(2)	1987	BG	6.8	152	0.7
Warminska(3)	1986	PL	6.8	138	0.7
Europe(1)	1986	F	7.0	155	0.7
Czernikowskaja(3)	1986	CS	7.0	156	0.7
Szaponex(3)	1986	H	7.2	151	0.7
FD-100(3)	1986	F	8.0	172	0.8
Maris Phoenix(3)	1986	GB	8.6	226	0.9
Verko(1)	1984	F	8.6	157	0.9
Pleven 2(2)	1987	BG	8.9	195	0.9
Iris(3)	1986	DK	9.0	211	0.9
MEV(1)	1984	FRG	9.1	167	0.9
Pleven 1(2)	1987	BG	9.3	205	0.9
Pleven 6(2)	1987	BG	9.5	209	1.0
DuPuits(3)	1986	F	9.6	204	1.0
Nadeshda 1(2)	1988	BG	10.0	224	1.0
Verko(1)	1986	GDR	10.1	186	1.0
Verko(1)	1985	GDR	10.3	189	1.0
Vertus(1)	1984	GDR	10.7	196	1.1
Nadeshda 2(2)	1988	BG	11.3	248	1.1
Kleszczewska(3)	1986	PL	11.6	264	1.2
Europa(1)	1984	GB	12.0	220	1.2
Pleven 13(2)	1987	BG	12.9	284	1.3
Ossam(2)	1988	BG	16.1	354	1.6
Obnova 10(2)	1988	BG	16.1	354	1.6

[a] The sources of the seeds are: (1) Institute of Seeds and Plants, Halle/S., Germany; (2) Christov Institute of Fodder, Sadovo, Bulgaria; (3) Institute of Soil Sciences and Plant Cult., Pulawy, Poland.

(1986) by variance (*F* test), comparison of average root length (Student's *t* test) and homogeneity (chi-square test).

Canavanine Analysis. This amino acid was quantified by colorimetric estimation with pentacyanoammoniumferrate (Fearon and Bell, 1955) with respect to results obtained by other groups (Rosenthal and Dahlman, 1982; Natelson,

1985a; Brune et al., 1988). The standard deviation was determined to be 15% at 5 μg/ml and 2% at 70 μg/ml sample.

Small amounts of canavanine were verified by TLC analysis of dansylated canavanine on polyamide sheets (Woods and Wang, 1967). The detection limit was found to be 10^{-12} M canavanine.

Amino Acid Analysis. Aliquots of extracts were subjected to amino acid analysis using an amino acid autoanalyzer (type AAA 881, Microtechna, Prague, CSFR) according to the method of Spackman et al. (1951). In general, the standard deviation was measured to 8–10%. The calculation of the amide asparagine and glutamine was based on the estimation of aspartic and glutamic acid before and after hydrolysis with 6 N NCl for 1 hr at 80°C. After that, the samples were evaporated in vacuo to dryness and the residues dissolved in 0.01 N HCl. In this case, the standard deviation was measured to 15–20%.

Extraction Procedure. Plant material (100 mg to 2 g) was dried at 80°C for 4 hr, powdered, and then extracted three times with 10 ml of a mixture containing 50% aqueous ethanol, 0.4 N with respect to H_2SO_4. Seeds and cotyledons were shaken three times with 20 ml diethyl ether to remove lipids. After that, neutralization of the extracts was carried out by the addition of 2 N NaOH. If protein was precipitated, the extracts were centrifuged at 2000*g* for 10 min, and the supernatant as well as the aqueous milieu of the paper disks or sandy beds was fractionated by cation exchange chromatography on Dowex 50 WX8 (NH_4^+), 100–200 mesh, according to Rosenthal (1977c). The resin-bound canavanine was eluated by 50% aqueous ethanol, 0.35 N with respect to NH_4OH. All eluates were evaporated to dryness in vacuo below 40°C, and the residues obtained were dissolved in 1–2 ml of double-distilled water.

For measuring amino acids by an automatical analyzer, the same extraction procedure was used except: (1) the cation exchange resin used was changed to Dowex 50 WX8 (H^+), 100–200 mesh, (2) the resin-bound amino acids were eluated by 2 N NH_4OH in 50% aqueous ethanol, and (3) the residues were dissolved in 4 ml 0.01 N HCl.

Feeding Experiments. Seedlings of alfalfa were grown under nonsterile conditions in Petri dishes on paper disks for two days as described above. Then, 10 seedlings were fed through the roots with one of the following compounds, [U−^{3}H]canaline, [6−^{3}H]glucose and [6−^{14}C]glucose, dissolved in 500 μl double-distilled water in a time range up to 20 hr at 20°C and 4000 Lux. After the feeding time, the seedlings were harvested and canavanine isolated as described under "Extraction procedure" above. Further purification of canavanine was obtained by TLC on silica gel with the mobile phase ethanol–25% NH_4OH 77:23 (v/v) as well as electrophoresis on cellulose sheets (Takahara et al., 1978). The canavanine area from chromatograms or pherograms was localized and scraped off directly into scintillation vials containing toluene–PPO–POPOP scintillator. To determine the uptake of canaline, aliquots of the feeding solution

were also added to scintillation vials. The radioactivity was measured in a Packard-Tricarb-Scintillation Spectrometer, model 3380.

Chemicals. [U−^{3}H]Canaline (specific activity: 346 MBq/mmol) was obtained by enzymatical degradation of Wilzbach-tritiated canavanine sulfate (Zentralinstitut für Kernforschung, Dresden, Germany) using liver arginase according to Rosenthal (1973). The isolation and purification procedure was described by Miersch (1967). [6−^{3}H]Glucose (specific activity: 6.73 GBq/mmol) and [6−^{14}C]glucose (specific activity: 1.98 GBq/mmol) were supplied by UVVVR, Prague, CSFR, and Radiochemical Centre, Amersham, England, respectively. All other chemicals are commercial products from Germany.

RESULTS AND DISCUSSION

Canavanine Level in Alfalfa Seeds and Seedlings. Values of canavanine obtained ranged from 5.7 to 16 mg/g dry seeds for 26 European varieties of alfalfa (Table 1). Furthermore, the amount of this toxic amino acid depends on the origin as well as the year of collection, as demonstrated with the cultivars Verko and Europa (Table 1). These quantitations are in agreement with experimental findings of Tschiersch (1961), Natelson (1985b), and our earlier data (Miersch et al., 1988, 1990). In particular the amount of canavanine obtained from different samples of the cultivar Weevlchek can be variable and ranged from 1.4 to 2% (Natelson, 1985a).

It can be shown that nearly the whole amount of canavanine was stored in the cotyledons, while this toxic compound was found to be 0.6% in the testa (Table 2). In the testa of *Canavalia ensiformis* (L.) DC seeds, the guanidinooxy compound was only measured to 0.3%.

Besides canavanine, mainly histidine reacts with pentacyanoammonium-

TABLE 2. DISTRIBUTION OF CANAVANINE IN SEEDS OF ALFALFA (*Medicago sativa* CV. VERKO)

	Dry seeds		Seeds soaked for 24 hr	
Sample	μg[a]	%	μg[a]	%
Seeds	5670	100	6800	100
Testa	34	0.6	538	9
Cotyledons[b]			4200	62
Hypocotyl and radicle[b]	5636	99.4	2017	29

[a] Canavanine, μg per 300 seeds; year of collection: 1985.
[b] In dry seeds the term means embryo and cotyledons.

ferrate (PCAF), yielding a pink complex (Rosenthal and Dahlman, 1982). This colorimetric method is more sensitive (eightfold) for the guanidinooxy compound (Natelson, 1985a, Brune et al., 1988) than histidine. Since the relative error of the PCAF reaction was measured for 5 μg and 70 μg/ml sample at 15% and 2%, respectively, the amount of histidine in biological samples is unimportant if the ratio of histidine to canavanine does not exceed 1:20 (Table 3). Additionally, a practical control of canavanine analysis was made by reducing the guanidinooxy compound using gaseous hydrogen on palladium oxide (Natelson and Bratton, 1984).

In young seedlings, the canavanine accumulation and degradation in relation to growth condition are shown in Figure 1. The catabolism and exudation of canavanine depends on the nutritional situation of growing seedlings (Figure 1C,D). In alfalfa plants cultivated under sterile conditions for 65 days, no canavanine was detectable using PCAF reagent (Miersch et al., 1988). This increase of the toxic amino acid during development of young seedlings (Figure 1, Table 3) is not explained by remobilization of a canavanine-containing compound because we could not find, e.g., higher levels of canavanylpeptides or canavaninosuccinic acid in seeds or seedlings. Feeding experiments have shown that lucerne seedlings cultivated for two days are capable of incorporating 24% of [^{3}H]canaline into canavanine in 12 hr (Table 4). The nonspecific incorporation of [6$-^3$H]- or [6$-^{14}$C]glucose is low (0.2%) under the same experimental conditions. Therefore, the incorporation of [^{3}H]canaline into canavanine was discussed via ureidohomoserine and canavanylsuccinate (Rosenthal, 1972). Besides [^{3}H]canavanine, a second ^{3}H-labeled compound was detected that was not homoserine. This unknown compound could be isoxazolidone, resulting from deamination, decarboxylation, and ring closure of canaline (Sugii et al., 1981).

The chromatographic separation of the main amino acids in extracts of mature seeds and seedlings is illustrated in Table 3. Extremely high levels of canavanine were measured in the seeds and far exceeded those of arginine and asparagine. Canavanine and arginine represented about 70% and 13%, respectively, which is in agreement with findings of *Canavalia* (Rosenthal, 1977b; Fujihara et al., 1986). The canavanine and arginine content increased during seedling development up to 29% and 23%, respectively, whereas asparagine and proline were enriched to 23% and 18%, respectively. Whereas canavanine was catabolized nearly completely, depending on cultural conditions (Figure 1), this amide is present for a long time in the lucerne plant (Bickoff et al., 1972; Girousse et al., 1991). During seedling growth, alfalfa represented a guanidino compound nitrogen-storage plant, which changed to an amide-nitrogen type. Proline accumulation that was observed can be also an auxiliary nitrogen storage in older plants or the result of environmental stresses (Nover et al., 1989).

Canavanine Exudation and Growth Inhibition. During the swelling of seeds

TABLE 3. FREE AMINO ACIDS IN ALFALFA SEEDS, GREEN SPROUTS, SEED AND ROOT EXUDATES (μg/100 SAMPLES)[a]

Amino acids	Mature seeds	Seeds soaked 24 hr in H_2O		Seedlings, 2 days			Seedlings, 4 days			Seedlings, 8 days			Seedlings, 16 days			Seedlings, 24 days		
		S	E	SH	R	E	SH	R	E	SH	R	E	SH	R	E	SH	R	E
Lys	44	59	11	221	141	64	431	85	76	183	48	46	251	29	20	370	79	84
His	26	39	6	415	265	43	908	96	46	586	22	–	587	43	19	589	55	22
Arg	180	114	15	1290	498	334	1816	301	376	1120	56	–	542	66	15	436	67	8
Asp	58	39	6	120	181	9	1081	72	12	84	–	–	1370	129	41	897	128	24
Asn	51	64	0	854	945	13	721	407	6	1574	–	–	2572	–	–	2252	–	–
Thr	–	–	–	–	–	–	–	+	–	59	–	–	388	–	–	393	–	–
Ser	–	–	–	–	–	–	–	+	–	100	–	–	645	–	–	613	–	–
Glu	32	170	13	98	80	2	78	15	5	127	45	4	466	74	65	302	63	35
Gln	29	–	0	62	15	3	56	9	4	36	–	–	396	–	–	435	–	–
Pro	12	19	3	133	125	–	+	37	+	129	+	+	2441	136	+	2273	+	+
Gly	–	–	3	23	64	7	40	22	8	25	3	1	99	5	8	124	+	+
Ala	–	–	9	96	351	4	161	31	3	69	4	4	567	73	12	520	32	0
Val	–	–	–	84	185	+	500	80	+	63	52	49	574	36	115	605	66	+
Ileu	–	–	3	57	112	+	349	54	+	48	5	+	249	31	14	289	62	+
Leu	–	–	2	49	78	+	199	31	+	30	3	+	146	13	4	206	31	+
Tyr	–	–	11	46	93	+	171	+	+	241	12	+	60	27	15	151	11	+
Phe	–	–	3	58	122	+	256	+	+	90	18	+	583	34	75	781	17	+
GABA	–	–	17	26	23	+	52	+	+	99	12	+	461	104	+	852	4	+
Can	994	931	138	1996	911	145	1734	441	143	1074	790	78	602	98	149	476	123	127
Total	1426	1435	240	5628	4189	624	8553	1681	679	5737	1040	182	12988	898	589	12564	788	426

[a] Year of collection: 1987. Germination, cultivation of seedlings, and standard deviations are described in Methods and Materials: weight of seedlings is shown in Figure 1. Abbreviation used: SH = shoots, S = seeds, R = roots, E = exudates, + = trace, – = not determined.

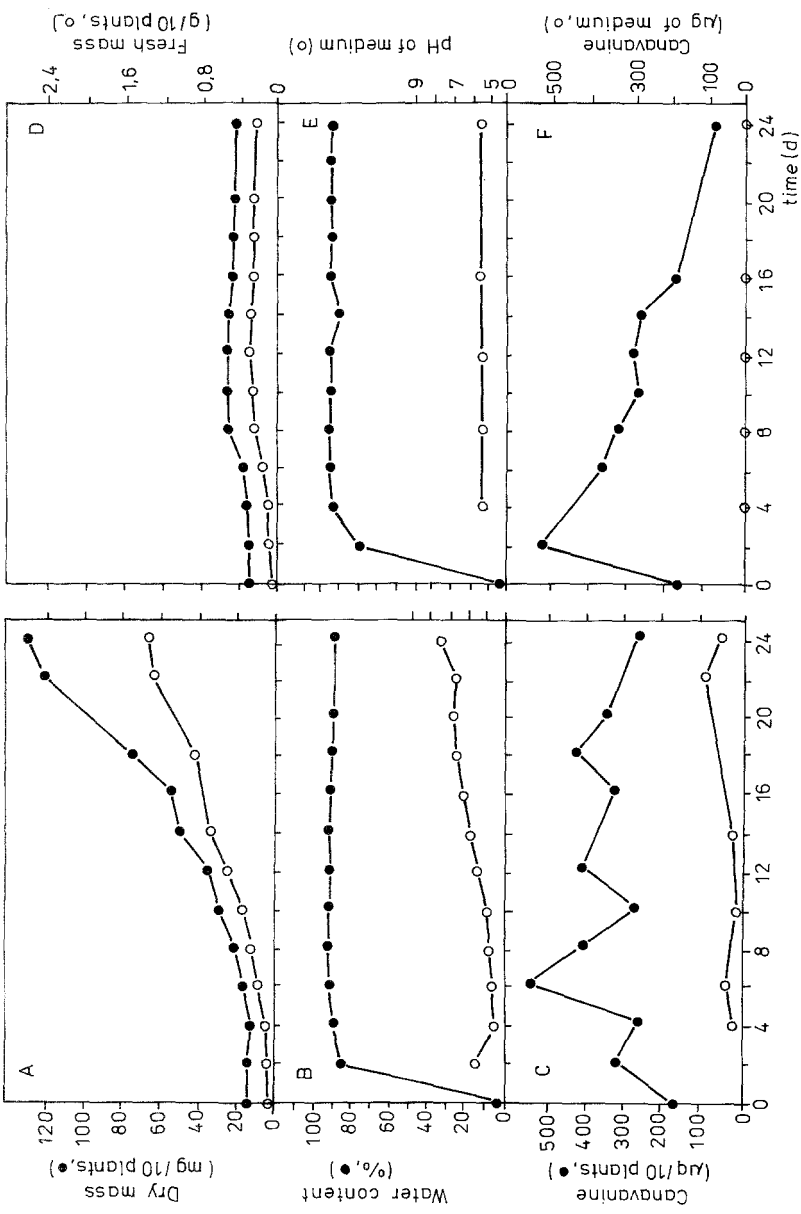

FIG. 1. Growth conditions and canavanine levels in seedlings and root exudations of alfalfa (*Medicago sativa* L. cv. Verko). Growth (A, D), water content, pH of medium (B, E), and canavanine levels as well as root exudates (C, F) of seedlings cultured in nutrition solution (A–C) or water (D, F).

and growth of seedlings, the storage compound was mobilized and released into the rhizosphere. Around seeds soaked in water, canavanine was found to be up to 8% of total amino acids or 13% of total canavanine in paper disks, but from roots of young seedlings this compound was released in nutrient solutions at a rate of about 1% of the total amount of amino acids (Table 3). The fluctuation of amino acids in root exudates of alfalfa (Richter, 1967) was also measured for canavanine, which ranged from 4% to 18% of total canavanine (Figure 1D, Table 3). This compound increased in the soil under experimental conditions to

TABLE 4. UPTAKE AND INCORPORATION OF TRITIATED CANALINE INTO CANAVANINE OF ALFALFA SEEDLINGS (*Medicago sativa* L. CV. VERKO)

Time (hr)	Uptake		Radioactivity of canavanine	
	Cpm[a] ($\times 10^3$)	%	Cpm[a] ($\times 10^3$)	%[a]
0	975.00	100		
4	280.71	29	24.48	8.7
8	350.85	36	74.00	21.1
12	397.47	41	97.58	24.6
16	426.96	44	90.07	21.1
20	545.00	56	112.35	20.6

[a] Per uptake of canaline.

TABLE 5. CANAVANINE SURROUNDING IMBIBED SEEDS AND IN ROOT SPACE OF ALFALFA SEEDLINGS

Variant	Number of seeds or seedlings	Volume (ml)	*Canavanine*	
			μg	μM
Seedlings				
Sand	150, 4 days	30	16	3
Sand	500, 4 days	30	42	8
Sand, sterile	500, 4 days	30	135	26
Knop-solution, sterile	10, 8 days	30	50	10
	10, 16 days	30	56	11
Knop-solution and humus, sterile	10, 8 days	30	40	8
	10, 16 days	30	35	7
	10, 24 days	30	100	19
Seeds				
Sand, sterile	500, soaked 1 day	50	46	9
Paper disk	500, soaked 1 day	10	100	57

such concentrations (Table 5) as to inhibit the growth of receptor plants. From experimental data, we could calculate a concentration of canavanine of 3–57 μM in the aqueous milieu of sandy bed or paper disks; 500 seedlings of alfalfa cultivated for four days under sterile conditions formed a concentration of canavanine of about 26 μM in the aqueous milieu of the sandy bed (Table 5). In the presence of microorganisms, the concentration of canavanine increased only to 8 μM (Table 5). With nonsterile conditions, no canavanine exudate was detected from roots of seedlings cultivated on water for 24 days (Figure 1F). Because canavanine at the 10 μM level inhibited the growth of tomato cells by 35% at the fourth day (Figure 2), we assume an allelochemical influence of canavanine on other plants surrounding the imbibed as well as germinated seeds. We also found growth inhibition in experiments with cabbage (*Brassica oleracea* L.) during seedling growth of seven days. In this case, the radicle length was significantly decreased to 51% and 81%, depending on the amount of alfalfa seedlings present (Table 6), and decreased to 41% by using 50 μM canavanine. Because canavanine was found in the solution, the sand, or on the paper disk, these inhibitory effects seem to be caused by canavanine or some other substances rather than by saponins (Gorski et al., 1991). Further experiments are in progress to demonstrate the release of root exudates by alfalfa grown under sterile as well as field conditions.

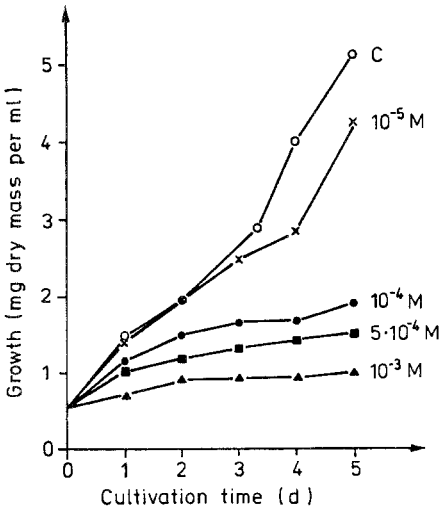

FIG. 2. Growth inhibition of cell suspension culture of tomato (*Lycopersicon esculentum* MILL. cv. Lukullus) by various concentrations of canavanine. Standard deviations were covered by symbols.

TABLE 6. INHIBITION OF RADICLE GROWTH OF CABBAGE (*Brassica oleracea* L.) BY ALFALFA (*Medicago sativa* L. CV. VERKO)

(days)	Time control (40 cabbage seeds) radicle length ± SD (mm)	Experiment 1 (40 cabbage and 40 alfalfa seeds) radicle				Experiment 2 (40 cabbage and 80 alfalfa seeds) radicle			
		Length ± SD (mm)	Inhibition (%)	F^a	χ^{2a}	Length ± SD (mm)	Inhibition (%)	F^a	χ^{2a}
2	3.33 ± 1.03	2.73 ± 0.67	18	2.4	4.56	3.30 ± 0.99	1	1.1	0.22
3	11.09 ± 5.32	4.31 ± 2.24	61	5.7	31.39	5.59 ± 3.09	50	3.0	21.94
4	23.03 ± 9.93	8.16 ± 4.75	65	4.4	32.26	7.83 ± 5.23	66	3.4	30.68
7	51.14 ± 21.62	25.88 ± 17.69	51	1.5	14.81	9.97 ± 7.89	81	7.4	36.53

[a] Significance level of $F = 1.8$ (5%) and $\chi^2 = 13.8$ ($\alpha = 0.01$).

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DISRUPTION OF FORAGING BY ARGENTINE ANTS, *Iridomyrmex humilis* (MAYR) (HYMENOPTERA: FORMICIDAE), IN CITRUS TREES THROUGH THE USE OF SEMIOCHEMICALS AND RELATED CHEMICALS

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Abstract—Foraging in trees by the Argentine ant, *Iridomyrmex humilis* (Mayr), was disrupted by a variety of synthetic chemicals, with the most effective chemical being farnesol. Testing of substrates for presentation of the disruptant chemicals gave some success with rubber or Tygon tubing, although best results were obtained through incorporation of the material into Stikem, which was then banded around tree trunks. Amounts of farnesol used for effective, long residual ant control were between 0.8 and 2 g per tree.

Key Words—Argentine ants, *Iridomyrmex humilis*, Hymenoptera, Formicidae, semiochemicals, ant repellents, farnesol.

INTRODUCTION

Ants are highly organized social insects whose communication with others of their species and with their environment is mediated largely through behaviorally

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active chemicals (Hölldobler and Wilson, 1990). Some of the chemicals cause repellency or inhibition of ongoing behavior. For example, an alarm pheromone released from an ant that is injured or attacked may cause a variety of specific behaviors, including repellency, among nearby sister workers.

The alarm pheromones of some ants have received considerable study. An example is the genus *Atta*, in which the mandibular glands contain a rich medley of chemicals, many presumably functioning as alarm pheromone components. The discovery of citral in the mandibular glands of *Atta sexdens* dates back to Butenandt et al. (1959). Blum et al. (1968) later found nerol and geraniol in the mandibular glands of *Atta* species. The content of these glands was further studied by Schildknecht (1976), who found β-pinene, citral, farnesol, and 14 other compounds.

Another example of a chemical medley constituting ant alarm pheromones was identified by Lloyd et al. (1989), who found methyl salicylate to be a principal alarm pheromone component produced by workers of several species of honey ants, *Myrmecocystus* spp. A number of terpenoids found in the workers, including neral, geranial, citronellol, and limonene, presumably also possess ant alarm-pheromone activity.

The various species of *Formica*, when alarmed, spray mixtures of formic acid and Dufour's gland secretion toward the enemy, with the mixtures serving simultaneously as defensive substances and alarm pheromones (Maschwitz, 1964). In some genera of ants, alarm pheromones may originate simultaneously from as many as three different glandular sites, serving both a defensive and an alarm function (Hölldobler, 1977).

It is of interest that, although workers of the Argentine ant, *Iridomyrmex humilis* (Mayr), the species which serves as the object of the present study, contain many volatile chemicals, including a number of terpenoid compounds, such as iridomyrmecin, which have insecticidal properties (Cavill and Houghton, 1974), there is apparently no alarm pheromone reaction exhibited by workers of this species to their crushed sisters (Key et al., 1981).

Chemical-structure–biological-activity relationships have been investigated for alarm pheromone components and related chemicals for several ant species. Blum et al. (1966) tested a series of 49 ketones related to one of the natural alarm pheromone chemicals (2-heptanone) on *Iridomyrmex pruinosus*. The more the structure was varied from that of 2-heptanone in terms of numbers of carbon atoms or position of functional groups, the lower the activity of alarm behavior elicited by the chemicals. A similar result was obtained by Regnier and Wilson (1968) for *Acanthomyops claviger*; they found alkanes between 10 and 13 carbons in length elicited good alarm response (the natural alarm pheromone of this species is an 11-carbon alkane). Enantiomeric specificity of antennal receptor response to alarm pheromones has been demonstrated for *Atta texana* and *A. cephalotes* (Riley et al., 1974) and for *Pogonomyrmex barbatus* (Benthuysen and Blum, 1974).

Many behavior-inhibiting or repellent chemicals are kairomones, which are released by members of other species and ward off ants. Honda (1983) demonstrated a defensive potential against ants of the secretion of the larval osmeteria of papilionid caterpillars; they determined the active principal to be α-pinene. Scheffrahn et al. (1983) reported on a mix of six sesquiterpene hydrocarbons that is secreted onto the head surface of the North American termite, *Amitermes wheeleri*; a defensive function was proposed for the mixture, due to the avoidance behavior seen in foraging Argentine ants. A Nearctic desert termite, *Amitermes minimus*, produces 4,11-epoxy-*cis*-eudesmane, which was found to be repellent to the ant, *Crematogaster californica* (Scheffrahn et al., 1984a).

The ant, *Forelius foetidus*, secretes 2-heptanone which, when used singly, functions as an alarm pheromone. It also secretes *cis, trans*-iridodial, which, when mixed with 2-heptanone, elicits defensive behavior in *F. foetidus* and repellent behavior in two other ants, *Solenopsis maniosa* and *Crematogaster californica* (Scheffrahn et al., 1984b).

Ants prey on the brood of tropical social wasps (Jeanne, 1975; Post and Jeanne, 1981). The wasps' defenses include application of repellent substances to the nest support. One of the active components in the defensive secretion of *Polistes fuscatus* is methyl palmitate. Ants of several species are reluctant to cross barriers of methyl palmitate, or its methyl ester homolog, methyl myristate (Post and Jeanne, 1981; Post et al., 1984; Henderson and Jeanne, 1989).

Kistner and Blum (1971) found that staphylinid beetles of the genus *Pella* mingle with workers of the ant, *Lasius spathepus*, from which they take food and dead ants. They are generally tolerated by the ants, but, if attacked, they release citronellal, which has a dispersal-inducing effect. Huth and Dettner (1990) examined the abdominal gland secretions of 13 staphylinid species and found them to contain a rapidly polymerizing iridoid dialdehyde, as well as α-pinene, neral, geranial, nerol, and citronellol; the role of these chemicals presumably is to inhibit aggression and cause dispersal of ants.

Some secondary plant chemicals are potent ant repellents, and the occurrence of such compounds is widespread in nature. Various terpenoid chemicals are the most important chemical factors in determining plant deterrency to leaf-cutting ants (Hubbell et al., 1983, 1984; Howard, 1987; Howard et al., 1988, 1989; Bueno et al., 1990).

Ants must be considered as serious pests of a number of agricultural crops. The action of the ants is often indirect, in that they tend homopterous honeydew-producing insects such as aphids, soft scales, and mealybugs. Through their transporting of these homopterous insects to new growth on the plants, and through their role in warding off potential parasites and predators, ants may create situations where the tended insects assume serious pest status, whereas, in the absence of the ants, the insects would often be regulated through the action of beneficial predatory and parasitic species.

It is surprising that, with so much fundamental research having been conducted to elucidate the many naturally occurring compounds that repel or otherwise inhibit the activities of ants, these chemicals have received essentially no attention as possible agents for pest management. The research discussed here was initiated to investigate the degree of disruption of Argentine ant trail-following on the trunks of citrus trees that could be obtained through the use of a variety of behaviorally active chemicals, including many of those mentioned above.

METHODS AND MATERIALS

The research was conducted in a planting of young, nonbearing lemon trees at the Limoneira Company Ranch, Santa Paula, Ventura County, California. Trees were about 2 m high, and stem diameter ranged from 3 to 5 cm at 10 cm above the soil surface. The trees were heavily infested with a variety of honeydew-producing Homoptera, and the Argentine ants made trails from their nests in the ground, up the trunks to the vicinities of the insects they were tending.

A double layer of duct tape was wrapped around the trunks of trees, at about 10 cm above the soil surface. Candidate chemicals tested for disrupting ant trail following were placed on specified lengths of cotton twine or Tygon or rubber tubing, which were then wrapped two or three times around the tree trunk, over the duct tape. The tape was intended to protect the tree trunks from possible phytotoxic effects of the tested chemicals. Under most circumstances, the twine or tubing was held in position by staples driven through the duct tape and into the tree trunk. An exception to this procedure occurred in the case of Stikem or Tree Tanglefoot-impregnated cotton twine, the ends of which were tucked under the loops of twine wrapped around the trunk.

Chemicals tested were obtained from a commercial supplier (Aldrich). They are listed in Tables 1–5 below. When a given chemical was tested in conjunction with a carrier of sticky material (Stikem Special, Seabright Enterprises, Emeryville, California; Tree Tanglefoot, The Tanglefoot Company, Grand Rapids, Michigan) the sticky material was first thinned through heating; the specified amount of sticky material was then mixed with the candidate chemical; finally, this sticky material–chemical mixture was placed in a glass jar containing a specified length of Puritan cotton garden twine and was continuously agitated until essentially all of the mixture was incorporated in and on the twine.

Efficacy of disruption of ant trail following and foraging was evaluated on specified days following placement of the chemical-impregnated twine or tubing around tree trunks, by counts of the numbers of ants walking across the twine or tubing during a 2-min interval. Ants were counted without regard to whether they were proceeding up or down the trunk. Untreated control counts were made of ants crossing over a band of untreated twine around control tree trunks.

RESULTS AND DISCUSSION

Nineteen chemicals were tested for efficacy through the inoculation of 1 g of each onto 30-cm lengths of cotton twine, which were then wrapped around the trunks of test trees (Table 1). Each tree was regarded as a plot, and treatments were replicated three times in a randomized complete-block design. Counts of ants passing over the test twine were made on the sixth day following application. It was evident that farnesol had a high degree of ant repellent activity, with several other chemicals (methyl eugenol, β-citronellol, and bornyl acetate) also possessing good activity. A difficulty in interpreting these results is apparent in that it is not clear whether a lack of activity of a chemical after six days of exposure in the field is due to that chemical having an inherent low level of

TABLE 1. EVALUATION OF CHEMICALS FOR DISRUPTION OF TRAIL FOLLOWING BY ARGENTINE ANTS ON CITRUS IN VENTURA, CALIFORNIA[a]

Chemical	Mean no. of ants/2 min passing barrier on day 6[b]
Farnesol	0.0 a
Methyl eugenol	0.6 ab
β-Citronellol	0.7 abc
Bornyl acetate	0.9 abc
Eugenol	1.7 bcd
Methyl myristate	2.1 cde
Citral	4.6 def
Methyl salicylate	4.7 def
Safrole	5.4 efg
Limonene	5.7 efg
Linalool	6.1 fgh
Benzaldehyde	8.7 fgh
Methyl anthranilate	10.0 fgh
Limonene oxide	10.1 fgh
α-Terpineol	11.5 fgh
Myrcene	11.5 fgh
α-Pinene	12.1 fgh
β-Pinene	13.5 gh
3-Carene	16.0 h
Untreated	10.1 fgh

[a] Lengths of (30 cm) cotton twine permeated with 1 g of candidate chemical were wrapped around tree trunks 10 cm above the soil surface; 1991.

[b] Each treatment chemical was replicated three times in a randomized complete-block design. Counts of ants crossing the barriers in 2 min were transformed to $\ln(X + 1)$ for ANOVA. Duncan's multiple-range test was performed on transformed means. Means in the same column followed by a common letter are not significantly (5% probability) different. Means presented here are transformed back to the original scale.

activity, or to the chemical having volatilized, oxidized, or otherwise become unavailable to the ants.

Testing conducted to evaluate the efficacy of methyl eugenol incorporated into Tygon tubing of three different sizes or into one size of rubber tubing, and of citral incorporated only into the rubber tubing, is summarized in Table 2. Through occasional agitation over a 24-hr period, approximately 1:1 incorporation of the chemicals into the Tygon or rubber was accomplished. Based on the efficacy of both chemicals on rubber tubing, methyl eugenol was seen to be more active in disrupting the foraging of Argentine ants than was citral. However, even at the heaviest application of methyl eugenol in Tygon, at about 13 g per tree, complete deterrence of foraging by ants was only seen for about one week.

Some of the better candidate chemicals found through earlier testing were mixed as either 10 or 40% solutions in Stikem, which was then incorporated at the rate of 2 g formulated material into 50-cm lengths of cotton twine (Table 3). The treated lengths of twine were placed around tree trunks as described above. Complete disruption of ant foraging for seven days after application was seen with the 40% farnesol treatment (giving 0.8 g farnesol per tree). By three

TABLE 2. EVALUATION OF TYGON OR LATEX RUBBER DISPENSERS CHARGED WITH METHYL EUGENOL OR CITRAL FOR DISTRUPTION OF TRIAL FOLLOWING BY ARGENTINE ANTS IN CITRUS IN VENTURA, CALIFORNIA[a]

Chemical	Tubing	Mean no. of ants/2 min passing barrier on indicated day[b]		
		2	6	9
Methyl eugenol	Tygon[c]	2.4 ab	0.9 ab	2.4 abc
	Tygon[d]	0.6 ab	0.7 ab	1.5 ab
	Tygon[e]	0.0 a	0.0 a	0.6 a
	Rubber[f]	0.2 a	0.0 a	1.0 a
Citral	Rubber[f]	0.3 a	2.7 bc	8.2 bc
Untreated		4.6 b	7.4 c	18.8 c

[a] Lengths (30 cm) of tubing permeated with weights of candidate chemical equal to the tubing weight were wrapped around tree trunks 10 cm above the soil surface; 1991.

[b] Each treatment chemical was replicated three times in a randomized complete block design. Counts of ants crossing the barriers in 2 min were transformed to $\ln(X + 1)$ for ANOVA. Duncan's multiple-range test was performed on transformed means. Means in the same column followed by a common letter are not significantly (5% probability) different. Means presented here are transformed back to the original scale.

[c] Tygon tubing 0.80 mm ID × 2.38 mm OD. Tube holds 2.1 g of chemical.

[d] Tygon tubing 1.59 mm ID × 4.76 mm OD. Tube holds 5.6 g of chemical.

[e] Tygon tubing 4.76 mm ID × 7.94 mm OD. Tube holds 13.2 g of chemical.

[f] Latex rubber tubing 6.35 mm ID × 7.94 mm OD. Tube holds 3.0 g of chemical.

TABLE 3. EVALUATION OF COTTON TWINE DISPENSERS CHARGED WITH STIKEM CONTAINING VARYING PERCENTAGES OF FOUR CANDIDATE CHEMICALS FOR DISRUPTION OF TRAIL FOLLOWING BY ARGENTINE ANTS ON CITRUS IN VENTURA, CALIFORNIA[a]

Chemical	Percent	Mean no. of ants/2 min passing barrier on indicated day[b]		
		3	7	14
Farnesol	10	0.0 a	0.9 ab	9.9 ab
	40	0.0 a	0.0 a	2.4 a
β-Citronellol	10	5.7 b	9.0 bcd	9.0 ab
	40	0.0 a	2.7 abc	49.8 b
Methyl eugenol	10	9.0 b	16.2 cd	13.3 ab
	40	0.6 a	4.2 abcd	7.4 a
Citral	10	6.1 b	14.3 cd	26.5 ab
	40	1.6 ab	28.2 d	30.2 ab
Stikem alone		3.2 ab	5.5 bcd	12.4 ab
Untreated		12.5 b	18.8 cd	21.6 ab

[a] Lengths (50 cm) of twine permeated with a total of 2 g of formulated material were wrapped around tree trunks 10 cm above the soil surface; 1991.

[b] Each treatment chemical was replicated three times in a randomized complete-block design. Counts of ants crossing the barriers in 2 min were transformed to $\ln(X + 1)$ for ANOVA. Duncan's multiple-range test was performed on transformed means. Means in the same column followed by a common letter are not significantly (5% probability) different. Means presented here are transformed back to the original scale.

days after application, and continuing through 14 days, farnesol is seen to be more active as a disruptant than methyl eugenol, citral, or β-citronellol, although, as discussed earlier, this comparison may be confounded by differing rates of volatilization or degradation of the tested chemicals.

Farnesol and Stikem were tested at higher rates (Table 4). A farnesol-Stikem mixture was placed at 2 g of each material onto 50-cm lengths of cotton twine wrapped around tree trunks. Also tested were 30-cm lengths of rubber tubing, each containing 3 g farnesol. Farnesol alone, at 2 g per twine per tree, provided effective (over 90%) disruption of foraging by ants for about four weeks; rubber tubing containing 3 g farnesol per tree provided effective disruption for about seven weeks; and 2 g of farnesol in 2 g of Stikem provided effective disruption for 17 weeks (the last recorded reading).

Additional chemicals were tested as mixtures in Stikem (2 g of chemical in 2 g of Stikem) (Table 5). These were compared for efficacy with a farnesol-Stikem mix. Also tested were a farnesol–Tree Tanglefoot mix, Tree Tanglefoot alone, and Stikem alone. No significant difference is seen between Stikem and Tree Tanglefoot alone in disrupting ant foraging. Both materials were penetrated

TABLE 4. EVALUATION OF COTTON TWINE DISPENSERS IMPREGNATED WITH VARYING AMOUNTS OF STIKEM PLUS FARNESOL AND OF LATEX RUBBER TUBING DISPENSERS IMPREGNATED WITH FARNESOL FOR DISRUPTION OF TRAIL FOLLOWING BY ARGENTINE ANTS ON CITRUS IN VENTURA, CALIFORNIA[a]

	Material/tree (g)		Mean no. of ants/2 min passing barrier on indicated day[b]					
Dispenser	Stikem	Farnesol	7	14	27	48	77	122
Twine	0	2	0.0 a	0.6 a	4.9 a	0.0 a	0.6 a	4.9 a
Twine	2	2	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Rubber tubing[c]	0	3	6.0 b	1.3 a	1.5 a	6.0 b	1.3 a	1.5 a
Untreated			21.9 b	23.0 b	52.2 b	21.9 b	23.0 b	52.2 b

[a] Lengths (50 cm) of twine and 30-cm lengths of tubing were wrapped around tree trunks 10 cm above the soil surface; 1991.
[b] Each treatment chemical was replicated three times in a randomized complete-block design. Counts of ants crossing the barriers in 2 min were transformed to $\ln(X + 1)$ for ANOVA. Duncan's multiple-range test was performed on transformed means. Means in the same column followed by a common letter are not significantly (5% prob.) different. Means presented here are transformed back to the original scale.
[c] Latex rubber tubing, 6.35 mm ID × 7.94 mm OD.

TABLE 5. EVALUATION OF COTTON TWINE DISPENSERS CHARGED WITH 2 g OF STICKEM OR TREE TANGLEFOOT PLUS 2 g OF VARIOUS CHEMICALS, FOR DISTRIBUTION OF TRAIL FOLLOWING BY ARGENTINE ANTS IN CITRUS IN VENTURA, CALIFORNIA[a]

Chemicals	Mean no. of ants/2 min passing barrier on indicated day[b]				
	7	14	21	50	95
Stikem + 3,6-dimethyl-4-octyne-3,6-diol	1.4 ab	4.3 ab	4.1 ab		
Stikem + abietic acid	26.0 c	35.6 b	65.3		
Stikem + nerol	1.8 ab	8.7 ab	4.8 ab		
Stikem + linalyl acetate	1.3 a	14.8 b	11.5 b		
Stikem + citral dimethyl acetate	1.4 ab	13.0 b	9.8 b		
Stikem + farnesol	0.0 a	0.0 a	0.0 a	0.0 a	0.0 a
Tree tanglefoot + farnesol	0.0 a	0.0 a	0.0 a	0.5 a	33.5 a
Stikem alone	17.5 c	11.6 b	22.9 b	52.7 b	48.7 a
Tree Tanglefoot alone	1.34 bc	24.5 b	26.1 b	56.5 b	155.8 b
Untreated	27.3 c	33.8 b	33.8 b	58.3 b	135.6 b

[a] Lengths (50 cm) of twine permeated with the tested materials were wrapped around tree trunks 10 cm above soil surface; 1991.

[b] Each treatment chemical was replicated five times in a randomized complete-blocks design. Counts of ants crossing the barriers in 2 min were transformed to $\ln(X + 1)$ for ANOVA. Duncan's multiple-range test was performed on transformed means. Means in the same column followed by a common letter are not significantly (5% probability) different. Means presented here have been transformed back to the original scale.

almost immediately (within seven days) by significant numbers of foraging ants. However, farnesol mixed with Tree Tanglefoot gave effective disruption of foraging for seven weeks, and farnesol mixed with Stikem gave effective disruption for about 14 weeks. None of the other tested materials gave ant foraging disruption comparable to that provided by farnesol.

These experiments have shown that foraging in trees by the Argentine ant can be disrupted by a variety of synthetic chemicals. The most effective chemical found to date is farnesol, although an extensive, systematic search for disruptants has not been undertaken. Our evaluation of substrates for presentation of the disruptant chemicals had indicated some success with rubber or Tygon tubing, although best results to date have been obtained through incorporation of the material into Stikem-impregnated cotton twine, which was then banded around tree trunks. Presumably, the Stikem decreases the rate of volatilization and/or degradation of the chemicals. The use of chemical plus Stikem has some appeal, because Stikem is presently used to prevent certain insects from climbing tree trunks; farnesol apparently greatly extends the effective life of Stikem. Amounts of farnesol used to afford effective ant control were between 0.8 and 2 g per tree. These amounts should be subject to reduction through determining single chemicals or mixtures of chemicals that possess higher levels of activity than farnesol and determining more effective substrates for presentation of the materials in the field. Antioxidants also may prove useful in prolonging the activity of the most promising materials.

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Book Review

Chemical Communication: The Language of Pheromones. William C. Agosta. New York: Scientific American Library, 1992. 179 pp.

Chemical ecology has come of age. Professor Agosta, head of the Laboratory of Organic Chemistry at Rockefeller University is the perfect author to present the field to a larger audience. He works on the cutting edge of the field himself and combines an amazing breadth of biological and chemical knowledge with the clear, enticing style of a good journalist. This superbly written book moves along like a fast-paced video. It brings together members of the diverse discipline of chemical ecology. Laid out taxonomically, in seven chapters, the book features the key players in a vivid history of discovery.

Dr. Agosta highlights some landmark research, such as the identification of the first bark beetle pheromone by R.M. Silverstein and D.L. Wood. The golden hamster's mating pheromones, studied in Dr. Agosta's laboratory, are covered in detail: Among other facets, only 200 molecules of dimethyl disulfide suffice to attract the male to a female odor source!

Pheromones can signal established fecundity. David Crews found that the female red-sided garter snake makes a pheromone from vitellogenin. The liver sends vitellogenin not only to the ovary for eggs, but also to the skin. Here it remains until the following year to form a pheromone that exudes through the skin. Thus, this year's amount of pheromone signals to a male last year's number of eggs. A male chooses a large female with a high level of pheromone and thus many eggs.

The chemical language of insects can be complex: ants use 10–20 different signals to coordinate their colony, most of them chemical (E.O. Wilson, B. Hölldobler). The honeybee uses more than 30 chemical signals. In invertebrates, odor–response relationships are more direct, "hardwired" than in vertebrates. In Agosta's example, three pinched sea slugs will all recoil and secrete their alarm pheromone. Pinch three dogs and you can expect all kinds of responses.

Other complexities challenge the investigator. Simple stimulus–response relationships between one compound and a specific behavior are only one of many ways to code information. Pheromones often are mixtures of compounds. In garter snakes, the male recognition pheromone contains squalene, which is absent in females, but other compounds enhance the effect of squalene. In the golden hamster, one compound, dimethyl disulfide, accounts for 60% of the attractive power of vaginal secretion, but full activity requires most or all of the

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other volatiles (A.G. Singer, R.J. O'Connell, and W.C. Agosta). Several pheromones can control behavior sequentially. For instance, the mating behavior of certain ticks is guided by three pheromones. First, dichlorophenol from the female attracts the male. Then, cholesteryl oleate (from blood diet) induces the male to mount the female. Finally, a mixture of fatty acids with 14, 16, 18, and 20 carbon atoms from the digestive tract of the female facilitates actual copulation. In goldfish, a steroidal sex pheromone from the female stimulates milt production in the male. After that, a prostaglandin attracts the male.

We have been weaned from the notion that pheromones have to be unique compounds. Common, even ubiquitous compounds, can still be pheromones. Brown algae use several hydrocarbons; the soybean cyst nematode, vanillic acid; mites, 2-hydroxy-6-methylbenzaldehyde; and garter snakes, squalene. Furthermore, the same or closely related compounds crop up in vastly different species: prostaglandins as hatching pheromone in barnacles and as sex attractant and hormone in goldfish. *exo*-Brevicomin is found in the aggregation pheromone of bark beetles and dehydro-*exo*-brevicomin in male mouse urine. Finally, two related animal species may use the very same mixture of compounds. The dog ticks *Dermacentor variabilis* and *D. andersoni* share the same mixture of fatty acids as copulatory pheromone, but *D. andersoni* females have 12 times as much pheromone as *D. variabilis*.

Pheromones may derive from the diet, either directly or via precursors. The western pine beetle *Dendroctonus brevicomis* uses myrcene from ponderosa pine directly as a constituent of its aggregation pheromone. Female ticks take up cholesteryl oleate with their blood meal. This compound signals to the male that a host is available. The male then mounts the female. The male bark beetle *Ips paraconfusus*, aided by gut bacteria, converts myrcene into ipsenol and ipsdienol; α-pinene is converted into *cis*-verbenol. These together form the aggregation pheromone. The male queen butterfly's danaidone is a pyrrolizidine alkaloid, made from ingested plant alkaloids. The male stores 80% of the toxic alkaloids in his reproductive accessory glands and transfers them to the female during copulation. The female in turn incorporates these compounds into her eggs, where they deter predators.

From hormone to pheromone or vice versa? How certain animals use chemical signals may shed some light on the evolution of their communication system. In the goldfish, a steroidal hormone first serves as hormone to stimulate egg production and later as a pheromone that stimulates milt production in males, as mentioned above. Likewise, a prostaglandin as hormone stimulates spawning and as pheromone attracts the male. Another "hormone-to-pheromone" is testosterone when it serves as sex attractant in the urine of the male sea lamprey. Conversely, as unicellular organisms evolved into metazoa, they may have incorporated their original intercellular, interorganismal pheromones into their tissues as hormones for now internal use.

Practical applications of pheromones have scored some spectacular successes. One is the treatment of California cotton fields with gossyplure, the female pink bollworm's sex attractant, to disrupt mating. The other is monitoring the populations of pest insects such as bark beetles or the medfly over vast areas by attracting them to sticky traps scented with sex or aggregation pheromones. At the same time, Dr. Agosta clearly points out how biological, political, and commercial factors limit the applications of pheromones.

Tantilizing leads abound in this still-young field. Examples: (1) the female pheromone in urine of the economically important common blue crab has not been identified yet. (2) Little is known about pheromones in spiders. (3) The dog pheromones are still elusive.

To produce this slender volume that ranges over a broad area of chemical ecology, many topics are inevitably omitted. Thus, Agosta does not use the term "priming pheromones," although he discusses priming effects in goldfish and house mice. He discusses geometric isomers but does not mention enantiomers at all. Another minor point: Amphibia also have a vomeronasal organ.

The illustrations are superb. It is here that the only (merely minor) typos and inconsistencies occur. The final editing of the figure captions probably was beyond the control of the author. The index is much too short. One cannot find Bruce effect, pregnancy block, or priming pheromones, or many other topics discussed in the text. A very clear glossary will be most helpful to the unitiated. In all, I highly recommend this excellent book. It is very worthwhile for specialist and lay reader alike, and real fun to read.

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Book Review

Microbial Mediation of Plant–Herbivore Interactions. P. Barbosa, V.A. Krischik, and C.G. Jones (eds.). New York: John Wiley & Sons, 1991. $98.00 hardcover, 530 pp. + xiii.

The study of plant–herbivore interactions in a bitrophic context had been qualified as too narrow a view in 1980 by Price and coworkers. This influences many researchers to include the third trophic level, represented by predators and parasitoids of herbivores. The study of three-trophic-level interactions involving macroorganisms had flourished since the stimulating paper of Price et al. (1980) and has yielded a wealth of information on a wide variety of phenomena. However, most of the information is still rather idiosyncratic. This should not surprise anyone, because it is difficult to find patterns in systems in which both direct and indirect interactions occur. It is very important that facts are gathered, but it is as important to incorporate the facts into theory. Recently, attempts have been made to predict patterns instead of merely "collecting stamps" in macroorganismal tritrophic interactions (Price, 1991a, Hunter et al., 1992; Vet and Dicke, 1992). No one will be surprised that, apart from macroorganisms such as parasitoids and predators, microorganisms also affect plant–herbivore interactions. However, these usually invisible organisms have received much less interest in tritrophic studies of plant–herbivore interactions than macroorganisms. Yet, unraveling the influence of microorganisms on interactions between plants and herbivores may reveal that characteristics imputed to macroorganisms in fact reflect activities of microorganisms. The book *Microbial Mediation of Plant–Herbivore Interactions* seems to be a logical step in the development of integrated knowledge on microorganisms as mediators of herbivore–plant interactions. Previous steps were chapters in books with a wider scope: Jones (1984) in the book *A New Ecology. Novel Approaches to Interactive Systems*, edited by Price, Slobodchikoff, and Gaud; and Berenbaum (1988) and Dicke (1988) in the book *Novel Aspects of Insect–Plant Interactions*, edited by Barbosa and Letourneau. The present book runs the risk of being accused of making plant–herbivore interactions more difficult to understand than they already are, but its appearance is more likely to stimulate many researchers to unravel phenomena not yet understood.

Microbial Mediation of Plant–Herbivore Interactions deals with an enormously varied scope of effects of microorganisms. The book has five parts: an integrative prologue, and four parts on plant mutualists, plant pathogens, insect

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mutualists, and insect pathogens, respectively. Thus, the book has the potential of being a manual of collected stamps, where the readers cannot see the wood for the trees. However, two of the editors, C.G. Jones and V.A. Krischik, provide a framework that gives a firm structure to the book. This is done in the first chapter by Jones, and also in the introductions to the different parts of the book by Krischik and Jones. In the first chapter, Jones stresses the importance of determining overall effects on performance of the insect to deduce the general ecological importance and evolutionary role of microorganisms. He subsequently develops an approach based on net effects to provide the book with a framework. In doing so, Jones hypothesizes on major selection pressures on plants, herbivores, and microorganisms. The value of his approach is evident from the sometimes counterintuitive predictions. Thus, this chapter is of great importance to the value of the book: the relatively limited knowledge of microorganisms as mediators of plant–herbivore interactions is presented in a conceptual framework that will stimulate future research to investigate the predictions made. In fact, this first chapter can be read as an excellent synopsis of the complete volume.

Despite this good framework, the main body of the book has chapters that are of varying interest, and the book could certainly have been less voluminous without losing essential information. The length of the chapters is rather variable (14–42 pages), with the longer chapters not always being the best ones.

The second part of the book (Plant Mutualists) is by far the longest one 270 pages). It contains chapters on the effect of symbiotic nitrogen-fixing bacteria, mycorrhizae, endophytes, and phylloplane microorganisms. The chapters by N.D. Johnson and B.L. Bentley (N_2-fixing bacteria) and by C.G. Jones and F.T. Last (ectomycorrhizae) are firmly based on the plant defense theory as developed by Coley et al. (1985) in which plant resources are the basis of plant defense. These are interesting chapters that strengthen my feeling that plant physiologists should be more involved in the study of plant–herbivore interactions. The chapters by K. Clay (endophytes) and by G.C. Carroll (plant-mutualistic fungi as antagonists of insects) are informative reviews and place plant defense in a tritrophic context involving microorganisms. The chapter by J.H. Benedict et al. points to interesting phenomena on the phylloplane, where the microflora may influence the production of plant volatiles that affect herbivore behavior. Despite some very exciting chapters, it is my feeling that this part of the book could have easily been shortened. For example, the chapter by Jones and Last had many repetitions; chapters 5 and 6 (by S.C. Rabatin and B.R. Stinner and by E.R. Ingham and R. Molina, respectively) have considerable overlap while chapter 4 (J.C. Moore et al.) is much more superficial in nature than several other chapters in this part of the book and lacks a clear message.

Part three, on plant pathogens, has a very exciting chapter by V.A. Krischik on specific or generalized plant defense. She points to the general nature of

plant defense that is effective against both plant pathogens and herbivores. The effects of this generalized defense on insect pathogens through sequestration may imply that plant defense aimed directly at herbivores has a boomerang effect through the effects on herbivore pathogens. It is interesting that defensive characteristics of plants that affect the behavior of natural enemies of herbivores through attracting these members of the third trophic level appear to be highly specific (Vet and Dicke, 1992). It seems that the specificity vs. generalized nature of plant defense is a topic that will appear in many future investigations of multitrophic level interactions.

Currently there is much interest in the effects of plant stress and plant vigor on herbivore performance (e.g., Price, 1991b; Mopper and Whitham, 1992). This issue is the topic of the chapter by P. Barbosa on plant pathogens and nonvector herbivores. However, it is remarkable to see that most literature cited in this chapter was published between 1960 and 1980, while relatively few citations are made to literature of the past decade. This is again a chapter (the longest of the book) that could have easily been shortened.

Part four, on insect mutualists, contains a very interesting and well-written chapter by R.A.I. Drew and A.C. Lloyd on the various aspects of the role of bacteria in the ecology of tephritid fruit flies. The chapter on symbiont-mediated detoxification in insect herbivores by P.F. Dowd provides food for thought about the potential abilities of insect herbivores to adapt to secondary plant chemicals. This adaptation may be much quicker than generally thought if, in fact, the detoxification is accomplished by symbiotic microorganisms.

Part five, on insect pathogens, has only two chapters; the one by J.C. Schultz and S.T. Keating on host-plant-mediated interactions between the gypsy moth and a baculovirus is among the best chapters of the book. These authors present both mechanistic data as well as the consequences for population dynamics, viral evolution, and gypsy moth evolution. This chapter again shows that a good understanding of interactions between different trophic levels may yield unanticipated effects: the influence of leaf chemistry on herbivores and their population dynamics may be far greater via an interaction with herbivore pathogens than by acting directly on the insect itself.

This book will be an eye-opener to many researchers who investigate plant–herbivore interactions. It will certainly stimulate those researchers to be alert for effects of microorganisms on herbivores, on plants, and especially on the interactions between the two. However, ideally it should also stimulate cooperation among entomologists, microbiologists, phytophatologists. In fact, it is striking that this book is dominated by entomologist authors. Yet, when seen in the development of the field of microorganisms as mediators of insect–plant interactions and given the stimulating contribution of the majority of this book to this development, I am certain that we will hear more exciting facts and theories. The information presented in this book is not only of interest for those

who study the interactions among plants, herbivores, and microorganisms; the book is worthwhile reading for all those interested in plant–herbivore interactions in general. In fact, there seem to be several parallels between patterns presented in this volume and patterns in tritrophic interactions involving macroorganisms exclusively. Comparing patterns in microorganismally mediated herbivore–plant interactions and macroorganismally mediated herbivore–plant interactions will be a fruitful effort that will contribute to our understanding of systems that comprise more than two interacting organisms.

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Announcement

PHEROMONE TECHNOLOGY IN EUROPE AND THE DEVELOPING COUNTRIES

The International Organization for Biological and Integrated Control of Noxious Animals and Plants—West Palearctic Regional Section will hold a meeting at the Natural Resources Institute, Chatham, England, on May 10, 1993. Topics will include: basic technologies in pheromone research and development, case studies of pheromone application, and general issues such as mechanisms for multinational collaborations, problems of registration of pheromones, etc. For further information write to: OILB Pheromone Meeting, Peter Beevor or Lawrence McVeigh, Natural Resources Institute, Central Avenue, Chatham Maritime, Chatham, Kent, ME4 4TB, England; Phone: (44) 634 880088; FAX: (44) 634 880066/77.

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CAPTURE OF GYPSY MOTH, *Lymantria dispar* (L.), AND *Lymantria mathura* (L.) MALES IN TRAPS BAITED WITH DISPARLURE ENANTIOMERS AND OLEFIN PRECURSOR IN THE PEOPLE'S REPUBLIC OF CHINA[1]

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Abstract—Pheromone traps baited with (+)-disparlure, *cis*-7,8-epoxy-2-methyocta-decane, captured males of *Lymantria dispar*, *L. monacha*, and *L. mathura* in northeastern People's Republic of China. *L. dispar* responded to the addition of olefin to (+)-disparlure-baited traps in a negative dose–response manner. Observations on site and seasonal capture of *L. dispar* and *L. mathura* are discussed.

Key Words—*Lymantria dispar*, gypsy moth, *Lymantria mathura*, Lepidoptera, Lymantriidae, pheromone, disparlure, *cis*-7,8-epoxy-2-methyocta-decane.

INTRODUCTION

Under a 1982 scientific agreement between the Chinese Academy of Forestry and the U.S. Department of Agriculture, Wallner et al. (1984) tested the attrac-

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[1]Lepidoptera: Lymantriidae.

tiveness of (+)-disparlure and its olefin precursor to gypsy moth, *Lymantria dispar* L., in two widely separated sites in the People's Republic of China (PRC). Their results indicated that the male response of Chinese gypsy moth was similar to that of North American gypsy moth, although there were significant differences between sites in male responses to the olefin precursor. During these 1982 tests no other lymantriid species were captured, although *L. mathura* (L.) had been observed feeding in one site (Dunhua) and *L. monacha* (L.) was known to be native to the area. *L. mathura* has not been reported as being attracted to (+)-disparlure and its analogs, whereas *L. monacha* has (Kovalev et al., 1980; Klimetzek et al., 1976; Vité et al., 1976; Schneider et al., 1974).

In 1983, under the same scientific agreement, a team of U.S. and PRC scientists traveled to Heilongjiang Province, in northeastern People's Republic of China, to investigate the natural enemy complex, biology, and behavior of the gypsy moth, *L. dispar*, and to continue the pheromone investigations initiated in 1982 (Wallner et al., 1984). We report here on a further test to clarify the effect of the olefin component and (+)-disparlure on capture of *L. dispar* in northeastern China, and on attempts to detect populations of *L. mathura* and *L. monacha* using (+)-disparlure baited traps.

METHODS AND MATERIALS

U.S. Department of Agriculture 3.8-liter milk carton pheromone traps (Schwalbe, 1981) were used in all tests. Traps were placed in six forest sites at the Provincial Forest Production Farm, Mengjiagang, Hunan County, Heilongjiang Province, PRC, in 1983. Gypsy moth larvae were collected in these sites three to four weeks prior to trap placement.

To determine the relative attractiveness of (+)- and (±)-disparlure and its *cis* olefin precursor [2-methyl-Z-7-octadecane], 36 traps were hung on July 21 in a 12-year-old *Larix* sp. plantation. The traps were arrayed in a complete randomized design with 15 m between each trap. Traps were hung from wood lathing nailed to tree trunks so that they were 0.6 m from the tree and 2 m above the ground. This test included seven disparlure/olefin treatments: 0.1 mg (+)-disparlure; 0.1 mg (+)-disparlure with 0.001 mg olefin; 0.1 mg (+)-disparlure with 0.01 mg olefin; 0.1 mg (+)-disparlure with 0.1 mg olefin; 0.1 mg (±)-disparlure; 0.1 mg (−)-disparlure; and 0.1 mg olefin. Each treatment was replicated five times. One unbaited trap completed the 36-trap design. Each trap contained a Vapona pesticide strip. The solutions of disparlure-related compounds were provided by B.A. Leonhardt, U.S. Department of Agriculture, Beltsville, Maryland. They were transported from the United States to the PRC in sealed ampules. In the Mengjiagang Biological Control Laboratory, the given amounts of each treatment were applied, each in 100μl of hexane, to cotton

wicks (1 cm long × 1 cm diam.) on the day they were placed in the field; the wicks remained in place throughout the experiment. Fresh disposable rubber gloves were used while baiting traps for each treatment.

To survey for lymantriid species in other sites in Mengjiagang, single traps were hung in five wooded sites where gypsy moth larvae previously had been collected (July 6–15). The five sites were 1–16 km apart and had the following composition: site 1, mixed second growth of *Q. mongolica*, *Betula*, *Corylus*, *Ulmus*, and *Tilia*; site 2, mixed second growth of *Q. mongolica*, *Corylus*, *Betula*, and *Malus*; site 3, mixed *Q. mongolica*, *Betula*, and *Salix* adjacent to a small *Larix* plantation; site 4, primarily *Q. mongolica* forest, with trees ca. 12.7 cm DBH; site 5, *Larix* spp. plantation, containing 18- to 20-year-old trees with a DBH of ca. 17 cm. Traps were hung (July 20–21) from branches of *Quercus mongolica* or *Larix* spp. Each trap contained a Hercon 4 × 25-mm PVC laminate wick with an inner polymer layer containing 0.5 mg (+)-disparlure (Paszek, 1984) and a 3 × 3× 0.03-cm piece of Vapona pesticide 7 (dichlorvos).

All traps were inspected at six- to seven-day intervals beginning on July 25 and ending September 8. On each visit, moths were collected and placed in containers labeled by trap number and date and returned to the laboratory for identification.

RESULTS

Table 1 and Figure 1 summarize the test to determine the relative attractancy of (+)-disparlure and olefin. *L. dispar* was found in 20 different traps representing all treatments except those baited with (−)-disparlure, olefin, or the unbaited trap. Traps containing only the (+)-disparlure had 44.2% of the 120 gypsy moths captured. With the addition of olefin, moth captures declined; traps with (+)-disparlure and 0.001 mg, 0.01 mg, and 0.1 mg olefin, respectively captured 29.2%, 11.7%, and 12.5% of all gypsy moths found in the 36-trap array. Seasonally, 84% of the 120 *L. dispar* trapped were caught in the two-week period of August 4–17. No moths were captured after August 24; traps were removed September 2.

A total of 1043 *L. dispar* were captured in the single traps baited with a Hercon (+)-disparlure dispenser; 333, 181, 154, 254, and 142 in the five sites, respectively. Peak capture (57%) of *L. dispar* in all five traps occurred August 11–17. The last *L. dispar* trapped ($N = 12$) occurred August 25–September 3. Traps were removed September 8.

Populations of *L. mathura* were detected in five of the six trap sites. The *L. monacha* were captured in site 5 during the second week of trapping; the trap also contained five *L. dispar*. *L. mathura* ($N = 23$) was captured in sites 1, 2, 3, 5, and 6. Ten were found in eight traps in the (+)-disparlure–olefin

TABLE 1. NUMBER OF *L. dispar* CAPTURED DURING 1983 IN PHEROMONE TRAPS BAITED WITH DISPARLURE ENANTIOMERS AND OLEFIN PRECURSOR, MENGJIAGANG, HUNAN COUNTY, HEILONGJIANG PROVINCE, PEOPLE'S REPUBLIC OF CHINA

Treatments/traps[a]	Traps (*N*)	*L. dispar* (*mathura*)					
		1	2	3	4	5	Total
1. 0.1 mg (+)-disparlure	5	9	0 (1)	26	9 (1)	9 (1)	53 (3)
2. 0.1 mg (+)-disparlure, 0.1 mg olefin	5	5	1	3 (1)	2	4 (2)	15 (3)
3. 0.1 mg (+)-disparlure, 0.01 mg olefin	4	3	4	3	4 (1)	lost	14 (1)
4. 0.1 mg (+)-disparlure, 0.001 mg olefin	5	4	3 (1)	4 (2)	3	21	35 (3)
5. 0.1 mg (±)-disparlure	5	0	0	1	0	2	3

[a] Traps were arrayed in complete randomized design with 15 m between each trap. Trap 5, treatment 3, was missing after first trap week. No lymantriids were caught in traps containing 0.1 mg (−)-disparlure, 0.1 mg olefin, or in the unbaited trap.

experiment; these traps contained either (+)-disparlure or (+)-disparlure and olefin. Seasonally, 20 *L. mathura* were caught in the first week of trapping (July 29–August 3), the other three were captured the following week, when the first *L. dispar* were captured. No *L. mathura* were found in traps with *L. dispar*.

DISCUSSION

Trap catches of *L. dispar* in (+)-disparlure- and olefin-baited traps in Mengjiagang, People's Republic of China, indicate a dose–response effect in which trap catch decreases as olefin concentration increases. This supports and further defines the response of *L. dispar* to disparlure-related compounds in northeastern China reported by Wallner et al. (1984). The response to the addition of olefin to (+)-disparlure-baited traps appears to be similar to the effect of the olefin on the attractancy of (±)-disparlure (racemic)-baited traps to North American gypsy moth (Cardé et al., 1973). They reported that when sticky "sectar" traps (model XC-26; 3M Company) were baited with both racemic disparlure and olefin, low concentrations of olefin suppressed attraction and higher concentrations "effectively inhibited attraction." In a subsequent study, Cardé et al. (1975) described olefin as having a dual effect depending on the "context of presentation"; when presented together with racemic disparlure, suppression of attraction occurs; when olefin is presented around any attraction source, an increase in searching occurs. The milieu of presentation of attractant

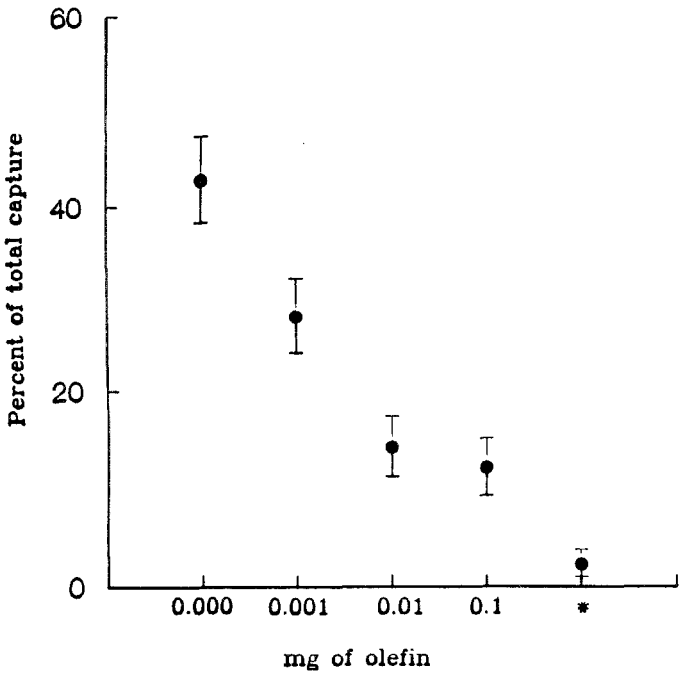

FIG. 1. The influence of olefin on trap capture of *Lymantria dispar* L.* (±)-Disparlure (0.1 mg).

and olefin in our study was random, and since searching behavior was not observed, the effect of olefin on searching cannot be addressed. However, suppression of attraction to individual traps containing both (+)-disparlure and olefin is apparent.

Only 120 moths were caught in the 36-trap array, with 53 of these found in traps baited with (+)-disparlure. In the five survey traps baited with Hercon (+)-disparlure dispensers, 1043 *L. dispar* were captured; each trap had more than the total caught in the test area. The difference in the emission rate of (+)-disparlure between cotton and Hercon PVC laminate wicks likely accounts for the disparity in captures. The apparent low emission rate of cotton wicks may also help explain the low capture of adults reported by Wallner et al. (1984); only 43 males were captured, but the 2500+ late-instar gypsy moth larvae collected in the same area suggests that population density in the predominantly oak forest was greater than moth capture indicated.

In our 1983 investigations in Mengjiagang, larval density was very low. In approximately 400 man hours of searching for and collecting *L. dispar* larvae, only 594 *L. dispar* and 1 *L. mathura* (on *Quercus* sp.) were collected. Larval

collections were made in all six sites in which traps were hung, but 71% were collected in the three *Larix* sp. plantations, sites 3 and 5, and the disparlure–olefin test site. Traps in these sites had the lowest seasonal capture of *L. dispar*. The success in finding larvae in *Larix* plantations may reflect the ease in finding them under bark flaps as compared to searching in the mixed hardwood areas with many other kinds of larval resting sites. The time spent searching in each of the six sites was approximately the same, but searching under bark flaps on *Larix* trees planted in rows, with sparse undergrowth, made our efforts more efficient and productive.

This is the first reported capture of *L. mathura* in disparlure-baited traps. However, the low number of *L. mathura* captured prevents definite statements on their relative attractancy to disparlure and olefin, or on other aspects of flight behavior. Our results do provide information that may be useful in designing other trapping experiments for evaluating *L. mathura*'s response to attractants and/or its seasonal and temporal flight periodicity. In contrast to the relatively low (35% of total) capture of *L. dispar* in *Larix* plantations, 78% of *L. mathura* were trapped in these sites, so trapping site may be important. This may be why the previous investigation (Wallner et al., 1984) was not successful in trapping this species: their traps were placed in predominantly hardwood areas. The low number and seasonal timing of *L. mathura* captures suggests that late July/early August was the latter part of its flight period and that traps probably should be arrayed earlier. Finally, although only one *L. mathura* larva was found, adult males were captured in five of the six trapping sites and in 12 of the 24 traps containing (+)-disparlure, so it is likely that this lymantriid species can be detected at relatively low population densities using the milk-carton trap and an appropriate attractant, perhaps (+)-disparlure.

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CONTENTS OF POISON APPARATUS AND THEIR RELATION TO TRAIL-FOLLOWING IN THE ANT *Daceton armigerum*

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Abstract—The poison gland, which is the source of the trail pheromone in *Daceton armigerum*, contains a mixture of dimethylpyrazine, trimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine in approximately equal proportions, similar to that of the ant *Manica rubida*. The cross-activity in trail-following tests with several other species can thus be explained. The Dufour gland of *D. armigerum* contains a mixture of 26 identified hydrocarbons, chiefly 9-tricosene, tricosane, and 9-pentacosene. It also contains very small amounts of two sesquiterpene aldehydes, tetramorine-2 and tetramorine-3, already known in other myrmicine Dufour glands. No volatile substances were detected in the pygidial gland.

Key Words—Ants, Hymenoptera, Formicidae, poison gland, Dufour gland, alkylpyrazines, tetramorine, trail following.

INTRODUCTION

The neotropical ant *Daceton armigerum* (Latreille), the only species in this myrmicine genus, is strictly arboreal and has been considered the most primitive species of the tribe Dacetini (Brown and Wilson, 1959). The division of labor

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within a *Daceton* colony has been studied by Wilson (1962) and more recently Hölldobler et al. (1990) concluded a behavioral analysis of chemical communication signals from exocrine glands of *D. armigerum* workers. These latter authors demonstrated that workers were recruited to foraging trails when termite food was provided for them, and they observed workers extending their stings at irregular intervals. In further tests, they showed that the trail-following response came from the poison gland and to a lesser extent from a sternal gland.

These results were different from those of Blum and Portocarrero (1966), who failed to demonstrate trail-following in *D. armigerum*, but found that three attine ant species could follow trails drawn with *D. armigerum* poison gland secretion. Wilson (1962) found that *Solenopsis invicta* followed trails of *D. armigerum* Dufour gland secretions. Hölldobler et al. (1990) confirmed these interspecific responses by showing that *Atta cephalotes* workers followed *D. armigerum* poison gland secretions but ignored their Dufour gland secretions, while *Solenopsis invicta* workers ignored *D. armigerum* poison gland secretions but followed those of Dufour glands, although the species always had a preference for their own trails. *Daceton*, however, did not follow the other species' trails. *Daceton* workers clearly perceived their Dufour gland secretion, but did not follow trails of it, nor were they more than mildly attracted to it.

In order to learn more about the secretions of *D. armigerum*, we have made a chemical examination of the pygidial, poison, and Dufour glands of workers. The results given here explain the unusual interspecific effect in trail-following tests with the other species described above.

METHODS AND MATERIALS

A sample of workers from a colony of *Daceton armigerum* collected in 1988 at Imataca Forest Reserve, Venezuela, were dissected as described by Morgan (1990), and either the Dufour glands or the whole poison apparatus (including both glands) were sealed in glass capillary tubes (approx. 1.5 × 0.2 cm.) and sent to Keele where they were stored in a refrigerator for analysis.

The sealed samples of glands were subjected to gas chromatography by crushing them in a solid sampling device (Morgan and Wadhams, 1972), which transferred the volatile material directly to the chromatography column, which in turn was linked to a mass spectrometer. Gas chromatography was carried out as described by Bagnères et al. (1991). For analysis of Dufour and pygidial glands, the sample was heated for 3 min in the injection area (220°C) before crushing. The oven temperature was initially 120°C and increased at 5°C/min to 270°C. For the poison glands, the injection area was maintained at 140°C. The oven temperature was initially at 35°C for 2.5 min and then increased at 5°C/min. For the early samples, it was continued to 270°C, but for later samples, it was stopped at 150°C, when all the compounds of interest had eluted.

For location of double bonds in alkenes, a sealed capillary containing 10 Dufour glands was crushed in a small tissue grinder made from glass tubing and a glass rod, and the hydrocarbons extracted with hexane (70 μl), of which 50 μl was transferred to a Keele Microreactor (Attygalle and Morgan, 1986). The solution was treated with iodine and dimethyl disulfide as described by Bagnères et al. (1991) to obtain the mass spectra of dimethylthioether derivatives of the alkenes.

RESULTS

The poison glands of *D. armigerum* contain very small amounts of low mass, volatile compounds, readily identified from their mass spectra and retention times (cf. Jackson et al., 1990a) as 2,5-dimethylpyrazine (DMP), trimethylpyrazine (TMP), and 3-ethyl-2,5-dimethylpyrazine (EDMP). The amounts were very small (of the order of 1 ng each per gland) and very variable. The least detectable amount with any reliability is 1 ng for each of these compounds. In only six of 10 samples were all three compounds visible, and sometimes the EDMP was present in smaller quantity than the other two. No higher mass compounds were observed, as expected for an aqueous protein venom.

The Dufour glands of *D. armigerum* contain chiefly a mixture of linear alkanes and alkenes, with tricosene and pentacosene the major ones (Table 1). By the methylthioether method, these were shown to be 9-tricosene (strong ions at *m/z* 173 and 243) and 9-pentacosene (characteristic ions at *m/z* 173 and 271). Traces of two separate isomers of heneicosene and of three isomers of heptacosene were evident from the gas chromatogram (Figure 1). These, no doubt, corresponded to isomers with the double bond at different positions in the chain, but there was too little for them to be seen in the methylthioether chromatograms. Two methyl-branched pentacosanes were also present. Also detected were very small amounts ($< 1\%$ of total) of the sesquiterpenoid aldehydes tetramorine-2 and tetramorine-3 (Ali et al., 1987).

The examination of the pygidial glands did not reveal the presence of any volatile substances by gas chromatography.

DISCUSSION

The behavioral work with *D. armigerum* (Hölldobler et al., 1990) was carried out with a large queenright colony. This was apparently a crucial factor for simulating natural conditions in the laboratory and for designing the appropriate bioassays during which the ants could be tested in foraging motivation. Unfortunately, we failed twice to obtain another queenright colony of *D. armigerum*. Myrmecologists are well aware that it is quite difficult to collect entire

TABLE 1. PERCENTAGE COMPOSITION OF DUFOUR GLAND SECRETION OF *D. armigerum*, EXPRESSED AS MEAN (± SAMPLE STANDARD DEVIATION) FOR TEN INDIVIDUAL SAMPLES

Label in Figure 1	Compound	%	± SSD
T-2	Tetramorine-2	t[a]	
T-3	Tetramorine-3	t	
A	Heneicosene isomer 1	1.8	1.0
B	Heneicosene isomer 2	1.3	0.7
C	Heneicosane	7.0	2.3
D	Tricosadiene	t	
E	9-Tricosene	27.0	4.8
F	Tricosane	11.4	1.6
G	11-Methyltricosane and tetracosene	t	
H	Tetracosane	t	
I	Pentacosadiene	3.5	0.8
J	9-Pentacosene	25.5	3.3
K	Pentacosene isomer	t	
L	Pentacosane	9.5	3.1
M	13-Methylpentacosane	1.6	0.7
N	5-Methylpentacosane	t	
O	3-Methylpentacosane	1.4	0.6
P	Hexacosane	t	
Q	Heptacosadiene	5.2	1.7
R	Heptacosene isomer 1	1.0	0.3
S	Heptacosene isomer 2	1.6	1.4
T	Heptacosene isomer 3	0.9	0.2
U	Heptacosane	1.2	0.9
V	13-Methylheptacosane	t	
W	7-Methylheptacosene	t	
X	5-Methylheptacosane	t	
Y	3-Methylheptacosane	t	

[a] t = trace, i.e., < 1% of total.

colonies of this species. We were therefore unable to bioassay the chemical compounds for their pheromone function. Nevertheless, we feel it is important to report our chemical results, because *Daceton* is a phylogenetically very interesting species within the subfamily Myrmicinae. Although a wealth of chemical data exist about other myrmicine species, this is the first chemical analysis of exocrine gland secretions for any dacetine ant. Furthermore, the findings given here also explain the unusual interspecific effects in trail-following tests with the other species described above.

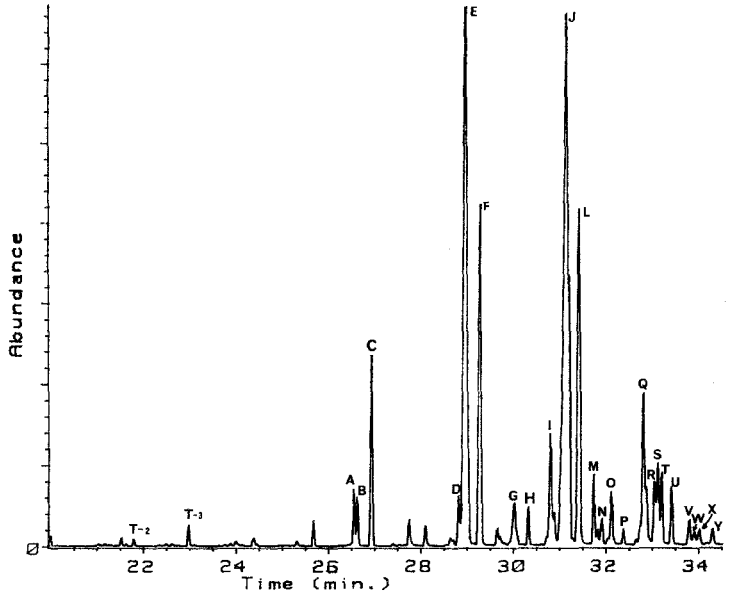

FIG. 1. Gas chromatogram of the Dufour gland contents of a single worker of *D. armigerum*.

The discovery of a mixture of simple alkylpyrazines in the venom gland of *D. armigerum* is not surprising, since these compounds, and EDMP in particular, have been identified as the trail pheromone in a number of species of myrmicine ants (Attygalle and Morgan, 1985). The venom gland of *Manica rubida* contains these same three pyrazines, although in behavioral tests, only EDMP was found to be active (Attygalle et al., 1985, 1986). These three substances were also found in *Tetramorium caespitum* venom glands (Attygalle and Morgan, 1983), where initially DMP and EDMP were found to act synergistically and later TMP was also found to have activity (Attygalle et al., 1986). A mixture of indole with traces of alkylpyrazines were shown to provide the pheromone in *Tetramorium meridionale* (Jackson et al., 1990a). We hope to carry out behavioral tests with these substances when next a colony of *D. armigerum* becomes available.

The trail pheromone of *Atta cephalotes* has been shown to be methyl 4-methylpyrrole-2-carboxylate (M4MPC) (Riley et al., 1974), but Robinson et al. (1974) have demonstrated cross-activity between *A. cephalotes* and *A. sexdens* in which EDMP is the trail pheromone (Cross et al., 1979; Evershed and Morgan, 1983), and Evershed and Morgan (1983) have shown that *A. cephalotes* venom glands contain both the pyrrole and EDMP. This alone can explain the observation that *A. cephalotes* workers will follow a trail of *D. armigerum*

venom gland secretions. Moreover, in just one of the 10 specimens of *D. armigerum* venom gland analyzed, we also detected M4MPC in quantity comparable to the pyrazines that were also present. We have no explanation for this isolated result. The exact blend of pyrazines must be critical in *D. armigerum* since its workers did not follow trails of *Atta cephalotes* or *T. caespitum*. In all cases where EDMP and M4MPC have been detected in poison glands they have been in the range of 1–5 ng. See, for example, Evershed and Morgan (1983) where an average of 1.1 ng of M4MPC per worker poison gland in *A. cephalotes* was reported.

The ability of *Solenopsis invicta* to follow trails of Dufour gland secretions of *D. armigerum* cannot be explained yet. Although sesquiterpenes have been found in *S. invicta* and they provide the trail pheromone of that species (Vander Meer, 1986), they are different in structure from the tetramorines reported here. The tetramorines were first discovered in the Dufour glands of *Tetramorium caespitum* and *T. impurum* (Billen et al., 1986) and later found also in *Leptothorax acervorum* and *Leptothorax nylanderi* (Ali et al., 1987; Jackson, 1991). We now know that these compounds are homologs of 2,3-dihydrofarnesal ($C_{15}H_{26}O$), which can be regarded as tetramorine-0. Tetramorine-1 ($C_{16}H_{28}O$), tetramorine-2 ($C_{17}H_{30}O$), and tetramorine-3 ($C_{16}H_{32}O$) have, respectively, one, two, and three extra CH_2 groups added to dihydrofarnesal but their exact position is yet to be determined.

The main constituents of the Dufour gland are a mixture of linear alkanes and alkenes, with 9-tricosene and 9-pentacosene the major components. There are small amounts of methyl-branched alkanes also present. These are commonly found in formicine ants, but up to now have only been found among harvester ants (*Messor* and *Pogonomyrmex*) in myrmicines (Ali et al., 1989). It is possible that the linear hydrocarbons act as a solvent and "keeper" for the more volatile and odorous tetramorines.

There have been few reports of the identification of volatile substances in the pygidial glands of ants, other than the iridoid-containing anal pygidial glands of the Dolichoderinae. Isogeraniol, *m*-hydroxybenzaldehyde, and two C_{17} hydrocarbons were identified in the pygidial glands of *Rhytidoponera metallica* (Meinwald et al., 1983), and we have found a variety of volatile substances recently in the pygidial glands of the Australian ants *Nothomyrmecia macrops* (Billen et al., 1988) and *Myrmecia nigriceps* (Jackson et al., 1990b). Attygalle et al. (1988) have identified (3*R*,4*S*)-4-methyl-3-heptanol in the pygidial gland of *Leptogenys diminuta*, where it functions as a trail pheromone. We were unable to identify any volatile substances in the pygidial glands of *D. armigerum* in the present work and are unable at present to account for the behavioral response to this gland described by Hölldobler et al. (1990).

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CHEMISTRY OF FRUIT FLIES: GLANDULAR SECRETION OF *Bactrocera (Polistomimetes) visenda* (HARDY)

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Abstract—The major component (>90% of volatiles) of the male rectal glandular extract of the nonpest species *Bactrocera visenda* (Hardy) is 3-methyl-2-butenyl acetate, with minor components being the isomeric 3-methyl-3-butenyl acetate, the homologous esters, 3-methyl-2-butenyl propanoate and 3-methyl-2-butenyl formate, along with 3-methyl-2-buten-1-ol, 3-methyl-2-butenal, and 3-methylbutyl acetate. None of these compounds has been identified previously from a *Bactrocera* species, supporting the view that *Bactrocera visenda* is taxonomically distant from other *Bactrocera* species identified from the Australian mainland. This collection of compounds adds to the known types utilized by dipteran species and emphasizes their extensive biosynthetic capability.

Key Words—*Bactrocera visenda*, Diptera, Tephritidae, fruit fly, 3-methyl-2-butenyl acetate.

INTRODUCTION

Within the identified species of fruit flies, a significant number are destructive pests of many forms of horticulture, and methods of monitoring and control are

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being sought that are based on the chemical cues that regulate phases of fruit fly biology (Drew et al., 1982). In this connection, the volatile compounds secreted and released from male rectal glands have been studied extensively and shown to be behavior-mediating in certain cases. On the other hand, many species are not of pest status, but study is still warranted to define the considerable biosynthetic capability exhibited by these insects (Blomquist et al., 1987; Blum, 1981; Bradshaw, 1985), as well as to delineate taxonomic features (Perkins et al., 1990a). In previous reports, we described studies of male rectal glands secretions from species that fall into each of these pests (Kitching et al., 1989) and nonpest categories (Perkins et al., 1990b; Krohn et al., 1991). *Bactrocera* (*Polistomimetes*) *visenda* (Hardy) is a medium-sized species (Drew, 1989) that is taxonomically very different from all other species identified from mainland Australia, but does resemble *Bactrocera mesonotochra* (Drew, 1989), a species localized in Morobe Province in Papua New Guinea. In view of this taxonomic isolation, it appeared that the chemistry of the male rectal gland secretion would be of interest, as previous studies (Perkins et al., 1990a) had shown that the gland contents can provide significant chemical taxonomic information. *Bactrocera visenda* is widespread in southern coastal areas of Papua New Guinea, northeastern coastal areas of Queensland, and the Torres Strait islands. It infests only native rain-forest hosts such as the "mangosteen" (*Garcinia warrenii* and *Garcinia gibbsiae*). In this report we describe the identification of the volatile chemical constituents of the male rectal gland, which are unlike the constituents of any other fruit fly species examined.

METHODS AND MATERIALS

Bactrocera visenda specimens were bred out in the laboratory at the Department of Primary Industries, Indooroopilly, Queensland, from infested mangosteen fruit (*Garcinia warrenii* and *Garcinia gibbsiae*) obtained from the rainforests of North Queensland.

Gland Secretion Analysis and Identification. Sexually mature adult male flies were chilled briefly (0°C) and their rectal glands were excised and stored in spectrograde pentane, as described previously (Kitching et al., 1989). Combined GC-MS examinations were performed using Hewlett-Packard HP5970 MSD, Finnigan Mat 1020 and double-focusing VG 70-750SE instruments with polar (BP21, FFAP) and nonpolar columns (DB5). Proton magnetic resonance spectra were recorded at 400 MHz (FT mode) on JEOL JNM-GX400 and Bruker WP400 spectrometers. Deuterochloroform was employed as solvent and chemical shifts (δ values) are relative to residual chloroform at δ 7.24. ^{13}C NMR spectra were recorded at 100 MHz utilizing $CDCl_3$ as solvent, and chemical shifts are relative to the central component of the $CDCl_3$ triplet at 77.00 ppm.

Synthesis of Glandular Components. 3-Methyl-2-buten-1-ol **(5),** 3-methyl-2-butenal **(6),** methylbutylacetate **(7),** and 3-methyl-3-buten-1-ol were obtained from the Aldrich Chemical Company and exhibited NMR and mass spectra in agreement with the structures. Compounds **1, 2, 3,** and **4** have been characterized previously (Babin et al., 1980; Aljancic-Solaja et al., 1987; Nazarov et al., 1952).

3-Methyl-2-butenyl Acetate (1). A stirred solution of 3-methyl-2-buten-1-ol **(5)** (1 g) in dry pyridine (20 ml) at 0°C was treated with acetyl chloride (0.94 g), and after about 12 hr was poured into ice–water and then extracted thoroughly with ether. The ether layer was washed with dilute HCl, $NaHCO_3$ solution, and water. The dried ($MgSO_4$) ether solution was concentrated under reduced pressure and the ester purified by flash chromatography (hexane) to provide **1** (0.89, 54%). ^{13}C NMR ($CDCl_3$): 170.96, 138.92, 118.54, 61.27, 25.64, 20.90, 17.87. ^1H NMR ($CDCl_3$): 1.66 (s, 3H), 1.71 (s, 3H), 1.99 (s, 3H), 4.51 (d, 2H, J = 7 Hz), 5.29 (t, 1H, J = 7 Hz). These ^1H NMR data are in excellent agreement with that reported (Babin et al., 1980). Mass spectrum: 128 ($M^{+\cdot}$, 0.4), 86 (7), 85 (4), 71 (15), 69 (21), 68 (49), 67 (36), 53 (20), 43 (100), 41 (54), 39 (24).

3-Methyl-3-butenyl Acetate (2). 3-Methyl-3-buten-1-ol was treated with acetyl chloride as described above in the preparation of **1.** Flash chromatography followed by preparative gas chromatography afforded a pure sample of **2.** ^{13}C NMR ($CDCl_3$): 171.04, 141.67, 112.16, 62.64, 36.65, 22.44, 20.92. ^1H NMR ($CDCl_3$): 1.73 (s, 3H), 2.01 (s, 3H), 2.31 (t, 2H, J = 7 Hz), 4.15 (t, 2H, J = 7 Hz), 4.71 (br s, 1H), 4.78 (br s, 1H). These ^1H NMR data agree well with that reported (Aljancic-Solaja et al., 1987). Mass spectrum: 128 ($M^{+\cdot}$, 0), 73 (2), 69 (4), 68 (52), 67 (31), 53 (10), 43 (100), 41 (12), 39 (15), such data being in agreement with a reported mass spectrum (Aljancic-Solaja et al., 1987).

3-Methyl-2-butenyl Propanoate (3). Treatment of alcohol **5** with propanoyl chloride, in the manner described for acquiring **1**, provided the propanoate **3**. [Lit. bp 162–164°/710 mm (Nazarov et al., 1952)]. ^{13}C NMR ($CDCl_3$): 174.33, 138.67, 118.65, 61.09, 27.45, 25.59, 17.81, 8.98. ^1H NMR ($CDCl_3$): 1.07 (t, 3H, J = 7.6 Hz), 1.64 (s, 3H), 1.69 (s, 3H), 2.25 (q, 2H, J = 7.6 Hz), 4.50 (d, 2H, J = 7 Hz), 5.27 (br t, 1H). Mass spectrum: 142 ($M^{+\cdot}$, 1), 114 (1), 100 (3), 85 (5), 71 (3), 69 (42), 68 (58), 67 (41), 57 (100), 53 (16), 41 (71), 39 (22).

3-Methyl-2-butenyl Formate (4). ^{13}C NMR ($CDCl_3$): 161:13, 139.91, 117.89, 60.74, 25.65, 17.93. ^1H NMR ($CDCl_3$): 1.70 (s, 3H), 1.75 (s, 3H), 4.64 (d, 2H, J = 7 Hz), 5.34 (br t, J = 7 Hz), 8.04 (s, 1H). These ^1H NMR data are in agreement with literature values (Babin et al., 1980). Mass spectrum: 114 ($M^{+\cdot}$, 1), 85 (9), 83 (1), 71 (38), 69 (31), 68 (59), 67 (50), 53 (36), 43 (28), 41 (100), 39 (56), such data being in agreement with that reported (Babin et al., 1980).

3-Methyl-2-butenal ***(6).*** Oxidation of 3-methyl-3-buten-1-ol with pyridinium chlorochromate provided **6,** identical with the commercially available material. Distillation [bp 135°, lit. 133°/730 mm (Pauling et al., 1976)] followed by careful preparative gas chromatography yielded **6.** ^{13}C NMR ($CDCl_3$): 190.96, 160.50, 128.03, 27.12, 18.80. ^1H NMR ($CDCl_3$): 1.92 (s, 3H), 2.11 (s, 3H), 5.82 (d, 1H, J = 8 Hz), 9.89 (d, 1H, J = 8 Hz). These ^1H NMR data are in agreement with literature values (Pauling et al., 1976). Mass spectrum: 84 ($M^{+\cdot}$, 100), 83 (48), 69 (6), 65 (2), 55 (82), 53 (18), 41 (76), 39 (80).

RESULTS AND DISCUSSION

Combined GC-MS examination of a pentane extract of approximately 20 rectal glands from *Bactrocera visenda* males showed the presence of one dominant component (>90% of volatiles), four minor components (each 1–2%), and a number of trace components (<1%) (Figure 1).

A library mass spectra search tentatively identified the major component as 3-methyl-2-butenyl acetate **(1),** and this was confirmed by synthesis and

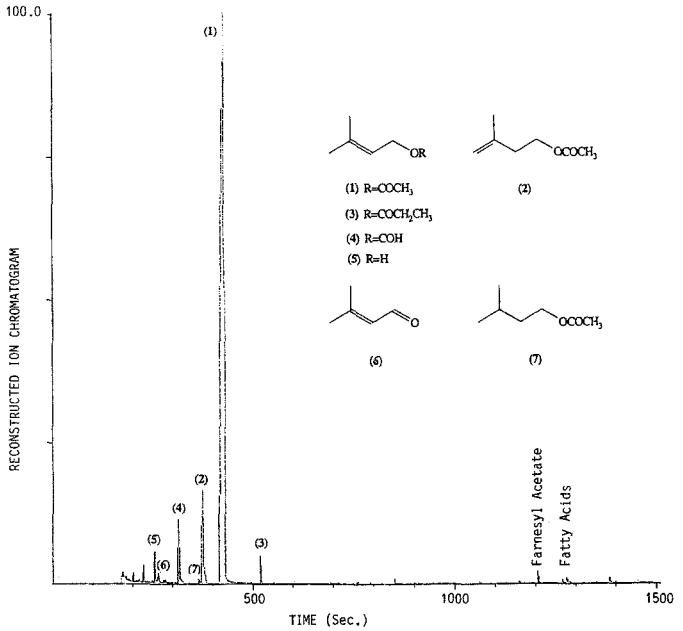


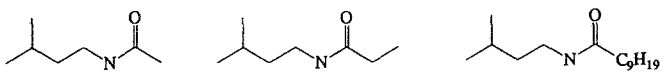
FIG. 1. Gas chromatograph of rectal gland extract form adult male *Bactrocera (Polistomimetes) visenda* (Hardy). *Column and conditions:* 30-m DB5 capillary column (J&W). Temperature programmed, 40°C, 2 min then 10°/min to 260°C.

coinjection of the authentic sample with the *B. visenda* extract. Three of the minor gland components showed significant mass spectral similarities with that of **1,** with ions at 53 (C_4H_5), 67 (C_5H_8), and 69 (C_5H_9) being important in this regard. GC-MS comparisons with authentic compounds enabled the identification of these components as the isomeric acetate, 3-methyl-3-butenyl acetate **(2),** and two other esters of **1,** 3-methyl-2-butenyl propanoate **(3)** and 3-methyl-2-butenyl formate **(4).** The fourth minor component showed a mass spectral library match with 3-methyl-2-buten-1-ol **(5),** and the assignment was confirmed upon examination of a commercial sample of the alcohol and coinjection with the gland extract.

An unsaturated C_5-aldehyde was deduced as the most likely structure of one of the trace components based on mass spectral interpretation. 3-Methyl-2-butenal **(6)** proved to be identical with the natural component (coinjection and GC-MS). An additional trace component was identified as 3-methylbutyl acetate **(7)** on the basis of library matching and coinjection of an authentic sample. A number of less volatile trace components were tentatively identified and included farnesyl acetate and C_{16} saturated and unsaturated acids.

Some of these compounds are known as components of the aroma of certain fruits with, for example, **1, 2** and **5** in pepino and other fruits (Shiota et al., 1988) and **1, 2, 5,** and **7** as minor headspace constituents of intact tree-ripened nectarines (Takeoka et al., 1988). α,β-Unsaturated aldehyde **6** is a volatile constituent of commercial proteinaceous baits for fruit flies (Morton and Bateman, 1981).

With respect to insect derivation, compounds **1, 2** and **7** have been identified (Jang et al., 1989) as minor or trace headspace components from the odor of calling male Mediterranean fruit flies (*Ceratitis capitata*). Compounds **2** and **7** have also been identified from the venom of the European hornet (Wheeler et al., 1983), while **7** is an important component of the alarm pheromone of honeybee workers (Morse et al., 1967; Blum et al., 1978). Long-chain fatty acid esters of **5** are present in the Dufour's gland of halictid bees (Duffield et al., 1981, 1982). Interestingly, aza-analogs of **7** are also known from insects (Figure 2); **8** is the alarm pheromone of the wasp, *Vespula squamosa* (Heath and Landolt, 1988) and was also found in *Dacus cucurbitae* (Baker et al., 1982; Perkins et al., 1990b). The same compound and related amides, including the corresponding propanamide **9,** were found in the rectal glands of *Dacus tryoni*



(8) (9) (10)

FIG. 2. Aza-analogues of **7** found in other insect species.

and *Dacus neohumeralis* (Bellas and Fletcher, 1979). Other volatiles showing a 1-aza-4-methylpentyl substructure, such as **10**, are known from ponerine ants (Fales et al., 1984). To the best of our knowledge, and propyl ester **(3)** and formate **(4)** have not been identified previously from a natural source.

The biosynthetic routes to volatile short-chain compounds present in insect species are unclear, but those with branched structures may be derived from isoprenoid units or amino acids (Blomquist et al., 1987; Bradshaw, 1985; Blum, 1981). In this regard, Attygalle et al. (1991) have demonstrated recently that $^2H_8$-L-valine is incorporated with high efficiency into methacrylic and isobutyric acids in the defensive gland of a carabid beetle, *Scarites subterranous* and that the isotope distribution pattern is consistent with the saturated acid being the precursor of the unsaturated analog.

Male rectal glands from other *Bacterocera* species generally contain amides (Bellas and Fletcher, 1979) and/or C_9–C_{13} spiroacetals, ketoalcohols, or diols (Kitching et al., 1989; Krohn et al., 1991; Perkins et al., 1990a,b). Thus the identification of the C_5 alcohols, esters, and aldehydes **(1–7)** in *B. visenda* supports the conclusion that this species is taxonomically quite distinct from other Australian species, and examination of *B. mesonotochra* (Drew, 1989) would be worthwhile from this point of view. In addition, the characterization of these compounds indicates a very diverse biosynthetic capability for Dipteran species (Tumlinson, 1989). Although no assessment of the biological activity of these components has been undertaken, it would be surprising if they lacked a role in the communication system of the species.

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PERFORMANCE CHARACTERISTICS OF A COMMERCIAL CONTROLLED-RELEASE DISPENSER OF SEX PHEROMONE FOR CONTROL OF CODLING MOTH (*Cydia pomonella*) BY MATING DISRUPTION

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Abstract—Performance characteristics of polyethylene tube dispensers containing a mixture of (*E*,*E*)-8,10-dodecadien-1-ol **(1),** dodecan-1-ol **(2),** and tetradecan-1-ol **(3)** were evaluated for suitability as a mating disruptant for codling moth control. The rate of loss of pheromone component from a dispenser at any time was found to be described by the equation:

$$-dP/dt = (k_1k_2 + k_d)P$$

where P is the amount of pheromone component in the dispenser well; t is time; k_1 is the ratio of the amount dissolved in the dispenser wall to the amount in the dispenser well; k_2 is the ratio of the evaporation rate to the amount dissolved in the dispenser wall; k_d is the rate constant for chemical decomposition. The evaporation rate, E, of a pheromone component at any time was given by:

$$E = k_1k_2P$$

For all three components during the first three weeks, k_1 decreased from ca. 0.25 to ca. 0.10 and was approximately constant thereafter. The decrease of k_1 with time may have been caused by weather-induced cross-linking of the polyethylene. Over time k_2 was constant and was $1.27 \pm 0.26 \times 10^{-3}$/hr for **1,** $1.96 \pm 0.33 \times 10^{-3}$/hr for **2,** and $0.31 \pm 0.05 \times 10^{-3}$/hr for **3.** The k_d was zero for **2** and **3** and 6.96×10^{-4} for **1.** After 150 days in an orchard in 1991, 95% of **1** was lost from the dispensers (61% of the loss was by chemical decomposition and 39% by evaporation). The heat summation units in a Yakima valley orchard during 1991 were 4.7% above the average for the 1980–1991 period, while during 1990 they were the highest for this

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period (26% above average). After the first three weeks of dispenser aging, the regression line half-lives for **1** for 1990 and 1991 were 31.0 and 35.1 days, respectively. The difference in temperature between 1990 and 1991 did not affect the half-life of **1** very much because so much of the loss was from photochemically induced decomposition. Based on an estimate of the required minimum evaporation rate for mating disruption of 2 mg/ha-hr and a half-life of 35 days for **1**, 2345 dispensers/ha would be required for one application per season; 944 dispensers/ha for two applications per season; and 734 dispensers/ha for three applications per season. If a different emission rate of **1** is required for reliable mating disruption, then the number of dispensers required would be changed proportionately.

Key Words—Sex pheromone, codling moth, *Cydia pomonella*, Lepidoptera, Tortricidae, Olethreutinae, controlled release, mating disruption, dodecan-1-ol, tetradecan-1-ol, (*E*,*E*)-8,10-dodecadien-1-ol.

INTRODUCTION

Since the early 1970s, the principle of insect control by application of sex pheromones to the host crop has been demonstrated numerous times (Roelofs, 1979). In the last few years this technique has become more practical, and renewed interest has developed (Charmillot, 1990; Rice and Kirsch, 1990; Kirsch, 1988).

Controlled-release dispensers are critical to the development of practical control programs. Commercial dispensers that have shown promise in some context and for which release characteristics have been studied, include microcapsules (Campion et al., 1981), Hercon Environmental Co. dispensers (Leonhardt and Moreno, 1982), Conrel Corp. hollow fibers (Weatherston et al., 1985a,b) and other types (Kraan and Ebbers, 1990).

Recently, the Biocontrol Ltd. dispenser has shown promise in commercial applications. This dispenser consists of a hollow plastic tube sealed at both ends and filled with pheromone. Imbedded longitudinally in the plastic wall is a wire so that the dispenser can be manually tied to a stem of crop foliage. This dispenser is used in Australia and the United States for control of the Oriental fruit moth (Rice and Kirsch, 1990), in Japan for control of a tea-tortrix complex (Ogawa, 1990), and extensively in management of pink bollworm (Baker et al., 1990).

Biocontrol Ltd. is also exploring the use of their dispenser for the sex pheromone of the codling moth. Their formulation consists of a mixture of (*E*,*E*)-8,10-dodecadien-1-ol (*E*8,*E*10–12:OH), dodecan-1-ol (12:OH), and tetradecan-1-ol (14:OH) stabilized with vitamin E. In a study of the evaporation rate characteristics of an earlier dispenser of these components, Brown et al. (1992) showed that the evaporation rate could be empirically correlated with a

combination of degree-hours and temperature during the summer of 1989 in the Yakima valley.

Weatherston (1990) suggested that pheromone release from the Biocontrol dispenser follows first order kinetics. The experience of Brown et al. (1992) with the codling moth formulation suggested a more complex situation with this pheromone. Our study was undertaken to identify factors controlling the loss mechanism of the codling moth formulation from this dispenser. The results indicate that the dispenser does follow first-order kinetics, but weathering of the dispenser and decomposition of 8,10–12:OH are modifying factors.

METHODS AND MATERIALS

Dispensers. The dispensers were obtained from Biocontrol Ltd., Davis, California. The dispensers were ca. 20 cm long and 1 × 3 mm in diameter, and the total weight of components in the dispensers was about 195 mg. Some of the liquid from one of the dispensers was weighed and made to a concentration of 12.27 μg/ml in heptane. External standards were used for gas chromatographic analysis (DB-WAX capillary column), which indicated a total pheromone titer of 11.48 μg/ml (93.6%); 58.8% 8,10–12:OH; 29.5% 12:OH; 5.3% 14:OH. According to the company, 5% vitamin E is added as a stabilizer (Phillip Kirsch, personal communication), which therefore accounts for most of the remaining 6.4%.

In 1991 and 1990, the dispensers were tied to apple tree branches on April 22 in a commercial orchard located 20 km from Yakima. Dispensers were placed in the upper third of the canopy of 15-year-old red delicious apple trees. At appropriate time intervals, three dispensers were individually collected, placed in plastic seal-top bags, and stored in a freezer.

Determination of Evaporation Rates. Dispensers were removed from the freezer and allowed to equilibrate at room temperature (ca. 22°C) for 24 hr. Then, immediately prior to determination of the evaporation rates, the lengths of the liquid wells were measured. The evaporation rates were determined by the method of McDonough et al. (1989), except 50-ml syringes were used for the dispenser chamber and silica gel Sep-Pak cartridges (Millipore Corp., Marlborough, Massachusetts) were used for collecting evaporated pheromone. Prior to insertion of a dispenser into the syringe, it was bent around a small beaker and the ends were crimped so that only a small part of the dispenser touched the side of the syringe wall. The dispensers were placed within 3 mm of the end of the syringe. The flow rate of nitrogen was 16.2 cm/sec (6 liters/min), and the temperature was 20°C in 1991 and 22.5°C in 1990.

Determination of Contents of Dispenser Liquid Well. The 1991 dispensers 0, 16, 36, and 86 days old were weighed. Then a hole was made in each end

of the dispenser and the contents drained into a 125-ml flask. The dispensers were cut laterally into two pieces, cotton wads were twice pushed through the dispensers with straightened paper clips, and both wads and clips added to the flask. The dichloromethane solution was decanted from the cotton wads and paper clips. The flask contents were then extracted three times with dichloromethane. Dichloromethane was removed from the pooled solution with a rotary evaporator, and the residue was weighted. The initial liquid well contents of the dispensers were 154 ± 3.5 mg.

Determination of Titer of Pheromone Components in Dispenser Wall. After the well contents were removed, the 1991 dispensers were each placed into 125-ml flasks containing 50 ml dichloromethane. The contents were shaken for 1 hr with a mechanical shaker. The extracts were poured into another flask, and the original flask was rinsed three times with dichloromethane. The extraction and rinsing procedures were repeated two more times. The combined extracts were partially evaporated to a volume of about 75 ml; 75 ml heptane was added, and the solution was again evaporated to a volume of 75 ml and made to an appropriate volume for gas chromatographic analysis. The dispensers were allowed to dry overnight and then weighed so the total weight of components dissolved in the plastic wall could be determined. The weight of nonpheromone extractable constituents of the plastic wall were constant over time at 13.3 ± 2.2 mg/dispenser.

Control Experiments. Controls were run for all analytical procedures. Recoveries were typically 90–100%, and no corrections were applied to analytical data.

Mathematical Relationships. If the evaporative loss from the dispenser were first order, then (assuming evaporation was the only loss process) the evaporation rate (E) of a pheromone component at any time would be:

$$E = -dP/dt = kP \tag{1}$$

where P is the amount of the pheromone component in the liquid well, t is time, and k is a proportionality constant (the ratio of the evaporation rate of a component to the amount of that component in the liquid well).

The process of evaporation involves two steps: the solution of the components of the well into the plastic wall and the evaporation out of the wall. In order to evaluate these processes separately, new constants can be defined: k_1 is the ratio of the amount of pheromone in the plastic wall to that in the liquid well, and k_2 is the ratio of the evaporation rate to the amount in the plastic wall. It follows that $k_1k_2 = k$.

If a first-order chemical decomposition were to occur concurrently with the first-order evaporative loss, then $k = k_1k_2 + k_d$, where k_d is the rate constant for chemical decomposition. Note that when decomposition occurs, the rate of

loss ($-dP/dt$) is no longer equal to the evaporation rate, E, which is still equal to k_1k_2P.

The integral form of the equation for a first order loss is:

$$\ln\left(\frac{P_0}{P}\right) = kt \text{ or } \ln\left(\frac{k_1k_2P_0}{k_1k_2P}\right) = kt$$

which is equivalent to

$$\ln\left(\frac{E_0}{E}\right) = kt \tag{2}$$

Where P_0 is the amount of pheromone when $t = 0$ and E_0 is the evaporation rate when $t = 0$. Therefore, plots of ln P versus time and ln E versus time will give parallel straight lines with a slope of $-k$.

Statistical Calculations. Regression lines and 95% confidence limits were calculated with NCSS 5.03 Software (Hintze, 1991).

RESULTS

Change in Evaporation Rate with Time Versus Loss of Dispenser Content with Time. Figure 1 shows a plot of logarithm of dispenser content in terms of both length of liquid well (millimeters) and weight of liquid well content (mil-

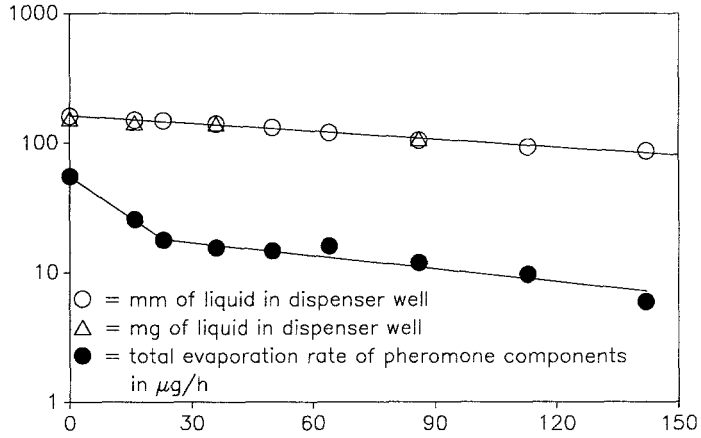

FIG. 1. Loss of dispenser well contents as measured by well length and weight of well components vs. time, and loss of evaporation rate, E (total of all components) vs. time during 1991. Ordinate numbers represent logarithm of titer in millimeters and milligrams, and logarithm E in micrograms per hour. Abscissa numbers are days.

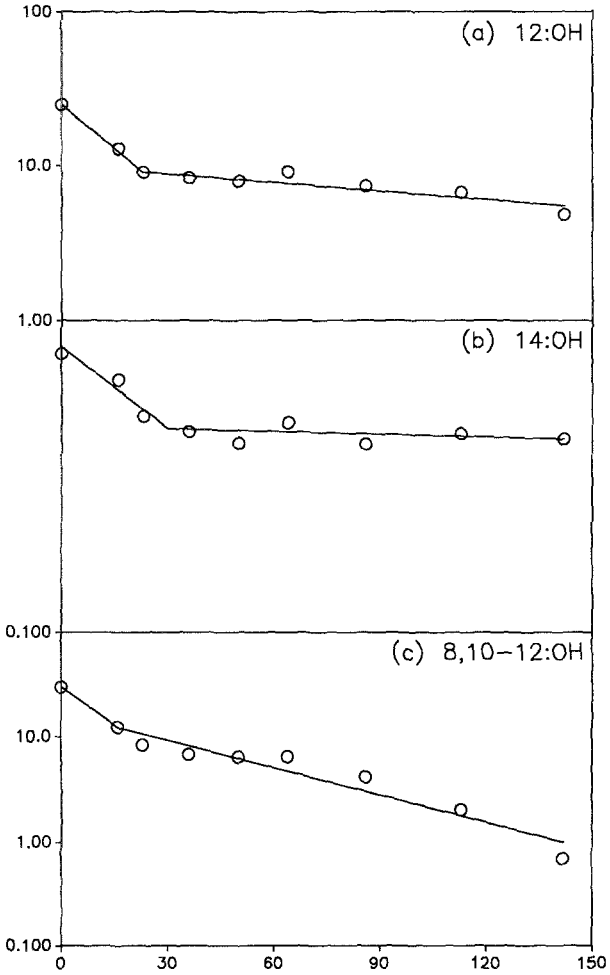

FIG. 2. Logarithm of evaporation rate, E (μg/hr), versus time (days) for 1991 dispensers for each pheromone component.

ligrams) with time (days) compared to the logarithm of total evaporation rate (micrograms per hour) of all three components with time during 1991. The slope of the line for logarithm of liquid well content (millimeters) corresponded to a half-life of 148.5 days (R^2 = 0.987). The slope for the first leg of the line for logarithm of evaporation rate corresponded to a half-life of 14.2 days (R^2 = 0.9997) and for the second leg, to a half-life of 81.3 days (R^2 = 0.910). Therefore, these lines are not parallel as would be required for a simple first-order process.

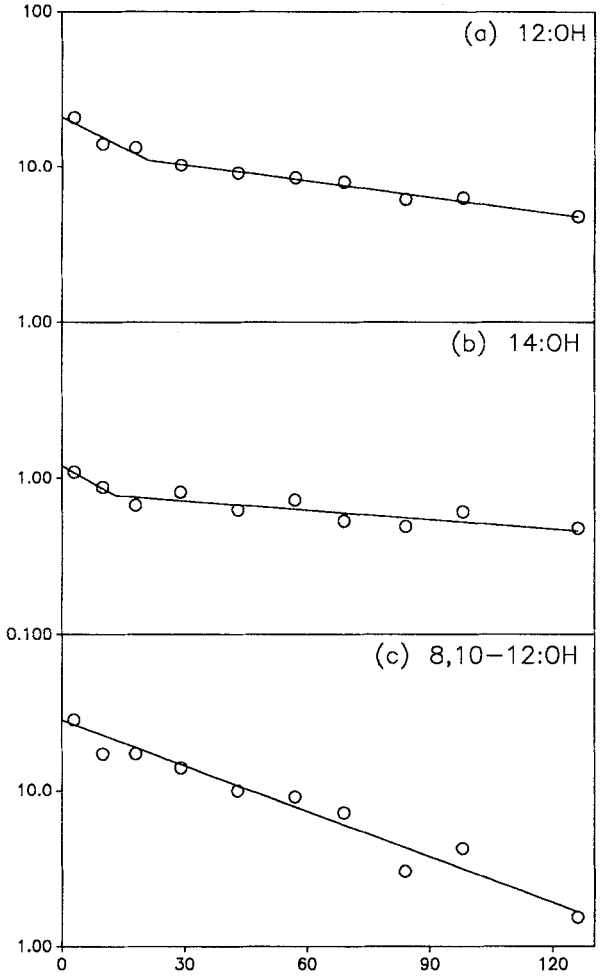

FIG. 3. Logarithm of evaporation rate, E (μg/hr), versus time (days) for 1990 dispensers for each pheromone component.

Change in Evaporation Rates of Individual Components with Time. Figure 2a–c shows plots of logarithm E versus time for each pheromone component in the 1991 dispensers. Each curve shows a faster loss during the first three weeks followed by a slower loss. The pattern is the same as that shown for the composite in Figure 1. Figure 3a–c shows the same plots for the 1990 dispensers. Again, for 12:OH and 14:OH the usual pattern is evident: a fast loss of E during the first three weeks followed by a slower loss. The pattern is not clear for 8,10–12:OH because of its fast loss after three weeks. Comparison of losses

TABLE 1. RATIO (k_2) OF EVAPORATION RATE TO TITER IN DISPENSER WALL[a]

Age (days)	$k_2 \times 10^3$ (SE)			k_2(12:OH)/ k_2(14:OH)	k_2(12:OH)/ k_2(8, 10–12:OH)
	12:OH	14:OH	8, 10–12:OH		
0	2.22 (0.37)	0.33 (0.03)	1.51 (0.22)	6.62	1.47
16	1.95 (0.40)	0.32 (0.05)	1.18 (0.27)	6.01	1.65
36	1.74 (0.19)	0.28 (0.07)	1.02 (0.14)	6.28	1.71
86	1.85 (0.22)	0.29 (0.03)	1.28 (0.16)	6.49	1.45
Overall average	1.96 (0.33)	0.31 (0.05)	1.27 (0.26)	6.37 (0.64)	1.56 (0.14)

[a]On day 0 there were 4 replications; on other days there were 3.

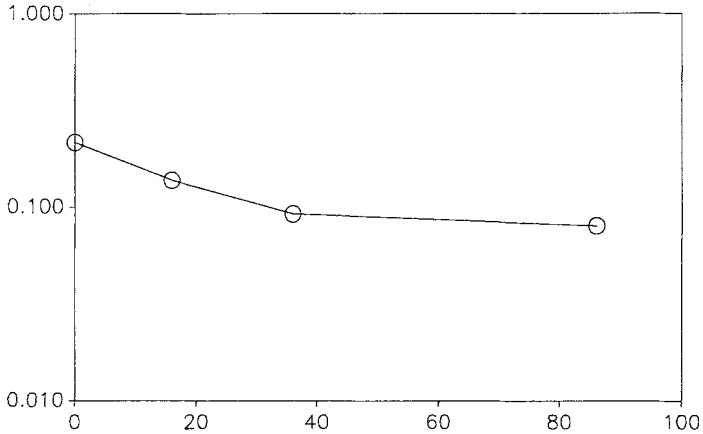

FIG. 4. Logarithm of k_1 (ratio of titer of pheromone components in plastic wall to the weight in dispenser well) versus time (days) for 1991 dispensers.

of each compound during the first three weeks (first three data points) shows that they are about the same. In terms of half-life, the values are: 12:OH, 23.7 days; 14:OH, 21.5 days; 8,10–12:OH, 21.7 days. The similarity of these values indicates that the slopes of the first leg of these curves were being dictated by a factor other than volatility.

Change in k_2 *Values with Time.* The k_2 values for each pheromone component in the 1991 dispensers were evaluated at four different dates from 0 to 86 days in the field and are summarized in Table 1. For each compound the k_2 values are constant across dates within $\pm 20\%$ of the averages. A regression analysis of the k_2 values versus time indicates slightly negative slopes with low correlation coefficients. Values of slopes and R^2 values for each compound are: 12:OH, −0.0039, 0.156; 14:OH, −0.0006, 0.189; 8,10–12:OH, −0.0020,

0.065. These data show that the evaporation rate of each pheromone component is proportional to the concentration of that pheromone component in the plastic wall.

Change in k_1 *Values with Time.* The k_1 values (total titer of pheromone components in the plastic wall: weight of total well contents) for 1991 dispensers are given in Figure 4. The values were not constant but decreased by a factor of about 2.5. In Figure 1, E values decreased by a factor of 3.1 during the first three weeks. Therefore, most of the fast decrease in E values shown in Figure 1 can be explained by changing k_1 values. Evaporative loss and chemical decomposition would also contribute to loss in evaporation rate during the first three weeks.

DISCUSSION

Evaluation of Steps in Evaporative Release. For the 1991 dispensers, Figure 1 shows that the change in evaporation rate, E, with time does not parallel the change in pheromone content with time. Figure 2 shows that the pattern of evaporation rate changes in Figure 1 applies to each individual component, and this pattern also exists for the 1990 dispensers (Figure 3).

The discrepancy between change of pheromone content and change of evaporation rates cannot be explained by variation in k_2, since k_2 values were virtually constant for each component. The k_1 values (Figure 4), however, decreased by a factor of 2.5, closely paralleling the changes in E values. Since k_1 values represent the partitioning of the components between the well and the wall, k_1 values could change because of changes in either medium. Oxidation of vitamin E or 8,10–12:OH would increase the polarity of the well contents (Ideses and Shani, 1988). Because pheromone components are of moderate polarity, higher polarity of well contents would probably not favor increased solubility of the components in the well over the wall. More probable is a decrease in the solubility of the components in the wall. Perhaps the combined effect of oxygen and sunlight caused cross-linking of the polyethylene (Smith et al., 1977). Cross-linking would decrease the solubility of the components (Smith et al., 1977).

Amount of 8,10–12:OH Lost by Chemical Decomposition. The ratio of k_2 values for 12:OH/8,10–12:OH (Table 1) was 1.56 ± 0.14. Based on data from rubber septa where the $t_{1/2}$ of 8,10–12:OH is 26.5 days at room temperature (Maitlen et al., 1976) and the $t_{1/2}$ of 12:OH at 20°C is 17.5 (McDonough, 1991), the ratio of rate constants (inverse of $t_{1/2}$ values) is 1.51. Moreover, the ratio of k_2 values for dispensers for 12:OH/14:OH is 6.37 ± 0.64 compared to 7.50 for rubber septa (McDonough, 1991). Therefore, ratios of evaporation rate constants from these polyethylene dispensers are similar to those from rub-

ber septa. This is reasonable because both polyethylene and rubber are hydrocarbons and therefore of very similar polarity. The ratio of k_2 values shows that 12:OH is more volatile and would be expected to be lost faster than 8,10–12:OH from these dispensers, but because 8,10–12:OH was lost faster, concurrent chemical degradation of 8,10–12:OH must have been occurring.

The percent of 8,10–12:OH lost by chemical decomposition in the 1991 dispensers can be estimated by comparing the theoretical loss curve based on evaporation as the only loss process to the observed loss from a combination of evaporation and decomposition. The changes of ln E values versus time for 12:OH and 8,10–12:OH are shown in Figure 2a and c. Because k_2 values are constant and k_1 values are approximately constant after the third week, the second leg of each curve can be analyzed in terms of equation 2:

$$\ln E = -kt + \ln E_0$$

E_0 is the value of E that would be obtained by extrapolating the regression line to time zero. For t in hours and E in micrograms per hour, the regression lines are: for 12:OH:

$$\ln E = -0.0001939t + \ln 10.43 \quad (3)$$

$R^2 = 0.67$ and the 95% confidence limits of k were -0.000260 to -0.000128; for 8,10–12:OH:

$$\ln E = -0.0008225t + \ln 16.47 \quad (4)$$

$R^2 = 0.89$ and the 95% confidence limits of k were 0.000964 to 0.000681. In the absence of chemical decomposition $k = k_1k_2$, and therefore the value of k for 12:OH from the regression equation 3 can be compared with k_1 and k_2 for 12:OH obtained from Figure 4 and Table 1. From Table 1 the value of k_2 is 1.96×10^{-3} compared to a value of k from the regression equation 3 of 1.94×10^{-4}. Therefore, k_1 should be about 0.1, which is a reasonable value based on the data of Figure 4. Because these two methods of estimating k agree, 12:OH must have been lost from the dispensers exclusively by evaporation.

Therefore, a value of $k_1 = 0.1$ can be used as an approximation for 8,10–12:OH, also. Then, since k_2 for 8,10–12:OH $= 1.27 \times 10^{-3}$ (Table 1) and if evaporation were the only loss process, k for 8,10–12:OH would be $k_1k_2 = 1.27 \times 10^{-4}$. When this value of k was substituted in place of the experimentally determined k in the regression equation 4 for 8,10–12:OH (keeping the same E_0 value), the calculated theoretical value of E after 150 days (3600 hr) was found to be 10.43 μg/hr compared to the observed value of 0.85 μg/hr (equation 4). Since the evaporation rate is proportional to the amount of material remaining in the well, the difference between observed and theoretical final evaporation rates is a measure of the amount lost because of decomposition. Therefore, the percentage of 8,10–12:OH lost by decomposition was 100(10.43

$- 0.85)/(16.47 - 0.85) = 61$. This is an approximate value because the value of k_1 was approximate. However, the result is not very sensitive to the value of k_1. For example, for k_1 values of 0.09 and 0.11, the percent decomposition would be 64.5 and 58.2, respectively.

Comparison of 1991 and 1990 Dispensers. Heat summation units for the period 1980–1991, except for 1982, and from April 1 to September 30 each year were calculated with lower and upper thresholds of 10°C and 31.1°C. The 11-year average was 26,131 ± 3179 deg-hr. In 1991 there were 27,347 deg-hr, and in 1990 there were 32,280 deg-hr.

Evaporation rate data from 1990 and 1991 dispensers allows us to compare data from a fairly average year, 1991 (with heat summation units 4.7% above average), with 1990, the hottest year of this 11-year period (with heat summation units 26% above average). Plots of logarithm E versus time for the 1991 and 1990 dispensers for each component are shown in Figures 2a–c and 3a–c, respectively. The second leg of the 1990 curves shows faster losses than in 1991. The half-lives in days and 95% confidence limits for each compound for 1991 and 1990 (Figures 2 and 3) were: 12:OH, 149 (111–226) and 89.1 (74.2–112); 8,10–12:OH, 35.1 (30.0–42.4) and 31.0 (23.3–46.5); 14:OH, 812 (282–infinity) and 148 (78–1456). The values of E for 14:OH were small and changed slowly with time. Consequently, its half-life values are imprecise. Half-life values for 12:OH were 40% smaller in 1990 compared to 1991, but only 12% smaller for 8,10–12:OH. The main factor in the loss curve for 8,10–12:OH is chemical decomposition. The rate of decomposition is mainly determined by the presence of oxygen and sunlight (Ideses and Shani, 1988) and is not affected very much by temperature; therefore the overall half-life should not be expected to show as large a percentage decrease as does the half-life for 12:OH. Because of the similarity of half-lives of 8,10–12:OH for these two dissimilar years, calculations based on the 1991 data should be broadly applicable to other seasons.

Efficiency of Dispensers. The minimum evaporation rate of *E*8,*E*10–12:OH needed for control of codling moth is not known with the desired precision. The value of the minimum needed evaporation rate will probably vary with moth population, size of trees, and other environmental factors. Charmillot (1990) estimated the value at 10–40 mg/ha-hr, Hathaway et al. (1979) estimated the value at 15 mg/ha-hr, and Cardé et al. (1978) estimated the value at 2 mg/ha-hr. If a value is assumed, equation 4 can be used to determine the number of dispensers needed for various application protocols.

Assuming a minimum needed evaporation rate of 2 mg/ha-hr and one application per season (150 days), the needed number of dispensers is this rate divided by the rate per dispenser on day 150 (2000 μg/ha-hr ÷ 0.853 μg/dispenser-hr) which is 2345 dispensers/ha-season. Fewer dispensers would be needed if more applications were made. If two applications were used per season, the second

application would only have to be enough to replace the amount lost by the time of the second application. After 75 days (from equation 4), the evaporation rate per dispenser is 3.75 μg/hr. The first application would require 533 dispensers (2000/3.75). After 75 days, the evaporation rate of *E*8,*E*10–12:OH had decreased by 77.2% [100 × (16.47 − 3.75)/16.47], and therefore the second application would require 411 (533 × 0.772) for a total of 944 dispensers/ha-season. Similarly, three applications per season would require 326 + 204 + 204 (total: 734) dispensers/ha-season. Clearly, two or three applications are much more efficient than one. If the minimum required evaporation rate is different from 2 mg/ha-hr, the number of required dispensers for each application protocol would be changed in direct proportion.

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ALLELOPATHIC ACTIVITY IN WHEAT-CONVENTIONAL AND WHEAT-NO-TILL SOILS: DEVELOPMENT OF SOIL EXTRACT BIOASSAYS[1]

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Abstract—The primary objective of this research was to determine if soil extracts could be used directly in bioassays for the detection of allelopathic activity. Here we describe: (1) a way to estimate levels of allelopathic compounds in soil; (2) how pH, solute potential, and/or ion content of extracts may modify the action of allelopathic compounds on germination and radicle and hypocotyl length of crimson clover (*Trifolium incarnatum* L.) and ivy-leaved morning glory (*Ipomoea hederacea* L. Jacquin.); and (3) how biological activity of soil extracts may be determined. A water-autoclave extraction procedure was chosen over the immediate-water and 5-hr EDTA extraction procedures, because the autoclave procedure was effective in extracting solution and reversibly bound ferulic acid as well as phenolic acids from wheat debris. The resulting soil extracts were used directly in germination bioassays. A mixture of phenolic acids similar to that obtained from wheat-no-till soils did not affect germination of clover or morning glory and radicle and hypocotyl length of morning glory. The mixture did, however, reduce radicle and hypocotyl length of clover. Individual phenolic acids also did not inhibit germination, but did reduce radicle and hypocotyl length of both species. 6-MBOA (6-methoxy-2,3-benzoxazolinone), a conversion product of 2-*o*-glu-

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cosyl-7-methoxy-1,4-benzoxazin-3-one, a hydroxamic acid in living wheat plants, inhibited germination and radicle and hypocotyl length of clover and morning glory. 6-MBOA, however, was not detected in wheat debris, stubble, or soil extracts. Total phenolic acids (FC) in extracts were determined with Folin and Ciocalteu's phenol reagent. Levels of FC in wheat-conventional-till soil extracts were not related to germination or radicle and hypocotyl length of either species. Levels of FC in wheat-no-till soil extracts were also not related to germination of clover or morning glory, but were inversely related to radicle and hypocotyl length of clover and morning glory. FC values, solute potential, and acidity of wheat-no-till soil extracts appeared to be independent (additive) in action on clover radicle and hypocotyl length. Radicle and hypocotyl length of clover was inversely related to increasing FC and solute potential and directly related to decreasing acidity. Biological activity of extracts was determined best from slopes of radicle and hypocotyl length obtained from bioassays of extract dilutions. Thus, data derived from the water-autoclave extraction procedure, FC analysis, and slope analysis for extract activity in conjunction with data on extract pH and solute potential can be used to estimate allelopathic activity of wheat-no-till soils

Key Words—Cover crops, wheat, *Triticum aestivum*, soybean, *Glycine max*, soil extracts, germination bioassays, phenolic acids, hydroxamic acids, allelopathy, slope analysis, ivy-leaved morning glory, *Ipomoea hederacea*, crimson clover, *Trifolium incarnatum*.

INTRODUCTION

As growers in the United States implement the conservation requirements of the 1985 and 1990 Food Security Acts, many will turn to conservation or no-till agriculture (Worsham, 1990). Understanding how conservation tillage will impact crop production and sustainability of various crop management systems is therefore of considerable interest to U.S. farmers. Although many benefits are cited for no-tillage (i.e., control of soil erosion, soil water retention, reduced use of fossil fuel, etc.), reductions in crop yield and a perceived increased use of herbicides (potential for weed resistance, pollution, etc.) continue to be concerns. The development of effective management options using plant residues could, however, minimize the negative impacts on crop yield and lead to early weed control and a reduction in the use of preemergence herbicides (Worsham, 1991a,b).

Inhibition of weed germination and seedling growth by small grain mulches may be due to: (1) the physical barriers and shading associated with the residues (Worsham, 1989), (2) the immobilization of nitrogen (Broadbent and Tyler, 1962; Kimber, 1973), (3) a reduction in soil disturbance (Worsham, 1989), and/or (4) allelopathic compounds (Chou and Patrick, 1976; Liebl and Worsham, 1983; Shilling et al., 1985, 1986a,b; Barnes et al., 1986; Waller et al., 1987).

Potential allelopathic compounds that have been identified in living and

decomposing tissue of small grains include phenolic acids (Chou and Patrick, 1976; Liebl and Worsham, 1983; Barnes et al., 1986; Blum et al., 1991), organic acids (Patrick, 1971; Chou and Patrick, 1976; Lynch, 1977; Tang and Waiss, 1978; Shilling et al., 1985), hydroxamic acids and related compounds (Willard and Penner, 1976; Niemeyer et al., 1989; Nair et al., 1990), and volatiles (Buttery et al., 1985; Bradow, 1991). The presence of such inhibitors in living and decomposing plant tissue does not, however, always translate into actual allelopathic activity, since the level of inhibition observed for a given weed or crop species depends upon: (1) the sensitivity of the species, (2) an adequate rate of release of inhibitors, (3) the activity and strengths of various "sinks" (i.e., clays, organic matter, microbes, seeds, roots) for inhibitors within the soil, and (4) environmental factors (i.e., temperature, soil nutrition, soil pH, etc.). These observations have led researchers to use soil extracts and various chromatographic techniques to identify and quantify potential inhibitors in soils. The identified potential inhibitors, usually in pure form, have then been tested for toxicity (Rice, 1984). Soil solutions, however, contain a complex mixture of inorganic and organic ions and/or molecules that affect the action of any given organic molecule in solution. The use of soil extracts to simulate the soil solution might thus be more meaningful in determining allelopathic potential. The primary objective of this research was, therefore, to determine if soil extracts could be used directly in bioassays for the detection of allelopathic activity.

We have chosen wheat (*Triticum aestivum* L. Coker 916) –soybean (*Glycine max* L. Merill Deltapine) no-till and conventional-till systems (Cecil and Appling gravelly sandy loam) for exploratory studies. The focus was primarily on phenolic acids and secondarily on 6-methoxy-2,3-benzoxazolinone, a conversion product of 2-*o*-glucosyl-7-methoxy-1,4-benzoxazin-3-one, a hydroxamic acid found in living wheat plants.

METHODS AND MATERIALS

Soil Extraction Procedures. Samples from A_1 and B_1 horizons (from the same pedon) of a Cecil (Typic Kanhaludults, clayey, kaolinitic, thermic) soil from the Piedmont of North Carolina were air-dried, sieved (0.25 mm), and characterized (Dalton et al., 1987). Air-dried soil was stored at room temperature in a laboratory until used. One gram of soil was placed in a 15-ml glass centrifuge tube, capped (Bacti-Capall, Sherwood Medical Industries), and autoclaved three times (15 min at 1.2 kg/cm^2 and 121°C) at three-day intervals. One milliliter of filter-sterilized (0.2-μm membrane filter) ferulic acid (1000 μg/ml), adjusted to pH 5 with NaOH, was added to each tube aseptically. The soil–ferulic acid mixtures were incubated at 24°C in a dark and high relative humidity environment. After 70 days, half the test tubes received 0.5 ml of filtered (What-

man No. 42) soil extract obtained from the appropriate Cecil soil material and 0.5 ml double-strength Hoagland's solution (Hoagland and Arnon, 1950) (pH 5). This reinfected the soil with microorganisms. The other half received 0.5 ml filter-sterilized soil extract and 0.5 ml filter-sterilized double-strength Hoagland's solution.

Soil extracts (300 ml of water per 150 g soil material) for reinfestation were obtained from soil–sand mixtures (1 : 2 soil–sand by weight) that had been treated with 5 ml of a phenolic acid mixture (1230 μg/ml; composition of the seven phenolic acid mixtures is described subsequently) and 30 ml of double-strength Hoagland's solution and incubated in the dark at 30°C for three days (Blum and Shafer, 1988). Thirty days after reinfestation, soil samples were extracted. Three extraction procedures were used: (1) immediate water extraction, (2) water-autoclave extraction (45 min) (Blum et al., 1991), and (3) EDTA extraction (0.5 M EDTA adjusted to pH 8, 5 hr extraction time) (Dalton et al., 1987). The final solution volume in each tube was approximately 4 ml. Actual water content was determined gravimetrically on a subset of test tubes (105°C). Tubes were centrifuged for 10 min at 12,100*g*. The resulting supernatants were filter-sterilized (0.2 μm membrane filters) and injected into an HPLC to quantify extracted ferulic acid (Blum et al., 1991).

Field Soil Samples and Extracts. Soil samples (0–2.5 cm depth) were obtained from soybean plots of a low-input sustainable agriculture (LISA) study located 5 km south of North Carolina State University campus. Soybean plots selected included plots with wheat (*Triticum aestivum* L. Coker 916) stubble (no-till), plots in which the wheat stubble had been tilled into the soil, and plots without wheat. Detailed information on the field plots used has been described by Blum et al. (1991). Soil [Cecil and Appling gravelly sandy loam (Typic Kanhaludults)] samples covering a range of treatments, harvests, and soil depths were chosen from 371 samples collected during July–October, 1989, and 72 samples collected during June–November, 1990. Upon collection, soil samples were sieved (3-mm sieve), placed into plastic bags, and stored at −20°C. Soil samples to be extracted were thawed overnight at 10°C. Water content was determined gravimetrically on 10–15 g of soil (105°C).

To obtain extracts, 50–75 g of soil, depending on the experiment, was placed in a 500-ml Erlenmeyer flask with 100 ml deionized water. Changes in the amount of soil extracted per milliliter of water were made to adjust the baseline concentrations of the extracts. Loosely capped flasks were autoclaved for 45 min, removed from the autoclave, and cooled to room temperature over 20–30 min. The slurry in the flask was centrifuged for 10 min at 27,200*g*, and the resulting supernatant was filtered (Whatman No. 42). Except for the final bioassay experiment, filtered supernatants were adjusted with NaOH or HCl to pH 5 (extract 1) or adjusted to pH 2 with HCl, centrifuged for 10 min at 27,200*g* to remove humic acid, and then adjusted to pH 5 with NaOH (extract 2).

Determination of phenolic acid content in extracts is described below. Freezing point depression of extracts was determined with a Osmette Precision Osmometer (Precision Systems Inc., Natick, Massachusetts). No-till soils for final soil extract bioassay were also analyzed for nitrate, ammonium (Cataldo et al., 1974), phosphorus (Taussky and Shorr, 1953), and potassium (atomic absorption spectroscopy).

Solute Potentials of Extracts. Data from Lang (1967) for sodium chloride (20°C) and a model from Steuter (1981) for polyethylene glycol (PEG) 6000 in conjunction with freezing point depression data were used to estimate the solute potential of salt and PEG solutions. This was done only to identify the range of solute potentials of extracts. Regressions for bioassays, to determine the relationship between soil extract solute potential and seed response, were based on freezing point depression values (milliosmoles, mosmol).

Phenolic Acid Determinations. Five milliliters of ferulic acid (standard phenolic acid) solutions (pH 5) or extracts 1 or 2 (0.5 ml) plus 4.5 ml deionized water were mixed with 0.75 ml of 1.9 M Na_2CO_3 and 0.25 ml Folin and Ciocalteu's phenol reagent. This mixture was incubated in the dark at room temperature for at least 1 hr before absorption at 750 nm was measured (Box, 1983; Blum et al., 1991). This procedure will be referred to subsequently as the FC method. Units of total phenolic acid are in micrograms ferulic acid equivalents per gram of soil or milliliter of soil extract. Individual phenolic acids were determined by HPLC analysis (Blum et al., 1991). Folin and Ciocalteu's phenol reagent and phenolic acids, with the exception of sinapic acid, were purchased from Sigma Chemical Company (St. Louis, Missouri). Sinapic acid was purchased from Aldrich Chemical Company (Milwaukee, Wisconsin).

6-MBOA Determinations. Folin and Ciocalteu's phenol reagent also reacted with 6-methoxy-2,3-benzoxazolinone (6-MBOA). 6-MBOA was purchased from Sigma Chemical Company. The amount of 6-MBOA dissolved in water (pH 5) could be estimated by the FC method and the following equation: μg 6-MBOA $= -1.427 + (98.341 * \text{ABS})$, $R^2 = 0.998$, $P \leq 0.0001$, where ABS = the optical density at 750 nm. The HPLC procedure used to detect and quantify phenolic acids was also used for 6-MBOA. 6-MBOA was detected and quantified, however, at 290 nm instead of 254 nm. 6-MBOA concentrations before and after autoclaving procedures were essentially identical. Since 6-MBOA and *p*-coumaric acid have very similar HPLC retention times, thin-layer chromatography of extracts was also used to confirm the absence or presence of 6-MBOA.

Germination Studies. Twenty crimson clover seeds (*Trifolium incarnatum* L. Jacquin. Tibbee; Funk Seeds International, Bloomington, Illinois) inoculated with *Rhizobium* (Nitragin Co., Milwaukee, Wisconsin) or 10 ivy-leaved morning glory seeds (*Ipomoea hederacea* L.; F&J Seed Service, Woodstock, Illinois) were placed in 9-cm-diameter petri dishes containing Whatman No. 1 filter paper and 5 ml of a solution to be tested. Petri dishes were incubated at 30°C for 48

hr before germination, and the total length of the radicle and hypocotyl were determined.

The following solutions were tested for inhibitory effects: (1) soil extracts adjusted to pH 5, unless otherwise stated; (2) PEG 6000 solutions (pH 5) ranging from 0 (distilled water) to 10%; (3) Hoagland's solution (pH 5) ranging from 0 strength (distilled water) to double-strength; (4) a phenolic acid mixture (12, 11, 17, 12, 33, 10, and 5% of the *p*-hydroxybenzoic, caffeic, vanillic, syringic, *p*-coumaric, ferulic, and sinapic acids, respectively; at total concentrations of 0–394 μg/ml; initial solution pH was 5) with various concentrations of PEG or strengths of Hoagland's solution; (5) individual phenolic acids (caffeic, ferulic, *p*-coumaric, *p*-hydroxybenzoic, vanillic, sinapic, or syringic) or 6-MBOA solutions ranging from 0 to 2 mM (pH 5); or (6) 6-MBOA (0–1 mM) with various concentrations of PEG or various strengths of Hoagland's solution. Molecular weights of phenolic acids and 6-MBOA ranged from 138.1 for *p*-hydroxybenzoic acid to 224.2 for sinapic acid. The proportions of the phenolic acids in the mixture were based on the average values for soil extracts (extract 2) from wheat–no-till plots (Blum et al., 1991). Hoagland's solution of various strengths and PEG solutions of various concentrations were used to generate different solute potentials.

General Data Analyses. Data were analyzed by analysis of variance, regressions (PROC GLM), and response surface (PROC RSREG) analysis (SAS Institute Inc., 1988). An alpha value of 0.05 was used in all instances.

Analysis for Final Soil Extract Bioassay. Experimental details for preliminary bioassays, which varied somewhat from the final soil extract bioassay, are given in the results section. Each of the 33 soil extracts from no-till plots was tested at full-, half-, quarter-, and zero-strength using three subsamples of each extract. All extracts showed a trend towards a greater inhibition at higher concentrations. For the data from each extract, the simple linear regression of clover germination (and, separately, of radicle and hypocotyl lengths) on extract strength (0, 0.25×, 0.5×, 1×) was obtained using a common intercept. The resulting slope for an extract served as a measure of its biological activity (e.g., ability to inhibit germination or radicle and hypocotyl length); the steeper the slope, the higher the activity. For completeness, an overall test for quadratic effects was run and found to be not significant. Thus, the use of simple linear slopes was deemed to be adequate. The slopes were used as input (dependent variable) to a further analysis.

Other characteristics of the extracts were also obtained including pH, FC value, freezing point depression, NO_3^-, NH_4^+, K, and P. Model-building tools such as stepwise regressions (SAS PROC LEAPS) and response surface analysis (SAS PROC RSREG) were used to determine which combinations of these characteristics best accounted for the variability in slopes. The resulting regression model related the extract slopes to the measured characteristics of the extracts.

RESULTS

Soil Extraction Procedures. One hundred days after the addition of 1000 μg/g ferulic acid; water, water-autoclave, and EDTA procedures recovered 28, 32, and 37%, respectively, from sterile Cecil A_1, and 17, 30, 52% from sterile Cecil B_1 soil materials, respectively (Table 1). For soils reinfested with microorganisms, recovery was reduced to less than 2%.

Seventy-two and 83% of the ferulic acid added to sterile Cecil A_1 and B_1 soil materials, respectively, could not be recovered by the water-extraction procedure after 100 days (Table 2). Ferulic acid has been shown to be both reversibly and nonreversibly fixed in Cecil soils (Dalton et al., 1987). Six and 16% of the sorbed ferulic acid was recovered from Cecil A_1 and B_1 soil materials, respectively, by the water-autoclave procedure and 13 and 43% by the EDTA procedure. More than 96% of the sorbed ferulic acid recovered from sterile soil by the EDTA procedure was absent in soil extracts from reinfested soils. This was also true for the sorbed ferulic acid recovered from Cecil B_1 soil material by the water-autoclave procedure. Only 55%, however, of the sorbed ferulic acid recovered from the Cecil A_1 soil material by the water-autoclave procedure was accessible to microbes. The Cecil A_1 and B_1 soil material contained 37 and 2 g organic matter/kg, respectively (Dalton et al., 1987). This suggested that at least part of the sorbed ferulic acid recovered by the water-autoclave procedure came from the organic matter in the soil.

The EDTA procedure recovered 2–2.6 times more sorbed ferulic acid than the water-autoclave procedure. EDTA soil extracts, however, could not be used directly for germination bioassays, because clover and morning glory seeds (our bioassay species) would not germinate in the presence of these extracts. In

TABLE 1. RECOVERY OF ADDED FERULIC ACID FROM CECIL SOIL MATERIAL BY THREE EXTRACTION PROCEDURES

Soil and horizon	Microorganisms[a]	Solution pH just before extraction[b]	Recovery (μg/g ± standard error)		
			Water	Autoclave	EDTA
Cecil A_1	Absent	5.0	277 ± 3	322 ± 10	373 ± 19
	Present	7.4	0 ± 0	20 ± 4	4 ± 0.4
Cecil B_1	Absent	4.6	167 ± 12	304 ± 9	524 ± 6
	Present	5.2	0 ± 0	4 ± 0.4	0.4 ± 0.2

[a] Soil materials were reinfested with microbes 70 days after addition of ferulic acid; extracts of soil were made 30 days after reinfestation; three replicates.

[b] Because of the small volume, solutions from five test tubes were combined for pH measurements. Initial pH values of soils were 5.5 and 5.0 for Cecil A_1 and B_1, respectively.

TABLE 2. ESTIMATES OF FERULIC ACID ADDED TO CECIL SOIL MATERIAL SORBED IN SOIL AFTER 100 DAYS RECOVERED BY AUTOCLAVE AND EDTA PROCEDURES, AND AVAILABLE TO SOIL MICROBES

	Ferulic acid (μg/g)				
		Autoclave procedure		EDTA procedure	
Soil and horizon	Sorbed in soil[a]	Recovered[b]	Utilized by microbes[c]	Recovered[b]	Utilized by microbes[c]
Cecil A_1	723 (72%)[d]	45 (6%)[e]	25 (55%)[f]	96 (13%)[e]	92 (96%)[f]
Cecil B_1	833 (83%)	137 (16%)	133 (97%)	357 (43%)	357 (100%)

[a] Ferulic acid, 1000 μg, was added to 1 g of soil material. Sorbed in soil = amount not recovered by water extraction from sterile soil materials.
[b] For sterile soils. The difference in recovery between the water and autoclave or EDTA procedures.
[c] Difference between sorbed recovered by autoclave or EDTA procedures from sterile soils and soils reinfested with microbes.
[d] Percentage of 1000 μg initially added.
[e] Percentage of sorbed amount that was recovered.
[f] Percentage of recovered amount that was utilized by microbes.

addition, Folin and Ciocalteu's phenol reagent was reduced by EDTA immediately upon contact, thus EDTA soil extracts could not be used to estimate total phenolic acid equivalents in soil extracts. The EDTA extraction procedure also was very ineffective in extracting phenolic acids from plant debris. The EDTA extraction procedure extracted no detectable phenolic acids (HPLC analysis) from ground (60 mesh) wheat stubble (500 mg/100 ml), whereas extracts from the water-autoclave procedure contained 11 distinct peaks (HPLC analysis). Concentrations for ferulic, vanillic, *p*-coumaric, and *p*-hydroxybenzoic were 33, 22, 1034, and 47 μg/g dry weight, respectively. The water-autoclave procedure was, therefore, used in all subsequent studies.

Preliminary Bioassays of Soil Extracts. In an experiment using 1989 soil samples, the phytotoxicity of various concentrations of soil extract (0, 10, 20, 40, 80%) was determined. Data for germination (radicle emergence), mean radicle and hypocotyl length based on all seeds in a Petri dish (RAD), and mean radicle and hypocotyl length based on germinated seeds in a Petri dish (ARAD) of crimson clover were regressed against dilutions of extracts used (see "Analysis for Final Soil Extract Bioassay" in Methods and Materials section for details). Significant slopes for all individual extracts were linear. Six, 50, and 62% of the slopes for extract 1 (crude extract) and 38, 94, and 100% of the slopes for extract 2 (humic acid removed) were significantly greater than zero for germination, RAD, and ARAD, respectively. Thus the removal of humic acid by acidification and subsequent adjustment to pH 5 with NaOH apparently

changed the toxicity of the organic compounds in extract 2 and/or the solute potential of the solution. We suspect it was primarily the solute potential, since the milliosmole values (determined by freezing point depression) of extracts 1 and 2 were 1.25 ± 0.58 and 38.5 ± 2.40, respectively, and the removal of humic acid resulted in only minor changes in the free phenolic acid concentrations in the extract (Blum et al., 1991).

When slopes representing the activity of each extract for germination, RAD or ARAD, were regressed against the FC (total phenolic acid expressed as ferulic acid equivalents) and the milliosmole values of the original extract, only the extract 2 slopes were significantly greater than zero. An analysis based directly on germination, RAD, and ARAD values (not slopes) and FC and milliosmole values of the original extract (no extract dilutions were included in the analysis) resulted in F values that were 50% smaller than the F values from the slope (activity) analysis.

The pellet of humic acid produced by acidification of extract 1 was redissolved in the original volume from which it was recovered by adjusting the water solution to pH 5 with NaOH. The resulting solutions were tested against clover seeds. No significant effects on germination or radicle and hypocotyl length were observed.

For the experiments just discussed, each 0.42 ± 0.005 g of soil was extracted with 1 ml of water (2.35 ml/g). For the following experiment, 0.67 ± 0.005 g (wheat–conventional till) and 0.65 ± 0.006 g (wheat–no till) of soil were extracted with 1 ml of water (1.49 and 1.65 ml/g). These changes were made to adjust the baseline concentrations of the extracts. Extract 1 solutions of 1990 soils from wheat–no-till plots (extract dilutions were not included in this experiment) inhibited RAD and ARAD of clover and ARAD of morning glory (Table 3). The importance of FC and/or milliosmole values in determining RAD and ARAD responses depended on which of these variables was first placed in the response surface analyses (Table 3). This suggested that a colinearity existed between FC values and solute potential of the extract solutions. Extract 1 solutions of 1990 soils from conventional wheat–tilled plots did not affect germination or radicle and hypocotyl length of clover or morning glory.

Solute Potentials of Extracts. Modeling of NaCl solution freezing point depression values with solute potentials from Lang (1967) resulted in the following equation: J/kg of NaCl solution $= -2.133 \times 10^{-5} - (2.0924 \times 10^{-6} \times \text{mosmol}) - [1.3706 \times 10^{-9} \times (\text{mosmol})^2]$; $P \leq 0.0001$; $R^2 = 0.99$. Modeling of freezing point depression values for PEG 6000 with solute potential values generated from the PEG 6000 model of Steuter (1981) resulted in the following equation: J/kg of PEG $= 3.843 \times 10^{-6} - (1.426 \times 10^{-6} \times \text{mosmol})$; $P \leq 0.0001$; $R^2 = 0.99$. J/kg and mosmol in both equations represents joules per kilogram and milliosmoles, respectively.

Applying these equations, we found that NaCl (representing Hoagland's

TABLE 3. SEQUENTIAL MEAN SQUARE VALUES FROM GENERAL LINEAR MODELS PROCEDURE FOR RADICLE AND HYPOCOTYL LENGTH OF CLOVER AND MORNING GLORY SEEDS GERMINATED IN SOIL EXTRACTS FROM WHEAT/NO-TILL PLOTS[a]

Source	*df*	Clover		Morning glory
		RAD[b]	ARAD	ARAD
FC[c]	1	26.5244 (0.035)[d]	97.7233 (0.001)	622.3217 (0.004)
Milliosmoles	1	4.3415	2.6400	7.9543
$(FC)^2$	1	45.1372 (0.007)	18.1270	320.9215 (0.034)
$(Milliosmoles)^2$	1	0.3406	0.0022	2.0604
FC × Milliosmoles	1	0.0761	2.6921	1.5955
Error	34	5.5056	7.7218	66.1608
Milliosmoles	1	23.4226 (0.047)	55.2458 (0.011)	315.2734 (0.036)
FC	1	7.4433	45.1175 (0.021)	315.0026 (0.036)
$(Milliosmoles)^2$	1	14.9904	7.6883	159.1491
$(FC)^2$	1	30.4874 (0.024)	10.4408	163.8328
FC × Milliosmoles	1	0.0761	2.6921	1.5955
Error	34	5.5056	7.7218	66.1608

[a] Analysis run twice using different order of sources.
[b] RAD = sum of radicle and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (seeds per Petri dish: 20 for clover and 10 for morning glory); ARAD = sum of radicle and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in the dish.
[c] FC values (estimated phenolic acid values) ranged from 49 to 174 μg/ml ferulic acid equivalents. Milliosmole values ranged from 8 to 24. These values do not describe the factor space because they are not entirely independent.
[d] Values in parentheses are *P* values; if no *P* values are given then $P > 0.05$.

solution) and PEG solutions of 20 mosmol, for example, would equal -6.4×10^{-5} J/kg (−0.064 MPa) and -2.6×10^{-5} J/kg (−0.026 MPa), respectively. The solute potential of a 20 mosmol NaCl solution was thus 2.46 more negative in value than a 20 mosmol PEG solution. The J/kg values for 80 mosmol NaCl and PEG solutions were -2.0×10^{-4} (−0.20 MPa) and -1.1×10^{-4} (−0.11 MPa), respectively. In this case the NaCl solution was 1.82 times more negative than the PEG solution.

PEG and PEG–Phenolic Acid Mixtures. As the milliosmoles of the PEG solutions increased, germination, RAD, and ARAD declined (Table 4; Figure 1). A solution of 20 mosmol, for example, reduced clover germination, RAD and ARAD by 0, 13, and 5%, respectively. Morning glory germination, RAD, and ARAD were reduced 28, 44, and 36%, respectively.

As the milliosmoles of the PEG solutions increased, the effects of the phenolic acid mixture declined for RAD and ARAD of clover (Table 5; Figure 2). RAD and ARAD of morning glory were not affected by the concentrations

TABLE 4. PARTIAL REGRESSION COEFFICIENTS AND R^2 VALUES FOR GERMINATION AND RADICLE AND HYPOCOTYL LENGTH OF CLOVER AND MORNING GLORY SEEDS GERMINATED IN POLYETHYLENE GLYCOL SOLUTIONS[a]

Variable[b]	Intercept	Linear	Quadratic	Cubic	R^2
Clover					
GERM	0.7992	0.0018	-7.48×10^{-5}		0.57[c]
RAD	26.8512	−0.1799			0.46
ARAD	30.9000	−0.0848			0.15
Morning glory					
GERM	0.7871	−0.0124	7.44×10^{-5}		0.47
RAD	27.4575	−0.7162	0.0053		0.52
ARAD	36.8790	−1.0335	0.0213	-1.4×10^{-4}	0.24

[a] Milliosmole values ranged from 0 (distilled water) to 84 for clover and from 0 to 90 for morning glory; 10 replicates.
[b] GERM = germination; RAD = sum of radicle and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (seeds per Petri dish: 20 for clover and 10 for morning glory); ARAD = sum of radicle and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in the dish.
[c] $P \leq 0.0001$ for all regressions.

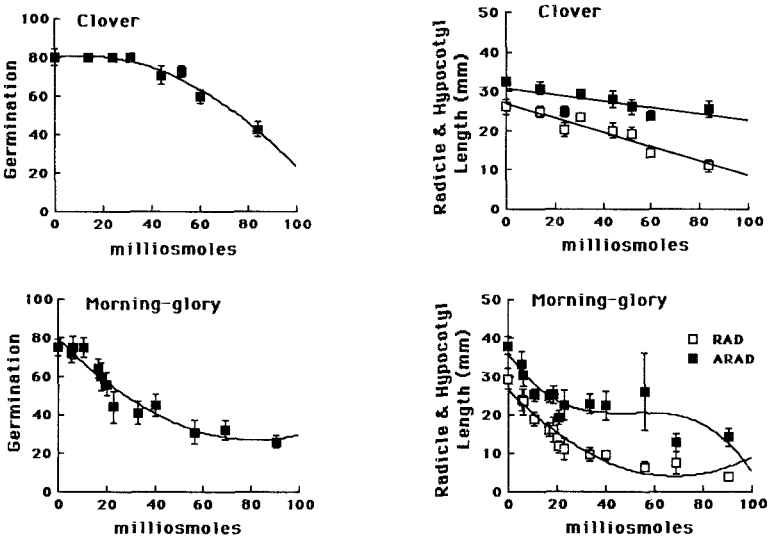

FIG. 1. Effects of PEG 6000 solutions with different freezing point depressions (milliosmoles) on germination and radicle and hypocotyl length (RAD, ARAD) of clover and morning glory. Means ± standard errors.

TABLE 5. PARTIAL REGRESSION COEFFICIENTS AND R^2 AND P VALUES FOR RADICLE AND HYPOCOTYL LENGTH OF CLOVER SEEDS GERMINATED IN VARIOUS CONCENTRATIONS OF PHENOLIC ACID MIXTURE AND POLYETHYLENE GLYCOL SOLUTION[a]

	RAD[b]	ARAD
Intercept	26.0402	31.8511
Phenolic acid mixture (PM)	-2.5703×10^{-2}	-3.433×10^{-2}
Milliosmoles	-0.1194	-0.1253
$(PM)^2$	-4.2733×10^{-7}	9.8475×10^{-6}
$(\text{Milliosmoles})^2$	-5.6096×10^{-4}	-1.2369×10^{-4}
PM × milliosmoles	2.8964×10^{-4}	3.3072×10^{-4}
R^2	0.61	0.61
P	0.0001	0.0001

[a] Milliosmole values for PEG solutions ranged from 0 (distilled water) to 90. Phenolic acid mixtures ranged from 0 to 393.6 μg/ml of solution. Mixtures were composed of 15% *p*-hydroxybenzoic, 10% caffeic, 17% vanillic, 10% syringic, 35% *p*-coumaric, 9% ferulic and 4% sinapic; 5 replicates.

[b] RAD = sum of radicles and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (20 seeds); ARAD = sum of radicles and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in the dish.

of the phenolic acid mixture used. The phenolic acid mixture consisted of seven phenolic acids proportioned to that observed for soils from no-till wheat–soybean plots (see Table 5 for composition) (Blum et al., 1991). Germination of both species was not altered by the concentrations of the phenolic acid mixture used. The increasing milliosmoles of the PEG solution did, however, reduce germination of both species as noted earlier (Table 4; Figure 1).

Hoagland's, Hoagland's–Phenolic Acid Mixtures. Clover germination, RAD, and ARAD were reduced with increasing strength of Hoagland's solution (Table 6; Figure 3). A solution of 20 mosmol reduced clover germination, RAD, and ARAD by 64, 71, and 35%, respectively. Morning glory germination was not affected by the zero to double-strength Hoagland's solutions. RAD and ARAD values of morning glory increased and then decreased (Table 6; Figure 3) as the strength of Hoagland's solution increased.

Since morning glory germination and radicle and hypocotyl length were not affected by the concentrations of the phenolic acid mixtures used, only clover seeds were treated with concentrations of the phenolic acid mixture in various strength of Hoagland's solution. Germination of clover was not affected significantly by the concentrations of the phenolic acid mixture. The effects of zero (distilled water) to double-strength Hoagland's solution on RAD and ARAD

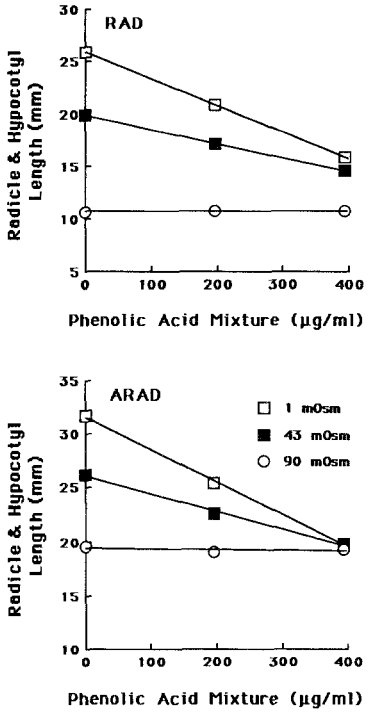

FIG. 2. Modification of the effects of a phenolic acid mixture on radicle and hypocotyl length (RAD, ARAD) of clover by PEG 6000 solutions with different freezing point depressions (milliosmoles). Lines derived from models in Table 5.

were independent of the effects of the phenolic acid mixture, i.e., the effects were additive (Table 7; Figure 4).

Individual Phenolic Acids. None of the seven phenolic acids (0–2 mM; *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, or sinapic) tested affected germination of clover or morning glory (data not shown). Clover RAD and ARAD were reduced by the cinnamic acid derivatives ferulic acid and *p*-coumaric acid and the benzoic acid derivatives *p*-hydroxybenzoic acid and vanillic acid (Table 8; Figure 5). RAD of morning glory was reduced only by *p*-hydroxybenzoic acid and ARAD of morning glory was reduced only by *p*-coumaric acid (Table 8; Figure 6). ARAD of clover, for example, was reduced 26, 11, 10, and 5% by 0.5 mM *p*-coumaric, ferulic, vanillic, and *p*-hydroxybenzoic acids, respectively. ARAD of morning glory was reduced 7% by 0.5 mM *p*-coumaric acid.

6-MBOA, PEG- or Hoagland's-6-MBOA Mixtures. Germination, RAD, and ARAD of both species were reduced by 6-MBOA (Table 9; Figure 7). For

TABLE 6. PARTIAL REGRESSION COEFFICIENTS, R^2, AND P VALUES FOR GERMINATION AND RADICLE AND HYPOCOTYL LENGTH OF CLOVER AND MORNING GLORY SEEDS GERMINATED IN VARIOUS STRENGTHS OF HOAGLAND'S SOLUTION[a]

Variable[b]	Intercept	Linear	Quadratic	R^2	P
Clover					
GERM	0.5999	−0.0231	0.0002	0.69	0.0001
RAD	10.8237	−0.4946	0.0054	0.72	0.0001
ARAD	19.0369	−0.4040	0.0034	0.55	0.0001
Morning-glory					
RAD	32.4311	1.1830	−0.0163	0.23	0.031
ARAD	44.3235	1.2762	−0.0189	0.33	0.005

[a] Milliosmoles of the various strengths of Hoagland's solution ranged from 0 to 61; 5 replicates.
[b] GERM = germination; RAD = sum of radicle and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (seeds per Petri dish: 20 for clover and 10 for morning glory); ARAD = sum of radicle and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in the dish.

example, a 0.5 mM 6-MBOA solution reduced clover germination, RAD, and ARAD values by 24–28%. Morning glory germination, RAD, and ARAD were reduced 14, 23, and 22%, respectively.

As the milliosmoles of the PEG solutions increased, the effects of 6-MBOA on clover germination and clover and morning glory RAD values declined (Table 10; Figures 8 and 9). The effects of mixtures of PEG and 6-MBOA on clover and morning glory ARAD were independent, i.e., the effects were additive.

As the milliosmoles of the various Hoagland's solutions increased, the effects of 6-MBOA on germination and RAD values of clover declined (Table 11). The various strengths of Hoagland's solution did not alter clover ARAD or morning glory RAD and ARAD values. Germination of morning glory was also not affected by 0–1 mM 6-MBOA.

Final Bioassay of Extracts. A subset of 1990 wheat–no-till soil samples was extracted by the water-autoclave procedure. The 33 soil samples chosen represented a cross section of the soil samples collected. Soil extract (extract 1) characteristics were as follows: pH 5.7 ± 0.058, freezing point depression 11.35 ± 0.757 mosmol, FC 121.94 ± 4.26 μg/ml ferulic acid equivalents, NO_3^- 6.016 ± 0.878 μg/ml, NH_4^+ 5.27 ± 0.1267 μg/ml, K 109.72 ± 5.496 μg/ml, and P 17.81 ± 0.951 μg/ml (number of extracts = 33). Since each milliliter of extract represented 0.60 ± 0.01 g soil, 1.68 ml of extract represented 1 g of soil. Five milliliters of extract or diluted extract were used in each Petri dish, thus the extract in each dish represented ≤3 g of soil or ≤0.15 g per crimson

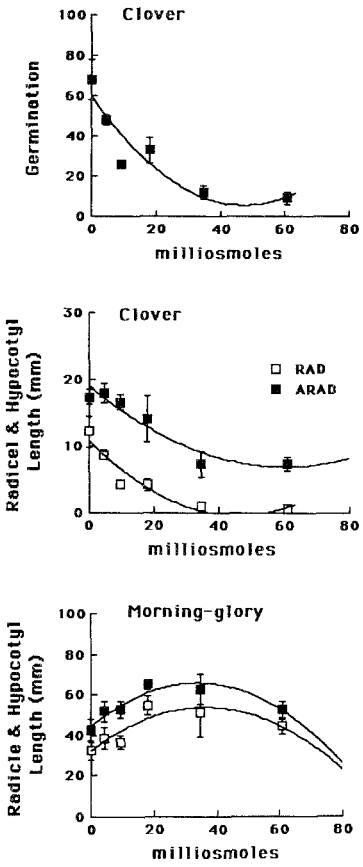

FIG. 3. Effects of Hoagland's solutions with different freezing point depressions (milliosmoles) on germination and radicle and hypocotyl length (RAD, ARAD) of clover and/or morning glory. Means ± standard errors.

clover seed (20 seeds per dish). See "Analysis for Final Soil Extract Bioassay" in Methods and Materials section for additional details.

The phytotoxicity of various dilutions of extract (0, 0.25×, 0.5×, and 1× strength) was determined on clover seeds. Sixty-three, 97, and 100% of the slopes were significantly greater than zero for clover germination, RAD and ARAD, respectively. Slopes were regressed against FC (μg/ml ferulic acid equivalents); milliosmoles; concentrations (μg/ml) of K, P, NO_3^- and NH_4^+, and pH of original soil extracts. PROC LEAPS, PROC RSREG, and PROC GLM procedures of SAS were used to identify the significant independent variables and to generate models (Tables 12 and 13; Figure 10). Partial regres-

TABLE 7. PARTIAL REGRESSION COEFFICIENTS, R^2, AND P VALUES FOR RADICLE AND HYPOCOTYL LENGTH OF CLOVER SEED GERMINATED IN VARIOUS CONCENTRATIONS OF PHENOLIC ACID MIXTURE AND VARIOUS STRENGTHS OF HOAGLAND'S SOLUTION[a]

	RAD[b]	ARAD
Intercept	8.5955	16.7421
Phenolic acid mixture	−0.0062	−0.0139
Milliosmoles	−0.3125	−0.0754
(Milliosmoles)^2	0.0038	
R^2	0.37	0.19
P	0.0001	0.0001

[a] Milliosmole values for the various strengths of Hoagland's solution ranged from 0 (distilled water) to 61. Phenolic acid mixture ranged from 0 to 393.6 μg/ml of solution. See Table 3 for composition of the phenolic acid mixture; 5 replicates.

[b] RAD = sum of radicles and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (20 seeds); ARAD = sum of radicles and hypocotyl lengths in a Petri dish divided by the number of germinated seed in the dish.

sion coefficients for FC values, milliosmoles, and pH were significant for clover RAD slopes, but only the partial regression coefficient for FC values was significant for clover ARAD slopes.

Clover RAD slopes, representing extract activity, became more negative with increasing FC values (linear), milliosmole values (concave), and pH values (linear). The more negative the slope, the more inhibitory the factor. The relationship of FC, milliosmoles, and acidity on clover RAD were independent, i.e., the effects were additive.

A separate analysis based on actual clover germination, RAD, and ARAD values (not slopes) and undiluted extracts confirmed the earlier observation (based on F values) that slope analysis was a more powerful tool in detecting the significant extract characteristics and their effects on germination and radicle and hypocotyl length.

DISCUSSION

An effective protocol for the identification of a functional allelopathic cover crop should provide: (1) an estimate, preferably a single integrated value, of the levels of active compounds in the soil; (2) an estimate of the biological activity of the observed action fraction; and (3) an estimate of how physical and chemical soil factors (e.g., pH, water potential, fertility, etc.) may moderate the effects of this active fraction. In addition, the relationship between the cover crop and crop performance must also ultimately be determined.

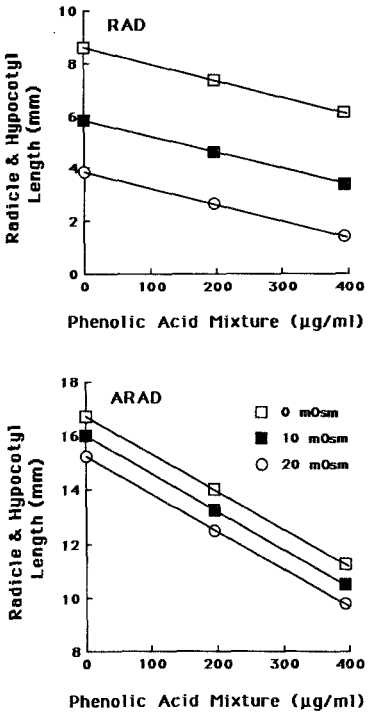

FIG. 4. Modification of the effects of a phenolic acid mixture on radicle and hypocotyl length (RAD, ARAD) of clover by Hoagland's solutions with different freezing point depressions (milliosmoles). Lines derived from models in Table 7.

Estimating Levels of Active Compounds in Soil. All productive agricultural soils contain a large number of organic substances, many of which can affect germination and growth of weeds and crops. Experience and the literature, however, suggest that certain types of compounds are more likely than others to inhibit germination and growth. In wheat systems, these include phenolic acids (Chou and Patrick, 1976; Liebl and Worsham, 1983; Barnes et al., 1986; Blum et al., 1991), organic acids (Patrick, 1971; Chou and Patrick, 1976; Lynch, 1977; Tang and Waiss, 1978; Shilling et al., 1985), hydroxamic acids and related compounds (Willard and Penner, 1976; Niemeyer et al., 1989; Nair et al., 1990), and volatiles (Buttery et al., 1985; Bradow, 1991).

Based on the recovery of ferulic acid, a phenolic acid, from sterile and nonsterile Cecil soils (Tables 1 and 2), water (immediate) and EDTA (0.5 M; pH 8; 5-hr extraction) extractions will provide a reasonable and adequate estimate of the active fraction of free or readily exchangeable phenolic acids in soils, but not phenolic acids in plant debris and/or soil organic matter. The

TABLE 8. PARTIAL REGRESSION COEFFICIENTS, R^2 AND P VALUES FOR GERMINATION AND RADICLE AND HYPOCOTYL LENGTH OF CLOVER AND MORNING GLORY SEEDS GERMINATED IN PHENOLIC ACID SOLUTIONS[a]

Phenolic acid	Variable[b]	Intercept	Linear	Quadratic	R^2	P
Clover						
FER[c]	RAD	29.1632	−5.6626		0.47	0.0046
	ARAD	37.4753	−7.9734		0.73	0.0001
PCO	RAD	39.1294	−28.3452	7.9082	0.84	0.0001
	ARAD	43.5217	−26.2946	6.7138	0.88	0.0001
POH	RAD	27.9264	−3.2470		0.29	0.0374
	ARAD	36.9121	−3.7198		0.29	0.0392
VAN	RAD	25.1801	−4.6527		0.28	0.0445
	ARAD	31.1549	−6.0399		0.53	0.0020
Morning glory						
POH	RAD	55.0781	−7.4888		0.23	0.0415
PCO	ARAD	61.4919	−8.1800		0.47	0.0018

[a] Phenolic acid concentrations ranged from 0 to 2 mM; 3 replicates.
[b] RAD = sum of radicles and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in a dish (seeds per Petri dish: 20 for clover and 10 for morning glory); ARAD = sum of radicles and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in the dish.
[c] FER = ferulic; PCO = *p*-coumaric; POH = *p*-hydroxybenzoic; VAN = vanillic.

immediate water extraction will provide an estimate of phenolic acid concentrations in soil solution, and the difference in the recovery of phenolic acid concentrations by the water and EDTA extraction will provide an estimate of that which is immediately available to go into soil solution. EDTA extracts of soil, which contain soil solution and reversibly bound phenolic acids, however, could not be used directly in germination bioassays, because 0.5 M, pH 8 EDTA solutions completely inhibited clover and morning glory germination. Longer-term soil extractions with water, to recover at least some of the reversibly bound phenolic acids in soils, could not be used because microbes utilize phenolic acids as a carbon source during the extraction period (Blum and Shafer, 1988). We therefore chose to use the water-autoclave procedure. This procedure is rapid, eliminates microbial activity in the extract, and changes extract acidity within acceptable limits (soil pH 6.5 $\pm$ 0.06, extract pH 5.6 $\pm$ 0.05, $N = 72$). Adjustments of extracts to a common pH were not desirable due to the variations this generated in solute potential for soil extracts. Soil extracts produced by the water-autoclave procedure were thus used directly in germination bioassays.

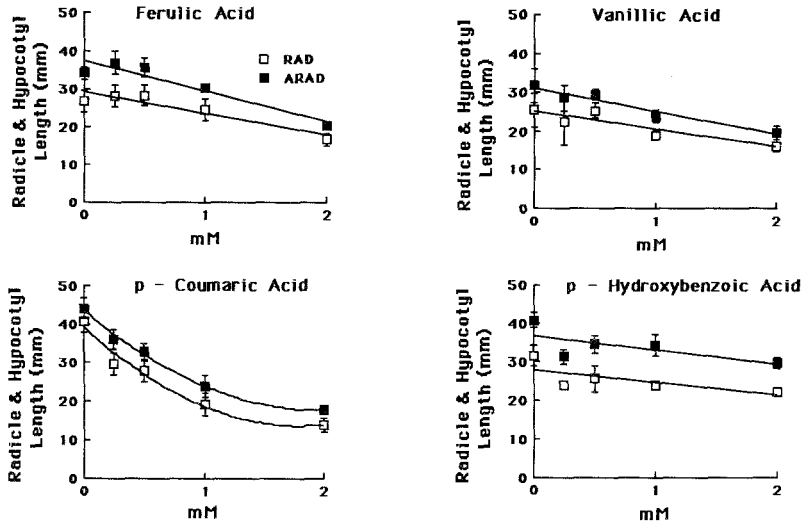

FIG. 5. Effects of individual phenolic acids on radicle and hypocotyl length (RAD, ARAD) of clover. Means ± standard errors.

Identification and quantification of individual phenolic acids in soil extracts by HPLC analysis were very time-consuming (approximately 1 hr per sample); so a rapid means of estimating phenolic acid content was required. An estimate of total phenolic acid, expressed as ferulic acid equivalents, in soil extracts was obtained by the use of Folin and Ciocalteu's phenol reagent (FC method). The resulting values were correlated ($r \geq 0.95$ for 1989 data and $r \geq 0.85$ for 1990 data) with the sum of seven phenolic acid concentrations in water-autoclave extracts as determined by HPLC analysis (Blum et al., 1991). Average values estimated by the FC procedure were 16.75 (1989) and 18 (1990) times greater than those determined by HPLC analysis. The phenol reagent used in the FC procedure was immediately reduced by EDTA, and thus EDTA extracts of soil could not be used to estimate total phenolic acid as ferulic acid equivalents. The phenol reagent is also reduced by a number of other organic and inorganic substances (Box, 1983) including 6-MBOA. 6-MBOA, however, was not detected by HPLC analysis or thin-layer chromatography in any of the wheat debris and soil extracts. This does not, however, preclude the presence of other hydroxamic acids or related products. Hydroxamic acids are constitutive secondary metabolites in several grasses (Zuniga et al., 1983), including wheat, maize, and rye (Willard and Penner, 1976). Wheat contains primarily 2-*o*-glucosyl-7-methoxy-1,4-benzoxazin-3-one (DIMBOA glucoside) (Willard and Penner, 1976; Nair et al., 1990). Upon cell injury, this glucoside is hydrolyzed and spontaneously converted to 6-MBOA. Since DIMBOA does not appear

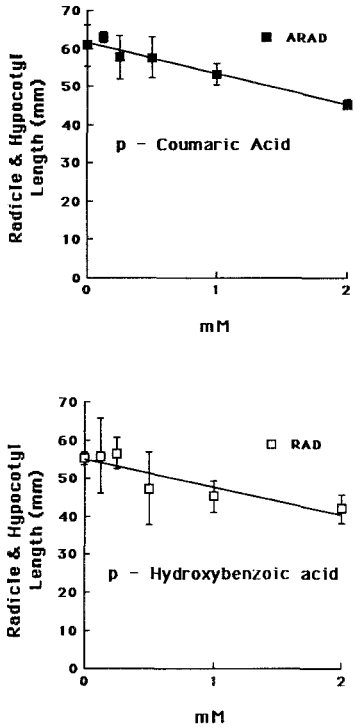

FIG. 6. Effects of individual phenolic acids on radicle and hypocotyl length (RAD, ARAD) of morning glory. Means ± standard errors.

to be released by living wheat plants (Argandona et al., 1987; Perez and Ormeno-Nunez, 1991), we assumed that injury of tissue with associated conversion to 6-MBOA was required. 6-MBOA was, however, not detected in soil or wheat debris extracts.

Potential Soil Extract Characteristics and Germination Bioassays. Germination and radicle and hypocotyl growth of weed and crop seeds are affected by many physical and chemical characteristics of soil extracts, such as pH, solute potential, nutrient content, and organic matter content. Effects of soil extracts on seed germination and radicle and hypocotyl length thus are not the exclusive property of allelopathic agents present in the extract, but are a product of all such factors acting independently or in concert with each other.

Crimson clover and ivy-leaved morning glory were used as bioassay species to characterize the role of some of these factors and their interactions. Crimson clover was chosen because of frequently poor early fall stands of clover seeded into our wheat–no-till soybean plots. Ivy-leaved morning glory was chosen

TABLE 9. PARTIAL REGRESSION COEFFICIENTS AND R^2 VALUES FOR GERMINATION AND RADICLE AND HYPOCOTYL LENGTH OF CLOVER AND MORNING GLORY SEEDS GERMINATED IN 6-MBOA[a]

Variable[b]	Intercept	Linear	R^2	P
Clover				
GERM	0.5347	−0.2774	0.66	0.0001
RAD	9.1971	−5.1132	0.58	0.0002
ARAD	18.3101	−9.4975	0.75	0.0001
Morning glory				
GERM	0.8732	−0.2502	0.47	0.0016
RAD	44.6090	−20.7662	0.65	0.0001
ARAD	54.0485	−23.7037	0.75	0.0001

[a]6-Methoxy-2-benzoxazolinone (6-MBOA) concentrations ranged from 0 to 2 mM; 3 replicates.
[b]GERM = germination; RAD = sum of radicle and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (seeds per Petri dish: 20 for clover and 10 for morning glory); ARAD = sum of radicle and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in the dish.

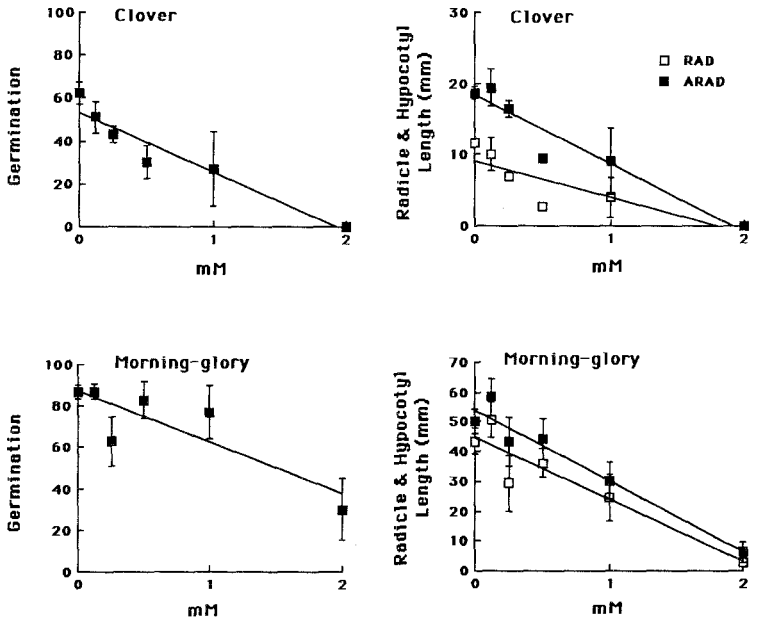

FIG. 7. Effects of 6-MBOA on germination and radicle and hypocotyl length (RAD, ARAD) of clover and morning glory. Means ± standard errors.

TABLE 10. PARTIAL REGRESSION COEFFICIENTS, R^2, AND P VALUES FOR GERMINATION AND RADICLE AND HYPOCOTYL LENGTH OF CLOVER AND MORNING GLORY SEEDS GERMINATED IN VARIOUS CONCENTRATIONS OF 6-MBOA AND OF PEG SOLUTIONS[a]

	Clover			Morning glory	
	GERM[b]	RAD	ARAD	RAD	ARAD
Intercept	0.6376	10.1441	16.8827	40.0192	46.9574
6-MBOA	−0.8015	−17.3058	−6.6683	−15.8548	−12.6862
$(6\text{-MBOA})^2$	0.4318	10.1467			
Milliosmoles	−0.0045	−0.0795	−0.0785	−0.7226	−0.8198
$(\text{Milliosmoles})^2$				0.0042	0.0056
6-MBOA × milliosmoles	0.0040	0.0723		0.1308	
R^2	0.63	0.73	0.42	0.84	0.84
P	0.0001	0.0001	0.0001	0.0001	0.0001

[a] Milliosmole values for PEG solutions ranged from 0 (distilled water) to 84; 6-MBOA ranged from 0 to 0.87 mM; 5 replicates.

[b] GERM = germination; RAD = sum of radicles and hypocotyl lengths (millimeters) in Petri dish divided by number of seeds in dish (seeds per Petri dish: 20 for clover and 10 for morning glory); ARAD = sum of radicle and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in dish.

because it is a noxious weed, and because Liebl and Worsham (1983) reported a significant reduction in morning glory in field studies involving wheat mulch.

Germination (i.e., radicle emergence) of clover and morning glory was not affected by the seven individual phenolic acids (0–2 mM) tested. Clover radicle and hypocotyl length was inhibited more than morning glory radicle and hypocotyl length by individual phenolic acids. A mixture of phenolic acids that simulated the composition of phenolic acids in wheat–no-till soil extracts inhibited radicle and hypocotyl length of clover but not of morning glory. Crimson clover thus became our primary bioassay species.

Although experiments with seven individual phenolic acids, 6-MBOA, and a mixture of phenolic acids that simulated wheat–no-till extracts were included in these studies, the effects of complex mixtures of organic compounds on germination and radicle and hypocotyl length were not addressed directly. Since FC values of soil extracts represent the Folin and Ciocalteu's phenol reagent-reactive portion of such organic complexes, identifying the composition of these reactive organic complexes would appear to be an important area for future study. Attempts to understand the effects of simple mixtures of phenolic acids on germination and plant growth have been made by a number of researchers (Rasmussen and Einhellig, 1977; Williams and Hoagland, 1982; Blum et al., 1984, 1985, 1989; Einhellig, 1987; Gerig and Blum, 1991), but these represent

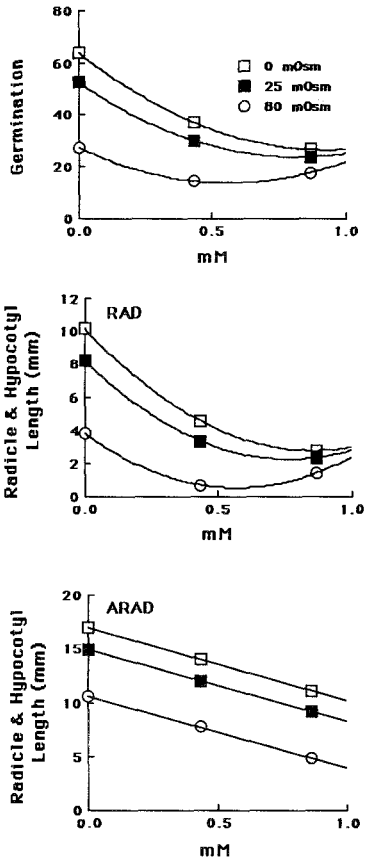

FIG. 8. Modification of the effects of 6-MBOA on germination and radicle and hypocotyl length (RAD, ARAD) of clover by PEG 6000 solutions with different freezing point depressions (milliosmoles). Lines derived from models in Table 10.

only a first step in understanding the role of complex organic mixtures in allelopathic interactions. Apparently, concentrations of individual phenolic acids in mixtures required for a given growth inhibition decline as the number of phenolic acids in a mixture increases (Gerig and Blum, 1991).

The effects of extract acidity on germination and radicle and hypocotyl length were also not determined in this study, but Blum et al. (1984, 1985) noted that effects of phenolic acids on radicle and seedling growth of some crop species were greater under acidic conditions than under neutral conditions. Changes in the acidity of phenolic acid solutions did not, however, result in any detectable modification of ladino clover (*Trifolium repens* L. Tillman) radicle length.

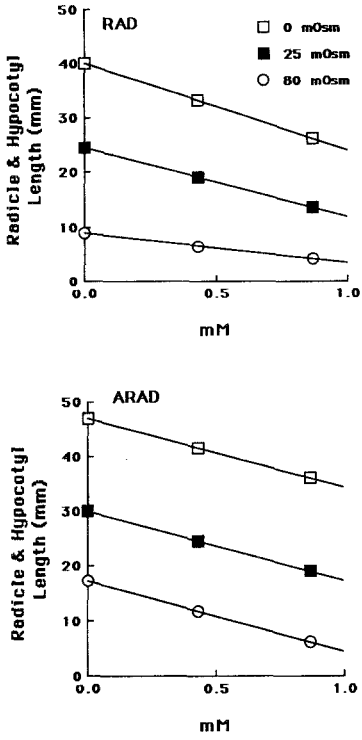

FIG. 9. Modification of the effects of 6-MBOA on radicle and hypocotyl length (RAD, ARAD) of morning glory by PEG 6000 solutions with different freezing point depressions (milliosmoles). Lines derived from models in Table 10.

The response of crimson clover and/or ivy-leaved morning glory seeds to PEG and Hoagland's solutions were substantially different. For example, PEG and Hoagland's solutions with freezing point depressions of 20 mosmol reduced clover RAD 13 and 71%, respectively. Morning glory RAD was reduced 44% by PEG and stimulated 52% by the Hoagland's solution. In addition, inhibition of clover RAD by the phenolic acid mixture declined as the milliosmoles for the different PEG concentrations was increased, whereas the inhibition of the phenolic acid mixture on clover RAD was independent of the milliosmoles for the different strengths of Hoagland's solution. These observations suggest the possibility of a series of complex interactions among clover or morning glory seeds, nutrient ions, and solute potentials of soil extracts. These differences in response may have been due to the nature of the osmoticum (i.e., organic vs. inorganic, nonionic vs. ionic) and/or the biological activity of the PEG molecules and/or the ions in the Hoagland's solution as perceived by the two species.

TABLE 11. PARTIAL REGRESSION COEFFICIENTS, R^2, AND P VALUES FOR GERMINATION AND RADICLE AND HYPOCOTYL LENGTH OF CLOVER AND MORNING GLORY SEEDS GERMINATED IN VARIOUS CONCENTRATIONS OF 6-MBOA AND VARIOUS STRENGTHS OF HOAGLAND'S SOLUTION[a]

	Clover			Morning glory	
	GERM	RAD	ARAD	RAD	ARAD
Intercept	0.6277	9.2035	14.7970	38.6440	43.9725
6-MBOA	−0.4073	−7.1105	−7.5534	−17.2815	−16.2401
Milliosmoles	−0.016	−0.3712			
(Milliosmoles)^2		0.0061			
6-MBOA × milliosmoles	0.0128	0.1597			
R^2	0.65	0.63	0.32	0.28	0.29
P	0.0001	0.0001	0.0001	0.0001	0.0002

[a] Milliosmole values for the various strengths of Hoagland's solution ranged from 0 to 31; 6-MBOA ranged from 0 to 1 mM; 5 replicates.

[b] GERM = germination; RAD = sum of radicles and hypocotyl lengths (millimeters) in Petri dish divided by number of seeds in dish (seeds per Petri dish; 20 for clover and 10 for morning glory); ARAD = sum of radicle and hypocotyl lengths in a Petri dish divided by the number of germinated seeds in dish.

TABLE 12. PARTIAL REGRESSION COEFFICIENTS, R^2, AND P VALUES FOR ASSAY SLOPES OF RADICLE AND HYPOCOTYL LENGTH OF CLOVER SEEDS GERMINATED IN SOIL EXTRACTS

	RAD[a]	ARAD
Intercept	4.864	−3.825
FC[b]	−0.058	−0.0470
Milliosmoles	0.663	
(Milliosmoles)^2	−0.024	
pH	−1.657	
R^2	0.70	0.36
P	0.0001	0.0002

[a] RAD = sum of radicle and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (20 seeds). ARAD = sum of radicle and hypocotyl lengths (millimeters) in a Petri dish divided by the number of germinated seeds in the dish.

[b] FC values (estimated phenolic acid values) ranged from 67 to 162 μg/ml ferulic acid equivalents; milliosmole values ranged from 2.8 to 18.6; pH ranged from 4.9 to 6.4.

TABLE 13. MEAN SQUARE VALUES FROM GENERAL LINEAR MODELS PROCEDURE FOR ASSAY SLOPES OF RADICLE AND HYPOCOTYL LENGTH OF CLOVER SEEDS GERMINATED IN SOIL EXTRACTS FROM WHEAT–NO TILL PLOTS[a]

Source	*df*	RAD[b] Sequential	RAD[b] Partial
FC[c]	1	47.8983 (0.0001)[d]	43.9812 (0.0001)
Milliosmoles	1	12.1635 (0.0025)	9.9138 (0.0057)
(Milliosmoles)^2	1	2.6285	5.9481 (0.0278)
pH	1	8.5858 (0.0094)	8.5858 (0.0094)
Error	28	1.105	
Milliosmoles	1	0.7258	
(Milliosmoles)^2	1	4.6637 (0.0493)	
FC	1	57.3008 (0.0001)	
pH	1	8.5858 (0.0094)	
Error	28	1.105	
pH	1	15.5246 (0.0008)	
Milliosmoles	1	0.2437	
(Milliosmoles)^2	1	11.5264 (0.0032)	
FC	1	43.9812 (0.0001)	
Error	28	1.105	

[a] Analysis run several times using different order of sources.
[b] RAD = sum of radicle and hypocotyl lengths (millimeters) in a Petri dish divided by the number of seeds in the dish (20 seeds).
[c] FC values ranged (estimated phenolic acid values) from 67 to 162 μg/ml ferulic acid equivalents; milliosmole values ranged from 2.8 to 18.6; pH values ranged from 4.9 to 6.4. These values do not describe the factor space because they are not entirely independent.
[d] Values in parentheses are *P* values; if no *P* values are given, then $P > 0.05$.

The solute potential of a NaCl solution (simulation of the Hoagland's solution) at 20 mosmol was 2.46 times more negative than a 20 mosmol PEG solution.

In summary, these observations suggest that before allelopathic effects of soil or plant extracts can be identified, the action of other physical and chemical properties (e.g., pH, solute potential, nutrient ion content, etc.) of the extracts and the interactions among such properties and allelopathic agents must be identified for any given bioassay species. One way of identifying potential active properties of extract solutions is to apply regression procedures to biological responses and extract characteristics.

Final Soil Extract Bioassay. In an attempt to estimate the activity of soils, extracts from soils were tested at various dilutions. Germination and radicle and hypocotyl length of clover were then regressed against the dilutions of these extracts. The resulting slopes, which represented activity of these extracts, were

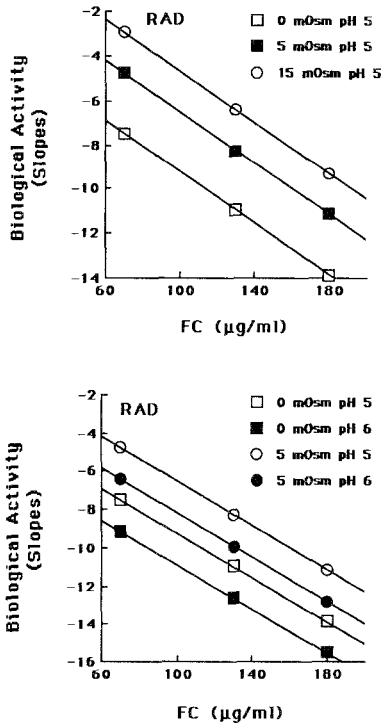

FIG. 10. Activity (slopes) versus FC (micrograms per gram ferulic acid equivalents), freezing point depression (milliosmoles), and pH in wheat–no-till soil extracts on radicle and hypocotyl length (RAD) of clover. The more negative the slope the greater the inhibition of radicle and hypocotyl length. Lines derived from models in Table 12.

then regressed against various characteristics of the extracts, including FC values, pH, freezing point depression, and ion content. This approach appeared to be a useful technique for the identification of the role and potential importance of allelopathic agents in soils.

We chose to estimate primarily the role of phenolic acids and secondarily 6-MBOA, in wheat–no-till and conventional till systems because numerous researchers have suggested that these were the important allelopathic compounds in wheat systems (see Rice, 1984; also see Introduction). Since 6-MBOA was not detected, the FC method was used primarily to estimate phenolic acid content in extracts (Box, 1983; Blum et al., 1991, also see Methods and Materials). Since the effects of allelopathic compounds are concentration-dependent, the amount of soil extracted per unit of water is important. Average dry weights of soil extracted for preliminary bioassay experiments by 1 ml water for 1989 and 1990 and the final 1990 soil extract bioassay were 0.42, 0.65 and 0.67 (no till

and conventional till), and 0.60 g, respectively. Petri dishes with undiluted extracts (5 ml) thus contained extracts derived from 2.10, 3.25 and 3.35, and 3 g of soil. Mean FC values (micrograms of ferulic acid equivalents per milliliter), and minimum and maximum values were as follows: (1) 1989 preliminary experiment, crude extracts—44, 19, and 70 (extracts with humic acid removed were approximately 10% lower); (2) 1990 preliminary experiment, wheat–conventional till—88, 40, and 154; wheat–no till—119, 49, and 174; and (3) 1990 final experiment, wheat–no-till—121, 67, and 162.

No significant FC trends for germination of both clover and morning glory seeds were observed for the 1989 and 1990 soil extracts. However, trends for FC values of extracts from wheat–no-till soils were observed for radicle and hypocotyl length of both species and slopes of radicle and hypocotyl length for clover; slopes for morning glory were not determined. The amount of soil extracted per unit of water for 1989 soil samples and for 1990 wheat–conventional-till soil samples resulted in FC concentration gradients that were too low for detectable trends by clover or morning glory.

Slopes for clover RAD (mean radicle and hypocotyl length based on all seeds in a Petri dish) became more negative with increasing FC, milliosmoles, and pH values; the more negative the slope, the more inhibitory the factor. Other soil extract characteristics such as NO_3^-, NH_4^+, K, and P were not found to make a significant contribution in characterizing the RAD response. The relationships of increasing soil extract FC, milliosmoles, and acidity on clover RAD were independent; i.e., the "effects" were additive.

Extract FC relationships for clover RAD were consistent with the effects observed with individual or the mixture of phenolic acids. The independent and additive actions of extract FC and solute potentials (represented here by milliosmoles) on clover RAD were consistent with the results observed for the phenolic acid mixture–Hoagland's solution experiment, but not for the phenolic acid mixture–PEG experiment. We suspect that the solute potentials produced by various dilutions of Hoagland's solutions more closely mimic solute potentials of soil extracts than the solute potentials produced by various dilutions of PEG. The effects of 6-MBOA on clover germination and the interactions between 6-MBOA and milliosmoles of Hoagland's solutions on clover RAD were not consistent with the activity of the soil extracts. The importance of other hydroxamic acids and other phytotoxins present in the extracts are presently unknown.

As the extract acidity increased, the toxicity (the slope) associated with FC decreased. We do not know how phenolic acid solutions with different pH values may affect radicle and hypocotyl length of crimson clover, but the effects of phenolic acids on radicle length of another clover species (*Trifolium repens* cv. Tillman) were not modified by solution pH (Blum et al., 1984). We do know, however, that the higher the acidity of the soil at the time of extraction, the lower the amount of phenolic acid recovered from soil (Dalton et al., 1983).

Furthermore, the lowest recovery of phenolic acids from soils occurs with acidic extractants when compared to neutral or basic extractants (Whitehead et al., 1981). We suspect that these may explain the relationships noted for extract pH, especially since soils were extracted with water, and thereby the initial soil pH and the extractant pH were determinants of the soil sample.

Trends for clover ARAD (mean radicle and hypocotyl length based on germinated seeds in a Petri dish) were noted only for FC values of the extract. ARAD values represent growth of active seeds, while RAD values represent growth associated with all seeds in a population. The lower R^2 values for ARAD, when compared to RAD, most likely were a result of genetic variation associates with clover seed lots. For example a few insensitive seeds within a Petri dish affected ARAD values much more than RAD values.

Summary. The water-autoclave procedure in conjunction with the FC method can provide a single estimate of the level of available phenolic acids in wheat–no-till and conventional till soil samples. The use of appropriate species and slope analyses of extracts should provide a measure of biological activity. The resulting slopes may be regressed against appropriate characteristics of the extract, such as solute potential, pH, etc., to study their relationships to biological activity. Thus soil extracts may be used directly to identify potential allelopathic activity in wheat soils.

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USE OF ODOR BAITS IN TRAPS TO TEST REACTIONS TO INTRA- AND INTERSPECIFIC CHEMICAL CUES IN HOUSE MICE LIVING IN OUTDOOR ENCLOSURES

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Abstract—House mice (*Mus musculus*) living in outdoor enclosures were tested for urinary chemical cue preferences using odor-baited traps. In the first experiment, with only volatile cues available, odors from conspecific males and females of various age classes and reproductive conditions were tested; no preferences were exhibited. In the second experiment mice had both nonvolatile and volatile cues available from the same sources as in experiment I. All age and sex class and female reproductive condition groups exhibited odor cue preferences except juvenile females. There were no specific odor cue preferences exhibited by any of the responder types with regard to odors from juvenile females. In the third and fifth experiments, mice were presented with nonvolatile plus volatile or only volatile urine odor cues, respectively, from four genera, *Mus*, *Peromyscus*, *Microtus*, and *Homo*. Mice of all age classes and both sexes preferred *Mus musculus* odor, were neutral toward *Microtus ochrogaster* odor, and avoided odors from *Peromyscus leucopus* and *Homo sapiens*; these patterns were the same regardless of whether only volatile or both volatile and nonvolatile cues were presented. The fourth and sixth experiments involved testing volatile cues only and volatile cues plus nonvolatile cues from human sweat or feces from dogs, cats, or shrews. Mice avoided the human sweat and feces from cats and shrews, but were neutral toward the odor of dog feces. There were effects on whether mice were trapped in the interior of the enclosure or on the perimeter for some odors tested in these six experiments. The findings provide insights regarding possible functions of odor cues in the behavioral ecology of house mice. Odor-baiting traps can be an effective tool with respect to testing some, but not all questions pertaining to olfactory cues and house mouse social biology.

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Key Words—House mouse, *Mus musculus*, odor baits, chemical cues, chemosignals, pheromones, *Microtus ochrogaster*, *Peromyscus leucopus*, social behavior, *Homo sapiens*, *Canis familiaris*, *Felis domesticus*, predators, competition.

INTRODUCTION

Olfactory cues play key roles in the behavioral biology of many rodent species (Vandenbergh, 1983a; Albone, 1984; Bronson, 1989), and house mice (*Mus musculus*) in particular (Bronson, 1979; Drickamer, 1986; Vandenbergh and Coppola, 1986). For house mice, odor cues may affect reproductive condition and puberty (e.g., Vandenbergh, 1983b; Drickamer, 1986), recognition of deme membership, age, sex, and reproductive condition (e.g., Bowers and Alexander, 1967; Cox, 1989), mate selection (e.g., Yamazaki et al., 1976; Lenington and Egid, 1985), and urine marking (e.g., Bronson, 1976; Hurst, 1987, 1989, 1990a–c). Similar functions have been reported for olfactory communication in other rodent taxa, including *Peromyscus* (e.g., Doty, 1972) and *Microtus* (e.g., Ferguson et al., 1986). In particular, Doty (1972) reported that female *Peromyscus maniculatus bairdi*, given a choice between odors from males of two species, their own and *P. leucopus noveboracensis*, preferred the homospecific odor when they were in estrus, but exhibited no preference when they were in diestrus.

Recently, Hurst (1990a–c) used laboratory enclosures to study responses by house mice to urine marks made by conspecifics. Dominant and subordinate males use urine marks to orient toward their own territory and away from territories of other males (Hurst, 1990a). Rates of investigation of urine marks and of countermarking indicate that odor communication between females is significant; females mark at high rates to signify their dominant breeding status (Hurst, 1990b). Dominant males countermark all types of female urine (Hurst, 1990c); this is in agreement with an earlier report concerning investigation of olfactory cues by males (Drickamer, 1989). Lastly, Hurst (1990c) reported that although countermarking responses by females were more variable than those for males, females did mark more frequently in response to males not from their own territory than when presented with urine marks from the home territory.

The behavior of many rodents is difficult to study under natural conditions because (1) many are nocturnal and/or (2) they are small and live in habitats where observation is limited or impossible. Odor-baiting of traps has been employed with some success in assessing the significance and possible functions of olfactory cues in rodent behavioral and social biology. Soiled traps are entered more frequently than clean traps by a variety of rodents, including *Microtus townsendii*, *M. agrestis*, *Apodemus sylvaticus*, *A. flavicollis*, and *Clethrionomys*

glareolus (Boonstra and Krebs, 1976; Stoddart, 1982; Gurnell and Little, 1992). *P. leucopus* preferred traps baited with odors from conspecifics of the opposite sex (Mazdzer et al., 1976), confirming a laboratory study of Doty (1973) on the same species. Daly et al. (1978) noted that there were seasonal differences in the tendency of *Peromyscus maniculatus gambelli* to enter traps containing conspecific odors. In a similar study, California voles (*Microtus californicus*) exhibited strong preferences for conspecific odors (Heske, 1987). Daly et al. (1980) showed that for *P. m. gambelli*, traps baited with conspecific odors were preferred by mice in reproductive condition, but mice not in reproductive condition preferred neutral, unbaited traps. In the same study, Daly et al. (1980) reported interspecific responsiveness to rodents in reproductive condition for deer mice, *Perognathus fallax*, and *Dipodomys merriami*. Drickamer (1984) found that *Peromyscus leucopus noveboracensis* and *P. maniculatus gracilis* avoided heterospecific odors and preferred homospecific odors, and also, within each species, there was a strong preference for heterosexual odors.

To our knowledge, while behavioral interactions of house mice and other species have been studied by trapping (Caldwell, 1964; Quandagno, 1968), no one has employed the odor-baiting technique to explore house mouse social behavior or interspecific reactions under natural or seminatural conditions. Rowe (1970) reported that wild stock house mice tested in small laboratory arenas responded differentially to live traps soiled by odors from mice of the opposite sex. Intraspecific odor cues may be important to mice in terms of a number of functions including finding mates, approaching or avoiding conspecifics, individual or deme recognition, and possible interspecific interactions and predator avoidance. Certainly the results of investigations by Hurst (1990a–c), Bronson (1976), Davies and Bellamy (1974), and others indicate that odor cues play key functional roles in the lives of house mice living in commensal situations. Are odor cues significant signals for house mice living under more feral conditions in field enclosures?

The three rodent species (house mice, *Peromyscus leucopus*, and *Microtus ochrogaster*) chosen for investigation in some of these experiments are sympatric over a broad range of the central United States (Hall, 1981). These species exhibit some degree of habitat overlap, either with pairs present in the same location or all three being caught in the same fields in some regions (Linduska, 1950; Whitaker, 1967; Drickamer, unpublished data from southern Illinois). Where interspecific relations involving voles, deer mice, and house mice have been examined in more detail, on species may dominate with the other going to extinction (e.g., *Mus musculus* and *Peromyscus polionotus*) (Caldwell, 1964; Caldwell and Gentry, 1965); one species may increase more rapidly than the other (e.g., *Mus musculus* and *Microtus californicus*) (Quadagno, 1968); or there may be the appearance of general coexistence with numbers for the different species varying slightly over time, but exhibiting no particular directional

patterns (Whitaker, 1967). For most reports of interspecific competition involving these genera and species of rodents, there are no reported observations of actual contact interactions or fighting behavior. The competition between the species must be indirect. While there may be some visual contact, it seems probable that there would be considerable communication via olfactory signals. It is thus of some interest to know whether odor cues may mediate some interactions between house mice, white-footed deer mice, and prairie voles.

Olfactory cues may also be important to rodents in terms of detecting the presence of and avoiding contact with potential predators. Several investigators (Eadie, 1952; Barbehenn, 1958; Fulk, 1972) have shown that activity and spatial relations in voles of several species are influenced by the presence of shrews (*Blarina brevicauda*). The shrews can prey upon the voles and thus we would expect voles to avoid them. Other predators, e.g., foxes and weasels (Madison et al., 1984) also affect the activity and spatial distribution of voles. It seems likely that house mice would also respond to the presence of predators by shifting either their activity and/or their use of space. Olfactory cues could play a significant role in the ability of the mice to detect the presence of potential predators.

To determine the manner in which olfactory cues influence house mouse behavior under natural conditions, we tested the following questions using free-living house mice maintained in outdoor enclosures: (1) Do male and female mice of different age and sex classes exhibit particular preferences for volatile and/or nonvolatile urinary cues from conspecifics of various age and sex classes? (2) Do house mice exhibit differential responses to volatile and/or nonvolatile odor cues from their own species, *Peromyscus leucopus*, *Microtus ochrogaster*, or *Homo sapiens*? (3) Do house mice exhibit differential responses to volatile and/or nonvolatile odor cues from potential predators?

METHODS AND MATERIALS

Enclosures. Eight 0.1-ha enclosures located in an abandoned horse pasture were used for these experiments (see Drickamer and Mikesic, 1990, for full description of the enclosures and their construction). Each enclosure measures 25 × 40 m, has wire mesh and aluminum side walls, and contains four symmetrically placed 2-m-diameter structures, called "mouse cities" (King, 1983), made of 2-m-high aluminum affixed to wooden posts. These mouse cities have a plywood roof, two entrances on opposite sides just above ground level, nesting cavities in cinder blocks located in the interior, and are supplied weekly with food and water. We mowed a 1.5-m strip around the interior perimeter of each enclosure and around each mouse city approximately once each month; in all other areas of each enclosure we allowed the vegetation to grow uncut, attaining

heights of 1–1.5 m. Each enclosure contained a 6 × 9 trapping grid with 5-m intervals between trap stations. The trapping grid thus had 26 trap stations located along the walls of each enclosure and seven rows of four traps each, for a total of 28, in the interior.

Start-Up Procedures. We initiated the enclosure populations with second- and third-generation stock from wild mice originally captured at Little Egypt Grain Co. (Crab Orchard National Wildlife Refuge, Williamson County, Illinois). The pens were started on April 1, 1990, and April 7, 1991, by placing groups of four females and one male into each mouse city (total of four males and 16 females into each enclosure). All eight enclosures were used in 1990 and four enclosures were used in 1991. Starter mice were all 110–160 days of age at the time the enclosures were stocked, and they had been held in unisexual groups of 3–5/cage since weaning at 25–30 days of age. One week prior to placement in the enclosures, mice were housed with one male and four females together. At this time we weighed each mouse to the nearest 0.5 g with a Pesola scale, checked its reproductive condition, and gave it a unique toe-clip ear-punch number. During the first week in the enclosures, mice were restricted to living inside the mouse city where they were placed. We then opened the cities, checked for the presence of the mice, and removed the rubber stoppers from the entrances so that mice had free access to the entire enclosure. Populations were allowed to grow freely for 30 weeks. The first three experiments commenced nine weeks after the pens were started, in early June 1990, and continued for 20 consecutive weeks, until early October. The fourth through sixth experiments were conducted between June and October 1991. The procedures and results are presented in the order in which the experiments were conducted.

Experiment I: Volatile Cues from Conspecifics. The initial experiment was designed to explore the responses of house mice to volatile urine odor cues from various age and sex classes of conspecifics. Eight different urine odor sources were used: adult males, juvenile males, adult estrous females, adult nonestrous females, adult pregnant females, adult lactating females, adult grouped females, and adult juvenile females. Juvenile males were ≤ 15 g body mass and did not have scrotal testes. Juvenile females were ≤ 13 g body mass and had not attained vaginal introitus. Adult males and females were ≥ 100 days of age; males were scrotal and female reproductive condition varied according to the treatment, although all had experienced first vaginal estrus. Grouped females were housed 5–6/cage; all others were housed individually, with their litters, or with a mate. We collected urine using metabolism cages (Maryland Plastics Corp.) with wild stock mice housed in the laboratory colony maintained at 22 $\pm$ 2°C and 50–80% relative humidity with overhead fluorescent lights on from 0800 to 2200 hr. Designated mice were placed in metabolism cages between 1200 and 1300 hours. We provided them with water, but not food, and removed them approximately 20 hr later at 0800 hr. The apparatus was then cleaned thoroughly.

Urine was collected fresh from at least 20 different subjects for each source type. It was pooled, diluted with 3 parts water to 1 part urine, placed in labeled 2-ml vials, and stored in a freezer at −20°C until used in the field enclosures. Freezing does not influence most urinary chemosignals (Drickamer, 1982; Drickamer and Mikesic, 1990) and urinary chemosignals are effective in very small doses (Drickamer, 1984).

The experimental design involved eight enclosures, eight urine odor sources, and eight weeks of testing. A complete Latin-square design was employed with each urine odor treatment occurring in each enclosure for one week. In each pen, 20 of the interior grid stations were used for the experimental test; these stations were the four traps in each of five of the seven rows. The same trap locations were used in each enclosure. Two Sherman live traps (9 × 10 × 25 cm long) were placed at each of these 20 locations; all other locations had a single Sherman live trap. One of each pair of traps was designated as a control trap, and the other as an odorized trap. Thus, there were 74 traps in each enclosure, 26 along the perimeter walls and 48 in the interior, 40 at stations with pairs, and 8 as individual traps. Every live trap was placed inside a half-gallon waxed milk carton, providing additional insulation and protection against the elements. Each week the traps and cartons designated for a particular odor source were rotated to the next designated pen.

Our procedures for control traps in this and subsequent experiments should be explained in detail. All traps were removed from the field, cleaned thoroughly with soap and water, and returned to the field in new, previously unused milk cartons after experiments II, III, IV, and V. The only time this procedure was not followed was between the first two experiments and in that instance we deliberately wanted to have the same set of traps used for both tests. Since all traps, control and experimental traps at the 20 designation stations in each grid, as well as all other traps could acquire some odors at or near their entrances from the passage of other mice, we have made the general assumption that control traps represent a baseline with accumulated odors possible near the entrance. This is then contrasted with odorized traps that have the same possible accumulation of incidental odors near the entrance plus the designated odor that we have supplied. Incidentally, this assumption would be true for all other previous studies of the effects of residual odors as detailed in the Introduction even to the point that within the same night mice could pause outside any trap long enough to deposit some odor, but without entering, although possibly influencing the selection of a trap to enter by any other mouse encountering that trap on that or subsequent nights. We should also note that under our field conditions, house mice did not leave visible urine marks or collections of feces on the traps, or the milk cartons in which the traps were placed, like those described by Hurst and Berreen (1985) for mice living in poultry houses.

The odors were supplied to the traps by placing 0.5 (±0.1) cc of a urine–

water mixture on a cotton square (2.5 × 2.5 × 0.3 cm thick; Ancare Corp.). The urine–water mixture was obtained by thawing appropriate vials from the freezer and mixing the solution with 3 parts water, thus making the final dilution 9 parts water to 1 part urine. The mixture was applied to the cotton square using a hypodermic syringe; different syringes were used for each urine source. Cotton squares were taken to the enclosures and placed into the traps as described below. Since the cotton squares were placed to the rear of the trap, on the treadle, only volatile cues from the odor source would be available to a mouse approaching the entrance of the trap. To obtain more stimulus, including non-volatile odor cues, the mouse was required to go to the rear of the trap.

We live-trapped on two days each week; traps were opened and baited between 1600 and 1800 hr on Tuesday and Thursday afternoons and checked and closed between 0600 and 0800 the next morning. On Tuesday afternoons, we collected all odorized traps and shifted them to the next designated pen in the design. Cotton squares from the previous week were removed and fresh ones were placed in the traps and odorized, then traps were distributed to appropriate stations in the "new" enclosure. All nonodorized traps (including control and perimeter traps) contained a clean cotton square. All traps in each enclosure were opened and baited with a pinch of rolled oats. When a mouse was caught in a control or odorized trap, that trap was removed to the laboratory for thorough cleaning. A clean trap with either a clean cotton square or one with appropriate urine odor was put in its place. On Thursdays we replaced all traps where captures had occurred during the previous trapping session and opened and baited all traps. Also on Thursdays, the cotton square in each odorized trap was refreshed with 0.2 cc of the appropriate urine–water mixture, made up as described previously and placed on the cotton in the traps using the same series of hypodermic syringes.

For each mouse that was captured, we recorded its sex, body mass, and reproductive condition. For males, reproductive condition was recorded as scrotal or nonscrotal. For females, if the vagina was open (all postpubertal female house mice generally have the vagina open continuously), we did a vaginal lavage with a pipet and approximately 0.2 cc of water. The wet-mount vaginal smears were examined in the field with a light microscope to determine the proportion of cornified cells in the smear. Females were classified as estrus (90% or more of the cells in the smear being cornified epithelial cells) or nonestrus (all other combinations of epithelial cells and leukocytes). Pregnancy and lactation were judged by body mass, condition of the nipples, and gentle palpation of the abdomen.

We analyzed the trapping results to determine whether there were any differential responses to the various odors, any differential responses by mice of particular age or sex classes, and any effects on capture rates in the interior versus perimeter locations. Chi-square tests (Sokal and Rohlf, 1981) were used with $\alpha = 0.05$.

Experiment II: Nonvolatile and Volatile Cues from Conspecifics. The second experiment was designed to test responses to both nonvolatile and volatile conspecific urine odor cues. The urine collection procedures, experimental design, field procedures, and analyses were identical to those used in experiment I except that the method of applying the urine to the cotton and traps was altered. Instead of a syringe to place the urine on the cotton square, we employed plastic spray bottles, of the type used for watering house plants. Urine was applied to the cotton square already in the trap and to the open trap door, thus putting odor(s) at the entrance to the trap as well as on the cotton, which was in the rear of the trap, on the treadle. Thus, in contrast to experiment I, mice could obtain either nonvolatile or volatile cues without entering the trap. Separate spray bottles were used for each urine odor type. The concentration of the urine sprayed into the traps was approximately the same as that used for experiment I; 12 cc of the urine–water mixture was obtained from thawed vials and was mixed with 35–40 cc of water, creating an overall dilution of about 1 part urine to 9–10 parts water. The amount of spraying used for each odorized trap in this experiment resulted in the use of the same initial amount of water and urine mixture (0.5 ± 0.1 cc) per trap as in experiment I. We then refreshed each trap entrance and cotton in the odorized traps with ca. 0.2 cc of the appropriate urine–water mixture at the time the traps were opened and baited on Thursday.

The odorized cotton squares in the traps were not changed each week when traps were shifted between pens, except, as noted earlier, when a trap caught a mouse. In the latter case, we always replaced the trap and cotton for the next trapping session.

Experiment III: Nonvolatile and Volatile Cues from Heterospecific Rodents and Human Urine. The third experiment was designed to test the responses of the mice to volatile plus nonvolatile odors from conspecifics and from three other species. Prior to the start of this experiment, all traps were removed to the laboratory and cleaned thoroughly to remove any residual odors from previous experiment; new milk cartons were placed at all stations when the clean traps were replaced into the enclosures. For this test, there were four urine odor sources consisting of pooled urine from males and females of four species: *Mus musculus*, *Peromyscus leucopus*, *Microtus ochrogaster*, and *Homo sapiens*. All but the human urine were collected in the same manner as described previously for experiment I. Mice and voles used for urine collection were first- or second-generation wild stocks being held in colonies in the laboratory (six males and six females—all adults—were used for each species). Human urine was collected from two adult males. As before, all urine was pooled, diluted with 3 parts water to 1 part urine and frozen in 2-ml vials. The experimental design involved four urine sources, four weeks, and all eight enclosures; each urine type was placed into two enclosures in each week. The four urine sources were rotated through the eight pens in four weeks. In all other respects, the design, proce-

dures, and analyses were like those for experiment II, including the fact that urine was sprayed onto the cotton and front door of designated traps and these odorized traps were not changed, except when a mouse was captured. Both odorized and control traps were exchanged for fresh traps when a mouse was captured.

Experiment IV: Volatile Cues from Potential Predators and Human Sweat. The fourth experiment involved testing volatile odors only from potential predators. Four enclosures were used; each of four treatments was tested in each enclosure on two separate occasions. Four odor bait types were rotated through the four enclosures over a four-week period and then the process was repeated for an additional four weeks. The four odor sources consisted of (1) human sweat, collected from an adult male *Homo sapiens*, (2) a homogenate of fecal material obtained from an adult male dog (*Canis familiaris*) mixed with water, (3) a homogenate of fecal material obtained from an adult male and an adult female cat (*Felis domesticus*) mixed with water, and (4) a homogenate of fecal material obtained from two short-tailed shrews (*Blarina brevicauda*) of unknown sex, mixed with water. The homogenates were prepared and frozen in 2-ml vials until used. Procedures for providing the odor baits to the traps on cotton squares and the trapping scheme were identical to experiment I. All traps and milk cartons were clean at the start of this experiment.

Experiment V: Volatile Cues from Heterospecifics and Human Urine. In this experiment we tested the effects of using only volatile odor cues from the four species used for experiment III, *Mus musculus*, *Microtus ochrogaster*, *Peromyscus leucopus*, and *Homo sapiens*. The design was similar to experiment IV, with four enclosures each tested twice in an eight-week period. The procedures for providing the cotton squares with odors and the trapping scheme were identical to those in experiment I.

Experiment VI: Nonvolatile and Volatile Cues from Potential Predators and Human Sweat. The final experiment involved testing responses to volatile plus nonvolatile odor cues from the potential predators tested in experiment IV. The design involved only four weeks and a single placement of each odor type into each of four enclosures. The methods used for odor-baiting the traps followed those for experiment II and all other procedures were as in experiment I.

Assumptions. We examined several assumptions inherent in the experimental methods just described to ensure the validity of the results. The number of misfired traps, those that were closed on a trap-check monitoring but did not contain mice, could be a problem if there were a large number of them. Furthermore, traps that had the mechanism set such that the trap would not fire, even with a mouse inside, could lead to loss of information. Across the eight enclosures and over the 20 weeks of testing there were 592 traps open each session for two sessions each week (74 traps/enclosure), providing a total of

23,680 trap nights in 1990. For 1991, there were four enclosures used for 20 weeks of testing with two trapping sessions each week and 296 traps open for each session, giving a total of 11,840 trap nights (74 traps/enclosure). There were, per trapping session, on average, less than five misfires and less than four traps that likely would not close if a mouse had entered. Both of these values are less than 1% of the available traps. (We should note that each time either problem was detected, the trap was adjusted or replaced as necessary.) Thus, it would appear that neither of these issues caused any measurable bias in our data.

A second issue is that some mice may get caught on both nights in a given week. Concerning the independence of these data points, we ascertained whether such occurrences comprised a significant percentage of the total captures and whether, when mice were caught twice in the same week, they were captured at different types of traps. Recaptures could be in the same type of trap, e.g., odorized (first capture)–odorized (second capture), control–control, nonexperimental–nonexperimental, or some combination of different sources, e.g., odorized–control, etc. The rate of recaptures varied from 10% to 19% across the six experiments (Table 1). This might be cause for some concern. However, of the various total recaptures within experiments, only very small proportions (<2%) were mice caught twice in the same type of trap (Table 1). Thus, we feel justified in using each capture as an independent event for our analyses.

A third issue concerns whether mice coming to grid stations with two traps, one control and one odorized, faced the problem of having both traps already closed, containing mice. If this problem arose at a high proportion of traps, we

TABLE 1. TOTAL CAPTURES BY EXPERIMENT AND TESTS OF ASSUMPTIONS ASSOCIATED WITH ODOR-BAITING EXPERIMENTAL PROCEDURES

	Experiment					
	I	II	III	IV	V	VI
Duration (weeks)	8	8	4	8	8	4
No. enclosures used	8	8	8	4	4	4
Total captures	586	667	315	423	296	169
Recaptures in same week (%)	113 (19%)	90 (14%)	31 (10%)	47 (11%)	34 (8%)	24 (14%)
Recaptures at the same bait type (%)	14 (2%)	7 (1%)	3 (1%)	8 (2%)	8 (2%)	65 (4%)
Two captures at same grid station on same night (%)	32 (5%)	26 (4%)	11 (4%)	11 (3%)	9 (2%)	4 (2%)

would lose information and also possibly create a bias in the data set, particularly at the stations that were part of the experimental test. Fortunately, the rates of pairs of captures, with both traps at a station closed were only 2–5% (i.e., only one station of the 20 experimental stations in each enclosure had both traps closed on a given trapping night), not of significant magnitude to bias results.

RESULTS

Experiment I: Volatile Cues from Conspecifics. For the 586 captures in this experiment, there were no significant differences in the numbers of mice captured with the various odor types present in the enclosures: $\chi^2 = 4.74$, $df = 7$; $P > 0.20$ (Table 2). We first analyzed the data with respect to the eight odor types used as attractants with expected captures based upon a 50:50 distribution between odor and control. There were no significant preferences for any of the eight test odors using volatile cues only (Table 2). With a contingency test, there also were no significant effects when considering captures in perimeter versus interior traps ($\chi^2 = 3.97$; $df = 7$; $P > 0.20$; Table 2). However, when capture data were analyzed using the numbers of trap stations (28 interior and 26 perimeter) to generate expected values, significantly more captures occurred in interior traps ($\chi^2 = 365.49$; $df = 7$; $P < 0.001$). In this test and subsequent tests of this type, we have assumed that because there were such low rates of double captures at those stations with two traps (see Assumptions), the use of all 28 interior stations as if they contained a single trap is justified and does not introduce any undue bias into the analyses. There was no significant effect (χ^2

TABLE 2. TOTAL CAPTURES, CAPTURES BY ODOR SOURCE TYPES, AND CAPTURES BY TRAP LOCATION WITH VOLATILE ODORS ONLY IN TRAPS—CONSPECIFIC ODORS (EXPERIMENT I)

Urine odor source	Total captures	Treatment		χ^2	P	Trap location	
		Odor	Control			Interior	Perimeter
Adult male	74	32	25	0.86	NS	67 (67.7)[a]	7 (6.3)
Juvenile male	63	25	26	0.02	NS	60 (57.6)	3 (5.4)
Nonestrous female	76	37	25	2.32	NS	67 (69.5)	9 (6.5)
Estrous female	74	35	26	1.33	NS	69 (67.7)	5 (6.3)
Grouped females	79	36	22	3.38	NS	71 (72.3)	8 (6.7)
Pregnant female	81	30	38	0.94	NS	76 (74.1)	5 (6.9)
Lactating female	62	30	23	0.92	NS	57 (56.7)	5 (5.3)
Juvenile female	77	23	34	2.12	NS	69 (70.4)	8 (6.6)

[a] Expected values in parentheses are from the chi-square contingency analyses—see Results.

= 0.01; $df = 1$; $P > 0.20$) involving age (young and adult) versus treatment (odor and control). There also was no significant effect ($\chi^2 = 0.98$; $df = 1$; $P > 0.20$) with sex versus treatment (odor and control).

We also analyzed the data according to the seven possible responder types—the different age and sex classes and reproductive conditions of mice that could enter the traps (Table 3). Because mice of different age and sex classes were present in the pens in differing numbers, we calculated expected values for total captures in each responder category by using available data regarding the composition of each enclosure population each week. For adult females, the four categories were combined because the reproductive condition of the female is not determined until capture and using that information to generate expected values would be circular. The mean numbers of each responder type that occurred in each of the enclosures during the eight weeks of this experiment were averaged to obtain a grand mean for all enclosures. These means were, in turn, used to generate expected values as proportions of the total number of captures. This same procedure was carried out for each of the other five experiments; when the duration of an experiment was only four weeks, we used a similar procedure, but computed the grand mean based on the enclosure means calculated for just those four weeks. Using expected values computed in this manner resulted in a significant difference in the proportional rate of capture for mice in the seven responder categories ($\chi^2 = 11.50$; $df = 6$; $P < 0.01$; Table 3). More adult females (all reproductive conditions combined) than expected were captured and fewer adult males, juvenile males, or juvenile females were caught than expected. Using expected values based on a 50:50 distribution between odor and control traps, only one of seven analyses, that for pregnant females, was significant; pregnant females preferred odorized traps over control traps (Table 3). For pregnant females, entry into odorized traps was not differentially distributed in favor of any particular odor type.

When data for responders were analyzed for location of capture using a contingency test, there was no significant effect ($\chi^2 = 5.82$; $df = 6$; $P > 0.20$; Table 3). When expected values were based on the numbers of interior vs. perimeter trap stations, significantly more mice were caught in interior than perimeter traps ($\chi^2 = 383.38$; $df = 6$; $P < 0.001$; Table 3).

Experiment II: Nonvolatile and Volatile Cues from Conspecifics. For the 687 captures in this experiment, there were no significant differences in the numbers of mice captured with the various odor types present in the enclosures: $\chi^2 = 8.34$; $df = 7$; $P > 0.20$ (Table 4). Again, we first analyzed the data with respect to the eight odor types. For seven of the eight odors there was a significant effect when both volatile and nonvolatile cues were available (Table 4); in each case more mice than expected were captured in odorized traps and fewer in control traps. With a contingency chi-square test, there was no significant differential capture of animals in the interior versus the perimeter with any of

TABLE 3. CAPTURES ACCORDING TO RESPONDER TYPES: TOTAL CAPTURES, ODOR VS. CONTROL, AND CAPTURES BY LOCATION WITH VOLATILE CUES ONLY IN TRAPS—CONSPECIFIC ODORS (EXPERIMENT I)

		Treatment				Trap location	
Responder type	Total	Odor	Control	χ^2	P	Interior	Perimeter
Adult male	129 (146)[a]	51	62	0.74	NS	122 (119.1)[b]	7 (9.9)
Juvenile male	42 (53)	21	14	1.40	NS	37 (38.8)	5 (3.2)
Nonestrous female	210 (325)	92	73	1.75	NS	190 (193.9)	20 (16.1)
Estrous female	90	35	41	0.47	NS	87 (83.1)	3 (6.9)
Pregnant female	51	25	10	6.43	<0.025	47 (47.1)	4 (3.9)
Lactating female	14	4	6	0.40	NS	13 (12.9)	1 (1.1)
Juvenile female	50 (62)	21	24	0.20	NS	45 (46.2)	5 (3.8)

[a] Proportionalized expected values (see text) in parentheses—single expected value for adult females includes all four categories of adult females.

[b] Expected values in parentheses are from the chi-square contingency analyses.

TABLE 4. TOTAL CAPTURES, CAPTURES BY ODOR SOURCE TYPES, AND CAPTURES BY TRAP LOCATION WITH NONOVOLATILE AND VOLATILE ODORS IN TRAPS—CONSPECIFIC ODORS (EXPERIMENT II)

Urine Odor Source	Total captures	Treatment		χ^2	P	Trap location	
		Odor	Control			Interior	Perimeter
Adult male	87	45	18	11.57	<0.001	72 (69.7)[a]	15 (17.3)
Juvenile male	84	39	21	5.40	<0.025	74 (67.3)	10 (16.3)
Nonestrous female	93	43	18	10.25	<0.005	75 (74.5)	18 (18.5)
Estrous female	70	32	13	8.02	<0.025	51 (56.0)	19 (14.0)
Grouped females	88	39	22	5.92	<0.025	74 (70.5)	14 (17.5)
Pregnant female	78	31	12	8.40	<0.001	60 (62.4)	18 (15.6)
Lactating female	77	34	14	8.33	<0.005	57 (61.6)	20 (15.4)
Juvenile female	84	28	22	0.72	NS	74 (67.3)	19 (17.9)

[a]Expected values in parentheses are from the chi-square contingency analyses—see Results.

the odor types as baits, $\chi^2 = 8.92$; $df = 7$; $P > 0.20$ (Table 4). However, using numbers of trap stations to generate expected values did produce a significant result; mice were captured more frequently than expected in the interior traps and less frequently in perimeter traps ($\chi^2 = 209.71$; $df = 7$; $P < 0.001$; Table 4). There was a significant interaction of age and treatment (Table 5; $\chi^2 = 4.82$; $df = 1$; $P < 0.01$); more adults and fewer young than expected were caught at odor-baited traps and fewer adults and more young than expected were caught in control traps. There was no significant effect considering sex vs. treatment (odor and control) ($\chi^2 = 0.49$; $df = 1$; $P > 0.20$).

When we analyzed overall captures according to the seven possible responder types using the method described in experiment I for generating proportional expected values, there were no significant differences in capture rates for the various categories ($\chi^2 = 7.04$; $df = 3$; $0.01 > P > 0.05$; Table 6). Using expected values based on a 50:50 capture rate for odorized and control traps, five of the seven responder types preferred odor-baited traps; only lactating females and juvenile females failed to exhibit significant preferences. Within each responder type, the binomial test (Siegel, 1956) was used to analyze the distribution of responses to the various odors used as baits. Adult male mice exhibited significant ($P < 0.05$) responses in favor of traps containing odors from adult males, pregnant females, and lactating females. Juvenile males were caught more than expected only in traps containing the odor of nonestrous females. Nonestrous females entered the odorized trap more than the control trap when the attractant odor was from juvenile males. Estrous females exhibited a significant choice of odorized traps with respect to both estrous female urine and urine from lactating females. Finally, pregnant females chose odorized traps over controls when the urine was from pregnant females. No other tests were significant.

Considering location of capture (perimeter vs. interior) for all seven responder types, there was no significant effect ($\chi^2 = 12.84$; $df = 6$; $0.05 <$

TABLE 5. INTERACTION OF AGE (YOUNG AND ADULT) VERSUS EXPERIMENTAL TREATMENT (ODOR AND CONTROL) WITH BOTH NONVOLATILE AND VOLATILE ODOR CUES PRESENT IN TRAPS (EXPERIMENT II)[a]

	Age	
Treatment	Young	Adult
Odor	119 (129.6)	172 (161.4)
Control	73 (62.4)	67 (77.6)

[a] Expected values from a chi-square contingency analysis are shown in parentheses.

$P < 0.10$; Table 7). When these same data were subjected to a chi-square analysis with the expected values generated based upon the numbers of trap stations, then significantly more mice were caught in the interior than on the perimeter, $\chi^2 = 186.15$; $df = 6$; $P < 0.001$ (Table 6).

Experiment III: Nonvolatile and Volatile Cues from Heterospecific Rodents and Human Urine. For the 315 captures in this experiment, there were significant differences in the numbers of house mice captured with the various odor types present in the enclosures ($\chi^2 = 11.43$; $df = 3$; $P < 0.001$; Table 7); fewer mice than expected were captured when *M. ochrogaster* odor was used and more than expected were captured when the homospecific odor was in the traps. For three of the four odor types there were significant effects with regard

TABLE 6. CAPTURES ACCORDING TO RESPONDER TYPES: TOTAL CAPTURES, ODOR VS. CONTROL, AND CAPTURES BY LOCATION WITH NONVOLATILE AND VOLATILE CUES IN TRAPS—CONSPECIFIC ODORS (EXPERIMENT II)

		Treatment				Trap location	
Responder type	Total	Odor	Control	χ^2	P	Interior	Perimeter
Adult male	178 (187)[a]	70	32	14.16	<0.001	133 (139.6)[b]	45 (38.4)
Juvenile male	135 (124)	67	39	7.40	<0.01	116 (105.9)	19 (29.1)
Nonestrous female	93 (203)	42	15	12.79	<0.001	73 (72.9)	20 (20.1)
Estrous female	69	30	11	8.80	<0.005	51 (54.1)	18 (14.9)
Pregnant female	33	17	4	8.05	<0.005	29 (25.9)	4 (7.1)
Lactating female	29	13	5	3.56	NS	26 (22.7)	3 (6.9)
Juvenile female	130 (153)	52	34	3.77	NS	95 (101.9)	35 (28.1)

[a] Proportionalized expected values (see text) in parentheses—single expected value for adult females includes all four categories of adult females.
[b] Expected values in parentheses are from the chi-square contingency analyses.

TABLE 7. TOTAL CAPTURES, CAPTURES BY ODOR SOURCE TYPES, AND CAPTURES BY TRAP LOCATION WITH VARIOUS SPECIES ODORS IN TRAPS (EXPERIMENT III)

		Treatment				Trap location	
Urine odor source	Total captures	Odor	Control	χ^2	P	Interior	Perimeter
Microtus ochrogaster	59	19	19	0.00	NS	45 (30.7)[a]	14 (28.3)
Homo sapiens	80	11	26	6.08	<0.025	47 (41.6)	33 (38.4)
Peromyscus leucopus	75	12	26	5.16	<0.025	55 (39.0)	20 (36.0)
Mus musculus	101	49	18	14.34	<0.001	80 (52.5)	21 (48.5)

[a] Values in parentheses are from the chi-square contingency analyses—see Results.

to the odor treatment (Table 7). Fewer captures than expected occurred in the odorized traps when either *H. sapiens* or *P. leucopus* odor was used as bait and more than expected were captured when *M. musculus* odor was used to odorize the traps. There was no significant effect for urine from *M. ochrogaster*.

For capture locations, testing interior versus perimeter, both the contingency test ($\chi^2 = 10.21$; $df = 3$; $P < 0.01$) and the test using numbers of trap stations to generate expected values ($\chi^2 = 59.01$; $df = 3$; $P < 0.001$) were significant (Table 7). A greater number of mice were captured at perimeter traps when *H. sapiens* odor was used as the bait than with the other three bait-odor treatments. For the *M. musculus*, *M. ochrogaster*, and *P. leucopus* odors, the general pattern seen in early tests—more mice caught in the interior traps—prevailed.

There was no significant effect ($\chi^2 = 3.27$; $df = 1$; $0.05 < P < 0.10$) when the data were analyzed with a contingency table involving age (young and adult) versus treatment (odor and control). A contingency test also revealed no significant effect ($\chi^2 = 0.05$; $df = 1$; $P > 0.20$) for sex (male and female) versus treatment (odor and control).

With respect to the seven possible responder types, there were no significant differences in the overall capture rates ($\chi^2 = 6.30$; $df = 3$; $P > 0.10$; Table 8). Where there were sufficient data to conduct tests (Table 8), there were no significant odor versus control effects for any responder types.

When we considered bait-odor type and analyzed capture locations in terms of interior versus perimeter traps, both the contingency test ($\chi^2 = 17.62$; $df = 3$; $P < 0.01$) and the test using numbers of trap stations to generate expected

TABLE 8. CAPTURES ACCORDING TO RESPONDER TYPES: TOTAL CAPTURES, ODOR VS. CONTROL, AND CAPTURES BY LOCATION WITH VARIOUS SPECIES ODORS IN TRAPS (EXPERIMENT III)

		Treatment				Trap location	
Responder type	Total	Odor	Control	χ^2	P	Interior	Perimeter
Adult male	87 (77)[a]	18	15	0.27	NS	48 (61.3)[b]	39 (25.7)
Juvenile male	54 (44)	13	18	0.81	NS	37 (38.1)	17 (15.9)
Nonestrous female	67 (131)	23	23	0.00	NS	54 (47.2)	13 (19.8)
Estrous female	13	4	3			9 (9.2)	4 (3.8)
Pregnant female	11	4	4			8 (7.8)	3 (3.2)
Lactating female	24	11	5	2.50	NS	21 (16.9)	3 (7.1)
Juvenile female	56 (63)	14	23	2.19	NS	43 (39.5)	13 (16.5)

[a] Proportionalized expected values (see text) in parentheses—single expected value for adult females includes all four categories of adult females.

[b] Expected values in parentheses are from the chi-square contingency analyses.

values ($\chi^2 = 54.58$; $df = 3$; $P < 0.001$) were significant (Table 8). The contingency test indicated that adult males were trapped more often than expected at the perimeter, whereas nonestrous females and lactating females were trapped more often than expected at interior stations; the other responder types did not differ significantly from expected values. Basing the expected values on the numbers of trap stations, all of the responder types except adult males were caught more often than expected at the interior stations; adult males were caught about equally at both locations (Table 8).

Experiment IV: Volatile Cues from Potential Predators and Human Sweat. For the 423 captures in this experiment, there were no significant differences with respect to the total numbers of mice captured with each of the four types of odor sources in the enclosures ($\chi^2 = 2.98$; $df = 3$; $P > 0.20$). When we analyzed the data with respect to odor versus control capture frequencies within each odor-bait type, using a prediction of equal rates of capture at the two types of traps, there were significant differences for all but the dog odor (Table 9). A contingency test ($\chi^2 = 9.25$; $df = 3$; $P < 0.005$) revealed significant differential captures at the perimeter versus the interior traps (Table 9). More mice than expected were caught at the interior traps when the bait odor was from a dog and fewer than expected were caught at interior stations when the bait odor was from shrews. Using the numbers of trap stations to generate the expected values for analyzing interior versus perimeter stations, we found that for human sweat, cat fecal odor, and shrew fecal odor, there were significantly more mice caught on the perimeter than expected ($\chi^2 = 48.63$; $df = 3$; $P < 0.001$; Table 9); only for the dog fecal odor was there no significant deviation from the expected pattern. There were no significant differences when the data were analyzed for age (young and adult) versus treatment (odor and control) ($\chi^2 = 1.62$; $df = 1$; $P > 0.20$) or when the data were analyzed for sex (male and female) versus treatment (odor and control) ($\chi^2 = 2.35$; $df = 1$; $P > 0.20$).

TABLE 9. TOTAL CAPTURES, CAPTURES BY ODOR SOURCE TYPES, AND CAPTURES BY TRAP LOCATION WITH VOLATILE ODOR CUES ONLY FROM POTENTIAL PREDATORS IN TRAPS (EXPERIMENT IV)

Odor source	Total captures	Treatment		χ^2	P	Trap location	
		Odor	Control			Interior	Perimeter
Dog (*Canis familiaris*)	109	18	29	2.57	NS	52 (39.9)[a]	57 (69.1)
Human sweat (*Homo sapiens*)	106	5	23	11.57	<0.001	37 (33.8)	69 (67.2)
Cat (*Felis musculus*)	114	9	29	10.53	<0.005	40 (41.8)	74 (72.2)
Shrew (*Blarina brevicauda*)	94	3	19	11.64	<0.001	26 (34.4)	68 (59.6)

[a] Expected values in parentheses are from the chi-square contingency analyses—see Results.

When we analyzed the data according to the possible responder types, there were significant differences from the proportionalized expected values ($\chi^2 = 16.60$; $df = 3$; $P < 0.001$; Table 10). Fewer females and more adult males and juvenile males and females were caught than expected. For four of the seven responder types there were significant deviations from a 50:50 expected ratio between control and odorized traps (Table 10).

When the data for capture location were analyzed with a contingency test, no significant deviations from the expected values were recorded ($\chi^2 = 0.61$; $df = 6$; $P > 0.20$). However, when the same data were examined using the numbers of interior and perimeter trap stations to generate the expected values, the test was significant ($\chi^2 = 40.37$; $df = 6$; $P < 0.001$; Table 10). Many more mice of each responder type were caught at perimeter stations than expected.

Experiment V: Volatile Cues from Heterospecifics and Human Urine. For the 429 captures in this experiment, there were no significant differences in the numbers of house mice captured with the various odor types present in the enclosures ($\chi^2 = 2.10$; $df = 3$; $P > 0.20$; Table 11). When we analyzed the data with respect to the odor versus control capture frequencies within each odor-bait type, using a prediction of equal rates of capture at the two types of traps, there were significant differences for all but urine from *Microtus ochrogaster* (Table 11). A contingency test ($\chi^2 = 49.39$; $df = 3$; $P < 0.001$) revealed significant differential captures at perimeter versus interior traps (Table 11). More mice than expected were caught at the interior traps when the bait odor was from voles and fewer than expected were caught at interior stations when

TABLE 10. CAPTURES ACCORDING TO RESPONDER TYPES: TOTAL CAPTURES, ODOR VS. CONTROL, AND CAPTURES BY LOCATION WITH VOLATILE ODOR CUES ONLY FROM POTENTIAL PREDATORS IN TRAPS (EXPERIMENT IV)

		Treatment				Trap location	
Responder type	Total	Odor	Control	χ^2	P	Interior	Perimeter
Adult male	98 (108)[a]	7	22	7.76	<0.001	37 (35.9)[b]	61 (62.1)
Juvenile male	49 (53)	5	14	4.26	<0.05	18 (18.0)	31 (31.0)
Nonestrous female	129 (202)	10	18	2.29	NS	49 (47.3)	80 (81.7)
Estrous female	35	4	11	3.27	NS	13 (12.8)	22 (22.2)
Pregnant female	29	2	11	6.23	<0.025	10 (10.6)	19 (18.4)
Lactating female	37	3	10	3.77	NS	12 (13.6)	25 (23.4)
Juvenile female	46 (70)	4	14	5.56	<0.025	16 (16.9)	30 (29.1)

[a] Proportionalized expected values (see text) in parentheses—single expected value for adult females includes all four categories of adult females.

[b] Expected values in parentheses are from the chi-square contingency analyses.

TABLE 11. TOTAL CAPTURES, CAPTURES BY ODOR SOURCE TYPES, AND CAPTURES BY TRAP LOCATION WITH VOLATILE CUES ONLY FROM VARIOUS SPECIES IN TRAPS (EXPERIMENT V)

Urine odor source	Total captures	Treatment		χ^2	P	Trap location	
		Odor	Control			Interior	Perimeter
Microtus ochrogaster	104	35	33	0.06	NS	89 (75.4)[a]	15 (28.6)
Homo sapiens	117	25	55	11.25	<0.001	57 (84.8)	60 (32.2)
Peromyscus leucopus	111	33	51	3.86	<0.05	83 (80.5)	28 (30.5)
Mus musculus	97	42	20	7.81	<0.01	82 (70.3)	15 (26.7)

[a]Expected values in parentheses are from the chi-square contingency analyses—see Results.

the bait odor was human urine. Using the numbers of trap stations to generate the expected values for analyzing interior versus perimeter stations, we found a significant effect ($\chi^2 = 114.33$; $df = 3$; $P < 0.001$; Table 11); more mice than expected were caught at interior traps for *Microtus*, *Peromyscus*, and *Mus* urine, and capture frequencies were close to expected values when human urine was the bait-odor source in the enclosure. There were no significant differences when the data were analyzed for age (young and adult) versus treatment (odor and control) ($\chi^2 = 2.43$; $df = 1$; $P > 0.20$) or when the data were analyzed for sex (male and female) versus treatment (odor and control) ($\chi^2 = 0.95$; $df = 1$; $P > 0.20$).

When we analyzed the data according to the possible responder types, there were no significant differences from the proportionalized expected values ($\chi^2 = 6.01$; $df = 3$; $P > 0.10$; Table 12). There were no significant deviations from a 50:50 expected ratio between control and odorized traps for any of the responder types (Table 12).

When the data for capture location were analyzed with a contingency test, no significant deviations from expected values were recorded ($\chi^2 = 1.16$; $df = 6$; $P > 0.20$; Table 12). However, when the same data were examined using the numbers of interior and perimeter trap stations to generate the expected values, the test was significant ($\chi^2 = 73.10$; $df = 6$; $P < 0.001$; Table 12). More mice of each responder type were caught at interior stations than expected.

Experiment VI: Nonvolatile and Volatile Cues from Potential Predators and Human Sweat. For the 169 captures in this experiment, there were significant differences in the numbers of house mice captured with the various odor types present in the enclosures ($\chi^2 = 15.18$; $df = 3$; $P > 0.001$; Table 13). More mice were captured when the bait odor was from dogs and fewer than expected were captured overall when the bait odor was from cats. When we analyzed the data with respect to the odor versus control capture frequencies

TABLE 12. CAPTURES ACCORDING TO RESPONDER TYPES: TOTAL CAPTURES, ODOR VS. CONTROL, AND CAPTURES BY LOCATION WITH VOLATILE ODOR CUES ONLY FROM VARIOUS SPECIES IN TRAPS (EXPERIMENT V)

Responder type	Total	Treatment		χ^2	P	Trap location	
		Odor	Control			Interior	Perimeter
Adult male	116 (106)[a]	29	44	3.08	NS	84 (84.1)[b]	32 (31.9)
Juvenile male	61 (50)	22	24	0.09	NS	43 (44.2)	18 (16.8)
Nonestrous female	103 (191)	40	30	1.43	NS	74 (74.7)	29 (28.3)
Estrous female	26	10	7	0.53	NS	18 (18.8)	8 (7.2)
Pregnant female	22	6	9	0.60	NS	17 (15.9)	5 (6.1)
Lactating female	33	10	14	0.67	NS	23 (23.9)	10 (9.1)
Juvenile female	68 (82)	18	31	3.45	NS	52 (49.3)	16 (18.7)

[a] Proportionalized expected values (see text) in parentheses—single expected value for adult females includes all four categories of adult females.
[b] Expected values in parentheses are from the chi-square contingency analyses.

TABLE 13. TOTAL CAPTURES, CAPTURES BY ODOR SOURCE TYPES, AND CAPTURES BY TRAP LOCATION WITH VOLATILE AND NONVOLATILE ODORS FROM POTENTIAL PREDATORS IN TRAPS (EXPERIMENT VI)

Urine odor source	Total captures	Treatment		χ^2	P	Trap location	
		Odor	Control			Interior	Perimeter
Dog (*Canis familiaris*)	57	13	14	0.04	NS	28 (33.1)[a]	29 (23.9)
Human sweat (*Homo sapiens*)	41	9	19	3.57	NS	27 (23.8)	14 (17.2)
Cat (*Felis musculus*)	23	1	9	6.40	<0.01	7 (13.3)	16 (19.7)
Shrew (*Blarina brevicauda*)	48	12	18	1.20	NS	36 (27.8)	12 (20.2)

[a] Expected values in parentheses are from the chi-square contingency analyses—see Results.

within each odor-bait type, using a prediction of equal rates of capture at the two types of traps, there were significant differences for human sweat and cat; in both instances fewer than expected captures occurred at the traps with odor baits (Table 13). A contingency test ($\chi^2 = 15.73$; $df = 3$; $P < 0.001$) revealed significant differential captures at perimeter versus interior traps (Table 13). More mice than expected were caught at the interior traps when the bait odor was from human sweat or shrews and fewer than expected were caught at interior stations when the bait odor was from dogs or cats. Using the numbers of trap stations to generate expected values for analyzing interior versus perimeter stations, we found a significant effect ($\chi^2 = 17.80$; $df = 3$; $P < 0.001$; Table

TABLE 14. CAPTURES ACCORDING TO RESPONDER TYPES: TOTAL CAPTURES, ODOR VS. CONTROL, AND CAPTURES BY LOCATION WITH VOLATILE AND NONVOLATILE ODORS FROM POTENTIAL PREDATORS IN TRAPS (EXPERIMENT VI)

		Treatment				Trap location	
Responder type	Total	Odor	Control	χ^2	P	Interior	Perimeter
Adult male	39 (39)[a]	7	32	16.02	<0.001	24 (22.6)[b]	15 (16.4)
Juvenile male	30 (20)	9	21	4.80	<0.05	17 (17.4)	13 (12.6)
Nonestrous female	41 (81)	7	34	17.78	<0.001	24 923.8)	17 (17.2)
Estrous female	16	3	13	6.25	<0.025	10 (9.3)	6 (6.7)
Pregnant female	11	2	9	4.45	<0.05	5 (6.4)	6 (4.6)
Lactating female	14	3	11	4.57	<0.05	7 (8.2)	7 (5.8)
Juvenile female	18 (29)	4	14	5.56	<0.025	11 (10.4)	7 (7.6)

[a] Proportionalized expected values (see text) in parentheses—single expected value for adult females includes all four categories of adult females.
[b] Expected values in parentheses are from the chi-square contingency analyses.

13); more mice than expected were caught at interior traps for human sweat and shrew; fewer than expected were caught at interior traps with cat odor, and capture frequencies were close to expected values when dog was the bait-odor source in the enclosure. There were no significant differences when the data were analyzed for age (young and adult) versus treatment (odor and control) ($\chi^2 = 1.63$; $df = 1$; $P > 0.20$) or when the data were analyzed for sex (male and female) versus treatment (odor and control) ($\chi^2 = 3.18$; $df = 1$; $0.05 < P < 0.10$).

When we analyzed the data according to the possible responder types, there were significant differences from the proportionalized expected values ($\chi^2 = 9.18$; $df = 3$; $P < 0.05$; Table 14). Adult males and females were captured at about the expected rates, but juvenile males were caught more often than expected and juvenile females less often. There were significant deviations from a 50:50 expected ratio between control and odorized traps for all of the responder types (Table 12).

When the data for capture location were analyzed with a contingency test, no significant deviations from expected values were recorded ($\chi^2 = 1.61$; $df = 6$; $P > 0.20$; Table 14). When these data were examined using the numbers of interior and perimeter trap stations to generate the expected values, the test also was significant ($\chi^2 = 73.10$; $df = 6$; $P < 0.001$; Table 14).

DISCUSSION

The foregoing experiments support several general conclusions: (1) House mice respond to conspecific odor cues when both volatile and nonvolatile odors are present, but not when only volatile cues are available. (2) There is some

discrimination of specific age and sex classes and of reproductive condition based on odor cues, particularly by adult mice. (3) House mice reacted differently to heterospecific odors from deer mice and prairie voles, although, when given the opportunity, they displayed a strong preference toward their own species odor. (4) House mice avoided odors from cat and shrew feces, but not dog feces. (5) House mice avoided odors from human urine, but not human sweat. Because of the large number of statistical tests that we conducted and in order to avoid possible type I errors, we have confined our conclusions to the general and consistent patterns observed within and across the six experiments.

Conspecific Odors. The lack of any recorded preferences in experiment I, followed by the consistent preferences exhibited in experiment II suggests strongly that the mice can distinguish conspecific odors, but not based on volatile odors. They apparently must be able to obtain direct contact with the odor substance in order to make discriminations and exhibit preferences. This finding suggests involvement of the vomeronasal organ and is in agreement with earlier reports concerning vomeronasal reception of many chemosignals that affect house mouse social and reproductive biology (Keverne, 1979; Johns, 1980; Drickamer and Assmann, 1981; Lepri et al., 1985).

The present finding of a general preference for conspecific odor over control traps without odor is reminiscent of the earlier work on voles by Boonstra and Krebs (1976), in which traps that had become soiled by previous captures were more likely to catch additional animals than clean traps. We conclude that house mice, like other rodents tested (*Peromyscus*, Mazdzer et al., 1976; Daly et al., 1980; and *Microtus*, Heske, 1987), will make use of urinary odor cues placed in live traps.

How specific are these odor cue preferences and how much detailed information are the mice obtaining from the urine? In the second experiment, adult mice were more likely to exhibit specific preferences than young mice. In general, we did not detect any particular patterns with respect to particular odor cue preferences by specific responder types. Three responder types exhibited significant preferences for urine of their own age or sex class and reproductive condition: adult males, estrous females, and pregnant females. This suggests the ability for specific recognition of a mouse's own condition, possibly by some sort of matching mechanism, but additional information would be needed to confirm this. It is also not clear why mice should differentially prefer urine from their own age or sex class and reproductive condition. Juvenile males and nonestrous females exhibited reciprocal preferences for the opposite urine odor. Again, there is no clear rationale concerning why this should occur. Adult males also preferred the urine odor of pregnant and lactating females, but, interestingly, they were not attracted to estrous or nonestrous females. If the odor-baiting technique proves useful, then, in conjunction with other techniques, e.g., DNA fingerprinting, a number of additional questions can be tested involving members of the same deme, neighbors and strangers, kin recognition, and mate finding.

In the first two experiments, significantly more mice were trapped at stations in the interior of the enclosures than on the perimeter. We attribute this result to the presence of the 1.5-m mowed strip of grass around the inside walls of each enclosure; these strips were removed approximately once each month. If mice had been trapped more often than expected along the walls, this would be good evidence for the effect of whatever urine odor was used to bait traps in the interior having a repelling effect on the mice.

Heterospecific Odors. With respect to interspecific interactions, we recorded more house mouse captures at odor-baited traps with conspecific urine than for any of the other treatments. This result is not surprising in light of the findings from the first two experiments. However, house mice avoided urine from *P. leucopus*. For deer mice, the effect was only one of avoidance; mice entered control traps more often than odor-baited traps. There was no effect on overall activity as measured by the captures in the interior versus those on the perimeter of the enclosures. This result is in accord with two previous field studies on interspecific interactions involving house mice and deer mice (Caldwell, 1964; Caldwell and Gentry, 1965). *P. polionotus* were generally able to outcompete house mice in an old field setting and in a one-acre enclosure. In contrast, King (1957) reported that house mice were more aggressive toward and dominant over *P. maniculatus*. To our knowledge, no one has yet studied the direct interactions of house mice and *P. leucopus* in a field setting. One of us (L.C.D.) has unpublished laboratory data in which *P. leucopus* were dominant to *M. musculus*. Those laboratory data and earlier field studies would suggest a prediction that house mice would avoid *P. leucopus* odors in the enclosures; this is what we observed.

In the third experiment, the house mice were caught less frequently when *M. ochrogaster* odor was present as a bait. We do not know exactly to what to attribute this finding, particularly since there was no effect on the location of mouse captures with regard to the interior versus perimeter trap sites in the enclosures with *M. ochrogaster* urine odor as bait. The picture is further complicated by the fact that no preference was exhibited between odor-baited and control traps for *M. ochrogaster* urine. Based upon previous work involving *M. musculus* and *Microtus* spp. (Lidicker, 1966; DeLong, 1966; Quadagno, 1968), we might have expected a clearer reaction from the house mice. In the previous studies of interactions between *Microtus* and *Mus*, voles generally displaced house mice. Further work will be necessary to more completely delineate the effects of urine odors from *M. ochrogaster* on house mouse behavior.

Predator Odors. Although there were some differences based on whether volatile only or volatile and nonvolatile cues were available, house mice avoided cat fecal odors and, to a lesser degree, shrew fecal odors. Cat odor and shrew odor also resulted in some shift in the activity of the mice; more were captured near the perimeter of the pens when these odors were presented in the traps in

the interior. These data on cats and particularly on shrews indicate, in a preliminary way, that house mice, like voles (Eadie, 1952; Barbehenn, 1958; Fulk, 1972) respond to odors from *Blarina* by avoiding them, both in terms of immediate choices of where to go, measured by negative responses to odor-baited traps, and in terms of general use of space, measured by their shift to the perimeter of the pens. In none of these tests did the mice avoid the odor of dog feces. We do not have a sound explanation for this apparent deviation from what we would have predicted based on the logic that dogs (canids), like cats (felids), represent potential predatory threats for the mice.

Human Odors. With human urine, there was both an avoidance of odor-baited traps relative to control traps and a general effect on activity. With human urine, the mice were caught more often on the perimeter than expected, suggesting that they shifted a significant proportion of their daily activity to the perimeter of the enclosure, even with the presence of the 1.5-m strip of mowed grass. The mice exhibited no differential response to human sweat odors, either in terms of their frequency of capture at odor- versus control-baited traps or with regard to interior versus perimeter trap stations. It is puzzling why house mice should avoid one odor source and not the other. House mice have lived in the presence of *H. sapiens* for many thousands of years and thus should be accustomed to any of the variety of odors from humans. It is also not clear that humans represent a threat of predation to house mice, yet something of this sort must be the source of the observed pattern of activity. It is possible that the mice are habituated to the odor of human sweat and thus failed to react to it in the same manner that they reacted to human urine. This habituation may come about because we leave sweat odors on virtually all traps as we work with them in the summer heat in southern Illinois. Hence, the mice have regularly encountered the sweat-odor cues, but not those from human urine.

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ODORS AS CUES FOR ORIENTATION TO MOTHERS BY WEANLING VIRGINIA OPOSSUMS

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Abstract—Three experiments were conducted to investigate whether whole-body and pouch odors facilitate social cohesion between young Virginia opossums and their mothers just prior to weaning. In experiment 1, young oriented toward plastic buckets containing their mothers, directing significantly higher levels of investigative behavior and more distress vocalizations toward them than toward buckets containing unrelated lactating females. In experiment 2, young oriented toward and investigated empty buckets containing whole-body odors of their mothers more than empty buckets containing odors of other females. Similarly, more investigative behavior was directed toward plastic bucket lids containing pouch odors from subjects' mothers than toward pouch odors from unrelated females in experiment 3. These results suggest that social odors help young didelphid marsupials maintain contact with their mothers, as in other mammals, and that whole-body and pouch gland odors are important chemical signals in this nongregarious species.

Key Words—*Didelphis virginiana*, Didephidae, maternal–young recognition, maternal odors, pouch glands, Virginia opossum.

INTRODUCTION

The importance of odors in facilitating maternal–young recognition and the orientation of dependent offspring to mothers has been established for a wide range of mammalian species (for reviews, see Stoddart, 1980; Brown and Macdonald, 1985). Scent glands are common in the New World marsupial family

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Didelphidae, as in other mammalian groups, and olfaction has been suggested to function as a primary modality for social communication in this group (McManus, 1970; Gardner, 1982). While there is a growing body of evidence of the importance of scent gland secretions and other chemical signals in didelphid reproduction and behavior (Boggs, 1969; Fadem, 1989; Fadem and Cole, 1985; Fadem and Schwartz, 1986; Holmes, 1987, 1990, 1992; Holmes Meisner, 1986), the role of olfactory cues in opossum social systems is generally not well understood. This is particularly true for odors that may facilitate social cohesion, including the maintenance of contact between mothers and offspring.

Virginia opossums are born after 12–13 days gestation and complete their postnatal development in the mother's pouch, where they remain firmly attached to nipples for 45–50 days (Reynolds, 1952). They are carried in the pouch until about day 80, after which the mother either leaves them in the den or carries them on her back while foraging (Eisenberg, 1975; Gillette, 1980; Reynolds, 1952; Seidensticker et al., 1987; Wright, 1989). During the period between detachment from the nipples and complete weaning, mothers and young use vocalizations to maintain social contact when they become separated: mothers emit a click, which sounds like a lip smack, and young produce a birdlike distress call (McManus, 1970; Reynolds, 1952). Mothers often respond to vocalizations of their offspring by approaching and allowing them to climb onto the body or into the pouch.

In the period between parturition and weaning, the skin of the maternal pouch produces a brownish orange, musky exudate, suggesting the development of specialized apocrine or sebaceous scent glands (Reynolds, 1952; and personal observation). By the time young have their eyes open and begin to move around the nest on their own, these secretions have become copious enough to coat their fur and discolor it (personal observation). Although odorous pouch secretions have been described briefly in other marsupials (Russell, 1984, 1985), and olfactory cues have been suggested to aid marsupial neonates in their climb to the pouch at birth (Hartman, 1928; Sharman and Calaby, 1964), the possible role of pouch gland scents in mediating interactions between *Didelphis* mothers and young in later development has never been investigated experimentally.

Young opossums begin to show an interest in solid food after about day 85 but are not completely weaned until between 96 and 108 days (Reynolds, 1952; Seidensticker, 1987; Wright, 1989). Although captive young of about 85 days of age may wander away from their mothers for brief periods, they often show signs of distress, including vocalizations, if separated long enough to become hungry (personal observation). In the wild, some young may continue denning with the mother or littermates for up to 120 days, after which they become independent and largely solitary (Wright, 1989; personal observation).

To explore the potential role of maternal odors in maintaining contact between young opossums approaching weaning and their mothers, I conducted

a series of pairwise choice experiments. The first was designated to determine if young oriented to restrained mothers when mothers were presented in combination with restrained unrelated lactating females in the absence of any physical solicitation of contact on the part of "stimulus" females. In the second, I tested whether young oriented toward whole-body odors of mothers when they were presented in combination with those of unrelated lactating females. The third experiment was performed to determine whether mothers' pouch odors alone elicited more orientation and investigative behavior by young than did pouch odors of unrelated females.

METHODS AND MATERIALS

Subjects and Test Setting. Experiments were conducted over a three-day period in early September, after the end of the 1991 breeding season (opossums breed from January to August), during the animals' most active nocturnal period (dusk to 2400 hr EST) (Holmes, 1991; McManus, 1971). The test arena consisted of a clean galvanized metal cattle watering tank (1.52 m diameter and 0.6 m deep), with a 3-cm-deep layer of clean pine shavings on the bottom. Testing took place in a quiet, well-ventilated room under diffuse fluorescent light.

Subjects were 17 young (10 females, 7 males) between 85 and 95 days of age from three litters (one of five, one of four, and one of eight young). Their ages had been determined earlier as pouch young (between 30 and 50 days of age) to an accuracy of one week using standard developmental landmarks for this species (Gardner, 1982; Hartman, 1923; Reynolds, 1952). The mothers of these three litters were used as stimulus females and odor donors; all were unrelated and had been live-trapped during their first breeding year in South Carolina or Georgia. All mothers had been captive for at least two months and housed individually with their litters in adjacent outdoor kennels. Olfactory and social contact were possible between animals in adjacent kennels. Subjects and stimulus females were accustomed to the presence of humans, but had not been handled except as required for routine care. Diet consisted of dry dog food supplemented with vitamin E, liver, eggs, vegetables, and fruit, and water was supplied ad libitum.

All 17 subjects and all three stimulus females were used in each of the three experiments. Young were removed from their mothers' nestboxes 9–10 hr before each test session, placed in clean 1.89-liter plastic buckets with their littermates, and deprived of food and water until testing was completed. Before beginning a test session, all subjects were combined in a single bucket and allowed to acclimate for 20 min. Subjects were tested in random order by selecting them "blindly" from this communal bucket (all were eartagged for

individual identification, but could not be identified except by very close inspection of tags). To further reduce the potential for observer bias, I performed all observations myself, but had another person code the labels for stimulus females and odors and select appropriate coded, paired combinations of stimulus females or odors for each test session. I did not decode these until testing was finished for each night.

Experiment 1. The three stimulus females were removed from their nestboxes several hours before testing and placed gently in separate clean 1.89-liter plastic buckets with lids, with 1-cm holes drilled at 3-cm intervals into both buckets and lids. A coded, preassigned pair of stimulus females—the subject's mother and one of the two unrelated lactating females—were placed 1 m apart in the test arena in their respective buckets. Each trial consisted of a 5-min test period that began when the subject was placed in the center of the test arena between the two stimulus buckets.

Young had ample space to move around the perimeter of the test arena and ignore both stimulus buckets, or to approach and actively investigate either at will. Only behaviors performed directly on or in the immediate vicinity of one of the buckets was recorded. This protocol made it very unlikely that avoidance of one stimulus object could be confused with attraction to the other. Responses to stimulus objects in each 5-min trial were quantified using a "one-zero" score (Bernstein, 1991; Rhine and Flanigan, 1978), consisting of the number of 5-sec intervals during the test period in which the subject performed a specified orientation or investigative behavior at least once. In experiment 1, behavior patterns that were scored included approaching within 5 cm of the stimulus bucket and sniffing, biting, licking, or otherwise contacting it. I scored vocalizations emitted by the young in the vicinity of a bucket in the same way.

When alarmed, adult opossums exude a distinctive smelly secretion from their anal glands (Gardner, 1982), therefore I handled stimulus females as little as possible and did not remove them or clean stimulus buckets between trials in experiments 1 or 2. However, the unrelated female used for each subject, as well as the position of the two stimulus females in the test arena in each trial, was alternated in a random fashion among tests for each evening. The pine shavings lining the floor of the tank were stirred up thoroughly between trials, thereby randomly redistributing any olfactory cues left on them by the subjects.

Experiment 2. The protocol outlined above was repeated in experiments 2 and 3, but each of the latter tests involved an alteration of the two stimulus objects used. In experiment 2, I used empty buckets that had been occupied for 4 hr by one of the three stimulus females and still contained any odors they had deposited during this time (probably including urine, saliva, vaginal and pouch secretions, as well as other body odors). Buckets were placed in the test arena on their sides without lids (rather than upright, as in experiment 1), and subjects were free to climb on or into either bucket. The behavior patterns scored in

experiment 1 were expanded to include these. As in experiment 1, buckets were not cleaned between trials in order to preserve the odors they contained, but their positions were randomized among trials.

Since the possible confounding influence of subjects' investigation of stimulus buckets on responses of subsequent subjects was not readily controlled, I addressed this possible source of error statistically (see Analysis section below).

Experiment 3. Pouch odors were collected from each of the three stimulus females by thoroughly swabbing the inside of the empty pouch with clean, dry tissues, which were then sealed in clean plastic bags until testing (approximately 4 hr). I attempted to collect only pouch secretions, to avoid picking up saliva, vaginal or anal gland secretions, and to alarm females as little as possible during this procedure. Secretions collected from pouches in this manner had a distinct odor, but I could not detect them visually. Immediately before testing, each of the tissues carrying a pouch odor was unfolded and sandwiched between two plastic bucket lids (30 cm in diameter with 30 2-cm holes drilled in each) stacked on top of one another. Lids were cleaned thoroughly with 70% alcohol before loading them with odors and between trials. I wore disposable plastic gloves when handling all odors and stimulus objects. and took care not to mix odors from different females. During testing, lids containing odors collected from the appropriate females were placed flat in the test arena in the same positions as were the buckets used in the first two experiments. The same observational protocol was used as in experiments 1 and 2, but behavior patterns scored were expanded to include walking on the lids as an additional form of orientation or investigative behavior.

Analysis. One-tailed Wilcoxon paired-samples tests (median tests) (Zar, 1984) were performed on data from each of the three experiments to test the hypothesis that subjects would orient or display higher levels of investigative behavior toward odors of their mothers than toward those of unrelated females. For experiments 1 and 2, the variance in each subject's response attributable to investigation by previous subjects was examined by regressing the differences between investigation scores directed toward the two buckets in each trial on the cumulative differences between responses to those buckets by subjects run previously (simple linear regression) (Zar, 1984).

RESULTS

All subjects thoroughly explored the entire test arena, and they approached a stimulus object in the first 20 sec in 42 (82%) of the 51 trials. In experiment 1, young directed higher levels of investigative behavior toward the bucket containing their mothers in 15 of 17 trials; one failed to investigate either stimulus bucket and was excluded from the analysis. Median scores for behavior

(i.e., number of 5-sec intervals during the test period in which one or more of the specified behaviors occurred) directed by the remaining 16 subjects toward mothers and unrelated lactating females were 32.0 and 3.0, respectively, and were significantly different ($T_{(\text{one-tailed})} = 5.5$, $N = 16$, $P < 0.0001$); means and standard deviations are shown in Figure 1. Vocalizations were directed toward the bucket containing the mother by six of 17 young and never toward the bucket containing the unrelated female. Medians for vocalizations toward mothers and nonmothers were 2.5 and 0, respectively, and were also significantly different ($T_{(\text{one-tailed})} = 0$, $N = 6$, $P = 0.025$). Stimulus females emitted no vocalizations that were audible to me during testing.

In experiment 2, 15 of 17 subjects oriented and directed more investigative behavior toward whole-body odors of mothers than those of unrelated females. Median scores were 12.0 and 6.0, respectively, for maternal and nonmaternal odors and were significantly different ($T_{(\text{one-tailed})} = 6$, $N = 16$, $P < 0.0005$; means in Figure 1). One subject failed to investigate either bucket. Similarly, in experiment 3, young investigated objects containing pouch odors alone from mothers significantly more than those containing odors from unrelated females. Of the 17 subjects, 12 investigated lids containing mothers' pouch odors more than those containing the odor of an unrelated female; medians were 11.0 and 9.0, respectively ($T_{(\text{one-tailed})} = 36.5$, $N = 17$, $P < 0.05$; means in Figure 1).

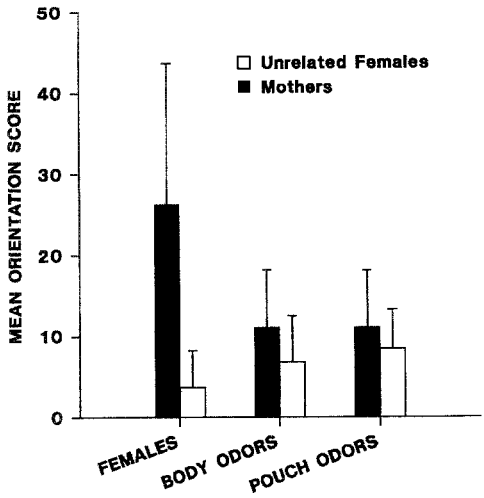

FIG 1. Responses of weanling opossums to odors of mothers and unrelated lactating females. Orientation scores consist of the number of 5-sec intervals in the 5-min test period (one-zero score) in which subjects engaged in specified investigative behaviors on or in the immediate vicinity of the stimulus object. Bars represent standard deviations. See text for details of experimental methods.

Neither young nor adult females vocalized audibly in experiments 2 or 3. The variance in investigative behavior explained (i.e., potentially confounded) by previous subjects' investigation of each bucket was nonsignificant in both experiments ($R^2 = 0.21$, $P = 0.08$ and $R^2 = 0.006$, $P = 0.78$ for experiments 1 and 2, respectively). This indicates that subjects were responding primarily to odors of stimulus females and not those deposited by subjects tested previously.

DISCUSSION

Weanling opossums consistently oriented toward and investigated odors of their mothers when odors were presented with those of unrelated lactating females. Subjects actively investigated their own mothers' odors in preference to those of strange females, rather than demonstrating an avoidance of unrelated females. This study did not employ odor "blanks" (clean, empty stimulus buckets or lids), hence I cannot conclude that young responded to odors of unrelated females differently than they might have to novel stimulus objects. Nonetheless, young clearly can discriminate their mothers from other lactating females using whole-body odors, and pouch odors alone contain sufficient chemical information to enable young to discriminate their mothers from other females. These results indicate that maternal odors, including pouch odors, have the potential to facilitate contact during the period preceding dispersal by juvenile opossums. It is not clear, however, if the enhanced production of pouch secretions during late postnatal development actually represents a specialized chemical mechanism for promoting contact between mothers and young. Whether pouch secretions are more effective cues for maternal recognition than odors from other specific body areas also remains to be investigated.

While the adaptive value of chemical cues for orientation to mothers by young opossums still suckling and denning with the mother is obvious, it is unknown how often young at this developmental stage may actually encounter and need to discriminate odors of unrelated lactating females in the wild. Recent radiotracking data show that home ranges of adult female *Didelphis* overlap (Fitch and Shirer, 1970; Gillette, 1980; Ryser, 1990; Sunquist et al., 1987), and observations of female captives generally have shown a greater capacity for social tolerance than was earlier suspected for this "solitary" species (Holmes, 1987, 1991). Adult females are more philopatric than males, and often disperse little from maternal home ranges; hence related as well as unrelated females with their litters may encounter one another on contiguous ranges. Since opossums are nongregarious, however, and usually live less than two years in the wild (Sunquist et al., 1987; Austad, 1992), olfactory recognition of individual conspecifics may be of minimal importance in the social structure of adults.

In sum, these findings corroborate past work indicating the importance of

chemocommunication in didelphid opossums, a group in which social behavior remains largely unexplored, and suggests that affiliative behaviors are better developed in this group than previously thought.

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2-METHYLTHIAZOLIDINE AND 4-ETHYLGUAIACOL, MALE SEX PHEROMONE COMPONENTS OF THE COCKROACH *Nauphoeta cinerea* (DICTYOPTERA, BLABERIDAE): A REINVESTIGATION

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Abstract—In *Nauphoeta cinerea*, male calling behavior is associated with sex pheromone release by the sternal glands. The male pheromone that attracts females from a distance is a mixture of 2-methylthiazolidine and 4-ethylguaiacol. It is active at very low concentrations, 0.05 and 0.01 ng, respectively. Two other compounds, 3-hydroxy-2-butanone and 2-methyl-2-thiazoline, act at close range, keeping the female in the vicinity of the male. The function of the volatile pheromone and those of previously described contact pheromones are discussed in regard to their possible involvement in the establishment of male dominant–subordinate relationships.

Key Words—Cockroach, Dictyoptera, Blattaria, *Nauphoeta*, 2-methylthiazolidine, 4-ethylguaiacol, male sex pheromone, calling behavior.

INTRODUCTION

Studies on the biology of the cockroach *Nauphoeta cinerea* have taken several approaches. Since Kramer (1964) described aggressiveness in males, about 20 papers have been devoted to the dominant–subordinate relationship and hier-

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archy (see Schal and Bell, 1983; Moore 1990b). In the same way, after Roth and Dateo (1966) demonstrated the existence of a male sex pheromone, Menon (1981) and Sreng (1984, 1985) studied the morphology of the exocrine glands involved in the production of this pheromone. Takahashi and Fukui (1980, 1983) and Sreng (1983, 1991) identified several compounds specific to the males that trigger different sequences of sexual behavior. Our aim was to study this hierarchy and its relationship to male sex pheromone titer and release, using as a basis the chemical data from Sreng (1991). We came to the conclusion that the methodology employed for extraction was unsatisfactory, leading to questionable results on the quantification and biological activity of the three compounds identified as the sex pheromone (3-hydroxy-2-butanone, 2-methylthiazolidine, and 4-ethylguaiacol), even though their presence and activity are indisputable. It was then necessary to reinvestigate the composition of the male sex pheromone, before investigating its possible function in agonistic behavior and a dominance hierarchy.

METHODS AND MATERIALS

Insects. Colonies of *N. cinerea* were reared in glass aquaria (40 × 25 × 25 cm) maintained at a constant temperature of 26± 2°C and 70% relative humidity with a 12 : 12 dark–light photoperiod. Insects had free access to dry dog food and water.

Adults used for bioassays were isolated from colonies at adult ecdysis (dates were recorded). Males and females were kept in separate plastic boxes (9 × 6 × 2 cm); males were kept alone and the females in groups (5 insects/box) until tested.

Preparation of Extracts. For GC analysis and quantification of the pheromonal components, five pooled samples of 10 adult males were taken at random in colonies at the third hour of the dark period. Sternal glands (sternites 3–7) and tergal glands (tergites 2–8) were dissected with microscissors and forceps. Glands, separated from adjacent tissues (hemolymph, fat body) with a paper towel, were transferred to a vial of double-distilled methylene chloride (4 ml/sternal or tergal gland) and allowed to soak for 3 hr at ambient temperature. Phenylethanol internal standard was added (200 ng/sternal or tergal gland). The pooled extracts (200 ml) were concentrated to a small volume ($\approx$300 μl) by the method of Kuderna-Danish (Perry, 1961) and kept in a deep freezer at −20°C.

For GC-MS analysis, two pooled samples (130 sternal and 130 tergal glands) were prepared with the same procedure as above, but without internal standard.

For a comparative GC analysis of the sternites of males, females, and last-instar males (L7) and female nymphs (L8), we used the same extraction procedure as above, using for each category three pooled samples of five insects.

Chemical Analysis. Gas chromatographic analyses were carried out with a Packard 437 gas chromatograph fitted with a split–splitless injector and a flame-ionization detector. The capillary column used was a Chrompack CP-Wax 58CB column (25 m × 0.25 mm ID, film thickness 0.22 μm) with temperature programming from 50°C to 220°C at 2°C/min. Injector and detector temperatures were, respectively, 270°C and 290°C. Helium was used as the carrier gas at a flow rate of 2 ml/min. The GC traces were recorded and integrated with a Shimadzu CR-4A integrator.

Gas chromatographic–mass spectrometric analyses were carried out with a Nermag R 10-10-C quadrupole mass spectrometer coupled to a Girdel 31 gas chromatograph fitted with a split–splitless injector and the same capillary column as above. The GC conditions were the same as described above. The column was directly connected to the ion source of the spectrometer through a heated transfer line maintained at 260°C. Electron impact mass spectra were obtained at 70 eV.

Identifications were made by comparison of the mass spectra of unknowns to those of the EPA-NIH mass spectral data base (Heller and Milne, 1980) and the mass spectra from the library of the Laboratoire de Recherches sur les Arômes de l'I.N.R.A. (Dijon).

Compounds 3-hydroxy-2-butanone (acetoin), (2*R*,3*R*)-butanediol, and (2*S*,3*S*)-butanediol were purchased from Pfaltz & Bauer; 4-methylphenol (*p*-cresol), 4-ethylphenol, and thiazolidine from Aldrich-chemie; 2-piperidone and phenylethanol (internal standard) from Fluka; acetic acid, propanoic acid, butanoic acid, octanoic acid, and nonanoic acid from Polyscience Corp.; guaiacol, 4-ethylguaiacol (4-ethyl-2-methoxyphenol), and 2-methyl-2-thiazoline from Interchim. 2-Methylthiazolidine was synthesized according to the procedure described by Sheehan et al. (1956).

Correct identification was confirmed if the GC characteristics and mass spectrum of the unknown compound were the same as those of the reference compound analyzed under the same conditions on a CP Wax 58 CB column. Identification was also confirmed by coinjection of glandular extracts and pure compounds. All chemicals were >98% pure as determined by capillary GC analysis and were used without further purification for bioassays.

Observations: Sexual Behavior, Reactions of Virgin Females toward Different Chemical Stimuli. Observations were made in glass aquaria (20 × 20 × 15 cm) between the second and sixth hour of darkness under a dim red light. The virgin males and females used (isolated in separate boxes since the imaginal molt) were 12 days old. Sexual behavior was observed until mating occurred on ten pairs. Pure chemicals, identified as male-specific compounds, were presented to virgin females on pieces of filter paper (see below) and the reactions of the females were recorded.

Bioassays (Y-Maze Olfactometer). Olfactory stimuli were tested on virgin

females (12 ± 4 days old) in an Y-maze olfactometer. The olfactometer was a glass tube (2 cm ID) with a 40-cm-long common arm (A), splitting into two 30-cm-long choice arms, B and C (the angle between B and C was 70°). The samples tested were living insects or filter papers (1 × 2 cm, folded in V) impregnated with the stimuli source. Samples were placed at the ends of arms B or C in a box (9 × 6 × 5 cm). An airstream (10 ml/min, controlled by flowmeters) flowed through the boxes and the arms B and C to convey the chemical stimuli through the common arm A to the "starting box" containing the test insect. Each insect was used once. The sample was changed after each insect was tested. We compared the time of discovery of the arm B and of the arm C (time taken by the insect to go to intersection and chose either arm B or C) by Wilcoxon's test of differences between two matched samples. The time of each test was 5 min. We recorded the first choice made. If the insect did not respond in 5 min, the test was terminated.

Each of the 10 virgin females to be tested was kept in a separate adaptation box (6 × 9 × 5 cm) for at least 1 hr before each test. Each box could be connected with the common branch A. Boxes had a screen door that could be raised to allow the female to enter the common arm at the starting point. Ten virgin females were used for each test. All tests were conducted in a dark room, under photographic red light between the second and the sixth hour of the scotophase.

Different chemical stimuli were tested: 1 μl CH_2CL_2 (solvent/control); extracts corresponding to: 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2} male sternal gland equivalents; 1, 10, 100, and 1000 ng of 3-hydroxy-2-butanone and thiazolidine; 1, 10, and 100 ng of 2-methyl-2-thiazoline, (2*R*,3*R*)-butanediol, (2*S*,3*S*)-butanediol, and a racemic mixture of (2,3)-butanediol; 0.05, 1, 10, 100, and 1000 ng of 2-methylthiazolidine; 0.01, 0.1, 1, 10, 100, and 1000 ng of 4-ethylguaiacol; different mixtures of 3-hydroxy-2-butanone, 2-methylthiazolidine, 4-ethylguaiacol (100:0.05:0.01; 100:5:1; 100:100:1; 100:100:20; 0:0.005:0.001; 0:0.05:0.01; 0:0.5:0.1; 0:5:1; 0:50:10 ng).

Virgin females were given the choice between the stimuli (extracts or chemicals) and the airstream alone. Ten virgin females were given the choice between three living males and mixtures of pure chemicals. The olfactometer was cleaned with methylene chloride after each test.

We recorded and compared the time spent by the females before entering branch A or B of the olfactometer by Wilcoxon's test. The time of each test was 5 min. If the insect did not respond in 5 min, the test was terminated.

RESULTS

The chromatogram shown in Figure 1 is representative of male sternal gland extracts revealing the presence of more than 30 compounds, with major ones being peaks 1, 4, 8, 9, 18, and 22, From the 31 compounds studied, 23 were

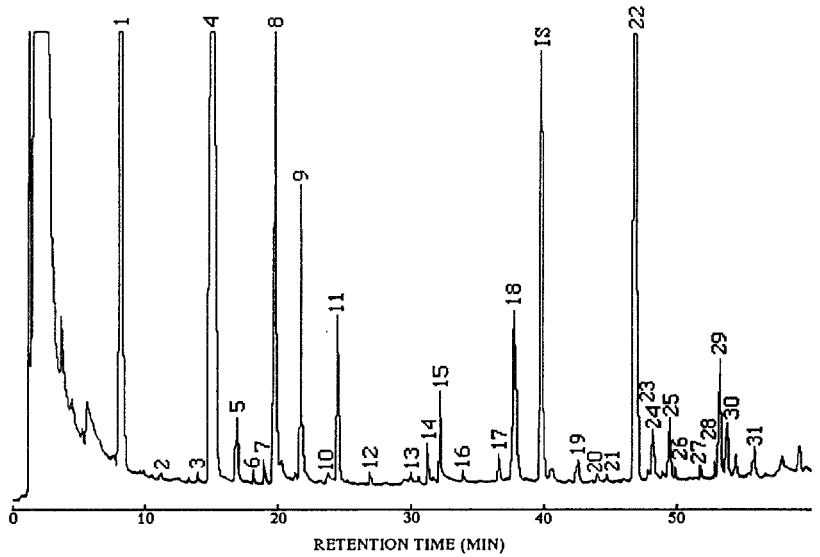

FIG. 1. Capillary gas chromatogram of the sternal gland secretion from males of *Nauphoeta cinerea*. Conditions: CP-Wax 58 CB fused silica column (25 m × 0.25 mm ID); temperature 50–220°C (2°C/min); carrier gas, helium at flow rate of 2 ml/min. Compounds are listed below with their molecular weight (MW) and the relative abundance of the eight main peaks [*m/z* (%)] observed on their EI mass spectrum.

Peak	Compound	RI[a]	MW	Mass spectrum [*m/z* (%)]
1	3-Hydroxy-2-butanone	a	88	45(100), 43(60), 88(M^+,11), 41(6), 89(3), 73(2), 55(1), 71(1)
2	2-Methyl-2-thiazoline	a	101	45(100), 101(M^+,41), 59(36), 86(34), 55(15), 45(14), 42(13), 27(7)
3	Acetic acid	a	60	43(100), 45(79), 60(M^+,47), 42(16), 29(11), 44(6), 41(5), 31(4)
4	2-Methylthiazolidine	a	103	88(100), 103(M^+,80), 56(65), 44(51), 57(50), 59(33), 42(32), 61(25)
5	Thiazolidine	a	89	89(M^+,100), 43(63), 88(35), 42(33), 59(16), 45(22), 60(19), 61(15)
6	2-Ethylthiazolidine	b	117	88(100), 70(21), 41(94), 117(M^+,13), 56(12), 58(9), 61(7), 45(7)
7	Propanoic acid	a	74	29(100), 74(M^+,80), 45(72), 73(44), 57(36), 55(14), 56(12), 43(10)
8	(2*R**,3*R**)-Butanediol	a	90	45(100), 43(15), 57(14), 47(6), 75(4), 44(4), 72(1), 55(1)
9	(2*R**,3*S**)-Butanediol	a	90	45(100), 43(14), 57(10), 47(14), 75(3), 44(3), 72(2), 55(1)
10	Dihydro-2-(3H) furanone	b	86	42(100), 23(47), 41(45), 86(M^+,28), 56(27), 29(26), 39(21), 35(11)

FIG. 1. Continued

Peak	Compound	RI[a]	MW	Mass spectrum [m/z (%)]
11	Butanoic acid	a	88	60(100), 73(34), 41(31), 43(23), 55(8), 27(8), 47(7), 88(2)
12	3-Methylbutanoic acid	b	102	60(100), 41(90), 43(57), 87(48), 74(47), 29(32), 27(29), 45(19)
13	Acetamide	b	59	44(100), 43(95), 59(M^+,79), 42(29), 57(10), 45(15), 41(10), 27(6)
14	*N,t*-Butylthiopipéridine	b	173	84(100), 82(34), 101(34), 59(33), 173(M^+,23), 114(22), 129(14), 144(7)
15	*N,n*-Butylthiopipéridine	b	173	84(100), 82(29), 101(34), 59(33), 173(M^+,18), 114(18), 130(9), 144(7)
16	Isomer unknown 18?		?	102(100), 42(25), 61(15), 59(16), 104(7), 56(4), 43(4), 103(4)
17	Guaiacol	a	124	109(100), 124(85), 81(45), 53(16), 39(9), 52(9), 51(7), 50(5)
18	Isomer unknown 16?		?	102(100), 42(26), 61(14), 44(8), 104(7), 59(6), 70(3), 27(3)
19	Unknown?		?	87(100), 130(67), 56(17), 70(14), 43(14), 42(12), 45(10), 102(4)
20	2-Methoxy-4-methylphenol	b	138	138(M^+,100), 123(95), 95(50), 77(32), 67(25), 39(18), 41(23), 65(13)
21	Unknown?		?	116(100), 87(73), 56(55), 42(38), 126(24), 45(23), 71(20), 43(20)
22	4-Ethylguaiacol	a	152	137(100), 152(M^+,35), 91(13), 122(11), 77(11), 65(6), 51(7), 39(7)
23	*p*-Cresol	a	108	107(100), 108(M^+,92), 77(37), 79(25), 90(18), 5(10), 53(9), 39(19)
24	2-Butylthiazolidine	b	145	88(100), 43(90), 56(63), 145(47), 102(29), 45(27), 117(21), 130(14)
25	Octanoic acid	a	144	43(100), 60(80), 73(72), 55(40), 101(25), 57(16), 115(4), 69(5)
26	2-Piperidone	a	99	99(M^+,100), 43(92), 30(80), 42(58), 41(39), 55(30), 70(22), 98(16)
27	Nonanoic acid	a	158	60(100), 73(76), 41(70), 43(69), 55(62), 69(32), 115(18), 39(17)
28	4-Ethylphenol	a	122	107(100), 122(M^+,33), 77(20), 91(6), 108(6), 53(5), 51(3), 65(3)
29	2-Ethylphenol	b	122	107(100), 122(M^+,54), 77(39), 91(16), 108(9), 121(5), 39(5), 79(5)
30	Unknown?		?	30(100), 60(69), 43(67), 150(15), 135(14), 77(8), 107(6), 119(4)
31	Methyl ester?		?	74(100), 87(69), 43(43), 88(21), 55(20), 41(20), 75(17), 143(10)

[a]RI-reliability of identification: a: mass spectrum and GC retention time identical with reference compounds; b: mass spectrum identical with published spectrum.

TABLE 1. AMOUNT AND PERCENTAGE OF 31 COMPOUNDS FROM MALE STERNAL AND TERGAL GLANDS OF *Nauphoeta cinerea*

Compound		Sternal gland amount		Tergal gland amount	
		(ng/M)[a]	%	(ng/M)[a]	%
1. 3-Hydroxy-2-butanone	M	6785 ± 1133	40.2	46 ± 11	16.5
2. 2-Methyl-2-thiazoline	M	traces	traces		
3. Acetic acid		traces	traces	traces	traces
4. 2-Methylthiazolidine	M	4667 ± 1092	35.8	84 ± 17	29.7
5. Thiazolidine	M	124 ± 13	0.7		
6. 2-Ethylthiazolidine	M	traces	traces		
7. Propanoic acid		traces	traces	traces	traces
8. (2*R**, 3*R**)-Butanediol	M	753 ± 168	4.8	39 ± 6	13.5
9. (2*R**, 3*S**)-Butanediol	M	455 ± 64	2.3	26 ± 4	9.1
10. Dihydro-2-(3H) furanone		12 ± 1	0.2	traces	traces
11. Butanoic acid		67 ± 11	1.2	14 ± 3	4.9
12. 3-Methylbutanoic acid		traces	traces	traces	traces
13. Acetamide		traces	traces	traces	traces
14. *N*, t-Butylthiopiperidine?		13 ± 4	0.2	21 ± 10	7.4
15. *N*, n-Butylthiopiperidine?		traces	traces	traces	traces
16. Unknown isomer 18?	M	26 ± 2	0.5		
17. Guaiacol		28 ± 5	0.3	22 ± 5	7.6
18. Unknown isomer 16?	M	138 ± 25	2.4		
19. Unknown?	M	traces	traces	traces	traces
20. 2-Methoxy-4-methylphenol		traces	traces	traces	traces
21. Unknown?		traces	traces	traces	traces
22. 4-Ethylguaiacol	M	644 ± 146	7.9	traces	traces
23. *p*-Cresol		traces	traces	traces	traces
24. 2-Butylthiazolidine	M	traces	traces	traces	traces
25. Octanoic acid		49 ± 13	0.9	traces	traces
26. 2-Piperidone		traces	traces	traces	traces
27. Nonanoic acid		traces	traces	traces	traces
28. 4-Ethylphenol		traces	traces	traces	traces
29. 2-Ethylphenol		51 ± 4	0.9	traces	traces
30. Unknown?		35 ± 6	0.6	31 ± 8	10.7
31. Methyl ester?		traces	traces	traces	traces
Total		≈13,900 ± 2700	100	≈290 ± 60	100

[a]The data are means of five pools (10 males/pool) ± SE expressed in nanograms of given compounds per male (ng/M). M: male-specific compounds; traces: less than 10 ng.

identified (listed in the legend of Figure 1). The identifications of two further compounds, the two butylthiopiperidines need confirmation. Six compounds remain unidentified; three of them are specific to males (Table 1). Compound 19 is only present in trace amount. Compounds 16 and 18 are present in noticeable amounts; 18 always represents more than 2% of the secretion. Compounds 16 and 18 display identical mass spectra (legend of Figure 1) and are probably isomers. Apart from 2-ethylthiazolidine and 2-butylthiazolidine, which are present in trace amounts, all other compounds specific to males are also major peaks: peak 1, 2-hydroxy-3-butanone; peak 4, 2-methylthiazolidine; peak 5, thiazolidine; peak 8, (2*R**,3*R**)-butanediol; peak 9, (2*R**,3*S**)-butanediol, and peak 22, 4-ethylguaiacol.

From 31 studied compounds, 19 were also found in females and larvae. From the 12 male-specific compounds found in the sternal gland extracts, only four were also found in the tergal gland extracts: 2-hydroxy-3-butanone, 2-methylthiazolidine, and the two isomers of butanediol. Table 1 shows that 2-methylthiazolidine is as abundant in sternal glands (36%) as in tergal glands (30%). Whereas their relative percentages are similar in the sternal and tergal secretions, that of 3-hydroxy-2-butanone is less than half as abundant in the tergal secretions. The two isomers of butanediol, on the other hand, were three times more abundant in tergal secretions.

The amount of each compound and its relative percentage in the sternal gland secretion varied widely between individuals. These individual variations are so significant that even the use of pooled samples instead of individuals did not eliminate them. The quantitative analysis of five pooled samples (10 males/sample) of sternal glands extracts showed that the amounts of the five main compounds vary from 4300 ng to 9300 ng for 3-hydroxy-2-butanone, 2200 to 7000 ng for 2-methylthiazolidine, 370 to 1130 ng for (2*R**,3*R**)-butanediol, 300 to 600 ng for (2*R**,3*S**)-butanediol, and 300 to 970 ng for 4-ethylguaiacol.

Bioassays. The biological activity of male sternal gland extracts was tested at different concentrations, from 10^{-6} sternal gland equivalents (St. Eq.) to 1 St. Eq. (Table 2). The male-specific compounds were tested separately or in mixture [3-hydroxy-2-butanone, 2-methyl-2-thiazoline, 2-methylthiazolidine, thiazolidine, (2,3)-butanediol, (2*R*,3*R*)-butanediol, (2*S*,3*S*)-butanediol, 4-ethylguaiacol, and mixtures of 3-hydroxy-2-butanone, 2-methylthiazolidine, and 4-ethylguaiacol].

Male sternal gland extract attracted receptive females with a dose of 10^{-5} St. Eq. (80% of positive responses). For 10^{-4} St. Eq. or more, 100% of the females respond positively.

Preliminary tests showed that only some of the male-specific compounds elicited responses from the females, including 3-hydroxy-2-butanone, 2-methyl-2-thiazoline, 2-methylthiazolidine, thiazolidine, the two enantiomers of butanediol, and 4-ethylguaiacol.

TABLE 2. FEMALE RESPONSES TO DIFFERENT CHEMICAL STIMULI IN Y-MAZE OLFACTOMETER[a]

	air	Time	%[b]	odor stimulus	Time	%[c]	W[d]
a	air	233 ± 24	50	air + 1 µl CH2CL2	274 ± 15	30	NS
b	air	228 ± 27	40	St.Ext. 10^{-6} Eq.	186 ± 34	60	NS
	air	282 ± 15	10	St.Ext. 10^{-5} Eq.	171 ± 24	80	*
	air	237 ± 30	0	St.Ext. 10^{-4} Eq.	102 ± 18	100	**
	air	256 ± 15	0	St.Ext. 10^{-3} Eq.	128 ± 14	100	**
	air	206 ± 22	0	St.Ext. 10^{-2} Eq.	75 ± 09	100	**
	air	262 ± 21	0	St.Ext. 10^{-1} Eq.	90 ± 15	100	**
	air	231 ± 17	0	St.Ext. 1 Eq.	85 ± 17	100	**
c	air	251 ± 33	40	3H2B 1 ng	198 ± 27	40	NS
	air	232 ± 29	40	3H2B 10 ng	196 ± 28	60	NS
	air	214 ± 30	10	3H2B 100 ng	109 ± 24	90	*
	air	228 ± 23	60	3H2B 1000 ng	267 ± 21	40	NS
d	air	241 ± 23	30	2M2T 1 ng	205 ± 27	50	NS
	air	246 ± 29	30	2M2T 10 ng	270 ± 16	30	NS
	air	277 ± 17	0	2M2T 100 ng	220 ± 25	60	*
e	air	221 ± 28	40	2MT 0.05 ng	238 ± 22	50	NS
	air	208 ± 27	10	2MT 1 ng	114 ± 14	90	**
	air	263 ± 25	10	2MT 10 ng	142 ± 13	90	**
	air	271 ± 20	0	2MT 100 ng	177 ± 24	100	**
	air	180 ± 34	40	2MT 1000 ng	181 ± 28	50	NS
f	air	233 ± 24	20	4EG 0.01 ng	274 ± 15	60	NS
	air	241 ± 27	40	4EG 0.1 ng	242 ± 23	40	NS
	air	252 ± 20	0	4EG 1 ng	117 ± 16	100	**
	air	272 ± 20	20	4EG 10 ng	168 ± 22	80	*
	air	191 ± 30	40	4EG 100 ng	165 ± 35	60	NS
	air	163 ± 27	40	4EG 1000 ng	148 ± 27	60	NS
g	air	292 ± 05	0	100/0.05/0.01	145 ± 25	100	**
	air	284 ± 11	0	100/5/1	112 ± 18	100	**
	air	277 ± 12	0	100/100/1	89 ± 15	100	**
	air	217 ± 21	0	100/100/20	74 ± 07	100	**
	air	223 ± 27	30	0/0.005/0.001	183 ± 24	70	NS
	air	223 ± 25	0	0/0.05/0.1	104 ± 13	100	**
	air	267 ± 17	0	0/0.5/0.1	141 ± 22	100	**
	air	187 ± 19	0	0/5/1	77 ± 08	100	**
	air	236 ± 26	0	0/50/10	70 ± 13	100	**
h	3 males	212 ± 28	40	0/0.5/0.01	192 ± 27	60	NS

[a] Virgin females ($N = 10$) were given a choice between an airstream and an odor stimulus (extracts or pure chemicals). Time: time spent by a female before entering the branch A or B of the olfactometer (mean ± SE). Eight different stimuli were tested: a, 1 µl CH_2Cl_2 (solvent/control); b, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1 male sternal gland extract equivalents (St.Ext. 10^{-x} Eq.); c, 1, 10, 100, 1000 ng of 3-hydroxy-2-butanone (3H2B); d, 1, 10, 100 ng of 2-methyl-2-thiazoline (2M2T); e, 0.05, 1, 10, 100, 1000 ng of 2-methylthiazolidine (2MT); f, 0.01, 0.1, 1, 10, 100, 1000 ng of 4-ethylguaiacol (4EG); g, different mixtures of 3H2B/2MT/4EG (100/0.05/0.01; 100/5/1; 100/100/1; 100/100/20; 0/0.005/0.001; 0/0.05/0.01; 0/0.5/0.1; 0/5/1; 0/50/10 ng); h, 3 males vs 3H2B/2MT/4EG (0/0.5/0.01).

[b] Percentage of females first chosing the branch with the airstream.

[c] Percentage of females first chosing the branch with the stimulus.

[d] NS: $P > 0.05$; $*P < 0.05$; $**P < 0.01$.

All of our attempts to trap the compounds 16 and 18 by semipreparative GC failed; very likely, these compounds decomposed during collection because the two trapped peaks resulted in several small peaks when reinjected in the GC.

Eight pure compounds were tested on mature females at different concentrations. Thiazolidine and (2,3)-butanediol (racemic mixture or pure *R,R* or *S,S* forms) did not attract females from a distance. These two compounds were tested at 1, 10, 100, and 1000 ng; females remained in the test box or did not significantly chose arms B or C.

3-Hydroxy-2-butanone attracted virgin females only at high concentrations. With 100 ng, 90% of the females were attracted. At a concentration of 1000 ng, the females avoided the arm of the olfactometer containing this stimulus.

2-Methyl-2-thiazoline, present in trace amount in the sternal glands (< 10 ng), attracted 60% of the females at a concentration of 100 ng, a concentration that was never found in the gland.

2-Methylthiazolidine at a concentration of 1 ng or 10 ng attracted 90% of the females, and 100% at a concentration of 100 ng. Beyond this concentration, the females were neither attracted nor repelled.

4-Ethylguaiacol attracted 100% of the females at a concentration of 1 ng, but the percentage of responding females decreased as the concentration increased (80% at 10 ng, 60% at 100 ng).

From these tests on pure compounds, it appeared that only 2-methylthiazolidine and 4-ethylguaiacol were consistently attractive alone at a concentration of 1 ng. When tested together, the amount necessary to obtain 100% attraction of females dramatically decreased: 0.05 ng and 0.01 ng, respectively. Amounts ten times lower (0.005 ng and 0.001 ng) still attracted 70% of the females even though the attractiveness was not statistically significant. This mixture remained attractive in a broad range of concentrations, since at concentrations of 50 ng and 10 ng, respectively, 100% of the females were still attracted.

These results are consistent with the fact that crude extracts from the sternal glands, at a concentration of 10^{-4} St. Eq., could attract 100% of the females. Of all the male-specific compounds, 2-methylthiazolidine and 4-ethylguaiacol are the only two that considering their threshold of activity and their relative abundance in the glands, could match this test.

The addition of 100 ng of 3-hydroxy-2-butanone (concentration at which this compound is active) to different mixtures of 2-methylthiazolidine and 4-ethylguaiacol did not change to their attractiveness.

When tested on virgin females at short range (20 cm), most of the male-specific compounds [2-hydroxy-3-butanone, 2-methyl-2-thiazoline, 2-methylthiazolidine, thiazolidine, (2*R*,3*R*)-butanediol, (2*S*,3*S*)-butanediol, 4-ethylguaiacol] elicited searching behavior and, when the impregnated paper was contacted, palpation. Whatever the compound tested, the attention of the female

was always very brief, the chemical stimulus triggering an exploration of the area and search for a male. The quantification of the time spent near the paper was not significant as compared to the blank.

Of these seven male-specific compounds, only two are very attractive at a distance and at very low thresholds—2-methylthiazolidine and 4-ethylguaiacol—and they constitute the two components of the volatile sex pheromone.

3-Hydroxy-2-butanone and 2-methyl-2-thiazoline are attractive at a distance only when they are used at high concentrations, but under natural conditions, where they are emitted in "normal" amounts, they probably act as close-range attractants, sexual stimulants, or arrestants, increasing the excitation of the female and preventing her from leaving the area near the male.

DISCUSSION

The most obvious sequence of the sexual behavior of *N. cinerea* is the wing raising posture of the males, and when Roth and Dateo (1966) demonstrated the existence of a male sex pheromone, they correlated its emission with this very characteristic behavior. Sreng (1979) demonstrated that the male sex pheromone was produced by the sternal glands and described the calling posture associated with its emission. Using more than 2000 virgin males, two compounds, 3-hydroxy-2-butanone and 2-methylthiazolidine, were identified, both being long-range attractants for females (Sreng, 1983; Brossut and Sreng, 1985). Recently, it was reported (Sreng, 1991) that the sex pheromone is a mixture of three compounds, 3-hydroxy-2-butanone, 2-methylthiazolidine, and 4-ethyl-2-methoxyphenol (4-ethylguaiacol) in a ratio of 4:4:1 (2700 ng). However, the inadequate amount of solvent used (100 μl) allowed only a limited extraction, leading to subsequent inaccuracy in quantification. The calling posture can last 2 hr or more, with many short interruptions. A constant emission in microgram amounts, as suggested by Sreng (1991), would imply tremendous biosynthetic activity. On the contrary, the thresholds estimated from the present match this extended pheromone release behavior. Study of the diel periodicity of male sex pheromone titer and release throughout adult life (Sirugue, 1992) demonstrated that the amounts of each compound in the secretion change during the life of the male, and that the wide variations noticed by Sreng (1991) are not due to the "presence of hierarchies" but a diel pattern in pheromone biosynthesis. 2-Methylthiazolidine and 4-ethylguaiacol are actively synthesized during the scotophase, while the amount of 3-hydroxy-2-butanone synthesized progressively falls during the scotophase, which fits with our results on their biological activity (Sirugue et al., unpublished data; Sirugue, 1992).

Nitrogen-containing compounds are fairly common in vertebrate and insect semiochemicals (Wheeler, 1977; Blum, 1981), but sulfur-containing compounds

are unusual in insects (mandibular glands of fire ants, Longhurst et al., 1980; pygidial glands of aquatic beetles, Dettner and Schwinger, 1980). 4-Ethylguaiacol is very common in plants (tea-plant, Yamanishi et al., 1970; tobacco-plant, Irvine and Saxby, 1969; rhubarb, Frattini et al., 1976), and less in insects where it has been reported (with other closely related compounds) as an attractant for the northern corn rootworm, *Diabrotica barberi* (MacGovern and Ladd, 1981). 3-Hydroxy-2-butanone is found in many plants (*Helianthus annuus*, Etievant et al. 1984; *Vitis rotundifolia* Welch et al., 1982; *Carica papaya*, *Psidium guajava*, Idstein and Schreier, 1985a,b) and in red clover, *Trifolium pratense*, where it is one of the volatiles that attract the beetle *Hylastinus obscurus* (Kamm and Buttery, 1984; Kamm et al., 1984).

We have no real indication of the origin of the main compounds found in the sternal secretions. 3-Hydroxy-2-butanone and butanediol may be simultaneously produced by fermentation of glucose by bacteria and fungi (MacFaddin, 1980; Gottshalk, 1986). In the sternal glands, where it assumes no obvious function, butanediol may be a precursor of 3-hydroxy-2-butanone.

Biosynthesis of volatile semiochemicals in insects remains, in many cases, very perplexing. In arctiid moths, the methyl-branched hydrocarbon sex pheromone is biosynthesized from leucine via methyl-branched fatty acids, but these fatty acids were detected in small amounts or were absent in the pheromone gland (Charlton and Roelofs, 1991). This suggests that once "initiated, biosynthesis of the pheromone proceeds rapidly with little or no accumulation of intermediates and/or that the pheromone is biosynthesized elsewhere in the insect and transported to the gland" (Charlton and Roelofs, 1991). Such a process would explain why the only compounds found in abundance in the sternal glands of *N. cinerea* are the components of the sex pheromone.

During courtship, male *N. cinerea* display two successive very characteristic postures, the first being a calling posture when the long-range sex pheromone is emitted (Sreng, 1979; Sirugue, 1992), and the second, after antennal contact with a female, being the wing-raising posture in which the tergal glands are exposed (Roth and Willis, 1952). Usually, as soon as the male adopts the wing-raising posture, the female climbs on its back, "feeding" on the tergal secretions while the male grasps the female's genitalia. We observed that if the female fails to respond to the wing raising, even if she is in contact with the male, the male adopts the calling posture again, then switches again to the wing-raising posture and so on. This behavior demonstrates that the sternal secretions, basically used at long range, can also act at short range. The tergal secretions (aphrodisiacs) are complex mixtures (Brossut et al., 1975) in which the volatile compounds represent only a few percent of the mixture. In *N. cinerea*, in terms of the two components of the long-range pheromone, only 2-methylthiazolidine is still present in detectable amounts, but at a concentration 50 times less than

in the sternal glands. 4-Ethylguaiacol is present in trace amounts. 3-Hydroxy-2-butanone is 150 times less abundant than in the sternal glands. When emitted by the tergal glands, these compounds could enhance the attraction of the aphrodisiac and promote the female climbing on the male's back.

Females of *Leucophaea maderae* are not attracted by males of *N. cinerea*. The volatile male sex pheromone of *L. maderae*, attractive at a distance, is 3-hydroxy-2-butanone. Two other compounds, senecioic and (*E*)-2-octenoic acids, act as short range arrestants (Sirugue, 1992). These three main compounds are also found in the tergal secretions and, in contrast with *N. cinerea*, are present in large amounts, the pheromone 3-hydroxy-2-butanone being only 10 times less abundant than in the sternal glands.

Aggressive behavior, dominant–subordinate relationships, hierarchy, and territoriality have been extensively studied in male *N. cinerea* (Ewing, 1972, 1973; Ewing and Ewing, 1973; Bell et al., 1979; Bell and Gorton, 1978, Breed at al., 1980). The use of olfactory cues in discrimination among individuals has been suggested in the establishment of male dominance hierarchies and in the choice of a male by females (Smith and Breed, 1982; Schal and Bell, 1983; Moore, 1988, 1989, 1990a; Sreng, 1991). Our data (Sirugue, 1992) clearly show that the presence of dominant males inhibits the calling posture in subordinate males, which results in the accumulation of great amounts of pheromone in the latter. Until now, we have failed to demonstrate the existence of chemicals specific to dominant or subordinate males.

N. cinerea presents a rather complex sexual behavior with its series of sequences. The first is the emission of a volatile sex pheromone, which promotes the encounter of the sexes. The following recognized sequences are controlled by chemical signals perceived through contact chemoreception. The female sex pheromone of *Blattella germanica*, consisting of four methylketones, is one example of these contact sex pheromones (Nishida et al., 1976; Schal et al., 1990). A similar pheromone, eliciting male wing-raising posture, was studied in *N. cinerea* by Takahashi and Fukui (1980), but the hydrocarbon fraction isolated was also present in females. We are reinvestigating its composition. In this species, several contact pheromones are involved in social interactions. The "nauphoetin" [octadecyl (*Z*)-9-tetracosenoate] described by Fukui and Takahashi (1983) as an intermale recognition pheromone is not connected with aggressive behavior. Our current research indicates the existence of another contact pheromone that triggers male agonistic behavior through antennal contact.

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CHEMICAL CUES ASSOCIATED WITH VISUALLY ELABORATE AGGRESSIVE DISPLAYS OF THREE-SPINE STICKLEBACKS

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Abstract—A two-choice chamber was used to determine if three-spine sticklebacks could distinguish between water conditioned by a displaying male and water conditioned by a male that was active but not displaying. Both nonterritorial males and gravid females appeared to recognize which male was displaying; i.e., males and females were more likely to bite or bump the substrate in front of a chamber receiving water from a displaying male. Nonterritorial males were more likely to enter the chamber receiving water from a male that was not displaying; gravid females entered the chamber that received water from the displaying male more often than males but not more often than expected by chance. The aggressive displays of male sticklebacks may have both visual and chemical components; alternatively, the fish may have been capable of detecting chemical by-products associated with different rates of physical activity.

Key Words—Chemical signals, aggression, three-spine sticklebacks, *Gasterosteus aculeatus*, communication.

INTRODUCTION

A major goal of research in animal communication is to determine how and why signals influence perceivers. One of the first steps in determining how displays work is to examine the way in which they engage a perceiver's sensory

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system. Displays often influence more than one of the perceiver's senses at the same time; for example, many signals include both visual and acoustic components. However, relatively little effort has been directed toward determining whether vertebrates use chemical cues in concert with the visual or acoustic components of their displays.

Determining how signals influence a perceiver's sensory system and its behavior is particularly crucial for species that are used frequently in studies of behavior. The elaborate displays of three-spine sticklebacks (*Gasterosteus aculeatus*) have held the attention of ethologists for decades (Tinbergen, 1951; Assem, 1967; Wootton, 1976, 1984); however, studies of nonvisual signals in sticklebacks are rare.

Many studies of chemical signals in fish have neglected to consider whether cues from passive donors differ from those released by donors that are actively courting or behaving aggressively, even though such differences might influence the way in which the cues affect perceivers (Colyer and Jenkins, 1976). Researchers also have neglected to evaluate how the same chemical cues influence both sexes.

Here we determine whether nonterritorial males and gravid females: (1) detect differences between water conditioned by a territorial male that is displaying and water conditioned by a male that is not displaying, and (2) prefer to approach water from a displaying male or water from a nondisplaying male in a two-choice chamber.

METHODS AND MATERIALS

Sticklebacks were seined from Priest Creek, Quebec (45°48′N, 75°43′W) in the late spring. Males and females were kept in separate tanks and fed brine shrimp and blood worms twice a day. All subjects had bred at least once in the laboratory. Fish were maintained on a 8:16-hr light–dark cycle and 16°C for one month before being shifted to a 16:8-hr cycle; we did this to synchronize subjects that had experienced different lighting conditions.

The test apparatus consisted of four tanks (Figure 1). The top tank (51 × 26 × 30 cm high) fed water through plastic tubes (4 mm in diameter) into two lower "stimulus" tanks (30 × 16 × 21 cm high). These tanks had a mirror mounted on one end wall and a cover plate that could be raised and lowered to expose or hide the mirror (Figure 1).

A second pair of tubes connected the stimulus tanks to a "choice" tank (77 × 32 × 31 cm high). This tank contained a U-shaped partition that rested flat against the bottom of the tank. The partition left a 10-cm-wide opening between the central compartment and each side compartment; subjects were free to swim through the opening.

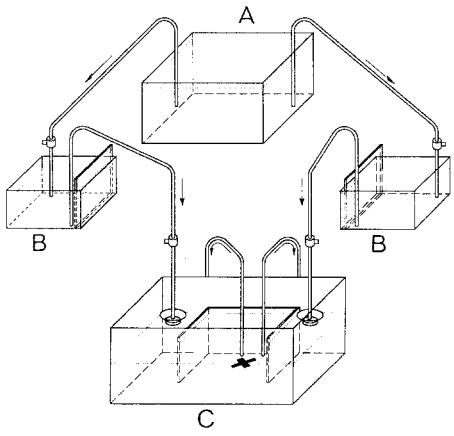

FIG. 1. Experimental apparatus used to determine whether nonterritorial males and gravid females distinguished between water from displaying and nondisplaying males. X marks the spot where the test fish was introduced. A, reservoir of aged water; B, stimulus tanks with mirror covers and mirrors mounted on inner walls; C, test tank with U-shaped partition. The arrows show the direction of water flow through the apparatus.

Behind each arm of the partition, a tube from one of the stimulus tanks poured water over a plastic mesh, which broke the flow of water so that it dripped into the side compartments (Figure 1). This severed any acoustic link that a continuous flow of water might create between tanks (Crapon de Caprona and Ryan, 1990).

A third pair of tubes, mounted along the back wall of the partition, provided an outflow. To ensure that the flow patterns on each side of the tank were identical, we carefully measured the position of the partition arms and connecting tubes so that they were perfectly symmetrical within the tank. The tubes on each side of the apparatus were cut to the same length. Plastic valves were used to set the water flow on each side; we used a graduated cylinder to ensure that water flow through each tube was exactly 80 ml/min. The flow of water was checked regularly.

At the beginning of each test, the tubes were clasped and a "stimulus male" was placed into each of the stimulus tanks. These males occupied territories prior to the tests and were capable of "creeping through" their nests (i.e., this behavior signifies a complete nest; see Wootton, 1976) in the presence of gravid females. Stimulus males were held in isolation prior to the experiments.

After a 30-min settling period, the cover plate in one stimulus tank (selected randomly) was raised to expose the mirror; the cover plate in the other tank was jiggled but not removed. Both stimulus males remained active during most of

their time in the tanks; occasionally, the males' activities were punctuated with brief periods of hovering or resting. Males that were exposed to the mirror displayed (using "head downs" and "charges"; see Wootton, 1976) and bit at their mirror image; the other males swam and bumped the walls of their tank but rarely displayed.

After 10 min, the tubes were unclasped. One minute later, a dip-net was used to place the test fish in front of the outflow tubes (Figure 1). From behind a blind, the observer recorded: (1) the direction(s) in which the fish bumped or bit the substrate; (2) which side of the tank the fish entered first (hereafter called "approached"); and (3) the time elapsed before the fish left the central compartment.

Tests ended when the fish passed either arm of the partition; the mirror cover was then replaced. After removing the test fish, the water in the choice tank was drained and the tank refilled with clean water. We only partially drained the stimulus tanks (the stimulus males were not removed), and then allowed water to flow through them for 15 min before filling them again and starting the next test with a new fish. This time the opposite stimulus fish was exposed to the mirror; this process was continued until we had tested 10 individuals.

The next day we ran the experiment again, using the same test fish but switching stimulus fish between tanks. We did this to determine if we could reverse the pattern of results recorded on the previous day and to assess how the previous day's experience influenced the choices made by males and females.

The experiment consisted of 10 two-day trials; each trial consisted of 20 tests (10 on the first day and 10 on the second). Trials were one week apart. In five trials we tested 10 gravid females; in the other five trials we tested 10 nonterritorial males (note, however, that two of the males died before the last trial). A new pair of stimulus males was used in each trial. We also ran a control trial on nine males and nine females to determine how the fish responded to tests in the absence of stimulus fish.

Replicated tests of goodness of fit (Sokal and Rohlf, 1981) were used to determine whether: (1) males or females were more likely to bump or bite the substrate in front of the partition that separated them from the water of a displaying male, and (2) males or females were more likely to approach the water from a displaying male.

RESULTS

After each subject entered the choice tank, it remained motionless for 5–15 sec before exploring the central compartment. During this time, subjects often bit at or bumped the substrate near each partition. Because individual bumps and bites occurred in rapid succession, we simply recorded whether the animal bumped or bit to the right, left, or to both sides of the choice chamber.

Bumps and bites were directed toward the base of the partition arms (which were not sealed to the tank floor) or one of the two openings. We did not include cases where a test fish bumped or bit the substrate as it entered one of the two side chambers. Only one of nine females and two of nine males bumped or bit the substrate when no stimulus fish were present in the apparatus; during actual trials, the activities were more common. Gravid females bit or bumped the substrate during more tests than males [round 1: females 78.0% (39 of 50 tests), males 45.8% (22 of 48 tests); round 2: females 82.0% (41 of 50 tests), males 52.1% (25 of 48 tests); $\chi^2 > 9.90$, $df = 1$, $P < 0.005$].

During the first round, males were more likely than females to bump or bite toward only one side of the arena [females 36.0% (14 of the 39 cases), males 90.9% (20 of the 22 cases); $\chi^2 = 17.3$, $df = 1$, $P < 0.001$]; this was not the case during the second round [females 43.9% (18 of 41 cases), males 56.0% (14 of 25 cases); $\chi^2 = 0.9$, $df = 1$, NS].

Males that bumped or bit toward only one side of the chamber were most likely to direct their activities toward water from the displaying male (Table 1). However, a test of heterogeneity indicated variation between trials in round 1 ($G_H = 11.5$, $df = 4$, $P < 0.05$); this was mainly the result of the last trial (i.e., the only trial where more males displayed toward the covered-mirror side). Data from trials in the second round and all other rounds listed below were homogeneous ($G_H < 5.7$, $df = 4$, NS).

Females that bit or bumped toward only one side of the chamber were also more likely to direct the activity toward water from the displaying male; however, the difference was only significant during the second round (Table 1).

Males were most likely to enter the side compartment receiving water from the nondisplaying animal; this trend was significant during the first round but not the second (Table 1).

TABLE 1. NUMBER OF INDIVIDUALS APPROACHING OR BITING TOWARD WATER FROM DISPLAYING AND NONDISPLAYING MALES IN TWO-CHOICE CHAMBER

		Nonterritorial males			Gravid females		
Activity	Round	Male displaying	Male not displaying	G_P[a]	Male displaying	Male not displaying	G_P[a]
Bump/Bite	1	17	3	10.8***	8	5	1.2,NS
	2	11	3	4.9*	14	4	5.9*
Approach	1	13	35	10.5**	30	20	2.0,NS
	2	19	29	2.1,NS	28	22	0.7.NS

[a] $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, not significant.

Gravid females approached water from the displaying animal more often than males (round 1: $\chi^2 = 10.8$, $df = 1$, $P < 0.001$; round 2: $\chi^2 = 2.6$, $df = 1$, NS) but not significantly more often than expected by chance (Table 1).

In the control situation, fish left the central chamber in less than a minute ($X \pm$ SE: males 27.56 sec $\pm$ 10.38; females 52.00 sec $\pm$ 19.28). In the experimental situation, males and females took much longer to leave the central chamber (males 160.5 sec $\pm$ 18.28; females 133.3 sec $\pm$ 10.70; Wilcoxon-Mann-Whitney tests, $P < 0.01$). In neither case did one sex take significantly longer than the other to leave the central chamber (Wilcoxon-Mann-Whitney tests, NS). In the experimental situation, there were no major differences in the time taken to enter either side of the choice tank (males: mirror 126.7 sec $\pm$ 14.0, other 177.4 sec $\pm$ 26.3; females: mirror 127.0 sec $\pm$ 13.9, other 141.0 sec $\pm$ 16.9; Wilcoxon-Mann-Whitney tests, NS).

DISCUSSION

Chemical cue(s) from displaying male sticklebacks were detected by nonterritorial males and gravid females. Nonterritorial males avoided water conditioned by displaying males; gravid females approached the water more often than males but not more often than expected by chance (ca. 10%). Both males and females were most likely to bite or bump in the direction of water from the displaying male.

The bites and bumps performed by test fish were very similar to the "head down" threats used by male sticklebacks defending territories. The test fish would orient toward a partition, lower its snout, and drawback along the substrate. We observed the same type of behavior when fish were exposed to territorial males behind a glass partition; gravid females often attempted to engage in courtship activities with such males shortly after bumping or biting the substrate (Waas, unpublished observation). The activities are probably best considered displacement behaviors, reflecting a state of internal conflict (Sevenster, 1961).

Tests of heterogeneity showed that a similar proportion of animals bumped and/or bit toward or approached water conditioned by displaying males in all five trials; this suggested that animals did not habituate to the test apparatus between trials. By swapping stimulus males between tanks on the second day of each trial, we found that we could reverse the sequence of results obtained on the first day. However, fewer animals distinguished between the two stimuli, suggesting that the fish habituated to the conditions when tested on subsequent days with the same two stimulus males.

Only two other studies have determined how chemicals emitted by a displaying fish influence the behavior of conspecifics. Colyer and Jenkins (1976)

found that *Betta splendens* males placed in water conditioned by displaying males "air gulped" (an act associated with arousal; Simpson, 1968) more often than males in water conditioned by nondisplaying fish. However, work by Ingersoll et al. (1976) on the same species showed no difference. Neither of these studies examined the responses of females.

The chemicals released by male sticklebacks may constitute a signal similar to birdsong. However, females did not approach water from the displaying male more often than expected by chance. Females may not use chemical cues in mate choice. Alternatively, females may have chosen between males on the basis of chemical cues that were not influenced by whether the individual was displaying; i.e., the cues emitted by displaying males may make them more obvious but not more attractive. Furthermore, some females may not have been motivationally or physiologically ready for courtship even though they appeared gravid.

Male sticklebacks prefer to nest near other males (Assem, 1967). Thus, we may have expected males to be attracted to, instead of repelled by, cues from displaying males. However, a signal's effect may depend on the context in which it is performed. For example, a songbird's call may repel birds that trespass on their territory but attract more distant animals searching for breeding areas. In our study, chemical cues were released by an unseen, nearby male; thus, the test males may have perceived themselves as trespassers and avoided the displaying animal. This does not negate the possibility that males approach cues from distant males when searching for breeding areas.

Finally, it is possible that the chemical cues detected by males and females were a simple by-product of metabolic processes (e.g., associated with movement during displays); for example, Sevenster (1961; see also van Iersel, 1953) found that male sticklebacks exposed to brief CO_2 baths performed fewer sexual and more aggressive activities than males exposed to tap water. Perceivers may be using cues that are not "actively conveyed" by the donor (Liley, 1982). In some cases, the cues may be precursors of true pheromones; for example, it has been suggested that sexual pheromones in fish evolved from excreted hormones and their metabolites (Doving, 1976; Colombo et al., 1982).

Sticklebacks have been described as having a very limited ability for chemosensory perception (e.g., Bardach and Todd, 1970; Wootton, 1976). The present study and other recent work (e.g., van Havre and FitzGerald, 1988; FitzGerald and van Havre, 1987 suggests that this may not be the case. Despite their small olfactory epithelium (which has only two folds; Teichmann, 1959), sticklebacks detect and react to chemical cues associated with their visually elaborate displays.

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ALLELOPATHIC SUBSTANCES AND INTERACTIONS OF *Delonix regia* (BOJ) RAF[1]

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Abstract—A unique pattern of weed exclusion was found under the canopy of *Delonix regia*, which was planted in many places as an ornamental tree in the south of Taiwan. A quadrat method was employed to examine the botanical composition between the area underneath *D. regia* and its adjacent control grassland. The number of species and coverage of understory species were significantly lower in the area of the *D. regia* than that of the grassland, indicating the growth of understory species was suppressed by *D. regia*. A series of aqueous extracts of leaves, flowers, and twigs of *D. regia* were bioassayed against three species to determine their phytotoxicity, and the results showed highest inhibition in the flowers. A water-culture experiment indicated the aqueous extract of flowers of *D. regia* on two local understory species (*Isachne nipponensis* and *Centella asiatica*) inhibited growth of both species by more than 70%. The phytotoxicities of fallen leaves and flowers of *D. regia* were not significantly affected by temperature. When the plant material was subjected to temperatures above 70°C, however, phytotoxicity was decreased, indicating that the allelopathic nature of *D. regia* could easily be decomposed by fire. By means of paper, thin-layer, and high-performance liquid chromatography, and UV–visible spectrophotometry, responsible phytotoxins present in leaves, flowers, and twigs of *D. regia* were identified as 4-hydroxybenzoic, chlorogenic, 3,4-dihydroxybenzoic, gallic, 3,4-dihydroxycinnamic, 3,5-dinitrobenzoic, and L-azetidine-2-carboxylic acids, and 3,4-dihydroxybenzaldehyde. The findings of bioassays and the number and amount of responsible allelopathic compounds found in *D. regia* are well correlated, thus permitting the conclusion that the exclusion of understory plants under the canopy of *D. regia* trees was due primarily to the allelopathic

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effect of the fallen flower, leaves, and twigs of the *D. regia*. A possible mechanism of action is discussed.

Key Words—*Delonix regia*, ornamental tree, allelopathy, phytotoxic phenolics, L-azetidine-2-carboxylic acid, understory species, mechanism of action.

INTRODUCTION

Delonix regia (Boj.) Raf., a deciduous tropical tree distributed in the Philippines, Indonesia, Africa, and many other parts of the world, was introduced to Taiwan many centuries ago as an ornamental tree. The trees were planted along roadsides and at school campuses and parks in the south of Taiwan. A relatively thick mat of fallen flowers, leaves, seed pods, and twigs of *D. regia* is found under the canopy of *D. regia*, but understory plants are almost entirely lacking. The unique pattern of the bare area underneath the trees has been observed by the senior author for several decades, yet the mechanism of formation of the pattern has not been studied. In the past, systematic and pathologic investigations of *D. regia* were carried out (Ather and Mahmood, 1980; Gill and Husaini, 1982; Ghouse and Hashmi, 1977; Hodges and Tenorio, 1984). Biochemical ecological studies of the plant were scant, with Sung and Fowden (1969) isolating azetidine-2-carboxylic acid from seedlings of *D. regia*, which would interfere with the biosynthesis of proline. The physiological and ecological significance of L-azetidine-2-carboxylic acid and its related natural products have not been well understood, although much allelopathic research of leguminous plants has been carried out in many parts of the world (Chou and Waller, 1980, 1989; Chou and Kuo, 1986; Chou, 1992; Rice, 1984; Waller, 1987.) This study was thus aimed at elucidating the allelopathic interaction of the understory species with *D. regia* and the mechanism thereof.

METHODS AND MATERIALS

Study Site. Delonix regia was planted in many places in the southern parts of Taiwan. When the trees bloom in the summer, they are beautiful in appearance. The present study sites were selected from the campuses of the Taiwan Livestock Research Institute and the National Cheng Kung University (NCKU), at Tainan, Taiwan. Adjacent to the *Delonix regia* trees, a control grass area was also selected for comparison. The physical environments for the two study areas were not significantly different.

Field Measurements. The botanical composition of understory species under *D. regia* and the controlled grass area was investigated by using a quadrat method, 2 × 2 m each; coverage and presence of each species present in the two plantations was measured. Ten quadrats were used along a transection of

D. regia trees and in the grassland. All of the species appearing in the quadrats were identified. The measurements were done quarterly for two years (1990–1992), in order to determine seasonal succession.

Materials. Plant materials, including leaves, flowers, and twigs, were collected from each study site. Some of the samples were air-dried at room temperature, and the remainder were stored at −5°C prior to use. Under the canopy of *D. regia*, litter was collected from the surface of the ground (designated as A_{00}); soil beneath the litter at a depth of 0–5 cm from the soil surface (designated as A_0), and rhizosphere soil (A_1) at depths of 5–10 cm in 34 × 34-cm sections were also sampled in each quadrat. The soils were air-dried and screened through a 2-mm sieve to remove all visible plant residue. After preparation, plant and soil samples were used for bioassay, chemical extraction, and identification of some components.

Preparation of Aqueous Extracts of D. regia *Leaves, Flowers, Twigs, and Soil.* A series of aqueous extracts of air-dried leaves, flowers, and twigs was separately prepared by adding 2, 4, 6, 8 and 10 g to 198, 196, 194, 192, and 190 ml of distilled water, respectively, shaking the mixture for 2 hr, and then subjecting to vacuum filtration. Another series of extracts was prepared by adding 1 g of air-dried plant material to 99 ml dichloromethane, shaking for 2 hr, after which the CH_2Cl_2-soluble extract was collected; the plant residue was dried at room temperature (15–20°C). The same procedure was used in preparing the methanol extraction. To the dry residues from the dichloromethane and methanol, 99 ml distilled water was added to prepare the extracts. Soil extracts were prepared by shaking 150 g of each soil with 500 ml distilled water for 2 hr and centrifuging the mixture at 3000 rpm (2369 *g*). All plant and soil extracts were stored at −5°C before bioassay.

Bioassay Techniques. Two bioassay techniques were employed to determine the phytotoxicity of the leaf, flower, twig, and soil extracts. A modified sponge bioassay described by Chou and Young (1975) and a modified chromatographic bioassay described by Chou and Chung (1974) were employed using test seeds as follows: *Lactuca sativa* Linn. (lettuce), *Medicago sativa* Linn. (alfalfa), *Brassica chinensis* Linn. (Chinese cabbage), *Ageratum conyzoides*, *Isachnne nipponensis*, and *Centella asiatica*. Some of these test plants were also found to occur in the experimental sites. Distilled water served as a control for the bioassays. Tests and controls were set up in triplicate.

The authentic compounds, azetidine-2-carboxylic acid, and phenolic acids were prepared in a series of aqueous solutions, 10, 20, 40, 80, 160, 320 and 500 ppm for bioassay, using the same technique as described to determine phytotoxicity. The radicle growth of seeds was recorded 72 hr after incubation at 25°C in an incubator. The bioassay data were subjected to statistical analysis using Student's *t* test and Duncan's multiple range test (Gomez and Gomez, 1976).

Temperature Treatment on Plant Materials of D. regia. In order to determine the effect of temperature on the phytotoxic nature of leaves, flowers, and twigs of the plant, these materials were treated at temperatures of 37°C, 70°C, and 110°C, respectively. The samples were then extracted with distilled water, using the same techniques as described earlier. The 1% aqueous extracts were bioassayed against lettuce seeds.

Greenhouse Experiments Using Pots. Young seedlings of *Ageratum conzoides* transplanted from the field into pots were allowed to grow to 10 cm height. The seedlings then were separately watered with 1%, 2%, or 3% aqueous extracts from the flowers of *D. regia* using tap water as a control. The seedling growth of *A. conzoides* as affected by the test solutions was compared daily until the fifteenth day after treatment.

Water Culture. Three stolon cuttings (about 15 cm long, cut between two young nodes) of each tested plant (*Centella asiatica* and *Isachne nipponensis*) were used and grown in a 15-cm glass tube (3 cm internal diameter) containing either distilled water as a control or aqueous extracts of *D. regia* plant parts. Each treatment was run in triplicate, and the tubes were placed in an incubator at 25°C for seven days; then the root length of each plant was measured (Leu, 1991).

Quantitative Determination of Allelopathic Substances. The allelopathic substances present in the aqueous extracts of the *D. regia* plant parts were obtained by using extraction and purification techniques as described by Chou and Yao (1983) and Leu (1991). The phytotoxins from the *D. regia* rhizosphere soil were obtained using extraction techniques developed by Wang et al. (1967a) and Tang (1986). Compounds were identified by paper chromatography (Wang et al., 1967b) and a high-performance liquid chromatograph (HPLC), using an LDC/Milton Roy ConstaMetric 3000, SpectroMonitor 3000, flow rate of 1 ml/min, wavelengths at 254 nm and 280 nm, a column of Lichrospher 100 RP-18 (250 mm, 4 mm ID), and using the solvent methanol–distilled water–glacial acetic acid (30:70:1, v/v/v). The amount of L-azetidine-2-carboxylic acid was determined by techniques described by Leu (1991): an aliquot of ninhydrin solution and the isolated compound were mixed, then determined by a UV–visible spectrophotometer at OD 570 nm, and the same technique was used to compare the authentic standard compound for reference material.

RESULTS

Botanical Composition in Study Sites of D. regia. The area under the canopy of *D. regia* trees lacks understory plants (Figure 1A), but outside the canopy there is luxuriant growth of weeds (Figure 1B). The floristic composition in the

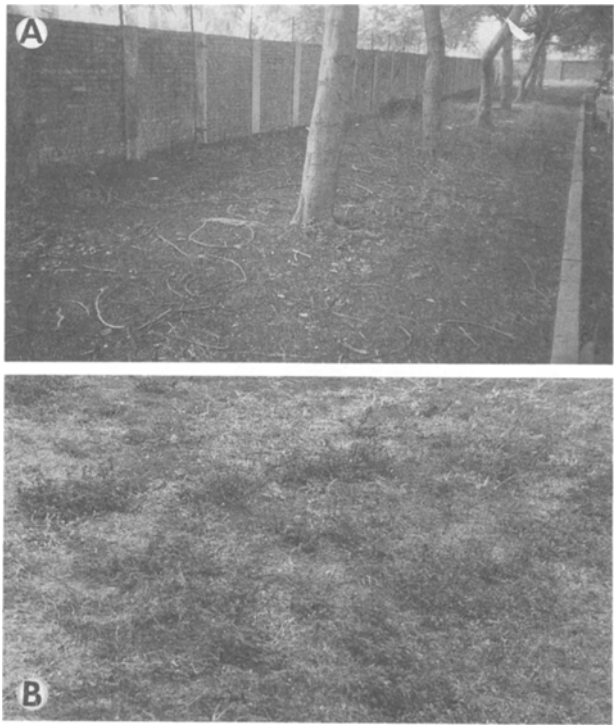

FIG. 1. A pattern of vegetation difference of understory plants shown on the tree of *Delonix regia* floor (A) and the adjacent grassland control (B) at the campus of the National Cheng Kung University, Tainan, Taiwan.

D. regia understory at the NCKU site included 29 species, while in the adjacent area of grassland control there were 38 species (Table 1). Of them, *Alysicarpus vaginalis*, *Borreria medium*, *Cyperus rotundus*, *Desmodium renifolium*, *Gnaphalium purpureum*, *Indigofera trifoliata*, *Ipomoea acuminata*, *Mimosa pudica*, *Oxalis corymbosa*, *Phyla nodifolia*, *Sida rhombifolia*, *Sporobolus hirta*, and *Zoysia* sp. were the most abundant in the control site. These species were either absent or less abundant on the floor of the *D. regia* tree site (Table 1), showing that the coverage of the plants at each site was significantly different.

Although climatic changes that occurred during the four seasons were not distinctly different, summer and winter seasons were different enough that there was variation in botanical composition. There was a significantly different number of species affected by seasons, being sharply decreased from spring to winter; significant differences of the number of species also was observed in the areas of control and the *D. regia* sites. The amount of coverage of understory

TABLE 1. COMPARISON OF BOTANICAL COMPOSITION OF UNDERSTORY SPECIES BETWEEN SITES OF *Delonix regia* AND GRASSLAND CONTROL, 1990–1992

Species name	Coverage (%)									
	Control (grassland)					*Delonix regia*				
	Spr.	Sum.	Aut.	Win.	Mean	Spr.	Sum.	Aut.	Win.	Mean
Ageratum conyzoides	+[a]	+	+	+	+	+	–[b]	–	–	–
Alysicarpus vaginalis	40	41	–	43	43	2	–	+	–	2
Blechum pyramidatum	3	–	–	–	3	+	–	–	–	+
Borreria articularis	20	–	–	–	20	–	–	–	–	–
Cardamine arisanensis	1	–	–	–	1	6	–	–	–	6
Centella ariatica	+	+	–	–	+	–	–	–	–	–
Chloris barbata	+	–	–	–	+	+	–	–	–	+
Cyperus rotundus	5	2	2	–	3	1	+	–	–	+
Cyperus sp.	–	13	1	13	9	+	–	+	–	+
Dactyloctenium egyptiuam	–	–	+	–	+	–	–	+	–	+
Delonix regia	–	–	–	–	–	–	–	–	+	+
Desmodium triflorium	–	40	28	33	33	–	+	–	–	+
Digitaria sanguinalis	+	–	–	–	+	–	–	–	–	–
Eleusine indica	2	2	2	–	2	–	–	–	–	–
Eragrostis amabilis	–	–	–	+	+	–	–	–	–	–
Erigeron bonariensis	1	–	–	–	1	+	–	–	–	+
Eucalyptus sp.	–	–	+	–	+	–	–	–	–	–
Gnaphalium purpureum	27	–	–	–	27	2	–	–	–	2
Gomphnena celosioides	–	–	–	–	–	+	–	–	–	+

Hedyotis diffusa	1	–	4	–	3	–	–	–	–	–
Humulus scandens	–	+	–	–	+	–	–	–	–	+
Indigofera trifoliata	28	–	–	–	28	–	–	–	–	–
Ipomoea acuminata	+	+	–	–	+	1	+	–	–	+
Isachne nipponensis	1	–	–	–	1	+	–	–	–	+
Ixeris chinensis	+	–	–	–	+	+	–	–	–	+
Kyllinga sp.	–	–	2	–	2	–	–	–	–	–
Lantana camara	+	–	+	+	+	–	–	+	+	+
Malvastrum coromandelianum	5	–	+	–	3	+	–	–	–	+
Merremia sp.	–	3	+	18	7	–	–	+	–	+
Mimosa pudica	18	–	–	31	25	+	–	–	–	+
Mosla formosana	–	–	–	+	+	–	–	–	–	–
Oxalis corymbosa	–	13	13	21	15	–	+	–	+	+
Paederia scandens	–	–	–	4	4	–	–	–	–	–
Pennisetum setosum	1	–	–	–	1	–	–	–	–	–
Phyla nodiflora	7	25	27	42	23	3	+	+	–	1
Sida rhombifolia	30	12	12	16	18	+	–	–	–	+
Sida veronicaefolia	–	–	–	–	–	+	–	–	–	+
Sporobolus fertilis	4	–	–	–	4	1	–	–	–	1
Synedrella nodiflora	–	–	+	–	+	–	–	+	–	+
Urtica sp.	–	+	–	–	+	–	+	–	–	+
Vernonia parviflora	+	–	–	–	+	+	–	–	–	+
Zoysia spp.	50	–	–	–	50	–	–	–	–	–
Total	244	151	91	221	326	16	+	+	+	12
Total number of species					39					29

[a] + indicates the species present but the average number is less than 1.
[b] – indicates the species absent.

species was drastically lower on the *D. regia* plot than in the control site (Figure 2), indicating that both physical competition and biological interaction of allelopathy could possibly be involved. However, most of the species appearing on the ground under *D. regia* were neither light sensitive nor light tolerant (Chou and Yang, 1982; Chou and Kuo, 1986). Since the two study sites were situated close together, their physical properties, such as soil characteristics, moisture, and texture were approximately the same. Thus, the relatively bare ground with little survival of weeds was probably not due to physical competition among species.

Phytotoxic Effects of Aqueous Extracts of Delonix regia *Plant Parts.* Aqueous solutions comprised of 1%, 2%, 3%, 4%, 5%, of flowers, leaves, and twigs of *D. regia* were bioassayed against three test plants, namely alfalfa (*Medicago sativa*), lettuce (*Lactuca sativa*), and Chinese cabbage (*Brassica chinensis*). Radicle growth of lettuce and Chinese cabbage was most significantly inhibited by the extracts, while the growth of alfalfa was inhibited to a lesser extent (Figure 3). Among the extracts, the phytotoxicity was remarkably higher in the flowers than in the others, ranging from 20% to 80% inhibition for the 1% extract and from 25% to 90% for the 5% extract. The degree of inhibition increased with an increase of concentration of the extracted plant material.

The increasing inhibition of the extracts upon the tested plant material might be caused by an osmotic and/or pH effect. The extracts were analyzed for osmotic concentration using a Fiske osmometer. The osmotic concentrations of 3% extracts of leaves, flowers, and twigs were all below 50 mosmol (Table 2). Neutral solutions, such as polyethylene glycol, sucrose or manitol solution,

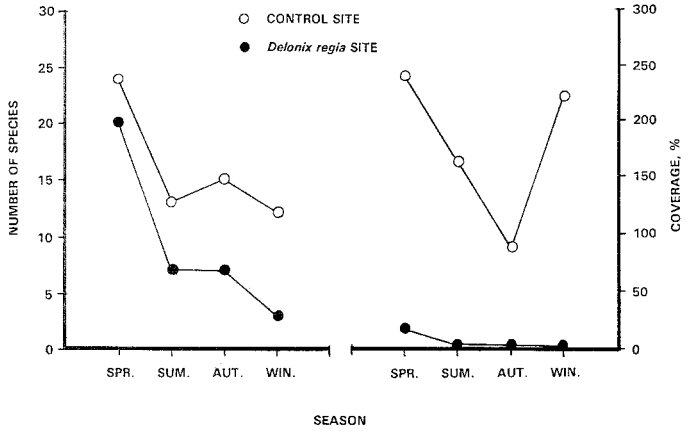

FIG. 2. A comparison of seasonal variation on the number of species and coverage by understory species on the *D. regia* floor and its adjacent grassland control sites.

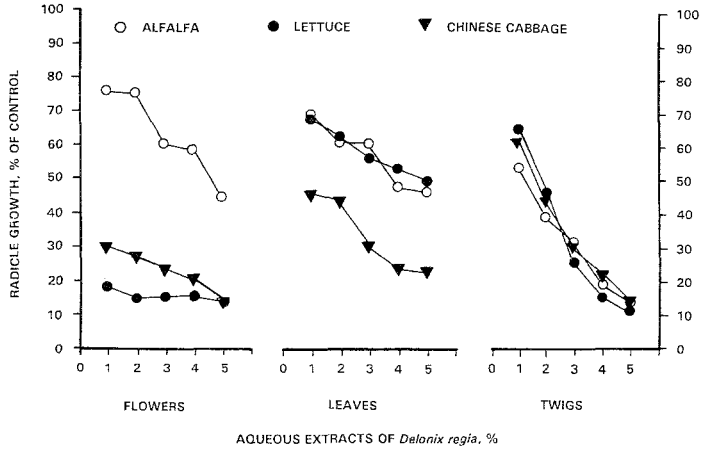

FIG. 3. The effects of aqueous extracts of flowers, twigs, and leaves of *D. regia* using a series of concentrations on the radicle growth of alfalfa, lettuce, and Chinese cabbage.

TABLE 2. COMPARISON OF OSMOTIC CONCENTRATION AND pH OF AQUEOUS EXTRACTS OF FLOWERS, LEAVES, AND TWIGS OF *D. regia* COLLECTED IN 1990–1992

Concentration (%)	Osmotic concentration (mosmol/kg)			pH		
	Flower	Leaf	Twig	Flower	Leaf	Twig
1	16	12	12	4.47	6.08	6.19
2	31	22	30	4.43	6.16	6.15
3	48	33	45	4.37	6.19	6.08
4	67	42	62	4.34	6.19	6.04
5	83	52	72	4.32	6.28	6.04

cause less than 25% osmotic inhibition in the lettuce bioassay at or below 50 mosmol (Chou and Young, 1974). In the present study, the lowest pHs of the extracts ranged from 4.32 to 4.47, which should give about 20% inhibition if neutral solutions adjusted to the same pH and osmotic concentration were employed (Chou and Young, 1974). This degree of inhibition is here called "osmotic and pH inhibition." Thus, if the inhibition yielded by *D. regia* is above 50%, the inhibition after subtracting 20% osmotic and pH inhibition will still be 30%. Thus, the inhibition is due naturally to phytotoxic inhibition. In the present study, the inhibition of plant extracts was significantly higher than 50%, which means that the inhibitory effects of aqueous extracts of flowers,

leaves, and twigs are due to the phytotoxic compounds present in the extracts, and the effects of osmolality of pH can be neglected. The degree of inhibition is shown to be in the order: flower > leaves > twig.

To ascertain if the growth inhibitors were hydrophilic or hydrophobic, the leaves, flowers, and twigs were extracted with dichloromethane and methanol. The aqueous solutions were bioassayed against lettuce and the results obtained are shown in Figure 4. The findings indicated that the water extract that followed the CH_2Cl_2 extraction exhibited nearly the same degree of inhibition (Figure 4B) as the original water extract (Figure 4A), while the aqueous extract that followed after methanol extraction showed only a little inhibition (Figure 4C). It was concluded that the inhibitor was hydrophilic in nature.

Leachability of Phytotoxins from Plant Parts and Soil of D. regia. A 5% aqueous extract of flowers, leaves, twigs, and soils of *D. regia* was obtained. The first extract was designated as extract I, and excluded the soil; the residues were then reextracted with water twice and designated as extracts II and III. All extracts were bioassayed against lettuce to compare their phytotoxicities. The results shown in Figure 5 indicated that most of the phytotoxic substances are

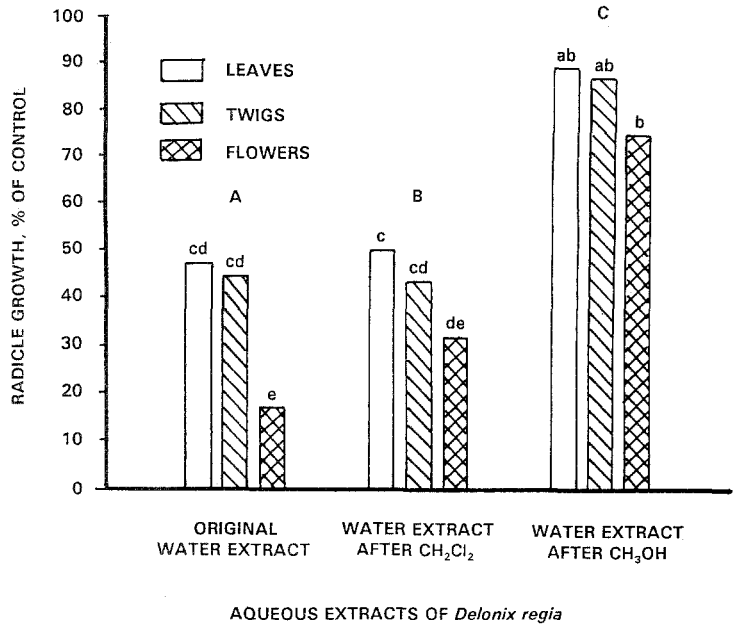

FIG. 4. A comparison of phytotoxicity of 1% aqueous extracts of plant parts of *D. regia*. The aqueous extracts were obtained: (A) by the original water extract, (B) after extraction with dichloromethane, and (C) after extraction with methanol. Effects are on the radicle growth of *Brassica campestris* L.

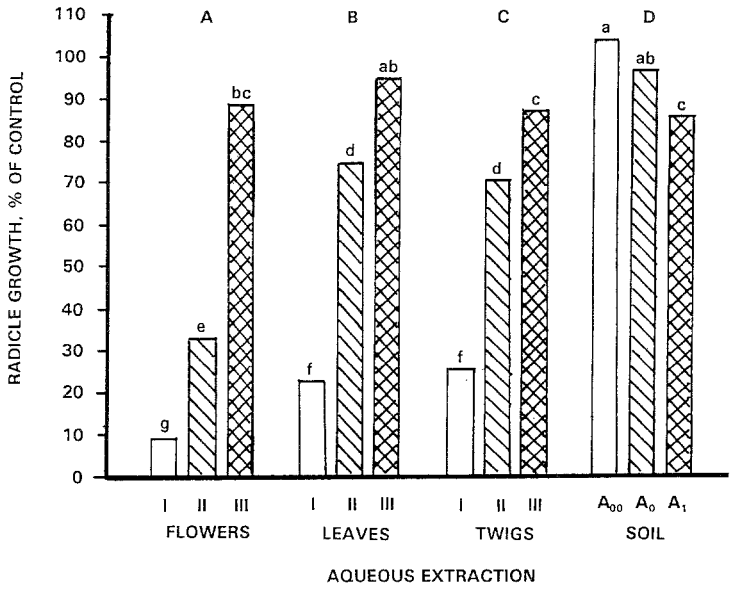

FIG. 5. The effects of aqueous extracts of plants of *D. regia* and its soil on the radicle growth of lettuce. The plant extracts were obtained by sequential extraction: 5% aqueous extracts of flowers, leaves, and twigs of *D. regia* were obtained (I), the residues were extracted with water twice and designated as extracts II and III. The soil was collected from different depths of the soil profile; the litter collected from the soil surface is designated as A_{00}, soil collected beneath the litter layer of surface soil to a depth of 0–5 cm is designated as A_0, and the soil collected beneath A_0 layer to 10 cm depth is designated A_1.

present in extracts I and II, and little toxicity remains in extract III. This finding suggests that the leachability of allelopathic substances in plant parts of *D. regia* is high.

Regarding the bioassay of soil extracts, A_{00} (litter on ground), A_0 (soil beneath litter at depth 0–5 cm), and A_1 (rhizosphere soil at depth 5–10 cm), the results show that A_{00} revealed stimulation, while soil A_0 exhibited neither stimulation nor inhibition, and soil A_1 had an inhibitory effect (Figure 5). The findings indicated that the allelopathic substances could be water soluble and leached out from the litter through the soil surface and down to the deep soil by rainfall.

Effects of Temperature on Phytotoxicity of Plant Parts of D. regia. During the summer in Tainan, the surface soil temperature may go up to 40–50°C. The plant material exposed to such temperatures may be deleteriously affected,

resulting in denaturation of chemical constituents that lead to a change of phytotoxic properties. The bioassay results of aqueous extracts of leaves, flowers, and twigs treated by temperatures of 37°C, 70°C, and 110°C are given in Figure 6. It shows that about 60% phytotoxicity remains in the leaves, flowers, and twigs at 37°C, but the toxicity in twigs is greatly reduced at 70°C and lost at 110°C for leaves and twigs. Nevertheless, 60% of the toxicity was still retained in the flowers treated at 110°C, suggesting that phytotoxic constituents present in the plant parts of *D. regia* are different.

Inhibitory Effect of Aqueous Extracts of D. regia *Flowers on Plant Growth in Pot.* Since most inhibitory agents acting upon the growth of understory species of *D. regia* were due to dropping flowers, a further investigation was undertaken to determine the effect of the extract on the growth of *Ageratum conyzoides*. One percent, 2%, and 3% extracts of the flower of *D. regia* were used on 10-cm-high seedlings of *A. conyzoides* that were transplanted into pots in the greenhouse; the results are shown in Table 3. When the *A. conyzoides* were watered with tap water (control) and the 1% extract of flowers, they grew well during a period of 15 days. However, the plants grew quite poorly when they were watered with 2% or 3% flower extract. The plants began to show chlorosis at the seventh day with 2% extract and at the third day with 3% extract. The plants began to wilt at the ninth day with 2% extract and the seventh day with

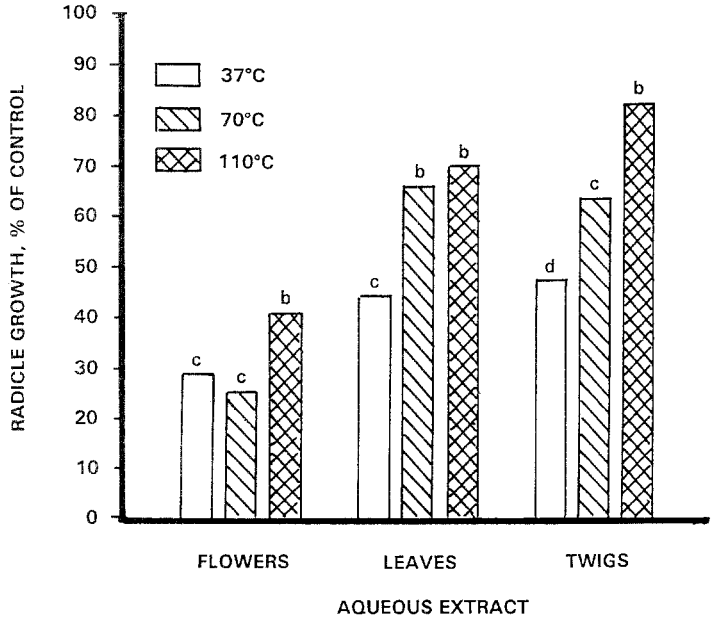

FIG. 6. The effects of temperature on the phytotoxicities of aqueous extracts of flowers, leaves, and twigs of *D. regia* on the radicle growth of lettuce.

TABLE 3. INHIBITORY EFFECTS OF AQUEOUS EXTRACTS OF FLOWERS OF *D. regia* ON SEEDLING GROWTH OF *Ageratum conyzoides* PLANTED IN AUTUMN 1990

	Concentration (%)					
DAI[a]	0 (control)	1	2		3	
1	A[b]	A	A		A	
3	A	A	A		1B	
5	A	A	A		3B	
7	A	A	1B		3B	2C
9	A	A	1B	2C	2B	5C
11	A	A	2B	10C	2B	11C
15	A	A	3B	10C	1B	12C

[a] Day after irrigation with extract.
[b] A = normal growth, B = leaf chlorosis, C = leaf wilt.

3% extract, suggesting that the leachate of *D. regia* flowers could significantly suppress the growth of understory species.

In addition to that, water culture experiments were employed to assist in gaining an understanding of the response of *Isachne nipponensis* and *Centella asiatica* to the flower extracts. Both species were commonly found in the university campus, but the latter species was occasionally found in the control site and was absent under the canopy of *D. regia* trees. In the 1% extract experiment, the root growth of both plants was severely retarded and the inhibition was 65% for *I. nipponensis* and 73% for *C. asiatica* as compared to the distilled water control. In the 2% extract, the inhibition increased up to 82% for the former and 100% for the latter species. Moreover, when the extract concentration was increased to 3%, the inhibition went up tremendously to about 100% for both species (Figure 7). The findings suggested that the low percentage of both species under the canopy of *D. regia* was apparently due to the allelopathic interaction of the species.

Identity of Allelopathic Substances in D. regia. The responsible allelopathic substances present in aqueous extracts of flowers, leaves, and twigs of *Delonix regia* were identified by paper chromatography and quantitatively estimated by HPLC, using standard compounds. The compounds identified were chlorogenic, 4-hydroxybenzoic, 3,4-dihydroxybenzoic, gallic, 3,4-dihydroxycinnamic, 3,5-dinitrobenzoic, L-azetidine-2-carboxylic acids and 3,4-dihydroxybenzaldehyde (Table 4). Of them, chlorogenic acid and 3,4-dihydroxybenzaldehyde were absent in leaf; L-azetidine-2-carboxylic acid was not found in twigs; and four compounds, 4-hydoxybenzoic, 3,4-dihydroxycinnamic, 3,5-dinitrobenzoic, and L-azetidine-2-carboxylic acids were missing in the flowers (Table 4).

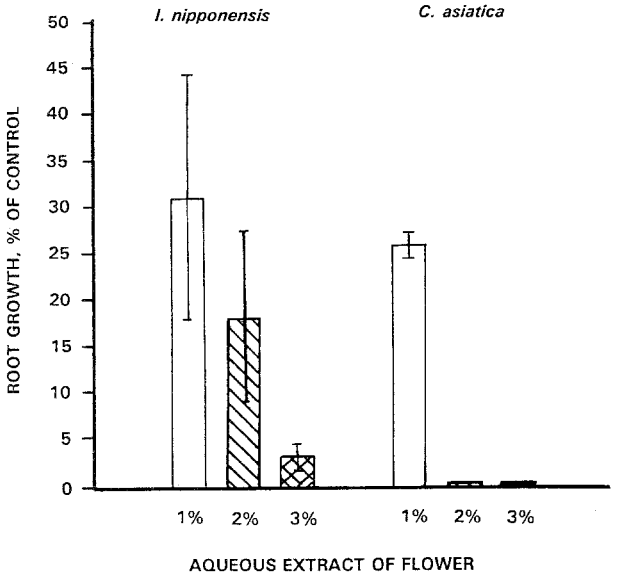

FIG. 7. Comparison of phytotoxic effect on a series of aqueous extracts, 1%, 2% and 3%, of *D. regia* on the seedling growth of *I. nipponensis* and *C. asiatica*. The experiment was conducted in water culture by using extracts as tests; tap water served as a control.

TABLE 4. QUANTITATIVE DETERMINATION OF SOME ALLELOPATHIC COMPOUNDS PRESENT IN FLOWERS, LEAVES, AND TWIGS OF *D. regia*

Compound[b]	Concentration (10^{-3} mg/g sample)		
	Flower	Twig	Leaf
Chlorogenic acid	2.15	0.03	
3, 4-Dihydroxybenzoic acid	5.24	0.22	0.13
Gallic acid	0.25	0.20	0.04
3, 4-Dihydroxybenzaldehyde	1.78	0.01	
4-Hydroxybenzoic acid		0.05	1.62
3, 4-Dihydroxycinnamic acid		0.10	0.11
3, 5-Dinitrobenzoic acid		0.82	1.17
L-Azetidine-2-carboxylic acid[c]			0.55
Total	9.42	1.43	3.62

[a] The plant materials were collected in summer of 1990.
[b] Amounts of phenolic compounds were quantitatively identified by means of HPLC, while that of L-Azetidine-2-carboxylic acid was determined by UV spectrophotometer at OD 570 nm.
[c] From fresh leaves of *Delonix regia*.

Chromatographic Bioassay of Compounds Isolated from Leaf, Flower, and Twig of D. regia. By using a chromatographic bioassay as described by Chou and Chung (1974), spots appearing on paper chromatograms developed in 2% acetic acid were cut and bioassayed against lettuce seeds. The same chromatographic paper without spotting with extract was used as control. The bioassay results for chromatographic segments according to R_f values showed both stimulation and inhibition (Table 5). The chromatograms from flowers and twigs showed higher inhibition than leaves, indicating that most allelopathic compounds were released from these two parts.

To further confirm the allelopathic properties of the compounds isolated, chlorogenic, L-azetidine-2-carboxylic, gallic, 4-hydroxybenzoic, 3,4-dihydroxybenzoic, 3,4-dihydroxycinnamic, and 3,5-dinitrozenzoic acids and 3,4-dihydroxybenzaldehyde prepared in a series of concentrations of aqueous solutions (10, 20, 40, 80, 160, 320, and 500 ppm) were bioassayed with lettuce seeds. The bioassay results are given in Figure 8 and indicate that the significant inhibition above 30% of the control was shown at 10 ppm. The inhibition increased when the concentration of compounds increased. Most of these compounds have been confirmed to be allelopathic by many scientists (Chou and Lin, 1976; Chou and Muller, 1972; Chou and Lee, 1991; Tang, 1986; Waller,

TABLE 5. CHROMATOGRAPHIC BIOASSAY OF INDIVIDUAL FRACTIONS OF AQUEOUS EXTRACTS FROM LEAVES, FLOWERS, AND TWIGS OF *D. regia*

R_f (×100%, in 2% HOAC) Spot zone	Radicle growth (% inhibition of control)		
	Leaf	Flower	Twig
0.00–4.7	66[a]	88	71
4.7–6.0	−5	−3	−3
6.0–10.0			21
18–28		18	28
28–35		5	59
35–42		22	14
42–52	17	22	46
52–58			72
58–66	3	28	38
66–72	−11	0	−21
72–82	14	7	0
82–90	33		
90–96	40		
Solvent front (96–100)			

[a]Positive data indicate percent inhibition, while the negative data are percent stimulation.

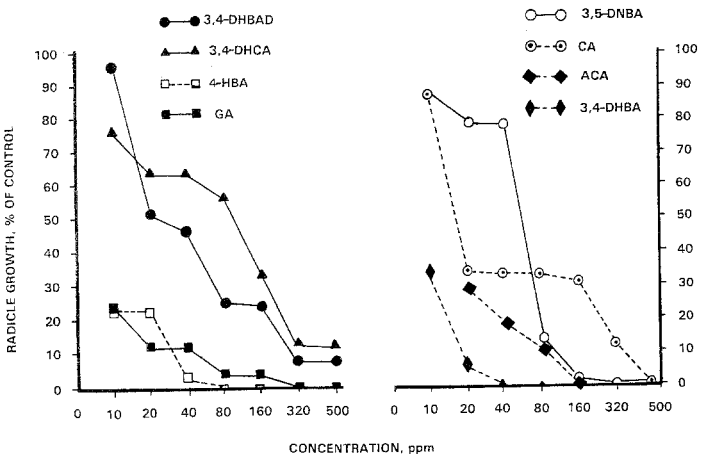

FIG. 8. A comparison of a series of concentrations of phytotoxic substances on the radicle growth of lettuce. The abbreviations of compounds are: CA = chlorogenic acid; 3,4-DHBA = 3,4-dihydroxybenzoic acid; GA = gallic acid; 3,4-DHBAD = 3,4-dihydroxybenzaldehyde; 4-HBA = 4-hydroxybenzoic acid; 3,4-DHCA = 3,4-dihydroxycinnamic acid; 3,5-DNBA = 3,5-dinitrobenzoic acid; ACA = Azetidine 2-carboxylic acid.

1987). L-azetidine-2-carboxylic acid was first reported to be an allelochemical compound.

DISCUSSION

A unique pattern of weed exclusion under the canopy of *Delonix regia* has been found in the tropical areas of Taiwan. In autumn, the flowers of *D. regia* trees blossom and a substantial numbers of flowers, leaves, twigs, and pods fall to the ground. These fallen plant parts could play an important role in establishing the unique pattern. On the other hand, competition for physical factors, such as light, soil moisture, and nutrients between weeds and *D. regia* could not be ruled out from the interaction, and some attention in examining these and other pertinent factors should be carefully investigated. The competition for light was not important between trees of *D. regia* and its understory species because the light intensity measured under the canopy of *D. regia* was sufficient for the growth of understory species; in addition, the soil moisture and nutrient contents were much higher in the soil of *D. regia* (Leu, 1991), reflecting that the physical competition for light, soil moisture, and nutrients were not significant between the *D. regia* and its understory species. Moreover, the relatively bare ground underneath *D. regia* was not due to mammal activities, because the

area has been fenced (Figure 1A) since the university was established and was taken care of by the university gardeners.

However, the gardeners regularly collected and burned the litter (photos shown in Leu, 1991). In the burn area under the *D. regia* trees, there is an apparent lack of weeds appearing for up to a year. Chou (1973) indicated that burning does not completely denature the phytotoxins present in the soil layer below 5 cm deep of the California chaparral, such as *Arctostaphylos glandulosa* var. *zacaensis*. McPherson and Muller (1969) indicated that the removal of *Adenostoma fasciculatum* in the chaparral vegetation of southern California resulted in luxuriant growth of weeds, which appeared in the first year following the fire. Some of the allelopathic compounds could be decomposed and lose their phytotoxic activity when the temperatures rise to 110°C and higher by surface fires. This suggests that different allelopathic constituents have different responses to the fire. Chou further examined the plantation of *D. regia* on the campus of the Taiwan Livestock Research Institute and found that a substantial number of weeds appeared under the canopy of *D. regia* trees one year after the trees were removed (Chou, 1989 personal observation). The finding suggested that the removal of the toxic source from *D. regia* resulted in reappearance of weeds in the site.

The accumulation of litter, leachates, and metabolites formed from biodegradation were found to be allelopathic agents suppressing the growth of plants under the canopy of *D. regia* trees. The present bioassay findings strongly support the allelopathic hypothesis for the unique pattern of weed exclusion. Aqueous extracts of the *D. regia* plant parts revealed significant phytotoxicity, with the flowers of the plants exhibiting the highest inhibition of tested plant parts. It was found that the phytotoxins present in *D. regia* were water soluble and thus were leached by rain. Consequently, a continuous supply of fallen leaves, twigs, pods, and flowers ensures that allelopathic compounds are constantly provided to suppress the growth of understory species.

In the present study, eight phytotoxins were identified. Of them, L-azetidine-2-carboxylic acid was first reported here as an allelopathic substance. We found that the aqueous extract of flowers of *D. regia* caused the highest phytotoxicity among extracts of plant parts; however, only four phenolics were present and four were missing in flowers, suggesting that other important water-borne phytotoxin(s) should be identified. Casinovi et al. (1983) and Heisey (1990a,b) indicated that most Simarubaceae plants possess water-borne phytotoxins that are methanol-soluble quassinoids.

In conclusion, our results suggest that the exclusion of plants under the canopy of *Delonix regia* was due primarily to allelopathic interaction. Some of the responsible allelopathic substances were identified; however, some other phytotoxins, such as anthocyanins, flavonoids, or quassinoids which may be present in plant parts of *D. regia*, need to be positively identified before the mechanism of action can be established.

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THE ROLE OF LANIERONE IN THE CHEMICAL ECOLOGY OF *Ips pini* (COLEOPTERA: SCOLYTIDAE) IN CALIFORNIA

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Abstract—Five doses of lanierone (2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one) were tested with one dose of enantiomerically pure [99.4% (4*R*)-(−)] ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) for activity as an aggregation pheromone of *Ips pini* (Say) in California. The response of *I. pini* to 1 mg/day ipsdienol + 20 μg/day lanierone was significantly greater than the response to ipsdienol alone, but the response pattern did not demonstrate a clear dose–response relationship. The response to the highest dose of lanierone (2 mg/day) was significantly lower than the response to ipsdienol alone. Ipsdienol attracted significantly more *I. pini* than a male-infested log. Lanierone did not alter the percentage of male *I. pini* responding to ipsdienol alone. Neither sex of *I. pini* or *Dendroctonus brevicomis* LeConte from California produced detectable amounts of lanierone, but myrcene-aerated male *D. brevicomis* produced 97.8%-(4*S*)-(+)-ipsdienol. The black-bellied clerid, *Enoclerus lecontei* (Wolcott) (Coleoptera: Cleridae) was attracted to lanierone when released with ipsdienol. Neither compound was attractive when released

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alone, proving synergism for the kairomone of this predator. Lanierone did not influence the response of the predators *Temnochila chlorodia* (Mannerheim) (Coleoptera: Trogositidae) and *Enoclerus sphegeus* (F.) (Coleoptera: Cleridae), which were attracted to all treatments containing ipsdienol. *Tomicobia tibialis* Ashmead (Hymenoptera: Pteromalidae) responded in significantly greater numbers to the male-infested log than it did to ipsdienol or ipsdienol + 20 μg/day lanierone.

Key Words—Coleoptera, Scolytidae, Cleridae, Trogositidae, Pteromalidae, *Ips pini*, *Dendroctonus brevicomis*, *Enoclerus lecontei*, *Enoclerus sphegeus*, *Temnochila chlorodia*, *Tomicobia tibialis*, ipsdienol, lanierone, aggregation pheromone, kairomone.

INTRODUCTION

Ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) was identified as the aggregation pheromone of the pine engraver beetle, *Ips pini* (Say) (Coleoptera: Scolytidae), from populations in California (Birch et al., 1980) and New York (Stewart, 1975; Lanier et al., 1980). Prior to the identification of ipsdienol from *I. pini*, populations from California, Idaho, and New York were known to respond preferentially to their regionally specific pheromones (Lanier, 1972; Lanier et al., 1972), yet populations from California (and Arizona) and Ontario were shown to be interfertile (Lanier, 1972). In lab and field tests in California, (4*R*)-(−)-ipsdienol [95% (−)-lab, 97.8% (−)-field] was found to be attractive to *I. pini* while the (4*S*)-(+)-enantiomer [87% (+)-lab, 94.1% (+)-field], which was inactive alone, interrupted the response to (−)-ipsdienol (Birch et al., 1980). In contrast, in New York, Lanier et al. (1980) found the maximum response to racemic ipsdienol and demonstrated synergism between the two enantiomers [91% (−) and 80% (+)] in a field bioassay. Raffa and Klepzig (1989) provided additional evidence that eastern populations of *I. pini* prefer intermediate enantiomeric blends of ipsdienol when they showed maximum trap catches to 50% (−) and 25% (−) in two separate field studies in Wisconsin. The high activity of (−)-ipsdienol in western populations was further documented in one population in southeastern British Columbia [98% (−)-ipsdienol, Miller, 1990] and in another population in California [99.9% (−)-ipsdienol, Seybold et al., 1992d]. Miller (1990) also demonstrated a preferential response to the racemate by three other populations in southern British Columbia. In a study of production of ipsdienol by individual *I. pini*, Miller et al. (1989) found that inter- and intrapopulational variation of enantiomeric composition of ipsdienol ranged from 91% (−) in California and 89% (−) in southeastern British Columbia to 43% (−) in New York and 34% (−) in southwestern British Columbia.

Recently Teale and Lanier (1991) found considerable variability in the

response of a New York population to ipsdienol; in spring and early summer this compound had no activity. Subsequent investigations with the New York population resulted in the identification of lanierone (2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one), which was shown to act as a synergist with synthetic racemic ipsdienol in the lab and in the field (Teale et al., 1991).

In the present study we investigated a California population of *I. pini* for the presence (or absence) and activity of lanierone to determine whether lanierone is a factor in the interpopulational specificity observed by Lanier et al. (1972). Because we have found that the black-bellied clerid, *Enoclerus lecontei* (Wolcott) (Coleoptera: Cleridae), is attracted to ipsdienol and lanierone and since *E. lecontei* is the principal predator of the western pine beetle, *Dendroctonus brevicomis* LeConte (Person, 1940), we also investigated a California population of *D. brevicomis* for the presence or absence of lanierone.

METHODS AND MATERIALS

Laboratory Studies. For analysis of lanierone production, *I. pini* were collected on June 13, 1991 in ponderosa pine, *Pinus ponderosa* Laws., and Jeffrey pine, *Pinus jeffreyi*, Grev. & Balf. logging debris from area I of the field study site. For analysis of lanierone and ipsdienol production, *D. brevicomis* were collected on September 20, 1991 in a recently cut stem of *P. ponderosa* and by flight-trapping. The collection site was the University of California Blodgett Experimental Forest (El Dorado County, California). Insects were allowed to emerge from the infested material and stored at 4°C until utilized (Browne, 1972). Volatiles were collected separately from male and female *I. pini* and *D. brevicomis* by introducing the insects individually into freshly cut logs of *P. ponderosa*. *I. pini* males (741) and females (636) were introduced into the logs through holes drilled through the outer bark, secured in the artificial tunnels by screening, and allowed to feed in the phloem for 24 hr. After this feeding period, the logs (one set of three, 8–13 cm diameter × 28 cm length, for each sex) were placed into 19-liter silylated glass carboys (Sylon CT, Supelco Inc., Bellefonte, Pennsylvania) and aerated for 168 hr (July 12–19, 1991) with charcoal-filtered air (4 liters/min). Volatiles from each carboy were trapped on 15 g Porapak Q (50/80 mesh) (Supelco Co., Bellefonte, Pennsylvania) (Byrne et al., 1975) held in 25-cm × 2.4-cm (ID) glass columns. Each Porapak sample was extracted with 350 ml pentane. The same procedure was used with *D. brevicomis* males (166) and females (267), which were aerated October 15–22, 1991 (one set of two, 8- to 11-cm-diameter × 28-cm-length logs for each sex).

Flight-trapped and newly emerged *D. brevicomis* of each sex were introduced into freshly cut *P. ponderosa* logs, allowed to feed for 48 hr, and recovered

alive from the logs. Mid- and hindgut tissue was removed from fed and unfed insects and extracted for 2 hr in 200 μl pentane with 5% ethyl acetate for analysis of lanierone production. *D. brevicomis* of each sex were also placed on glass wool in tightly sealed 19-liter glass carboys and incubated for 18 hr at room temperature with or without 200 μl β-myrcene (Byers, 1982). Mid- and hindgut tissue from these insects as well as from insects recovered from the Porapak Q aeration was removed and extracted for 24 hr in 200 μl diethyl ether for analysis of ipsdienol enantiomeric composition. Total guts dissected for ipsdienol analysis were as follows: unfed, no myrcene: 31 males, 44 females; unfed, myrcene: 78 males, 60 females; fed: 31 males; 37 females.

The Porapak extracts of the *I. pini* and *D. brevicomis* male and female aerations were analyzed for lanierone by GC-MS using multiple ion detection (*m/z* 109, 124, 137, 152) in a Finnigan model 4500 fitted with a 30-m SPB-1 capillary column (Supelco, Inc., Bellefonte, Pennsylvania), with the injection temperature set at 250°C. The samples were run at 70 eV and the detection level for lanierone was ~0.5 ng. Porapak extracts of *D. brevicomis* male and female aerations were analyzed for ipsdienol by fractionation on HPLC (Seybold et al., 1992b,c). These fractions were assayed by GC for detection of ipsdienol (conditions below). The crude male *D. brevicomis* Porapak extract was also analyzed for the presence of ipsdienol by GC-MS using the conditions described above. Diethyl ether gut extracts of *D. brevicomis* were dried on sodium sulfate and filtered through sodium sulfate and glass wool before concentration to 20 μl. Concentrated crude extracts were analyzed for ipsdienol enantiomeric composition by direct injection on capillary GC [Shimadzu GC-9A; column: 50 m Cydex-B (50 m × 0.22 mm ID × 0.25 μm film), SGE Inc., Austin, Texas; He carrier gas; flow rate: 1.4 ml/min (2.7 kg/cm^2), linear velocity: 24.2 cm/sec, column temperature: 110°C isothermal, injector and detector temperatures: 250°C]. The presence of each enantiomer of ipsdienol on GC was verified by coinjection with authentic enantiopure (+)- and (−)-ipsdienol (Seybold et al., 1992a,c).

Residue analysis of ipsdienol in membrane release devices was carried out by Consep Membranes Inc. (Bend, Oregon) using capillary GC. For analysis, each lure was cut into 1-cm strips and extracted in 10 ml acetone with 1 mg/ml hexadecane as internal standard. An aliquot of each extract (2 μl) was analyzed by capillary GC [column: HP-5 (25 m × 0.2 mm ID × 0.33 μm film); He carrier gas; flow rate: 1 ml/min, linear velocity: 26.1 cm/sec; temperature program: 140°C for 5 min followed by 20°C/min to 220°C and held for 8 min; injection temperature: 250°C; and detector temperature: 300°C]. Under these conditions, ipsdienol had a retention time of ~3.8 min and hexadecane had a retention time of ~9.5 min.

Field Studies. A field study to determine the flight response of *I. pini* and associated fauna to varying doses of lanierone released with a standard dose of

ipsdienol was conducted between July 26 and August 3, 1991, in a recently thinned stand of *P. ponderosa* and *P. jeffreyi* located near Jelly Spring, Lassen County, California (19 km east of Hat Creek, Shasta County, California). This site is located 70 km southeast of that utilized by Birch et al. (1980) and Lanier et al. (1972) for *I. pini* pheromone isolation and field testing. The 13-km^2 site was divided into three test areas, each separated by at least 2 km. Area I was located in the $SE\frac{1}{4}$ $NE\frac{1}{2}$ S26 T34N R6E, area II was located in the $SW\frac{1}{4}$ S23 T34N R6E, and area III was located in the $NE\frac{1}{4}$ S15 and $NW\frac{1}{4}$ S14 T34N R6E. In each area, eight-unit funnel traps (Pherotech Inc., Delta, British Columbia) (Lindgren, 1983) and associated treatments were randomly assigned to positions located at least 100 m apart to minimize treatment interactions. This was a randomized block design, in which a block consisted of nine trap positions in a test area and a single day's trap catch. There were 24 blocks in the study (eight days at each of three test areas). Each day, the funnel traps were emptied, rebaited, and rerandomized between 7:30 and 11:30 AM. The treatments in the study were: (1) blank trap, (2) *P. jeffreyi* log infested with 20 male *I. pini*, (3) synthetic ipsdienol (1 mg/day), (4) synthetic lanierone (20 μg/day), (5) ipsdienol (1 mg/day) + lanierone (0.1 μg/day), (6) ipsdienol (1 mg/day) + lanierone (1 μg/day), (7) ipsdienol (1 mg/day) + lanierone (20 μg/day), (8) ipsdienol (1 mg/day) + lanierone (100 μg/day), and (9) ipsdienol (1 mg/day) + lanierone (2 mg/day). Ipsdienol (neat) was released from membrane lures (Consep Membranes, Inc.), and lanierone (in hexane) was released from capped, 2.5-ml polyethylene vials (Kartell, Fisher Scientific). Each ipsdienol membrane lure was allowed to elute 1 mg for 24 hr, and then replaced with a fresh lure. The initial amount of racemic ipsdienol loaded in the device was 7.93 mg. The load was delivered volumetrically based on the specific gravity of pure ipsdienol (0.9) and adjusted for the chemical purity of the synthetic ipsdienol. Prior laboratory analysis[6] had shown that these lures release an average of 1.39 mg/day over a four-day period, 1.31 mg/day over a five-day period, and 1.02 mg/day over a seven-day period (conditions: 25°C $\pm$ 1°C, wind speed 0.3 m/sec, and relative humidity 20–30%). The daily release rate of ipsdienol during the study was determined from residue analysis of the used membrane lures. Each polyethylene vial was charged with a 100-μl or 200-μl hexane solution of lanierone in concentrations calibrated to give the desired release rates [100 μl of 250 μg/ml for 0.1 μg/day; 100 μl of 500 μg/ml for 1 μg/day; 100 μl of 1 mg/ml for 20 μg/day (two vials); 100 μl of 10 mg/ml for 100 μg/day; and 200 μl of 50 mg/ml for 2 mg/day (two vials)]. The male-infested logs ($\sim$10 cm diameter $\times$ 12 cm length) were incubated for 36 hr prior to placement in the test and dissected following the test to determine how many males constructed feeding galleries. Throughout the course of the study, the mean maximum temperature

[6]Data on file at Consep Membranes Inc., Bend, Oregon.

on the site was 32.7°C and the mean minimum temperature was 8.9°C with a range of 4.4°C to 36.1°C.

Because hexane was used as a solvent for lanierone, a second study was conducted to determine if the presence of hexane alone would influence the response of *I. pini* and associated fauna to ipsdienol. This test was conducted on July 31, 1991 near area I of the lanierone dose study. The two treatments were ipsdienol (1 mg/day) and ipsdienol (1 mg/day) + hexane (100 μl/day = 66 mg/day). Release devices were identical to those used in the lanierone dose study. Each treatment was replicated nine times. Because lanierone is a solid at room temperature, the effect of neat lanierone on the response of *I. pini* to ipsdienol could not be tested.

Chemicals. (−)-Ipsdienol [99.39% ± 0.02% (−), 97.86% ± 0.61% chemical purity, mean of 10 injections ± SE] used in field studies was purchased from Pherotech Inc. Technical grade β-myrcene was purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. Lanierone was synthesized in Syracuse (Teale et al., 1991). All solvents were HPLC grade.

Statistical Analysis. Data from tests 1 and 2 were analyzed using a nonparametric randomized blocks procedure with aligned ranks to test for equality of all treatments (Lehmann, 1975, Chapter 6, pp. 270–273). Because we knew at the outset that our trap-catch data would not be normally distributed (trap catches are integer data), we chose to use this nonparametric analysis. Data were aligned by subtraction of the mean catch in each block. Selected pairwise comparisons of treatments were performed using the Wilcoxon signed-rank test (Lehmann, 1975, Chapter 3, pp. 123–132) and the Bonferroni method was used for pairwise comparisons to control overall error rate (procedurewise $\alpha = 0.05$). Where results of tests are represented with an inequality sign, this indicates a one-sided test. Comparisons frequently involved the treatment ipsdienol (1 mg/day) + lanierone (20 μg/day), because this represents an approximation of the ratio in which the two compounds occur in New York populations of *I. pini* (Teale et al., 1991). In some cases, we performed one-sided pairwise comparisons in order to determine whether our data showed the same trends as reported for this insect in eastern North America (Teale et al., 1991). Regression analyses of percentage of male *I. pini* and the number of *E. lecontei* responding on the logarithm of lanierone concentration were performed as described in Draper and Smith (1981, Chapter 1, pp. 8–33). In the regression analysis, a log $[Y + 1]$ transformation was performed on the number of *E. lecontei*. An analysis of equality of proportions of male *I. pini* caught for all treatments as well as pairwise comparisons of proportions of males caught for selected pairs of treatments were performed using Pearson's goodness-of-fit test. The Bonferroni method was also used to control overall error rate for these pairwise comparisons (procedurewise $\alpha = 0.05$).

RESULTS

Laboratory Studies. The multiple ion detection mass spectra (*m/z* 109, 124, 137, 152) of the *I. pini* male and female aerations revealed ipsdienol and no detectable level of lanierone in the male extract, but neither compound in the female extract. Lanierone and ipsdienol were not detected in gut extracts from fed or unfed male or female *D. brevicomis*. Neither compound was detected in Porapak Q aeration extracts from male or female *D. brevicomis*. However, ipsdienol was present in gut extracts from male (but not female) insects that had been aerated with myrcene. The enantiomeric composition of the ipsdienol present in males was 97.79 ± 0.04% (+) (mean of five injections ± SE).

Laboratory residue analysis of used membrane lures from the field study (Table 1) showed that the mean daily release rate of ipsdienol throughout the course of the study was 2.56 ± 0.26 mg (grand mean of differences between day 0 and other days, nine measurements). This estimate of release rate should be viewed as a maximum estimate. The lures analyzed for day 0 of the study were not taken in the field at the time that the test was begun and then shipped for analysis. Rather, they were prepared in Berkeley and shipped to Bend for analysis. Thus, these devices had representative expected loads for day 0 and were prepared using the same equipment and materials as the other devices, but were not treated identically.

Field Studies. With the exception of one dose (2 mg/day), the lanierone dose study shows that *I. pini* responds in larger numbers to the combination of ipsdienol and lanierone than it does to ipsdienol alone (Figure 1). The response

TABLE 1. QUANTITY OF IPSDIENOL REMAINING IN MEMBRANE LURES FIELD TESTED 19 km EAST OF HAT CREEK, CALIFORNIA, JULY 26–AUGUST 3, 1991

Day of study	Ipsdienol (mg) (mean ± SE)	Lures analyzed (*N*)	Field temperature (°C) (minimum–maximum)
0	7.77 ± 0.05	12	
1	4.69 ± 0.18	18	8.9–34.4
2	5.68 ± 0.07	18	10.0–35.6
3	5.24 ± 0.12	9	10.0–36.1
4	5.44 ± 0.09	9	10.0–35.6
5	4.17 ± 0.26	9	11.1–35.0
5[a]	3.97 ± 0.07	18	11.1–35.0
6	5.60 ± 0.14	9	8.9–31.1
7	5.99 ± 0.10	9	4.4–24.4
8	6.14 ± 0.05	9	7.8–28.9

[a] Hexane test.

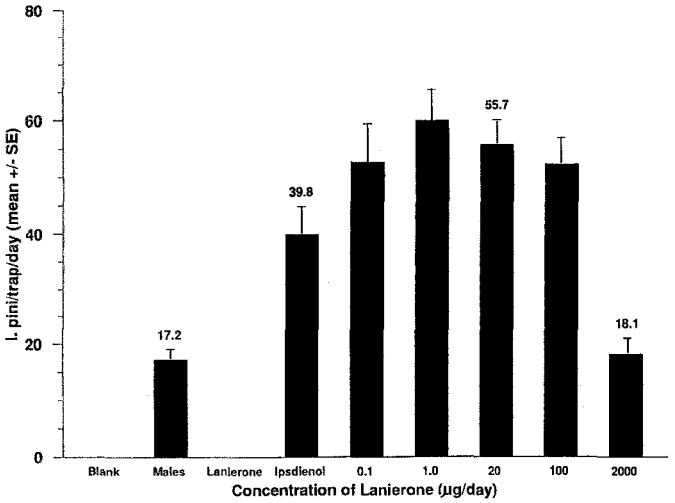

FIG. 1. The effect of varying the release rate of lanierone with a constant release rate [1 mg/day, 99.4% (−)] of ipsdienol on the mean number of *Ips pini* trapped 19 km east of Hat Creek, California, July 26–August 3, 1991. Male-infested logs, ipsdienol alone (1 mg/day), lanierone alone (20 µg/day), and blank traps served as controls. Treatment means (per trap per day) given above bars represent treatments involved in pairwise comparisons. The following pairwise comparisons were tested:

Comparison	Type of test	Significant
H_O: Ipsdienol ≥ ipsdienol + 20 µg lanierone H_A: Ipsdienol ≤ ipsdienol + 20 µg lanierone	One-sided	Yes ($P = 0.0055$)
H_O: Ipsdienol ≤ ipsdienol + 2 mg lanierone H_A: Ipsdienol ≥ ipsdienol + 2 mg lanierone	One-sided	Yes ($P = 0.0004$)
H_O: Ipsdienol ≤ male-infested log H_A: Ipsdienol ≥ male-infested log	One-sided	Yes ($P = 0.0003$)

The test for equality of all treatments was significant ($P < 0.0001$). The pairwise comparisons were analyzed using the Wilcoxon signed-rank test and the Bonferroni method was used to control for overall error rate (procedurewise $\alpha = 0.05$).

of *I. pini* to ipsdienol + lanierone (20 µg/day) was significantly greater than the response to ipsdienol alone. The response of *I. pini* to the combination of ipsdienol and the highest dose of lanierone (2 mg/day) was significantly lower than the response to ipsdienol alone. In a third significant comparison, the response of *I. pini* to ipsdienol alone was more than twice the response to the male-infested log.

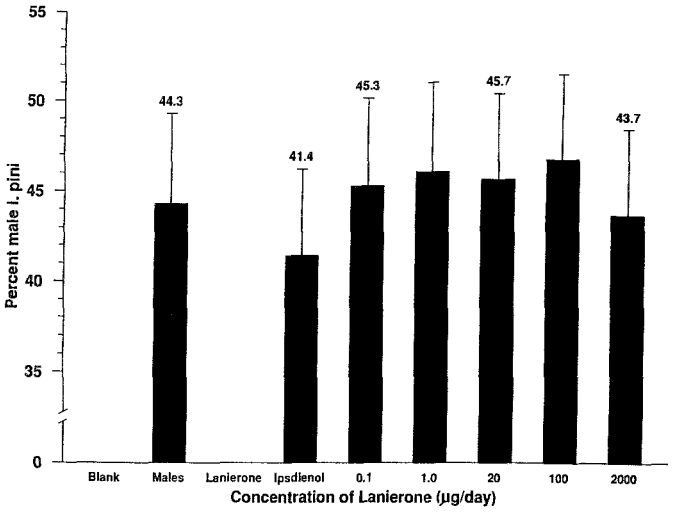

FIG. 2. The effect of varying the release rate of lanierone with a constant release rate [1 mg/day, 99.4% (−)] of ipsdienol on the percentage of male *Ips pini* trapped 19 km east of Hat Creek, California, July 26–August 3, 1991. Male infested logs, ipsdienol alone (1 mg/day), lanierone alone (20 µg/day), and blank traps served as controls. Treatment means (per trap per day) given above bars represent treatments involved in pairwise comparisons. The following pairwise comparisons were tested:

Comparison	Type of test	Significant
H_O: Ipsdienol = male-infested log H_A: Ipsdienol ≠ male-infested log	Two-sided	No ($P = 0.3209$)
H_O: Ipsdienol ≥ ipsdienol + 20 µg lanierone H_A: Ipsdienol ≤ ipsdienol + 20 µg lanierone	One-sided	No ($P = 0.0202$)
H_O: Ipsdienol + 20 µg lanierone ≥ male-infested log H_A: Ipsdienol + 20 µg lanierone < male-infested log	One-sided	No ($P = 0.6942$)
H_O: Ipsdienol + 2 mg lanierone ≤ ipsdienol + 0.1 µg lanierone H_A: Ipsdienol + 2 mg lanierone > ipsdienol + 0.1 µg lanierone	One-sided	No ($P = 0.7242$)

The test for equality of all treatments was not significant ($P = 0.2400$). The pairwise comparisons were analyzed using the Pearson's goodness-of-fit test and the Bonferroni method was used to control for overall error rate (procedurewise $\alpha = 0.05$).

The proportion of male *I. pini* responding was not influenced by the presence of lanierone (Figure 2). Regression analysis of percentage of males on logarithm of concentration of lanierone [log (mg)] did not fit the model [R^2 = 0.0026, $Y = -0.37$ (log X) + 45.17 (where Y = percentage of males trapped and X = mg lanierone), slope not significantly different from 0, $P = 0.58$, $\alpha = 0.05$]. The percentage of male *I. pini* responding to the male-infested log (44.3%) was not significantly different from the percentage of male *I. pini* responding to (−)-ipsdienol (41.4%) (Figure 2). The addition of lanierone at 20 μg/day did not cause a significant increase in the percentage of male *I. pini* responding to ipsdienol (45.7% vs. 41.4%) and the addition of 2 mg/day lanierone did not cause a significant increase over 0.1 μg/day lanierone (43.7% vs. 45.3%). There was no significant difference between the responses of *I. pini* to ipsdienol and to ipsdienol + hexane (mean response to ipsdienol ± SE: 44.5 ± 9.5; mean response to ipsdienol + hexane ± SE: 48.3 ± 10.2; two-sided Wilcoxon signed-rank test, $P = 0.9528$).

Insect predators and parasitoids of *I. pini* responded to various treatments in these studies. The proportion of each sex of these associates responding was not determined. None of these insects appeared to be attracted to lanierone alone. However, some were attracted to ipsdienol alone, and one, *Enoclerus lecontei*, responded to the combination of these compounds. In selected paired comparisons, the responses of the predators *Temnochila chlorodia* (Mannerheim) (Coleoptera: Trogositidae) and *Enoclerus sphegeus* (F.) (Coleoptera: Cleridae) to ipsdienol + lanierone (20 μg/day) did not differ significantly from their responses to ipsdienol alone (Figures 3 and 4). The responses of both species to ipsdienol alone were significantly greater than they were to an unbaited trap. This study indicated a significant difference between the responses of *T. chlorodia* to ipsdienol and to the male-infested log (Figure 3). In this case, ipsdienol was more attractive than the male-infested log. The response of *E. sphegeus* to ipsdienol was not significantly different from the response to the male-infested log (Figure 4).

E. lecontei exhibited a clear dose-dependent response to lanierone when delivered with ipsdienol (Figure 5). The response of this predator to ipsdienol + lanierone (20 μg/day) did not differ significantly from its response to the male-infested log, while the response to ipsdienol + lanierone (20 μg/day) was significantly greater than it was to a blank trap. The catch at traps baited with ipsdienol alone was not significantly different from the response to the blank trap. Although we could not analyze the comparison between the response to the blank trap and the response to lanierone (in hexane) alone, no *E. lecontei* were trapped coming to either treatment (Figure 5). The estimated slope in the regression analysis for *E. lecontei* was significant [$R^2 = 0.52$, log $(Y + 1) = 0.17$ (log$X$) + 0.69 (where Y = trap catch and X = mg lanierone), slope significantly different from 0, t test, $P = 0.0001$, $\alpha = 0.05$].

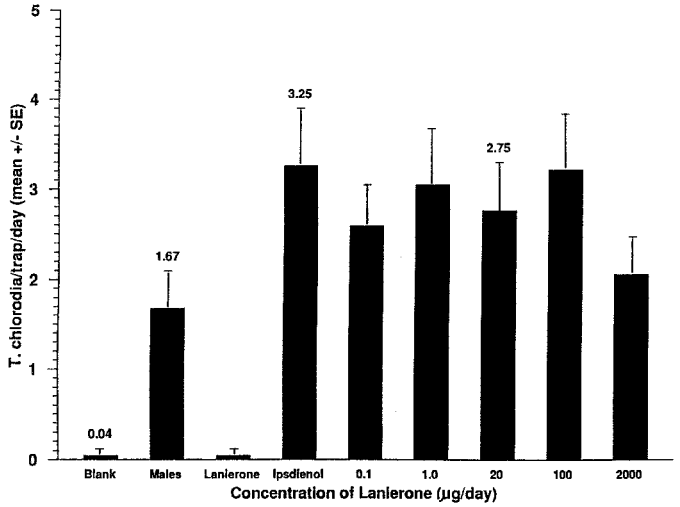

FIG. 3. The effect of varying the release rate of lanierone with a constant release rate [1 mg/day, 99.4% (−)] of ipsdienol on the mean number of *Temnochila chlorodia* trapped 19 km east of Hat Creek, California, July 26–August 3, 1991. *Ips pini* male-infested logs, ipsdienol alone (1 mg/day), lanierone alone (20 µg/day), and blank traps served as controls. Treatment means (per trap per day) given above bars represent treatments involved in pairwise comparisons. The following pairwise comparisons were tested:

Comparison	Type of test	Significant
H_O: Ipsdienol ≤ blank H_A: Ipsdienol > blank	One-sided	Yes ($P < 0.0001$)
H_O: Ipsdienol = male-infested log H_A: Ipsdienol ≠ male-infested log	Two-sided	Yes ($P = 0.0026$)
H_O: Ipsdienol = ipsdienol + 20 µg lanierone H_A: Ipsdienol ≠ ipsdienol + 20 µg lanierone	Two-sided	No ($P = 0.3830$)

The test for equality of all treatments was significant ($P < 0.0001$). The pairwise comparisons were analyzed using the Wilcoxon signed-rank test and the Bonferroni method was used to control for overall error rate (procedurewise $\alpha = 0.05$).

A mean of 2.4 *Tomicobia tibialis* Ashmead (Hymenoptera: Pteromalidae) (Figure 6) were trapped at male-infested logs each day while a mean of less than 0.2 *T. tibialis* were trapped at lanierone, ipsdienol, or the combination of these compounds. The response to the male-infested log was significantly greater than it was to the blank trap, to ipsdienol, or to ipsdienol + lanierone (20 µg/day).

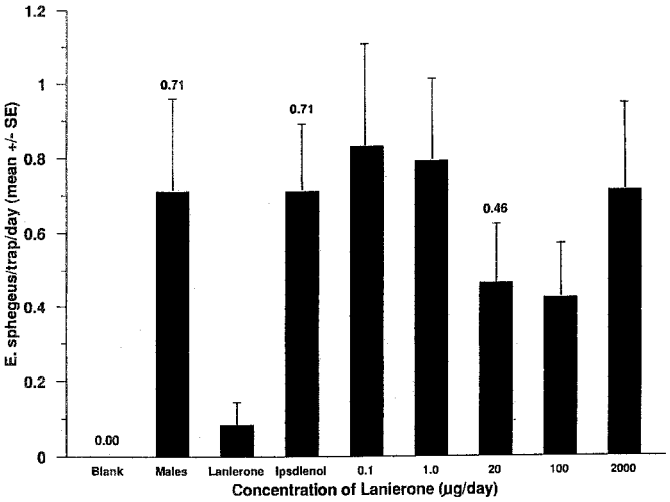

FIG. 4. The effect of varying the release rate of lanierone with a constant release rate [1 mg/day, 99.4% (−)] of ipsdienol on the mean number of *Enoclerus sphegeus* trapped 19 km east of Hat Creek, California, July 26–August 3, 1991. *I. pini* male-infested logs, ipsdienol alone (1 mg/day), lanierone alone (20 μg/day), and blank traps served as controls. Treatment means (per trap per day) given above bars represent treatments involved in pairwise comparisons. The following pairwise comparisons were tested:

Comparison	Type of test	Significant
H_O: Ipsdienol $\leq$ blank H_A: Ipsdienol $\geq$ blank	One-sided	Yes (P = 0.0005)
H_O: Ipsdienol = male-infested log H_A: Ipsdienol $\neq$ male-infested log	Two-sided	No (P = 0.8557)
H_O: Ipsdienol = ipsdienol + 20 μg lanierone H_A: Ipsdienol $\neq$ ipsdienol + 20 μg lanierone	Two-sided	No (P = 0.2775)

The test for equality of all treatments was significant (P = 0.0010). The pairwise comparisons were analyzed using the Wilcoxon signed-rank test and the Bonferroni method was used to control for overall error rate (procedurewise α = 0.05).

A total of 40 *Pityogenes carinulatus* (LeConte) (Coleoptera: Scolytidae) were trapped during the study and 38 of the specimens responded to treatments involving lanierone and ipsdienol. The test for equality of all treatments was significant (P = 0.002) for *P. carinulatus* and the response to ipsdienol + 0.1 μg/day lanierone (0.33 insects/trap/day) was significantly greater than the response to the blank trap (0 insects/trap/day)(P = 0.0072).

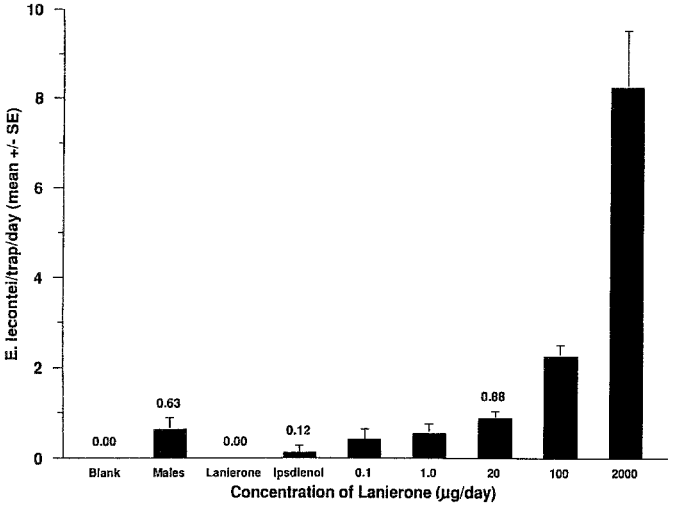

FIG. 5. The effect of varying the release rate of lanierone with a constant release rate [1 mg/day, 99.4% (−)] of ipsdienol on the mean number of *Enoclerus lecontei* trapped 19 km east of Hat Creek, California, July 26–August 3, 1991. *I. pini* male-infested logs, ipsdienol alone (1 mg/day), lanierone alone (20 µg/day), and blank traps served as controls. Treatment means (per trap per day) given above bars represent treatments involved in pairwise comparisons. The following pairwise comparisons were tested:

Comparison	Type of test	Significant
H_O: Ipsdienol = blank H_A: Ipsdienol ≠ blank	Two-sided	No ($P = 0.0833$)
H_O: Lanierone = blank H_A: Lanierone ≠ blank	Two-sided	Could not test
H_O: Ipsdienol + 20 µg lanierone ≤ blank H_A: Ipsdienol + 20 µg lanierone ≥ blank	One-sided	Yes ($P < 0.0001$)
H_O: Ipsdienol + 20 µg lanierone = male-infested log H_A: Ipsdienol + 20 µg lanierone ≠ male-infested log	Two-sided	No ($P = 0.1786$)

The test for equality of all treatments was significant ($P < 0.0001$). The pairwise comparisons were analyzed using the Wilcoxon signed-rank test and the Bonferroni method was used to control for overall error rate (procedurewise $\alpha = 0.05$).

The predators *Enoclerus barri* Knull (Coleoptera: Cleridae) and *Othnius* spp. (Coleoptera: Othniidae), and the associates *Lasconotus subcostulatus* Kraus (Coleoptera: Colydiidae), *Corticeus* spp. (Coleoptera: Tenebrionidae), *Dendroctonus valens* LeConte (Coleoptera: Scolytidae), *Pityophthorus jeffreyi* Blackman (Coleoptera: Scolytidae), *Aulonium* spp. (Coleoptera: Colydiidae),

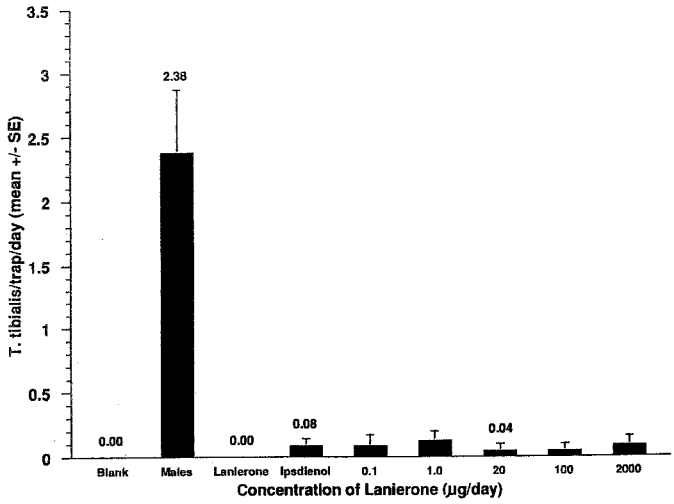

FIG. 6. The effect of varying the release rate of lanierone with a constant release rate [1 mg/day, 99.4% (−)] of ipsdienol on the mean number of *Tomicobia tibialis* trapped 19 km east of Hat Creek, California, July 26–August 3, 1991. *Ips pini* male-infested logs, ipsdienol alone (1 mg/day), lanierone alone (20 µg/day), and blank traps served as controls. Treatment means (per trap per day) given above bars represent treatments involved in pairwise comparisons. The following pairwise comparisons were tested:

Comparison	Type of test	Significant
H_O: Male-infested log ≤ blank H_A: Male-infested log ≥ blank	One-sided	Yes ($P < 0.0001$)
H_O: Male-infested log ≤ ipsdienol H_A: Male-infested log ≥ ipsdienol	One-sided	Yes ($P < 0.0001$)
H_O: Male-infested log ≤ ipsdienol + 20 µg lanierone H_A: Male-infested log ≥ ipsdienol + 20 µg lanierone	One-sided	Yes ($P < 0.0001$)

The test for equality of all treatments was significant ($P < 0.0001$). The pairwise comparisons were analyzed using the Wilcoxon signed-rank test and the Bonferroni method was used to control for overall error rate (procedurewise $\alpha = 0.05$).

Lyctocoris spp. (Hemiptera: Anthocoridae), and *Cylistix* spp. (Coleoptera: Histeridae) were trapped only in small numbers, and thus conclusions cannot be drawn about their responses to ipsdienol or lanierone.

Dissection of the 24 male-infested logs used in this study revealed that a mean of 18.04 (range 15–20) males had constructed feeding galleries in each of these logs.

DISCUSSION

The California population of *I. pini* that we studied behaves differently than the New York population in several ways. Ipsdienol elicits a proportionately higher level of response from California *I. pini* than a male-infested log (Figure 1). However, New York *I. pini*, in both lab and field studies, responds in significantly greater numbers to male-produced volatiles or a male-infested log than it does to ipsdienol (Teale et al., 1991). Teale et al. (1991) used a field-study dose of ipsdienol (10 mg for a six-day test period) similar to that used in California (2.6 mg/day). Birch et al. (1980) discovered the importance of the (−)-enantiomer of ipsdienol to the field attraction of this California population of *I. pini*. Response of *I. pini* to 99.4% (−)-synthetic ipsdienol in our study may exceed the response to the male-infested log because of the higher release rate from the devices or because the optical purity of the synthetic compound exceeds that of a log infested with 20 male *I. pini* [mean 98.2% (−), range 96.6–98.9% (−), 12 replicates]. The dose of (−)-ipsdienol released in 24 hr from these male-infested logs ranges from 0.7 to 1.9 mg/day (Seybold et al., 1992d).

In California, *I. pini* responds to ipsdienol at levels comparable to its response to a male-infested log from May through August (Seybold et al., 1992d), while in New York *I. pini* responds to ipsdienol in greater numbers (although still lower than to the male-infested log) only after the beginning of August (Teale and Lanier, 1991). Similarly, in Wisconsin, Raffa (1991) reported trapping the most beetles late in the season, but the level of response relative to male-produced attractants was not tested. Nonetheless, in two of three years where the flight behavior of *I. pini* was studied from spring through fall, Raffa (1991) demonstrated a response to ipsdienol in April and May. Wisconsin and New York populations are similar because they produce and respond to similar enantiomeric blends of ipsdienol (Seybold et al., unpublished; Raffa and Klepzig, 1989; Teale, 1990).

Another difference between California and New York populations is that the response of California *I. pini* to increasing doses of lanierone in the presence of ipsdienol does not demonstrate the clear dose-dependent response elicited from New York *I. pini* (Figure 1 and Teale et al., 1991). In addition, the percentage of male *I. pini* responding in California to the male-infested log is not significantly different from the percentage of male *I. pini* responding to (−)-ipsdienol alone. Adding lanierone to ipsdienol does not affect the proportion of males responding in California. In two different field studies, Birch et al. (1980) also found that the male to female sex ratio of *I. pini* responding to (−)-ipsdienol was not different from the sex ratio of *I. pini* responding to a male-infested log. However, in New York, Teale et al. (1991) have found a positive relationship between the percentage of males in the responding population and the quantity

of lanierone added to a fixed amount of ipsdienol. They also found that the percentage of male responders was highest (48%) to the male-infested log. In California, the percentage of males responding to the male-infested log (44.3%) was not significantly greater than the percentage of males responding to ipsdienol and 20 μg/day lanierone (45.7%).

There is a considerable difference between the enantiomeric composition of ipsdienol produced by New York and California populations of *I. pini*. Birch et al. (1980) isolated ipsdienol from benzene extracts (Young et al., 1973) of California *I. pini* male-produced frass, as well as from volatiles condensed in a liquid nitrogen trap. The enantiomeric composition of the ipsdienol from this isolation was not determined, but Stewart (1975) found that California *I. pini* produce (−)-ipsdienol, and Miller et al. (1989) obtained a mean of 91% (−) from a population collected near Hat Creek, California. Lanier et al. (1980) reported the isolation of ipsdienol from male New York *I. pini* by trapping volatiles on Porapak Q. Earlier, the enantiomeric composition of this ipsdienol had been found to be 35% (−) (Stewart, 1975). Miller et al. (1989) measured a mean of 43% (−) from a population collected in New York. The isolations by Birch et al. (1980) and Lanier et al. (1980) were both guided by lab and field assays.

In addition to the differences in the stereochemistry of ipsdienol, we have found that a California population of *I. pini* does not produce detectable levels of lanierone. Based on the smallest detectable peak in the GC (FID) trace, lanierone production would have to be much less than 0.002% of the amount of ipsdienol produced by male *I. pini* in California. This is one-hundredth of the amount of lanierone (0.2%, relative to ipsdienol) produced by *I. pini* in New York (Teale et al., 1991). The absence of lanierone in this California population of *I. pini* and its presence in New York populations may be related to differences in the hosts (*P. ponderosa* vs. *Pinus resinosa* Ait.) from which volatiles were collected. Since Teale et al. (1991) did not find lanierone in Porapak extracts from female New York *I. pini* in *P. resinosa*, it is unlikely that lanierone is present in the host itself. However, precursors for biosynthesis of lanierone may be present in *P. resinosa*, but not in *P. ponderosa*. It should be noted that the host species does not appear to affect ipsdienol production (including enantiomeric composition), which was unchanged when males from New York populations reared in *P. resinosa* were allowed to feed in *P. ponderosa* (Seybold et al., unpublished).

Besides the interfertility of eastern (Ontario) and western (California and Arizona) populations of *I. pini* (Lanier, 1972), there are also some similarities in the flight behavior of western (California) and eastern (New York) populations. Neither responds to lanierone alone, and both appear to be inhibited at high lanierone concentrations [10 mg for a six-day test[7] period for eastern pop-

[7]Log[mg] doses in Teale et al. (1991) Figure 11 should read left to right, −3, −2, −1, 0, and 1.

ulations (Teale et al., 1991) and 2 mg/day for western populations (this study)]. Although western populations of *I. pini* do not produce detectable amounts of lanierone, the ipsdienol-elicited flight response of *I. pini* in California can be increased by its presence. This latter result would seem to suggest a relictual behavioral link between the eastern and western forms of *I. pini*.

Much remains to be learned about the evolutionary history of the pheromone variants of *I. pini* [formerly *I. oregonis* (Eichhoff) = western and *I. pini* (Say) = eastern; Hopping, 1964]. Based on glacial history and production and response profiles of enantiomeric blends of ipsdienol, Miller (1990) suggested that eastern populations of *I. pini* originated from a southeastern refugium in the Appalachian Mountains, that eastern-like populations currently in British Columbia originated from a southwestern refugium, and that western populations in British Columbia derived from populations similar to those presently in northern California. However, there are other possible explanations for the origin of the pheromone types noted in British Columbia. These include introduction from eastern localities (J.W.A. Volney, personal communication); blending of eastern and western forms originating from eastern and western glacial refugia, respectively (D.E. Bright, personal communication); and post-Pleistocene colonization from the Beringia (= Yukon) refugium (D.E. Bright, personal communication).

It is possible that an eastern population of *I. pini* was introduced and established in British Columbia. Movement of insects in logs, timbers, and rough-wood crates by human transport is a well-known current problem (Ciesla, 1988), but it has probably also occurred during the past century. For example, there is an endemic population of *I. calligraphus calligraphus* in central California that probably originated from the southeastern United States and was present at least by 1914 (Herbert, 1916, Wood and Stark, 1968, and Lanier et al., 1991).

The eastern form of *I. pini* may have moved naturally into northwestern Canada from a southeastern refugium (Matthews, 1979) or from midcontinental refugia (Critchfield, 1985) by following the post-Pleistocene migration of jack pine, *Pinus banksiana* Lamb. *P. banksiana* is a rapidly colonizing species that invaded western Canada during the last 10,000 years where it occurs in a hybridization zone with *P. contorta latifolia* in northern Alberta and the southern Yukon Territory (Critchfield, 1957, 1985). *P. contorta* appears to have experienced a more sedentary post-Pleistocene colonization pattern, originating from refugia south of the Cordilleran glacial ice mass and, perhaps, in the Yukon (Critchfield, 1985). This zone of host hybridization prompts the question whether eastern and western forms of *I. pini* also form a zone of sympatry in this region. The rapid migration of *P. banksiana* through Canada suggests that the eastern form of *I. pini* may have become established in British Columbia in *P. contorta latifolia* prior to or contemporaneously with the advent of the western form,

which may have also utilized *P. ponderosa*, *P. contorta murrayana*, or *P. contorta contorta* as its refugial hosts. Ross (1955) also noted the transcontinental distribution of *P. banksiana* as a major factor in the evolution of the *Neodiprion* sawflies. Despite the continuity of genetic variation in *P. banksiana* across Canada, it is possible that it also occupied one or more western refugia during the Pleistocene (Critchfield, 1985). Similarly, host islands of *P. contorta* may have been available for *I. pini* in ice-free corridors between the Cordilleran and Laurentide ice sheets (Critchfield, 1985).

Miller (1990) discounted Beringia as a source of present-day *I. pini* in British Columbia based on the meager and highly localized modern-times collection record of *I. pini* in coastal southeastern Alaska (Wood, 1982). However, in addition to indications that the host, *P. contorta*, may have been in a Yukon refugium (Critchfield, 1985), there is one record of *I. pini* from interior Alaska (Bright, 1976). Moreover current distribution does not always reflect historical distribution [e.g., the scolytid, *Carphoborus andersoni* Swaine, occurs only in northern Alberta, the Northwest Territories, and Alaska, but fossils indicate its former presence in Minnesota, New York, and Ontario between 10,000 and 70,000 years ago (Bright, 1976; Wood, 1982; D.E. Bright, personal communication)].

Gahan (1938) and Reid (1957) documented the association of the solitary parasitoid wasp *T. tibialis* with *I. pini*. *T. tibialis* has also been shown to respond to logs infested with male California five-spined ips, *Ips paraconfusus* Lanier (Bedard, 1965) and to logs infested with male and not female *I. pini* (Rice, 1968). In a field study in California, Lanier et al. (1972) demonstrated that *T. tibialis* preferred California and Idaho male *I. pini*-infested logs over New York male *I. pini*-infested logs. In our study we have found that *T. tibialis* responded to logs infested with male *I. pini*, but not to ipsdienol, lanierone, or any combination of these compounds. This indicates that the kairomone that attracts *T. tibialis* to *I. pini* probably involves other chemicals, although it may include ipsdienol, which is produced by males of both *I. paraconfusus* and *I. pini* (Silverstein et al., 1966; Birch et al., 1980). In the present study, *T. tibialis* was often observed and collected from the white surface of the top of the funnel traps beneath the male-infested logs, but it was never observed or collected from this surface or near the release devices on traps baited with synthetic chemicals. Nonchemical behavioral stimuli associated with the presence of male frass, the pine log, and the attracted live bark beetles may also have contributed to the arrestment of *T. tibialis* on the surface of traps baited with male-infested logs.

In contrast to its influence on *I. pini*, lanierone had no effect on the responses of *T. chlorodia* or *E. sphegeus* to ipsdienol. As with California *I. pini*, the response of these predators to the male-infested log appears to be fully explained by their response to ipsdienol. Despite many prior studies of bark beetle pheromones where *T. chlorodia* has been shown to respond to various natural and

synthetic substrates, it has never been found to respond to ipsdienol alone [*D. brevicomis*-infested log (Vité and Gara, 1962); male *I. paraconfusus*-infested log (Vité and Gara, 1962; Rice, 1969); synthetic *exo*-brevicomin (Bedard et al., 1969, 1980; Vité and Pitman, 1969); synthetic ipsenol and male *I. pini*-infested log (Furniss and Livingston, 1979); and mixture of ipsenol, ipsdienol, and *cis*-verbenol (Wood et al., 1968; Wood, 1970)].

The red-bellied clerid, *E. sphegeus*, is a principal predator of the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Struble, 1942), and also preys on *Ips* spp. (Furniss and Carolin, 1977). Furniss and Livingston (1979) reported low numbers of *E. sphegeus* responding in Idaho to synthetic ipsenol and combined with logs infested with male *I. pini*, while Miller and Borden (1990) presented clear evidence from British Columbia that *E. sphegeus* is attracted to synthetic, racemic ipsdienol. In California we have confirmed the response of *E. sphegeus* to ipsdienol and further demonstrated that *E. sphegeus* will respond to almost optically pure (99.4%) (−)-ipsdienol.

The black-bellied clerid, *E. lecontei*, is the principal predator of *D. brevicomis* (Person, 1940) and is known to aggregate rapidly on trees freshly attacked by *D. brevicomis* (Stephen and Dahlsten, 1976). It also preys on *Ips* spp. (Furniss and Carolin, 1977). Vité and Gara (1962) reported attraction of *E. lecontei* to *P. ponderosa* logs infested with any of five species of scolytids, including *I. paraconfusus* and *D. brevicomis*. Wood et al. (1968) and Wood (1970) trapped *E. lecontei* responding to *P. ponderosa* logs infested with male *I. paraconfusus* and to various mixtures of synthetic ipsenol, ipsdienol, and *cis*-verbenol. Lanier et al. (1972), Birch and Wood (1975), and Furniss and Livingston (1979) have reported response of *E. lecontei* to natural aggregation pheromones produced by male *I. pini*. *E. lecontei* responded in a dose-dependent fashion to synthetic, racemic ipsdienol (Miller and Borden, 1990). In that study the pine monoterpene, β-phellendrene, had no effect on the response of *E. lecontei*.

In our study, *E. lecontei* did not respond to one release rate of 99.4% (−)-ipsdienol or lanierone individually above control levels; however, together the two compounds represent a synergistic kairomone for a significant bark beetle natural enemy. This pattern of activity is similar to that discovered for *I. paraconfusus*, where three terpene alcohols, including ipsdienol, were inactive individually, but together they elicited the attractant response (Silverstein et al., 1966; Wood et al., 1968). The dose-dependent response (Figure 5) stands in contrast to the response of *I. pini* (Figure 1) to these two compounds, and to the results obtained by Miller and Borden (1990) in British Columbia. Indeed, *E. lecontei* in California responds to the ipsdienol–lanierone combination as *I. pini* does in New York (Teale et al., 1991). It would be interesting to determine the response pattern for *E. lecontei* to increasing doses or variable enantiomeric compositions of ipsdienol in the presence of a fixed dose of lanierone.

Lanier et al. (1972) found that *E. lecontei* in California and Idaho exhibited

a strong preference for logs infested with male *I. pini* from New York over those infested with males from California and Idaho. The strong response of *E. lecontei* to lanierone and ipsdienol, which are both produced by New York *I. pini*, and the absence or very low amounts of lanierone in California *I. pini* in this study are sufficient to explain the preference observed by Lanier et al. (1972). In this study, the lower response of *E. lecontei* to ipsdienol alone than to the male-infested log (Figure 5) may indicate the release of additional kairomonal compounds from the male-infested log.

The strong response of *E. lecontei* to lanierone + ipsdienol may have evolved because of a close association between *E. lecontei* and eastern populations of *I. pini*, for which lanierone plays a major role in aggregation (Teale et al., 1991). The current distribution of *E. lecontei* is reported to range eastward from the Pacific coast to Ontario and Michigan (Person, 1940; Papp, 1960), and *I. pini* is the principal bark beetle attacking pines in northeastern and north central North America. In contrast, *E. sphegeus* and *T. chlorodia*, neither of which discriminated between the combination of lanierone + ipsdienol and ipsdienol alone, have entirely western distributions (Person, 1940; Barron, 1971). *T. chlorodia* is replaced by the closely related *T. virescens* (F.) and *T. acuta* LeConte in eastern and southern North America (Barron, 1971).

Another possibility is that the strong response of *E. lecontei* to lanierone + ipsdienol in western North America may have evolved in response to production of lanierone and ipsdienol by a western ancestral form of *I. pini* that resembled its present-day eastern counterpart in this trait. Selection pressure exerted by *E. lecontei* on *I. pini* may have resulted in the loss or reduction of the biosynthetic capacity for lanierone production and a reduced response to lanierone and ipsdienol by the present population of *I. pini* in California relative to other populations in North America. Miller et al. (unpublished) have found that the relative increase in response of California *I. pini* to lanierone and ipsdienol compared to ipsdienol alone is the lowest of six populations tested in North America. It has been suggested that selection pressure by the predaceous clerid *Thanasimus dubius* (F.) results in a shift in response to the enantiomers of ipsdienol by populations of *I. pini* in north-central North America (Raffa and Klepzig, 1989; Herms et al., 1991). In the same fashion, Payne et al. (1984) suggested that selection pressure by *T. dubius* on *D. frontalis* may result in a production–response shift in the enantiomeric composition of frontalin.

Alternatively, *E. lecontei* may have evolved a strong kairomonal response to ipsdienol and lanierone because these compounds are produced by other prey species in western North America. Male *D. brevicomis* have been reported to produce (+)-ipsdienol (Byers, 1982), and this compound was present in the extracts of hindgut tissue from males that were collected live from a pheromone-baited tree (Byers et al., 1984). In the latter study, the amount of ipsdienol was maximal when the males were exposed to elevated levels of oleoresin exuded

by the attacked tree (day 2, 80 ng/male). Ipsdienol remained detectable later in the colonization process (days 8 and 11, 5 and 7 ng/male, respectively). The high, but slightly delayed response of *E. lecontei* to an aggregation of *D. brevicomis* (Byers et al., 1984) may be explained by lanierone production in either or both sexes of *D. brevicomis* several days after arrival on the tree. This would also explain the increase in response of *E. lecontei* when a *D. brevicomis*-infested log is added to a male *I. paraconfusus* log (Byers and Wood, 1980). Therefore, we hypothesized the presence of lanierone in *D. brevicomis*, but did not detect it. It is possible that lanierone is produced by *D. brevicomis* when both sexes are together in the standing tree or that it is produced by *E. lecontei* itself as an aggregation pheromone component whose activity is dependent on ipsdienol, a bark beetle-produced synergist.

We have shown for the first time that the enantiomeric composition of ipsdienol produced by male *D. brevicomis* is 97.8% (+), which is slightly greater than that produced by the cohabiting *I. paraconfusus* from the same locality (Seybold et al., 1992b,c). This enantiomeric composition of ipsdienol may limit aggregations of *D. brevicomis*. Byers (1982) has shown that racemic ipsdienol interrupts response of *D. brevicomis* to synthetic aggregation pheromones. However, various enantiomeric blends of ipsdienol remain to be tested against the natural pheromone produced in trees under attack by *D. brevicomis*. This enantiomeric composition of ipsdienol would also interrupt aggregations of *I. pini* in areas where *I. pini* and *D. brevicomis* co-occur in *P. ponderosa* (e.g., eastern California, Oregon, Washington, Idaho, and southeastern British Columbia). The absence of male-produced ipsdienol in gut extracts of fed and unfed insects, as well as in the extract from the Porapak Q aeration may be a result of low myrcene content in the *P. ponderosa* logs used to feed *D. brevicomis* males. Byers et al. (1984) observed the correlation of ipsdienol production in male *D. brevicomis* with oleoresin exudation from *P. ponderosa* and myrcene is present in *P. ponderosa* oleoresin.

Kohnle and Vité (1984) have classified the prey location strategies of bark beetle predators as generalist or specialist based on the number of kairomones used for prey location. Several members of the Trogositidae, including *T. chlorodia*, were grouped among the specialists (one kairomone), while members of the Cleridae were grouped among the generalists (several kairomones). However, we have demonstrated that, in addition to *exo*-brevicomin, *T. chlorodia* also responds to ipsdienol, which is produced by at least 21 species of *Ips* in North America (Seybold et al., 1992b) and by male *D. brevicomis* (Byers, 1982). *E. lecontei* and *E. sphegeus*, the principal predators of *D. brevicomis* and *D. ponderosae*, respectively, also respond to ipsdienol + lanierone and ipsdienol, respectively. In contrast to species-specific aggregation pheromones of their prey, these bark beetle predators have evolved the capacity to recognize multiple kairomone signals that are important components of the aggregation

pheromone of different prey species. Vité and Gara (1962) observed the response of these three predators to five scolytid species. The ability to locate aggregations of several bark beetle species occurring throughout its geographic range increases the size of the possible food base for the predator and thus increases the probability of survival and reproduction.

The strong response of *E. lecontei* to ipsdienol and lanierone released at 2 mg/day and the inhibition of *I. pini* response at this dose suggest a novel control strategy using behavioral chemicals. Nascent aggregations of *I. pini* could be treated with a proportionately high dose of lanierone compared to ipsdienol, which would minimize further bark beetle aggregation while recruiting additional predators. Together with lanierone, the use of (+)-ipsdienol, a strong inhibitor for *I. pini* (Seybold et al., 1992d) and possibly for *D. brevicomis* (Byers, 1982), may also decrease the probability of further bark beetle aggregation.

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BEHAVIORAL AND ELECTROPHYSIOLOGICAL ACTIVITY OF (*Z*,*E*)-7,9,11-DODECATRIENYL FORMATE, A MIMIC OF THE MAJOR SEX PHEROMONE COMPONENT OF CAROB MOTH, *Ectomyelois ceratoniae*

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Abstract—The behavioral and electrophysiological activity of a mimic [(*Z*,*E*)-7,9,11-dodecatrienyl formate] of the major sex pheromone component [(*Z*,*E*)-9,11,13-tetradecatrienal] of carob moth was assessed. Wind-tunnel bioassays demonstrated that the formate was as effective as natural gland extracts, and significantly more effective than the trienal alone or than the trienal blended with two minor pheromone components, in evoking source contact. Dispensers containing the formate were as effective as trienal-containing blend lures in attracting males when placed at the same dosage in traps in date gardens. Single-cell recordings showed that at least two olfactory neurons, differentiated by spike amplitude, are located in the long trichoid hairs on male carob moth antennae. Dose–response relationships indicated that puffs from cartridges loaded with at least 0.1 μg of the formate or of the trienal were necessary to elicit spiking by either the small- or the large-spiking cell within a sensillum. Cross-adaptation studies demonstrated that both compounds stimulated the same large-spiking cell. The frequencies of spikes evoked from the large cell when stimulated by emissions from 0.1-μg, 1-μg, or 10-μg cartridges of either the formate or the trienal were not significantly different, suggesting that the formate is an effective mimic of the trienal at the antennal receptor cell level.

Key Words—Lepidoptera, Pyralidae, sex pheromone, (*Z*,*E*)-9,11,13-tetradecatrienal, mimic, (*Z*,*E*)-7,9,11-dodecatrienyl formate, wind tunnel, attractant, electrophysiology, receptor cell.

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INTRODUCTION

In the desert valleys of southern California, the carob moth, *Ectomyelois ceratoniae* (Zeller), is a serious pest of dates (Warner, 1988). If its range in this state expands northward towards the Central valley, economically important nut crops such as almonds and pistachios may be threatened, as the carob moth is a pest of these crops in other parts of the world (Dhouibi, 1982; Gothilf, 1984). In an effort to detect and monitor carob moth populations in southern California date gardens, synthetic lures placed in traps have been used with some success (Baker et al., 1991). The carob moth sex pheromone consists of a blend of three unsaturated aldehydes isolated and identified from female gland extracts (Baker et al., 1989b, 1991): (*Z,E*)-9,11,13-tetradecatrienal (trienal), (*Z,E*)-9,11-tetradecadienal (dienal), and (*Z*)-9-tetradecenal (monoenal). All three compounds evoked EAG activity from male antennae, and the major trienal component elicited upwind flight by males in the wind tunnel, with either minor component improving flight response when blended with the trienal (Baker et al., 1991). An 8:1:1 (trienal–dienal–monoenal) synthetic blend, approximating the ratio found in female gland extracts, was as effective as the natural gland extract in eliciting upwind flight and source contact (Baker et al., 1991).

In the field, Baker et al. (1991) demonstrated that the 8:1:1 blend loaded into a polyethylene Beem capsule, and placed unsealed in a Pherocon 1C trap captured significant numbers of carob moth males, although the numbers were low when compared to those trapped by live females. They suggested that this discrepancy might be due to decomposition of the trienal by oxidation and/or photodegradation under field conditions; without the trienal, attraction to the synthetic lure was negligible. Similar degradation problems have been reported for two other recently identified conjugated triene pheromones, 10*E*,12*E*,14*Z*-hexadecatrienal, a pheromone component of *Manduca sexta* (L.) (Doolittle et al., 1990), and 10*E*,12*E*,14*Z*-hexadecatrienyl acetate, a pheromone component of the mulberry pyralid, *Glyphodes pyloalis* Walker (Honda et al., 1990).

The likelihood of decomposition of the carob moth trienal in the field over periods of days or weeks prompted us to examine a potentially more stable mimic of this component that would successfully attract males when placed in traps. In the present study, we report the results of wind-tunnel bioassays and field-trapping studies that compare the attractiveness of (*Z,E*)-7,9,11-dodecatrienyl formate (formate) with that of the trienal. A formate compound was chosen as the trienal mimic because Mitchell et al. (1975) suggested that formates may be more stable under field conditions than unsaturated aldehyde pheromones, and because formates have been shown to disrupt mating communication in some moth species whose pheromones consist of blends of aldehydes (Mitchell et al., 1975, 1976). We also describe the single-cell responses of male carob moth olfactory neurons to the three sex pheromone components,

and to the formate, in order to determine if the formate is an effective mimic of the trienal at the antennal receptor cell level.

METHODS AND MATERIALS

Chemical Synthesis. The monoenal and dienal were prepared from the corresponding alcohols (available from Aldrich Chemical Co.) by oxidation with pyridinium dichromate molecular sieve (Herscovici et al., 1982) and by Swern oxidation (Mancuso et al., 1978). The crude products were purified by HPLC (RP-18; low-pressure gradient; methanol–water) or by flash chromatography on silica gel (5% ether in hexane) followed by Kugelrohr distillation (0.1 mm Hg, oven temperature 100°C). Syntheses of the trienal have been published elsewhere (Baker et al., 1989b, Millar, 1990). The triene formate was synthesized as described below (Figure 1).

Proton NMR spectra ($CDCl_3$) were recorded on a GE-300 NMR spectrometer at 300 MHz. Infrared spectra were recorded from films of neat compounds on NaCl plates with a Perkin-Elmer 727 spectrometer. Unit resolution mass spectra (electron impact at 70 eV) were recorded with a Hewlett-Packard (Avondale, Pennsylvania) 5970 mass selective detector interfaced to an H-P 5890 capillary GC. An HP-1 capillary column (20 m × 0.2 mm, Hewlett-Packard) was used. Routine GC runs were made on an H-P 5890 GC fitted with a DB-5 column (20 m × 0.32 mm, J & W Scientific, Folsom, California). Flash chromatography was run with 230–400 mesh silica gel (Aldrich Chemical Co.). Unless otherwise specified, reaction work-up solutions were dried over anhyd. Na_2SO_4 and concentrated under partial vacuum (ca. 100 mm Hg) on a rotary evaporator.

(1E)-1-Chloro-dec-1-en-3-yn-10-ol ***(3).*** 7-Octyn-1-ol **(2)** was prepared from 3-octyn-1-ol **(1)** in 84% yield by the modified acetylene zipper reaction (Abrams and Shaw, 1988). A dry 2-liter 3-neck flask was loaded with bis(triphenylphosphine)palladium(II) chloride and CuI (1 g each) and flushed

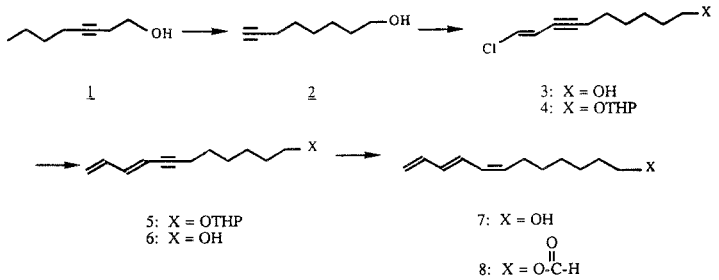

FIG. 1. Synthetic route to (*Z,E*)-7,9,11-dodecatrienyl formate.

with Ar. Alkynol **(2)** (20 g, 159 mmol), freshly distilled *trans*-1,2-dichloroethylene (31 g, 356 mmol), and THF (200 ml) were added to the reaction flask via a 5-cm column of 3 Å activated molecular sieve. To the stirred mixture were added dropwise 40 ml of diisopropylamine (distilled from CaH_2), and the initially pale yellow solution rapidly turned brown, then black. The mixture was stirred overnight at room temperature, after which time the reaction was ca. 50% complete. A further 1 g each of CuI and bis(triphenylphosphine)-palladium(II) chloride was added, and stirring was continued until the reaction was complete (4 hr). Hexane (400 ml) was then added, and the mixture was stirred a further 10 min before filtering with suction. The filtrate was extracted with saturated aqueous NH_4Cl (3 × 100 ml), dried, and passed through a column of coarse silica gel (70–230 mesh), eluting with 40% EtOAc in hexane. The eluate was concentrated and distilled, bp 100°C (0.03 mm Hg), yielding 25.2 g (85%) of chloroalcohol **(3)**. NMR δ: 6.43 (d, 1H, J = 13.7 Hz, H1), 5.92 (dt, 1H, J = 13.5, 2.2 Hz, H2), 3.64 (m, 2H, H10), 2.30 (td, 2H, J = 6.8, 2.2 Hz, H5), 1.5–1.65 (m, 4H, H6,9), 1.35–1.48 (m, 4H, H7,8), 1.22 (br. s, 1H, OH). IR cm^{-1}: 3360 (s, br.), 3100 (w), 3050 (w), 2965 (s), 2880 (s), 2250 (m), 1590 (m), 1240 (m), 1065 (m), 930 (s), 860 (m), MS *m/z*: 151 (2, $M-Cl)^+$, 114 (72), 112 (52), 105 (45), 91 (84), 79 (100), 77 (57), 65 (55), 51 (37), 41 (99).

(9E)-Dodeca-9,11-dien-7-yn-1-ol ***(6)***. Chloroalcohol **(3)** was protected as the THP ether by treatment with dihydropyran (18 ml) and a few crystals of *p*-toluenesulfonic acid in ether overnight. The mixture was worked up by extraction with sat. aq. $NaHCO_3$ and brine, drying, concentration, and removal of solvent traces under vacuum. The protected alcohol **(4)** gave one spot on TLC (5% EtOAc in hexane) and was used without further purification.

Tetrakis(triphenylphosphine)palladium(0) and chloride **(4)** (36.92 g, 134 mmol) were added to 300 ml of degassed toluene under Ar. The mixture was cooled in an ice-bath while vinyl magnesium bromide (150 ml of a 1 M solution in THF) was added dropwise over 1 hr. The mixture was warmed to room temperature with stirring overnight. [Caution: the starting material and product were indistinguishable by TLC (5% EtOAc in hexane)]. The reaction mixture was poured into hexane (500 ml), extracted thoroughly with 2 M NH_4Cl and brine, dried, and concentrated. The residue was dissolved in MeOH (100 ml), 100 mg of *p*-toluenesulfonic acid was added, and the mixture was stirred at room temperature until transetherification was complete (36 hr). $NaHCO_3$ (2 g) was added, and the mixture was concentrated on a rotary evaporator. The residue was partitioned between water and hexane (200 ml each). The hexane layer was washed with brine, dried, and partially fractionated by passage through a 2.5-cm × 20-cm column of silica gel, eluting with 40% EtOAc in hexane, and cutting ca. 300-ml fractions. The fraction containing the purified product was concentrated and pumped under vacuum, giving dienynol **(6)** as a gold oil. NMR

δ: 6.51 (dd, 1H, J = 15.3, 10.8 Hz, H10), 6.35 (overlapped ddd, 1H, J = 16.7, 10.7, 9.8 Hz, H11), 5.62 (dt, 1H, J = 15.3, 2.0 Hz, H9), 5.26 (br. d, 1H, J = 16.3 Hz, H12 *cis*), 5.13 (d, 1H, J = 9.8 Hz, H12-*trans*), 3.65 (t, 2H, J = 6.5 Hz, H1), 2.32 (td, 2H, J = 6.7, 2.1 Hz, H6), 1.63–1.5 (m, 4H, H2,5), 1.5–1.32 (m, 4H, H3,4), 1.25 (br. s, 1H, OH). IR cm^{-1}: 3360 (s, br), 3110 (w), 3050 (w), 3025 (w), 2965 (s), 2880 (s), 2245 (m), 1630 (m), 1065 (s), 1040 (m), 1010 (s), 950 (m), 915 (m), 855 (m). MS *m/z*: 178 (2, M^+), 163 (1), 131 (4), 117 (19), 106 (27), 105 (25), 91 (100), 79 (46), 78 (37), 77 (32), 65 (42), 41 (45).

(7Z,9E,11)-Dodecatrien-1-yl formate ***(8).*** Zinc granules (30 mesh, 100 g) were washed with 200 ml of 0.1 M HCl for 20 min under Ar. The acid was pipetted off, and the cleaned zinc was rinsed twice with distilled water. THF–water (80:20, 500 ml) was added, followed by a solution of dienynol **(6)** (13.35 g, 75 mmol) in 50 ml THF. The mixture was stirred vigorously under Ar for five days, then filtered, and the THF removed by rotary evaporation. The residue was extracted with hexane, the hexane layer backwashed with brine, dried, and concentrated, yielding 13.6 g of trienol **(7)** as a yellow oil. This oil was used without further purification.

Formic acetic anhydride was freshly prepared by addition of formic acid (20 ml of 96%) to 40 ml of acetic anhydride at 0°C. When the addition was complete, the mixture was slowly warmed to 50°C, held at this temperature for 20 min, then cooled to room temperature, and used immediately.

The mixed anhydride (30 ml) was added dropwise to a solution of trienol **(7)** (13.6 g, 75 mmol) and pyridine (30 ml) in ether (250 ml) at 0°C. The mixture was warmed to room temperature overnight, then cooled to 0°C, and 125 ml of sat. aq. $NaHCO_3$ were added cautiously (effervescence!) The layers were separated, the ether layer was extracted again with aq. $NaHCO_3$ and brine, then dried and concentrated, yielding 14.2 g of crude product **(8)**. A portion (4 g) was purified by flash chromatography on silica gel (5 cm × 25 cm, 5% ether in hexane), yielding 2.47 g of purified product, which was contaminated with 4% of an unidentified isomer. The purified product showed signs of rapid degradation, with the formation of insoluble white deposits on the rims of the collection tubes. Consequently, the rest of the crude material was purified by rapid Kugelrohr distillation (oven temperature 110°C, 0.07 mm Hg). The distillate (8.79 g from 10.2 g of crude material) was immediately diluted to a concentration of 50 mg/ml with hexane, butylated hydroxytoluene (BHT) was added as an antioxidant (50 mg/g formate), and the solutions were flushed with Ar before storing in brown glass bottles at −20°C. Formate solutions prepared this way could be stored for several months with negligible deterioration. NMR δ: 8.06 (br. s, 1H, Formate H), 6.49 (dd, 1H, J = 14.8, 11.3 Hz, H9 or 10), 6.41 (dt, 1H, J = 16.8, 10.3 Hz, H11), 6.21 (dd, 1H, J = 14.8, 10.7 Hz, H10 or 9), 6.05 (br. t, 1H, J = 11.0 Hz, H8), 5.47 (dt, 1H, J = 10.7, 7.7

Hz, H7), 5.22 (d, 1H, J = 16.6 Hz, H12-*trans*), 5.08 (d, 1H, J = 10.2 Hz, H12-*cis*), 4.17 (td, 2H, J = 6.6, 0.5 Hz, H1), 2.20 (m, 2H, H2), 1.67 (m, 2H, H6), 1.5–1.3 (m, 6H, H3-5). ^{13}C NMR δ: 25.68, 27.72, 28.44, 28.73, 29.43, 64.02, 117.00, 128.49, 128.54, 133.06, 133.15, 137.17, 161.18. IR cm^{-1}: 3110 (w), 3040 (m), 2965 (s), 2880 (s), 1725 (s), 1625 (m), 1585 (m), 1475 (m), 1190 (s), 1015 (s), 950 (m), 910 (m). MS *m/z*: 208 (21, M^+), 133 (4), 119 (5), 105 (12), 93 (39), 91 (64), 80 (84), 79 (100), 77 (64), 65 (16), 41 (42).

Wind-Tunnel Bioassays. Moths were obtained from laboratory cultures started from individuals collected in 1985 from infested dates near Indio in the Coachella Valley of Riverside County, California. Larvae were reared on a honey and wheat-bran diet in 1-gallon jars (Warner, 1988). Pupae were segregated by sex, and adults emerged on a 14 : 10 hr light–dark photoperiod regime.

Bioassays were conducted in a 3.5 × 1.0 × 1.0-m wind tunnel described in detail in Kuenen and Baker (1982). Two- to four-day-old males were loaded singly into screen cages (4 cm long × 3 cm diameter) during the photophase and each cage was covered with a Petri dish lid. Cages with males were placed in an environmental chamber until being transferred to the wind tunnel 1 hr before bioassays in order to acclimate males to the wind-tunnel conditions (24–26°C, 0.3 lux, 30–70% relative humidity, 0.5 m/sec wind velocity). Bioassays were conducted 4–6 hr into the scotophase, the optimal sexual activity period for carob moth (Vetter, Tatevossian, and Baker, unpublished data). Two 8 : 1 : 1 blend ratios were used, representing 40 ng trienal, 5 ng dienal, and 5 ng monoenal (referred to as the trienal blend), or 40 ng formate, 5 ng dienal, and 5 ng monoenal, respectively, with the formate replacing the trienal (referred to as the formate blend). The trienal and the formate also were each tested alone at 40 ng. Solutions were checked on GC to confirm that the ratio and concentration of each treatment were correct. When freshly prepared, the trienal was >98% stereoisomerically pure by capillary GC (DB-5). However, the trienal was contaminated with 2–5% of a rearrangement product that coeluted with the trienal on silica gel flash chromatography or HPLC. The formate was >92% pure by capillary GC, and was contaminated with an uncharacterized impurity, also apparently due to rearrangement. A control consisted of 5 female equivalents (FE) obtained from excised pheromone glands according to methods described in Baker et al. (1991). For both the treatment and the control, 10 μl of a diluted solution prepared in hexane was delivered onto a 5-cm-diameter filter-paper disk (Whatman No. 1) affixed to a 6-cm-long metal clip that was stuck in a cork base. The cork wire holder for each disk was placed on a 15 × 15-cm sheet-metal platform 15 cm above the tunnel floor, and 30 cm from the tunnel's upwind end. After completing bioassays, clips were cleaned with acetone and air-dried overnight.

Males were released from their cages 1 m from the odor source. A cage

containing a male was placed on the metal platform in the plume until the male took flight or until 1 min elapsed. If males did not fly, they were checked for flight ability by forcibly dislodging them from the cage. Forty flight-capable males were tested for each treatment and for the control. Males were scored for: (1) locking onto the plume and progressing upwind, (2) flying to within 50 cm of the source, and (3) contacting the source. Tests were conducted using a randomized, complete-block design so that all treatments were presented on a given day. The numbers of moths performing each behavior were compared by using a χ^2 2 $\times$ 2 test of independence with Yates' correction (Steel and Torrie, 1960). Statistical significance was determined at the 0.05 level.

Field-Trapping Studies. Two field studies were conducted in Deglet Noor date gardens located in the Coachella Valley in Indio or in Oasis, California, to determine the attractancy of the formate compared to that of the three-component aldehyde blend of carob moth. One study was conducted from late August through late September, 1990. Four different lures were placed in separate Pheroocon 1C traps (Zoecon Corp., Palo Alto, California), and the traps were positioned in the date palm canopy, which was ca. 18 m above ground level, by using a rope and pulley system. Lures consisted of black, chopped, Celcon hollow fibers (1.5 cm $\times$ 0.02 cm ID) (Scentry Inc., Buckeye, Arizona) that were heat-sealed at one end and then vacuum-filled with either an 8:1:1 blend of the trienal–dienal–monoenal (three fibers), or with the formate (three and 10 fibers). Both the aldehyde blend and the formate were diluted by 90% with an inert diluent, methyl tridecanoate, before the fibers were filled. Fibers also were filled with neat formate (three fibers). Fibers were stuck into a 2-cm polyurethane foam cube and affixed to the bottom of a trap. Treatments were placed in a row in each of three replicates in a randomized, complete-block design. There were two trees separating treatments in each block and 10 trees between blocks. Traps were checked every three to four days, the males removed, and the traps rerandomized within blocks. Trap bottoms were replaced when they became too soiled or dusty. Trap counts were transformed using $(x + 0.5)^{1/2}$ before being subjected to analysis of variance (ANOVA). Treatment means were separated at the 0.05 level of significance using Tukey's HSD Studentized range test (Steel and Torrie, 1960).

Because this field study (as well as two others containing live females) seemed to indicate a significant interference with male capture even to females, and hence a general disruptive effect whenever a grid of more than a few traps was used, another field comparison was made between a single trap containing the synthetic lure (either trienal blend or formate), and a trap containing live females. This study was conducted in three Deglet Noor date gardens from mid-July through mid-August of 1990 and 1991. Both the aldehyde blend and the formate were diluted by 90% with methyl tridecanoate, and three fibers were filled as previously described and stuck into a 2-cm polyurethane foam cube

that was affixed to the bottom of a Pherocon 1C trap. Five 1- to 3-day-old virgin female carob moths were placed in a screen cage in a separate Pherocon 1C trap as a control. A 2-dram glass vial containing a water-saturated dental wick was placed inside the screen cage to provide a water source for females. Traps were placed in the date palm canopy, which varied between 2 and 15 m above ground level, depending on the garden, by using a rope and pulley system. In 1990, a trienal blend-baited trap was placed in a date palm in each of the three date gardens, with a female-baited trap placed 5–10 trees away (ca. 30-m spread). In 1991, a formate-baited trap was placed in the same date palm in each garden as the previous year, with a female-baited trap also placed in the same tree as the trap from the previous year. The number of males caught in female-baited or in treatment-baited traps was recorded every three to four days. In both years, traps with females were rebaited twice a week, and fiber traps were rebaited once every two weeks. The trap catches of female-baited or of treatment-baited traps from each date garden were combined to obtain the mean ($\pm$SD) number of males captured per trap for each year. Trap catches from treatment-baited traps, either trienal blend-baited or formate-baited, and from female-baited traps were totaled within each garden, and the ratio between the number of males captured in a treatment-baited trap and in a female-baited trap was computed for each of the three date gardens. These six ratios were compared using ANOVA with the two chemical treatments blocked by date garden site. Statistical significance was determined at the 0.05 level.

Single-Cell Recordings and Stimulation. Recordings from olfactory antennal neurons of 1- to 3-day-old male carob moths were obtained by using a cut-sensillum technique (Kaissling, 1974; Van der Pers and Den Otter, 1978) that has been used on several other species of Lepidoptera (Baker et al., 1989a; Hansson et al., 1987, 1990; Löfstedt et al., 1990; Todd et al., 1992). An antenna was cut from the head, and its base placed in a micropipet (50 μl FISHERbrand disposable micropipets) that was filled with Beadle-Ephrussi Ringer solution (Ephrussi and Beadle, 1936), and grounded with an Ag–AgCl wire. Using a Leitz stereo microscope at 320× magnification, a single sensillum trichodeum was positioned over the blade of a vertically oriented, stationary glass knife by using a micromanipulator. The tip of the hair was then cut using a second glass knife whose edge was brought down over the vertical knife edge by means of a Leitz joy-stick type micromanipulator. The cut end of the hair was immediately contacted with a saline-filled Ag–AgCl pipet recording microelectrode. To prevent evaporation of the saline, a 10% aqueous solution of polyvinylpyrrolidone (Sigma Chemical Co., St. Louis, Missouri) was added to the saline (1 : 10 parts by volume), and the tip of the recording microelectrode was coated with a thin film of petroleum jelly. Recordings of both the AC and DC components of the signal were amplified on a Hansson model 103 amplifier, and stored on VCR tape using a Vetter model 420F four-channel FM recorder. Responses were

displayed and printed using a Gould model 1604 (DSO) digital oscilloscope/printer.

The antenna was continuously bathed in a stream of purified, humidified air (10 ml/sec) that passed through a 17-cm-long glass tube (8 mm ID) whose outlet was positioned 2 cm from the antennal preparation. For each of the three sex pheromone components and the formate, 10 μl of a diluted solution prepared in hexane was pipetted onto a filter-paper strip held in a glass Pasteur pipet cartridge (15 cm long). Stimulus doses ranged from 10^{-3} to 10^{1} μg/cartridge in $\log_{10}$ steps. Solutions were checked by GC to confirm that the amounts of respective compounds at a particular concentration were equal. Prior to exposure to any of the test compounds, the spontaneous activities of the receptor cell(s) within a sensillum were monitored for 60 sec. Cells were exposed to 30 msec puffs (Roelofs and Comeau, 1969) of each compound in random order, beginning with the 10^{-3}-μg cartridges and working upward to the 10-μg cartridges, by manually injecting a 2-ml puff into the airstream through a hole in the glass tube 15 cm from the outlet. Interstimulus intervals ranged between 30 and 60 sec, or until the background firing approximated that observed prior to stimulus presentation. When action potentials (spikes) were elicited by a compound, their frequency was determined for 0.2 sec following the commencement of excitation, and peak-to-peak amplitude values (mV) of the bimodal spikes were obtained during this period. The spike frequencies of individual receptor cells that were excited by each dosage of the trienal and of the formate were compared using Student's t test at the 0.05 significance level.

Cross-Adaptation Studies. Preliminary recordings indicated that the trienal and the formate consistently stimulated a large-spiking cell within each sensillum. Therefore, cross-adaptation studies were conducted to determine if the spiking activity could be attributed to the same cell or to different cells that produced similar-sized spikes (Kaissling et al., 1989). Recordings were made from the cells within 15 sensilla using four antennae of 1- to 3-day-old male carob moths as previously described. A 1-μg filter-paper-loaded cartridge of the trienal and of the formate provided the stimulus sources. To deliver a uniform stimulus with a well-defined onset and offset, a stimulus flow controller (model SFC-2, Syntech Inc., Hilversum, The Netherlands) that has been described in detail by Vickers and Baker (1992) was used. The stimulus valve was open for 0.5 sec, and two interstimulus intervals, i.e., the time between the end of the first stimulus and the beginning of the second stimulus, were used, either 0.1 sec or 1 sec. The stimulus regime consisted of the following four stimulus pairs presented at both interstimulus intervals: (1) trienal–trienal; (2) formate–formate; (3) trienal–formate; and (4) formate–trienal. Between 30 and 60 sec elapsed between exposure of the preparation to each stimulus pair.

Scanning Electron Micrographs. Antennae from newly emerged males were used for scanning electron micrographs. For fixation, antennae were placed in

2% glutaraldehyde (in cacodylate buffer, pH 7.4). Postfixation involved placing the antennae in a 2% solution of osmium tetroxide for 24 hr, followed by dehydration to 100% EtOH. Glacial acetic acid was added to each dehydration step to maintain the natural size and shape of the antenna. Specimens were critical-point dried, mounted on stubs and coated under vacuum with 60:40 gold–palladium. Specimens were examined and micrographs prepared at 15 kV accelerating voltage, using 55 P/N film on a JEOL JSM-C35 SEM located in the Department of Nematology, University of California, Riverside.

RESULTS

Wind-Tunnel Bioassays. The 8:1:1 blend containing either the trienal or the formate, or either compound alone, was as effective as 5 FE of gland extract in eliciting upwind flight in male carob moths (Table 1). Similarly, all blends were equivalent to 5 FE of gland extract in causing males to fly to within 50 cm of the source ($P > 0.05$, χ^2 2 × 2 test of independence) (Table 1). The formate alone was significantly better than either the trienal alone or than the 8:1:1 trienal or formate blends in eliciting close approach to the source ($P < 0.05$, χ^2 2 × 2 test of independence) (Table 1). The formate alone also caused significantly more males to complete upwind flight and contact the source compared to either the trienal blend or to the trienal alone ($P < 0.05$, χ^2 2 × 2 test of independence). Both the formate alone and the formate-containing blend were as effective as the natural extract in evoking source contact (Table 1).

TABLE 1. PERCENTAGES OF MALE CAROB MOTHS FLYING IN WIND TUNNEL IN RESPONSE TO 8:1:1 BLEND OF TRIENAL, DIENAL, AND MONOENAL, OR OF FORMATE, DIENAL, AND MONOENAL, OR TO TRIENAL OR FORMATE ALONE (8:0:0), COMPARED TO 5 FE OF GLAND EXTRACT[a]

	(%) Males responding to blend ratios				
	trienal		formate		
Behavior	8:0:0	8:1:1	8:0:0	8:1:1	Gland extract, 5 FE
Upwind flight	50 a[b]	65 a	70 a	63 a	53 a
Flight to within 50 cm of the source	38 b	38 b	65 a	38 b	43 ab
Source contact	18 b	18 b	45 a	25 ab	40 a

[a] Source loading on filter paper was 40 ng of trienal or formate in 10 μl of hexane. $N = 40$ flight-capable males.

[b] Percentages in a row followed by the same letter are not significantly different at the 0.05 level according to a χ^2 2 × 2 test of independence with Yates' correction.

Field-Trapping Studies. In the field study comparing the attractancy of the four synthetic lures to male carob moths, traps containing the three-fiber diluted formate lure captured as many males (2.6 ± 3.8, mean ± SD) per three- to four-day trapping period as those containing the three-fiber diluted trienal blend lure (0.7 ± 1.2, mean ± SD) ($P > 0.05$, Tukey's test). Thus the formate was at least as active as the trienal blend in attracting males in the field. The 10-fiber diluted formate lure attracted significantly more males to traps (6.3 ± 6.8, mean ± SD) than the three-fiber neat formate lure (1.7 ± 2.2, mean ± SD), or the three-fiber diluted trienal blend lure (0.7 ± 1.2, mean ± SD) ($P < 0.05$, Tukey's test), but a similar number of males as the three-fiber diluted formate lure (2.6 ± 3.8, mean ± SD). There were no statistically significant differences between the mean numbers of males caught per trap for all the other treatment comparisons ($P > 0.05$, Tukey's test).

In the other field study, the female-baited traps caught significantly more males (42 ± 34.4, mean ± SD, $N = 33$) than the trienal blend-baited traps (3.7 ± 4.6, mean ± SD, $N = 33$) when placed in a date palm canopy ($P < 0.001$, Student's t test). Similarly, female-baited traps captured significantly more males (67.2 ± 36.5, mean ± SD, $N = 31$) than the formate-baited traps (16 ± 10.9, mean ± SD, $N = 30$) ($P < 0.01$, Student's t test). However, the formate was a significantly better lure relative to females than the trienal-containing blend, since the formate-to-female trap capture ratio (0.25 ± 0.07, mean ± SD) was significantly larger than the trienal-to-female trap capture ratio (0.07 ± 0.05, mean ± SD) ($P < 0.01$, ANOVA). Thus, the formate-baited traps caught 3–4 times as many male carob moths as the trienal-blend containing traps, relative to live-female traps.

Single-Cell Recordings and Stimulation. Single-cell recordings were obtained from 56 sensilla using 10 male antennae. The longer, curved trichoid hairs located on the ventral aspect of the antenna, and irregularly distributed over the surface of a subsegment, were easily accessible to cutting compared to the shorter, straighter hairs located closer to the dorsal scaled surface (Figure 2). Recordings from these longer trichoid sensilla showed that two spike sizes could be easily distinguished; there was a larger impulse of 13.6 ± 3.9 mV (mean ± SD), and a smaller impulse of 6.9 ± 0.8 mV (mean ± SD). The spike amplitude values obtained from cells responding to emissions from 10-μg cartridges of the dienal, trienal, and formate were not included in these mean values because the spike amplitudes of both cells were noticeably reduced by exposure to this dosage. Although transmission electron microscopy of male carob moth antennae has not yet been performed to confirm the number of receptor cells within the longer trichoid hairs, the two different spike amplitudes that we consistently observed indicate that at least two receptor cells are present, which we will refer to as the large and the small cell, respectively, even though at this point it would be possible for more cells of identical spike sizes to be present.

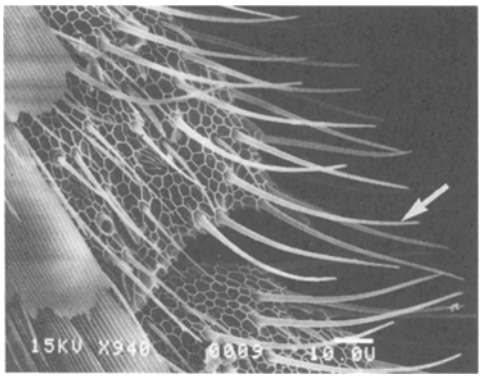

FIG. 2. Scanning electron micrograph of the middle subsegments (30–40) of the male carob moth antenna, showing the long trichoid hairs (see arrow) that were cut to obtain single-cell recordings.

In all but two of the sensilla, background firing by one or both receptor cells was observed prior to stimulus presentation. In 34 of the sensilla, both cells fired spontaneously. The background activity was 0.3 ± 0.1 spikes/sec (mean ± SD) for the smaller-spiking cell, recorded from 43 sensilla, and 0.5 ± 0.1 spikes/sec (mean ± SD) for the larger-spiking cell, recorded from 45 sensilla.

Dose–response curves indicated that puffs from cartridges loaded with less than 0.1 μg of a pheromone component or of the formate did not elicit spiking activity from either the large cell (Figure 3A) or the small cell (Figure 3B). A few of the sensilla sampled contained a cell or cells that were excited by puffs from 0.1-μg cartridges of the dienal, trienal, and formate, but not by the monoenal (Table 2). All three compounds stimulated the larger-spiking cell within a sensillum (Figure 4), although the smaller cell also was excitable in some sensilla (Table 2). There was no significant difference between the spike frequencies of the large-spiking cell when exposed to puffs from 0.1-μg cartridges of the trienal or of the formate ($P > 0.05$, Student's t test) (Figure 3A). A statistical comparison between the number of spikes evoked by the small cell when exposed to emissions from 0.1-μg cartridges of the trienal or of the formate could not be made because only one of the sensilla we sampled contained a small cell excited by the formate (Table 2).

The numbers of sensilla with receptor cells responding to puffs of the dienal, trienal, and formate increased most dramatically between the 0.1-μg and 1-μg dosages (Table 2). The first receptor cell responses to the monoenal also were recorded at the 1-μg dosage (Figure 3A), although the number of sensilla that we sampled that contained such cells was low (Table 2). The monoenal

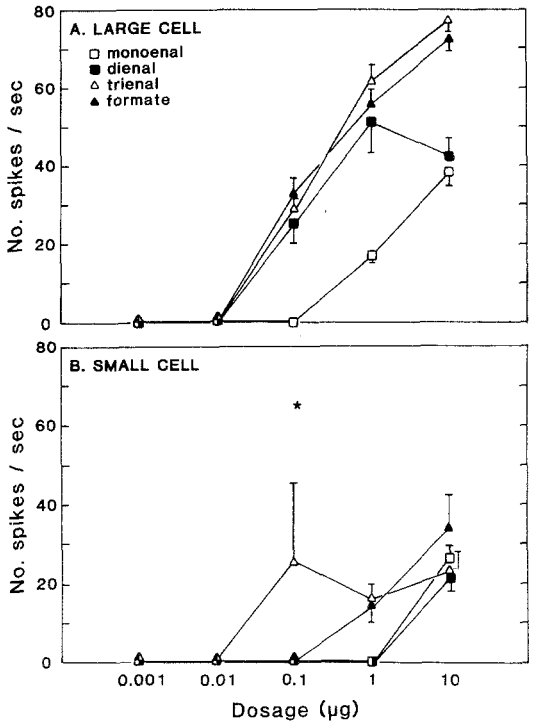

FIG. 3. Mean (± SE) dose-response relationship of the spiking activity of a large cell (A) and a small cell (B) in male carob moth antennal sensilla in response to puffs from cartridges loaded with graded amounts of one of the three sex pheromone components (monoenal, dienal, and trienal) or the trienal mimic (formate). Fifty-six sensilla were sampled. For the small-cell responses (B) at the 0.1-μg dosage, note the asterisk, which represents the spiking activity evoked by exposure to the dienal (65 spikes/sec, $N = 1$) and to the formate (65 spikes/sec, $N = 1$); no spiking activity was recorded from the small cell in the other 55 sensilla sampled.

typically stimulated the larger-spiking cell within a sensillum (Figure 4). The spike frequencies elicited by both the large and the small cell when exposed to puffs from 1-μg cartridges of the trienal were not significantly greater than those evoked by exposure to the formate ($P > 0.05$, Student's t test) (Figure 3A and 3B).

Exposure to puffs from 10-μg cartridges of the trienal or of the formate elicited single-cell responses from nearly all the sensilla sampled (Table 2). The spike frequencies of the small and the large cells when exposed to puffs from 10-μg cartridges of the trienal were not significantly different from those evoked by exposure to the formate (Student's t test, $P > 0.05$) (Figure 3A and 3B).

TABLE 2. PERCENTAGE OF SENSILLA ON MALE CAROB MOTH ANTENNAE WITH RECEPTOR CELL(S) RESPONDING TO EMISSIONS FROM CARTRIDGES LOADED WITH VARIOUS DOSAGES OF MONOENAL, DIENAL, AND TRIENAL (SEX PHEROMONE COMPONENTS), AND OF FORMATE (TRIENAL MIMIC)[a]

	Percentage of sensilla with a responding receptor cell									
	0.001 μg		0.01 μg		.1 μg		1 μg		10 μg	
Compound	SM	L	SM	L	SM	L	SM	L	SM	L
Monoenal	0	0	0	0	0	0	0	3.6	8.9	28.6
Dienal	0	0	0	0	1.8	3.6	0	12.5	33.9	67.9
Trienal	0	0	0	0	3.6	12.5	21.4	69.6	32.1	96.4
Formate	0	0	0	0	1.8	12.5	17.9	67.9	19.6	89.3

[a] A total of 56 sensilla was sampled. SM = small cell, L = large cell.

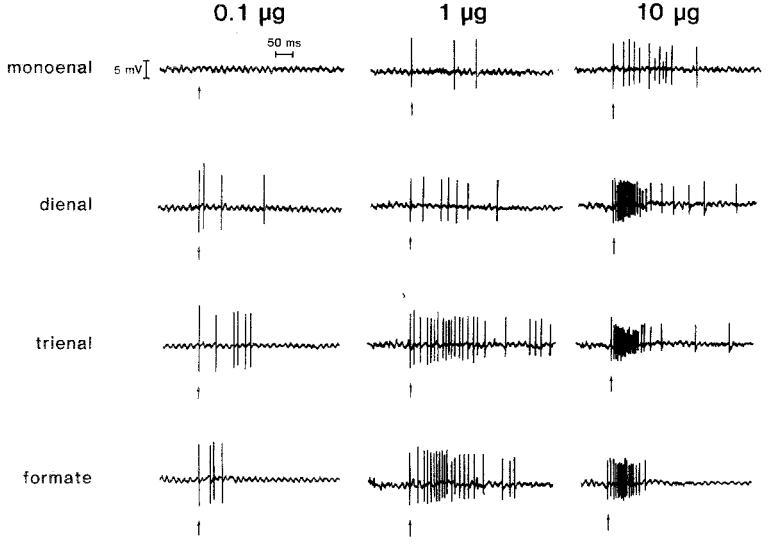

FIG. 4. Typical single-cell responses of male carob moth antennal cells to emissions from 0.1-μg, 1-μg, and 10-μg cartridges of the monoenal, dienal, and trienal (sex pheromone components) and of the formate (trienal mimic). Arrows represent stimulus presentation. Note the spikes (2) produced by the smaller-spiking cell interspersed among the large spikes for 10 μg of monoenal.

Puffs from 10-μg cartridges of the dienal, trienal, and formate appear to overdrive the larger-spiking receptor cell, as suggested by the reduced interspike intervals and diminished spike amplitudes immediately following stimulus presentation (Figure 4). However, the spike amplitude of the large cell was not diminished by exposure to puffs from 10-μg cartridges of the monoenal (Figure 4). We also noted increased spiking activity from the small cell within a sensillum after puffs from 10-μg cartridges of each compound, compared to the lower dosages (Figure 3B).

Cross-Adaptation Studies. The single-cell responses of a large-spiking neuron located within the antennal sensilla of male carob moths showed that this neuron could be partially adapted by exposure to the trienal–trienal pair, with a 0.1-sec interstimulus interval (Figure 5A). A large-spiking cell was similarly partially adapted by exposure to the formate–formate pair at a 0.1-sec interstimulus interval (Figure 5E). For both the trienal–trienal (Figure 5C) and the formate–formate (Figure 5G) stimulus pairs, the large cell was capable of becoming markedly more disadapted after a 1-sec interstimulus interval because the second stimulus of each pair evoked action potential bursts that were nearly as high in frequency as those to the first stimulus of the pair. Exposing the neuron to the trienal–formate or to the formate–trienal pairs resulted in a similar pattern of responses as those evoked by the homogeneous pairs, whether it was for the 0.1-sec interstimulus interval (Figures 5B and 5F) or for the 1-sec interstimulus interval (Figures 5D and 5H). Initial exposure to the trienal seems to adapt the large cell more completely to the second puff in the stimulus pair, regardless of whether it is the formate or the trienal (Figure 5A–D), compared to when initial exposure is to the formate (Figure 5E–H).

DISCUSSION

If a mating disruptant is to be developed against the carob moth, it would be helpful to extend the field life of the blend of aldehydes identified by Baker et al. (1989b, 1991) or to develop a more stable mimic. The first step in developing a more stable mimic is ensuring that its ability to attract males is as good as the identified pheromone components. In this initial assessment of the activity of a potential trienal pheromone mimic, our results indicate that (*Z*,*E*)-7,9,11-dodecatrienyl formate is an effective replacement for (*Z*,*E*)-9,11,13-tetradecatrienal, the major sex pheromone component of the carob moth. We do not know why the formate seems to be as effective as, or more effective than, the blend of the trienal plus two other aldehydes in attracting carob moth males in the wind tunnel and in the field. However, the greater activity of a mimic compared to the natural pheromone is not unprecedented. Mimics have been found in other studies that are more active in attracting males than the natural

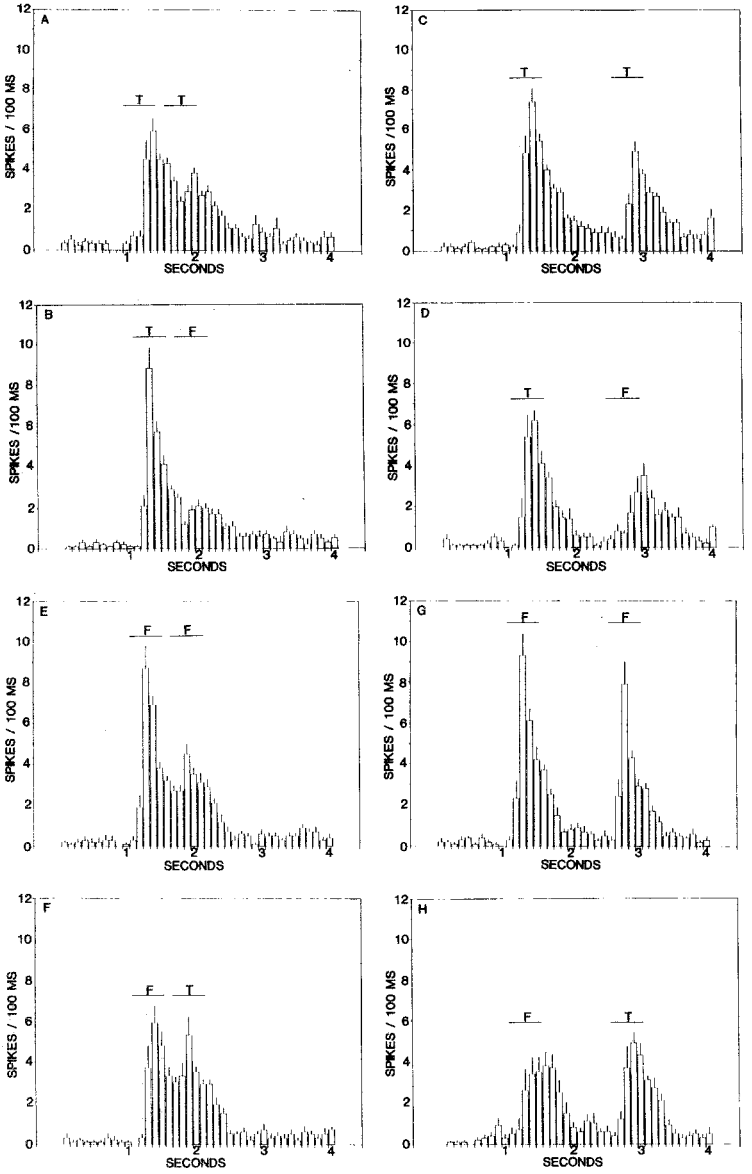

FIG. 5. Histograms showing the spike frequency (mean ± SE) of the large-spiking neuron to four stimulus pairs (T = trienal, F = formate) during cross-adaptation studies. Each histogram shows a 1-sec prestimulus period, followed by a 3-sec period during which two 0.5-sec long stimulus puffs are directed over the sensillum. Horizontal lines above the bars indicate that the stimulus valve is open, and odor molecules are entering the airstream that is passing over the antennal preparation. Interstimulus intervals are 0.1 sec and 1 sec. Recordings are continued for at least 1 sec after the end of the second stimulus.

pheromone component blend (Cardé and Roelofs, 1977) or that are more active than the natural components in stimulating antennal receptor cells (Löfstedt et al., 1990). The formate is as active on the same antennal receptor cells as the trienal; therefore, the mimic appears to be at least as active as the major pheromone component at both the neurophysiological and the behavioral levels.

Mitchell et al. (1975, 1976) showed that formate esters could act as mimics of aldehyde pheromones by demonstrating their disruptive effects on mating in species of *Heliothis*. Similarly, Landolt et al. (1982) showed that several diene formates, mimics of the dienal major pheromone component of the navel orangeworm, *Amyelois transitella* (Walker), dispensed in female-baited traps in almond orchards, reduced the number of males captured relative to untreated controls. Our study differs from these previous studies in that our initial goal has been to determine the attractancy of the formate as a trienal pheromone mimic, rather than first determining its disruptive capabilities. Compounds that are the best attractants singly or in blends have been shown to be the most efficacious disruptants of mating (Roelofs and Cardé, 1977; Minks and Cardé, 1988; Cardé, 1990). Also in our study, we have implicated the site at which the formate acts to mimic the aldehyde as being at the receptor cell level, something that most other studies, except for Grant et al. (1989), have heretofore not done.

Mitchell et al. (1975) suggested that replacing the aldehyde functional group of a pheromone with a formate ester enhances stability, so formate esters may be successful mimics of unsaturated aldehyde pheromones because of their increased field life relative to these aldehydes. It is well known that aldehydes are susceptible to degradation via a number of pathways, including oxidation to form peroxides (Kobayashi et al., 1990) or carboxylic acids (Verter, 1970; Shaver and Ivie, 1982), or by the formation of trimers (Starr and Vogel, 1978; Kubisa et al., 1980; Dunkelblum et al., 1984) or polymers (Bevington, 1952), especially when catalyzed by traces of protic or Lewis acids, or by heat. Substitution of the formate group for the aldehyde eliminates these degradation pathways, and provides an analog with a potentially longer field lifetime.

However, conjugated trienes readily isomerize and polymerize at ambient temperatures or above, especially when subjected to heat and sunlight under field conditions (Doolittle et al., 1990; Honda et al., 1990; Millar, 1990). Conjugated hydrocarbons also are prone to oxidative degradation to furans (Ideses and Shani, 1988). In a similar fashion to the carob moth trienal, the pure formate analog began to polymerize over a period of several hours when left at room temperature in air. However, unlike the trienal, the formate could be stored at $-20°C$ in dilute solution (hexane) under Ar for extended periods with no noticeable degradation, conditions under which the trienal often polymerized into an intractable gel.

We do not know if our formate remains biologically active longer than the

trienal under the field conditions in the date gardens. In the field study comparing the attractancy of four synthetic lures to male carob moths, our frequent replacement of the lures every three to four days was designed to test the attractancy of fresh formate and trienal lures without the complication of potential long-term degradation. In the other field study, the fiber lures were replaced every two weeks, and the formate outperformed the trienal blend, relative to live females, in attracting males, thus suggesting that the formate may have a longer period of optimal attractivity than the aldehyde blend. The assessment of relative field longevity needs to be performed in future field studies in which traps have much longer replacement intervals.

The coupling between female-emitted sex pheromone components and the olfactory receptor cells located in male antennal sensilla is generally highly specific for moths (Baker, 1989), with one receptor type very selective for a particular component of the sex pheromone blend. Although the electrophysiological activity of a compound may be detrimentally affected by altering the double-bond position or geometry (Liljefors et al., 1985; Bengtsson et al., 1987), or by modifying functional groups (Liljefors et al., 1984), pheromone mimics can substitute for the natural pheromone component at both the neurophysiological and the behavioral levels (Löfstedt et al., 1990).

The electrophysiological activity of formates on the antennal receptors of several noctuid species was demonstrated by Priesner et al. (1975) when they showed that formate compounds could evoke moderate EAG responses when compared to natural sex pheromone components. Our single-cell recordings from male carob moth antennal neurons showed that the formate was as electrophysiologically active as the trienal in evoking action potentials. The effective concentrations of the two compounds reaching the sensilla during stimulation were likely to be very similar, based on the comparable vapor pressures of the trienal (206 MW) and the formate (208 MW). At all dosages tested, the spike frequencies elicited from receptor cells were similar for the trienal and for the formate, and cross-adaptation studies demonstrated that both compounds stimulated the same large-spiking cell. Grant et al. (1989) also found that the aldehyde major sex pheromone component of *Heliothis zea* (Boddie) and a formate mimicking this component stimulated the same large-spiking neuron, although the aldehyde elicited significantly higher spiking activity than the formate when tested at several dosages. Based on GC–single-cell analysis, Baker et al. (1991) initially concluded that the trienal was stimulating a smaller-spiking cell within a sensillum, whereas the monoenal and the dienal were stimulating the larger-spiking cell. However, the present, more detailed examination of the antennal receptor cell responses of male carob moths to various dosages of the trienal indicates this conclusion was probably in error. Excessive amounts of the trienal may have issued from the GC effluent in the GC–single-cell study by Baker et al. (1991), thus overdriving the larger-spiking cell, and causing a reduction in

spike size to that more typical of the smaller-spiking cell (Figure 4). We now conclude that the major component and its mimic stimulate the larger-spiking cell within a male carob moth antennal sensillum, as is typical of all other moth species studied to date (Grant et al., 1989; Hansson et al., 1987, 1990; Löfstedt et al., 1990). Our results indicating that initial exposure to the trienal adapted the large cell more completely to the second puff in the stimulus pair, whether it was the trienal or the formate, may be explained by slight differences in the concentration emitted from the trienal pipets compared to the formate pipets, or perhaps to subtle differences in the time courses of adaptation–disadaptation in cells exposed to the trienal compared to the synthetic mimic. Nevertheless, our conclusion is that the two compounds stimulate the same receptor neuron based on the great similarities in adaptation of the large neuron.

Our data indicate that the behavioral and electrophysiological activity of the formate is equal, if not enhanced, compared to that of the trienal blend in attracting carob moth males. Although the formate has thus far outperformed the trienal blend under field conditions, more work needs to be done to improve the efficacy of the formate lure to make it competitive with females with regard to attractancy. We do not know why the ability of the formate to attract males in the wind tunnel is as good as the female extract, yet in the field it is only a fraction as good as live females.

In general, the high activity of synthetic blends in wind tunnels has been maintained in trapping tests in the field (Baker and Linn, 1984). However, full comparisons of the activity of synthetics against females in both the wind tunnel and the field are relatively rare, and so it is difficult to say how unusual such a decline in activity is, when the lures are taken into the field. It may be that the entire effect is due to degradation under field conditions, or perhaps some other inadequacy exists with respect to missing components that is not exposed in the relatively simple environment of the wind tunnel. In wind-tunnel bioassays with *Manduca sexta* (L.), Tumlinson et al. (1989) demonstrated that a synthetic blend of aldehydes evoked the same behavioral response level from males as gland rinses or calling females. One of the aldehydes necessary for completing the behavioral sequence, i.e., locating the source and attempting copulation, was (*E*,*E*,*Z*)-10,12,14-hexadecatrienal. Although a synthetic blend containing this trienal was not tested in the field, Tumlinson et al. (1989) suggested that aliphatic conjugated trienes are very labile and will decompose in nature when placed as components in lures or other pheromone dispensers.

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OVIPOSITION PREFERENCE OF THE DIAMONDBACK MOTH (*Plutella xylostella*) UNAFFECTED BY THE PRESENCE OF CONSPECIFIC EGGS OR *Bacillus thuringiensis*

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Abstract—Neither toxins from *Bacillus thuringiensis* Berliner nor conspecific eggs deterred oviposition by the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), in laboratory choice tests. The finding that toxins did not deter oviposition by moths from a susceptible line shows that oviposition preference and larval survival were not associated in this line. Selection for larval resistance to toxins did not significantly alter oviposition preference, which rules out a strong genetic correlation between larval performance and oviposition preference. Failure of conspecific eggs to deter oviposition may not represent lack of association of preference and performance because other evidence suggests that larval performance may not be greatly affected by larval density. These results suggest that the ability of refuges to slow evolution of physiological resistance to *B. thuringiensis* toxins will not be magnified by an oviposition preference for untreated foliage.

Key Words—Oviposition preference, preference-performance correlation, egg-load assessment, resistance, toxin, *Bacillus thuringiensis*, *Plutella xylostella*, Lepidoptera, Plutellidae.

INTRODUCTION

In phytophagous insects, the relationship between oviposition preference and larval performance affects many aspects of nutritional ecology, including host

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shifts and the evolution of host specialization (Slansky and Rodriguez, 1987; Thompson, 1988). This relationship also has important implications for the efficacy of plant protectants and the evolution of resistance (Gould, 1991; Caprio and Tabashnik, 1992). One might assume that host preference reflects relative larval fitness on hosts, but this is not always true (Tabashnik and Slansky, 1987; Thompson, 1988). Therefore, to understand host-plant use, one must determine whether preference and performance are associated and, more specifically, whether they are genetically correlated (Thompson, 1988; Jaenike, 1990; Via, 1990; Jaenike and Holt, 1991).

Oviposition by Lepidoptera is influenced by a variety of factors, including phytochemicals, presence of conspecific eggs, and host shape and texture (Thompson and Pellmyr, 1991). In this study, we investigated the effect of larvicidal proteins produced by the microbe *Bacillus thuringiensis* Berliner (Hofte and Whiteley, 1989) and the effect of conspecific eggs on oviposition behavior in the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), a worldwide pest of cruciferous vegetables (Talekar, 1986). Oviposition in this species is stimulated by glucosinolates, the mustard oils (Gupta and Thorsteinson, 1960; Reed et al., 1989), and can be deterred by other phytochemicals (Dover, 1985; Tabashnik, 1985).

We addressed three questions concerning oviposition behavior of the diamondback moth. First, do *B. thuringiensis* toxins deter oviposition in susceptible moths? Given the fact that survival of susceptible larvae is reduced in the presence of *B. thuringiensis* toxins, susceptible females should be deterred from ovipositing on leaves laden with toxins if oviposition preference and larval performance are associated. In field populations, adults encounter toxins on plants sprayed with commercial formulations containing toxins. The recent incorporation of toxin-coding genes from *B. thuringiensis* into genomes of several crop plants, and the planned commercial use of these genetically engineered plants (Gasser and Fraley, 1989; Boulter et al., 1990), means that field populations of insects will eventually be exposed to *B. thuringiensis* toxins produced by plant tissue. For both the current and anticipated field situation, one can view the toxins from *B. thuringiensis* as analogous to phytochemicals that could have deterrent effects on oviposition.

Second, we asked whether selection for increased larval survival on a diet including toxins would change the preference of ovipositing females as a correlated response. We addressed this question by determining whether moths derived from a field population resistant to *B. thuringiensis* (Tabashnik et al., 1990), and selected for a higher level of resistance in the laboratory (Tabashnik et al., 1991; Groeters et al., 1992), differed in their oviposition preference from susceptible moths. If so, performance and preference would be genetically correlated.

Third, do conspecific eggs deter further oviposition, as in another crucifer-

feeding lepidopteran, *Pieris brassicae*, (Rothschild and Schoonhoven, 1977; Klijnstra, 1986), and some other Lepidoptera (Thompson and Pellmyr, 1991)? Egg-load assessment is most common in species for which resource competition is likely, and so is consistent with an association of preference and performance (Thompson and Pellmyr, 1991). Diamondback moth populations appear to be regulated primarily by natural enemies, host nutrient status, and weather rather than resource competition (Harcourt, 1986). Therefore, failure to find an oviposition deterrent effect of conspecific eggs in the diamondback moth might be consistent with a positive association between preference and performance based on egg loads.

MATERIALS METHODS

Effect of Toxin on Oviposition. Oviposition preference was tested in females from a resistant (NO-QA) and a susceptible (NO-Q) line, both derived originally from diamondback moths sampled at a watercress farm near Pearl City, Oahu, Hawaii. Four generations of selection for resistance to *B. thuringiensis* resulted in a greater than 1000-fold difference in median lethal concentration (LC_{50}) between NO-QA and NO-Q (Groeters et al., 1992; selection procedures described in Tabashnik et al., 1991). The moths used in this study were the F_1 offspring of moths used to measure the aforementioned difference in resistance between lines. Although the resistant line had not undergone selection during the previous generation, the difference in resistance between the lines is unlikely to have declined dramatically over that time.

Larvae were raised on cabbage plants (C-G Cross, Takii and Company, Kyoto, Japan) in cylindrical cages made of acetate sheets. They were maintained at 28°C and 14:10 hr light–dark photoperiod. Upon adult eclosion, pairs of adults were placed into inverted Petri dishes (100 mm diam.). Food was provided by a 30-mm piece of dental wick saturated with 10% sucrose solution. Two cabbage leaf disks (20 mm diam.) served as potential oviposition sites. They were placed about 30 mm part, each on a strip of filter paper saturated with water to keep them turgid. One disk had been dipped for 5 sec in a distilled water dilution of the HD-1 strain of *B. thuringiensis* subsp. *kurstaki* (Dipel 2X; Abbott Laboratories, North Chicago, Illinois) at the recommended field rate of 0.0256 mg AI/ml. In bioassays of third-instar larvae from the previous generation, this concentration caused greater than 90% mortality in the susceptible NO-Q line and less than 1% mortality in the resistant NO-QA line at 120 hr after treatment. The other disk was dipped for 5 sec in distilled water. Following treatment, disks were air dried for 2 hr at about 23°C before being placed into cages. Most moths began ovipositing on the first night following adult eclosion. Leaf disks were collected the following morning, even if no oviposition had

occurred, and replaced with a fresh pair of disks. This procedure was repeated until we had obtained eggs laid on two separate days from each pair. Adult moths were maintained at 28°C and 14:10 hr light–dark photoperiod. Oviposition data were gathered from 37 susceptible females and 22 resistant females.

Oviposition preference was tested using randomized complete block models similar to the model employed by Jaenike and Grimaldi (1983). To test for an effect of toxins on oviposition preference of susceptible moths, we used a model that included a main effect of leaf dip and female as a block effect. A difference between susceptible and resistant moths for oviposition preference was tested with a model that included main effects for line and leaf dip, and female nested within line as a block effect. A significant line × treatment interaction would indicate that the lines differed in oviposition preference. Individual variation for preference would be indicated by a significant treatment × female interaction. Analyses were performed by the general linear models procedure of SAS (SAS Institute, 1985).

Effect of Conspecific Eggs on Oviposition. "Treated" leaf disks were obtained by placing 10 disks (20 mm diam.) and 50 two-day-old adult moths into a Petri dish (150 mm diam.) that was placed into a light-proof box at 28°C. Darkness in the box stimulated oviposition, and after 1–9 hr leaf disks with eggs on them were removed from the Petri dish. The number of eggs on each disk (its egg load) was recorded. One treated (with eggs) and one untreated (without eggs) disk were then placed into a Petri dish (100 mm diam.), 30 mm apart, along with a single female moth. A small slit made in the side signified the treated leaf disk. Females were maintained at 28°C and 14:10 hr light–dark photoperiod. Leaf disks were collected in the morning; eggs were laid the previous evening. We counted the total number of eggs laid on each disk and obtained the number laid over the course of the evening on the treated disk by subtracting the egg load for that disk. Varying the time females were allowed to oviposit on treated disks yielded a range in egg loads. The experiment was repeated three times with low, moderate, and high egg loads. In the replicate with a low egg load, a laboratory stock founded with individuals from Leeward, Oahu (LAB-L), was used. In the other two replicates, females from LAB-P, a stock founded with individuals from Pulehu, Maui, were tested. Data for each egg load level were analyzed using a t test for paired comparisons. The entire data set was analyzed by analysis of variance with main effects for egg load level (low, moderated, high) and conspecific eggs (eggs present or absent), and female nested within egg load level as a block effect.

Power Analysis. In all statistical analyses, the probability of type I error (α) was set at 0.05. Upon failure to reject a null hypothesis, we performed a power analysis to determine the detectable effect size in the experiment (Rotenberry and Wiens, 1985; Cohen, 1988). The detectable effect size is the smallest difference in a dependent variable that would be likely to result in rejection of

the null hypothesis, given the sample size (N), α, and the desired probability of type II error (β). The magnitude of the detectable effect size relative to an effect postulated to be reasonable for the biological phenomenon being investigated indicates whether or not power sufficient to detect the biological effect was present in an experiment. β was set at 0.05 to equal α. This allowed application of the same probability standard to a conclusion from a negative result (failure to reject the null hypothesis) as from a positive result (rejection of the null hypothesis).

RESULTS

B. thuringiensis toxins did not deter oviposition by susceptible diamondback moths. Susceptible moths laid 32.2 ± 2.90 (mean ± SE) eggs on leaf disks dipped in water and 32.1 ± 2.7 eggs on disks dipped in toxins. The difference was not significant (Table 1). Failure to find an effect of toxins on oviposition was not due to lack of power. The power of the F test for an effect of leaf dip is equivalent to the power of a paired t test. The detectable effect size for a paired t test with $N = 37$, $\beta = 0.05$ and $S = 33.3$ (standard deviation of the difference in number of eggs laid on the two types of leaf disk) is 18.6. The mean number of eggs laid on a disk was 32. Thus, we had sufficient power to detect even a weak deterrent effect, say nine fewer eggs than the mean on disks treated with *B. thuringiensis* and nine more eggs on control disks for a total difference of 18 eggs, a value equivalent to the detectable effect size. It can be concluded with a probability of less than 0.05 of being incorrect that *B. thuringiensis* toxins did not act as an oviposition deterrent.

Moths selected for resistance to *B. thuringiensis* toxins laid more eggs on leaf disks dipped in toxins (30.3 ± 3.4) than on disks dipped in water (22.5 ± 3.4), but the difference in preference compared with susceptible moths was not significant (effect of line × leaf dip interaction in Table 2). This suggests that preference and performance are not strongly genetically correlated. The power

TABLE 1. ANALYSIS OF VARIANCE FOR EFFECT OF *B. thuringiensis* TOXINS ON OVIPOSITION BY SUSCEPTIBLE DIAMONDBACK MOTHS

Source		*df*	SS	Ratio[a]	*F*	*P*
Leaf dip	[1]	1	6	[2]/[3]	0.01	0.92
Female	[2]	36	27,170	[2]/[3]	1.26	0.25
Leaf dip × female	[3]	36	21,525	[3]/[4]	1.23	0.22
Error	[4]	72	34,968			

[a] Ratio of mean squares used to construct F ratio.

of the F test for line × leaf dip interaction is equivalent to the power of a two-group t test (susceptible versus resistant lines) on the difference in number of eggs laid on the two types of leaf disks (Green, 1989). The detectable effect size for this t test, using an average N of 29 with $\beta = 0.05$ and $S = 29.7$ is 26 eggs. Because this effect is large, we cannot exclude the possibility of a weak genetic correlation between preference and performance. The leaf dip × female interaction was not significant (Table 2), which reflects an overall lack of variation in preference among individual females.

Conspecific eggs on leaf disks did not deter oviposition. For all three egg load levels, the number of eggs laid on disks without eggs was greater than the number laid on disks with eggs, but for no level was the difference significant (Table 3). Despite the tendency for fewer eggs to be laid on disks with conspecific eggs at each of the egg load levels, analysis of variance of the entire data set also indicated that the effect of conspecific eggs was not significant, either as a main effect ($F = 1.34$; $df = 1, 66$; $P = 0.25$) or through interaction with egg load level ($F = 0.10$; $df = 2, 66$; $P = 0.90$). The detectable effect sizes

TABLE 2. ANALYSIS OF VARIANCE FOR EFFECT OF *B. thuringiensis* TOXINS ON OVIPOSITION BY SUSCEPTIBLE AND RESISTANT DIAMONDBACK MOTHS

Source		*df*	SS	Ratio[a]	*F*	*P*
Line	[1]	1	1,565	[1]/[4]	2.48	0.12
Leaf dip	[2]	1	1,107	[2]/[5]	2.36	0.13
Line × leaf dip	[3]	1	927	[3]/[5]	1.98	0.17
Female (line)	[4]	57	36,016	[4]/[5]	1.35	0.13
Leaf dip × female	[5]	57	26,733	[5]/[6]	1.19	0.21
Error	[6]	112	44,057			

[a] Ratio of mean squares used to construct F ratio.

TABLE 3. EFFECT OF CONSPECIFIC EGGS ON OVIPOSITION BY DIAMONDBACK MOTH

	Egg load			Egg count (mean ± SE)				
Line	Range	Mean	*N*	Disks with eggs	Effect of eggs[a]	*t*	*P*	Detectable effect size[b]
LAB-L	1-17	3.6	30	13.3 ± 1.7	1.9 ± 3.4	0.57	0.57	11.4
LAB-P	5-36	15.8	20	11.9 ± 2.0	3.6 ± 3.0	1.21	0.24	10.1
LAB-P	15-69	39.5	19	15.4 ± 1.9	1.4 ± 3.4	0.40	0.69	11.5

[a] Number of eggs laid on disks without eggs − number laid on disks with eggs.
[b] Smallest difference in number of eggs likely to be detected as statistically significant with $\alpha = \beta = 0.05$.

were about 10–12 eggs (Table 3). If the level of a deterrent effect corresponded to the number of eggs already on a leaf disk, then the power to detect an effect in the "low" replicate may have been insufficient because there was only an average of 3.5 eggs on treated leaf disks, a value less than the detectable effect size. However, the power of the "medium" and "high" replicates to detect a deterrent effect was sufficient because the average number of eggs present initially on the treated leaf disk exceeded the detectable effect size.

DISCUSSION

The finding that toxins from *B. thuringiensis* did not deter oviposition by diamondback moths from a susceptible line shows that host preference was not positively correlated with larval performance in this line. The simplest explanation for this result is that females cannot detect the toxins. Other possibilities include lack of genetic variation in oviposition preference and lack of sufficient selection for avoidance of toxins. It has been suggested previously that oviposition on unsuitable hosts (Wiklund, 1975; Chew, 1977; Legg et al., 1986) may reflect recent associations in which the insect has not had enough time to evolve appropriate changes in behavior. In other cases, overriding effects of ecological factors, such as natural enemies, host phenology, microclimate, and foraging constraints, may cause poor correspondence between nutritional suitability and preference (Tabashnik and Slansky, 1987; Thompson, 1988).

The absence of a significant difference in preference between resistant and susceptible moths excludes a strong genetic correlation between preference and performance. However, in contrast to the lack of preference in susceptible females, resistant females laid slightly more eggs on leaf disks laden with toxins than on disks without toxins. Thus, we cannot rule out the possibility of a weak genetic correlation between preference and performance. Physiological resistance of diamondback moth larvae to *B. thuringiensis* toxins is associated with reduced fitness in the absence of toxins (Groeters et al., 1992). This type of trade-off in insect fitness on plants with and without toxins would favor host specialization; a genetic correlation between performance and preference would facilitate specialization (Via, 1990). Our results are consistent, however, with the general finding that such correlations are not common in phytophagous insects (Thompson, 1988; Jaenike and Holt, 1991).

We did not find phenotypic variation among females in oviposition preference in our laboratory lines, but intraspecific variation in preference is common in other species (Thompson and Pellmyr, 1991). The lines that we studied may have lost phenotypic and genetic variation that occurs in field populations of diamondback moth. Another important limitation of our study is that preferences for whole plants in the field may not be reflected by preferences for leaf disks in the laboratory.

In this study, the presence of conspecific eggs did not deter oviposition by diamondback moths. Although this finding might suggest that preference and performance are not associated, the diamondback moth's small size relative to its hosts and the importance of factors other than competition in regulating moth populations (Harcourt, 1986) suggest that selection for egg-load assessment is limited. Thus, our results for the diamondback moth do not contradict the principle of egg-load assessment in other Lepidoptera (Thompson and Pellmyr, 1991).

The absence of a deterrent effect of conspecific eggs has an important bearing on the test for a deterrent effect of *B. thuringiensis* toxins. A deterrent effect of conspecific eggs could have caused a uniform distribution of eggs across leaf tissue with and without toxins to be observed, suggesting lack of preference, when, in fact, the distribution was caused by a balance between a deterrent effect of toxins and a deterrent effect of previously laid eggs. The absence of a deterrent effect of conspecific eggs suggests that the failure of females to avoid ovipositing on leaf disks with *B. thuringiensis* toxins could not have been a result of the masking of a deterrent effect of toxins by an even stronger deterrent effect of conspecific eggs.

Because diamondback moths readily lay eggs on leaves treated with *B. thuringiensis* toxins, and larvae readily consume treated foliage (Schwartz et al., 1991), repeated applications of these toxins select intensely for physiological resistance (Gould, 1991). Thus, the evolution of physiological resistance to *B. thuringiensis* in diamondback moth (Ferre et al., 1991; Schwartz et al., 1991) is not surprising. Although the potential for evolution of behavioral resistance to *B. thuringiensis* toxins cannot be excluded, available evidence indicates that physiological resistance is the more likely outcome.

One of the most promising tactics for slowing evolution of resistance is to provide refuges of untreated plants (Tabashnik et al., 1991). Deterrence of oviposition by toxins would result in a disproportionately large fraction of eggs in refuges, which would enhance the ability of refuges to slow resistance development. If females do not avoid toxins, as these data suggest, the effectiveness of refuges will not be magnified by a behavioral preference for untreated foliage.

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CHEMICAL SEX DISCRIMINATION IN THE CRAYFISH *Procambarus clarkii*: ROLE OF ANTENNULES

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Abstract—Chemical sex discrimination of conspecifics by male and female crayfish (*Procambarus clarkii*) was tested. Intact female subjects were more attentive in the presence of stimulus water from males than from females and showed more aggressive postures in the presence of stimulus water from females than from males. Intact males showed more aggressive postures in the presence of stimulus water from males than from females. Females and males with only one inner antennule ramus and one outer ramus performed discrimination and localization almost as well as intact subjects. Subjects with only the two inner rami or only the two outer rami were disadvantaged in either responding to the stimuli or localizing them. The relevance of this outcome to previous conflicting reports on the use of antennules in sex discrimination in crayfish is discussed.

Key Words—*Procambarus clarkii*, Crustacea, crayfish, sex discrimination, antennule, chemical communication, aggression, discrimination.

INTRODUCTION

Studies of some decapod crustaceans have demonstrated conspecific sex recognition through their response to chemical factors in water (see Dunham, 1988, for a recent review). Studies of the lobster *Homarus americanus* have focused on the mating interaction between males and females, deducing the use of chemical cues (Atema and Engstrom, 1971; Dunham, 1979; Atema and Cowan, 1986). In studies of true crabs, the emphasis has been on response to female chemical stimuli (either water from females' holding tanks or diluted females'

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urine) by conspecific males (Ryan, 1966; Eales, 1974; Christofferson, 1978; Gleeson, 1980). In freshwater crayfish, this type of sexual recognition has been demonstrated in male *Orconectes virilis* (Hazlett, 1985) and in both male and female *Orconectes propinquus* (Tierney and Dunham, 1984; Tierney et al., 1984). The latter proposed that the aesthetascs on the outer rami of the antennules were essential sensilla for noncontact semiochemical transduction.

Evidence for sexual recognition through the presumed use of pheromones in the crayfish *Procambarus clarkii* has also been addressed in the literature, but not without controversy. Ameyaw-Akumfi and Hazlett (1975), and Ameyaw-Akumfi (1976), reported that males could chemically discriminate males from females. Males responded in an aggressive fashion to the water from a male's tank, but in a submissive fashion to the water from a female's tank. From incomplete ablation experiments, they concluded that the inner rami of the antennules mediated this discrimination. In contrast, Itagaki and Thorp (1981) found no such discrimination by male and female *P. clarkii* in their experiments with a flow-through system. Thorp and Ammerman (1978) found no significant chemical sex discrimination in male *Procambarus acutus*, with a flow-through system. The present study was undertaken in an effort to clarify the ability of *P. clarkii* to recognize conspecific sex and to determine what role the antennules play in chemical sexual discrimination.

METHODS AND MATERIALS

The subjects, form I, intermolt male and nongravid female *P. clarkii*, were obtained from a supplier in Louisiana. They were held in large aerated plastic holding tanks for five days and then housed in individual aerated glass jars containing 4 liters of distilled water for five days before testing. They were maintained on a light cycle of 12 hr dark–12 hr light, at 18–20°C, and fed commercial fish food every second day. The experiments took place in April, May, and June 1990.

Antennule ablations were of both inner rami, both outer rami, or a cross ablation of one inner and one contralateral outer ramus (determined randomly). They were performed after anesthesia by immersion in ice water. Twenty-four hours after this procedure, the animal was used in an experiment. Intact animals were not altered in any way. Stimulus water was obtained by gently scooping out 50 ml from a jar that had held one crayfish for five days (either male or female). Stimulus water came from a different crayfish for each trial. Stimulus source crayfish were not size matched. However, tests with female *P. clarkii* from the same population showed no difference in response to unfamiliar female stimulus water ($P > 0.25$) when diluted to the following concentrations with water from their own home tank (percent by volume): 100%, 75%, 50%, and 25% (Oh and Dunham, unpublished data).

The test procedure followed was similar to that used by Rose (1986) and consisted of first placing a subject crayfish and 2 liters of its own jar water in a 20 × 36 × 22 cm (high) aquarium. After 15 min, the stimulus water (from either male or female) was placed in a 50-ml burette, which released one drop every 2–3 sec into the bubble stream above an airstone in the aquarium. The behavior of the subject animal was infrared videotaped for 15 min from an adjacent room. The aquarium and the burette were thoroughly rinsed three times with distilled water between tests. The intertest interval was 20 min. The remaining 2 liters of the subject's own jar water was then placed in the aquarium along with the animal. The subject was once again left for 15 min, and then new stimulus water, which was from a different sex crayfish than in the preceding test, was presented. This test was also videotaped for a period of 15 min. The experiments were performed at night, when the animals were most active (2100 to 0200 hr). The order of the treatments (intact, inner ablates, outer ablates, cross ablates), the order of the individual animals used in each test, and the order of the source of the stimulus water were all randomized.

Twelve males and 12 females were utilized in each of the four treatments (a total of 96 crayfish), and each animal was tested once with a male's and once with a female's stimulus water. No crayfish was used more than once for a given treatment.

The cumulative duration of the two relevant groups of behaviors exhibited by the test animals was measured with stopwatches. The first is *chelae up*, which includes the raising of the chelae from the substrate, and also the cheliped displays of meral extension and meral spread (Bruski and Dunham, 1987). This set of behaviors is indicative of the perception of a stimulus. The second group of behaviors is *attention to stimulus source*, which includes being within 2 cm of the airstone, touching the airstone with either the head or a chela, and climbing on the airstone. This set of behaviors is indicative of the perception and localization of a stimulus.

RESULTS

The median scores for the two groups of behaviors, in the four test groups of 12 crayfish each, are shown in Figures 1–4. All animals responded in each test.

The raw scores for the two groups of behaviors during the male and female stimulus water tests, within the four treatment groups, were compared using the Wilcoxon signed-rank test (Castellan and Siegel, 1988), at the 5% significance level. The raw scores for the chelae up and the attention to stimulus source behaviors were also compared across treatment groups using the Wilcoxon-Mann-Whitney test (Castellan and Siegel, 1988), at the 5% significance level.

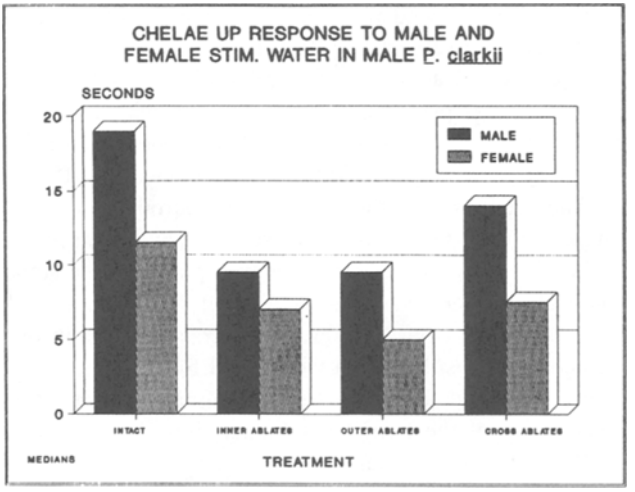

FIG. 1. Median chelae up response scores for the four groups ($N = 12$) of male crayfish *Procambarus clarkii* tested with the stimulus water from the same sex or the opposite sex conspecific. Upper, median, and lower interquartile values are given in parentheses, male stimulus test first, female stimulus test second: Intact animals (12, 19, 23; 8, 11.5, 16). Cross ablates (10, 14, 15; 4, 7.5, 9) had one inner and one outer antennule. Inner ablates (5, 9.5, 10; 5, 7, 8) had both outer antennules. Outer ablates (3, 9.5, 12; 2, 5, 6) had both inner antennules. Significance values are given in the text.

Response of Females. The intact, inner ablated (outer antennules intact) and the cross ablated (one inner and one outer ramus) female subjects displayed significantly more *chelae up* behavior in the presence of stimulus water from the same sex than in the presence of stimulus water from the opposite sex ($P = 0.007$ for intact females, $P < 0.02$ for female inner ablates, $P = 0.03$ for female cross ablates). There was no significant difference in chelae up behavior of female outer ablates in response to male versus female stimulus water ($P = 0.17$).

The female subjects differed significantly in their attention to the stimulus source. In the female trials, significantly more attention was paid to male stimulus water than to female stimulus water by the intact ($P = 0.006$), outer ablated ($P = 0.02$), and cross ablated subjects ($P = 0.01$). There was no significant difference in the attention to stimulus source by the inner ablated female subjects ($P = 0.51$).

The intact female scores for chelae up in response to both male stimulus water and female stimulus water were significantly greater than those of the inner ablates ($P < 0.03$ and $P = 0.05$, respectively). There were no significant differences between scores for the intact females and outer ablates ($P > 0.23$

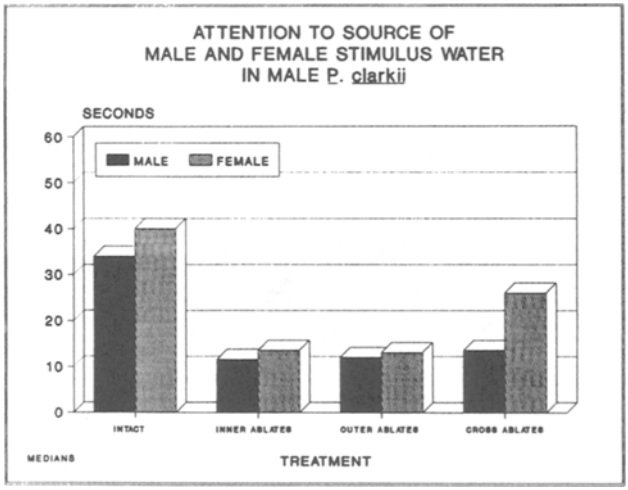

FIG. 2. Median attention to stimulus source response for the four groups ($N = 12$) of male crayfish *Procambarus clarkii* to stimulus water from the same sex or the opposite sex conspecific. Upper, median, and lower interquartile values are given in parentheses, male stimulus test first, female stimulus test second: Intact animals (19, 34, 43; 21, 40, 55). Cross ablates (8, 13.5, 18; 12, 26, 35) had one inner and one outer antennule. Inner ablates (4, 11.5, 14; 7, 13.5, 20) had both outer antennules. Outer ablates (6, 12, 17; 8, 13, 19) had both inner antennules. Significance values are given in the text.

and $P > 0.37$, respectively) or cross ablates ($P > 0.17$ and $P > 0.35$, respectively, the inner and outer ablates ($P > 0.10$ and $P > 0.09$, respectively) or cross ablates ($P > 0.06$ and $P > 0.10$, respectively), or between the outer and cross ablates ($P > 0.22$ and $P = 0.35$, respectively).

Both intact and cross ablated female subjects had significantly higher attention to the stimulus source scores, in response to male stimulus water, than those of the inner ablates ($P = 0.007$ and $P = 0.05$, respectively). There were no significant differences between the scores for intact females and outer ablates ($P > 0.13$) or cross ablates ($P > 0.49$). There were no significant differences between the scores for the outer ablates and those for the inner ($P > 0.11$) or cross ablates ($P = 0.16$).

In response to female stimulus water, the female subjects exhibited no significant differences across treatment groups in attention to the stimulus source scores. There were no significant differences in scores between the intact females and the inner, outer, and cross ablates ($P = 0.35$, $P > 0.27$, and $P > 0.22$, respectively), between the inner and the outer ($P > 0.38$) and cross ablates ($P = 0.25$), and between the outer and cross ablates ($P > 0.37$).

Response of Males. The intact, inner ablated (outer antennules intact) and

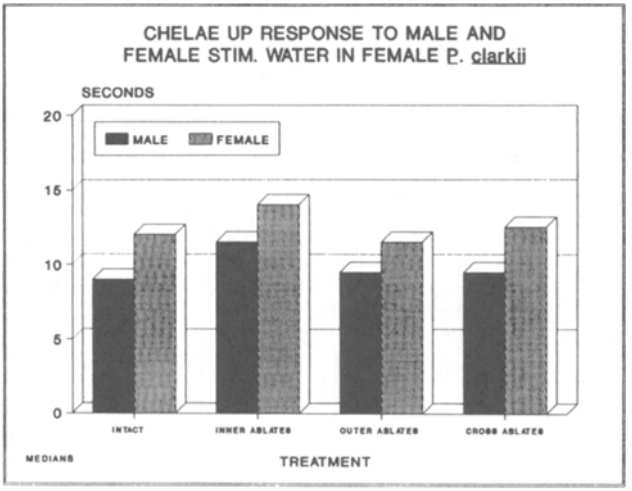

FIG. 3. Median chelae up response scores of the four groups ($N = 12$) of female crayfish *Procambarus clarkii* tested with stimulus water from male and female conspecifics. Upper, median, and lower interquartile values are given in parentheses, male stimulus test first, female stimulus test second: Intact animals (7, 11.5, 13; 11, 14, 16). Cross ablates (8, 9.5, 11; 10, 12.5, 14) had one inner and one outer antennule. Inner ablates (7, 9, 10; 10, 12, 14) had both outer antennules. Outer ablates (8, 9.5, 10; 8, 11.5, 13) had both inner antennules. Significance values are given in the text.

the cross ablated (one inner and one outer ramus) male subjects displayed significantly more chelae up behavior in the presence of stimulus water from the same sex than in the presence of stimulus water from the opposite sex ($P < 0.05$ for intact males, $P = 0.02$ for male inner ablates, $P = 0.0002$ for male cross ablates). There were no significant differences in chelae up behavior of male outer ablates in response to male versus female stimulus water ($P = 0.055$), although this difference was only marginally nonsignificant.

The male subjects did not differ significantly in their attention to the stimulus source water from males versus that from females. This result obtained in all four treatments (intact $P = 0.37$, inner ablate $P = 0.34$, outer ablate $P = 0.34$, and cross ablate $P = 0.34$).

Intact male subjects had higher chelae up scores in the presence of male stimulus water than those of the inner ablates ($P < 0.005$) and the cross ablates ($P < 0.01$), but not the outer ablates ($P = 0.055$), although the latter difference is only marginally nonsignificant. The scores of the cross ablates were significantly greater than those of the inner ablates ($P = 0.002$). There were no significant differences between the inner and outer ablates ($P > 0.08$) or the outer and cross ablates ($P > 0.12$). Intact male scores in the presence of female stimulus water were significantly greater than those of the inner ablates ($P <$

FIG. 4. Median attention to stimulus source response scores of the four groups (N = 12) of female crayfish *Procambarus clarkii* tested with the stimulus water from the same sex or the opposite sex conspecific. Upper, median, and lower interquartile values are given in parentheses, male stimulus test first, female stimulus test second: Intact animals (36, 47, 64; 21, 31, 35). Cross ablates (38, 51.5, 38; 24, 31.5, 47) had one inner and one outer antennule. Inner ablates (28, 32.5, 48; 16, 23, 42) had both outer antennules. Outer ablates (29, 44.5, 54; 17, 36, 38) had both inner antennules. Significance values are given in the text.

0.005), outer ablates ($P = 0.007$), and cross ablates ($P < 0.004$). The scores for the inner and cross ablates were significantly greater than those of the outer ablates ($P < 0.04$ and $P = 0.05$, respectively).

Attention to stimulus source scores for the intact males in the presence of the male stimulus water were significantly greater than those of the inner ablates ($P < 0.001$), outer ablates ($P < 0.001$), and cross ablates ($P = 0.006$). There were no significant differences between the inner and outer ablates, the inner and cross ablates, and the outer and cross ablates ($P > 0.31$, $P > 0.19$, and $P > 0.13$, respectively). In the presence of the female stimulus water, the scores for the intact males were significantly greater than those of the inner ($P < 0.004$), outer ($P < 0.0007$), and cross ablates ($P = 0.005$). The cross ablate scores were significantly greater than those of the outer ablates ($P < 0.02$).

DISCUSSION

Responses of Females. From the results, it is apparent that both intact and cross ablated female *Procambarus clarkii* are able to discriminate between the presence of male and female conspecifics and localize the source of stimulation

using chemical cues. The inner ablates were also able to make this discrimination, but did not localize the source of stimulation. The outer ablates did not perform chelae up behavior when demonstrating discrimination, but did differentially localize the source of male versus female stimulation.

Oh and Dunham (1991) found that female *P. clarkii* required both rami of the antennules (although not bilaterally) to clearly discriminate themselves chemically from other female conspecific and to localize the source of the stranger's water. However, their score (attention) included both chelae posture and localization of the chemical source. The present study separates these responses and shows a differential effect of outer versus inner ramus ablation on them.

Tierney and Dunham (1984) reported, after inner and outer antennule ablations, that in female crayfish *Orconectes propinquus*, the outer antennule rami were responsible for localization of the source of chemicals from opposite-sex conspecifics. They found that the inner antennule rami, while chemosensory in nature, did not play a part in conspecific localization. The contradiction between their results and those in the present study may be due to species (and generic) differences in detecting and transducing semiochemicals. Furthermore, differences in the source of stimulus water (four animals conditioned their stimulus water as opposed to one in the present study) and the type of test system used (flow-through in their study as opposed to a lentic system in the present study) may have played some unknown role in the results.

Itagaki and Thorp (1981) conclude that their female *P. clarkii* showed no evidence of chemical sex discrimination. However, their Figure 2 and Table 2 data (comparisons DE and DEF) do show sex discrimination (in chela wave) and discrimination and localization of the chemical source (time in upstream zone). Those are the only unconfounded comparisons (possible "stress pheromone" absent) that could give evidence of female sex discrimination in their design. We feel that low levels of chelae movements in isolation does not negate their value as a discriminandum here.

Responses of Males. The intact males, inner ablates, and cross ablates responded with more agonistic behavior to the male stimulus water than to the female stimulus water, demonstrating chemical discrimination based on sex. (The nonsignificant trends in median differences shown in Figures 1 and 2 suggest that additional experiments are perhaps warranted, with a larger sample of males.) The males did not demonstrate localization of the female stimulus, which may reflect a low priority for reproductive activity under the testing conditions.

Ameyaw-Akumfi and Hazlett (1975) demonstrated clear sex discrimination by male *P. clarkii* with the discriminanda of chela posture, telson posture, and feeding movements. As in the present study, they did not find a sex bias in male localization of the chemical source. Itagaki and Thorp (1981) also found no chemical source localization in male *P. clarkii*.

In the same study, Ameyaw-Akumfi and Hazlett (1975) concluded, after total antennule and inner ramus ablations with male *P. clarkii*, that chemical sexual discrimination was dependent on the presence of the inner rami of the antennules, because the above ablations resulted in loss of sex discrimination. They did not report outer ramus ablations. In the present study, the intact, inner ablated, and cross ablated male subjects discriminated between the male and female stimulus water in their chelae up scores, although not equally well. The outer ablate group score was marginally nonsignificant ($P = 0.055$). The reason for the discrepancy between our male inner ablate group performance and that from the Ameyaw-Akumfi and Hazlett (1975) study is not clear.

Tierney and Dunham (1984) reported, after inner and outer antennule ablations, that in male crayfish (*Orconectes propinquus*) the outer antennule rami were responsible for localization of the source of chemicals from opposite sex conspecifics, just as reported above for females. They found that the inner antennule rami, while chemosensory in nature, did not play a part in conspecific localization.

Itagaki and Thorp (1981) found no evidence of chemical sex discrimination or source localization in male *P. clarkii*. Thorp and Ammerman (1978) also found no evidence of chemical sex discrimination or source localization in male *Procambarus acutus*, but that could have been due to a confounding effect of "stress pheromone," as they point out.

Both Thorp and Ammerman (1978) and Itagaki and Thorp (1981) used a flow-through water system, but it is uncertain what relevance that may have had to the outcome. Much has been made of the fact that closed systems, as used by Ameyaw-Akumfi and Hazlett (1975), Rose (1986), Oh and Dunham (1991), and in the present study, probably more nearly resemble the average habitat of *P. clarkii* than does a flow-through system (Hazlett, 1985; Huner, 1988; Rose, 1984). However, given the complexity of the natural environment in which these animals interact, and the probable great variance of local concentrations of both crayfish odors and metabolite concentrations, it seems unreasonable to assume that chemical sex discrimination could occur only in a lotic or only in a lentic environment (see Hazlett, 1984; Thorp, 1984, for discussion).

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ROLE OF SEX PHEROMONE COMPONENTS IN BEHAVIORAL REPRODUCTIVE ISOLATION BETWEEN *Autographa gamma* (L.) AND EITHER *Trichoplusia ni* (Hübner) OR *Chrysodeixis chalcites* (Esp.) (LEPIDOPTERA: NOCTUIDAE: PLUSIINAE)[1]

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Abstract—The composition of the *Autographa gamma* sex pheromone was reexamined and only (Z)-7-dodecenyl acetate and (Z)-7-dodecenol were identified by capillary GC, GC-MS, and dimethyl disulfide derivatization and subsequent GC-MS analysis. The fatty acid content of the pheromone glands was also studied, and a series of saturated and unsaturated acids was identified. However, most of the related pheromonal compounds were not detected. The male response to the pheromone components was studied in a flight tunnel and compared with the response to calling females. The best synthetic baits evoked a response similar to that observed to the virgin females, but males spent significantly more time at calling females than at the synthetic baits. The preferred synthetic baits consisted of (Z)-7-dodecenyl acetate alone or of a blend with 5% (Z)-7-dodecenol. Increasing the relative amount of the alcohol caused a gradual reduction in male response, particularly in the last steps of the courtship sequence. The addition of the minor sex pheromone components of the sympatric Plusiinae species, *Trichoplusia ni* and *Chrysodeixis chalcites*, to the *A. gamma* pheromone was also investigated in the flight tunnel. Some of these components exhibited a significantly antagonistic effect on the *A. gamma* male courtship behavior. The most potent antagonists were (Z)-5-dodecenyl acetate and (Z)-9-tetradecenyl acetate. The response of *A. gamma* and *T. ni* males to conspecific and heterospecific females was also compared in the flight tunnel. Whereas *A. gamma* males were attracted only to their conspecific females, a small percentage of *T. ni* males were also

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attracted to *A. gamma* females and 11% performed the whole courtship sequence.

Key Words—*Autographa gamma*, *Trichoplusia ni*, *Chrysodeixis chalcites*, Lepidoptera, Noctuidae, sympatric Plusiinae, pheromone specificity, flight tunnel, attraction, inhibition.

INTRODUCTION

In many moths, and particularly in closely related species, overlapping female sex pheromone components have been found (Cardé and Baker, 1984; Baker, 1989). Most of the sex pheromones in the noctuid subfamily Plusiinae are characterized by (*Z*)-7-dodecenyl acetate (*Z*7–12 : Ac) as the main component (Arn et al., 1986), although there are a few exceptions (Heath and Landolt, 1987; Mazor et al., 1991). Despite the fact that in some locations several Plusiinae species have been trapped with *Z*7–12 : Ac (Alford and Hammond, 1982), generally speaking there is no interspecific cross-attraction between sympatric Plusiinae species under natural conditions in the field. To maintain premating and behavioral reproductive isolation among closely related moths, species-specific pheromones are utilized consisting usually of multicomponent blends containing attractive and antagonistic components (Landolt and Heath, 1987; Linn et al., 1988).

The gamma moth, *Autographa gamma*, abundant in Israel in the spring, is sympatric with *Cornutiplusia circumflexa* and *Trichoplusia ni* and sometimes with the later-appearing *Chrysodeixis chalcites* as well (Yathom and Rivnay, 1968). In the *A. gamma* sex pheromone, two components have been identified: *Z*7–12 : Ac and (*Z*)-7-dodecenol (*Z*7–12 : OH) (Dunkelblum and Gothilf, 1983; Toth et al., 1983). Field experiments indicated that lures consisting of *Z*7–12 : Ac with a low concentration of *Z*7–12 : OH were not specific to *A. gamma* males, and varying numbers of *T. ni* males were also trapped. Conversely, the six-component *T. ni* and the three-component *C. chalcites* lures were specific and never caught *A. gamma* males (Dunkelblum and Mazor, unpublished). These results prompted us to: (1) reinvestigate the composition of the *A. gamma* sex pheromone blend, and (2) study the effect of the addition of the minor *C. chalcites* and *T. ni* sex pheromone components to the *A. gamma* pheromone on the flight behavior of *A. gamma* males in a wind tunnel.

METHODS AND MATERIALS

Insects and Extracts. The colony was established from *A. gamma* moths that were captured in light traps. Larvae were reared on a modified semisynthetic diet medium (Shorey and Hale, 1965). Pupae were sexed; males were kept in

the flight-tunnel room and females in a separate room. Both rooms were maintained under the same conditions with a dark–light regime of 10:14 hr, at 26 ± 2°C and 60 ± 5% relative humidity. Adults were provided with a 10% sugar solution.

Batches of 25 pheromone glands were dissected from the ovipositors of 5- to 6-day-old females, 4 hr into scotophase, and then immersed for 15 min in hexane. The supernatant solution was pipetted into conical vials sealed with a Teflon-lined screw cap and then concentrated to the required volume by letting the samples stand in the fume hood. For quantification, 100 ng of 10-undecenyl acetate (10–11:Ac) was added as internal standard. Attempts to collect airborne pheromone from *A. gamma* virgin females, in a flow system described by Landolt and Heath (1987), failed. For the analysis of fatty acids, pheromone glands were extracted with chloroform–methanol (2:1) for 30 min and subjected to basic methanolysis with KOH in methanol, as described by Bjostad and Roelofs (1984).

Chemical Identification. Capillary GC analyses were conducted on a Carlo Erba 5300 instrument equipped with a FID detector and a splitless injector system. For pheromone analysis, a 30-m × 0.25-mm DB-5 column (J&W; Folsom, California) was kept for 2 min at 60°C and then programmed to 150°C at a rate of 10°C/min; after 21 min the temperature was raised to 200°C, at the same rate. For fatty acid methyl ester analysis, a 30-m × 0.25-mm DB-225 column (J&W) was kept for 2 min at 60°C and then programmed to 120°C at a rate of 10°C/min; after 35 min the temperature was raised at 150°C, at the same rate. The purge valve was opened 1 min after injection, and the carrier gas (helium) pressure was 1 atm (14.7 psi).

Dimethyl disulfide (DMDS) adducts were prepared from several standards (Z5–10:Ac, Z7–12:Ac, Z9–14:Ac, and Z7–12:OH) and gland extracts, as described by Dunkelblum et al. (1985). The DMDS adducts were analyzed by GC-MS on a Finnigan 5100 system, at 70 eV, coupled with a Superincos data system. A 30-m × 0.25-mm RTX 5 fused silica column (Restec; Bellefonte, Pennsylvania) was kept 2 min at 60°C and then programmed to 250°C at a rate of 20°C/min. The purge valve was opened 0.5 min after injection and the carrier gas (helium) pressure was 10 psi.

Behavioral Tests. The flight tunnel (195 × 60 × 60 cm) used in these tests was the same as described by Snir et al. (1986). The tests were conducted at a light intensity of roughly 0.5 lux with a wind speed of 0.5 m/sec, at 25 ± 2°C. All experiments were conducted with 7- to 10-day-old males during the second half of scotophase. Males were tested individually and the following behavior categories were recorded: starting flight (within 1 min), directed flight, landing, hairpencil display, and copulation attempts. The duration of source contact was recorded and males that stayed close to the bait more than 60 sec were removed from the flight tunnel. Synthetic baits were prepared by impreg-

nating rubber septa (5 × 9 mm, Arthur H. Thomas Co.), preextracted with dichloromethane in a Soxhlet apparatus, with the appropriate blends. The baits were used within 10 days after preparation. They were kept in closed vials as described by Snir et al. (1986). Individual 4- to 7-day-old virgin females (*A. gamma* or *T. ni*) were also used as baits. They were placed in cylindrical wire screen cages fitted with a plastic bottle filled with a sugar solution and plugged with a cotton wick. When needed for an experiment, a calling female was selected and introduced into the wind flight tunnel. Dispensers loaded with Z7–12:Ac and mixtures with Z7–12:OH were compared with calling females. The effect of the minor *T. ni* and *C. chalcites* pheromone components was tested by adding these compounds, in the relative ratios similar to those found in the conspecific females, to the *A. gamma* pheromone. The C_{14} acetates were added in a 10-fold amount in order to compensate approximately for their lower volatilities as compared to the C_{12} acetates (McDonough et al., 1989).

Results were analyzed by the chi-square 2 × 2 test of independence with the Yates' correction at $P < 0.05$ using the actual number of insects and are presented as percentage of response.

RESULTS

Chemical Identification. Capillary GC analysis of gland extracts indicated the presence of Z7–12:Ac and Z7–12:OH, as shown before (Dunkelblum and Gothilf, 1983). Despite the presence of additional peaks in the chromatograms, no additional pheromonal compounds could be positively identified. Maximal amounts of pheromone components, 3.4 ± 0.6 ng of Z7–12:Ac and 0.5 ± 0.3 ng of Z7–12:OH per female, were observed in 7-day-old females in the seventh hour into scotophase (Mazor and Dunkelblum, unpublished). Dimethyl disulfide (DMDS) derivatization of a gland extract and subsequent GC-MS analysis (Figure 1) confirmed the presence of these two compounds in the gland extracts with diagnostic ions of *m/e* 278, 161, 117 and 61 for Z7–12:OH/DMDS and *m/e* 320, 203, 143, 117 and 61 for Z7–12:Ac/DMDS. Monitoring the ion of *m/e* 61, characteristic of DMDS adducts (Francis and Veland, 1981), in the range between 10:Ac/DMDS and 14:Ac/DMDS, revealed no additional monounsaturated pheromonal components.

Analysis of fatty acid methyl esters, obtained by basic methanolysis of gland extracts, showed a series of C_{12}, C_{14}, C_{16}, and higher fatty acids. Considering that the same two pheromone components, albeit in an almost inverse ratio, were found also in *C. circumflexa* (Mazor et al., 1991), the fatty acid profile of pheromone glands of this Plusiinae moth was compared with that of *A. gamma* (Figure 2). The two profiles were very similar, with the same fatty acids present in both. The direct precursor of the pheromonal components, Z7–

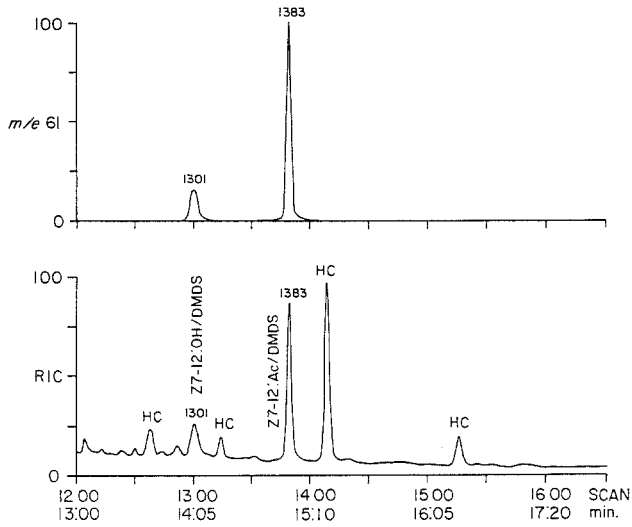

FIG. 1. Total ion chromatogram of dimethyl disulfide (DMDS) adducts of *Autographa gamma* sex pheromone gland extracts. The presence of DMDS adducts was monitored with the *m/e* 61 ion. Peaks marked with HC are hydrocarbons.

12:Ac and Z7-12:OH, was Z7-12:Acid. Most of the other fatty acids such as Z9-14:Acid and Z11-16:Acid are probably precursors of Z7-12:Acid (Roelofs and Bjostad, 1984). The saturated fatty acids, Z9-16:Acid and the C_{18} fatty acids (not shown) are common in most gland extracts (Dunkelblum et al., 1985). The only additional uncommon acid in both Plusiinae moths was Z7-14:Acid. However, no additional pheromonal compounds derived from any observed fatty acid, except Z7-12:Acid, could be detected in both *A. gamma* and *C. circumflexa* gland extracts.

Behavioral Tests. In the first part of these tests, the male response to Z7-12:Ac and different blends of Z7-12:Ac + Z7-12:OH was compared with the response to a calling female (Table 1). Lures consisting of Z7-12:Ac alone or with 5% Z7-12:OH, evoked the same strong response as that of a virgin female. However, there was a significant difference in the duration of source contact between males reaching the synthetic bait and males responding to a calling female. Males that approached the female stayed an average 57.5 sec near the bait (most stayed more than 60 sec) and only 19.9 sec near the best synthetic bait. Increasing the relative amount of the alcohol to 50% and 100% lowered the male response but only in the last behavioral stages, and particularly in hairpencil display, copulation attempts, and duration of source contact.

The influence of the addition of the minor *T. ni* components (Bjostad et al., 1984; Dunkelblum and Mazor, unpublished) to the *A. gamma* pheromone

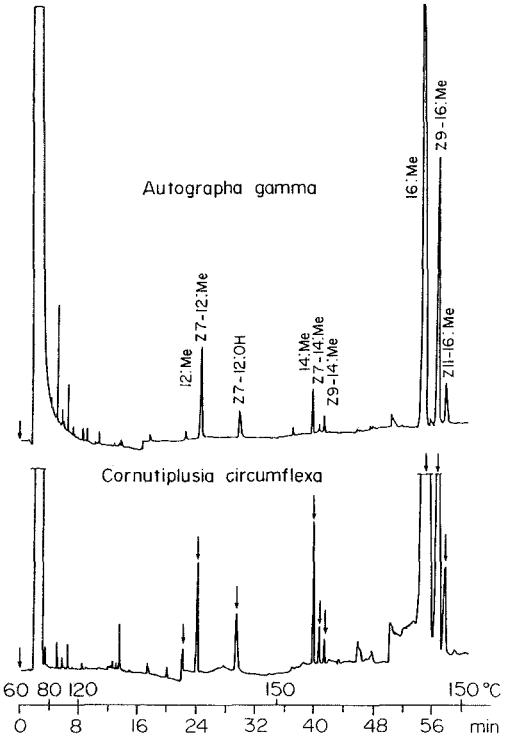

FIG. 2. Capillary GC traces of methanolized gland extracts of *Autographa gamma* (top) and *Cornutiplusia circumflexa* (bottom). The identified peaks were marked with the appropriate abbreviated formulas in the top trace and with arrows in the bottom trace.

was examined in the second part of the behavioral tests (Table 2). The blend of Z7–12 : Ac + 5% Z7–12 : OH was used as the standard *A. gamma* pheromone for these tests. Addition of either 12 : Ac or 11–12 : Ac had no effect on the flight behavior of the *A. gamma* males. Addition of Z7–14 : Ac had a moderately antagonistic effect, particularly on the three last behavioral stages. The antagonistic effect of Z9–14 : Ac was stronger and was perceived throughout the entire courtship sequence from the start of the flight to the copulation attempts. The strongest inhibitory effect was observed with the addition of Z5–12 : Ac, which reduced drastically the percentage of responding *A. gamma* males. Only 2/40 males landed on the lure consisting of the *A. gamma* pheromone + Z5–12 : Ac, as compared with 40/40 males that landed on the same lure without Z5–12 : Ac.

These behavioral tests indicate that baits containing the *T. ni* minor pheromone components can not attract *A. gamma* males. Conversely, field tests have shown that synthetic *A. gamma* baits attract both conspecific males and some

TABLE 1. RESPONSE (%) OF MALE *Autographa gamma* IN WIND TUNNEL TO SYNTHETIC PHEROMONE COMPONENTS AND CALLING FEMALE ($N = 60$)[a]

Bait on rubber septa (μg)	Starting flight	Directed flight	Source contact	Hairpencil display	Copulation attempts	Duration of source contact (sec)[b]
Z7-12:Ac (25.0)	100a	100a	96.7a	93.3ab	65ab	19.9b
Z7-12:Ac (25.0) Z7-12:OH (1.25)	100a	100a	95a	85bc	60b	17.9b
Z7-12:Ac (25.0) Z7-12:OH (12.5)	100a	96.7ab	91.7a	75c	41.7b	18.7b
Z7-12:Ac (25.0) Z7-12OH (25.0)	95a	88.3b	63.3b	46.7d	18.3c	8.9c
Calling female	100a	100a	100a	100a	80a	57.5a

[a]Within columns, percentages followed by a common letter do not differ significantly at $P < 0.05$ according to the chi-square test of independence with the Yates correction. Time of duration at the source was analyzed according to the Tukey test at $P < 0.05$.
[b]Most males returned to the source after the first contact. Many of those that responded to a calling female remained in contact with the female for more than 60 sec and were removed from the tunnel after 1 min.

TABLE 2. RESPONSE OF MALE *Autographa gamma* IN WIND TUNNEL TO ITS PHEROMONE BLEND (Z7-12:Ac 25 μg + Z7-12:OH 1.25 μg) WITH AND WITHOUT THE MINOR *Trichoplusia ni* PHEROMONE COMPONENTS ($N = 40$)[a]

Bait on rubber septa (μg)	Starting flight	Directed flight	Source contact	Hairpencil display	Copulation attempt
Blend	100a	100a	100a	100a	60a
Blend + 12:Ac (0.4)	100a	100a	100a	95a	57.5ab
Blend + 11-12:Ac (0.1)	100a	100a	100a	95a	67.5a
Blend + Z5-12:Ac (0.6)	37.5c	15b	5d	5d	2.5d
Blend + Z7-14:Ac (2.5)	100a	97.5a	77.5b	57.5b	35bc
Blend + Z9-14:Ac (2.5)	70b	35b	27.5c	27.5c	15cd

[a]Within columns, percentages followed by a common letter do not differ significantly at $P < 0.05$ according to the chi-square test of independence with the Yates correction.

T. ni males (Dunkelblum and Mazor, unpublished). With these results in view, the response of *A. gamma* and *T. ni* males to conspecific and heterospecific females was compared in the flight tunnel (Table 3). Both *A. gamma* and *T. ni* males responded very strongly to their conspecific females, and most of them completed the whole courtship sequence. However, *A. gamma* males responded only to *A. gamma* females. On the other hand, some of the *T. ni* males were also attracted to the *A. gamma* females, with 11% of them performing the complete courtship sequence.

The influence of the addition to the *A. gamma* pheromone of the minor *C. chalcites* components, Z9–12:Ac and Z9–14:Ac, at the level present in *C. chalcites* was tested in the last part of the behavioral work (Table 4). The influence of the other two minor *C. chalcites* pheromone components, 11–12:Ac and 12:Ac (Dunkelblum et al., 1987b), was tested in the previous experiment (Table 2). The antagonistic effect of Z9–12:Ac was moderate, but it affected

TABLE 3. RESPONSE (%) OF MALE *Autographa gamma* AND *Trichoplusia ni*[a] TO CONSPECIFIC AND HETEROSPECIFIC VIRGIN FEMALES[b] IN WIND TUNNEL[c]

Response of male to female	Number of males	Starting flight	Directed flight	Source contact	Copulation attempts
A. gamma to *A. gamma*	50	100a	96a	96a	80a
T. ni to *A. gamma*	80	67.1b	36.6b	18.3b	11b
T. ni to *T. ni*	50	100a	100a	100a	80a

[a] Males that did not respond within 2 min were removed.
[b] *Autographa gamma* males did not respond at all to *Trichoplusia ni* females.
[c] Within columns, percentages followed by a common letter do not differ significantly at $P < 0.05$ according to the chi-square test of independence with the Yates correction.

TABLE 4. RESPONSE (%) OF MALE *Autographa gamma* IN WIND TUNNEL TO ITS PHEROMONE BLEND (Z7–12:Ac 25 μg + Z7–12:OH 1.25 μg) WITH AND WITHOUT THE MINOR *Chrysodeixis chalcites* PHEROMONE COMPONENTS ($N = 40$)[a]

Bait on rubber septa (in μg)	Starting flight	Directed flight	Source contact	Hairpencil display	Copulation attempts
Blend	100a	100a	100a	97.5a	67.5a
Blend + Z9–12:Ac (0.4)	85a	82.5a	67.5b	62.5b	35b
Blend + Z9–14:Ac (25)	55b	52.5b	42.5c	32.5c	12.5c

[a] Within columns, percentages followed by a common letter do not differ significantly at $P < 0.05$ according to the chi-square test of independence with the Yates correction.

all behavioral stages. Addition of a relatively large amount of Z9–14 : Ac caused an inhibitory effect similar to that of the lower amount tested before (Table 2).

DISCUSSION

Several years ago we identified Z7–12 : Ac and Z7–12 : OH in the sex pheromone gland extracts of *A. gamma* (Dunkelblum and Gothilf, 1983). The same compounds were also identified independently by Toth et al. (1983). Reexamination of the *A. gamma* gland extracts by capillary GC and by DMDS derivatization with subsequent GC-MS (Figure 1) analysis did not reveal any additional pheromonal components. An attempt to use the fatty acid content of the pheromone glands as a means to predict the presence of trace amounts of pheromonal components (Roelofs and Bjostad, 1984) did not help. Interestingly, the same fatty acid content was found also in the closely related Plusiinae moth *C. circumflexa* (Figure 2), which utilizes the same pheromone components (Z7–12 : OH and Z7–12 : Ac), albeit in an almost inverse ratio (Mazor et al., 1991). The presence of some specific fatty acids may be an indication of traces of the corresponding pheromone components, which are very difficult to identify. Furthermore, not all pheromonal components, originating from potential precursors, are indeed found in the gland extracts. For example, many specific fatty acids were identified in the gland extracts of *Choristoneura* species, but the number of detected pheromone components was much smaller (Dunkelblum et al., 1985). It seems that Z7–12 : Ac and Z7–12 : OH are, in fact, the main pheromonal components of *A. gamma*. Their amount in the pheromone gland is low. Therefore, even if other minor components were present, their detection would be very difficult. Field tests conducted several years ago indicated that the alcohol has some synergistic effect on trap catch of *A. gamma* males, but its activity was not always evident (Dunkelblum and Gothilf, 1983). However, the presence of the alcohol in the blend was important as a compound antagonistic to the sympatric *T. ni* males (Tumlinson et al., 1972; Dunkelblum and Mazor, unpublished).

The response of *A. gamma* males to lures baited with Z7–12 : Ac alone or with the acetate and 5% of Z7–12 : OH was very similar to their response to a calling female. The only difference was the duration of source contact. Males responded significantly longer close to the female than to the synthetic blend. Addition of the alcohol, at a level of 5% relative to the acetate, had no influence on the response of the males, either synergistic or antagonistic. Increasing the level of the alcohol in the binary blend resulted in a gradual decrease of male response, particularly in the last stages of the courtship sequence. The response of the males to a 1 : 1 mixture of Z7–12 : Ac and Z7–12 : OH was significantly lower in most behavioral stages, including the duration of source contact, than

their response to the standard blend containing 5% of the alcohol (Table 1). This agrees with the observation that the sympatric Plusiinae moth *C. circumflexa* utilizes the same pheromonal components as *A. gamma* but with the alcohol as the predominant component (Mazor et al., 1991). Interestingly, these two species are systematically closely related. Both genera, *Autographa* and *Cornutiplusia*, belong to the same subtribe Plusiina of the Plusiini tribe (Boisduval). On the other hand, *C. chalcites* and *T. ni*, which utilize multicomponent sex pheromones, belong to the Argyrogrammatini tribe (Kitching, 1987).

Attempts to collect airborne pheromone from *A. gamma* females were not successful, probably due to the small amounts of pheromone, and it is not certain that Z7–12:OH is actually emitted by the virgin female. However, it seems to us that the alcohol is indeed a part of the *A. gamma* sex pheromone. This view is supported by the discovery of specialist cells to Z7–12:OH in the receptor system of *A. gamma* males (Priesner, 1980).

Examination of the effect of the minor pheromone components of *T. ni* on the flight behavior of *A. gamma* males indicated that the five compounds have a different influence on the male response. Addition of 12:Ac or 11–12:Ac to the binary blend of Z7–12:Ac and Z7–12:OH had no effect on the flight behavior of the males. The three other *T. ni* minor components. Z5–12:Ac, Z7–14:Ac, and Z9–14:Ac, revealed an antagonistic influence, with Z5–12:Ac causing the most pronounced inhibitory effect (Table 2). A combination of the antagonistic components, with Z5–12:Ac in the predominant role, excludes the approach of any *A. gamma* males to a *T. ni* female. Conversely, a small percentage of *T. ni* males do fly towards a *A. gamma* female, because the specificity of the *A. gamma* sex pheromone is not high enough. Traps baited with the *A. gamma* pheromone blend of Z7–12:Ac and 5% Z7–12:OH caught some *T. ni* males in the field, but far fewer than baits containing Z7–12:Ac alone (Dunkelblum and Mazor, unpublished). The present flight experiment with live *A. gamma* and *T. ni* moths (Table 3) confirmed the ability of the *A. gamma* females to elicit the courtship sequence from a small percentage of *T. ni* males. A similar relation between the response of *T. ni* and that of *Pseudoplusia includens* males to conspecific and heterospecific pheromone blends and females was observed by Landolt and Heath (1987) and Linn et al. (1988). The percentage of *T. ni* males that approached a *P. includens* female was much higher than that of *P. includens* males flying towards a *T. ni* female. The inhibitory effect of the *T. ni* sex pheromone to *P. includens* males was similarly attributed to the antagonistic effect of Z5–12:Ac and Z9–14:Ac.

The two minor components of the *C. chalcites* sex pheromone, Z9–12:Ac and Z9–14:Ac (at a level higher than in the *T. ni* pheromone), tested in a separate experiment, both inhibited the courtship sequence of *A. gamma* males (Table 4). These results are in accord with observations from many field tests indicating that the *C. chalcites* sex pheromone blend is species-specific (Dunkelblum et al., 1987a).

Usually, species possessing a common major sex pheromone component utilize unique minor components to provide species-specific signals. Some of the minor components are part of the conspecific attractive message, whereas others may function also as antagonists to closely related sympatric species. *Autographa gamma* females seem to use a simple two-compartment blend consisting of Z7–12:Ac, the major sex pheromone component in most Plusiinae moths, and Z7–12:OH. Plusiinae males that respond, even partially, to Z7–12:Ac alone, and are not inhibited strongly by relatively small amounts of Z7–12:OH, may be attracted to a certain extent to the *A. gamma* sex pheromone blend. It seems that the Israeli *T. ni* males are less sensitive to the antagonistic effect of small amounts of Z7–12:OH (Dunkelblum and Mazor, unpublished) than the American *T. ni* males (Bjostad et al., 1984) and, therefore, they respond moderately to the *A. gamma* pheromone. Conversely, *A. gamma* males are sensitive to several Plusiinae minor sex pheromone components and their courtship behavior is inhibited strongly by some of the components emitted by the *T. ni* and *C. chalcites* females (Tables 2 and 4). The presence of specialist cells in the *A. gamma* male antenna for Z5–10:Ac, Z5–12:Ac and Z9–14:Ac (Priesner, 1980) indicates that the *A. gamma* males are able to detect these compounds despite the fact that they are not part of their conspecific sex pheromone. This phenomenon seems to be common among related species and explains the antagonistic effects we observed in the flight-tunnel experiments. The specificity of the male response results from the enhanced sensitivity to its conspecific pheromone blend and to the capability to perceive antagonistic components emitted by the closely related species.

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ATTRACTION OF BARK BEETLES, *Tomicus piniperda*, *Hylurgops palliatus*, AND *Trypodendron domesticum* AND OTHER INSECTS TO SHORT-CHAIN ALCOHOLS AND MONOTERPENES

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Abstract—Several Scandinavian forest insects, *Hylurgops palliatus*, *Tomicus piniperda*, and *Trypodendron domesticum* (Coleoptera: Scolytidae), *Rhizophagus ferrugineus* (Coleoptera: Rhizophagidae) and *Pollenia* spp. (Diptera: Calliphoridae) were attracted to window traps baited with ethanol and placed on Scots pine trees (*Pinus sylvestris*) in May–June, 1986. Release of ethanol at increasing relative rates of 0, 0.01, 0.1 and 1.0 (800 mg/day) from the window traps on trees in 1987 caused *H. palliatus*, *T. domesticum*, and *R. ferrugineus* to be increasingly attracted, while *T. piniperda* was equally attracted at both 0.1 and 1.0 rates. The attraction of *T. piniperda* to ethanol was weak compared to attraction to a monoterpene mix, (±)-α-pinene, (+)-3-carene, terpinolene. The terpene mix plus ethanol was significantly more attractive to *H. palliatus* than ethanol alone, but terpenes significantly reduced the attraction of *T. domesticum* to ethanol. Baiting of pipe traps with a series of short-chain alcohols (methanol to hexanol) each alone showed that ethanol was greatly preferred by *H. palliatus*, *T. domesticum*, and *R. ferrugineus* over alcohols of one more or one less carbon, while longer-chain alcohols were not attractive. However, *Glischrochilus hortensis* (Col.: Nitidulidae) was attracted only to propanol. A series of 10-fold increasing release rates of ethanol (0.0001–1.0, where 1.0 = 800 mg/day) with either a "low" or "high" release of the terpene mix had various effects on the sexes during their attraction to pipe traps and subsequent entering of holes. Release of (−)-verbenone at 0.25 mg/day had no significant effect on *H. palliatus* or *R. ferrugineus* attraction to ethanol, but the response of *T. domesticum* to ethanol was reduced. Several theories on olfactory mechanisms of host selection by *T. piniperda* are integrated and placed in ecological perspective.

Key Words—Host-plant selection, Coleoptera, Scolytidae, Nitidulidae, Rhi-

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zophagidae, Calliphoridae, Diptera, semiochemical, monoterpenes, methanol, ethanol, propanol, terpinolene, α-pinene, 3-carene, verbenone.

INTRODUCTION

Bark beetles and associated beetles feeding on or living in trees must locate a suitable host from among the relatively few scattered widely in the forest during their dispersal from brood or hibernation sites. The host tree is restricted usually to one or a few species and in most cases the insects seek weakened, less-resistant trees, or trees that are in the initial stages of decay. Thus, it is expected that species have evolved behavioral responses to volatile host-plant chemicals that indicate the presence of a suitable host in which reproduction can occur. It is well known that ethanol, probably released by microorganisms in decaying woody tissue (Graham, 1968; Moeck, 1970; Cade et al., 1970) and stressed plants (Kimmerer and Kozlowski, 1982), is attractive to a wide variety of species of forest Coleoptera (Moeck, 1970, 1981; Kerck, 1972; Roling and Kearby, 1975; Magema et al., 1982; Montgomery and Wargo, 1983; Kohnle, 1985; Dunn et al., 1986; Klimetzek et al., 1986; Schroeder 1987, 1988; Schroeder and Eidmann, 1987; Witcosky et al., 1987; Atkinson et al., 1988; Phillips et al., 1988; Volz, 1988; Chénier and Philogène, 1989; Schroeder and Lindelöw, 1989). Similarly, various tree monoterpenes (e.g., α-pinene, myrcene, terpinolene, β-pinene) and turpentine are attractive to a large number of species (Fatzinger, 1985; Byers et al., 1985; Witcosky et al., 1987; Phillips et al., 1988; Schroeder, 1988; Chénier and Philogène, 1989; Schroeder and Lindelöw, 1989; Miller and Borden, 1990; Phillips, 1990). Synergism between ethanol and various monoterpenes (or turpentine) has also been widely reported (Nijholt and Schönherr, 1976; Kohnle, 1985; Tilles et al., 1986; Vité et al., 1986; Phillips et al., 1988; Volz, 1988; Schroeder, 1988; Chénier and Philogène, 1989; Schroeder and Lindelöw, 1989; Phillips, 1990). These compounds are not only important for primary attraction to plants but also may play a role in enhancing the bark beetles' response to aggregation pheromone (Bedard et al., 1969; Pitman et al., 1975; McLean and Borden, 1977; Borden et al., 1981; Paiva and Kiesel, 1985; Byers et al., 1988; Miller and Borden, 1990).

Bark beetle species that aggressively attack and kill living trees have been shown invariably to possess an aggregation pheromone, usually of two or more components, and are weakly, if at all, attracted by host volatiles (Byers, 1989). However, so-called "secondary" bark beetle species, those that colonize dying or decaying trees, less often use an aggregation pheromone, but generally are strongly attracted to host volatiles and ethanol (Kohnle, 1985; Klimetzek et al., 1986; Schroeder, 1988; Schroeder and Lindelöw, 1989). The pine shoot beetle, *Tomicus piniperda* (L.) (Scolytidae), sometimes kills living Scots pine (*Pinus*

sylvestris L.), but more often prefers recently fallen (still living) trees (Långström, 1984). Byers et al. (1985) used the subtractive-combination bioassay and fractionation method (Byers, 1992a) to rigorously identify the semiochemicals responsible for aggregation of *T. piniperda*. A combination of (−)-(*S*)-α-pinene, (+)-(*R*)-α-pinene, (+)-3-carene, and terpinolene, or each alone, was effective in attracting both sexes. During the isolation study, designed for detection of synergistic pheromone components, no evidence was found for beetle-produced compounds being attractive, in contrast to most bark beetles that aggregate en masse on hosts (Byers, 1989).

Following the paper of Byers et al. (1985), Vité et al. (1986) reported that ethanol plus two of the above monoterpenes (α-pinene and terpinolene) were about eight times more attractive than these terpenes to *T. piniperda*. Unfortunately, the release rates of neither the monoterpenes nor ethanol was reported. The activity of ethanol alone was also not determined. Earlier, Magema et al. (1982) had suggested ethanol is attractive to *T. piniperda*, although statistical evidence was lacking (and no release rates were given). However, the evidence of Vité et al. (1986) that ethanol could play a role in aggregation of *T. piniperda* is not in conflict with Byers et al. (1985), since the latter study used Porapak Q to collect organic volatiles, and this adsorbent is inefficient with small molecules like ethanol. In apparent contrast to the ethanol–monoterpene synergism reported by Vité et al. (1986), Klimetzek et al. (1986) tested different release rates of ethanol (24–125 mg/day) with an unreported release rate of α-pinene and terpinolene and found that the higher releases of ethanol inhibited attraction of *T. piniperda*. However, a control with ethanol alone, or terpenes alone, was not reported. Two other studies, also in seeming contrast to Vité et al. (1986), showed that ethanol at higher rates inhibited attraction of *T. piniperda* to α-pinene (Schroeder, 1988; Schroeder and Lindelöw, 1989).

Vité et al. (1986) proposed that ethanol in combination with monoterpenes are used by *T. piniperda* to locate diseased, and therefore susceptible host trees. Since chemical release rates in their study were not measured, and it is not known how much ethanol is released from diseased trees, this hypothesis remained in doubt. A way to probe the question is to assume that monoterpene releases from healthy trees are similar to diseased trees, but that ethanol releases are higher from the latter. Then, by placing ethanol on healthy trees, these trees will appear diseased and should attract more *T. piniperda*.

The objectives of the present study concerning *T. piniperda* were to determine: (1) whether ethanol is attractive when placed on standing Scots pine; (2) the relative attractiveness of ethanol, a more complete blend of monoterpenes alone, or a combination of monoterpenes with ethanol; and (3) the relative attractancy of various short-chain primary alcohols (C_1–C_6) and ethylene glycol. The attraction of other scolytids and associated fauna was also monitored. In addition, the attraction of *Hylurgops palliatus* (Gyll.) and *Trypodendron domes-*

ticum (L.), among other beetles, to ethanol alone, (−)-(*S*)-verbenone alone, or both in combination was tested. Verbenone also was tested with ethanol since the former compound is released as Scots pine logs age and has been shown to inhibit the attraction of *T. piniperda* to host volatiles (Byers et al., 1989).

METHODS AND MATERIALS

Both the window/barrier trap type (Byers et al., 1989) and the pipe trap with funnel (Bakke et al., 1983; Byers et al., 1988) were used for testing semiochemicals. The pipe trap can reveal behavioral differences between the sexes as they orient to the attractant source since the trap collects beetles in two bottles (wire screen bottoms), as they bounce off the pipe barrier into a large 33-cm-diameter funnel, and as they enter any of the 900 holes in the pipe.

Window traps of transparent plastic (17 × 35 cm high) with funnel and collection bottle were wired to Scots pine trees (about 25–35 cm DBH) at 2 m height (all experiments in a pine plantation, Sjöbo, southern Sweden). The traps on trees were spaced 90 m apart in two parallel lines 90 m apart. Ethanol was released from two open, plastic-vial dispensers per trap (total 800 mg/day) from 13 traps while 13 traps were unbaited controls. Collections of insects were done five times. The plastic vial dispensers (730 type, Kartell Inc., Italy) were made of polyethylene (0.6 cm diam. × 3 cm). Treatments were assigned at random and remained stationary during the test (April 29–May 12, 1986). A second test in 1987 (April 24–May 6) used the same traps and general methods; 11 traps/tree each were baited with either 0 (unbaited), 0.01, 0.1, or 1.0 ethanol release rates (1.0 equivalent to 800 mg/day). The diffusion-dilution method was used (Byers, 1988) to dilute ethanol with water based on mole percentages to obtain the desired rates.

A comparison of the relative attraction rates of ethanol alone (800 mg/day), the same dose plus a monoterpene mix, and the monoterpene mix alone was done using pipe traps with funnel (April 29–May 12, 1986). The monoterpene mix consisted of several monoterpenes and release rates: terpinolene (>97.3% GLC, Carl Roth KG) at 2.5 mg/day, (+)-3-carene (> 99%, $[\alpha]_D^{20} = 17°$) at 6 mg/day, and (−)-α-pinene (99%, $[\alpha]_{546}^{20} = 50°$) and (+)-α-pinene (99.5%, $[\alpha]_{546}^{20} = 57°$) each at 14 mg/day (Fluka AG). Each of the monoterpenes was placed in two plastic vial dispensers with 200 μl neat compound each, and all were placed inside the pipe. The three traps per treatments were placed 10 m apart in a line and were replicated in three areas on four dates for a total of 12 replicates. Baits were randomized after each test day.

In 1987 (April 23–May 11), two release rates of the above monoterpene mix, one at the same or "high" release rate and a "low" rate of about 3.3% that of the high rate (based on 3-cm-long tubes with an opening 3.3% that of

the dispenser above), were tested alone and in combination with a series of 10-fold increased release rates of ethanol: 0.0001, 0.001, 0.01, 0.1, and 1.0. Diffusion–dilution was again used to dilute the ethanol appropriately with water. Twelve pipe traps with funnel, six for each series, were placed in one line with traps 10 m apart. Treatments were randomized after each collection (once or twice a day) for a total of 15 replicates. In another test, eight pipe traps were baited with a series of increasing length primary alcohols (methanol, ethanol, propanol, butanol, pentanol, and hexanol) and ethylene glycol plus an unbaited trap. Exponential regression was used on vapor pressure statistics (Weast, 1971) for the primary alcohols to predict the tube opening diameters necessary to approximate the same release as the standard 1.0 ethanol rate (800 mg/day). Thus, methanol was released from one dispenser, ethanol from two, propanol from three, and butanol from seven, pentanol from 20, and hexanol from 35. For ethylene glycol, a glass container with an opening about 137 times that for methanol was used to obtain an equivalent rate of release.

The effects of verbenone on attraction of insects to ethanol was tested in 1987 (April 23–May 11) in two areas with tree traps each spaced 10 m apart in a line. The trap consisted of two window traps placed back-to-back at 1.2 m height on a metal pole. Ethanol was released at 800 mg/day from two of the traps, one of these also had two dispensers of (−)-verbenone releasing 0.25 mg/day (>99%, $[\alpha]_D^{20} = -246°$, 99.2% ee, Bedoukian). The third trap released only (−)-verbenone.

RESULTS

Window Traps Baited with Ethanol on Scots Pine Trees. Three species of bark beetle, *Tomicus piniperda*, *Hylurgops palliatus*, and *Trypodendron domesticum* were caught in the ethanol-baited window traps placed on the Scots pine trees (Table 1). The beetle *Rhizophagus ferrugineus* Payk. (Coleoptera: Rhizophagidae) and several cluster flies, *Pollenia* spp. (Diptera: Calliphoridae), were also caught only on the ethanol baited traps. Catches were relatively low in 1986 and not all of the 65 trap replicates caught insects, so use of conventional statistical tests for comparing catches was inappropriate. However, a chi-square test comparing the proportions of traps catching to those that caught none was used to compare the baits within a species between release rates. Using this method, all species above were significantly attracted to ethanol-baited traps on trees (Table 1). In 1987 a similar experiment had window traps on trees baited with either 0, 0.01, 0.1 or 1.0 ethanol equivalent (800 mg/day), and again the three bark beetle species and *R. ferrugineus* were principally caught on higher release rates of ethanol (Figure 1, Table 2). *H. palliatus* and *T. domesticum* were significantly attracted to relative rates of 0.01 ethanol and higher on the

TABLE 1. CATCH OF FOREST INSECTS ATTRACTED TO WINDOW TRAPS BAITED WITH ETHANOL (800 mg/day) OR UUBAITED AT BREAST HEIGHT ON SCOTS PINE TREES (APRIL 28–MAY 12, 1986)

Species[a]	Males	Females	Total	Percentage of traps catching[b]		*P* value chi square
				Baited traps	Unbaited traps	
H. palliatus	27	21	48	37	1.5	<0.001
T. domesticum	26	24	50	55	0	<0.001
T. piniperda	3	2	5	6	0	0.04
R. ferrugineus	—	—	82	46	0	<0.001
Pollenia spp.	14	4	18	20	0	<0.001

[a] *Hylurgops palliatus*, *Trypodendron domesticum*, *Tomicus piniperda*, *Rhizophagus ferrugineus*, and *Pollenia* spp. (see text).
[b] N = 65 trap replicates for each treatment.

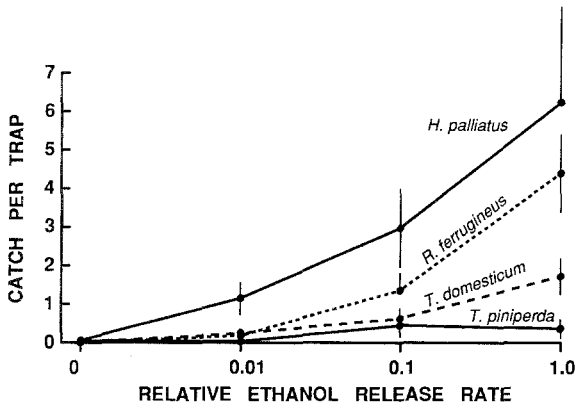

FIG. 1. Catch of *Hylurgops palliatus*, *Trypodendron domesticum*, *Tomicus piniperda*, and *Rhizophagus ferrugineus* attracted to window traps releasing different relative rates of ethanol at breast height on Scots pine trees (1.0 ethanol rate equivalent to 800 mg/day; April 24–May 6, 1987, Sjöbo, Sweden). Bars represent 95% confidence intervals (N = 66).

trees, while *T. piniperda* was not significantly attracted at 0.01 but appeared to be attracted at the 0.1 and 1.0 rates (Table 1), although attraction at the 1.0 rate was not increased as in the other three species (Figure 1, Table 1). In 1987, 10 male flies (*Pollenia* spp.) were caught only in the ethanol baited traps (one in 0.01, three in 0.1, and six in 1.0 baits). Nine of these individuals were separated to species by genitalia differences and were composed of one *P. amentaria*

TABLE 2. CATCH OF VARIOUS FOREST COLEOPTERA ATTRACTED TO WINDOW TRAPS BAITED WITH DIFFERENT RELATIVE RATES OF ETHANOL (1.0 RATE EQUIVALENT TO 800 mg/day) AT BREAST HEIGHT ON SCOTS PINE TREES (APRIL 24–MAY 6, 1987, SJÖBI, SWEDEN)

Species[a]	Catch				Catch range	Percent of traps catching[b]
	Male	Female	Male proportion	95% BCL		
Blanks						
H. palliatus	1	3	0.25	0.05–0.70	0–2	4.5
T. domesticum	0	0				0
T. piniperda	2	1	0.67	0.21–0.94	0–1	4.5
R. ferrugineus	0	0				0
0.01 Etnanol						
H. palliatus	35	40	0.47	0.36–0.58	0–6	51.5a
T. domesticum	9	7	0.56	0.33–0.77	0–1	24.2a
T. piniperda	0	2			0–1	3
R. ferrugineus	(6)[c]	(6)			0–2	12.1a
0.1 Ethanol						
H. palliatus	102	94	0.52	0.45–0.59	0–23	65.2a
T. domesticum	26	26	0.50	0.37–0.63	0–5	47ab
T. piniperda	8	20	0.29	0.15–0.47	0–7	19.7ab
R. ferrugineus	(44.5)[c]	(44.5)			0–9	54.5ab
1.0 Ethanol						
H. palliatus	218	193	0.53	0.48–0.58	0–61	83.3abc
T. domesticum	44	61	0.42	0.33–0.51	0–9	75.8abc
T. piniperda	13	11	0.54	0.35–0.72	0–4	15.2ab
R. ferrugineus	(145)[c]	(145)			0–15	84.8abc

[a] *Hylurgops palliatus*, *Trypodendron domesticum*, *Tomicus piniperda*, and *Rhizophagus ferrugineus*.
[b] $N = 66$ trap replicates for each treatment; letters indicate proportion of traps catching were significantly different ($P < 0.05$) from corresponding treatments, where a = blanks, b = 0.01, c = 0.1 ethanol.
[c] Assumes equal sex ratio.

(Scop.), two *P. rudis* (Fbr.), four *P. angustigena* Wainwr. and two *P. labialis* R.-D.

Attraction of Forest Beetles to a Series of Primary Alcohols. In the series of increasing length primary alcohols, only methanol, ethanol, and propanol caught insects (Figure 2). There appeared to be a subtle shift in the response spectrum of the species, in which *H. palliatus* preferred ethanol but sometimes was attracted to propanol, *T. domesticum* restricted its response to ethanol, *R. ferrugineus* also preferred ethanol but would orient to methanol, while *Glis-*

chrochilus hortensis Fourcroy was caught only on propanol (Figure 2). No insects were caught on butanol, pentanol, hexanol, ethylene glycol, or in the unbaited pipe trap.

Attraction of Forest Insects to Monoterpenes and Ethanol. The catches of the above beetle species and others on pipe traps baited with either ethanol (800 mg/day), monoterpenes mix (see methods), or both are shown in Table 3.

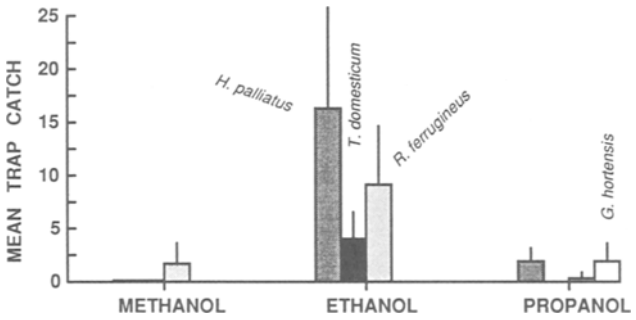

FIG. 2. Catch of *Hylurgops palliatus*, *Trypodendron domesticum*, *Rhizophagus ferrugineus*, and *Glischrochilus hortensis* attracted to pipe traps with funnel releasing either methanol, ethanol, or propanol at similar rates (about 800 mg/day; April 23–30, 1987, Sjöbo, Sweden). Bars represent 95% confidence limits ($N = 9$). Traps with either butanol, pentanol, hexanol, ethylene glycol, or unbaited caught no insects.

TABLE 3. CATCHES (MALE–FEMALE) OF VARIOUS FOREST COLEOPTERA ATTRACTED TO PIPE TRAPS WITH FUNNEL AND BAITED WITH EITHER ETHANOL (800 mg/day), MONOTERPENES (SEE TEXT), OR BOTH (APRIL 29–MAY 12, 1986, SJÖBO, SWEDEN)

	Total catch (male:female)					
	Ethanol		Terpenes		Ethanol + terpenes	
Species[a]	Entering pipe	Funnel	Entering pipe	Funnel	Entering pipe	Funnel
H. palliatus	0:2	3:7	0	0	24:19	21:22
T. domesticum	3:4	16:14	0	0	0:1	1:1
T. piniperda	0	0	1:1	17:21	2:2	26:16
R. ferrugineus	0	9	0	1	10	5
G. quadripunctatus	0	6	0	0	0	0
H. ater	0	1:0	0	0	0	5:2
H. abietis	0	0	0	0	0	4

[a] *Hylurgops palliatus*, *Trypodendron domesticum*, *Tomicus piniperda*, *Rhizophagus ferrugineus*, *Glischrochilus quadripunctatus*, *Hylastes ater*, and *Hylobius abietis*.

Wilcoxon signed-rank tests indicated a synergism of ethanol and monoterpenes in attraction of *H. palliatus* ($P < 0.01$, $N = 10$). The attraction of *T. domesticum* to ethanol was inhibited by the monoterpenes ($P < 0.001$, $N = 12$). *T. piniperda* was not attracted to ethanol but was caught on the terpene baits ($P < 0.01$, $N = 10$).

A second test with pipe traps in 1987 attempted to determine the effect of increasing release rates of ethanol combined with two release rates of the monoterpene mix, either a "high" rate or a "low" rate (3.3% of the high rate). At the higher monoterpene release rate, the attraction of *T. piniperda* was apparently unaffected by increasing release rates of ethanol over the ranges tested (Figure 3A). However, at the lower monoterpene release rate, the attraction of *T. piniperda* increased when ethanol rates increased from 0.01 to 1.0 (Figure 3A, the 1.0 rate was significantly different from the 0.01 rate, Wilcoxon test,

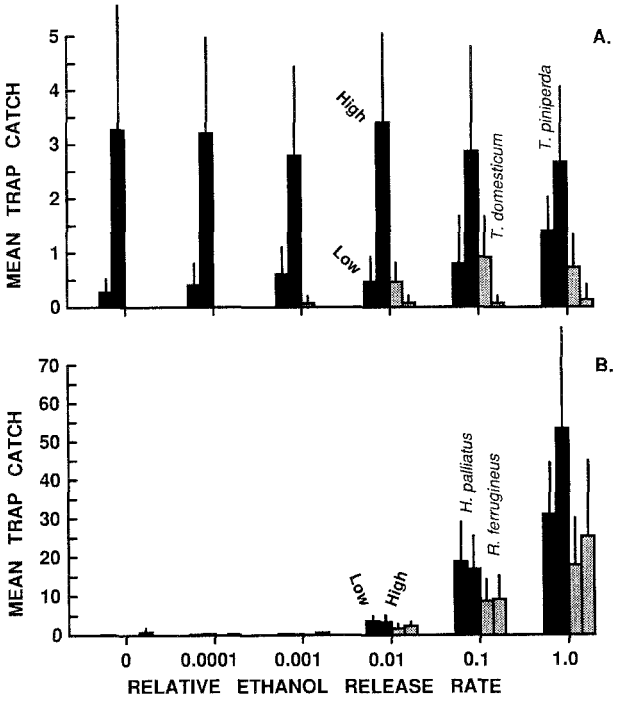

FIG. 3. (A) Catch of *Tomicus piniperda* and *Trypodendron domesticum* attracted to pipe traps with funnel releasing monoterpenes [terpinolene, (+)-3-carene, and (+)- and (−)-α-pinene] at either a "low" or "high" rate (see text) with various relative rates of ethanol (1.0 dose equivalent to 800 mg/day). (B) Catch of *Hylurgops palliatus* and *Rhizophagus ferrugineus* attracted to the same pipe traps (April 23–30, 1987, Sjöbo, Sweden). Bars represent 95% confidence limits ($N = 15$).

$N = 12$, $P < 0.05$). *T. domesticum* response increased with ethanol rates from 0.001 to 1.0 at the low monoterpene rate but remained low at the high monoterpene rate (Figure 3A), consistent with the inhibition by monoterpenes in earlier tests (Table 3). The attraction of *H. palliatus* and *R. ferrugineus* increased with ethanol rates from 0.01 to 1.0 at both the low and high monoterpene releases (Figure 3B). Although not significant, the higher rate of monoterpenes caught more *H. palliatus* at the highest ethanol release.

In the above test, beetles were collected in the outer large funnel or after they entered one of the 900 holes in the pipe. The percentage of *R. ferrugineus* entering holes was about twice as much as for *H. palliatus* at higher ethanol release rates regardless of monoterpene rate (Figure 4A). However, at the high monoterpene rate, both species increased their entering of holes as the ethanol rate was increased, but not at the low monoterpene rate (Figure 4A). At the

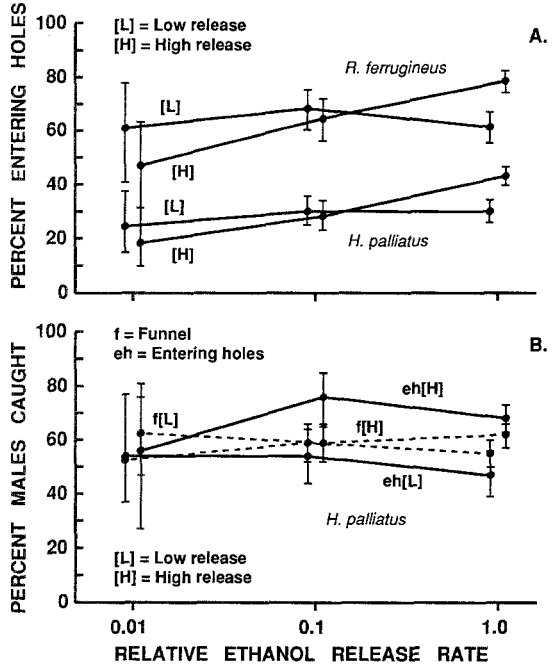

FIG. 4. (A) Percentage of *Hylurgops palliatus* and *Rhizophagus ferrugineus* entering holes in pipe traps with funnels that released monoterpenes [terpinolene, (+)-3-carene, and (+)- and (−)-α-pinene] at either "low" or "high" rates (see text) with various relative rates of ethanol (1.0 dose equivalent to 800 mg/day). (B) Male percentages of *Hylurgops palliatus* caught in the funnels or caught after entering holes in pipe traps as in Figure 4A (April 23–30, 1987, Sjöbo, Sweden). Bars with brackets represent 95% binomial confidence intervals for proportions.

higher ethanol rates (0.1 and 1.0) with monoterpenes, the percentage of males of *H. palliatus* that entered holes was significantly higher at the high monoterpene rate than at the low monoterpene rate (Figure 4B).

Effect of (−)-Verbenone on Attraction of Beetles to Ethanol. Window traps (back-to-back) baited with ethanol caught, as expected, *H. palliatus*, *T. domesticum*, and *R. ferrugineus* (Table 4). *T. piniperda* was not caught, consistent with its nonresponse to ethanol baits in Table 3. The addition of (−)-verbenone to ethanol did not decrease the attraction of *H. palliatus* but the response of *T. domesticum* was significantly reduced (Table 4). Fourteen *Rhinosimus planirostris* Fbr. (Coleoptera: Salpingidae) were caught on baits with ethanol (compared to none on verbenone). The pollen beetle, *Meligethes aeneus* Fbr.

TABLE 4. CATCH OF VARIOUS FOREST COLEOPTERA ATTRACTED TO WINDOW TRAPS (BACK-TO-BACK) BAITED WITH ETHANOL (800 mg/day), (−)-VERBENONE (0.25 mg/day), OR BOTH (APRIL 23–MAY 11, 1987, SJÖBO, SWEDEN)

Species[a]	Catch				Catch range	Percent of traps catching[b]
	Male	Female	Male proportion	95% BCL		
Ethanol						
H. palliatus	46	33	0.58	0.47–0.68	0–9	90
T. domesticum	24	26	0.48	0.35–0.61	0–6	85
R. ferrugineus	(70)[c]	(70)			0–23	90
M. aeneus	(1)[c]	(1)			0–1	10
G. quadripunctatus	(3)[c]	(3)			0–3	20
Ethanol +(−)-verbenone						
H. palliatus	64	48	0.57	0.48–0.66	0–22	85
T. domesticum	14	10	0.58	0.39–0.76	0–3[d]	70
R. ferrugineus	(103.5)[c]	(103.5)			0–38	90
M. aeneus	(11)[c]	(11)			0–4[d]	40
G. quadripunctatus	(5.5)[c]	(5.5)			0–3	45
(−)-Verbenone						
H. palliatus	1	2	0.33	0.06–0.79	0–1	15
T. domesticum	0	0			0	0
R. ferrugineus	(1)[c]	(1)			0–1	10
M. aeneus	(5.5)[c]	(5.5)			0–3	20
G. quadripunctatus	0	0			0	0

[a] *Hylurgops palliatus*, *Trypodendron domesticum*, *Rhizophagus ferrugineus*, *Meligethes aeneus*, and *Glischrochilus quadripunctatus*.
[b] $N = 20$ trap replicates for each treatment.
[c] Assumes equal sex ratio.
[d] Wilcoxon signed-rank tests indicated catch was different from that on ethanol alone ($P < 0.05$).

(Coleoptera: Nitidulidae), an important pest of oilseed rape (*Brassica napus*) was attracted to baits with verbenone (Table 4). No other insects were attracted to verbenone.

DISCUSSION

Primary alcohols other than ethanol have not been reported as being attractive to scolytids. However, only a few studies have tested methanol (Moeck, 1970; Montgomery and Wargo, 1983), while longer-chain alcohols were not investigated. It might be considered surprising that methanol (wood alcohol) had little activity (Figure 2). The fact that ethanol is a common by-product of glycolysis while methanol is not probably explains the evolution of the use of ethanol by forest insects. Moeck (1970) found methanol to be a minor constituent and ethanol a major constituent of extracts from Douglas-fir sapwood attractive to *Trypodendron lineatum*. Although *Tomicus piniperda* was not attracted by ethanol alone, the beetle was attracted when ethanol was placed on trees (Table 1, Figure 1) or combined with monoterpenes (Figure 3). Schroeder and Eidmann (1987) baited Scots pine trees with ethanol (1800 mg/day) and induced attacks by *T. piniperda*. Electroantennogram (EAG) responses of *T. piniperda* to a series of straight-chain alcohols indicated that the antennae respond increasingly with longer chain length, up to a maximum between pentanol and heptanol and then decrease (Lanne et al., 1987). The response spectrum can be due in part to differences in volatility. Thus, although ethanol plays a role in host selection (discussed subsequently), the EAG for ethanol is lower than for longer-chain alcohols (which probably are not involved in behavior). The attraction of the occasional crop pest *Glischrochilus hortensis* (Nitidulidae) (Alford, 1976) to propanol (Figure 2) is unusual since few insects are attracted to propanol. For example, in addition to the report here, only two nitidulid species in the genus *Carpophilus* are reported to be attracted to propanol in the Coleoptera (Lin and Phelan, 1991; Dowd and Bartelt, 1991).

Verbenone inhibited the response of *Trypodendron domesticum* but not *Hylurgops palliatus* to ethanol (Table 4). Response to ethanol by *T. domesticum* was also inhibited by the monoterpene mix, (±)-α-pinene, (+)-3-carene, and terpinolene, while the response of *H. palliatus* was enhanced (Table 3). α-Pinene has been shown earlier to inhibit *T. domesticum* response to an attractive bait of ethanol plus lineatin (Paiva and Kiesel, 1985), and Norway spruce resin or α-pinene has been shown to enhance response of *H. palliatus* to ethanol (Kohnle, 1985; Schroeder and Lindelöw, 1989). Verbenone has been shown to be increasingly released from aging Scots pine logs (Byers et al., 1989). *H. palliatus* is a "secondary" colonizing species and prefers moribund trees, hence the attraction to ethanol and insensitivity to verbenone. *T. domesticum* does not

feed in Scots pine but colonizes deciduous trees (in the study area: *Fagus sylvatica*, *Quercus* spp. *Betula* spp.) and is known to be attracted to ethanol (Magema et al., 1982; Paiva and Kiesel, 1985). Thus, conifer monoterpenes and verbenone (from decaying conifers) that are repellent would provide a mechanism for avoiding unsuitable colonization areas.

Verbenone is found in hindguts of the important pest bark beetles of the United States, *Dendroctonus frontalis* and *D. brevicomis* (Renwick and Vité, 1968), and the compound inhibited response of both beetles to their pheromone (Renwick and Vité, 1969, 1970). Verbenone from *D. brevicomis* was later shown to inhibit aggregation response of *Ips paraconfusus* to natural and synthetic pheromone (Byers and Wood, 1980), and verbenone inhibited *I. typographus* response to synthetic pheromone components (Bakke, 1981). A third genus of bark beetles was added to the list when *T. piniperda* response to the monoterpene mix above was inhibited (Byers et al., 1989). This led Byers (1989) to speculate that verbenone, as a consistent signal of microbial activity in decaying hosts, would be used by bark beetles earlier in evolution as a kairomone to avoid less suitable hosts and then subsequently serve additionally as a pheromone to reduce intraspecific competition and/or as an allomone to reduce interspecific competition. Verbenone production in beetles would coevolve in several species since the same chemical could serve as the signal for all three types of beneficial information. Recently, verbenone was found to inhibit the aggregation pheromone response of another important pest bark beetle of Europe, *Pityogenes chalcographus* (Byers, 1992b).

The attraction of *H. palliatus*, *T. domesticum*, *Glischrochilus quadripunctatus* L., and *Rhizophagus ferrugineus* to ethanol alone, with monoterpenes, and on trees (Tables 1–4, Figures 1–3) is consistent with earlier reports (Moeck, 1970; Nijholt and Schönherr, 1976; Magema et al., 1982; Kohnle, 1985; Paiva and Kiesel, 1985; Klimetzek et al., 1986; Schroeder, 1988; Volz, 1988; Schroeder and Lindelöw, 1989). The attraction of the cluster fly, *Pollenia rudis* (and related species above, Table 1), to ethanol was unexpected since this fly parasitizes earthworms (Thomson, 1973). However, it has been caught at baits with meat (Steinborn, 1981), where ethanol was probably released due to fermentation. Both sexes of *Pollenia* species may respond to ethanol, indicating food sources, but more males may have been caught due to their more extensive foraging patterns (typical of flies in Muscidae and Calliphoridae). Only one other report has proven ethanol, from fermenting sugar, attractive to pest flies in the genera *Musca*, *Muscina*, and *Fannia* (Hwang et al., 1978).

An explanation for the higher percentage of both *H. palliatus* and *R. ferrugineus* entering the holes in the pipe trap at the higher release rate of monoterpenes with the highest ethanol release is that the ratio of monoterpenes to ethanol may be closer to the natural ratio and indicates an appropriate host (Figure 4A). Host-tree compounds, monoterpenes and ethanol, elicited increased

entering rates of bark beetles *T. lineatum* and *P. chalcographus* into pipe traps baited with aggregation pheromone (Vité and Bakke, 1979; Bakke, 1983; Byers et al., 1988). The relatively large differences between the species in entering holes may not be due only to behavioral differences, but also to the fact that *H. palliatus* is easier to catch in the funnels than *R. ferrugineus*. The differences between the sexes of *H. palliatus* in entering holes at the high monoterpene release and higher ethanol releases (Figure 4B) are difficult to explain without more experiments and a deeper understanding of the biology.

The main purpose of the present experiments was to investigate the role of ethanol, in conjunction with monoterpenes, in the location of suitable hosts by *T. piniperda*. Byers et al. (1985) quantified the release rates of α-pinene, terpinolene, and 3-carene from a freshly cut log of Scots pine (28 cm $\times$ 13 cm diam.) and found them each to be about 15 mg/day (terpinolene somewhat less). Release of these amounts in the field competed favorably with a host log in attracting *T. piniperda*. Byers et al. (1985) theorized that since healthy trees were not expected to release appreciable amounts of these monoterpenes compared to broken tops and storm-felled trees where oleoresin wounds are numerous, the aggregation response to monoterpenes functioned in the beetle's recognition of both host species (other trees have less monoterpenes) and host susceptibility to colonization (wounded trees have less capacity to produce more oleoresin for defense).

Vité et al. (1986) presented evidence that ethanol enhanced the attraction of *T. piniperda* to α-pinene and terpinolene by about eight times, but as stated above chemical release rates were not given, making it difficult to replicate the results. They proposed that "contrary to" the theory of Byers et al. (1985) "we present evidence that aggregation and olfactory recognition of susceptible host material depends on the synergism between monoterpenes and ethanol." However, since ethanol release from aging Scots pine has not been quantified, there is some doubt to the validity of the theory. The two theories are not mutually exclusive, however. One way to test the hypothesis of Vité et al. (1986) would be to place ethanol baits on healthy trees as was done here (Table 1, Figure 1). The attraction of *T. piniperda* to these traps on trees (Table 1, Figure 1) supports the hypothesis. Schroeder and Eidmann (1987) also found that trees baited with ethanol (or each of the monoterpenes) were attacked by *T. piniperda*. Vité et al. (1986) did not test ethanol alone, but it has very little activity alone (Table 3, and none caught in Table 4 and Figure 2), a finding also made by Schroeder (1988) and Schroeder and Lindelöw (1989).

Klimetzek et al. (1986) "qualified" the ethanol–monoterpene synergism theory when they reported that increasing ethanol release rates from 24 to 120 mg/day reduced the response of *T. piniperda* to a mixture of racemic α-pinene and terpinolene. Since monoterpenes alone were not tested, it was not possible to confirm the synergism of ethanol and monoterpenes. In fact, the decline in

response did not support the theory that ethanol played a role in selection of susceptible hosts. Schroeder (1988) also attempted to confirm the theory of Vité et al. (1986) by increasing the release of ethanol in five steps over an even wider range from 0 to 50 g/day in combination with a 10 mg/day release of α-pinene. The attraction of *T. piniperda* linearly declined with the logarithm of ethanol release, supporting Klimetzek et al. (1986) but not Vité et al. (1986).

Schroeder and Lindelöw (1989) provided evidence that could explain the discrepancies between the above results. In their Figure 1 for *T. piniperda* it can be seen that a high release of α-pinene was most attractive to the beetle and that ethanol releases from 0 to 3 g/day had little, if any, effect on the attraction. Ethanol released at even higher rates, 120 mg/day (Klimetzek et al., 1986) or 50 g/day (Schroeder, 1988), would inhibit the response to monoterpenes. However, at a low release rate of monoterpene (2.4 or 22 mg/day), the lower releases of ethanol from 0 to 3 g/day had a synergistic effect on the weak response (Schroeder and Lindelöw, 1989). These latter results are similar to those found here (Figure 3B). Therefore, the beetle could find diseased, but physically uninjured, trees by a weak response to a synergism between low monoterpene release rates and moderate ethanol rates—the hypothesis of Vité et al. (1986). These trees would be tested occasionally by beetles and, if low in resistance, this would permit beetles to continue feeding. Resinosis and monoterpene release would elicit increased numbers joining in a mass-attack. Injured trees with wound oleoresin, and trees under attack with "pitch tubes," would have a higher monoterpene release and attract the most beetles, a scenario in line with the hypothesis of Byers et al. (1985). Trees with high ethanol release rates would indicate a tree in advanced decay and unsuitable for reproduction, thus to be avoided, as in the hypothesis of Klimetzek et al. (1986) (high monoterpenes releases would normally not occur with high ethanol rates). In addition, verbenone from decaying hosts would inhibit response to monoterpenes (Byers et al., 1989). Measurements of ethanol and monoterpene release rates from various host and nonhost substrates would be of interest and are necessary for further integration and refinement of these ecological perspectives.

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Book Review

Herbivores: Their Interactions with Secondary Plant Metabolites—Volume 2. Ecological and Evolutionary Processes, 2nd ed. Edited by G.A. Rosenthal and M.R. Berenbaum (eds.). San Diego: Academic Press, 1992. 493 pp.

The first edition of *Herbivores: Their Interaction with Secondary Plant Metabolites*, edited by G.A. Rosenthal and D.H. Janzen, played a major role in providing a conceptual framework for studying the chemical ecology of plant–herbivore interactions. In 1979, when the first edition was published, the study of plant–herbivore interactions was in a rapid stage of growth and change, and it was inevitable that *Herbivores* would be out-of-date very quickly. A revised second edition, edited by G.A. Rosenthal and M.R. Berenbaum, has now appeared. The second edition is a two-volume set, with the first volume, *The Chemical Participants*, corresponding to Part II of the first edition, and the second volume, *Ecological and Evolutionary Processes*, corresponding to part I of the first edition. A review of Volume 1 has already appeared in this journal (Martin, Volume 18, Number 6, pp. 929–930, 1992).

Volume 2 contains 493 pages divided into twelve chapters, which compares with 350 pages divided into eight chapters in Part I of the first edition. The chapter titles in the second edition are: The Evolution of Chemical Ecology (P. Feeny), Behavioral Responses of Insects to Plant Secondary Plant Compounds (E. Städler), How Animals Perceive Secondary Plant Compounds (J.L. Frazier), Allelochemical–Nutrient Interactions in Herbivore Nutritional Ecology (F. Slansky, Jr.), Metabolic Defenses against Plant Allelochemicals (L.B. Brattsten), Third Trophic Level Influences of Plant Allelochemicals (M. Rowell-Rahier and J.M. Pasteels), Microbial Mediation of Plant–Herbivore Ecology (P.L. Phelan and B.R. Stinner), Phototoxins in Plant–Insect Interactions (J.T. Arnason, B.J.R. Philogène, and G.H.N. Towers), Woody Plant–Mammal Interactions (J.P. Bryant, P.B. Reichardt, T.P. Clausen, F.D. Provenza, and P.J. Kuropat), The Chemical Ecology of Plant–Herbivore Interactions in Marine versus Terrestrial Communities (M.E. Hay and P.D. Steinberg), Genetics of Secondary Metabolism and Herbivore Resistance in Plants (M.R. Berenbaum and A.R. Zangerl), and Evolution and Coevolution of Plants and Phytophagous Arthropods (D.J. Futuyma and M.C. Keese). The individual chapters are uniformly well written, with bibliographies that include numerous references to articles published in 1989, 1990, and 1991.

As the editors state in the Preface, Volume 2 is "more of a sequel than a

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second edition—little of the subject material overlaps with the first edition." Of the 23 contributors to Volume 2 of the second edition, only one had a chapter in Part I of the first edition. Only three of the chapters in this volume have counterparts in the earlier version. Most of the new chapters in this volume fall into two broad categories: those that review the successful extension of the approaches used to study the chemical ecology of plant–insect interactions in terrestrial systems into new areas (e.g., three trophic level interactions, microbial mediation of plant–herbivore interactions, phototoxins, marine systems, and mammalian herbivory) and those that describe progress in areas that are crucial to the development of general principles that underlie plant–herbivore interactions (e.g., behavior, chemosensory physiology, the interactions of nutrients with allelochemicals, detoxification, the genetics of plant–herbivore interactions, and coevolutionary theory).

The opening chapter by Feeny falls into a category all its own. Feeny traces the roots of the various themes of contemporary research in the chemical ecology of plant–herbivore interactions (e.g., host specificity, food utilization, plant toxicity, chemical defense, and phytochemistry) to seminal papers dating from the 1800s through the first half of this century. He then recounts the explosion of the field in the 1960s and 1970s following the publication of a series of landmark papers by Dethier, Fraenkel, Gordon, Kennedy, Mittler, Self, Thorsteinson and a few others in the 1950s and early 1960s. Next he cites recent influential papers that exemplify current thinking in the field, and concludes by identifying some important issues that remain unresolved. This remarkable chapter is high-quality intellectual history, and it is fascinating reading.

It follows from Feeny's account that although the study of the influence of secondary plant metabolites on the ecology and evolution of plant–herbivore interactions may be a relatively new field, it is no longer a discipline in its infancy. It had a juvenile phase that ended sometime in the 1960s, followed by an adolescent phase that lasted about 20 years. Sometime in the 1980s it achieved maturity. The second edition of *Herbivores* reflects this maturity. The great contribution of the new edition is to present an important and vigorous subdiscipline within ecology that has both breadth and depth, and to show how the study of plant–herbivore interactions is influencing other areas of biology as much as its is being influenced by them. In revising *Herbivores*, the editors have produced an outstanding work that, for years to come, will serve both students new to the field and researchers already active in the field. Profs. Rosenthal and Berenbaum have surely done their part to "insure that the discipline continues to flourish in the next century."

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