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IDENTIFICATION, SYNTHESIS, AND FIELD EVALUATION OF THE SEX PHEROMONE OF THE CITRUS FRUIT BORER *Ecdytolopha aurantiana*

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Abstract— The sex pheromone of the citrus fruit borer *Ecdytolopha aurantiana* has been identified by gas chromatography coupled to an electroantennographic detector (GC-EAD). The electron impact mass spectral (EI-MS) fragmentation of the major EAD-active peak gave identifying features for a monounsaturated acetate. Further analyses by chemical ionization mass spectrometry (CI-MS), vapor-phase infrared spectroscopy (GC-IR), along with chemical derivatization (DMDS reaction), led to full characterization of the major component as (*E*)-8-dodecenyl acetate (*E*8–12 : Ac). The second constituent was identified as the related alcohol, (*E*)-8-dodecenol (*E*8–12 : OH). The two compounds were indistinguishable from the authentic synthetic standards in chemical and EAD analyses. Samples of the two compounds were obtained by a facile synthesis utilizing lithium chemistry. Field tests showed that captures in traps baited with a mixture of *E*8–12 : Ac and *E*8–12 : OH at 100 : 1 and 10 : 1 ratios were not significantly different from the catches in traps having two virgin females. Dosage tests showed better performance of traps baited with 1 mg than those with 0.1 mg of the pheromone blend, either in 100 : 1 or 10 : 1 ratio.

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Key Words—Lepidoptera, Tortricidae, *Ecdytolopha aurantiana*, sex pheromone, (*E*)-8-dodecenol, (*E*)-8-dodecenyl acetate, GC-EAD.

INTRODUCTION

The citrus fruit borer *Ecdytolopha aurantiana* Lima (Lepidoptera: Tortricidae) is an economically important pest in Latin America, in particular, in Brazil, where it causes crop losses to orange growers estimated at US$ 50 million/yr (Anonymous, 2000). Recent studies on the sexual behavior and field ecology of *E. aurantiana* on citrus trees suggest that a female-released sex pheromone may be useful for monitoring populations of the citrus fruit borer (Bento et al., 2001). A synthetic pheromone system would allow growers to minimize the number of insecticide sprays, which, without a reliable monitoring system, becomes an economically and environmentally costly way to prevent crop damage. This prompted us to undertake the identification, synthesis, and field evaluation of the citrus fruit borer pheromone.

METHODS AND MATERIALS

Analytical Procedures. Low-resolution electron impact mass spectrometry (EI-MS) was carried out with a HP 6890 gas chromatograph (GC; Agilent, formerly Hewlett-Packard, Palo Alto, California) linked to a mass selective detector (MSD 5973; Agilent). Chromatographic resolution was done on a HP-5MS column (30 m $\times$ 0.25 mm; 0.25 μm) that was operated at 70°C for 1 min, increased to 270°C at a rate of 10°C/min, and finally held at this temperature for 10 min. Chemical ionization (CI) was performed on a HP 5890 II Plus GC connected to a Mass Engine 5989B (Agilent). The reactant gas was 5% ammonia diluted in helium. Samples were separated on a HP-5MS column (30 m $\times$ 0.25 mm; 0.25 μm). The oven temperature was set at 60°C for 1 min, increased to 200°C at a rate of 5°C/min, increased again to 270°C at 10°C/min, and finally held at this temperature for 10 min. Vapor-phase Fourier transform infrared (FTIR) spectra were recorded with a HP 6890 GC coupled to a FTIR system equipped with a light pipe interface, FTS-40A, GC/C32 (Bio-Rad, Cambridge, Massachusetts). The GC was equipped with a HP-5 column (30 m $\times$ 0.32 mm; 0.25 μm) operated at 70°C for 1 min, increased to 250°C at a rate of 10°C/min, and finally held at this temperature for 10 min. The light pipe and the transfer line were set at 230°C and 250°C, respectively. In all GCs, helium was used as carrier gas at 1 ml/min, except for the GC-FTIR, for which the flow rate was 2 ml/min. GC with electroantennographic detection (EAD) was performed according to the method of Struble and Arn (1984). A HP 5890 II Plus GC was modified to have the effluent from the capillary column split into EAD and flame ionization detector (EAD/FID, 3 : 1).

E. aurantiana antennae were placed in the previously described acrylic stage (Leal et al., 1992). The stage was set inside the glass transfer line (2 cm away from the GC outlet) and connected with gold wires to an amplifier (gain 5) and filtered through a passive filter (cutoff frequency 0.12 Hz). The signal was fed into an A/D 35900E interface (Agilent). FID and EAD signals were acquired with a 3365 Series II Chemstation (Agilent). To obtain EAD responses for synthetic (*Z*)- and (*E*)-8-dodecenyl acetate (10 ng of each), six to eight samples of the two isomers were injected successively within 5–6 min in split mode.

Pheromone Extraction and Isolation. For extraction, hexane (Fisher, Pesticide Residue Grade) was distilled in an all-glass distillation apparatus. The last segments of the abdomen (segments VIII and IX) of 400 three-day-old virgin females were extracted with distilled hexane (4 ml). Extractions were done between 6:00 and 8:00 PM, coinciding with the mating time of *E. aurantiana*, as previously observed in a greenhouse (Bento et al., 2001). The crude extract was subjected to flash column chromatography on a Pasteur pipet filled with silica gel (Wakogel C-200; Wako, Tokyo, Japan) and eluted successively with solutions of ether in hexane (0, 1, 3, 5, 10, 15, 20, 50, and 100%). All fractions were concentrated to ca. 1 virgin female-equivalent per microliter.

Derivatization. An aliquot of the ether–hexane (5 : 95) fraction (50 μl) was concentrated in a 1-ml V-vial (Wheaton, Millville, New Jersey) by a slow stream of argon. The residual material was dissolved in distilled dimethyl disulfide (DMDS, 80 μl), and a small crystal of iodine (ca. 200 μg) was added. A magnetic spin vane was introduced, and the vial was sealed with a Teflon-lined cap. The reaction mixture was stirred at 60°C overnight. After cooling to room temperature, hexane (100 μl) was added and the reaction was quenched and washed with sodium thiosulfate (5%; 50 μl). The organic phase was dried over anhydrous sodium sulfate, concentrated to 50 μl, and analyzed by GC-MS.

Syntheses. For identification purposes, commercially available (*Z*)- and (*E*)-8-dodecenyl acetate (IPO-DLO, Wageningen, The Netherlands) were used. (*Z*)- and (*E*)-Dodecenol were obtained by alkaline hydrolysis (KOH/MeOH) of the corresponding acetates. For field experiments, samples of (*E*)-8-dodecenyl acetate **1** and (*E*)-8-dodecenol **2** were prepared utilizing lithium chemistry (Figure 1). A solution

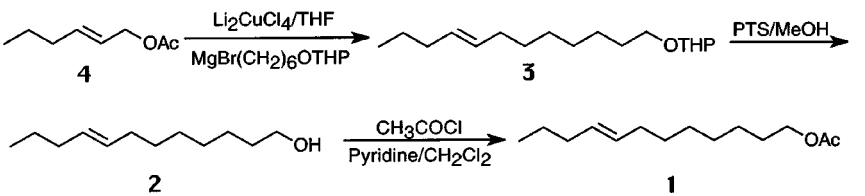

FIG. 1. Scheme for the preparation of (*E*)-8-dodecenol (**2**) and (*E*)-8-dodecenyl acetate (**1**) for the field evaluations.

of (*E*)-2-hexenyl acetate **4** (24 g, 90 mmol) and Li_2CuCl_4 (0.31 g, 1.44 mmol) in THF (14.4 ml) was added to 100 ml of a solution of $MgBr(CH_2)_6OTHP$ (26 g, 90 mmol) in THF at 10° (Figure 1). After stirring for 3 hr, the reaction mixture was poured into dilute HCl and extracted with ether. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated under vacuum to give **3**. The crude product was stirred with *p*-toluenesulfonic acid monohydrate (PTS; 0.86 g, 4.5 mmol) in methanol (MeOH; 100 ml). After stirring for 2 hr, K_2CO_3 (1.3 g, 9 mmol) was added and stirring was continued for 2 hr. The reaction mixture was filtered, concentrated, and distilled to give **2** (6.6 g, 36 mmol; bp 107°/1.5 mm Hg) in overall 40% yield. EI-MS data: 55(100%), 41(55), 42(10), 43(14), 53(10), 54(39), 56(15), 57(13), 66(8), 67(98), 68(55), 69(35), 70(8), 71(6), 79(9), 81(90), 82(84), 83(20), 95(57), 96(45), 97(9), 109(24), 110(18), 123(9), 124(6), 138(5), 166(8). IR ν_{max} (cm^{-1}): 2960 (s), 2925 (s), 2860 (s), 1740 (s), 1675 (w), 1240 (s), 965 (s).

To a solution of **2** (1.84 g, 10 mmol) and pyridine (1.19 g, 15 mmol) in CH_2Cl_2 (30 ml) at 10°, CH_3COCl (1.02g, 13 mmol) was added. After stirring for 2 hr, the reaction mixture was poured into water and extracted with ether. The ether layer was washed with brine, dried over anhydrous sodium sulfate, concentrated, and distilled to give **1** (2.21 g, 9.8 mmol; 98% yield; bp 104°/1.5 mm Hg). EI-MS data: 82(100%), 41(39), 43(63), 54(33), 55(70), 56(10), 61(8), 67(90), 68(45), 69(25), 81(87), 83(20), 95(58), 96(63), 97(10), 109(29), 110(26), 123(13), 124(9), 137(7), 138(6), 166(29). IR ν_{max} (cm^{-1}): 3640 (s), 2962 (s), 2926 (s), 2862 (s), 1675 (w), 1050 (s), 965 (s). The sample was devoid of the *Z*-isomer as determined by chemical derivatization with *m*-chloroperbenzoic acid (Attygale, 1998). Under the above GC conditions, the epoxide derived from *E*8–12 : Ac appeared at 13.95 min, and no trace peak was detected at 14.18 min, the retention time for the epoxide derived from *Z*8–12 : Ac.

Field Experiments. Tests were done in commercial citrus groves in São Paulo, Brazil. The ratio of the two components (1 mg) was tested at the Fazenda São João, in Altair, which has 13-year-old, 3.5-m-high trees (variety: Valencia) planted in a 5-×8-m fashion. On the other hand, two dosages (0.1 and 1 mg) and two ratios (10 : 1 and 100 : 1) of the pheromone components were tested at the Fazenda Entre Rios, in Gavião Peixoto, which has 5-year-old, 3-m-high trees (variety: Pera Rio) planted in a 7- ×3.5-m arrangement. The test compounds were incorporated in a Fuji Flavor slow-releasing device made of ES fiber (Chisso Co. Ltd, Tokyo, Japan) and covered with polyethylene film. The pheromone dispensers were loaded into Sticky Delta traps (Fuji Flavor Co. Ltd. Tokyo, Japan), which were suspended at 3 or 3.5 m. The treatments were replicated four times over three consecutive days in a completely randomized block design and with an intertrap distance of 40 m. All treatments, including 2-day-old virgin females, were renewed daily. To stabilize the variance, capture data were transformed to log $(x + 1)$, in particular, because of the occurrence of zero catches in the unbaited traps (Snedecor and Cochran,

1989). The figures were drawn with the original data (before transformation). The means of the transformed data were tested for significance by ANOVA with JMP Software, Version 2 (SAS Institute, Cary, North Carolina). Treatments followed by the same letters are not significantly different at the 5% level in the Tukey-Kramer honestly significant difference test.

RESULTS AND DISCUSSION

The response-guided strategy for the isolation of the citrus fruit borer sex pheromone, i.e., fractionating crude extract and monitoring the biological activity of each fraction with a bioassay, was unrewarding. Although observations of the typical behavioral responses of moth to pheromones, namely, wing-fanning, precopulatory behavior, and attraction, led us to the active fractions, activity was observed in multiple fractions for which GC-MS analyses showed many peaks, including homologous compounds. For example, the ether–hexane (5 : 95) fraction contained various monounsaturated acetates. It is unlikely these homologous compounds can be clearly separated by further purification by silica gel column so as to allow unambiguous identification of the pheromone components. On the other hand, gas chromatography–electroantennographic detection (GC-EAD), with the male *E. aurantiana* as the sensing element, allowed rapid identification of the active compounds. Analyses of the crude extract showed two EAD-active peaks, which appeared at 10.9 and 12.4 min (Figure 2). The results were consistent when GC-EAD was repeated either with the same or different antennae ($N = 16$). The

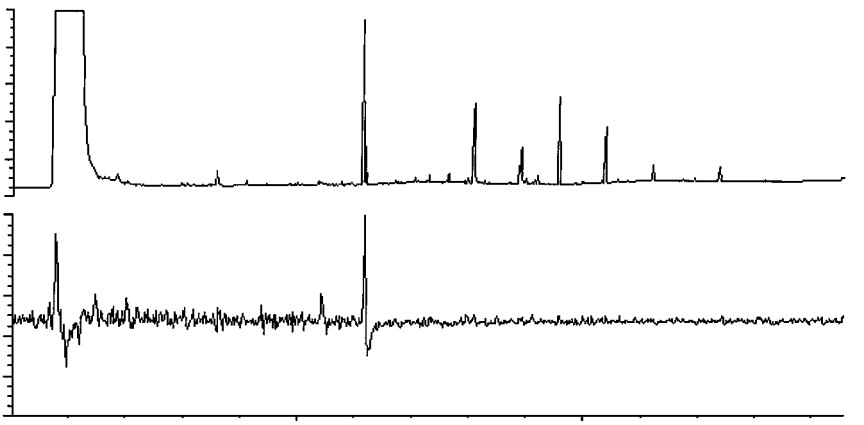

FIG. 2. Responses from flame ionization detector (FID, top) and electroantennographic detection (EAD, bottom; sensing element: male *E. aurantiana* antenna) to a hexane extract from abdominal tips of females (ca. 5 female-equivalents).

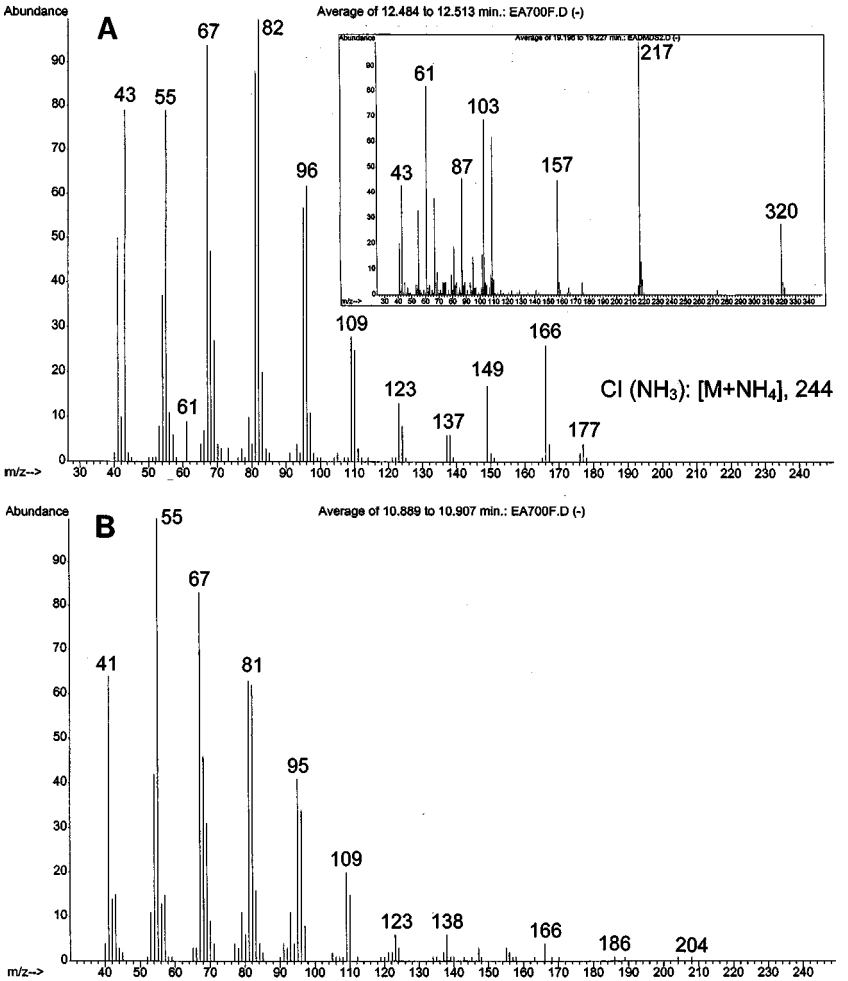

FIG. 3. Mass spectral data from the EAD-active peaks (Figure 2) at 12.4 min (A) and 10.9 min (B). Inset: MS of the major EAD-active peak after derizativation (DMDS adduct).

two EAD-active peaks were also detected in the fraction separated by silica gel column, the major compound being detected in the 3, 5, and 10% fractions and the minor compound in the 15 and 20% fractions. These are the biologically active fractions (above), but attraction was found only when two fractions (one of each group) were tested together.

The fragmentation pattern of the major EAD peak (Figure 3A) suggested that it was an acetate, as evidenced by the diagnostic peaks at *m/z* 61 from elimination

of the alkyl moiety and transfer of two hydrogen atoms to the fragment containing the oxygen atom. The quasimolecular ion (M^+-60) at *m/z* 166 indicated that the compound was a monounsaturated acetate with a molecular weight of 226. Chemical ionization mass spectrometry (CI-MS), with ammonia as the reactant gas, gave a base peak at *m/z* 244, which confirmed the molecular weight of 226. These findings suggested that the major EAD-active compound was dodecenyl acetate, in agreement with a library (Wiley) search, in which the best fit was 8-dodecenyl acetate. GC-MS analysis of the DMDS adducts showed the molecular weight at *m/z* 320 (Figure 3A, inset), an increment of *m/z* 94, suggesting the incorporation of two SCH_3 in one unsaturation in the parent compound. In addition, the base peak at *m/z* 217 and the peak at *m/z* 103 indicated that the unsaturation was located at position 8. Therefore, the compound was confirmed to be 8-dodecenyl acetate. The stereochemistry of the pheromone was determined by vapor-phase IR (Figure 4), which showed a typical *trans* band at 971 cm^{-1} (out of plane CH bending) and the lack of the =CH stretching (*cis* band) at ca. 3010 cm^{-1} (Leal, 1998). In conclusion, the major constituent was unambiguously identified as 8-(*E*)-dodecenyl acetate (**1**). In GC, GC-MS, GC-FTIR, and GC-EAD analyses, synthetic 8-(*E*)-dodecenyl acetate was indistinguishable from the natural product. Moreover, male antennae responded more strongly to the *E* than the *Z* isomer in EAD measurements (Figure 5), and the *Z* isomer was not detected in the gland extracts.

Although the FID peak of the EAD-active compound at 10.9 min (Figure 2) was very small, based on the structure of the major peak and the MS data of the minor component (Figure 3B), the second component was suggested to be the

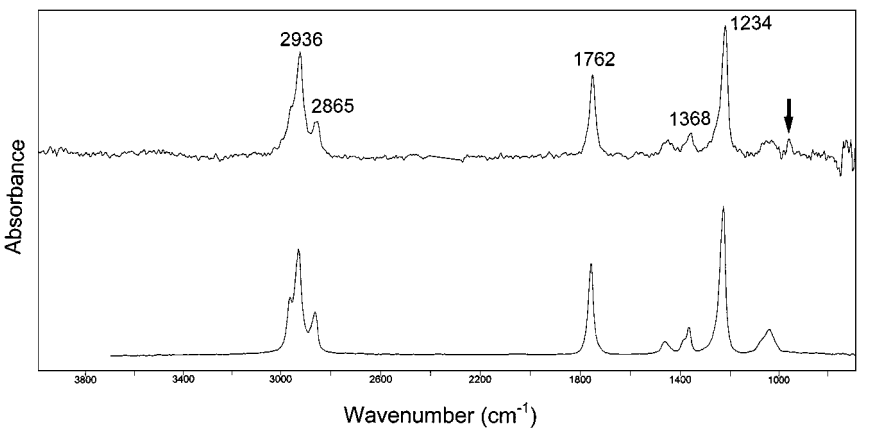

FIG. 4. Vapor-phase IR data generated with 10 female-equivalents of the major EAD-active peak (top). A library spectrum of a saturated homolog is given as reference (bottom). Arrow shows a characteristic *trans* band at 971 cm^{-1}.

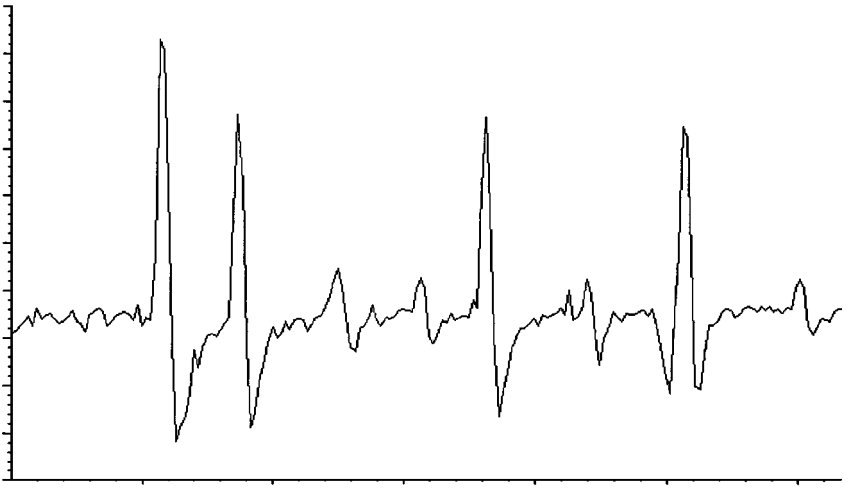

FIG. 5. Responses of a male *E. aurantiana* antenna to multiple injections of (*E*)- and (*Z*)-8-dodecenyl acetate introduced through a GC in split mode (50 : 1; ca. 0.2 ng/injection).

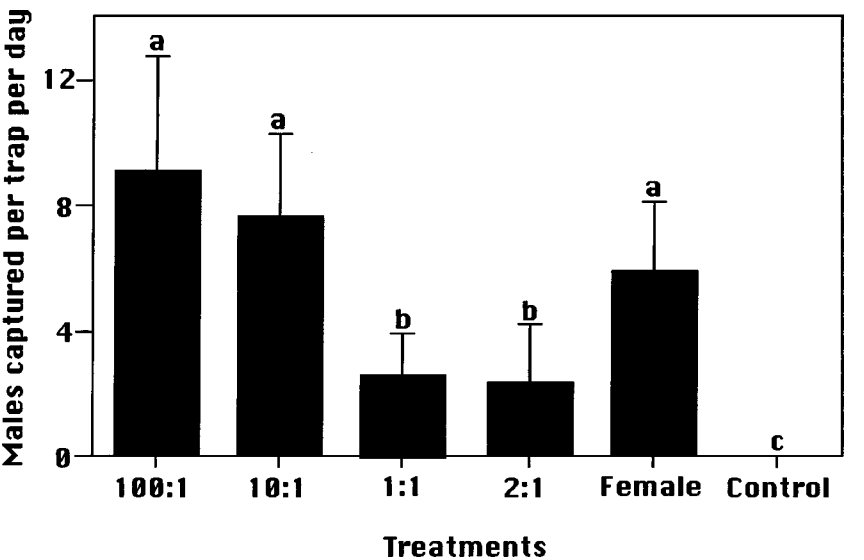

FIG. 6. Captures of *E. aurantiana* in sticky Delta traps baited with synthetic mixtures of the two pheromone components, (*E*)-8-dodecenyl acetate and (*E*)-8-dodecenol (1 mg of pheromone per trap).

related alcohol (**2**). A synthetic sample of the alcohol gave the same retention time as the minor peak, was EAD-active, and showed a similar MS profile. The MS of the synthetic compound devoid of impurities lacks some of the minor fragments, in particular, the ones at *m/z* 186, 189, 204, and 208, which are probably derived from an overlapping peak. Preliminary field experiments showed no significant catches of *E. aurantiana* in traps baited with only one of the two compounds, i.e., either (*E*)-8-dodecenol or (*E*)-8-dodecenyl acetate. However, captures in traps baited with the binary mixture were higher than control traps (Figure 6), regardless of the ratio of the two compounds. Captures in traps baited with high rates of *E*8–12 : Ac/*E*8–12 : OH (100 : 1 and 10 : 1) were not significantly different from the catches in traps baited with two virgin females. Performance of the traps with *E*8–12 : Ac/*E*8–12 : OH at 1 : 1 or 2 : 1 ratios were lower than the female traps, but better than the unbaited traps (control). Interestingly, there was no difference between the captures in traps having a 100 : 1 or 10 : 1 ratio of the two pheromone components. This is suggested to be due to the large number of treatments or because of the amount of pheromone (1 mg per device). In a separate set of experiments, the two ratios (100 : 1 and 10 : 1) were tested at different dosages. That traps baited with 1 mg of the pheromone blends captured more than those with

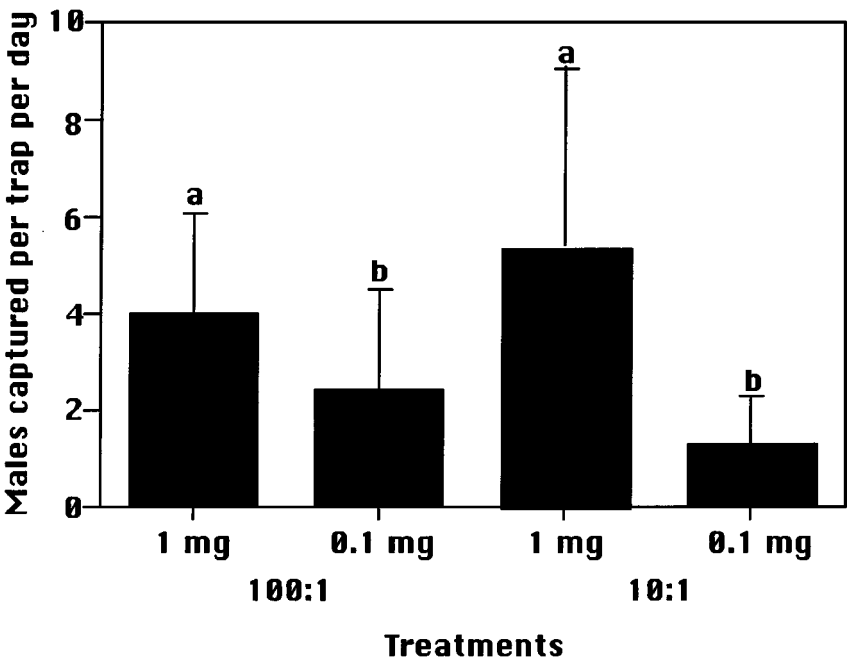

FIG. 7. Catches of *E. aurantiana* in sticky Delta traps with synthetic mixtures of the two pheromone components at two dosages and two different ratios.

0.1 mg (Figure 7) indicated that there was no saturation at the 1-mg level. In fact, the release rate of pheromone dispenser is very low as compared to the rubber septum. Furthermore, the catches in traps baited with 0.1 mg of the pheromone at the 100 : 1 and 10 : 1 ratios were not significantly different. It may be possible that the optimum ratio can be determined only in experiments under controlled conditions, such as wind tunnels. However, the optimal ratio for practical application in monitoring the insect population lies between 100 : 1 and 10 : 1 (*E*8–12 : Ac/*E*8–12 : OH). Although the scanty amount of the minor component prevented accurate analytical measurement of the ratio of the two compounds in the pheromone gland, it was closer to 100 : 1 than to 10 : 1.

Tortricid moths have been favorites of pheromone research. Sex pheromones have been characterized in over 60 species, and attractants of another 300 have been determined in field tests (Arn, 1991). The known female sex pheromones are all composed of primary aliphatic alcohols and their corresponding acetates and aldehydes. In general, they have 12 or 14 carbon atoms in the primary chain and one or two double bonds. (*E*)-8-Dodecenyl acetate and (*E*)-8-dodecenol have been identified as pheromone components of various moth species (Arn et al., 1998) and binary mixtures of **1** and **2** have been identified at least in two species, i.e., a sex pheromone from *Hedya chionosema* (Roelofs and Brown, 1982) and a sex attractant for *Strophedra nitidana* (Ando et al., 1977).

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