Electrophysiological Studies and Identification of Possible Sex Pheromone Components of Brazilian Populations of the Sugarcane Borer, *Diatraea saccharalis*

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Virgin female gland extracts of sugarcane moth *Diatraea saccharalis* (Fabricius) (Lepidoptera: Pyralidae), from three locations in Brazil, have been analyzed. By GC-MS analysis and comparison of the chromatographic retention time of the components of the pheromone gland with those retention times of synthetic standards, we observed the presence of (*Z*)-hexadec-11-enal (**1**), hexadecanal (**2**), (9*E*,11*Z*)-hexadecadienal (**4**), (9*Z*,11*Z*)-hexadecadienal (**5**) and (9*E*,11*E*)-hexadecadienal (**6**), as minor components besides the major constituent (9*Z*,11*E*)-hexadecadienal (**3**) already reported. We found no variations in the composition of the gland extracts deriving from the three Brazilian populations and only two compounds, (*Z*)-hexadec-11-enal (**1**) and (9*Z*,11*E*)-hexadecadienal (**3**), elicited antennal responses (GC-EAD). In electroantennography (EAG), however, pure compounds **1** and **3**, a binary mixture containing **1** and **3,** and a mixture containing all of the six synthetic compounds **1–6** elicited a depolarization in male antennae of *D. saccharalis*, without any statistically different delay. The EAG responses to the other isomers of 9,11-hexadecadienal were small and not significantly different from the control, except for the (9*Z*,11*Z*)-isomer (**5**) which showed an relatively strong electroantennal activity.

Introduction

The sugarcane borer, *Diatraea saccharalis* (Fabricius) (Lepidoptera: Pyralidae), is one of the most important pests of maize and sorghum crops and the key pest of sugarcane in the Americas (Cueva *et al*., 1980; Gallo, 1980; Macedo *et al*., 1993).

Various strategies for control of this pest have been developed, because insecticidal control is not efficient partly due to the simultaneous presence of all development stages of the pest throughout the year. Moreover, a control with insecticides is difficult, because the insects are protected in galleries inside the sugarcane plant at the larval and pupal stages. In Brazil, *D. saccharalis* is mostly controlled by mass release of the larval parasitoid *Cotesia flavipes* (Cameron), resulting in substantial reduction of the insect population (Botelho, 1992). However, the combination of the larval parasitoid *C. flavipes* with the egg parasitoid *Trichogramma pretiosum* also has a substantial effect on the reduction of *D. saccharalis* populations in the field (Botelho *et al*., 1999). The efficacy of the egg parasitoid is related to the timing of its release in the field with the *D. saccharalis* oviposition period. Therefore, the use of the sex pheromone of *D. saccharalis* for determining male flight phenology is another strategy that offers a high potential for monitoring an accurate timing of the release of egg parasitoids.

To date, only (9*Z*,11*E*)-hexadecadienal (**3**) has been reported as a sex pheromone component of the sugarcane borer (Carney and Liu, 1982). Various field tests have been carried out to verify the attractiveness of pheromone traps using (9*Z*,11*E*)-hexadecadienal and to compare it with that of

traps containing virgin females. In all tests the traps with virgin females have been significantly more attractive than those using synthetic pheromones (Hammond and Hensley, 1971; Patrick and Hensley, 1970; Perez and Hensley, 1973; Botelho *et al*., 1976; Botelho *et al*., 1978; Almeida *et al*., 1983). The use of *D. saccharalis* pupae as a source of the sex pheromone has been reported by Lima-Filho and Riscado (1988). Both methods are time-consuming and none of them has yet been completely optimized for monitoring *D. saccharalis*. A better knowledge of the total composition of the female sex pheromone may lead to an increase in the capture of *D. saccharalis* males in pheromone traps.

In order to increase the effectiveness of pheromone trapping, it would be necessary to know the complete pheromone composition. Therefore, we decided to investigate the composition of extracts of the sex pheromone glands of *D. saccharalis* females. In a pilot experiment, insects were sent to Sweden and the pheromone glands analyzes showed that all the four stereoisomers of 9,11-hexadecadienal were presented (E. M. Santangelo *et al*., unpubl.). In this paper we report a more extensive investigation of extracts of the sex pheromone glands of *D. saccharalis* females from three locations in Brazil. We also report the electroantennographic activity of the four isomers of 9,11-hexadecadienal and other constituents occurring in the extracts of the sex pheromone gland of *D. saccharalis* females.

Materials and Methods

Standards of (*Z*)-hexadec-11-enal (**1**) and hexadecanal (**2**) were obtained by pyridinium dichromate oxidation of the corresponding alcohols, which were purchased from Aldrich Chemical (Milwaukee, Wisconsin). The four isomers of 9,11-hexadecadienal (**3–6**) were prepared from 9-decen-1-ol as described by Santangelo *et al*. (2002).

The pupae of *D. saccharalis* used in our experiments originated from the Brazilian laboratories of two sugar companies (Usina Santa Adélia, Jaboticabal – SP and Usina Santa Cruz, Campos – RJ), and a co-operative (Copersucar, Piracicaba – SP). The insects were obtained from laboratory rearing on artificial diets and sexed at the pupal stage (Parra, 1986). For emergence of adults the pupae were placed individually in plastic vials (6 cm × 6 cm ∅). Male and female pupae were maintained in an incubation chamber under a reversed light cycle of L12:D12 h; 60 ± 5% r.h. and 25 ± 1 °C.

Twenty couples of 1–3 days old *D. saccharalis* were placed, at least 2 hours before the observations, in a Perspex cage (30 × 30 × 30 cm) maintained at 24 ± 1 °C, 65% r.h. and 12 h photophase. The mating behavior was recorded under 0.1 lux red light intensity by an infrared camera (Magnavox CCD-MS40) and using a video recorder (JVC model HR-J600U). The observations of the mating behavior began in the last hour of the photophase and were carried out thoughout the scotophase. Six replicates were carried out (n = 120 insects).

The pheromone glands of virgin females were extracted at the fifth hour of the 2nd scotophase. The number of glands excised during any collection period varied with the availability of calling females. The glands from Piracicaba were extracted in batches of 30, 30 and 33 glands; origin Campos, 29, 20 and 51 glands; origin Jaboticabal, 30, 27 and 36 glands. We have done three replicates of the gland extracts of every population of laboratory-reared females (n = 3), and these replicates were excised and extracted separately. The excisions of the glands were performed by gently squeezing the lateral-posterior abdominal section of the females, causing protrusion of the terminal abdominal segments, which were then clipped as units. The glands excised were all placed together in 100 µl hexane in a microvial. After 24 h the hexane extract was transferred into brown microvials with Teflon®-lined screwcaps. The gland extracts were stored at −20 °C until analysis. Peak areas of the possible pheromone compounds were normalized to the peak area of (9*Z*,11*E*)-hexadecadienal of each preparation, so that the gland extracts could be compared. Data of each normalized compound were subjected to ANOVA and a mean compared using the Tukey test ($p < 0{,}05$).

The extracts were analyzed by gas chromatography in a Shimadzu 17-A chromatograph equipped with DB-5 or DB-1 columns (30 m × 0.25 mm i.d. 0.25 µm film thickness J and W Scientific), using a temperature program of 70 °C/1 min, 7 °C/min to 160 °C/0 min, 2 °C/min to 200° and finally to 280 °C at 15 °C/min, held for 10 min.

GC-MS analysis were recorded on a Shimadzu QP5000 spectrometer, using helium as carrier gas and a DB-1 column. Injections of 1 μl of each splitless synthetic standard solution and each gland extract were made at 250 °C, using the same temperature program described above. Electron impact mass spectra (70 eV) were obtained in the *m/z* range of 33–250.

Electroantennographic detection (EAD) after the gas chromatographic separation was used to locate possible pheromone components. The GC-EAD instrument consisted of a Shimadzu 17-A chromatograph equipped with a flame ionization detector coupled to an electroantennography system (EAG) (Syntech, Hilversum, The Netherlands) and a DB-5 column. The column effluent (carrier gas hydrogen) was mixed with a nitrogen make-up gas flow (12 ml/min) before it was splitless to the FID and to the heated transfer capillary leading to the antennal preparation. The compounds in the transfer capillary were eluted into a humidified and purified air stream (1.2 l/min), which led them direct to the antennal preparation. The same chromatographic conditions as above were used for the chromatographic separation. The FID was kept at 280 °C, whereas the temperature of the transfer capillary was maintained at 290 °C to avoid condensation.

The antennae of 1–2 days old *D. saccharalis* males were used for the electroantennographic experiments (EAG) and the electroantennographic detection (EAD). The male antenna was excised (pulled out of the head) by means of a forceps and a few segments were cut off at the base and the tip (Bjostad, 1998). The antenna was then fixed between two stainless steel electrodes by pushing the base and tip into droplets of an electrically conductive gel (Spectra 360® electrode gel – Parker, Orange, New Jersey) applied onto the metal electrodes.

The specimen with the antennal preparation was placed in such a way that the humidified air directed the compounds eluted from the stimulus delivery over the antenna. The antennal responses were amplified and recorded with a data acquisition controller and a software EAG (Syntech).

We wanted to verify whether the male moth antenna was really more sensitive to (9*Z*,11*E*)-hexadecadienal than to the other compounds, or whether the response to this compound observed in EAD was stronger because of the higher concentration in the gland extracts. Hence, we tested the EAG responses to synthetic standards of the same concentration (1 mg/ml), to the binary combination of (9*Z*,11*E*)-hexadecadienal and (*Z*)-hexadec-11-enal and to the mixture of the six synthetic compounds with identical concentrations of every individual component (1 mg/ml).

The EAG response was evaluated in the following way: the compounds were released from Pasteur pipettes containing a piece of filter paper (*ca.* 0.8 cm^2), impregnated with 5 μl of each freshly prepared solution of the test compounds and passed over the antennae. In addition to the pipettes with the test preparations, one Pasteur pipette containing a filter paper impregnated with 5 μl of the hexane solvent was used as control. The puff containing the test substance was delivered into a continuously humidified and purified air stream of 1.2 l/min by purified air, passing for 0.3 seconds through the impregnated filter paper in the pipettes. Control stimulation was made at the beginning and at the end of every series of EAG experiments. The test compounds were then applied randomly at intervals of 30 seconds. The EAG amplitudes in response to the synthetic compounds were expressed in relation to the responses to the control (hexane), because of the large differences in overall sensitivity between individual antennae, and to compensate the decline in antennal sensitivity during a measuring session. In this normalization procedure, the responses to the control were defined as 100%. The values obtained between two references (controls) were corrected according to the values of the references by linear interpolation. The Syntech EAG software calculated the normalized values automatically. The compounds were tested using 20 antennae of *D. saccharalis* males from Jaboticabal. The mean normalized responses of the different compounds were submitted to ANOVA for statistical analysis and compared using the Tukey test ($p < 0.05$).

Results and Discussion

All matings, regardless of insect age, occurred between the 3rd and the 8th hour of the scotophase, with a peak of 38–39% occurring at the 5th hour (Fig. 1). These results suggested that the best time for extracting the sex pheromone glands would be between the 5th and the 6th hour of the scotophase.

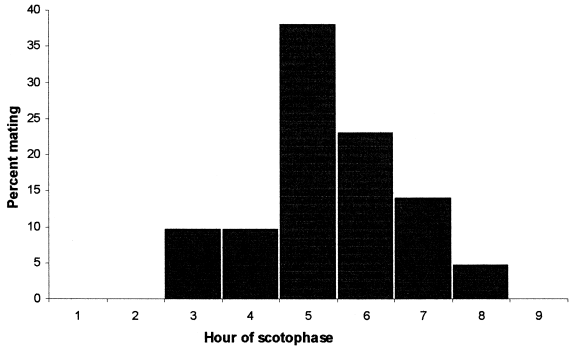

Fig. 1. Percentage of mating of *D. saccharalis* under laboratory conditions during the scotophase (24 ± 1 °C; 65% r.h.; 12 h photophase and n = 120 insects).

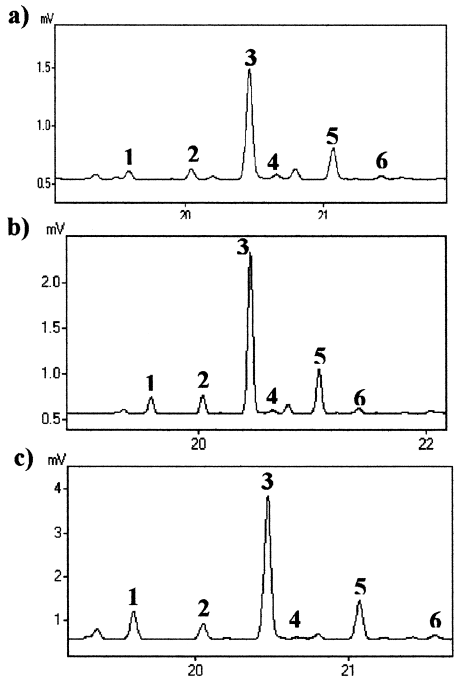

Fig. 2. Chromatograms of extracts of virgin females of *Diatraea saccharalis* from different locations in Brazil. Identification of peaks: (**1**) (*Z*)-hexadec-11-enal, (**2**) hexadecanal, (**3**) (9*Z*,11*E*)-hexadecadienal, (**4**) (9*E*,11*Z*)-hexadecadienal, (**5**) (9*Z*,11*Z*)-hexadecadienal and (**6**) (9*E*,11*E*)-hexadecadienal. a) 36 glands, origin Jaboticabal – SP. b) 51 glands, origin Campos – RJ. c) 30 glands, origin Piracicaba – SP.

Besides the previously identified pheromone component (9*Z*,11*E*)-hexadecadienal (**3**) (Carney and Liu, 1982), we found other isomers in minor quantities (Fig. 2). (9*Z*,11*E*)-Hexadecadienal was found as the major component of the gland extracts of insects from different locations. Six compounds could be identified by comparison of the retention times of their solutions with those of solutions of synthetic standards, and by analysis of the corresponding mass spectra: (*Z*)-hexadec-11-enal (peak 1), hexadecanal (peak 2), (9*Z*,11*E*)-hexadecadienal (peak 3), (9*E*,11*Z*)-hexadecadienal (peak 4), (9*Z*,11*Z*)-hexadecadienal (peak 5) and (9*E*,11*E*)-hexadecadienal (peak 6) (Figs 2a, b and c). (9*E*,11*Z*)-Hexadecadienal and (9*E*,11*E*)-hexadecadienal were present in traces and small quantities in the extracts, respectively (Fig. 3). The geometry of the dienes was corroborated by comparison of retention times on two GC columns with those of synthetic standards. Besides these compounds, the analysis of the mass spectra revealed the presence of straight chain alkanes and fatty acids with higher retention times, *i.e.* of lower volatilities.

Hedin *et al.* (1986) analyzed extracts of the sex pheromone glands of the southwestern corn borer, *D. grandiosella* Dyar, and verified the presence of six pheromone components, all being monoenic aldehydes with 16 and 18 carbon atoms: hexadecanal, (*Z*)-octadec-13-enal, (*Z*)-octadec-9-enal, (*Z*)-hexadec-11-enal, (*Z*)-hexadec-9-enal and (*Z*)-octadec-11-enal. Only (*Z*)-hexadec-9-enal, (*Z*)-hexadec-11-enal and (*Z*)-octadec-13-enal have proved to be as effective as the female in male attraction. Electrophysiological studies confirmed the requirement of these three compounds, but not hexadecanal, (*Z*)-octadec-9-enal and (*Z*)-octadec-11-enal. Gries *et al.* (1998) identified three aldehydes, (*Z*)-hexadec-11-enal, (*Z*)-hexadec-7-enal and (*Z*)-octadec-13-enal, as sex pheromone components in pheromone glands of the Pyralidae moth, *D. considerata* (Heinrich). Our comparison of the constituents of the pheromone glands of *D. saccharalis* with the compounds also found in the glands of the phylogenetically related species *D. grandiosella* and *D. considerata*, verifies that only (*Z*)-hexadec-11-enal is a common and effective pheromone component in the three species. Another difference is that (*Z*)-hexadec-11-enal is the major component in *D. grandiosella* and *D. considerata* while it occurs in small amounts in *D. saccharalis.*

There was only a slight variation, both in ratio and total quantities, in the gland contents of the three laboratory rearings. The gland extracts pre-

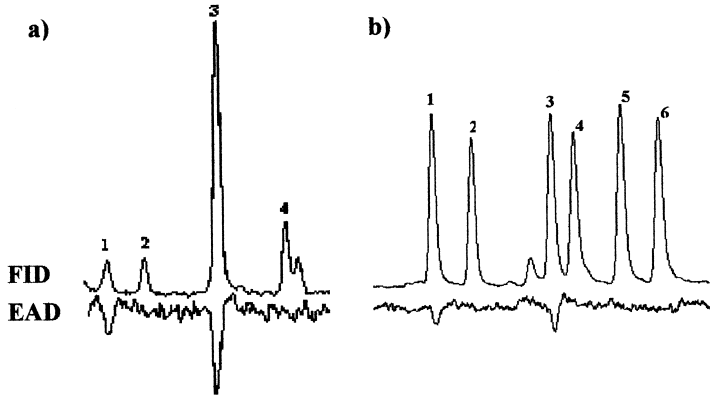

Fig. 3. Coupled gas chromatogram-electroantennogram (EAD) of a male *D. saccharalis* antenna, stimulated by compounds: (**1**) (*Z*)-11-hexadecenal, (**2**) hexadecanal, (**3**) (9*Z*,11*E*)-9,11-hexadecadienal, (**4**) (9*E*,11*Z*)-9,11-hexadecadienal, (**5**) (9*Z*,11*Z*)-9,11-hexadecadienal and (**6**) (9*E*,11*E*)-9,11-hexadecadienal. a) Extract of the pheromone glands of 51 virgin females (origin Campos). b) Mixture of synthetic compounds (0.1 mg/ml each).

sented no statistical difference of any component. The relative composition of the six candidate pheromone compounds found in the gland extracts of *D. saccharalis* from different origins (average of three gland extracts represented by three replicates each) was 4.3: 8.0:68.2:traces: 17.8:1.7 for (*Z*)-hexadec-11-enal (**1**), hexadecanal (**2**), (9*Z*,11*E*)-hexadecadienal (**3**), (9*E*,11*Z*)-hexadecadienal (**4**), (9*Z*,11*Z*)-hexadecadienal (**5**) and (9*E*,11*E*)-hexadecadienal (**6**), respectively.

The GC-MS verification of the presence of the isomers, in the female gland extracts, enabled us to analyze the gland extracts and standard solutions by GC-FID/EAD and, thereby, to determine which compounds elicited antennal response in male moths. GC-FID/EAD gave evidence of two reproducible antennal responses to all of the female pheromone gland extracts of different origins, as well as to the standard solution. As expected, the retention time of the compound, which elicited the strongest signal, coincided with that of (9*Z*,11*E*)-hexadecadienal (**3**) of the gland extract (Fig. 3a) and the standard solution (Fig. 3b), since compound **3** was the major pheromone component in this species and had proved attractive in field tests (Almeida *et al.*, 1983). A smaller but consistent antennal response was also observed from a minor component, whose retention time matched with that of (*Z*)-hexadec-11-enal (**1**). A response to the other isomers or to hexadecanal (**2**) was neither observed from any gland extract of the various origins, nor from the standard solution. The identities of the two active compounds in the pheromone gland extracts were confirmed by comparisons of retention times, elution patterns and by mass spectral analysis of the corre-

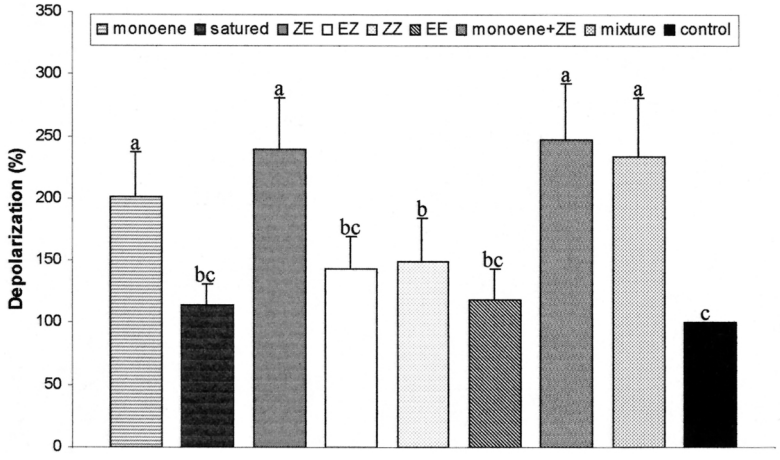

Fig. 4. Mean values (+S.D., $n = 20$) of EAG responses of *D. saccharalis* males to individual compounds [(**1**) (*Z*)-11-hexadecenal, (**2**) hexadecanal, (**3**) (9*Z*,11*E*)-9,11-hexadecadienal, (**4**) (9*E*,11*Z*)-9,11-hexadecadienal, (**5**) (9*Z*,11*Z*)-9,11-hexadecadienal and (**6**) (9*E*,11*E*)-9,11-hexadecadienal], binary mixture containing (**1**+**3**) and the mixture of synthetic compounds. Mean values marked with the same letter are not significantly different at $p < 0.05$ based on Tukey test.

sponding peaks. Electrophysiological recordings indicated that antennae from males of *D. saccharalis* distinguished between geometrical isomers of the two pheromone components, and invariably responded only to (*Z*)-hexadec-11-enal and (9*Z*,11*E*)-hexadecadienal.

The mean electroantennogram (EAG) responses to the possible pheromone compounds of *D. saccharalis* are shown in Figure 4. The experiments using the pure isomers **1** and **3**, the binary mixture of **1** and **3,** and a mixture of all of the synthetic compounds showed the highest depolarization in male antennae of *D. saccharalis*, demonstrating any statistical difference in these treatments. When we analyzed the responses elicited by others pure isomers, lower depolarization values were obtained with (9*E*,11*Z*)-, and (9*E*,11*E*)-hexadecadienal and hexadecanal, presenting no statistical difference when compared with the control, except with (9*Z*,11*Z*)-hexadecadienal, which showed an intermediate antennal activity. This corroborated the results observed in the EAD experiments with the gland extracts and the synthetic standard solution.

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