Journal of Chemical Ecology, Vol. 21, No. 11, 1995

ELECTROANTENNOGRAPHIC AND COUPLED GAS CHROMATOGRAPHIC–ELECTROANTENNOGRAPHIC RESPONSES OF THE MEDITERRANEAN FRUIT FLY, *Ceratitis capitata*, TO MALE-PRODUCED VOLATILES AND MANGO ODOR

ALLARD A. COSSÉ,[1,*] JULIE L. TODD,[1] JOCELYN G. MILLAR, LEE ANN MARTÍNEZ,[2] and THOMAS C. BAKER[1]

Department of Entomology, University of California, Riverside, California 92521

(Received March 17, 1995; accepted July 9, 1995)

Abstract—We have identified five compounds from the headspace of calling male Mediterranean fruit flies (medfly), *Ceratitis capitata* (Wiedemann), and three compounds from the headspace of ripe mango (*Mangifera indica* L.) using coupled gas chromatographic–electroantennographic (GC-EAG) recordings, coupled gas chromatographic–mass spectrometric (GC-MS) analysis, and electroantennographic (EAG) assays of standards. The male-produced volatiles eliciting responses from female antennae were ethyl-(*E*)-3-octenoate, geranyl acetate, (*E*,*E*)-α-farnesene, linalool, and indole. An EAG dose–response test of linalool enantiomers and indole with female medfly antennae showed relatively strong EAG activities, but no significant difference between (*R*)-(−)-linalool and (*S*)-(+)-linalool. The three mango volatiles were identified as (1*S*)-(−)-β-pinene, ethyl octanoate, and β-caryophyllene. In addition, a strong antennal response was recorded from a contaminant, α-copaene, present in a commercial sample of β-caryophyllene. The EAG response amplitudes from both male and female antennae to the above three mango volatiles were significantly greater than to a hexanol control. For both male and female medfly antennae, the greatest EAG responses were elicited by β-caryophyllene followed by ethyl octanoate. The mean EAG responses of female antennae to β-caryophyllene and (1*S*)-(−)-β-pinene were significantly greater than those of male antennae.

*To whom correspondence should be addressed.
[1]Present address: Department of Entomology, Iowa State University, Ames, Iowa 50011.
[2]Present address: Department of Biology, University of Southern Colorado, Pueblo, Colorado 81001.

0098-0331/95/1100-1823$07.50/0

Key Words—Diptera, Tephritidae, Mediterranean fruit fly, *Ceratitis capitata*, pheromone, attractants, mango volatiles, *Mangifera indica*, headspace analysis, gas chromatography–electroantennography.

INTRODUCTION

The medfly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is a notorious pest because of its broad host range, attacking over 250 different types of fruit and vegetables, and its importance to international quarantine (Hagen et al., 1981). The most widely used lure in trapping programs for medfly detection is trimedlure, a synthetic compound primarily attractive to male medflies (Beroza et al., 1961). The lack of an effective female attractant has prompted several studies to examine the male sex pheromone and host odors as lures for attracting female flies. Several researchers have reported on the isolation, identification, or attractancy of male-produced volatiles from calling male medflies, resulting in a total of 56 identified compounds (R.R. Baker et al., 1985; P.S. Baker et al., 1990; Jang et al., 1989, 1994; Heath et al., 1991; Landolt et al., 1992a; Flath et al., 1993). The focus of these studies has been mainly on the attractiveness of the most abundant male odor components. A three-component blend was shown to have limited attraction for medfly females in wind-tunnel bioassays and in field tests (Heath et al., 1991; Landolt et al., 1992a). Recently, Jang et al. (1994) reported on the attractiveness of a five-component blend in wind-tunnel experiments, including the three components tested by Heath et al. (1991) and Landolt et al. (1992a); they found that this blend was still less attractive than the natural male pheromone. These results indicate that improvements in the synthetic male pheromone blend may be possible. Analysis of volatile compounds with coupled gas chromatographic–electroantennographic (GC-EAG) assays may help eliminate compounds with no biological activity and suggest compounds for behavioral studies. Furthermore, host odor volatile analysis by GC-EAG assay could identify compounds that, in combination with male pheromone, may further enhance female attraction to male pheromone, as was shown with the papaya fruit fly, *Toxotrypana curvicauda* Gerstaecker (Landolt et al., 1992b).

By using GC-EAG and EAG recordings, we have examined the peripheral olfactory selectivity of female medflies to volatile compounds from males and from ripe mango in order to identify potentially attractive novel compounds. Volatiles of mango were analyzed because this fruit is a preferred host of several fruit fly species and the identity of many volatiles of intact mango have been determined (Engel and Tressl, 1983; MacLeod and Pieris, 1984; MacLeod and Snyder, 1985; Bartley and Schwede, 1987; Bartley, 1988; MacLeod et al., 1988). Our study represents the first use of the GC-EAG technique with medfly

and allowed us to pinpoint compounds present in trace amounts that may have gone undetected by using GC analysis and EAG recordings as separate techniques.

METHODS AND MATERIALS

Insects. Pupae were obtained from a medfly rearing facility operated by USDA-APHIS in Petapa, Guatemala. Upon arrival at the UCR Insect Quarantine Facility, the dyed (Day Glo Yellow) male and female pupae were placed in separate cages and held under natural light conditions. Adults flies used for the EAG and GC-EAG studies were fed honey and tested 3–30 days after emergence.

Collection of Male-Produced Volatiles. Extracts of volatiles produced by aeration of calling males were provided by R. R. Heath at the Insect Attractants, Behavior, and Basic Biology Research Laboratory, USDA-ARS, Gainesville, Florida, who used collection procedures previously described (Heath et al., 1991). Volatiles were collected from wild male medflies 5–10 days old in groups of 8–12 flies for 2-hr periods throughout the photophase. The males had access to a yeast hydrolysate food source, which was replaced with sugar water 24 hr prior to the collection of the volatiles. The male-produced volatiles were collected in glass tubes (4 cm long × 4 mm ID) containing 25 mg of Super-Q (Alltech Assoc. Inc., Deerfield, Illinois) placed between two stainless steel frits. The Super-Q traps were eluted with 200 μl of methylene chloride. Each sample was concentrated to ca. 50 μl under nitrogen prior to GC-EAG analysis.

Collection of Mango Fruit Volatiles. Ripe mangos (cv. Tommy Atkins) were obtained from a local supermarket and washed with water before being analyzed. A piece of mango peel with pulp attached (ca. 0.5 × 0.5 × 0.5 cm) was placed in a 25-ml fritted sparger, which was connected to a Tekmar 2000 purge and trap concentrator (Tekmar, Cincinnati, Ohio). The conditions for collecting volatiles on a Tenax trap were as follows: 5 min purge at room temperature (He, 20 ml/min), 5 min dry purge, and 4 min desorb at 180°C. The Tekmar 2000 was directly interfaced to the GC-EAG setup.

Chemical Analyses. One-microliter aliquots of the collected male-produced volatile extract were injected in splitless mode onto 30-m × 0.25-mm-ID fused silica capillary gas chromatographic columns, coated either with DB-5 or DB-1 (J & W Scientific, Folsom, California) or Ultra-2 (Hewlett-Packard Company, Palo Alto, California; equivalent to DB-5) for analyses by GC-EAG and GC–mass spectrometry (GC-MS). Column conditions were as follows: He carrier gas flow of 1.5 ml/min, injector temperature 200°C, oven temperature program 1 min delay on inlet purge, 2 min at 40°C, then 20°C/min to 220°C.

Mango volatiles were analyzed by interfacing the GC-EAG and GC-MS

with the Tekmar 2000 purge and trap collector, using the same columns with the following column conditions: He carrier gas flow of 1.5 ml/min, injector temperature 200°C, oven temperature program, 4 min at 0°C, 4 min at 40°C, 25°C/min to 220°C (GC-EAG); and injector temperature 250°C, oven temperature program, 4 min at 0°C, 10°C/min to 250°C (GC-MS).

GC-MS analyses were performed using a Hewlett-Packard 5890 gas chromatograph with a direct interface to a Hewlett-Packard 5970 mass selective detector (electron impact, 70 eV).

Electroantennogram Responses. Simultaneous GC-EAG and EAG analyses were performed as described by Baker et al. (1991) using a Varian model 3740 GC (Varian Associates, Sugarland, Texas). EAG recordings were made by placing a saline-filled (Beadle-Ephrussi Ringer) glass electrode in the back of an excised medfly head. The recording electrode was inserted into the tip of one of the antennae. To examine antennal sensitivity to the identified "minor" male-produced volatiles and to mango odor, EAGs, not coupled to GC, were recorded for a series of commercial or synthetic compounds. Serial dilutions of the tested compounds were made in redistilled HPLC-grade hexane such that the tested compounds were applied to filter-paper strips (0.5 × 3.0 cm, Whatman No. 1) in 10 μl of solvent. The filter-paper strips were placed inside Pasteur pipets (15 cm long). Control puffs of blank (filter paper plus solvent) and air (empty pipet) controls showed relatively low EAG activities, which were difficult to distinguish from the background activity. For this reason and to compensate for possible deterioration of the preparation, each 2-ml puff of test compound was preceded by a standard control compound, hexanol (10-μg dose). EAG amplitudes were standardized according to the responses to hexanol by dividing the amplitude of the EAG generated from the test compounds by that of hexanol. Within a particular series of test compounds, presentation of the test compounds was randomized. EAG data were subjected to ANOVA and mean responses were compared using a t test.

Chemicals. Racemic linalool, ethyl octanoate, indole, geranyl acetate, and (1*S*)-(−)-β-pinene were obtained from Aldrich Chemical Company (Milwaukee, Wisconsin) and were >98% pure (label information and GC analysis). (*R*)-(−)-Linalool and β-caryophyllene were purchased from K & K Laboratories (Cleveland, Ohio). The β-caryophyllene sample contained 1% α-copaene based on GC-MS comparisons with authentic samples of α-copaene isolated from cubeb oil (Millar et al., 1986) and 10% α-humulene based on GC-MS comparisons with commercially available material (Fluka Chemical Company, Ronkonkoma, New York). (*S*)-(+)-Linalool was synthesized according to Landolt et al. (1994) and ethyl-(*E*)-3-octenoate was prepared as described by Heath et al. (1991). (*E*,*E*)-α-Farnesene was obtained from R.R. Heath, USDA, Gainesville, Florida.

RESULTS

Analysis of Volatiles Collected from Calling Males. A total of 30 combined GC-EAG analyses of the volatiles collected from calling male medflies by aeration was obtained using 30 different female medfly antennae. Only those GC peaks that consistently revealed EAG activity were targeted for further analysis. The combined GC-EAG analysis consistently revealed five GC peaks on both DB-1 and DB-5 columns with corresponding EAG activity with female medfly antennae (Figure 1, compounds I, II, III, IV, and V). The retention times on both columns and GC-MS spectra of compounds I–V corresponded precisely to those of five compounds known to be produced by male medfly: linalool, ethyl-(*E*)-3-octenoate, indole, geranyl acetate, and (*E*,*E*)-α-farnesene, respectively. Moreover, combined GC-EAG recordings on both DB-1 and DB-5 columns demonstrated that linalool, ethyl-(*E*)-3-octenoate, indole, geranyl acetate, and (*E*,*E*)-α-farnesene had retention times identical to compound I–V, respectively, from the collected male volatiles and were EAG active.

Standardized EAG dose responses revealed that 100 ng and higher dosages of indole and (*S*)-(+)-linalool, and 1 μg and higher dosages of (*R*)-(−)-linalool elicited stronger EAG responses in female antennae than hexanol (Figure 2). Indole (10 μg) generated responses 2.1 times (±0.4 SD) as great as those elicited by hexanol, (*S*)-(+)-linalool (10 μg) 1.5 times (±0.5 SD) as great, and (*R*)-(−)-linalool (10 μg) 1.4 times as great (±0.4 SD). The 10-μg dosages of indole generated significantly stronger responses than either (*R*)-(−)-linalool or (*S*)-(+)-linalool ($t = 2.61$, $P < 0.05$, $t = 2.15$, $P < 0.05$, respectively), and the 100-ng dosage of indole generated significantly stronger responses than (*R*)-(−)-linalool ($t = 5.12$, $P < 0.05$). However, there were no significant differences in the EAG responses to (*R*)-(−)-linalool and (*S*)-(+)-linalool for the dosages tested. The mean EAG response of female medfly antennae to hexanol was 0.4 mV (±0.5 SD, $N = 9$).

Analysis of Mango Volatiles. Analyses of trapped mango volatiles by coupled GC-EAG on both DB-1 and DB-5 columns revealed three compounds that consistently elicited strong antennal responses from female medflies (Figure 3). These compounds were identified as β-pinene, ethyl octanoate, and β-caryophyllene, respectively, by comparison of GC-MS retention times and mass spectra with those of authentic compounds. Moreover, combined GC-EAG recordings of (1*S*)-(−)-β-pinene, ethyl octanoate, and β-caryophyllene on the same two capillary columns demonstrated that these retention times were identical to compounds VI, VII, and VIII, respectively, from the collected mango volatiles and that all three compounds were EAG active (Figure 4). Closer examination of the GC-EAG responses obtained with a mixture of commercially available compounds identified from mango revealed an additional minor peak with EAG

activity labeled with a question mark in Figure 4. Mass spectral and retention time analyses of the β-caryophyllene sample revealed the presence of β-caryophyllene, α-copaene, and α-humulene. The identities of the latter two compounds were confirmed by comparison of retention times and mass spectra with authentic standards. Combined GC-EAG analysis of β-caryophyllene consistently revealed that α-copaene elicited strong EAG responses in addition to the

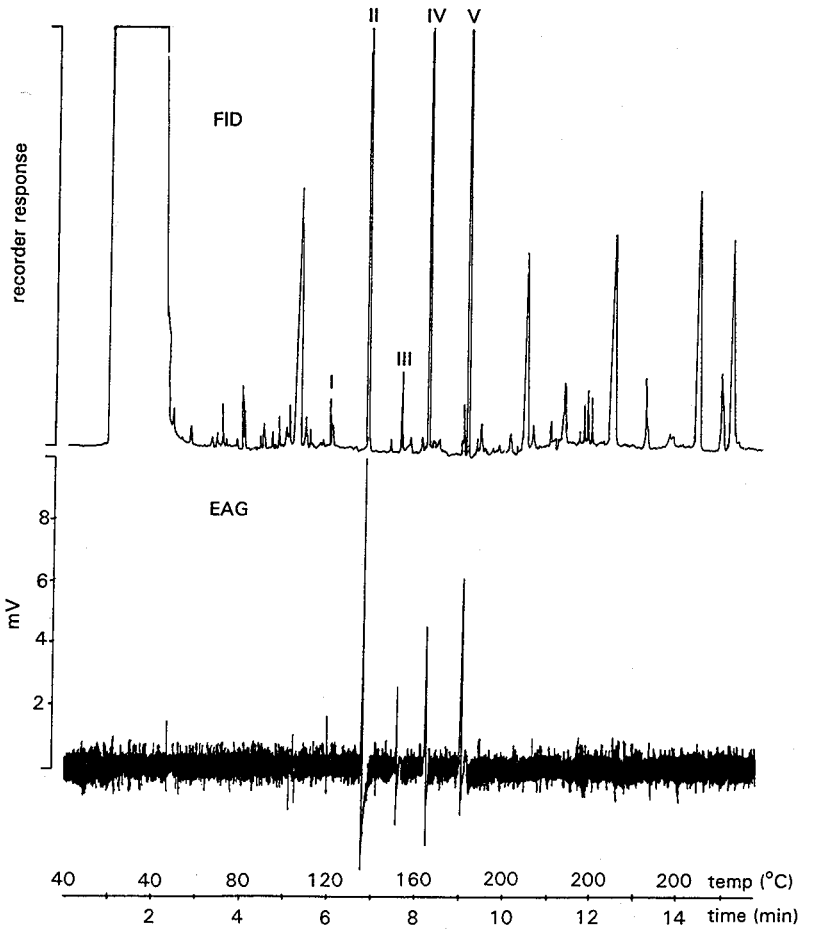

FIG. 1. Reconstructed simultaneously recorded gas chromatogram [flame ionization detector (FID)] of trapped volatiles of calling male medflies (top) and electroantennogram (EAG) of a female medfly antenna (bottom). EAG active compounds are labeled I, II, III, IV, and V (see text for details).

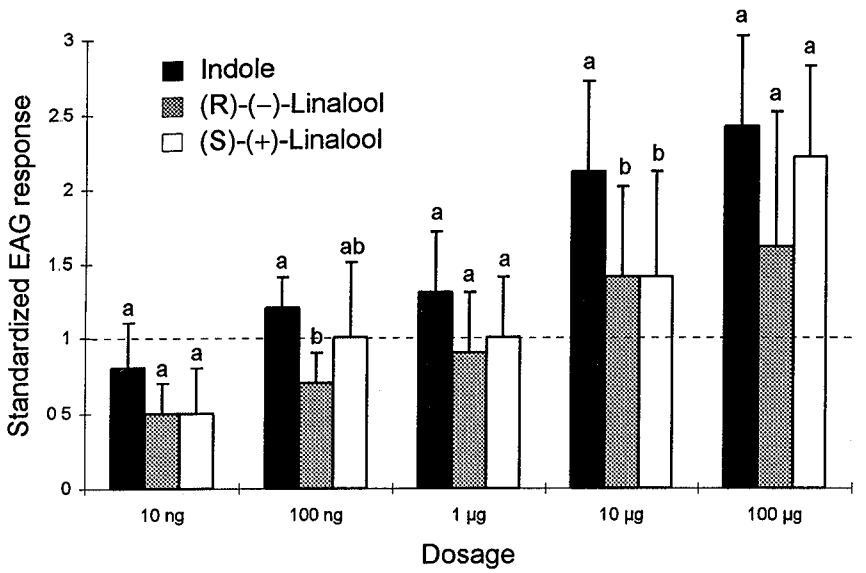

FIG. 2. EAG dose responses of female medfly antennae to selected known volatile compounds released by calling males. Stimuli were commercial or synthetic standards. Standard deviations of mean responses are presented as error bars. Dotted line indicates the mean response to the hexanol standard (10 μg). Dosage means with the same letter are not significantly different by Student's t test at $P < 0.05$ ($N = 9$).

EAG activity of β-caryophyllene, while α-humulene showed no activity on female antennae (Figure 5). No α-copaene was detected in the trapped mango volatiles. The following compounds were also identified by retention time and mass spectral matches with standards, but showed no GC-EAG activity: 1-butanol, ethyl propanoate, 3-methyl butanol, ethyl butanoate, ethyl-(*E*)-2-butenoate, α-pinene, *o*-cymene, myrcene, ocimene, *p*-cymene, limonene, terpinolene, and α-humulene.

Standardized EAG response measurements with 100-ng dosages of (1*S*)-(−)-β-pinene, ethyl octanoate, and β-caryophyllene showed greater EAG responses elicited by ethyl octanoate and β-caryophyllene than by hexanol for both male and female antennae (Figure 6). Average responses to (1*S*)-(−)-β-pinene, ethyl octanoate, and β-caryophyllene of female antennae were 0.78 $\pm$ 0.04 SD, 1.88 $\pm$ 0.25 SD, and 4.68 $\pm$ 0.70 SD, respectively, while average responses from male antennae were 0.41 $\pm$ 0.08 SD, 1.90 $\pm$ 0.28 SD, and 3.84 $\pm$ 0.42 SD, respectively. The mean EAG responses of female medflies to β-caryophyllene and (1*S*)-(−)-β-pinene were significantly greater than those of male flies ($t = 3.27$, $P < 0.01$, $t = 12.86$, $P < 0.01$, respectively). The

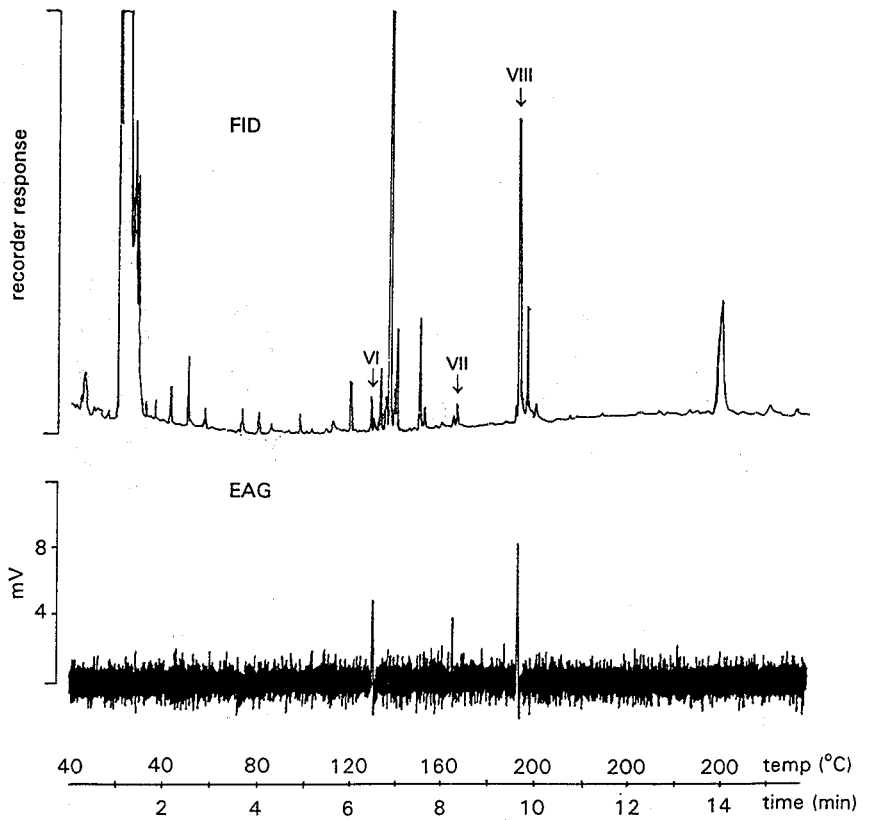

FIG. 3. Reconstructed simultaneously recorded gas chromatogram [flame ionization detector (FID)] of trapped volatiles of ripe mango (top) and electroantennogram (EAG) of a female medfly antenna (bottom). EAG active compounds are labeled VI, VII, and VIII (see text for details).

presence of a 1% α-copaene contamination in the 100-ng dosage of β-caryophyllene would have elicited at most a 40% increase in the EAG response to that of β-caryophyllene alone (see Figure 5). The mean EAG responses of female and male medfly antennae for hexanol were 0.3 mV ($\pm$0.3 SD, $N = 9$) and 0.3 mV ($\pm$0.2 SD, $N = 11$), respectively.

DISCUSSION

The goal of this research was to provide some focus regarding the many compounds that have been identified from volatile emissions of calling male

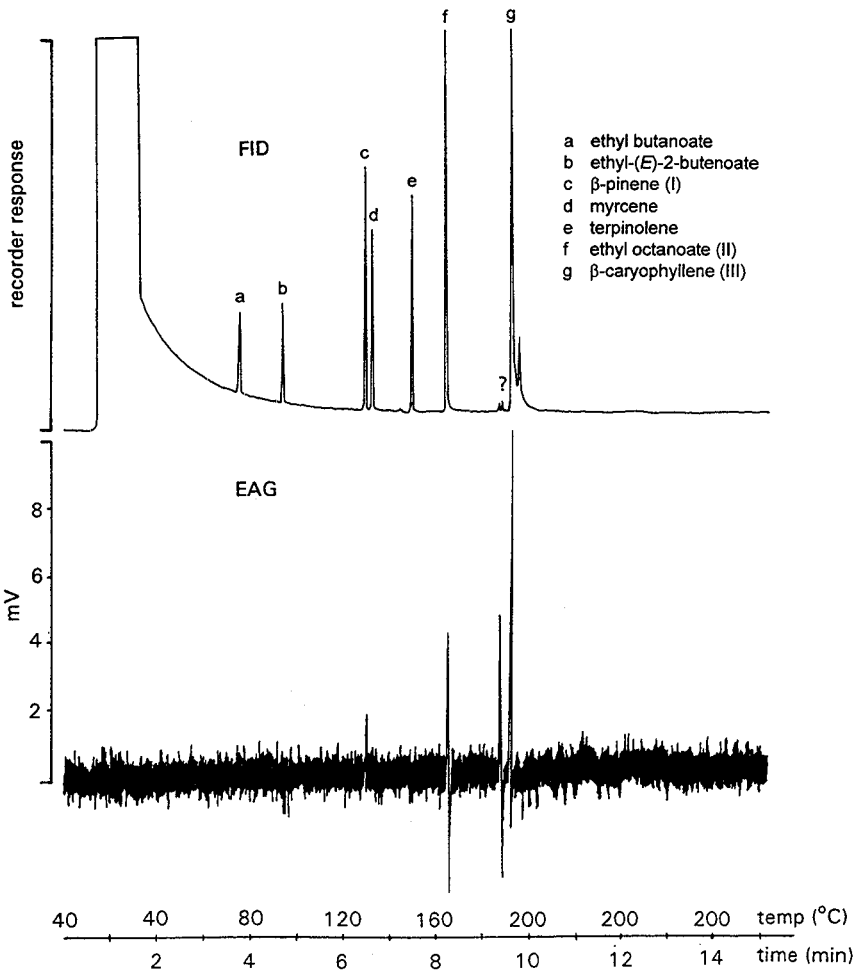

FIG. 4. Reconstructed simultaneously recorded gas chromatogram [flame ionization detector (FID)] of a mixture of commercial and synthetic compounds identified from ripe mango (top) and electroantennogram (EAG) of a female medfly antenna (bottom). EAG active compounds are labeled VI, VII, VIII, and ? (see text for details).

medflies and from host fruits. In particular, we wanted to see if some heretofore overlooked minor or trace compounds in these volatile emissions might stand out with regard to EAG amplitude and indicate that behavioral testing should be performed with those compounds added to the blend if such testing had not previously been done.

The coupled GC-EAG provides a real-time plot of the sequential responses

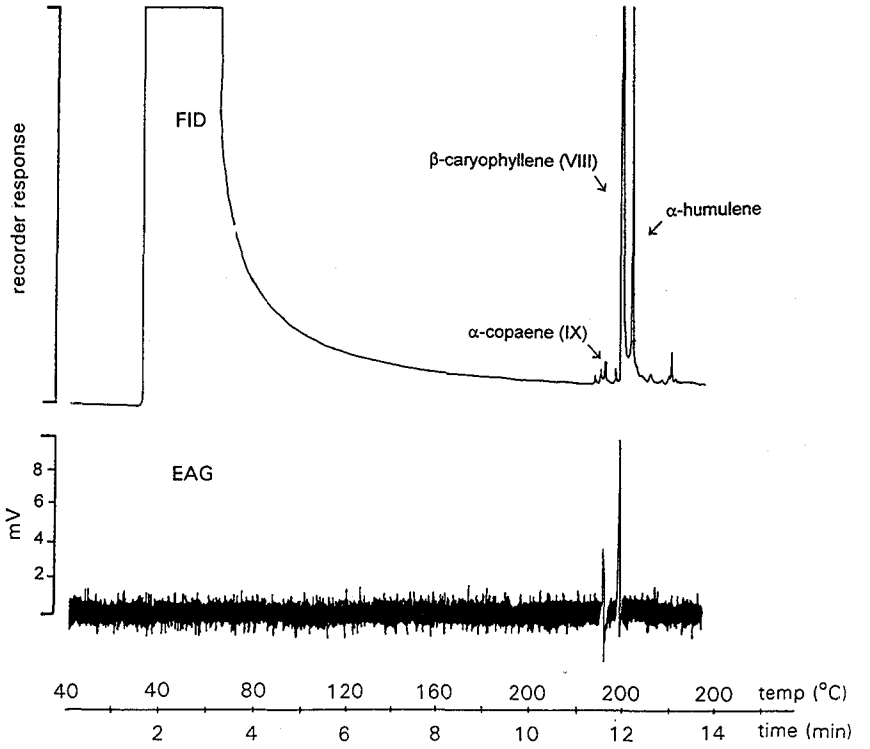

FIG. 5. Reconstructed simultaneously recorded gas chromatogram [flame ionization detector (FID)] of a mixture of α-copaene, β-caryophyllene, and α-humulene (top) and electroantennogram (EAG) of a female medfly antenna (bottom). EAG active compounds are labeled VIII and IX (see text for details).

of a single antenna to every compound in a natural extract. Thus, a single run provides comparative antennal response data for each compound and unequivocally highlights the compounds in the extract to which the antennae are most sensitive. Furthermore, possible problems of being misled by strong responses to trace contaminants in a synthetic sample that is puffed over the antenna are eliminated because the sample is separated into its component compounds, which are then passed over the antenna one at a time as they elute off the GC column. This technique requires that the preparations stay extremely stable over the course of the GC run in order to avoid spurious peaks from occurring and confounding the analysis. We were able to achieve this stability and sensitivity for our medfly preparations. This study, to the best of our knowledge, represents the first-ever medfly GC-EAGs.

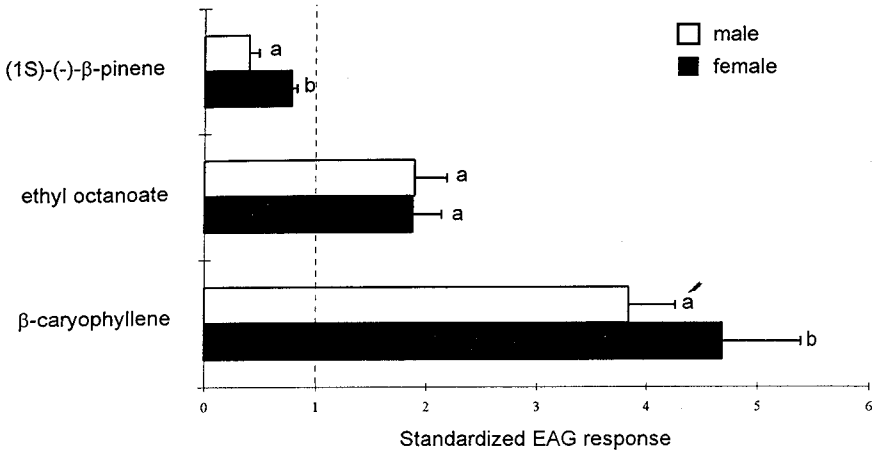

FIG 6. EAG responses of male and female medfly antennae to commercial or synthetic compounds (100 ng) identified from ripe mango. Standard deviations of mean responses are presented as error bars. Dotted line indicates the mean response to the hexanol standard (10 μg). Male and female response means from the same compound with the same letter are not significantly different by Student's t test at $P < 0.05$ ($N = 10$).

Our approach of combined GC-EAG analyses demonstrated that female medfly antennae selectively responded to only five of the many compounds that were present in the headspace of calling male medflies. We identified these compounds as ethyl-(*E*)-3-octenoate, geranyl acetate, (*E*,*E*)-α-farnesene, linalool, and indole by GC-MS analyses. All five compounds had been previously identified (Baker et al., 1985; Jang et al., 1989; Heath et al., 1991; Flath et al., 1993). Ethyl-(*E*)-3-octenoate, geranyl acetate, and (*E*,*E*)-α-farnesene represent three of the five most abundant male odor components (Jang et al., 1989; Heath et al., 1991; Flath et al., 1993), and linalool and indole have been previously quantified as "intermediate" and "trace" components, respectively, based on reconstructed gas chromatograms (Figure 1 and Jang et al., 1989; Flath et al., 1993). Jang et al. (1989), working with medflies from Hawaii, isolated and identified 56 compounds from the odor of calling males and, by using EAGs, ranked the response to linalool in the top half of all the compounds tested, whereas the response to indole was ranked as one of the two lowest responses recorded. In contrast, our EAG dose–response tests showed indole equaling or exceeding the responses elicited by either (*R*)-(−)-linalool or (*S*)-(+)-linalool. However, due to differences in methodology, no accurate comparison can be made between our results and those of Jang et al. (1989).

Several studies have reported on the field attractancy of female medfly to the identified compounds, individually or as mixtures, with the exception of

indole. Recently, Jang et al. (1994) reported on flight behavior of females in a wind tunnel to a synthetic blend of ethyl-(*E*)-3-octenoate, geranyl acetate, (*E*,*E*)-α-farnesene, ethyl acetate, and 1-pyrroline, as well as to the odor of calling males, and they found that the natural male pheromone was still the most active blend for female attraction. Indole and linalool were not included in these tests. Initial laboratory assay results showed a significant increase in attraction of a previously tested three-component sex pheromone blend when indole was added (R.R. Heath, personal communication).

Our results with mango volatiles have revealed potential new attractants. Of the previously reported 54 volatile compounds identified from mango (Bartley, 1988), we identified 16 compounds in the headspace of mango pieces. Only three compounds consistently evoked responses in combined GC-EAG analyses with female antennae. Our GC-MS analyses identified the compounds as β-pinene, ethyl octanoate, and β-caryophyllene. In addition, the results from our studies showed that the known attractant, α-copaene, a contaminant in our commercial sample of β-caryophyllene, was strongly EAG active, as might be expected from its behavioral activity (Flath et al., 1994a,b). β-Caryophyllene has been reported as a medfly attractant (Beroza and Green, 1963), but Warthen and McInnis (1989) were unable to reproduce these results and suggested that the β-caryophyllene samples tested were attractive because of the presence of α-copaene as an impurity. This conclusion possibly should be reexamined in light of our results showing highly selective GC-EAG responses to both α-copaene and β-caryophyllene by female antennae. The behavioral activity of ethyl octanoate either alone or as part of a blend has not been tested. Jang et al. (1989) did not find ethyl octanoate in medfly emissions, but they did test it as an analog of ethyl-(*E*)-3-octenoate and ranked it as giving an intermediate EAG response on both male and female medfly antennae. Ethyl octanoate does function as one component of a four-component host-fruit blend that attracts male and female Mexican fruit flies, *Anastrepha ludens* Loew (Robacker et al., 1992). Its structure is similar to that of the medfly sex phermone component, ethyl-(*E*)-3-octenoate.

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