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SEX PHEROMONE OF TOMATO FRUIT BORER, *Neoleucinodes elegantalis*

AIVLÉ CABRERA,[1,2,*] ALVARO E. EIRAS,[3,5] GERHARD GRIES,[4] REGINE GRIES,[4] NEUDO URDANETA,[2] BEATRIZ MIRÁS,[1] CESAR BADJI,[5] and KLAUS JAFFE[1]

[1]*Laboratòriò de Comportamiento*

[2]*Departamento de Química*
Universidad Simón Bolívar
Apartado 89000, Caracas 1080-A, Venezuela

[3]*Departamento de Parasitologia, ICB*
Universidade Federal de Minas Gerais (UFMG), Belo Horizonte
CEP 31270-901, MG, Brazil

[4]*Centre for Environmental Biology*
Department of Biological Sciences
Simon Fraser University
Burnaby, British Columbia V5A 1S6, Canada

[5]*Laboratório de Proteção de Plantas, CCTA*
Universidade Estadual do Norte Fluminense
CEP 28045-640, Campos, RJ, Brazil

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Abstract—Five candidate pheromone components were identified by analyzing pheromone gland extracts by gas chromatography (GC), coupled GC-electroantennographic detection (EAD), and coupled GC-mass spectrometry (MS): (*E*)-11-hexadecenol(*E*11–16 : OH), (*Z*)-11-hexadecenol (*Z*11–16 : OH), (*E*)-11-hexadecenal, (*E*)-11-hexadecenyl acetate, and (*Z*)-3,(*Z*)-6,(*Z*)-9-tricosatriene (*Z*3,*Z*6,*Z*9–23 : Hy). In electroantennogram (EAG) recordings, synthetic *E*11–16 : OH elicited stronger antennal responses at low doses than other candidate pheromone components. Field tests demonstrated that synthetic *E*11–16 : OH as a trap bait was effective in attracting males, whereas addition of *Z*11–16 : OH inhibited the males' response. *Z*3,*Z*6,*Z*9–23 : Hy strongly enhanced attractiveness of *E*11–16 : OH, but was not attractive by itself. A pheromone blend with synergistic behavioral activity of an alcohol (*E*11–16 : OH) and hydrocarbon (*Z*3,*Z*6,*Z*9–23 : Hy) component is most unusual in the Lepidoptera. The synthetic two-component pheromone is approximately 60 times more attractive than the female-produced blend and might facilitate the control of this pest.

*To whom correspondence should be addressed; e-mail: acabrera@usb.ve

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Key Words—*Neoleucinodes elegantalis*, Lepidoptera, Crambidae, sex pheromone, (*E*)-11-hexadecenol, (*Z*)-3,(*Z*)-6,(*Z*)-9-tricosatriene, (*Z*)-11-hexadecenol, GC-EAD, tomato.

INTRODUCTION

The tomato fruit borer, *Neoleucinodes elegantalis* (Lepidoptera: Crambidae), is one of the most important economic pests of tomato (*Lycopersicum esculentum*) crops in tropical South America. Current control tactics include frequent applications of pesticides (Reis and Souza, 1996) that may lead to the development of insecticide resistance. Moreover, once larvae have entered the fruit, pesticides and biological control agents are ineffective. Pheromone-based mating disruption represents an alternative tactic to manage this pest insect but requires identification of the female-produced sex pheromone. We know that virgin females attract males in the field (Mirás et al., 1997), and extracts of abdominal tips with pheromone glands from virgin females attract males in wind tunnel bioassays (Eiras, 2000). Here we report identification, electrophysiological studies, and field experiments of sex pheromone components of *N. elegantalis.*

METHODS AND MATERIALS

Insects. Tomato fruits infested with *N. elegantalis* larvae were collected in commercial tomato crops in the states of Lara and Aragua, Venezuela, and in Rio de Janeiro, Brazil. Fruits were kept in plastic containers until larvae emerged from the fruit to pupate. Adult insects were maintained according to Eiras (2000). During peak calling activity, abdominal tips with pheromone glands of 2-to 3-day-old virgin female moths were removed and extracted for 10–15 min in hexane. The supernatant was withdrawn and stored in microcapillaries at -15°C.

Chemical Analyses. Pheromone extracts were subjected to gas chromatographic (GC) and coupled GC-electroantennographic detection (EAD) analyses, using a Hewlett Packard 5890A gas chromatograph equipped with fused silica columns (30 m $\times$ 0.25 or 0.32 mm ID) coated with DB-5, DB-210, or DB-23 (J&W Scientific, Folsom, California). Forty female equivalents of pheromone gland extract were also analyzed by coupled GC-mass spectrometry (MS), employing a Perkin Elmer QMass-910 attached to a GC-Autosystem 2000, equipped with a DB-5 column (25 m $\times$ 0.18 mm ID, Quadrex, New Haven, Connecticut).

The position and geometry of a double bond in an EAD-active hexadecenol were determined by: (1) GC-EAD analyses of synthetic hexadecenols (*E*2 to *E*14 and *Z*2 to *Z*14); (2) derivatization of extract with *m*-chloroperbenzoic acid (Bierl-Leonhardt et al., 1980), followed by GC-MS analysis of the resulting epoxide; and (3) acetylation of pheromone extract with acetic anhydride and

pyridine, followed by GC analyses [HP-GC equipped with a SP-1000-coated column (30 m × 0.25 mm ID; Supelco, Bellefonte, Pennsylvania)] with improved chromatographic resolution of the acetylated alcohol and synthetic acetate standards.

Effluvia of calling, 1- or 2-day-old female *N. elegantalis* were adsorbed on a solid-phase microextraction (SPME) fiber coated with 100 μm polidimethylsiloxane (Supelco). The fiber was exposed to a female during the entire calling period and then desorbed at the capillary injection port (270°C) of the GC equipped with columns (30 m × 0.25 mm ID or 25 m × 0.18 mm ID; Quadrex, New Haven, Connecticut) coated with methyl–5% phenyl silicone or Carbowax 20 M. Retention characteristics of female-produced, fiber-desorbed compounds were compared with those of authentic standards.

Electrophysiology. Electroantennogram (EAG) recordings were carried out, using a Syntech EAG system (NL-1200 BM Hilversum; The Netherlands). Five compounds (*E*11–16 : OH, *Z*11–16 : OH, *E*11–16 : OAc, *E*11–16 : Ald, and *Z*3,*Z*6,*Z*9–23 : Hy) at increasing logarithmic doses (10 to 10^6 ng) were tested with each of five 48- to 72-hr-old male antennae. An antenna was stimulated by subjecting it to puffs (0.5 sec) of purified and humidified air (1.5 liters/min) delivered through a Pasteur pipet (15 cm long), containing a filter paper strip (1 × 5 cm) impregnated with 50 μl of a test solution. Air puffs from an empty pipet, pipet plus filter paper, or filter paper plus solvent served as control stimuli.

Syntheses. (*Z*)-9,(*Z*)-12,(*Z*)-15-octadecatrien-1-ol (99%) was synthesized from methyl linolenate after reduction with $LiAlH_4$. This alcohol was oxidized with pyridinium chlorochromate (PCC) to afford (*Z*)-9,(*Z*)-12,(*Z*)-15-octadecatrienal (98%). *n*-Pentyl magnesium bromide was added to a solution of this aldehyde in anhydrous ether to obtain 6-hydroxy-(*Z*)-14,(*Z*)-17,(*Z*)-20-tricosatriene. A solution of this compound in anhydrous pyridine was treated with methane sulfonyl chloride (Kocovský and Cerný, 1978). The obtained mesylate was reduced with zinc dust and sodium iodide (Kocovský and Cerný, 1978) to obtain (*Z*)-3,(*Z*)-6,(*Z*)-9-tricosatriene, which was purified (>99%) by flash silica column chromatography using *n*-hexane as eluent. ^{1}H NMR (ppm; δ): 5.35 (m), 2.80 (t), 2.05 (m), 1.25 (m), 0.95 (t) 0.85 (t); EI-MS [*m*/*z* (relative intensity)]: 79 (100); 108 (98); 67 (49); 93 (48); 262 (6); M^+ 318 (2).

Sources of Candidate Pheromone Components. (*E*)-11-Hexadecenol (*E*11–16 : OH) was obtained from the Research Institute for Plant Protection (IPO-DLO), Wageningen, The Netherlands; (*Z*)-11-hexadecenol (*Z*11–16 : OH) was purchased from Aldrich Chem. Co. (Milwaukee, Wisconsin); (*E*)-11-hexadecenyl acetate (*E*11–16 : OAc) was prepared by acetylation of *E*11–16 : OH with acetic anhydride and pyridine; (*E*)-11-hexadecenal (*E*11–16 : Al) was prepared by oxidation of E11-hexadecenol with PCC in dichloromethane; (*Z*)-3,(*Z*)-6,(*Z*)-9-tricosatriene (*Z*3,*Z*6,*Z*9–23 : Hy) was synthesized as described above. All compounds were >98% chemically pure.

Field Experiments. Field experiments were conducted in commercial tomato plantations in Aragua State, Venezuela, employing a complete randomized block design. Water traps (Mirás et al., 1997) were suspended ~1 m above ground at 20-m intervals and baited with red rubber septa (Aldrich Chemical Co., Milwaukee, Wisconsin, catalog No. *Z*12,434-6) that were Soxhlet-extracted with ethanol for 24 hr, and impregnated with candidate pheromone components in HPLC-grade dichloromethane. Location, experimental period, and number of replicates for each experiment are reported in the captions of Figures 3 and 4 below.

Experiment 1 tested traps baited with *E*11–16 : OH (1 mg) and *Z*11–16 : OH (1 mg), singly and in binary combination at ratios of 1 : 0.1 and 1 : 0.01 mg. Solvent-impregnated rubber septa served as baits in control traps. Both experiments 2 and 3 tested traps baited with two virgin females or *E*11–16 : OH (1 mg). Experiment 4 tested traps baited with *Z*3,*Z*6,*Z*9–23 : Hy (1 mg) or *E*11–16 : OH (1 mg) singly, and *E*11–16 : OH (1 mg) in binary combinations with *Z*3,*Z*6,*Z*9–23 : Hy (0.025, 0.05, or 0.1 mg) and in penternary combination with *Z*11–16 : OH (0.07 mg), *E*11–16 : Ald (0.02 mg), *E*11–16 : OAc (0.02 mg), and *Z*3,*Z*6,*Z*9–23 : Hy (0.04 mg) at ratios as determined in pheromone gland extract. Experiment 5 tested traps baited with *E*11–16 : OH (1 mg) in binary combinations with *E*11–16 : OAc (0.05 mg), *E*11–16 : Ald (0.05 mg), or *Z*3,*Z*6,*Z*9–23 : Hy (0.05 mg), in ternary combination with *E*11–16 : OAc (0.05 mg), and *E*11–16 : Ald (0.05 mg), and in quaternary combination with *E*11–16 : OAc (0.05 mg), *E*11–16 : Ald (0.05 mg), and *Z*3,*Z*6,*Z*9–23 : Hy (0.05 mg). Experiment 6 tested traps baited with *E*11–16 : OH (1mg) singly, and in combination with *Z*3,*Z*6,*Z*9–23 : Hy (0.05 mg).

Statistical Analysis. Multivariate analysis with the using Mann-Whitney test (Spiegel, 1991) was performed to analyze data from field tests.

RESULTS

Pheromone Identification. GC-EAD analysis of pheromone gland extracts of female *N. elegantalis* revealed six components that elicited an antennal response (Figure 1). The most abundant and EAD-active component **2** (Figure 1) was hypothesized to be a hexadecenol, based on its retention indices on DB-5 (1865), DB-210 (2078), and DB-23 (2406) columns. Position and geometry of the double bond was approximated by comparative GC and GC-EAD analyses of synthetic hexadecenols. (*E*)-11- and (*E*)-12-hexadecenol (*E*11- and *E*12–16 : OH) had retention times identical to, and EAD-activity comparable with, female-produced **2**. Epoxidation (Bierl-Leonhardt et al., 1980) of female-produced **2**, and GC-MS analysis of the epoxy alcohol revealed fragmentation ions at *m/z* 99 and 199 (due to α-cleavage of the epoxy group), confirming the double bond at C-11. GC-EAD analyses of pheromone extract on a DB-23 column, which separates the *E* and *Z* isomers, provided tentative evidence also for trace amounts of the *Z* isomer.

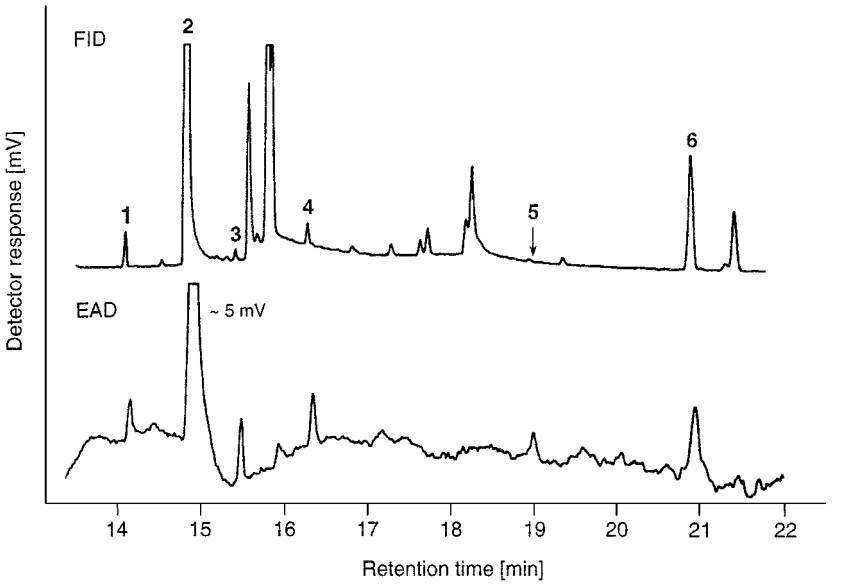

FIG. 1. Flame ionization detector (FID) and electroantennographic detector (EAD: male *N. elegantalis* antenna) responses to one female equivalent of pheromone gland extract, chromatograph on a DB-5 column; temperature program: 100°C (1 min) then 10°C/min to 240°C. Compound identity as follows: 1 = (*E*)-11-hexadecenal (*E*11–16 : Ald); 2 = (*E*)-11-hexadecenol (*E*11–16 : OH); 3 = unknown; 4 = (*E*)-11-hexadecenyl acetate (*E*11–16 : OAc); 5 = unknown; 6 = (*Z*)-3,(*Z*)-6,(*Z*)-9-tricosatriene (*Z*3, *Z*6, *Z*9-23 : Hy). Note: GC-EAD analyses of pheromone extract on a DB-23 column, which separated *E* and *Z* isomers of candidate pheromone components, provided tentative evidence also for trace amounts of (*Z*)-11-hexadecenol (*Z*11–16 : OH).

EAD-active **1** and **4** (Figure 1) were hypothesized and, through comparative GC analyses of authentic standards, confirmed to be *E*11–16 : Ald (**1**) and *E*11–16 : OAc (**4**). Mass spectrum and retention indices of EAD-active **6** were indicative of an triunsaturated C_{23} hydrocarbon, such as *Z*3,*Z*6,*Z*9–23 : Hy (Bell and Meinwald, 1986). Identical retention and mass spectrometric characteristics of an authentic standard and female-produced **6** confirmed this structural assignment. EAD-active compounds **3** and **5** occurred below detection threshold of the flame ionization detector and are yet to be identified.

In GC analyses of SPME-desorbed volatiles, small amounts of *E*11–16 : OH and *Z*3,*Z*6,*Z*9–23 : Hy could be detected.

Electrophysiology. Synthetic equivalents of candidate pheromone components evoked significant EAG responses. *E*11–16OH was most EAG-active and, unlike other test stimuli, elicited >5 mV EAG responses from male antennae already at a 10 ng dose (Figure 2). *Z*11–16 : OH, *E*11–16 : OAc, *E*11–16 : Ald,

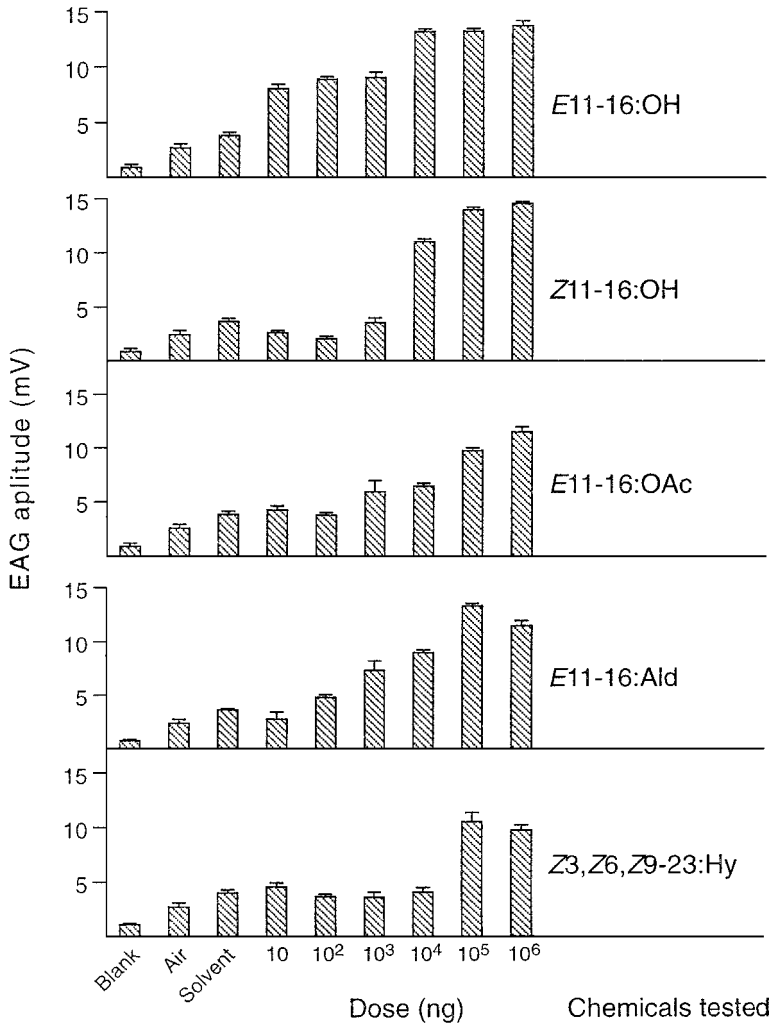

FIG. 2. Responses by male *N. elegantalis* antennae in electroantennogram (EAG) recordings to increasing amounts of five candidate pheromone components; compound nomenclature as in Figure 1.

and *Z*3,*Z*6,*Z*9–23 : Hy required doses of 10^4, 10^3, 10^2, and 10^5 ng, respectively, to induce EAG voltages greater than those of control stimuli (Figure 2).

Field Tests. Traps baited with *E*11–16 : OH captured significant numbers of male moths (Figure 3, experiment 1). *Z*11–16 : OH, in contrast, was not attractive and, when added to *E*11–16 : OH, strongly reduced captures of males. Traps baited

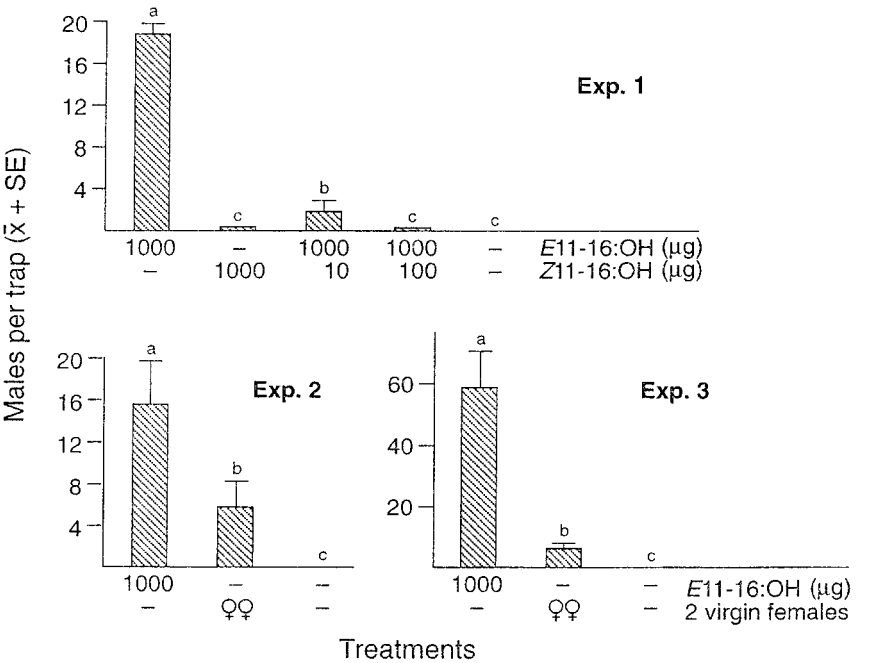

FIG. 3. Captures of male *N. elegantalis* in experiments 1–3 in water traps baited with two virgin female *N. elegantalis* or candidate pheromone components singly and in various combinations. Experiment 1: Casablanca, Aragua State; October 16–December 1, 1996; four replicates. Experiment 2: Casablanca, Aragua State; December 5–19, 1996; 7 replicates. Experiment 3: Múcura, Aragua State; February 15–20, 1997; 4 replicates. In each experiment, bars with the same letter superscript are not significantly different, $P > 0.05$.

with *E*11–16 : OH at 1 mg captured more males than those baited with two virgin female moths (Figure 3, experiments 2, and 3). *Z*3,*Z*6,*Z*9–23 : Hy enhanced attractiveness of *E*11–11 : OH, but was not attractive by itself (Figure 4, experiment 4). *E*11–16 : OH plus *Z*3,*Z*6,*Z*9–23 : Hy was the most attractive blend among several two-, three-, or four-component blends of candidate pheromone components (Figure 4, experiment 5). Synergistic activity of *Z*3,*Z*6,*Z*9–23 : Hy was confirmed in experiment 6 (Figure 4).

DISCUSSION

Our data show that *E*11–16 : OH and *Z*3,*Z*6,*Z*9–23 : Hy are sex pheromone components in *N. elegantalis*. Evidence in support of this conclusion includes the following: (1) both compounds were present and EAD-active in pheromone

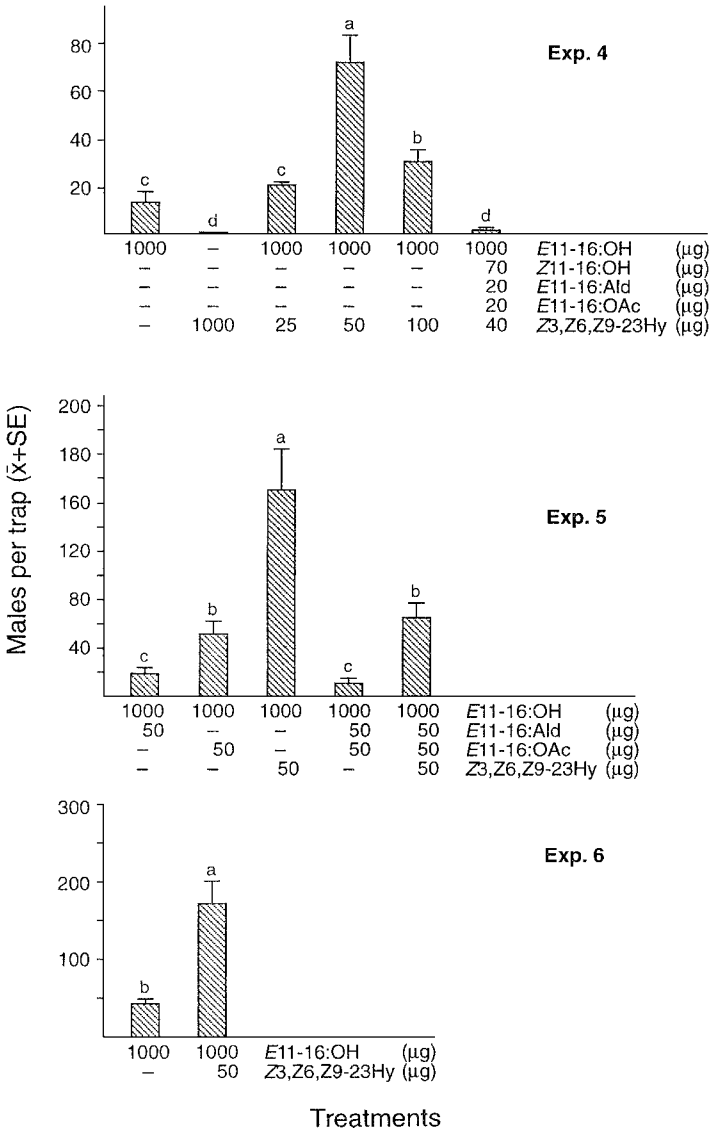

FIG. 4. Captures of male *N. elegantalis* in experiments 4–6 in water traps baited with candidate pheromone components singly and in various combinations. Experiment 4: Pao de Zárate, Aragua State; June 23–August 3, 1998; 5 replicates. Experiment 5: Valle de Tucutunemo, Aragua State; February 5–March 26, 1999; 5 replicates. Experiment 6: Pao de Zárate, Aragua State; July 3–25, 2000, 10 replicates. In each experiment, bars with the same letter superscript are not significantly different, $P > 0.05$.

gland extracts and effluviums of female moths (Figure 1); (2) identifications were based on comparative GC, GC-MS, and GC-EAD analyses of female-produced compounds and authentic standards; (3) both compounds elicited stronger antennal responses from male antennae in EAG recordings than did control stimuli (Figure 2); and (4) blends of *E*11–16 : OH and *Z*3,*Z*6,*Z*9–23 : Hy attracted significant numbers of males (Figure 4). *E*11–16 : OH is the major pheromone component, because it was most abundant in gland extracts (Figure 1), elicited the strongest EAG response at low doses (10 or 100 ng) (Figure 2), and, unlike *Z*3,*Z*6,*Z*9–23 : Hy, was attractive by itself as a bait (Figures 3 and 4).

*E*11–16 : OH has also been identified as a sex pheromone component in other moths. It is the major pheromone component of the pod worm, *Leucinodes orbonalis* (Zhu et al., 1987; Attygalle et al., 1988), and a minor pheromone component of female pickleworms *Diaphania hyalinata* and *D. nitidalis* (Raina et al., 1986; Klun et al., 1986). It was also present in pheromone gland extracts of eggfruit caterpillar, *Sceliodes cordalis* (Clearwater et al., 1986) and of sugarcane borer, *Sesamia grisescens* (Whittle et al., 1995), but behavioral activity of *E*11–16 : OH in these two species is yet to be determined.

*Z*3,*Z*6,*Z*9–23 : Hy is reported here for the first time as a sex pheromone component in the Lepidoptera. While it was identified as a trace component in pheromone glands of the arctiid moth *Creatonotos gangis* (Bell and Meinwald, 1986), behavioral activity was not determined. A sex pheromone blend with synergistic behavioral activity between a triene hydrocarbon (*Z*3,*Z*6,*Z*9–23 : Hy) and an alcohol (*E*11–16 : OH), as demonstrated in our study, is most unusual. Most triene hydrocarbons as pheromone components in the Lepidoptera are either attractive by themselves (Millar et al., 1992) or serve as synergists of corresponding diene epoxides (Tóth et al., 1994).

Further compounds (*Z*11–16 : OH, *E*11–16 : Ald, *E*11–16 : OAc) in pheromone gland extracts of female *N. elegantalis* seem to have no pheromonal activity. This conclusion is based on their low EAG activity at low doses (Figure 2) and/or lack of attractiveness in field experiments. The role of *Z*11–16 : OH is interesting. Its low EAG activity but strong behavioral inhibition at a low dose (Figures 2 and 3) suggests that mate-seeking males may select calling females based on the presence or absence of *Z*11–16 : OH in the pheromone effluvium, providing a basis for a highly efficient system of sexual selection (Jaffe, 1996, 1998, 1999), as only very fit females would produce *E*11–16 : OH with small amounts of *Z*11–16 : OH. Alternatively, *Z*11–16 : OH may serve as a bifunctional pheromone component (Linn and Roelofs, 1995) in a sympatric species to enhance the specificity of the communication signal, although no such sympatric species is known.

The two-component pheromone blend of *E*11–16 : OH and *Z*3,*Z*6,*Z*9–23 : Hy seems to work as a supernormal stimulus (Tinbergen and Perdeck, 1950), as it is approximately 60 times more attractive than the natural pheromone blend emitted by the females (Experiments 3 and 4, in Figures 3 and 4, respectively).

A synthetic mixture of *E*11–16 : OH and *Z*3,*Z*6,*Z*9–23 : Hy as a trap bait is currently used by Brazilian and Venezuelan tomato growers to reduce infestations of *N. elegantalis*, promising efficient control in pheromone-based mass trapping programs.

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