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A LONG-RANGE ATTRACTANT KAIROMONE FOR EGG PARASITOID *Trissolcus basalis*, ISOLATED FROM DEFENSIVE SECRETION OF ITS HOST, *Nezara viridula*

L. MATTIACCI,[4-5] S.B. VINSON,[1,*] H.J. WILLIAMS,[1,2] J.R. ALDRICH,[3] and F. BIN[4]

[1]*Department of Entomology*

[2]*Department of Chemistry*
Texas A&M University
College Station, Texas 77843-2475

[3]*USDA-ARS, Insect and Chemical Ecology Laboratory*
Beltsville, Maryland 20705-2350

[4]*Agricultural Entomology Institute*
Borgo XX Giugno, 06121 Perugia, Italy

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Abstract—A short-chain α,β-unsaturated aldehyde, (*E*)-2-decenal, present in the defensive metathoracic gland of *Nezara viridula* (L.) (Heteroptera: Pentatomidae), stimulates a behavioral response in the egg parasitoid *Trissolcus basalis* (Woll.) (Hymenoptera: Scelionidae). Preliminary studies showed that *T. basalis* are attracted to an area containing adult *N. viridula*, but we also found that female *T. basalis* would examine and probe glass beads coated with an acetone extract of the metathoracic gland from males or females. Using this bioassay, the kairomone was isolated by bioassay directed by preparative gas chromatography and identified by NMR and mass spectrometry as (*E*)-2-decenal. The biological activity of the identified aldehyde was compared with analogs to determine specificity. An unstable *Z* isomer was found to be more active but not present in detectable or behaviorly relevant levels in the host, based on the bell-shaped dose–response curve of the two isomers. An investigation was also designed to determine if the *E* isomer was also responsible for the egg recognition kairomone activity previously reported. However, no 2-decenal isomers were detected in host egg extracts and the chemical characteristics of the 2-decenal isomers differ from the unidentified egg recognition kairomone. The role of the (*E*)-2-decenal in attracting female *T.*

*To whom correspondence should be addressed.
[5]Present address: Department of Entomology, Wageningen Agricultural University, P.O. Box 8031, 6700 EH Wageningen, The Netherlands.

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basalis to *N. viridula* was demonstrated in a Y-tube olfactometer; this alk-2-enal appears to act as a long-range kairomone orienting *T. basalis* to *Nezara* populations.

Key Words—*Trissolcus basalis*, *Nezara viridula*, Hymenoptera, Scelionidae, Heteroptera, Pentatomidae, kairomone, parasitoid, identification, defensive glands, host location, (*E*)-2-decenal.

INTRODUCTION

The importance of kairomones as chemical cues to orient parasitoids to their host has been demonstrated for an increasing number of species of parasitic *Hymenoptera* (Vinson, 1985). Parasitoids often use different kairomones at various stages of the host selection process, so that these chemicals serve as either long-distance attractants, searching stimulants, or host-recognition or host-acceptance factors. Several chemicals involved in the host selection process have been identified (reviewed by Jones, 1981; Kainoh, 1990) from plants, hosts, and host-related sources (food, shelter, associated organisms). The southern green stink bug, *Nezara viridula* (L.) (Heteroptera: Pentatomidae) is an important worldwide pest (Clausen, 1978; Jones, 1988); therefore, we have been studying the chemical ecology of its principal solitary egg parasitoid *Trissolcus basalis* (Woll.) (Hymenoptera: Scelionidae). Bin et al. (1987) discussed the host selection behavior of *T. basalis* and reported that female parasitoids were attracted to adult *N. viridula* in an olfactometer. Sales et al. (1980) also reported that *T. basalis* responded to volatiles but from host egg masses in an olfactometer. Bin et al. (1993) provided evidence of a host-recognition factor in the adhesive that holds the eggs together in an egg mass, which elicits strong ovipositor probing by *T. basalis*. We report here the identification of a volatile in the defensive secretion of *N. viridula* that attracts *T. basalis* and the response of *T. basalis* to several structurally related compounds.

METHODS AND MATERIALS

Insect Cultures

The host *Nezara viridula* was reared in plastic cylindrical containers (15 × 20 cm) with screened lids, in an incubator at 28°C, 14:10 hr light–dark and 50% relative humidity, and fed on green beans and sunflower seeds. The colony was derived from individuals collected in soybean fields in south Texas.

The parasitoid *Trissolcus basalis* was maintained in glass test tubes (2 × 9 cm) with screen lids, provided daily with a mixture containing honey (10%), yeast (10%), benzoic acid (1%) in 100 g of sugar (Safavi, 1968). Egg masses of *N. viridula* were glued on strips of cardboard, exposed for parasitization to three to four *T. basalis* females for 24 hr, and incubated until adult emergence (13–15 days) at 27°C, 14:10 hr light–dark, 70% relative humidity.

Chemical Procedures

Isolation of Kairomone. Adult *Nezara* were frozen at −20°C and their abdomens immediately dissected, leaving the metathoracic gland reservoir attached to the metasternite. The gland contents were collected from the isolated metathoracic reservoir by inserting a fine-pointed glass capillary into the reservoir and taking up the contents by capillary action. The gland contents from either 1 or 10 adults were pooled in a 100-μl cone-bottomed vial with spectral grade *n*-heptane as solvent. The sample was fractionated by preparative GC (Tracor 550) using a 1.83 mm ID glass column with 3% OV-101 on Chromosorb 750 (100–120 mesh) with temperature program from 45°C to 280°C, at 15°C/min. The metathoracic gland contents of one *Nezara* male were separated in seven fractions, each of which was collected by a glass capillary cold trap connected to a splitter at the detection port of the GC (Brownlee and Silverstein, 1968). The collected fractions were bioassayed and the separations and bioassay were repeated twice. Once an active GC fraction was determined, this fraction was reisolated from an extract of 10 *Nezara* male glands and further purified by injecting 25 μl of the corresponding active fraction onto 3% OV-17 on Chromosorb 750, using a similar glass column as above, at 115°C. Three subfractions were collected and bioassayed.

Kairomone Identification. The composition of the metathoracic gland contents of *N. viridula* has already been elucidated (Gilby and Waterhouse, 1965), so we compared the retention time of the single active purified fraction and our mass spectral data with the data of Gilby and Waterhouse (1965). Based on this comparison, we obtained synthetic (*E*)-2-decenal (ICN Biomedicals) and compared the chromatographic, mass, and NMR spectroscope properties with our active fraction. We also conducted and compared bioassay results with both the natural and synthetic material. Since later studies suggested that (*Z*)-2-decenal might be present but unstable, we compared the chromatograms of both (*Z*)- and (*E*)-2-decenal with the chromatograms of the contents of the metathoracic gland removed from live males and immediately injected into the GC.

Synthesis of Analogs. To determine the specificity of the response of *T. basalis* to (*E*)-2-decenal, several analogs were synthesized or purchased and then tested. Six analogs [(*E*)-2-hexenal, *n*-octenal, (*E*)-2-nonenal, *n*-decanal, (*E*)-2-*n*-undecenal, *n*-undecanal] were obtained from commercial sources. The (*E*)-2-decenol was prepared from (*E*)-2-decenal by reaction with excess $NaBH_4$ in methanol at 0°C. The ethyl (*E*)-2-decenoate was prepared by the reaction of triethyl phosphonoacetate with octanal in benzene (Wadsworth and Emmory, 1973). The distilled product was purified by preparative gas chromatography

before testing. (*E*)-2-Decenoic acid was prepared from the ester by reaction with KOH in methanol for 20 min. The solution was washed with ether, acidified, and the acid was extracted with ether.

The *Z* isomer of 2-decenal was prepared as follows: 1-nonyne (4.5 g, 0.036 mol) in 20 ml ether was treated with 0.04 mol (30 ml 1.5 M) CH_3Li. The mixture was stirred for 1 hr, then formaldehyde (3.3 g, 0.11 mol) prepared by heating paraformaldehyde, was bubbled into the solution at 0°C. The solution was poured into 5 vol of water, extracted with two 20-ml portions of ether, and the combined ether extracts were dried over Na_2SO_4, rotary evaporated, and flash distilled (airbath 120°C, 10 mm Hg) to yield 2.4 g (42%) of 2-decyne-1-ol.

A solution of 200 mg (0.0013 mol) of the alcohol in 10 ml of ethyl acetate with 100 mg of 5% palladium on calcium carbonate poisoned with lead was stirred under 1 atm of H_2 gas until the theoretical amount of H_2 had been taken up. The solution was filtered through Celite, rotary evaporated, and treated with 0.4 g (0.002 mol) pyridinium chlorochromate in 4 ml CH_2Cl_2 (Corey and Suggs, 1975). Clean up was conducted in the usual manner followed by final purification using preparative gas chromatography on a 6 ft × 0.4 mm ID glass column packed with 3% SP2340 on Chromosorb G-AW to give material for testing. Approximately 60% isomerization to the *E* isomer took place during the oxidation procedure.

Dose Response. Once the active material was identified, the parasitoids response to different doses of both (*Z*)- and (*E*)-2-decenal and other isomers were determined. Five 10-fold dilutions of (*E*)- and (*Z*)-2-decenal were first bioassayed beginning at 1 μg/μl in acetone. For the other analogs, bioassay began at 0.01 μg/μl in acetone using a minimum of ten *T. basalis* females. Five microliters of solution were applied to the three glass beads as described below and the same four behaviors were recorded and evaluated utilizing a minimum of 15 *T. basalis* females.

Because the (*Z*)-2-decenal was unstable, we synthesized, purified, and bioassayed samples containing this isomer within 2 hr of the final synthetic step. Five stock solutions were prepared as follows: 1 μg/μl (*E*)-2-decenal, 0.1 μg/μl (*Z*)-2-decenal, 1.08 μg/μl *E* + 0.27 μg/μl *Z* (80% *E* to 20% *Z* isomer); 0.675 μg/μl *E* + 0.675 μg/μl *Z* (50% *E* to 50% *Z* isomer); and 0.27 μg/μl *E* + 1.08 μg/μl *Z* (20% *E* to 80% *Z* isomer). Each were 10-fold serially diluted eight times and 10 μl were applied to three glass beads as described, but only those eliciting antennation and ovipositor unsheathing were recorded. This test was replicated twice with five females per dose. After each bioassay, a stock sample applied to three glass beads as described and held during the bioassay period was extracted and injected into the GC to confirm the isomers in the ratios applied. The results were expressed as the percent of females exhibiting ovipositor unsheathing.

General Bioassay Procedures

Glass Bead Bioassay. Bioassays were conducted in a flat-bottomed depression slide (17 mm diam. × 3 mm deep), covered with a glass cover slip to provide a chamber. Artificial eggs consisting of 1.00-mm-diam. glass beads were cleaned with acetone and oven dried at 150°C for 10 min. The clean glass beads were coated with solvent (control), extract or solution (tests), by placing three beads in a small watch glass with 5 or 10 μl of solvent, extract, or a test fractions. Beads were rolled in the solvent until dry. The three coated beads were placed in the observation chamber on a 10 × 10-mm Parafilm square (American National Can, Greenwich, Connecticut 06836) to hold them in place. Parasitoid females, 72–96 hr after emergence, which had not been exposed to hosts, were used for all bioassays. Each test consisted of 9–15 observations per treatment. Responses were recorded for 10 min after releasing one female into the arena. For all the bioassays, except those involving (*Z*)-2-decenal, responses were recorded with a video camera (RCA model 2000, RCA Video Equipment, New Holland Av., Lancaster, Pennsylvania 17604) connected to a black and white monitor (RCA model TC1110). The duration and frequency of behaviors were recorded using an IBM personal computer XT model 286, programmed as an event recorder with "The Observer" installed as a software package for behavioral research (Noldus, 1991). From the observations, the following behaviors were recorded: (1) encounter: the wasp touched or walked over the beads even if it left in a few seconds; (2) antennation: upon encounter the wasp stopped and examined the beads with its antennae; (3) ovipositor unsheathing: the antennating wasp unsheathed her ovipositor; and (4) acceptance: the female attempted to drill the bead using her ovipositor. After a female was introduced into the arena, the number of times (frequency) she performed each of the four behaviors described and the duration (in seconds) of the period she spent antennating the beads (behavior 2) were recorded.

In tests comparing (*Z*)- and (*E*)-2-decenal, 10 μl of solvent was applied to a group of three glass beads as described, but only the number of females both antennating and unsheathing their ovipositor was recorded. Recording of data was interrupted after 5 min if no encounter occurred or after 5 min of the first encounter or upon acceptance.

Olfactometer Studies. The olfactometer consisted of a 4.0-cm-ID glass Y tube, with the 15-cm-long Y arms connected to a glass sample chamber and a 10-cm common stem. The air flow (100 ml/min) passed through activated charcoal and water before entering sample chambers. Two white neon lamps were placed on the two sides of the system. Ten microliters of a chemical solution applied to rubber septa were placed in one arm of the olfactometer. Solvent treated septa were used as control in the other arm. In studies with (*E*)-2-decenal the concentration used was 0.1 μg/μl in acetone. New septa were used each

time. In each test one *T. basalis* female was placed at the beginning of the common stem and the percentage time spent and the number of times that the insect walked in each arm, during 10 min of bioassay, were measured with the video camera and computer as described above. Fifteen replications, alternating the control arm, were also performed in order to test possible influence of uneven light or other environmental conditions.

Comparison with Egg Recognition Kairomone

Since we used glass beads as a convenient bioassay, we wanted to determine if the volatile kairomone isolated from adult *Nezara* was responsible for the egg recognition behavior reported by Bin et al. (1993) and, if not, to compare the two kairomones (see Discussion for more details concerning the egg recognition kairomone). Bin et al. (1993) demonstrated that a host-recognition kairomone is present in the adhesive covering the eggs, which is used by *N. viridula* to glue the egg mass onto the leaf surface. We determined the presence of the two isomers of 2-decenal in the adhesive. An egg extract was prepared by soaking newly laid eggs in acetone (1 μl/egg, for at least 12 hr) as described by Bin et al. (1993). Ten microliters of the sample was bioassayed for biological activity using glass beads as described above and 100 μl of an active extract was applied to a thin-layer chromatography (TLC) plate (Whatman Kc 18 F, chemically bonded reverse-phase) and developed with 100% acetone as mobile phase. One hundred microliters of (*E*)-2-decenal (the *Z* isomer was not stable enough for this procedure) at 1 μg/μl in acetone was applied to a different lane of the TLC plate to compare the R_f values. The separation was visualized using iodine or long wavelength UV light.

Since (*E*)-2-decenal could be present in the egg but not accumulate in the adhesive, extracts of eggs were prepared from an egg mass (1 μl acetone/egg). Solutions were bioassayed and equivalent active concentrations of acetone egg extract and (*E*)-2-decenal were compared by GC on 3% OV-101 as described. The solvent and fractions from the GC were collected and bioassayed. Equivalent active concentrations were also applied to reverse-phase thin-layer plates and developed in acetone (13 cm). After separation, the plate was divided into 1-cm strips, the plate material was removed from the support with a razor blade, extracted in acetone, and bioassayed.

Egg extracts were also prepared by soaking the eggs in heptane or methylene chloride (24 hr, 1 μl of solvent/egg) or macerating (1 hr, 1 μl solvent/egg). The extracts were immediately injected on a 3% OV-101, Chromosorb 750 column, temperature programmed from 45°C to 280°C at 15°C/min, for the detection of (*E*)- or (*Z*)-2-decenal.

Statistical Analysis

The behavior parameters were analyzed by Kruskall-Wallis ANOVA in order to test the overall variability (comparison with egg recognition kairomone and with analogs) and by the Mann-Whitney U test for significant differences in pairwise comparisons (dose–response test and antennation time in the comparison with analogs) (Siegel, 1956). Bonferroni inequality was applied to make the Mann-Whitney U test reliable for multiple comparisons (Harris, 1975). In the olfactometer bioassay Friedman two-way ANOVA was used (Siegel, 1956).

RESULTS

Previous research (Bin et al., 1987; Sales, 1979) had demonstrated that adult *Nezara viridula* release volatiles that attract female *T. basalis* in an olfactometer. Our preliminary studies indicated the responsible volatiles were associated with the metathoracic glands of adults; in fact, we found that *T. basalis* would respond by rapid location (encounter), antennation, ovipositor extrusion, and even ovipositor drilling (acceptance) of glass beads treated with the diluted contents (0.02–0.001 female equivalents) of the metathoracic gland. Through gas-chromatographic separation and bioassay using glass beads, we found one peak (fraction 5) eliciting equivalent activity to the crude metathoracic gland extract (Figure 1). All other fractions were inactive. Comparison of our gas-chromatographic patterns with published data (Gilby and Waterhouse, 1965) on the identity of the contents of the metathoracic gland of *Nezara viridula* indicated (*E*)-2-decenal as the kairomone responsible for the observed behavior of *T. basalis*. Synthetic (*E*)-2-decenal was found to elicit the same behavior of the crude metathoracic gland extract and had identical chromatographic, NMR, and mass spectral characteristics as the active fraction (Figure 1).

At a concentration less than 5×10^{-4} μg, less than 20% of the females responded with encounters and antennation to (*E*)-2-decenal, while between 5×10^{-3} and 5×10^{-1} μg provided the best response (Figure 2). At 5 μg applied, none of the females unsheathed their ovipositor, but they moved away from the treated beads and there was an increase in preening. Also from Figure 2 it appears that females respond to the different dose-treated beads by increasing their encounter frequency, antennation frequency, and antennation time rather than their frequency of ovipositor unsheathing or acceptance.

Figure 3 shows the response of *T. basalis* to nine of the analogs of (*E*)-2-decenal initially available at 0.01 μg/μl applied. Of these, (*E*)-2-decenoic acid ethyl ester resulted in a prolonged antennation of the few females that responded. While (*E*)-2-decenal resulted in an encounter frequency of over 50, encounter frequencies between 20 and 30 were obtained with all other compounds. Both the 2-decenoic acid ethyl ester and the 2-decenoic acid elicited antennation equal

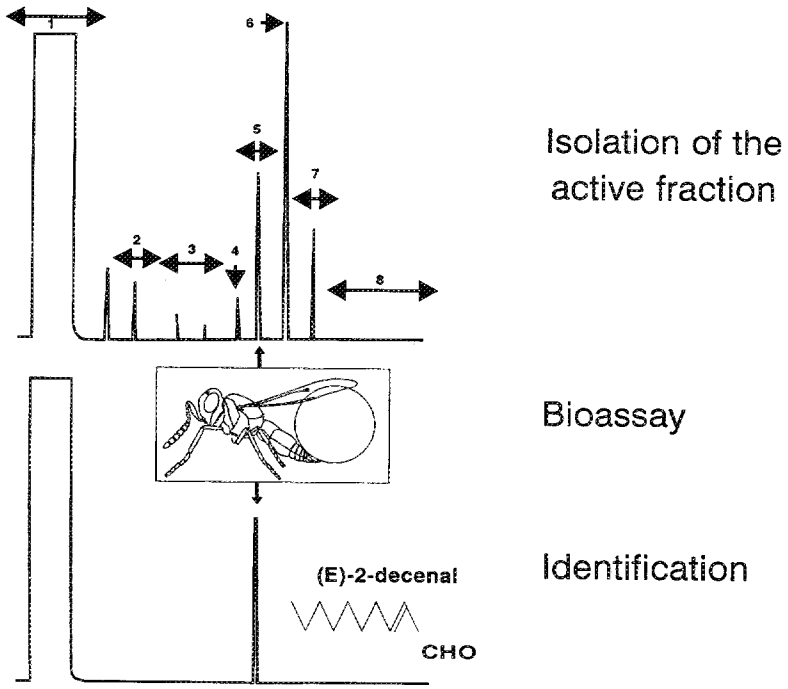

FIG. 1. General scheme for isolation and identification of (*E*)-2-decenal through gas chromatographic separation and a glass bead bioassay: of the fractions obtained by preparative GC from the crude metathoracic gland extract, only fraction 5 elicited acceptance behavior in the wasp.

to (*E*)-2-decenal at 0.01 $\mu g/\mu l$ (Figure 3), but showed no activity at lower doses. Furthermore, none of these compounds was detected in active samples.

(*Z*)-2-Decenal is unstable, converting to the *E* isomer at room temperature over a period of a day. Using freshly synthesized (*Z*)-2-decenal, we found that *T. basalis* is one order of magnitude more sensitive to (*Z*)-2-decenal. Combinations of *Z* and *E* are presented in Figure 4 along with the data of both isomers presented separately on glass beads. Activity of combinations appeared to be additive, the reduction of activity of the (*E*)-2-decenal at higher concentration if *Z* is due to repellency of the higher concentration of the *Z* or the combination.

A comparison of the (*E*)-2-decenal with egg extracts (the host recognition kairomone isolated in acetone from eggs), indicated that both solutions elicited all the behaviors associated with host recognition (Figure 5). The number of encounters is not significantly different for either solution, but the wasps showed a greater response for the egg extract; in fact, antennation time and ovipositor extrusion are significantly higher than for (*E*)-2-decenal or control. Antennation

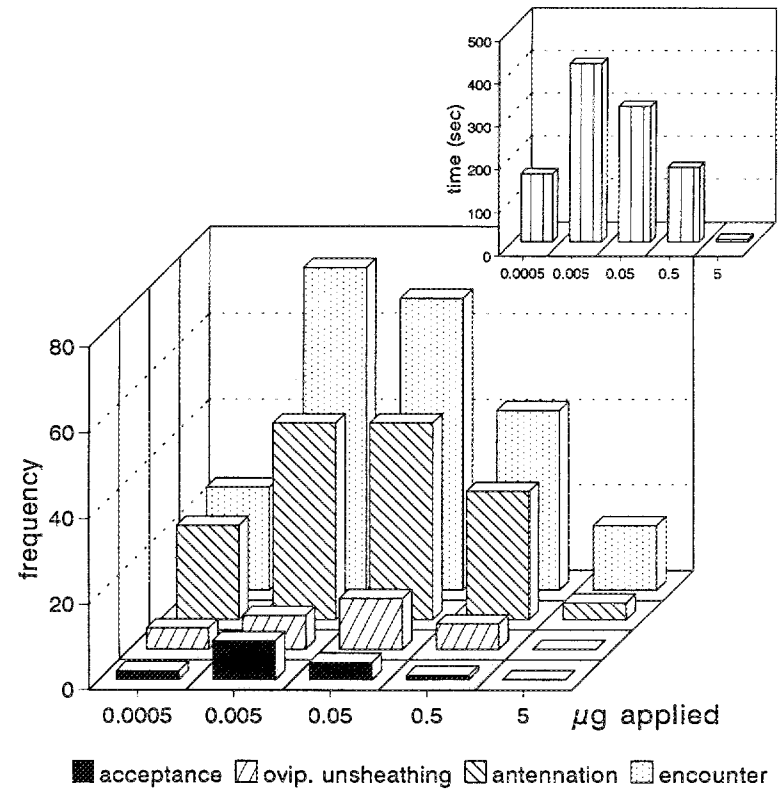

FIG. 2. Frequency of response of female *T. basalis* to different doses of (*E*)-2-decenal applied to glass beads (Kruskall-Wallis ANOVA, $P > 0.05$). The insert figure shows the antennation time (seconds) of females to glass beads treated with different doses.

frequency and acceptance of both kairomone-containing solutions are significantly higher than the solvent, but not different from each other.

(*E*)-2-Decenal or (*Z*)-2-decenal were not detected in heptane extract of egg masses or in active acetone extract of egg masses by gas-chromatographic analysis. We were also unable to detect (*Z*)-2-decenal in the metathoracic gland of either males or females. A response to the GC acetone egg extract was found near the solvent front, while all the analogs of (*E*)-2-decenal eluted much later. Thin-layer chromatography of (*E*)-2-decenal and the active acetone extract of *Nezara* egg revealed only one active band for each, but of different R_f values. These results suggest that the (*E*)-2-decenal is not responsible for the egg recognition activity and, if present on egg masses, it occurs below detectable levels.

The response of female *T. basalis* to (*E*)-2-decenal in a Y-tube olfactometer

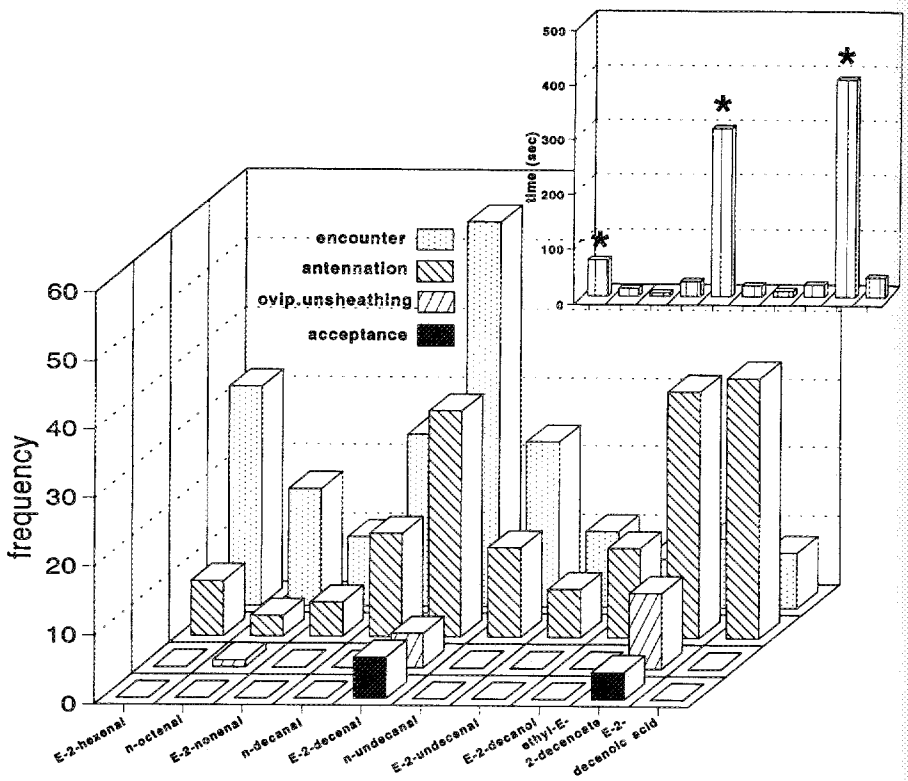

FIG. 3. Response of *T. basalis* to structurally related compounds at 0.01 $\mu g/\mu l$ applied. (*E*)-2-Decenal scored the highest frequency of the four recorded behaviors (the overall variability was tested by Kruskall-Wallis ANOVA at $P > 0.05$). The Mann-Whitney paired comparison showed significant different antennation time for (*E*)-2-hexenal, (*E*)-2-decenal, and ethyl (*E*)-2-decenoate ($*P = 0.05/9 = 0.005$).

(Figure 6) revealed that while the number of times that the wasp walked into the two arms was the same, they spent considerably more time in the (*E*)-2-decenal contaminated air (Friedman two-way ANOVA, $P > 0.01$).

DISCUSSION AND CONCLUSIONS

Several authors (Sales, 1979; Sales et al., 1980; Bin et al., 1987) have provided evidence that *T. basalis* responds to volatiles released by host adults and egg masses. The results of this study demonstrate that (*E*)-2-decenal from the metathoracic gland of *Nezara viridula* is responsible for this attraction and

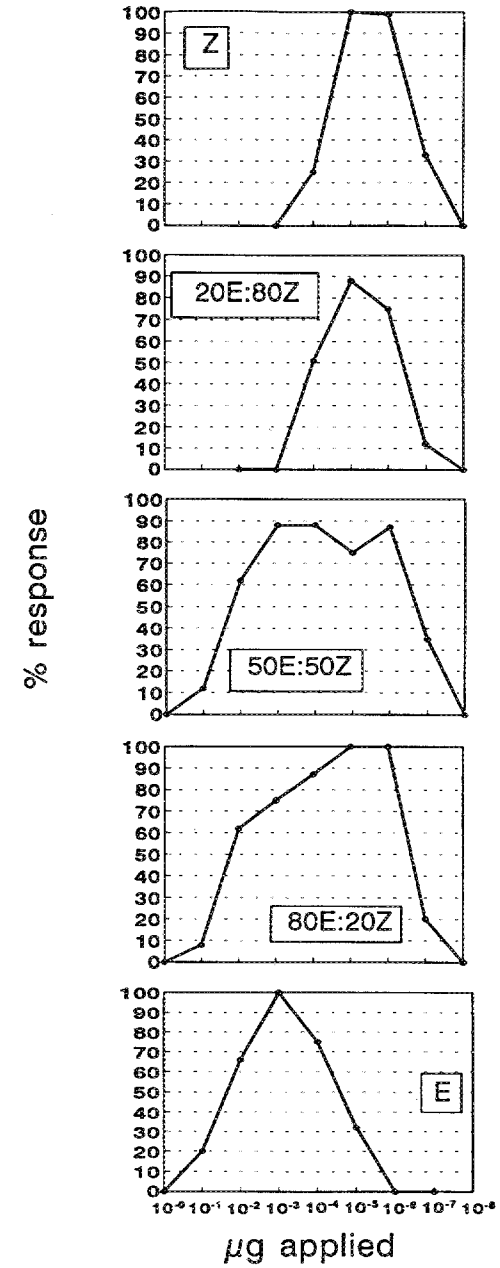

FIG. 4. Response of *T. basalis* to the two isomeric forms of 2-decenal: (*Z*)-2-decenal was found to be active at lower concentrations than (*E*)-2-decenal. The composition in of the mixture is given in the box in each graph.

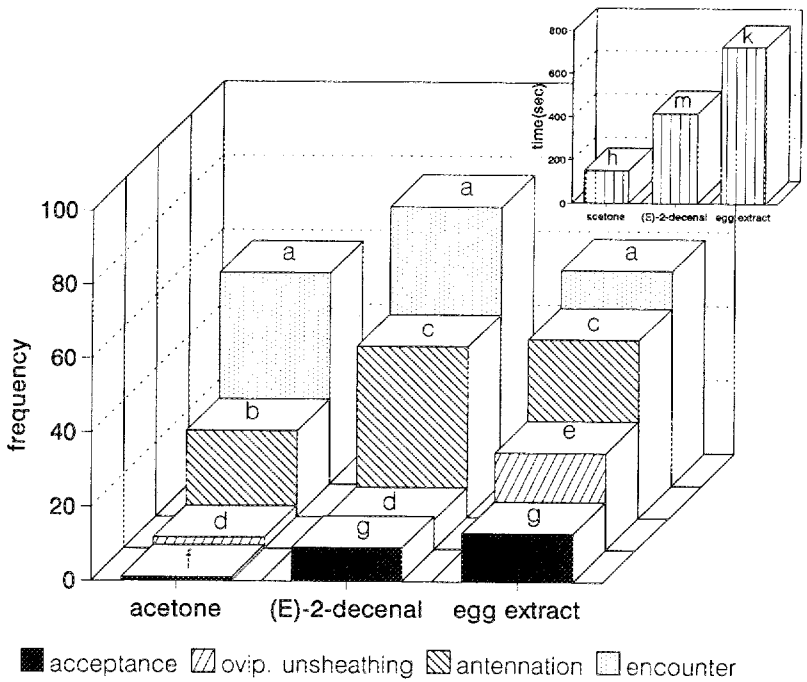

FIG. 5. Comparison of the responses of *T. basalis* between (*E*)-2-decenal and the kairomonally active egg adhesive. Acetone was used as control treatment (Mann-Whitney U test, applying Bonferroni inequality to one-tailed significance level. Different letters mean significant differences at $P = 0.05/3 = 0.017$). The insert figure shows the antennation time (seconds) of females to glass beads treated with the different extracts.

aggregation to adult contaminated areas. None of the other gas-chromatographic regions of the chromatograph eluent elicited activity. Furthermore, since we found that between 0.02 and 0.001 female equivalents were active in our bioassay (each female yielded between 1 and 2 μl of secretion), along with the information from Gilby and Waterhouse (1965) that (*E*)-2-decenal comprises 25.7% of the gland contents, our results with dilutions of pure (*E*)-2-decenal are comparable, suggesting that the other components of the gland are not only inactive, but they are not synergistic. The discovery that *T. basalis* were more sensitive to (*Z*)-2-decenal and the report that traces of the *Z* isomer are present in the defensive secretion of *Nezara viridula* (Gilby and Waterhouse, 1965) suggested the possibility that *T. basalis*, after reaching the community of adult *Nezara*, might respond to females due to the presence of *Z* isomer. However, we were unable to detect (*Z*)-2-decenal at a behaviorally significant level in the defensive secretion of female *Nezara*.

FIG. 6. A chart showing the response of female *T. basalis* in a Y-olfactometer to (*E*)-2-decenal. The "walks" chart shows the number of times a female entered the two arms. The "time" chart shows that females spend significantly more time in (*E*)-2-decenal-contaminated air (Friedman two-way ANOVA, $P < 0.01$).

Although we used glass beads to emulate eggs as a simple and convenient bioassay in the purification process, neither (*Z*)- or (*E*)-2-decenal appears to be present on eggs in sufficient quantities to elicit host-recognition behavior. Furthermore, the activity of an active host recognition extract isolated from eggs (Bin et al., 1993) has a different TLC R_f value than (*Z*)- or (*E*)-2-decenal, and the GC retention times differ. Thus, there appear to be at least two compounds involved in host selection by *T. basalis*. One functions in the possible attraction and aggregation of *T. basalis* female in areas where stink bug adults occur. The second unidentified kairomone appears to be associated with the adhesive used to glue the eggs together and to the substrate on which they are laid (Bin et al., 1993).

The results demonstrate also that the females respond in a dose-dependent way to (*E*)-2-decenal, which is one of the most toxic chemicals within the defensive secretion of *Nezara viridula* (Gilby and Waterhouse, 1965) and it is not tolerated by many insects at high concentrations (Blum, 1981).

T. basalis has a wide host range (14 genera among Pentatomidae and Scutelleridae) (Cumber, 1964; Orr, 1988): it would be interesting to investigate whether (*Z*)-2-decenal is present in any of these species or whether (*E*)-2-decenal is the only attractive compound present in the defensive secretions of all these species. When examining the response of *T. basalis* to related compounds of (*E*)-2-decenal, both 2-decenoic acid and its ethyl ester elicited strong antennation. These compounds were not detected on eggs or in the glue, but they may play a role in the recognition of other species. Aldrich (1988), reported that shield bugs (Heteroptera: Scutelleridae) resemble stink bugs in the arrangement and chemistry of their metathoracic glands and larval dorso abdominal glands. (*E*)-2-Decenal and 4-oxo-(*E*)-2-hexenal are common secretory components. Whether the response of *T. basalis* to Scutelleridae is only due to the (*E*)-2-decenal or also to 4-oxo-(*E*)-2-hexenal would be of interest.

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