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Identification (GC and GC-MS) of unsaturated acetates in *Elasmopalpus lignosellus* and their biological activity (GC-EAD and EAG)

Two insect colonies of *Elasmopalpus lignosellus* were reared in our laboratory, the first being initiated from pupae obtained from a cornfield in the region of Sete Lagoas, Minas Gerais and the second from a cornfield in the region of Goiânia, Goiás. From the two colonies, two extracts were prepared from the pheromone glands of virgin *E. lignosellus* females. The extract obtained from the first colony was designated as extract 1 while the extract obtained from the second colony was designated as extract 2. Extract 1 was analyzed by gas chromatography-mass spectrometry (GC-MS) with (*Z*)-9-hexadecenyl acetate [(*Z*)-9-HDA] and (*Z*)-11-hexadecenyl acetate [(*Z*)-11-HDA] being identified and confirmed by the formation of DMDS derivatives. In addition, a third acetate, which could be either (*E*)-8-hexadecenyl acetate [(*E*)-8-HDA] or (*E*)-9-hexadecenyl acetate [(*E*)-9-HDA] was detected by GC-MS. Extract 2 was analyzed by gas chromatography (GC) and gas chromatography-electroannetography (GC-EAD) revealing the presence of (*Z*)-11-HDA and (*Z*)-9-TDA. In addition, the same compounds elicited a response with the *E. lignosellus* male antenna obtained from the second insect colony. Electroantennography (EAG) screening with the male *E. lignosellus* antenna (obtained from the second insect colony) was conducted with the 23 possible tetradecenyl acetates (TDA) and 22 hexadecenyl acetates (HDA) as standards. Out of the 23 TDA isomers evaluated, only (*Z*)-9-TDA elicited a response and out of the 22 HDA [(*Z*) and (*E*) isomers Δ^2 to Δ^{13}] evaluated only (*Z*)-11-HDA elicited a response. The acetate compositions of two extracts obtained from insects originating from the two states (Minas Gerais and Goiás) of Brazil were different from one another as well as from that obtained from insects in Tifton, GA, USA. The bioactivity data (GC-EAD) of the extract 2 differed from those reported for the Tifton, GA, USA population. These data suggest polymorphism in relation to the insect populations found in Brazil and in the USA. The possibility of the existence of an *E. lignosellus* sub-species cannot be ruled out.

Short Communication

1 Introduction

Elasmopalpus lignosellus is a major pest that attacks corn in Brazil and granaries worldwide [1, 2] and is controlled by extensive use of toxic insecticides. Ecologically accepted methods for its control are highly desirable, and the identification of a sex pheromone in *E. lignosellus* [3] would open up such a possibility. Ten acetates and alcohols were identified by gas chromatography-mass spectrometry (GC-MS) in the pheromone gland of *E. lignosellus*. The composition of the pheromone mixture was determined through field tests and a good capture obtained [4].

However, the sex pheromone blend commercially available in the USA produced a very low response when evaluated in field experiments in Brazil [5]. This led to the hypothesis that the pheromone blend of *E. lignosellus* in Brazil might be different due to regional variations [6, 7].

In order to test the above hypothesis, the volatiles in the pheromone gland of *E. lignosellus* females were characterized by GC-MS. Two extracts obtained from insects from different regions were examined. Coupled gas chromatography-electroantennography (GC-EAD) and GC-MS takes full advantage of the separation and detection capabilities of the two techniques. They are therefore very useful in the identification of active compounds in insects [8–11]. Hence, the response of the active extracts obtained in the pheromone gland was determined by GC-

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EAD. In addition, the response of the males' antennae to 23-tetradecenyl acetate (TDA) and 22-hexadecenyl acetates (HDA) used as standards was evaluated by electroantennography (EAG) with puffs on the male *E. lignosellus* antenna.

2 Methods and materials

The 23 possible TDA and 22 HDA [(*Z*) and (*E*) isomers of Δ^2 to Δ^{13}] isomers (99% pure) were obtained from the Research Institute for Plant Protection (Wageningen, The Netherlands). Dimethyl disulfide was purchased from Aldrich Chemicals. Fused capillary columns (30 m × 0.25 mm; film thickness of 0.25 μm) coated with DB-1 and SP 2340 as the stationary phases, were purchased from J & W Scientific and Supelco, respectively.

2.1 Preparation and evaluation of pheromone gland extracts

Two insect colonies of *Elasmopalpus lignosellus* were reared in our laboratory on artificial diets [12], the first being initiated from pupae obtained from a cornfield in the region of Sete Lagoas, Minas Gerais, and the second from pupae obtained from a cornfield in the region of Goiânia, Goiás. Extracts from insects were prepared during the calling period [5] by removing the pheromone glands of 1–2-day-old virgin females and extracting with 100 μL of hexane for 2 h. The extract obtained from the first colony was designated as extract 1 while the extract obtained from the second colony was designated as extract 2. Extract 1 was analyzed by gas chromatography-mass spectrometry. Extract 2 was analyzed by gas chromatography (GC) and gas chromatography-electroantennography detector (GC-EAD) to determine its activity on the *E. lignosellus* male antenna (obtained from the second insect colony). Extract activity was determined by preliminary tests in wind-tunnel bioassays. The active extracts were concentrated to about 30 μL by a gentle stream of N₂, and 1 μL was injected for GC-MS (after the addition of internal standard tetradecane) and GC-EAD analysis.

2.2 GC-MS analysis

Data were obtained using a gas chromatograph-mass spectrometer fitted with an autosampler (Shimadzu, Kyoto, Japan, model QP 5000). To obtain analytical data with DB-1 and SP-2340 stationary phases, the GC oven temperature was programmed from 60°C to 280°C at a rate of 6°/min and from 60°C to 220°C at a rate of 6°/min, respectively. For the analyses of the DMDS derivatives (DB-1 capillary column), the GC oven temperature was programmed from 100°C to 280°C at 10°/min. Helium was the carrier gas for all the analyses with a flow rate of 1 mL/min. Electron ionization mass spectra (70 eV) were recorded by scanning from *m/z* 29 to 320 for acetate anal-ysis and *m/z* 29 to 470 for the DMDS derivatives. For all the analyses, the transfer line temperature was maintained at 280°C. Splitless injections were made in all cases.

The 22 individual HDA [(*Z*) and (*E*) isomers of Δ^2 to Δ^{13}] standards were mixed with 100 ng of the internal standard tetradecane (C₁₄) and injected through the auto sampler. The pheromone extracts were also mixed with C₁₄ but were manually injected due to the small volume. The monounsaturated acetates in the pheromone extracts were identified by comparing the retention times of the peaks with those of standards and mass spectra. The retention time (t_r) of each standard was provided by the GC-MS integration system. The relative retention time (rt_r) on the SP-2340 coated column was obtained by subtracting the t_r of C₁₄ from that of each compound.

2.3 Derivatization

The DMDS derivatives were prepared as described in the literature [13–16]. To an active glandular extract of *E. lignosellus* in hexane (30 μL), a solution of I₂ in freshly distilled ether (15 μL, 5%) and dimethyl disulfide (25 μL, Aldrich Chemicals) were added. After allowing the mixture to react overnight at room temperature, a solution of Na₂S₂O₃ (5%) was added until the brown color disappeared, and the mixture was extracted with hexane (3 × 20 μL). The hexane layers were combined and evaporated to dryness under a gentle stream of N₂; the residue was dissolved in 20 μL of hexane, and 1 μL of the solution was analyzed by GC-MS.

2.4 GC and GC-EAD

The system consisted of a gas chromatograph (Shimadzu, Kyoto, Japan, model 17A) with a FID detector and coupled to an electroannetograph (Syntec Stimulus Controller, CS-05, Hilversum, The Netherlands) via an effluent splitter (1:1), both connected to a computer and an integrating system. With the aid of a microscope the male *E. lignosellus* antenna was removed from the head and mounted between the electrodes. The base of the antenna was inserted into the reference electrode, and the tip of the antenna after removing the terminal segments, was inserted into the recording electrode. The antenna faced toward the gas flow from the GC effluent. The volatiles were analyzed using the conditions previously described. The monounsaturated acetates were identified in the extracts by comparing the retention times of the peaks with those of standards [23 possible TDA isomers and 22 HDA isomers].

2.5 EAG

The system consisted of a data acquisition interface (IDAC-02) and a single-ended probe (Synthec, Hilversum,

Table 1. Relative retention times (rt_r) [min] of hexadecenyl acetates (based on tetradecane) and acetates in the pheromone gland extract (extract 1) of *E. lignosellus.*

Standard	(E)-3-HDA	(E)-4-HDA	(Z)-3-HDA	(E)-5-HDA	(Z)-4-HDA	(E)-7-HDA	(E)-6-HDA	(E)-8-HDA	(E)-9-HDA	(Z)-5-HDA	(Z)-6-HDA	(E)-10-HDA	(Z)-7-HDA	(E)-11-HDA	(E)-12-HDA	(Z)-8-HDA	(Z)-9-HDA	(E)-13-HDA	(Z)-10-HDA	(Z)-11-HDA	(Z)-12-HDA	(Z)-13-HDA
rt_r	13.761	13.859	13.943	13.975	13.976	13.989	13.992	14.035	14.070	14.104	14.118	14.119	14.169	14.176	14.186	14.200	14.269	14.281	14.341	14.422	14.524	14.601
Peak No																						
1								14.038														
2																	14.272					
3																				14.422		

The Netherlands, model PRS-1). The recording and the reference electrodes consisted of silver-coated wire in glass micropipettes containing 0.5 M KCl. EAG data were recorded and stored in a microcomputer using software provided with the Synthec system (Version 2.2).

The response of each of the 23 TDA and 22 HDA isomers was evaluated by EAG (Synthec Stimulus Controller, Hilversum, The Netherlands, model CS-05) using male antennae obtained from the second insect colony. The system consisted of an aluminum tube with a small orifice in the middle. Carbon-filtered and humidified air (5 mL/min) was delivered continuously into the other arm of the tube via Teflon tubing, while acetate stimuli were delivered in puffs of 0.3 s through the small orifice with Pasteur pipettes. In all experiments the pipettes were filled with strips of Whatman No 1 filter paper (1.5 × 0.5 cm) and impregnated with 10 μg of each acetate. The time interval between stimulus puffs was 30 s. A total of 20 antennae were used for each acetate series.

3 Results and discussion

3.1 Acetate identification by GC-MS and GC

Figure 1 shows a section of the reconstructed gas chromatogram obtained from the volatiles present in the hexane extract (extract 1) of *E. lignosellus* glands (column coated with SP 2340). No peaks corresponding to TDA or the respective alcohols were detected. We looked for such compounds since they have been previously reported for *E. lignosellus* [4]. Instead, only one peak was recorded with the DB-1 column in the region where HDA isomers eluted.

Our experience with extracts from other insects has shown that a higher resolution was obtained with the polar column SP-2340 as compared to the non-polar column DB-1 [15, 16]. The same was true for *E. lignosellus*. On a non-polar column only one peak corresponding to a HDA isomer could be detected while on the SP-2340 column, three peaks corresponding to HDA isomers with very similar retention times and mass spectra were detected. The mass spectra of all these peaks showed a base peak at m/z 43, which along with m/z 61, indicated that they were acetates. The peak at m/z 222 resulting from loss of acetic acid from the (undetected) parent ion suggested that the compounds were HDA isomers. In order to identify the
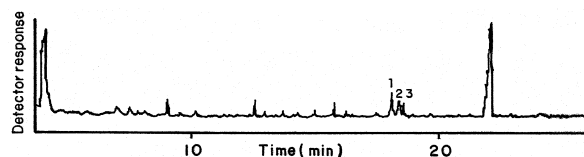

Figure 1. Section of the reconstructed gas chromatogram obtained from the volatiles present in the hexane extract (extract 1) of *E. lignosellus* glands using a fused silica column coated with SP-2340. Peaks 1, 2, and 3 were identified as (E)-8-HDA or (E)-9-HAD; (Z)-9-HDA; and (Z)-11-HDA, respectively.

correct HDA isomer, 22 hexadecenyl acetates standards were injected along with the internal standard C_{14} under the same conditions. Excellent reproducibility was obtained for rt_r with the variation being in the third decimal place (data not shown). Hence, to identify the compounds in the pheromone extract a difference of ± 0.003 min was utilized. The rt_r for the first peak in the pheromone extract was 14.038 min and hence based on the difference of ± 0.003 min there were two possibilities, i. e., (E)-8-HDA or (E)-9-HAD (**Table 1**). Similarly, for the second peak (rt_r = 14.272 min) there were two possibilities, i. e. (Z)-9-HDA or (E)-13-HDA. For the third peak (rt_r = 14.422 min) there was only one possibility, i. e., (Z)-11-HDA. The mass spectra of all these peaks confirmed that they were hexadecenyl acetates.

DMDS data led to the identification of 9-HDA and 11-HDA and in both cases the stereochemistry was confirmed by standards to be Z. Hence, peaks 2 and 3 were identified as (Z)-9-HDA and (Z)-11-HDA, respectively. Similarly, peak 1 was identified as (E)-8-HDA or (E)-9-HDA. However, it was not possible to identify the correct isomer since the DMDS derivative could not be prepared. It must be mentioned that it was possible to prepare the DMDS derivatives with (E)-8-HDA and (E)-9-HDA standards on a microgram scale. However there is no explanation as to why we could not prepare the DMDS derivative corresponding to peak 1. We have faced the same problem when working on the identification of the pheromone of *Tuta absoluta* [16]. In this study, we could not prepare the DMDS derivative of (E)-3-TDA in the pheromone extract but (Z)-8-TDA and (Z)-11-TDA presented no problems. We were able to prepare the DMDS derivatives with the standards of (E)-3-TDA (Z)-8-TDA and (Z)-11-TDA on as
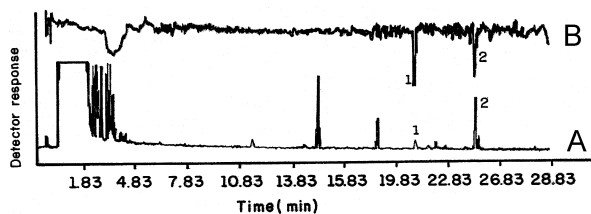
Figure 2. Parallel FID (A) and EAD (B) traces obtained with bioactive hexane extract (extract 2) of *E. lingosellus* females using the fused silica column coated with DB-1. Electrophysiologically active peaks 1 and 2 (upper trace) were identified as (*Z*)-9-TDA and (*Z*)-11-HDA, respectively.

little as 1 ng. We concluded at that time that some compound or factor prevented the DMDS derivative formation with (*E*)-3-TDA.

GC analysis of extract 2 (**Figure 2**) revealed the presence of (*Z*)-9-TDA (peak 1) and (*Z*)-11-HDA (peak 2). Thus, acetate compositions of two extracts obtained from insects originating from two states (Minas Gerais and Goiás) of Brazil differed from one another as well as from that obtained from insects in Tifton, GA, USA [4]. In that study the following ten compounds were identified by GC-MS in the pheromone gland of the same insect obtained in Tifton, GA, USA: 16.5% (*Z*)-7-TDA]/8.8% (*Z*)-9-TDA/ 3.8% tetradecyl acetate/28.8% (*Z*)-9-HDA/7.4% (*Z*)-11-HDA/9.6% hexadecyl acetate/2.1% (*Z*)-7-tetradecenol/ 7% (*Z*)-9-tetradecenol and 4.4% (Z)-11-hexadecenol.

3.2 Physiologically active acetates by GC-EAD

Parallel FID and EAD traces obtained with the active extracts using the capillary column coated with SP 2340 are presented in Figure 2. With the help of standards,

peak 1 was identified as (*Z*)-9-TDA while peak 2 was identified as one of the following four compounds: (*E*)-10-HDA, (*E*)-11-HDA, (*E*)-12-HDA, (*Z*)-11-HDA and (*Z*)-12-HDA. Since (*Z*)-11-HDA was confirmed by GC-MS, it was concluded that peak 2 was (*Z*)-11-HDA. Due to sample limitations, it was not possible to conduct GC-MS analysis with this extract.

3.3 Response to synthetic standards of male *E. lignosellus* antenna by EAG

Figure 3 shows male antennal responses with TDA and HDA standards. Among the TDA isomers, only (*Z*)-9-TDA elicited a response while in the HDA series, only (*Z*)-11-HDA did. This result was consistent with our chemical (GC-MS) and biological data (GC-EAG) as well as those of the literature report [4].

(*Z*)-9-TDA and (*Z*)-11-HDA identified as the active compounds of the US populations [4] were present in the Goiás population. Thus, the two-pheromone bouquets are not so different. Actually, the Minas Gerais population differs more as it does not contain TDA.

Our bioassays indicate that the composition of the bioactive compounds is very different from that reported in the literature [4]. In that study (*Z*)-7-TDA, (*Z*)-9-TDA, (*Z*)-11-HDA and (*Z*)-9-TDOH showed field activity. Our bioassays are different from those used in the literature and hence a strict comparison cannot be carried out. However, when the chemical composition and bioassay data are considered along with our field tests [5], there is a preliminary indication that *E. lignosellus* populations found in Brazil and in the US are different. Also, the possibility of the existence of sub-species cannot be ruled out.
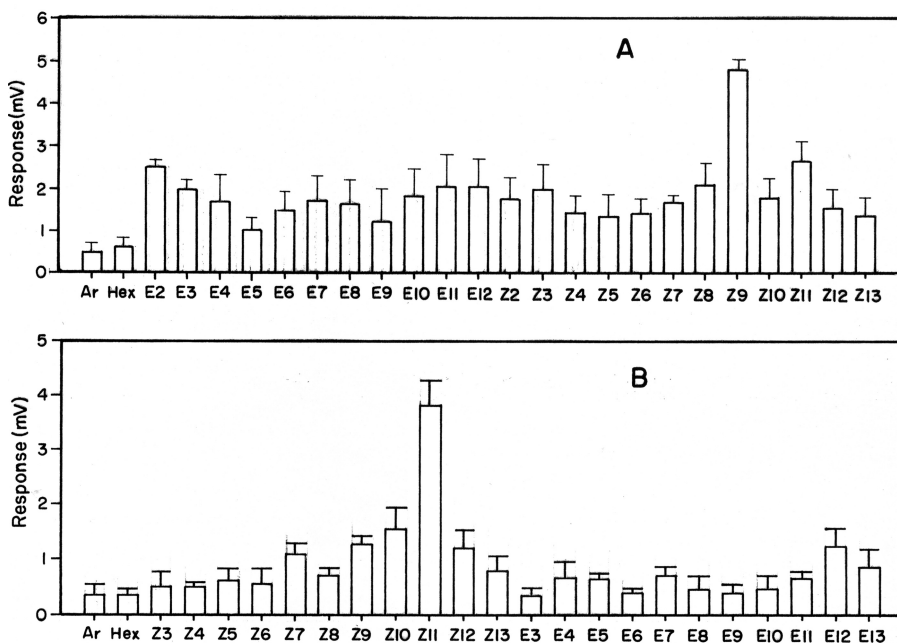

Figure 3. EAG response obtained with the male antenna by screening all possible 23 tetradecenyl acetates (A) and 22-hexadecenyl acetates [(*Z*) and (*E*) isomers of Δ^2 to Δ^{13}] (B) along with air and hexane (blank).

In moths, the relative proportions of sex pheromone components and the specificity of the male behavioral response are often decisive for reproductive isolation between closely related species that overlap in time and space [17–19]. Significant inter-population variation in pheromone blends and male response has been demonstrated in several moth species [20–23]. It appears that this is the case with *E. lignosellus* in relation to the population studied in the USA [4]. Recently, sex pheromone "dialects" have been reported in *Agrotis segetum* from different geographic regions [23].

4 Conclusions

Our results suggest polymorphism in *E. lignosellus*. In addition, the existence of sub-species cannot be ruled out. This may have important implications for the control of this insect since it occurs in several regions in Brazil. We are presently addressing this by collecting insects from several regions in Brazil and comparing the chemical compositions of volatiles contained in their pheromone glands.

Acknowledgements

References

[1] J.E. Funderburk, D.C. Herzog, R.E. Lynch, *J. Entomol. Sci.* **1987**, *22*, 159–168.

[2] P.A. Viana, In: 2° Simpósio Latino Americano de cana-de-açúcar, Maceió, Al, 1993. pp. 1–21.

[3] T.L. Payne, J.H. Smith, *Environ. Entomol.* **1975**, *4*, 355–356.

[4] R.E. Lynch, J.A. Klun, B.A. Leonhardt, M. Schwart, J.W. Garner, *Environ. Entomol.* **1984**, *13*, 121–126.

[5] C.S.S. Pires, E.F. Vilela, P.A. Viana, J.T.B. Ferreira, *An. Soc. Ent. Bras.* **1992**, *21*, 413–417.

[6] C.E. Linn Jr., W.L. Roelofs, *J. Chem. Ecol.* **1985**, *11*, 1583–1590.

[7] D.R. Thomson, N.P.D. Angerilli, C. Vincent, A.P. Gaunce, *Environ. Entomol.* **1991**, *20*, 935–938.

[8] D.L. Struble, H. Arn, In: H.E. Hummel, T.A. Miller (Ed.). *Techniques in Pheromone Research.* Springer-Verlag, New York 1984, p. 161.

[9] A. Cork, P.S. Beevor, A.J.E. Gough, D.R. Hall, In: F. McCaffery, I.D. Wilson (Ed.), *Chromatography and Isolation of Insect Hormones and Pheromones*. Plenum Press, New York 1990, p. 271.

[10] A.A. Cossé, J.L. Todd, J.G. Millar, L.A. Martínez, T.C. Baker, *J. Chem. Ecol.* **1995**, *21*, 1823.

[11] L.B. Bjostad, *Physiol. Ent.* **1998**, *13*, 139–145.

[12] R.B. Chalfant, *J. Ga. Ent. Soc.* **1975**, *10*, 33–37.

[13] G.W. Francis, K. Veland, *J. Chromatogr.* **1981**, *219*, 379–384.

[14] H.R. Buser, H. Arn, P. Guerin, S. Rauscher, *Anal. Chem.* **1983**, *55*, 818–822.

[15] A.B. Attygalle, G.N. Jham, A. Svatoš, R.T. Frighetto, J. Meinwald, E.F. Vilela, F.A. Ferrara, *Tetrahedron Lett.* **1995**, *36*, 5471–5474.

[16] A.B. Attygalle, G.N. Jham, A. Svatoš, R.T. Frighetto, J. Meinwald, E.F. Vilela, F.A. Ferrara, *Bioorg. Med. Chem.* **1996**, *4*, 305–314.

[17] C.J. Sanders, *Science* **1971**, *171*, 911–913.

[18] J.H. Tumlinson, C.E. Yonce, R.E. Doolittle, R.R. Heath, C.R. Gentry, E.R. Mitchell, *Science* **1974**, *185*, 614–616.

[19] R.T. Cardé, A.M. Cardé, A.S. Hill, W.L. Roelofs, *J. Chem. Ecol.* **1977**, *3*, 71–84.

[20] C. Löfstedt, J. Löfqvist, B.S. Lanne, J.N.C. Van Der Pers, B. Hansson, *Oikos* **1986**, *46*, 250–257.

[21] J.R. Byers, D.L. Struble, *J. Chem. Ecol.* **1990**, *16*, 2981–2992.

[22] J.R. Byers, D.L. Struble, C.E. Herlne, G.C. Kozub, J.D. Lafontaine, *J. Chem. Ecol.* **1990**, *16*, 2969–2980.

[23] B.S. Hansson, C. Löfstedt, G. Szöcs, M. Subchev, J. Löfqvist, *J. Chem Ecol.* **1990**, *16*, 1611–1662.