Development of pheromone-based trapping for the

Melaleuca quinquenervia* biological control agent, *Oxyops vitiosa

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Introduction

The estimation of insect density and distribution can be an inaccurate and time consuming activity that can be improved by taking advantage of the insects own system of chemical communication, namely pheromones. Some weevil pheromones may be sex-specific in that they are emitted by males (e.g., boll weevils) to attract females. However, many variations occur as females may attract males or both sexes may respond to aggregation pheromones. Weevil pheromones have been described for many species of agricultural pests and formulated into a trapping system to detect or monitor insect densities in the field. Insect pheromones have become one of the keystone technologies in the area wide pest management of many economically important pest species (e.g., boll weevil, codling moth, gypsy moth). The goal of this objective is to develop this promising technique for beneficial weed biological control agents to estimate field densities and distribution. This will serve as an indispensable tool in the management of the weed with biological control.

As more information is available describing weevil attractants it becomes clear that aggregation behavior frequently involves both host plant volatiles and insect-derived compounds (Bartelt 1999). Although weevil-produced attractants have been identified, they are frequently found to be synergized to a much greater level of activity when combined with host plant compounds (e.g., Oehlschlager et al. 1993; Giblin-Davis et al. 1997; Perez et al. 1997). Additionally, many of the weevil-derived compounds are modified forms of the ingested plant compound. The *Oxyops* weevil aeration results reported herein indicate that nearly all the compounds recovered and identified from mated and unmated individuals can be traced to the food ingested by the weevil as either a plant compound or one of its metabolites. To develop effective attractants of *Oxyops* consideration of the activity of host plant compounds needs to be included. Therefore, the results reported here include volatile collections and weevil responses to both plant- and insect-derived compounds.

Terpenoids, or essential oils, constitute the largest and most diverse group of organic compounds in plants (Gershenzon and Croteau, 1991). Secondary chemistry of plants of the Myrtaceae is dominated by the presence of terpenoids, namely essential oils. Mixtures of C_{10} and C_{15} terpenoids are generally called essential oils and can occur in many plant families. The essential oil constituents of two major Australian genera of the Myrtaceae, the *Eucalyptus* and the *Melaleuca* have been well characterized (Boland et al., 1991; Brophy et al., 1989) undoubtedly due to their considerable medicinal value (Lassak and McCarthy, 1983). The concentration of these compounds in leaves is relatively high (1-3% fresh mass) and among the 150 compounds present in *M. quinquenervia* foliage, 5-10 constitute > 95% of the solvent-extractable terpenoids (Ramanoelina et al., 1994; Wheeler et al., 2002; 2003). Of these, 1,8-cineole, α-pinene, limonene, *trans*-nerolidol, β-caryophyllene and viridiflorol constitute the major terpenoids present in the leaves of this species.

Many of these terpenoids have biological activity as herbivore attractants, repellents, or feeding stimulants (Gershenzon and Croteau, 1991; Harborne, 1991; Langenheim, 1994). Recent results indicate that the larvae of *O. vitiosa* sequester terpenoids from leaves of *M. quinquenervia* which coat their integument (Wheeler et al. 2002; 2003). The *O. vitiosa* larvae are well protected from the one of the most aggressive generalist invertebrate predators in the southeastern US (Elvin et al., 1983; Lofgren et al., 1975), the red imported fire ant (Wheeler et al., 2002; 2003). Additionally, there is some evidence of biological activity of *M. quinquenervia* heartwood that repels North American termites (Carter and Huffman 1982).

The list of essential oils in leaves from *M. quinquenervia* includes some of the best known mediators of insect/plant interactions. For example, the essential oils in the banana weevil *Cosmopolites sordidus,* primarily 1,8-cineole is electro-physiologically active and acts as an attractant (Ndiege et al., 1996). This same terpenoid is a feeding deterrent and oviposition repellent to mosquitoes (Klocke et al., 1987) and repellent and toxicant against stored-grain beetles (Obeng-Ofori et al., 1997). Other active *M. quinquenervia* terpenoids like *trans*-nerolidol function either as insect attractants (Aldrich et al. 1991; 1993; Binder et al., 1995) or as antifeedants (Doskotch et al., 1980). Additionally, the essential oil components α-pinene and β-pinene are well known mediators of insect behavior (Gershenzon and Croteau, 1991).

Variation in terpenoid quality and quantity is a common characteristic that can be attributed to a variety of genetic, seasonal, and ontogenetic factors (Langenheim, 1994). The genetics of a population is one of the most pervasive factors creating numerous chemotypes with distinct terpenoid profiles. At least two *M. quinquenervia* chemotypes have been identified based upon the relative composition of individual terpenoids (Ramanoelina et al., 1994; Moudachirou et al., 1996; Trilles et al., 1999; Ireland, 2002; Wheeler et al., 2002; 2003). Recent analysis (still underway) of the volatile chemistry of the weed *M. quinquenervia* from Florida populations indicates the existence of at least two distinct terpenoid variants or chemotypes (Wheeler et al., 2003). In Australia, the same two chemotypes exist (Brophy and Doran, 1996; Ireland, 2002). Apparently these chemotypes can only be distinguished by gas chromatography (GC). Chemotype I is characterized by only two predominant terpenoids β-caryophyllene and *trans*-nerolidol and chemotype II is characterized by seven or more predominant terpenoids including 1,8-cineole and viridiflorol (Table 1). Leaves from chemotype I plants contain trace to low levels of the terpenoid viridiflorol, whereas, leaves from chemotype II plants lack the terpenoid nerolidol. The objective of this research was to identify the volatile components from adult weevils of *O. vitiosa* that impart behavioral responses in this species.

Methods and Materials

Insects. Adult weevils were field-collected and reared on leaves under optimal conditions (27° C; 95% RH; 14:10 photoperiod). To obtain adults of a known age, origin, diet, and mating history, weevil larvae were field collected and reared individually until reaching the adult stage. To identify the leaf chemistry and chemotype upon which each larva was collected, GC analysis was conducted using standard procedures (see below).

Chemicals. Terpenoids were purchased from commercial sources, except where mentioned, and were of the highest purity available. The list included the primary compounds reported by Brophy et al. (1989), Ramanoelina et al. (1994), and Wheeler et al. (2002; 2003). These included (+ and -) α-pinene (C. P. = 98%), (+ and -) β-pinene (99%), α-terpinene (98%), (+ and -) limonene (92%), 1,8-cineole (99%), γ-terpinene (98%), terpinen 4-ol (97%), (+ and -) α–terpineol (98%), β-caryophyllene (98%), (-) perilla aldehyde (-) cis verbenol, and *trans*-nerolidol (95%). Viridiflorol (86%) was extracted from *M. quinquenervia* foliage and generously donated by I. A. Southwell, (Wollongbar Agricultural Institute Wollongbar, New South Wales, Australia).

Volatile collection. The volatile aeration system entrained purified laboratory air across insects and test plants for delivery to an adsorbent material (Heath et al. 1992. Collections occurred at 27° C (± 3°) under ambient laboratory lights. Purified and humidified air flowed (0.5 liter/min) into glass chambers (15 x 35 cm; Analytical Research Systems, Gainesville, Fla), each containing insects either with or without the host plant. Additionally, a control consisted of only the volatile collection chamber. All the volatile-laden air was drawn through the adsorbents (30 mg; Super Q, Alltech Inc., Deerfield, IL) at the end of each chamber for 12 - 24 h. Frequently, the adsorbent tube was also used to prefilter the air before it aerated the samples. The volatiles were eluted from the adsorbent with a minimum of methylene dichloride (200 µl). An internal standard (50 ng/µl), tridecane was added and a 1 µl subsample was injected into a gas chromatograph for identification and quantification. When possible, response factors were calculated and the amount of each compound present was quantified.

GC analysis. Insect and plant volatiles were analyzed by gas chromatography mass spectrometry using one of our capillary instruments (HP 6890 or 5890). Our routine protocol included He as a carrier gas (1 ml/min), oven temperature of 50° C, held for 2 min, then increased at 8° C/min to 250° C which was held for 10 min. Injector temperature was 250° C and flame ionization detector temperature was 250° C. Injections of 1 ul were made on two capillary columns, DB-5 (Hewlett Packard Corp., Palo Alto, CA), and a DB-17 (J & W Scientific, Folsom, CA; all 30 m x 0.32 mm ID with a 0.25 µm film thickness). Kovats indices were calculated using *n*-alkanes and values were compared with published descriptions of plant compounds (Adams 2001).

GCMS analysis. Compound identities were confirmed by GC-MS using a Hewlett Packard 6890 instrument fitted with a HP-5MS (30 m x 0.25 mm, 0.25 micron film thickness) FSOT column with helium (36 cm/sec) as a carrier gas, injector port (generally split 1:50; or 1:20, occasionally splitless) at 250°C, mass selective detector (HP 5973) at 250°C (source) and 150°C (quad) with transfer line 280°C and ion source filament voltage of 70 eV. The oven temperature profile was as described above.

Component identification was made on the basis of mass spectral fragmentation, retention index with *n*-paraffins and comparison with authentic constituents and mass spectral and retention matching with commercial (NIST, Wiley, and Adams) libraries. Metabolites of *M. quinquenervia* foliar terpenoids were identified according to Southwell et al. (2003).

Electrophysiology. Electrophysiological responses to insect and plant volatiles were measured with an electroantennogram (EAG) and a coupled GC/EAG. All recordings were conducted with recently excised antennae (< 3 min) suspended between two Ag-AgCl glass electrodes filled with saline solution (0.1 N KCl). Antennal responses to volatile cues were amplified (10x) with a high impendence amplifier (Syntech UN-06; Syntech Hilversum, The Netherlands), captured, and analyzed with AutoSpike software (Ver. 2, Syntech). Purified air (0.6 liter/min) that was saturated with water (HPLC grade) was blown continuously across the antennal preparation.

EAG analysis. Manual puffs (300 ml/min) of select volatiles were made across the antennal preparation through a disposable Pasteur pipette. This was accomplished by applying 25 µl solutions of test compounds dissolved in paraffin at 0.1, 1.0, and 10% concentrations to filter paper strips (1 x 5 cm). The EAG data were standardized and expressed as a percentage of the antennal responses to 1-hexanol (1% in paraffin).

EAD analysis. Gas chromatography/EAG was conducted on a HP 5890 equipped with the same DB-17 capillary column described above. Flow rates and temperature profiles follow the description above. The column effluent was split 75:25 between the antennal preparation and a flame ionization detector. The column effluent passed through a heated transfer line 250 °C before being mixed with the purified air passing over the antennal preparation.

Y-tube analysis. Y-tube bioassays included a glass tube (1 cm ID) with a stem that measured 15 cm long and 2 arms that were each 10 cm long (Harari and Landolt, 1999). Purified and humidified air (1 liter/min) flowed across the test material delivered at several concentrations or a control into each arm. Bioassays were conducted under laboratory light at 27 °C (± 3°). Adults were field collected from wild *M. quinquenervia* plants in Ft Lauderdale, Fla. Test compounds were tested individually dissolved in organic solvent ($CHCl_3$) and mixed with paraffin to retard evaporation (Brockerhoff and Grant, 1999). Responses by both males and females to the test compounds were compared with a control ($CHCl_3$ + paraffin). Individuals were starved for 24h prior to testing. These tests were conducted with 30 replications and the Y-tubes were inverted 180˚ between each replicate. The Y-tube was washed, rinsed in EtOH, and oven dried (100˚C) between testing of different compounds.

Results

Volatile collection. Weevil larvae were field-collected bimonthly beginning Nov. 2002 to June 2003 from two *O. vitiosa* populations, Estero and University & Griffin, Ft Lauderdale, representing plants of distinct *M. quinquenervia* chemotypes, either nerolidol or viridiflorol, respectively. Adults were also field collected of unknown ages, nutritional and mating histories. Generally, distinct weevil populations were collected from different chemotypes as pheromones may be modified plant compounds and thus different dietary components may lead to different pheromone chemistry and distinct *O. vitiosa* populations may occur and communicate with different pheromones. Weevil larvae (100/chemotype) were fed screenhouse-grown *M. quinquenervia* leaves of the proper chemotype. Volatile collections (n=18/diet/sex) occurred separately on unmated males and females during 24 or 48 h. Estimated terpenoid components of leaves from each chemotype are listed in Table 1.

TABLE 1. Comparison of Mean (± SE) Concentration of 11 Terpenoid Components from $CHCl_3$-Extracted *M. quinquenervia* leaves from two chemotypes (*N* = 10). Values Estimated by Gas Chromatography, Expressed as μg/mg of Fresh Leaf, and Adjusted with Appropriate Response Factors

	M. quinquenervia	
Compound	**Chemotype I μg/mg (SE)**	**Chemotype II μg/mg (SE)**
α-pinene	0.06 (0.01)	1.47 (0.35)
β-pinene	0.01 (0.001)	0.44 (0.08)
α-terpinene	0 (0)	tr[a]
Limonene	0.03 (0.01)	1.13 (0.16)
1,8-cineole	0.01 (0.001)	3.15 (0.41)
γ-terpinene	0.001 (0.001)	0.08 (0.01)
terpinen 4-ol	0.001 (0.001)	0.07 (0.01)
α-terpineol	0.004 (0.001)	0.89 (0.12)
β–caryophyllene	1.7 (0.2)	0.52 (0.09)
trans-nerolidol	28.30 (3.7)	nd [b]
viridiflorol	0.20 (0.04)	6.06 (0.86)

[a] tr: trace

[b] nd: not detected

Analysis of these volatile collections by GCMS indicated a large number of plant compound (Table 1) and their metabolites (Figs. 1 & 2), primarily modified forms of 1,8-cineole (eucalyptol). These included 4-OH, 2α-OH, 3α-OH, 3β-OH, 7-OH, and 9-OH cineole (Fig. 1). Additionally, octan-6-one may be a further oxidation produced of 2α or 2β-OH cineole. Apparent metabolites of α-pinene (myrtenol) and limonene (perilla alcohol and perilla aldehyde) were also found. Many of the plant compounds that had passed unchanged through the insect digestive system were also recovered.

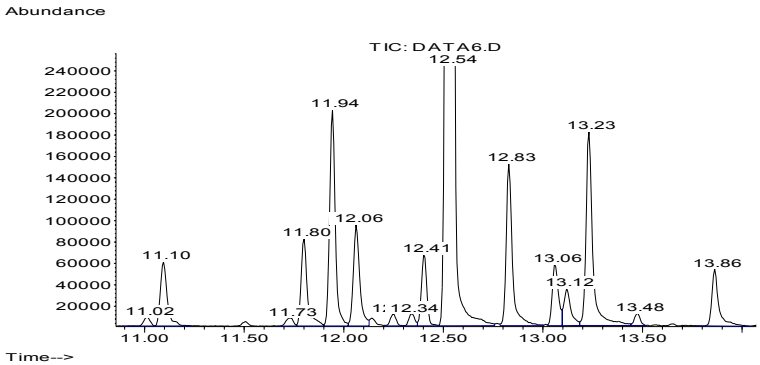

Fig. 1. Total ion count of volatiles collected from (n=12) *O. vitiosa* unmated males fed viridiflorol leaves. Identities include: retention time =11.8=4-OH-cineole, 11.94=α-terpineol; 12.06=myrtenol*; 12.41=cyclo hexanone*; 12.54=2α-OH-cineole; 12.83=3α-OH-cineole; 13.06=3β-OH-cineole; 13.12=7-OH-cineole; 13.23=9-OH-cineole; 13.48=perilla aldehyde; 13.86=perilla alcohol*. * unconfirmed assignment.

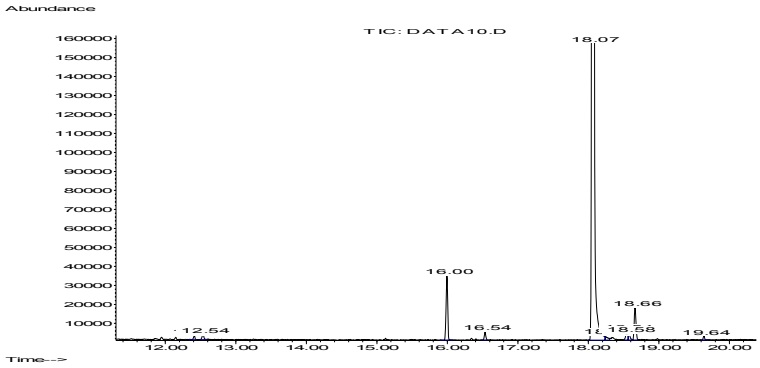

Fig. 2. Total ion count of volatiles collected from (n=23) *O. vitiosa* unmated males fed nerolidol leaves. Identities include: retention time =15.00=β-caryophyllene, 18.07=*trans*-nerolidol; 18.66=viridiflorol.

No differences in volatile profiles could be detected between those produced by males or females or by mated or purportedly non-mated individuals. However, nutritional differences influenced the volatiles collected (Figs. 1 & 2). The volatiles collected from the adults fed the viridiflorol chemotype plants produced volatiles that matched their diet and metabolites of these parent compounds (Fig. 1). Similarly, the volatiles collected from the adults fed the nerolidol chemotype plants produced volatiles characteristic of the leaves of this chemotype (Fig. 2). No plant metabolites were found from adults fed leaves of the nerolidol chemotype. These results suggest that if plant compounds are modified and used for pheromones, diet will have a dramatic influence on the pheromone produced.

EAG analysis. EAG analyses of select *M. quinquenervia* volatiles indicated that the *O. vitiosa* antennae detected the major terpenoids (in paraffin) found in the foliage and headspace of this species (Fig. 3). A detected compound will elicit a sharp negative deflection in voltage when puffed across an antenna.

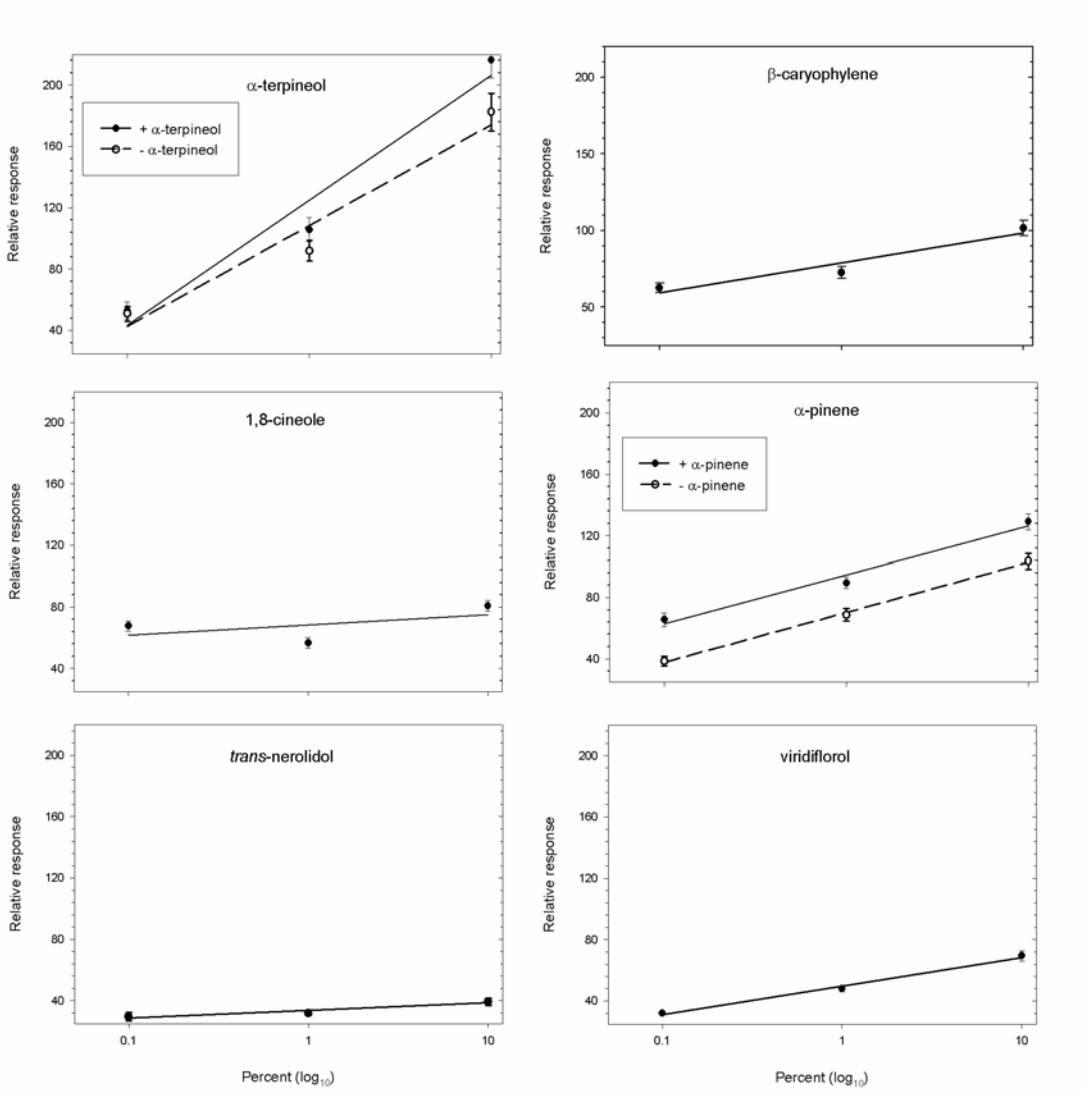

Fig. 3. EAG responses *M. quinquenervia* volatiles puffed across an excised *O. vitiosa* antennae. Data were standardized according to antennal responses to 1-hexanol (1% in paraffin) and expressed as a percentage relative to this standard.

EAD analysis. EAD analysis of plant and insect-derived volatiles indicated that *O. vitiosa* antennae respond (as indicated by a sharp negative deflection) to several *M. quinquenervia* volatiles (e.g., limonene, 1,8-cineole, and α-terpineol) as well as to several unknown possibly insect-produced compounds (Fig. 4). These responses indicate that these compounds are detected by the antennae. However, to determine behavioral relevance, these compounds are being tested in a Y-tube olfactometer.

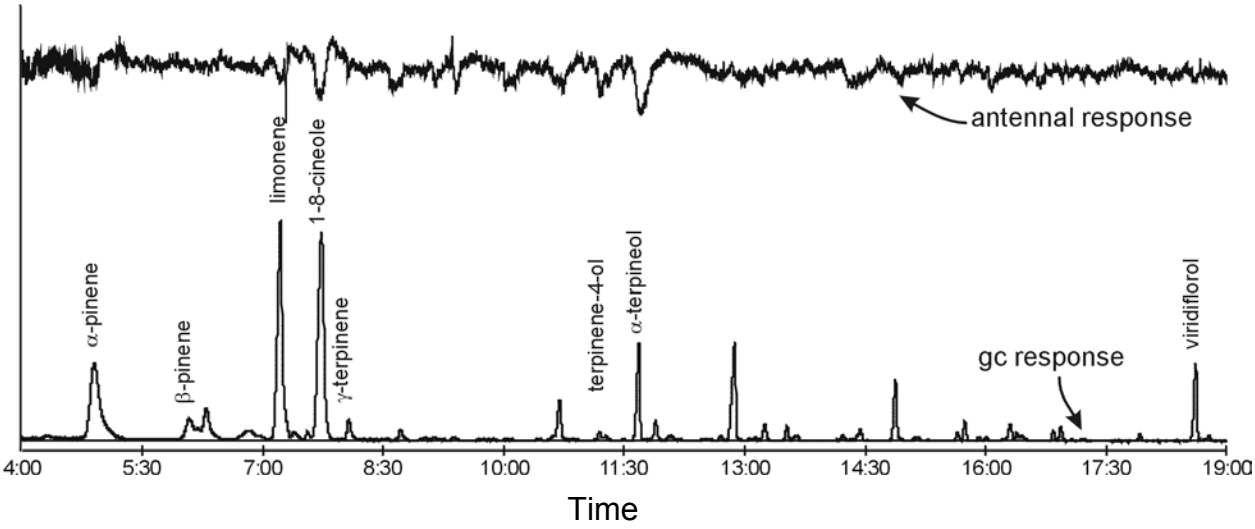

Fig. 4. Representative coupled GC-EAG chromatograph of *M. quinquenervia* and *O. vitiosa* volatiles collected (24 h) with Super Q adsorbent, eluted with 200 μl CH_2Cl_2 and injected into a gas chromatograph (GC). See text for GC conditions. *O. vitiosa* antennal response is amplified 10x and displayed at a range of 1 mV and the GC-FID response is displayed at 100 mV.

Y-tube analysis. The results of y-tube olfactometer where *O. vitiosa* adults of each sex were offered a choice between a solvent control and *M. quinquenervia* terpenoid dissolved in solvent indicate that the adults, regardless of sex, were attracted to several *M. quinquenervia* terpenoids (Table 2). Excised *Melaleuca* leaves were used to test the validity of the bioassay test, resulting in 85% of the responding adults attracted to the leaf odor compared with moistened filter paper. When testing individual components considerable variability occurred. However, at least 70% of the adults responded to one of the levels tested of (–) α-terpineol, 1,8-cineole, and *trans*-nerolidol suggesting that these components elicit an attraction response to the adults of this weevil. Additional testing of the compounds that were only recovered from weevils is presently restricted to those that can be purchased, which includes verbenol and perilla aldehyde. However preliminary results with these two insect-derived compounds suggest that these volatiles individually do not elicit a response from adult weevils. This work is still ongoing and presently is focused on the activity of these and other insect-derived compounds. Furthermore, once tentative activity is determined even these non-commercial insect-derived compounds are available from colleagues.

Table 2. Y-tube results of *O. vitiosa* adults tested with volatiles collected from con-specific adults and their host plant, *Melaleuca quinquenervia*.

compounds	Concentration (%)	% responding to test compound
(-)a-terpineol	0.1	83.3
(-)a-terpineol	1.0	66.7
(-)a-terpineol	10.0	40.0
(+)a-terpineol	0.1	70.0
(+)a-terpineol	1.0	55.6
(+)a-terpineol	10.0	60.0
(+)a-pinene	0.1	30.0
(+)a-pinene	1.0	50.0
(+)a-pinene	10.0	40.0
(-)a-pinene	0.1	60.0
(-)a-pinene	1.0	80.0
(-)a-pinene	10.0	50.0
(+)b-pinene	0.1	60.0
(+)b-pinene	1.0	60.0
(+)b-pinene	10.0	60.0
(-)b-pinene	0.1	70.0
(-)b-pinene	1.0	50.0
(-)b-pinene	10.0	70.0
(-)b-pinene	0.1	70.0
(-)b-pinene	1.0	60.0
(-)b-pinene	10.0	50.0
1,8-cineole	0.1	80.0
1,8-cineole	1.0	60.0
1,8-cineole	10.0	40.0
trans-nerolidol	0.1	70.0
trans-nerolidol	1.0	50.0
trans-nerolidol	10.0	40.0
viridiflorol	0.1	40.0
viridiflorol	1.0	60.0
viridiflorol	10.0	50.0
viridiflorol	0.1	50.0
viridiflorol	1.0	40.0
viridiflorol	10.0	50.0
viridiflorol	0.1	40.0
viridiflorol	1.0	30.0
viridiflorol	10.0	50.0

Modifications of this y-tube bioassay are being considered. One such modification incorporates a different delivery system of the test compound. One design includes specific amounts of test compounds loaded into capillary tubes (e.g., 5 ul) with

a known rate of compound delivery that can be determined gravimetrically. Additionally, a volatile profile that resembles the plant odor needs to be formulated and tested. Once y-tube responses are established to this profile, retesting the profile with systematic deletions may reveal the most relevant components imparting behavioral responses in these species.

Discussion

To speculate on the relevance of these findings would be premature as much of this research continues. However, the results demonstrate several important points. First, the methodology for the collection and identification of plant and insect-derived compounds from *M. quinquenervia* and *O. vitiosa* are well established and show progress toward achieving this goal. Moreover, by addressing this research on at least two levels, electrophysiolgical and behavioral, results of a test component can be verified with another method. This has been demonstrated with 1,8-cineole and α-terpineol which both elicited antennal (EAG & GC/EAG) and y-tube responses. The results also suggest that this species uses several volatile cues when finding both conspecifics and their host. Unlike the classical example of the crucifer feeding species that use only a token stimulus to find their host (Feeny 1992), *O. vitiosa* appears to use a mixture of several compounds to locate both conspecifics and their host *M. quinquenervia*. Similar mixtures of insect-derived compounds and mixtures of both insect and plant-derived compounds, possibly functioning synergistically, seem likely with *O. vitiosa*. Furthermore, as has been shown by other workers, EAD analysis may present an incomplete picture of the volatile cues that elicit behavioral responses in this species. More details on behavioral cues may be found by examining the reception of volatiles by specific sensillae on the antennal club with a single cell recording (SCR) technique. Although EAD results may indicate specific volatiles that elicit behavioral responses (Guerin and Visser, 1980), SCR is a more sensitive test (Bernays and Chapman, 1994) that measures the response of individual sensillae (Blight et al., 1997). Possibly distinct sensillae exist in *O. vitiosa* that are tuned separately to either pheromones or plant compounds as has been shown for other weevils (Dickens 1990). Restricting neurophysiological recording to specific sensillae with SCR may reveal this specificity in perception and function. Consequently, I am in the process of upgrading the EAG/EAD instruments (Syntech) to measure single cell responses of individual sensillae while coupled to the GC using research funds available through this Areawide project during upcoming funding cycles.

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