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MALE-PRODUCED AGGREGATION PHEROMONE OF THE AMERICAN PALM WEEVIL, *Rhynchophorus palmarum* (L.) (COLEOPTERA, CURCULIONIDAE): COLLECTION, IDENTIFICATION, ELECTROPHYSIOGICAL ACTIVITY, AND LABORATORY BIOASSAY[1]

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Abstract—Male American palm weevils (APWs), *Rhynchophorus palmarum* (L.) produced two sex-specific compounds, which were disclosed by volatile collections on Supelpak-2 and gas chromatography. One was a minor compound, not always detected. The major male-produced volatile was identified as (2*E*)-6-methyl-2-hepten-4-ol through coupled gas chromatography–mass spectrometry and gas chromatography–Fourier transform infrared spectrometry, proton nuclear magnetic resonance spectrometry, and rational synthesis. We propose the trivial name rhynchophorol for this new molecule, which proved to be the essential component of the APW aggregation pheromone by electroantennography, coupled gas chromatography–electroantennography and behavioral bioassays.

Key Words—Aggregation pheromone, Coleoptera, Curculionidae, *Rhynchophorus palmarum*, American palm weevil, (2*E*)-6-methyl-2-hepten-4-ol, synthesis.

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INTRODUCTION

The American palm weevil (APW), *Rhynchophorus palmarum* (L.), is a major pest of coconut and oil palm crops in tropical America and the West Indies (Lepesme et al., 1947; Genty et al., 1978). It is occasionally a pest on sugarcane (Restrepo et al., 1982). APW adults cause indirect damage to palm trees by vectoring the nematode responsible for red ring disease (Griffith, 1987). Direct damage is also caused by the larvae that develop in the stems, buds, and rachis of leaves and inflorescences of cultivated, ornamental, or wild palm trees (Lepesme et al., 1947; Wattanapongsiri, 1966). The only way to prevent red ring disease is to eliminate the adults and larvae of the APW since the difficulty and cost of nematicide treatments are extreme.

Despite its economic importance, few studies have been devoted to the chemical ecology of this weevil (Hagley, 1965). The APW is very large (35–50 mm long), hard to observe in the field, and difficult to manipulate in the laboratory. The endophytic development of the larvae (three to six months) in palm or sugarcane stems makes this species particularly difficult to rear in the laboratory, especially in temperate countries where natural food plants are not available.

Rochat et al. (1991) reported field and laboratory evidence documenting the existence of a male-produced aggregation pheromone in the APW. This species belongs to the second genus in the Rhynchophorinae for which pheromone-based communication has been reported. Pheromones in the Rhynchophorinae have been studied previously in the sole genus *Sitophilus* (Faustini et al., 1982; Phillips and Burkholder, 1981; Phillips et al., 1985, 1987, 1989; Walgenbach et al., 1983, 1987; Walgenbach and Burkholder, 1986).

This paper describes the collection, electrophysiological activity, and behavioral bioassays of the male-produced volatiles; and the identification, synthesis, and biological activity of the essential component of the APW aggregation pheromone.

METHODS AND MATERIALS

Insects and Volatile Collections. APWs were obtained from a laboratory colony reared on sugarcane. The colony originated from mixed strains from Colombia and Guadeloupe. Eclosed adults were separated by sex, kept individually ($27 \pm 2°C$ and $85 \pm 10\%$ relative humidity, in a 12:12 hr light–dark regime) in cylindrical plastic boxes (105×75 mm) with a wire mesh cover, and fed with sugarcane.

Five virgin APW males or females were placed in a cylindrical glass jar (6×22 cm) with three sugarcane pieces. Three pieces of the same size and from the same stem were placed in a similar jar as a control. The volatiles

emitted by APWs and/or sugarcane were simultaneously trapped in two glass cartridges connected downstream to the jars for two days (procedure 1) or one day (procedure 2). Each cartridge was filled with 0.5 g (procedure 1) or 0.2 g (procedure 2) of Supelpak-2 adsorbent (16/50 mesh; Supelco). The volatile collections were performed at 27 ± 2°C with a 12:12 hr light–dark regime. Air was passed at 500 ml/min through the glass systems. In procedure 1, the cartridges were eluted with 2.5 ml of methylene chloride (HPLC grade, Waters). The eluates were concentrated at 100 μl under nitrogen (150 ml/min) and used in pitfall bioassays. In procedure 2, the cartridges were eluted with 1 ml of methylene chloride and the extracts were used in electroantennography and for analytical investigations. Ten and 50 volatile collections were made using procedures 1 and 2, respectively. All extracts were stored at −30°C until use.

Analysis of Volatiles. Natural and synthetic volatiles were analyzed on a Carlo Erba Fractovap 2900 chromatograph equipped with 25-m × 0.32-mm-ID fused silica capillary columns coated with a polar phase (WCOT FFAP CB, Chrompack; temperature program: 35 to 55°C at 20°C/min, 8 min at 55°C and 55 to 220°C at 5°C/min) or a nonpolar phase (WCOT CPSil 8CB, Chrompack; temperature program: 35 to 60°C at 25°C/min, 5 min at 60°C, and 60 to 250°C at 5°C/min) with helium as carrier gas.

Gas chromatography–mass spectrometry (GC-MS) analyses were performed with a Girdel 32 chromatograph coupled to a R10-10C Nermag quadrupole mass spectrometer. The chromatograph was equipped with a fused silica capillary column (25 m × 0.32 mm ID; WCOT CPSil 5CB, Chrompack) and operated isothermally at 60°C. The electron impact (EI) was realized at 70 eV. The chemical ionization (CI) was obtained using ammonia as reactant gas at 92.5 eV.

Gas chromatography–Fourier transform infrared spectrometry (GC-FTIR) was carried out with a IFS-85 spectrometer coupled to a HRCG 5160 Carlo Erba chromatograph equipped with a fused silica capillary column (30 m × 0.32 mm ID; WCOT DB-5, J&W Scientific) heated from 30 to 170°C at 10°C/min. The light pipe was heated at 220°C.

Proton nuclear magnetic resonance spectrometry ([^{1}H]NMR) spectra of the natural major pheromone compound of APW males (R1) and synthetic compounds I–III (below) were recorded with a Brucker AC 400 spectrometer in C_6D_6 (99.93%, CEA, France). R1 (ca. 50 μg) was obtained by micropreparative capillary GC performed on a Girdel 300 chromatograph equipped with a 15-m × 0.53-mm-ID fused silica column (WCOT DB-5; J&W Scientific) and operated isothermally at 70°C. The splitting of crude extracts and the collections of R1 were performed with a Malosse (1990) fraction collector.

Chemical Synthesis. Racemic (2*E*)-6-methyl-2-hepten-4-ol (I), (2*E*)-5-methyl-2-hepten-4-ol (II), and (2*E*)-2-octen-4-ol (III) were synthesized by reacting crotonaldehyde at −10°C in THF with Grignard reagents of isobutyl

bromide, 2-bromobutane, and 1-bromobutane to give the corresponding alcohols I–III as confirmed by GC-MS and [^{1}H]NMR spectrometry (For proton number, see Figure 4).

Compound I showed EI ions at *m/z* (% relative abundance) 113 (2), 110 (3), 95 (8), 86 (8), 85 (6), 71 (100), 69 (8), 67 (11), 57 (10), 53 (8), 43 (20), 41 (21). I had [^{1}H]NMR δ (ppm) at 0.88 (d, 3H; H-8), 0.91 (d, 3H; H-7), 1.22 (ddd, 1H; H-5′) 1.44 (ddd, 1H; H-5), 1.5 (dd, 3H; H-1), 1.77 (m, 1H; H-6), 3.97 (m, 1H; H-4), 5.41 (m, 2H; H-2, H-3) with the following coupling constants (Hz): $J_{1-2} = 5$, $J_{1-3} = 0.8$, $J_{4-5} = J_{4-5'} = 8$, $J_{5-5'} = 13.5$, $J_{5-6} = J_{5'-6} = 6$, $J_{6-7} = J_{6-8} = 4.2$.

Compound II showed EI ions at *m/z* 110 (2), 100 (3), 95 (3), 81 (3), 71 (100), 69 (8), 57 (6), 53 (7), 43 (12), 41 (13). II had [^{1}H]NMR δ (ppm) at 0.89 (t, 3H; H-7), 0.95 (d, 3H; H-8), 1.25 (m, 2H; H-6), 1.55 (de, 3H; H-1), 3.75 (td, 1H; H-4), 5.45 (dd, 2H; H-2, H-3) with the following coupling constants (Hz): $J_{1-2} = 5.6$, $J_{1-3} = 2$, $J_{2-3} = 15$, $J_{3-4} = 5.4$, $J_{4-5} = 14.5$, $J_{4-OH} = 5.5$, $J_{5-8} = J_{6-7} = 6.8$.

Compound III showed EI ions at *m/z* 113 (2), 110 (3), 95 (2), 86 (12), 81 (8), 71 (100), 69 (9), 58 (8), 57 (7), 53 (9), 43 (14), 41 (17). III had [^{1}H]NMR δ (ppm) at 0.9 (t, 3H; H-8), 1.19–1.52 (m, 6H; H-5, H-6, H-7), 1.55 (de, 3H; H-1), 3.89 (m, 1H; H-4), 5.44 (dd, 2H; H-2, H-3) with the following coupling constants (Hz): $J_{1-2} = 5.9$, $J_{3-4} = 6.3$, $J_{4-5} = 6.4$, $J_{7-8} = 6.8$.

Compounds I–III were purified to >90% by distillation at 70°C and 10 mm Hg, as checked by GC-MS. I was further purified by HPLC using a Waters Associates model 510 chromatograph equipped with a 0.3 × 10-cm column (Silica normal phase, 8-μm particles; Chrompack). Compounds were detected with a differential refractometer R 401 (Waters Associates). The final purity of I was >99%. Racemic 6-methyl-4-heptanol (IV) was obtained by catalytic (PtO_2, methanol) hydrogenation of purified I.

Electroantennography. Electroantennograms (EAGs) were recorded from insects immobilized in a Plexiglas apparatus, using glass capillary electrodes filled with saline solution and connected to chloridized silver wires. The reference electrode was positioned at the base of the rostrum in a 0.5-mm-wide hole punched into the cuticle with an iron pin. The recording electrode was inserted into the antennal club (distal part and external area) on which the olfactory sensilla are located (Rochat, 1987). The electrical signal was fed into a 725-microprobe amplifier (WP Instruments inc.) and displayed on a Tektronix 5510 oscilloscope. Antennae of eight APWs of both sexes were subjected in randomized order to stimulation by air, solvent, six extracts containing APW and/or sugarcane volatiles, and eight synthetic chemicals (100 ng), including I tested also at 10 ng doses (Figure 2). All compounds except I–IV were commercial products of 99% purity. R1 was purified in an identical manner as I. One microliter of methylene chloride containing the natural or synthetic vola-

tiles was applied to a filter paper (17 × 17 mm) placed into a glass cartridge. Successive stimulations were separated by 2 min. EAG amplitudes were submitted to a two-way analysis of variance by stimulus (F1) and sex (F2). Interaction F1 vs. F2 would reveal sexual dimorphism in antennal responses to chemicals. Mean EAG amplitudes were compared using Newman-Keuls test ($\alpha = 0.01$).

Coupled gas chromatography–electroantennography (GC-EAD) was performed with a GIRA chromatograph modified according to Wadhams (1982). The chromatograph was equipped with a 25-m × 0.20-mm-ID fused silica column (WCOT HP-5, Hewlett Packard) heated from 60 to 220°C at 4°C/min. Helium was the carrier gas. The column effluents were split (50:50) between the FID and the antenna. A makeup flow of helium (20 ml/min) was added in the splitting device to accelerate the exit of the GC effluents towards the antenna. The effluents were driven from the chromatograph through a heated transfer line into a moistened airstream (1.4 l/min, 85% relative humidity, 26°C) focused onto the antennal preparation. The EAG signal was filtered (0.01–100 Hz bandpass) with an ERE amplifier (Ruy, France). GC and EAG signals were monitored on a two-channel chart recorder. Two APWs of both sexes were submitted, each one, to two extracts containing male (0.1 male-day-equivalent) + sugarcane volatiles.

Behavioral Bioassays. Insects were bioassayed using a two-choice pitfall olfactometer (Rochat et al., 1991) adapted from Pierce et al. (1981). The olfactometer consisted of a circular arena of 40 cm (diameter) × 14 cm (height) connected to two glass jars 9 cm (height) × 6.5 cm (diameter). According to preliminary investigations on the daily activity of the APW (Rochat, 1987), bioassays were run during the first half of the scotophase at 27 ± 2°C under red light at 40 lux. A single weevil was released at the center of the olfactometer arena and its position was recorded after 30 min. Weevils were classified as responding only if they fell into either the stimulus or the control jar or clung to the underside of the arena inside the jar.

Twenty to 32 weevils of both sexes were bioassayed in each of the following situations: (1) control treatment with two empty jars; (2) virgin male effluvia (0.1 male-day-equivalent) + sugarcane volatiles vs. sugarcane volatiles alone; and (3) 10 ng of (±)-I vs. solvent. One microliter of methylene chloride extract containing a natural or synthetic stimulus was applied to a filter paper (17 × 17 mm) placed into a glass jar just prior to the test. APW responses were quantified by the proportion of test weevils that responded after 30 min and the number of APWs choosing a stimulus jar. The first measure reflected the activity level of the population of test weevils (Walgenbach and Burkholder, 1986). The proportions of weevils that responded in situations 2 and 3 were compared with that obtained in absence of stimulus (situation 1) using the χ^2 test for two independent samples (Siegel, 1956). The numbers of weevils choosing a stim-

ulus were compared to the numbers of choices for the associated control jars using the nonparametric binomial test with the null hypothesis of an equal probability of choosing the control or the stimulus (Siegel, 1956).

RESULTS

Collection and Isolation of Male-Produced Pheromone. Comparisons of trapped volatiles from sugarcane, APW males + sugarcane, and APW females + sugarcane revealed the presence of two male-specific compounds: R1 and R2 (Figure 1). Females produced no detectible sex-specific volatiles. R1 was detected in 58 of 60 volatile collections analyzed by GC and was always the major of the two male-specific compounds. The mean release rate of R1 by APW males in 40 volatile collections was estimated by GC, using synthetic I as a standard, to be ca. 40 ng/male/hr. This rate varied between collections from ca. 0.5 to 140 ng/male/hr. R2 was detected in 48 of 60 extracts. The R1 : R2 ratio (based on GC peak areas) ranged most frequently between 100 : 15 and 100 : 5.

EAG Activity of Male-Produced Pheromone. Extracts containing male-produced + sugarcane volatiles gave very strong EAGs in both APW males and females (Figure 2). Extracts containing volatiles from sugarcane alone or from APW females + sugarcane induced EAGs as weak as those to air and solvent. Isolated R1 (40 ng) and a total extract containing R1 (15 ng) and R2 (R1 : R2 ratio of 100 : 18) induced EAGs of almost equal amplitudes.

All GC-EAD recordings from both male and female APWs showed high EAG responses to R1 (Figure 3). No EAGs were recorded in response to the sugarcane volatiles or to R2.

Pheromone Identification. The highest fragment ions observed in the EI mass spectrum of R1 were *m/z* 113 and *m/z* 110 (Figure 4). We assumed this compound to be an oxygenated aliphatic compound of mol wt 128. This assumption was confirmed by the CI − NH_3 mass spectrum, which showed ions at *m/z* (% relative abundance): 146 ($M + NH_4^+$, 2), 129 ($M + H^+$, 9), 28 ($M^{+\cdot}$, 100), 111 ($M + H - H_2O^+$, 7), and 71 (1). Thus the *m/z* 110 ion was due to the loss of water and the *m/z* 113 ion to the loss of a methyl group from the molecule ion of a compound with $C_8H_{16}O$ formula. Absorption at 3649 cm^{-1} in the FTIR spectrum of R1 assigned the oxygen function to an hydroxyl group, and absorption at 966 cm^{-1} indicated a *trans* C—C double bond. Catalytic (PtO_2, methanol) microhydrogenation of R1 saturated the C–C double bond according to the CI mass spectrum, which showed ions at *m/z*: 148 ($M + NH_4^+$, 100), 130 ($M^{+\cdot}$, 5), 112 ($M - H_2O^+$, 11) and 87 (81). The couples of 18 amu-distant ions at *m/z* 73/55 and *m/z* 87/69 in the EI mass spectrum of the hydrogenated R1 (i) located the hydroxyl group in a C-4 position. (i), a couple

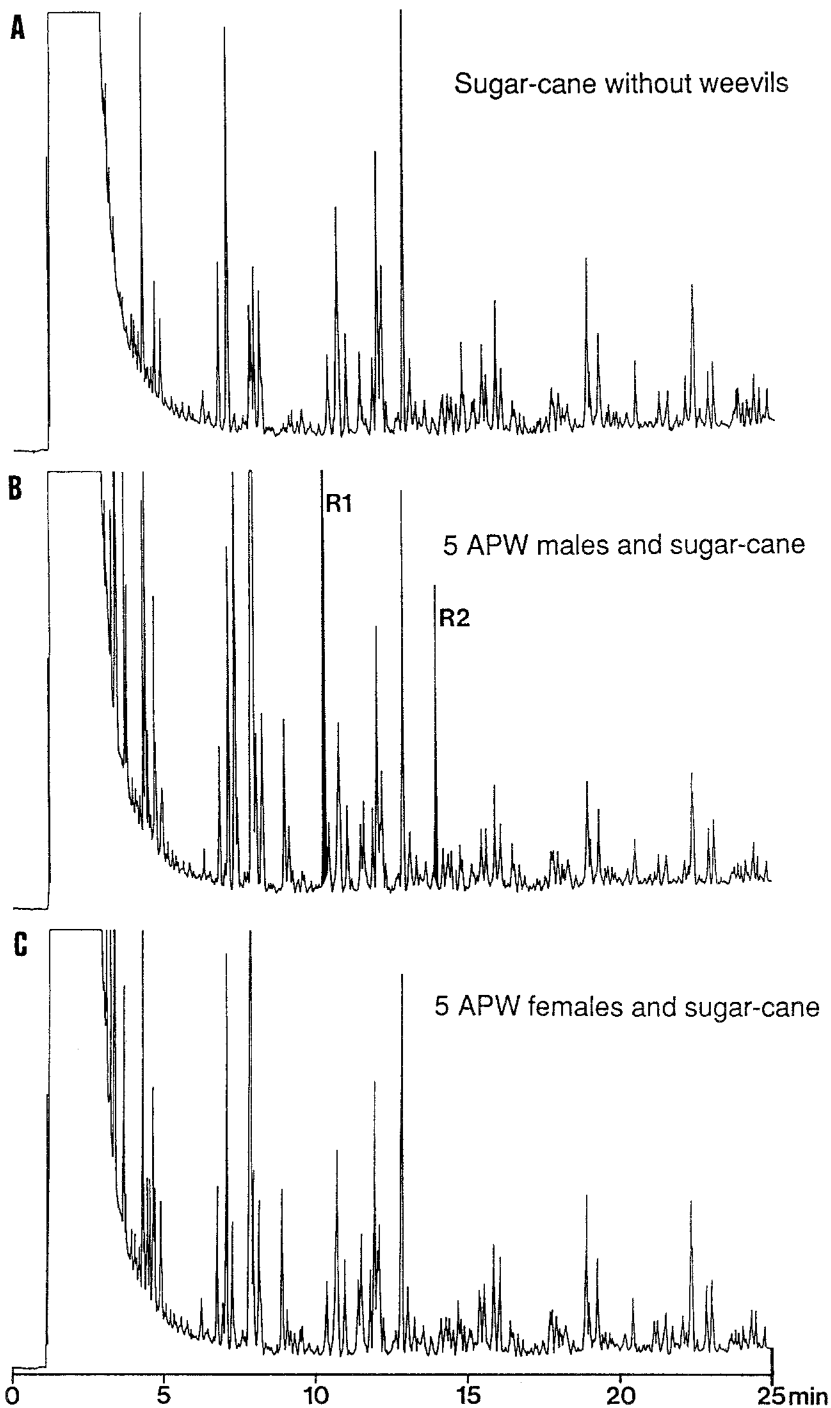

FIG. 1. Gas chromatograms of Supelpak-2-trapped volatiles from sugarcane (A), APW males and sugarcane (B), and APW females and sugarcane (C) showing two male-specific compounds: R1 and R2.

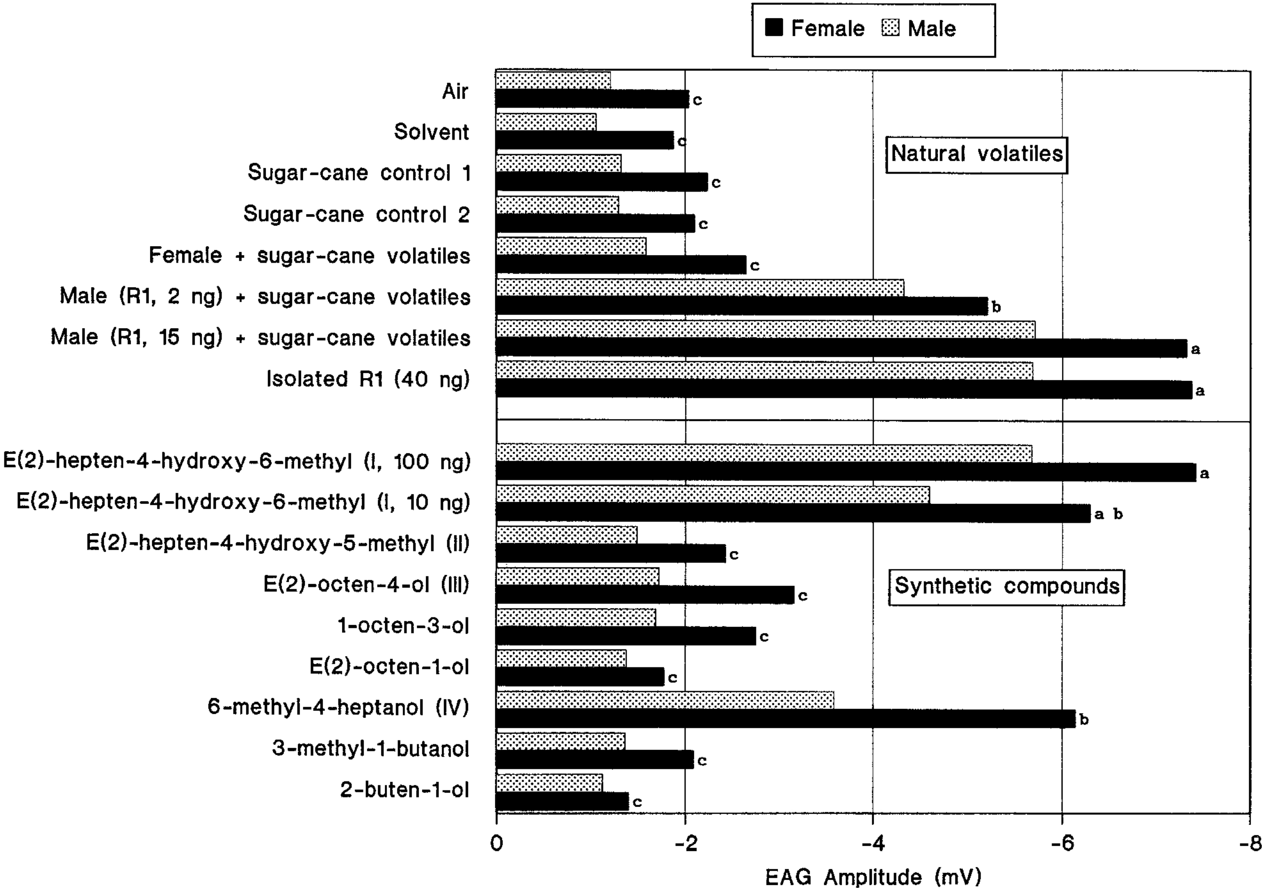

FIG. 2. Mean EAG responses from APW males and females ($N = 8$) to natural volatiles from APW and/or sugarcane and to synthetic pheromone-related compounds. Stimuli associated with the same letter do not differ significantly (Newman-Keuls test, $P < 0.01$) in terms of mean EAG amplitudes calculated on combined male and female responses.

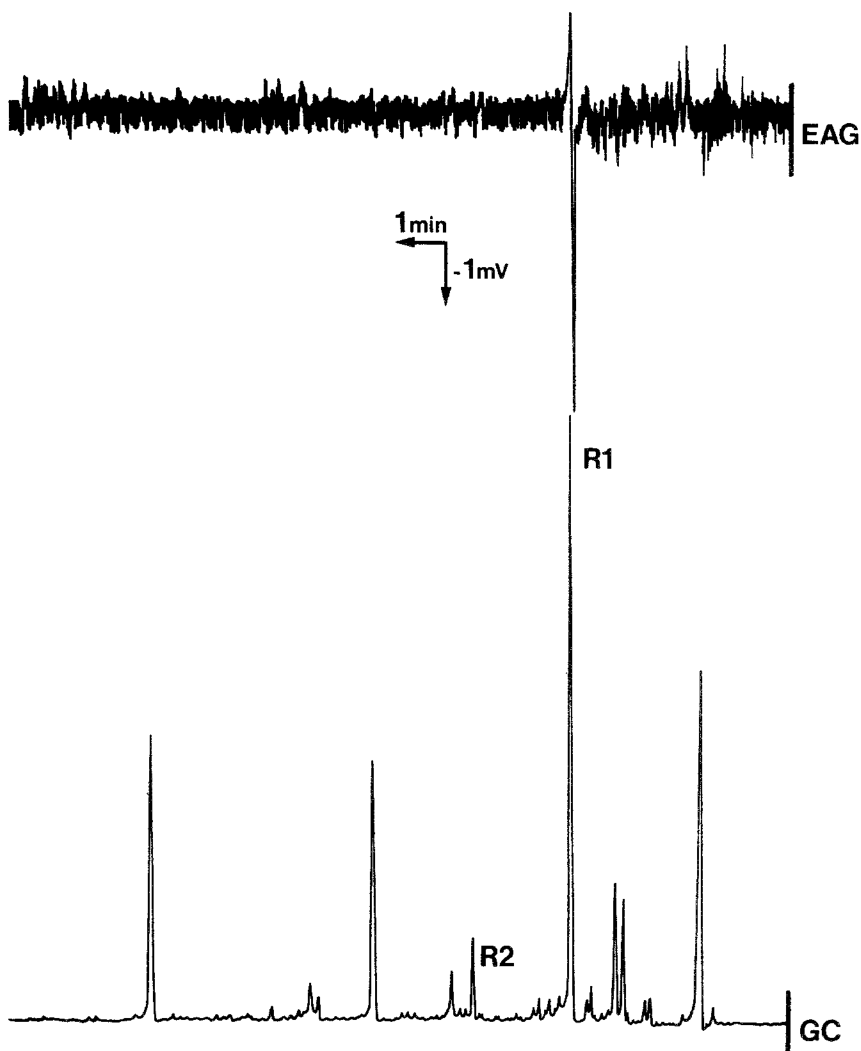

FIG. 3. GC-EAD recording from a female APW submitted to Supelpak-2-trapped volatiles from five APW males and sugarcane.

of 18 amu-distant ions at *m/z* 71/53 in the EI mass spectrum of R1 (ii) and GC-FTIR informations (iii) assigned the *trans* C–C double bond to be in C-2 position. The C-4 position of the hydroxyl group is in accordance with the presence of an even ion at *m/z* 86 resulting from a McLafferty rearrangement. Organization of the terminal four carbons of the chain to achieve complete structural identification of R1 could lead to three molecules: (2*E*)-6-methyl-2-hepten-4-

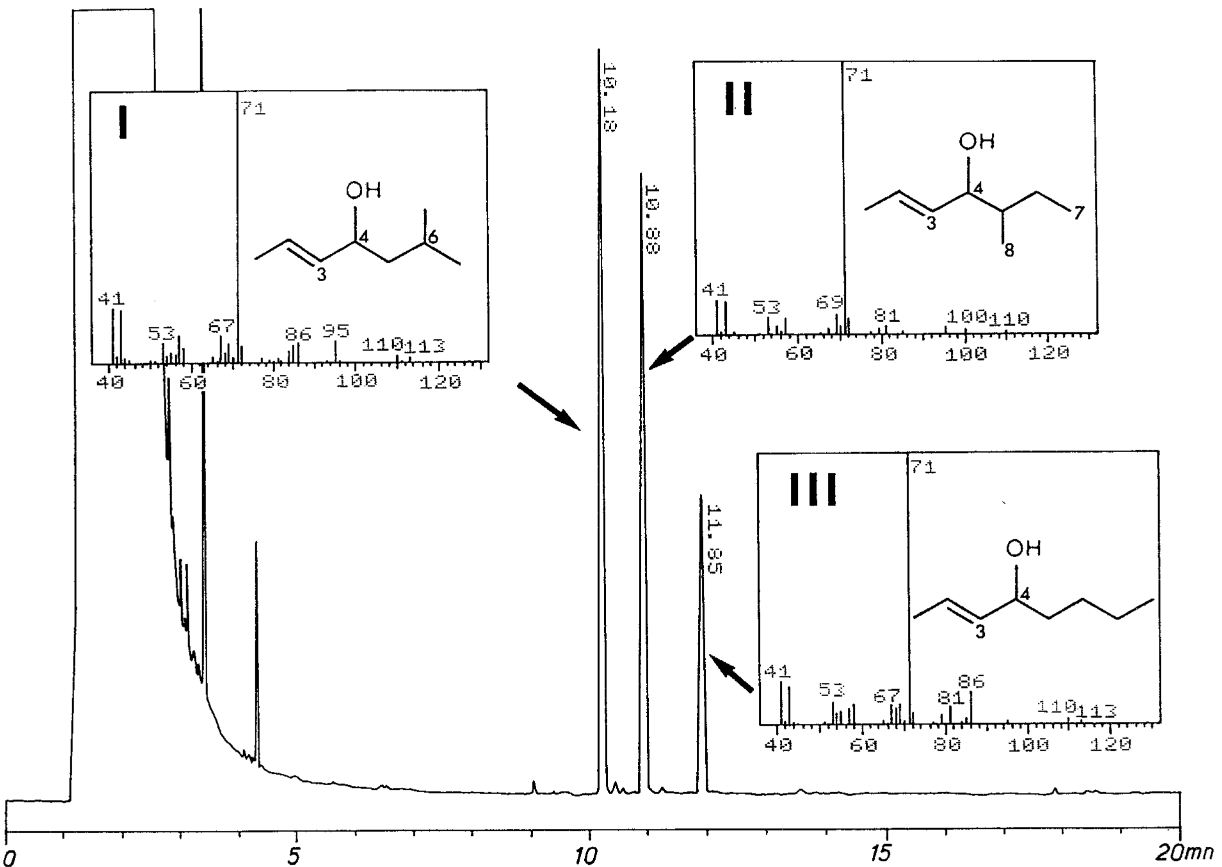

FIG. 4. Gas chromatogram (apolar column) and EI mass spectra of synthetic (2*E*)-5-methyl-2-hepten-4-ol (I), (2*E*)-5-methyl-2-hepten-4-ol (II), and (2*E*)-octen-4-ol (III).

ol (I), (2*E*)-5-methyl-2-hepten-4-ol (II), and (2*E*)-octen-4-ol (III). McLafferty rearrangement with II gave an even fragment ion at *m/z* 100 but not at *m/z* 86 as did R1, I and III. Comparisons of mass spectra; GC retention times of I, II, and III (Figure 4); and coinjection with R1, both on polar and apolar columns, led us to assign R1 to be (2*E*)-6-methyl-2-hepten-4-ol. The [^{1}H]NMR spectrum of R1 confirmed the identification of (2*E*)-6-methyl-2-hepten-4-ol. R1 shifts (ppm) were: 0.88 (d, 3H; H-8), 0.91 (d, 3H; H-7), 1.22 (ddd, 1H; H-5'), 1.44 (ddd, 1H; H-5), 1.5 (dd, 3H; H-1), 1.77 (m, 1H; H-6), 3.97 (m, 1H; H-4), 5.41 (m, 2H; H-2, H-3) with the following coupling constants (Hz): $J_{1\text{-}2} = 5$, $J_{1\text{-}3} = 0.8$, $J_{4\text{-}5} = J_{4\text{-}5'} = 8$, $J_{5\text{-}5'} = 13.5$, $J_{5\text{-}6} = J_{5'\text{-}6} = 6$, $J_{6\text{-}7} = J_{6\text{-}8} = 4.2$.

EAG Activity of Synthetic Pheromone and Analogs. Although the EAGs recorded from females were significantly greater in amplitude than those recorded from males ($F = 85.8$, $P < 0.001$), the EAG profiles disclosed no interaction stimulus by sex ($F = 1.27$, $P > 0.20$). Thus, there was no sexual dimorphism in the antennal responsness to the eight synthetic compounds tested. EAGs in response to I (10 and 100 ng) were significantly higher than to any octenol, including II and III (Figure 2) which in turn were not different from those to air and solvent. The mean EAG amplitudes obtained with 40 ng of isolated R1 and 100 ng of (±)-I were not statistically different. The mean EAG response to IV, a saturated analog of I, was significantly greater than to air and solvent and to any of the tested chemicals but I. However, IV had to be used at 10-fold higher doses than I to give EAGs of the same amplitude.

Behavioral Bioassay. In the two-choice pitfall olfactometer, (±)-I and volatiles from APW males induced similarly high numbers of weevils of both sexes to respond. Both males and females preferred significantly (±)-I to the solvent control (Table 1). Equivalent responses to (±)-I were obtained with wild APWs from Colombia and Guadeloupe (unreported data).

DISCUSSION

Rochat et al. (1991) showed that APW males produce a volatile aggregation pheromone. The strong EAGs in response to the male volatiles by APWs of both sexes corroborated the behavioral effect of the pheromone and showed that the response was due to a single major compound. The collection of variable amounts of this compound from male weevils may be due partly to the heterogeneity in age of the experimental weevils. Indeed, the age is a major factor influencing the production of aggregation pheromones in two *Oryzaephilus* species (Pierce et al., 1989).

Identification of (2*E*)-6-methyl-2-hepten-4-ol (I) as the essential pheromone compound was confirmed by both electrophysiological and behavioral data. We propose the trivial name rhynchophorol for this new pheromone.

TABLE 1. APW RESPONSES TO NATURAL AND SYNTHETIC MALE AGGREGATION PHEROMONE (RHYNCHOPHOROL) IN TWO-CHOICE PITFALL OLFACTOMETER AFTER 30 MIN

Treatment	Sex tested	Number tested	Number of responses[a]	Percent response		Binomial probability stimulus vs control[b]
				Stimulus	Control	
2 empty jars	Male	30	8	50	50	0.637
(control)	Female	20	2	50	50	—
Virgin male + food	Male	31	21**	71	29	0.021
volatiles vs. food volatiles	Female	21	14***	93	7	0.001
10 ng of (±)-I[c] vs	Male	32	20**	85	15	0.001
solvent	Female	31	20***	80	20	0.006

[a] Difference from the control (χ^2 test) indicated by:
n.s.: not significant, ** $P < 0.01$ and *** $P < 0.001$.
[b] Under the null hypothesis to respond to the stimulus and to the control with an equal probability of 0.5.
[c] (±)-I: racemic (2*E*)-6-methyl-2-hepten-4-ol (rhynchophorol).

(±)-Rhynchophorol was a potent lure for the APW in laboratory conditions without food volatiles or the minor male-specific compound. (±)-Rhynchophorol at 10-ng doses did not exhibit any inhibitory effect, and there was no evidence for a differential peripheral reception of (±)-rhynchophorol and natural rhynchophorol. Nevertheless, further investigations are underway to determine the enantiomeric composition of natural rhynchophorol since chirality in beetle communication is of major consequence (Mori, 1984; Borden, 1985; Walgenbach et al., 1987; Phillips et al., 1989).

Rhynchophorol is a new eight-carbon aliphatic and oxygenated pheromone. Such pheromones have been found in three coleopteran families: Cerambycidae (2,3-octanediol and 2-hydroxy-3-octanone; Sakai et al., 1984), Curculionidae (sitophilure, 5-hydroxy-4-methyl-3-heptanone; Phillips et al., 1985; 5-methyl-4,6-heptanedione; Blight et al., 1984) and Scolytidae (ipsenol, 2-methyl-6-methylene-7-octen-4-ol, and ipsdienol, 2-methyl-6-methylene-2,7-octadien-4-ol; Silverstein et al., 1966; sulcatol, 6-methyl-5-hepten-2-ol; Byrne et al., 1974; 4-methyl-3-heptanol; Pearce et al., 1975).

Although the genera *Rhynchophorus* and *Sitophilus* are very close, there is no obvious structural relationship between rhynchophorol and sitophilure. Because of its terpenoidic skeleton, rhynchophorol appears to be closely related to the scolytid pheromones ipsenol, ipsdienol, and sulcatol. This similarity suggests biogenesis of rhynchophorol from host-plant terpenes, as has been dem-

onstrated or assumed for many scolytid pheromones (Vanderwel and Oehlschager, 1987).

Field-trapping data (Rochat et al., 1991) and recent laboratory results (Rochat et al., unpublished data) support the hypothesis that APW males produce rhynchophorol only when they feed on the host plant. The biological significance of the male APW pheromone would thus be a signal to aggregate on a suitable food resource, as occurs in many other beetles (Walgenbach and Burkholder, 1986; Borden, 1985; Renwick, 1988).

The identification of the APW aggregation pheromone is a considerable step towards improving traditional methods used to control this major pest. APW adults have been caught in poisoned traps baited with palm stem pieces for many decades (Morin et al., 1988) without any attention paid to the role that the APWs themselves could play in attracting conspecifics. The use of synthetic rhynchophorol may lead, either alone or with host-plant kairomones, to a great improvement in the efficiency of APW trapping for survey and control of this major pest.

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