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Sex Pheromone of the Bud Borer *Epinotia aporema*: Chemical Identification and Male Behavioral Response

Paula Altesor · Carmen Rossini · Paulo H.G. Zarbin · Andrés González

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Abstract *Epinotia aporema* (Walsingham) is a Neotropical pest of legumes in southern South America. Its importance has increased during the last decade owing to the significant growth of soybean production in the region. Monitoring of *E. aporema* is difficult due to the cryptic behavior of the larvae, and hence, chemical control is carried out preventively. We analyzed the female-produced sex pheromone so as to develop monitoring traps and explore pheromone-based control methods. We analyzed pheromone gland extracts by combined chromatographic, spectrometric, and electrophysiological methods. Based on the comparison of retention indices, mass spectra, and electroantennogram (EAD) activity of the insect-produced compounds with those of synthetic standards, we identified two EAD-active compounds, (*Z*,*Z*)-7,9-dodecadienol and (*Z*,*Z*)-7,9-dodecadienyl acetate (15:1 ratio), as sex pheromone components of *E. aporema*. We also studied the behavior of males in wind tunnel tests using virgin females and different combinations of synthetic standards (15:1, 1:1, and 1:0 alcohol/acetate) as stimuli. A significantly greater percentage of males reached the chemical source with the 15:1 synthetic mixture than with any of the other treatments, indicating that these two compounds are pheromone components.

Keywords Sex pheromones · *Epinotia aporema* · Tortricidae · Wind tunnel · Lepidoptera

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P. Altesor · C. Rossini · A. González (✉)
Laboratorio de Ecología Química, Facultad de Química,
Universidad de la República,
Gral. Flores 2124,
Montevideo CP 11800, Uruguay
e-mail: agonzal@fq.edu.uy

P. H. Zarbin
Laboratorio de Semioquímicos, Departamento de Química,
Universidade Federal do Paraná,
Curitiba, PR, Brazil

Introduction

Soybean production in southern South America has increased dramatically in recent years. In Uruguay, for example, the planted area has expanded 30-fold during the period 2000–2007, causing a tenfold increase in the use of insecticides such as endosulfan and chlorpyrifos (Ferrari 2007). During the same period, the area of soybean production in Brazil grew to almost 60%, reaching more than 22 million hectares (Conab 2007).

Epinotia aporema (Walsingham, 1914) (Lepidoptera: Tortricidae) is a stem and bud borer that has become a major pest of legumes in Uruguay, Argentina, Chile, and southern Brazil (Iede and Foerster 1982; Sanchez et al. 1997; Pereyra and Sanchez 1998; Alzugaray et al. 1999). *E. aporema* originates from Costa Rica, but is now widely distributed throughout South America. The insect has five to six generations per year, mostly concentrated between November and April (Bentancourt and Scatoni 2006).

The economic importance of *E. aporema* is restricted to the south of its distribution due to the year-round availability of hosts (soybean, alfalfa, lotus, peas, flax, red clover) (Biezanko et al. 1957; Morey 1972; Bentancourt and Scatoni 1995; Alzugaray and Ribeiro 2000). In soybean, the larvae feed on vegetative plant parts, affecting the normal growth of the plant. In other legumes, larval feeding can severely affect the flower and prevent production of seeds, an important commodity in some forage

legumes (alfalfa, lotus) (Bentancourt and Scatoni 1995; Alzugaray and Ribeiro 2000).

E. aporema populations are monitored by direct observation of the larvae, a highly inefficient method with poor predictive capacity, and chemical control is, therefore, used in a prophylactic way. In order to develop a monitoring tool for the insect, we studied its sex pheromone. In this study, we report the characterization of the sex pheromone of *E. aporema* as a 15:1 mixture of (*Z*,*Z*)-7,9-dodecadienol and (*Z*,*Z*)-7,9-dodecadienyl acetate (hereafter referred to as Z7,Z9-12:OH and Z7,Z9-12:Ac, respectively).

Methods and Materials

Insects *E. aporema* adults were obtained from a laboratory population kept in an incubator, under a 14:10-h (L/D) photoregime, 21–23°C, and 60–70% relative humidity. The larvae were raised on an artificial diet based on bean homogenate and agar, either in glass tubes or in plastic cups. Virgin adults were obtained by separating male and female pupae.

Gland Extractions Female pheromone glands were dissected between the fifth and seventh hours of the scotophase. Virgin females (2–6 days old) were placed individually in glass tubes (10 cm length, 8 cm diameter; gauze-closed) during the photophase. Following the scotophase, calling females were removed from the vials, killed, and their glands exposed and excised with forceps. The glands of 83 females were accumulated in a 0.2-mL conical vial that was stored at −15°C. Pooled glands were extracted with 100 μL of distilled hexane for 20 min at ambient temperature, and the extract concentrated under a stream of N_2.

Chemical and Gas Chromatography–Electroantennogram Detection Analyses Gas chromatography–electroantennogram detection (GC-EAD) analyses were carried out using an HP 5890 Series II Gas Chromatograph adapted for simultaneous flame ionization (FID)/EAD detection. The column effluent was split with a fused silica outlet splitter (Alltech) with an extra make-up flow of N_2 (30 mL min^{-1}) added before the splitter. The column directing the flow to the EAD left the oven upwards into a condenser inner tube (1 cm diameter) where a stream of charcoal-filtered and humidified air (flow 300 mL min^{-1}) transported the volatile chemicals to the antennal preparation. The water in the condenser was kept cold by a circulating chiller. The antenna was located 1 cm downstream from the column exit and was held between antenna holders (Syntech, Hilversum, The Netherlands) with electrically conductive gel. The holders were in turn connected to a preamplifying probe (10X, Syntech), and the EAD response was further amplified with a high-impedance amplifier (Syntech). The analog signal was finally fed back to the GC motherboard for digitization and processing.

The GC-EAD was equipped with an Elite-WAX or an Elite-5 column (Perkin Elmer) (30 m×0.25 mm i.d., 0.25 μm film thickness), operated with a constant carrier gas flow of 2 mL min^{-1} (H_2). The temperature program for both columns was 70°C (kept for 1 min) to 250°C at 7°C min^{-1}. Injection was splitless with injector and detector temperatures of 220°C and 250°C, respectively.

GC–mass spectrometry (MS) analyses were carried out on a QP-2010 Shimadzu GC-MS, equipped with an AT™-WAX MS column (Alltech) (30 m×0.25 mm, 0.25 μm), operated with a constant carrier flow of 1 mL min^{-1} (He) and splitless injection. The initial temperature was 70°C (held 1 min), then increased to 250°C at 8°C min^{-1}. The injector temperature was 220°C, and the interface temperature was 250°C.

Retention indices (RIs) of the EAD-active components were determined by using the GC-EAD system by coinjecting a hexane solution of *n*-alkanes (even-numbered C8–C24, plus C19, 100 ppm each) with the gland extracts. RIs were calculated as defined for programmed temperature analysis (arithmetic index in Adams 2007).

Synthetic standards of the acetates were kindly provided by Tetsu Ando (Tokyo University of Agriculture and Technology) and Christer Löfstedt (Lund University). The alcohol standards were obtained by hydrolysis of the corresponding acetates (10% NaOH in ethanol, 100°C, 1 h).

Wind Tunnel Experiments The responses of males to virgin calling females and mixtures of synthetic standards were evaluated. The wind tunnel consisted of a cylindrical acrylic glass tube (150 cm length; 40 cm diameter) with a controlled suction pump, activated charcoal filters, and a metal screen to laminate the airflow (0.3 m s^{-1}, measured with a Testo 490 anemometer). Environmental conditions during wind tunnel tests were 20–25°C and 50–70% relative humidity. Dimmed red light was used for the observations. Males used in the experiments came from the laboratory colony. Four hours before testing, 1- to 5-day-old virgin males were placed in individual glass tubes (8 cm length, 4.5 cm diameter) with gauze enclosing both ends.

The stimuli (virgin females or pheromone septa) were hung at the tunnel entrance, centered, and inside a glass tube (10 cm length, 8 cm diameter; gauze-enclosed for the females). For the virgin female test, five females (2–5 days old) were placed in the tunnel 4 h before the experiments; for each male tested, at least one of the females was observed calling throughout the test period. Z7,Z9-12:OH and Z7,Z9-12:Ac (at mixtures of 15:1, 1:1, and 1:0 alcohol/acetate) were loaded (in 10 μL hexane) onto red rubber sleeve septa with a total dose of 1.6 μg per septum. Control septa were loaded

with 10 μL hexane. Septa were changed every day of the test with totals of ten males tested to each pheromone blend and 28 to the virgin females.

The tests were run between the third and seventh hours of the scotophase. Each tube containing a male was hung at the end of the tunnel, centered, and left closed for 1 min to allow the pheromone plume to reach the male. The gauze was then removed from both ends of the tube, and the behavior of the male was recorded continuously for 10 min. The behavioral elements observed were the time to initiate flight, response to the pheromone (including flying within the plume and/or wing fluttering), and reaching the odor source. Behavioral data were analyzed using analysis of variance and Dunnett's pairwise comparisons for the time of flight initiation [transformed to log (X+1)], and chi-square 2×2 contingency tables to compare the proportions of males arriving at the source and responding to the pheromone.

Results and Discussion

Chemical and GC-EAD Analysis Hexane extracts of pheromone glands [one female equivalent injected in 1 μL] elicited two consistent EAD responses from the male antennae. Only one of the EAD-active compounds was observed as a faint FID peak, while the other was only observed in the EAD trace (Fig. 1a; EAD responses shown with injection of synthetic compounds in Fig. 1b). Coinjection of the gland extracts and a mixture of *n*-alkanes in the GC-EAD system allowed us to calculate the RIs of both EAD-active compounds in the gland extract (2,076 and 2,155 on an Elite-WAX column; 1,529 and 1,664 on Elite-5). In addition, the GC-EAD analyses on both GC phases showed that the two EAD-active compounds inverted their relative elution order with the major compound eluting second on Elite-WAX (RI=2159) and first on Elite-5 (RI=1529). This inversion and the 135-unit difference in the RIs on Elite-5 suggested the presence of an alcohol/acetate pair in the mixture with alcohol as the major component. Moreover, the RI on both GC phases was around the region of RIs known for conjugated dodecadienyl alcohols (e.g., Δ5,Δ7: range 1,511–1,537 on DB5; 2,161–2,197 on DB-wax) and acetates (e.g., Δ5,Δ7: ranges 1,636–1,666 on DB5; 2,063–2,108 on DB-wax) (El-Sayed 2008). Previously, a 1:1 mixture of Z7,Z9-12:Ac and Z7,Z9-12:OH had been identified in the female gland of a European congener, *E. tedella* (Priesner et al. 1989), which prompted us to compare our pheromone extract with synthetic standards of these compounds. The standards were injected as a 100-ppm mixture in the GC-EAD system, giving strong EAD responses from *E. aporema* male antennae with identical retention times to the compounds in female extract that elicited the EAD signals (Fig. 1b).

GC-MS analysis of a concentrated (ten female equivalents per microliter) female gland extract revealed the presence of two peaks with identical retention times and mass spectra to those of Z7,Z9-12:Ac and Z7,Z9-12:OH standards (Fig. 2). The alcohol (both natural and synthetic) showed a clear M^+ peak (*m*/*z* 182) with a loss of 18 mass units and a weak fragment at *m*/*z* 31, both typical of primary alcohols. Both spectra showed the characteristic ions of aliphatic dienes (*m*/*z* 67, 82, and 96) and high abundance of the M^+ ion which, along with the long retention time on Elite-WAX (96 RI units larger than a

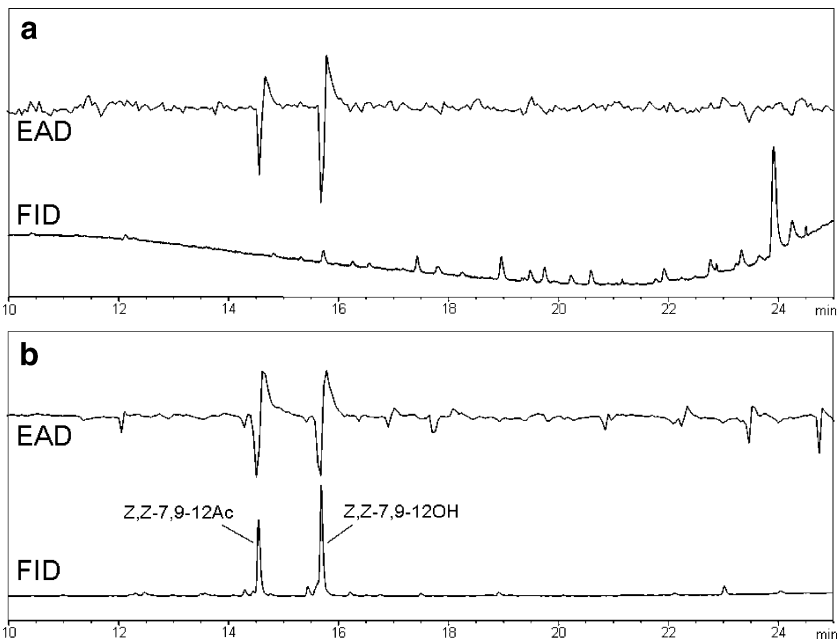

Fig. 1 Coupled flame ionization (*FID*)–electroantennogram detection (*EAD*) of **a** one female equivalent of *E. aporema* pheromone gland extract and **b** (*Z*,*Z*)-7,9-dodecadienyl acetate (*Z7,Z9-12:Ac*) and (*Z*,*Z*)-7,9-dodecadienol (*Z7,Z9-12:OH*) synthetic standards (100 ppm solution). Runs were performed consecutively on an Elite-5 column, each with a fresh male antenna, using identical temperature programs and splitless injection

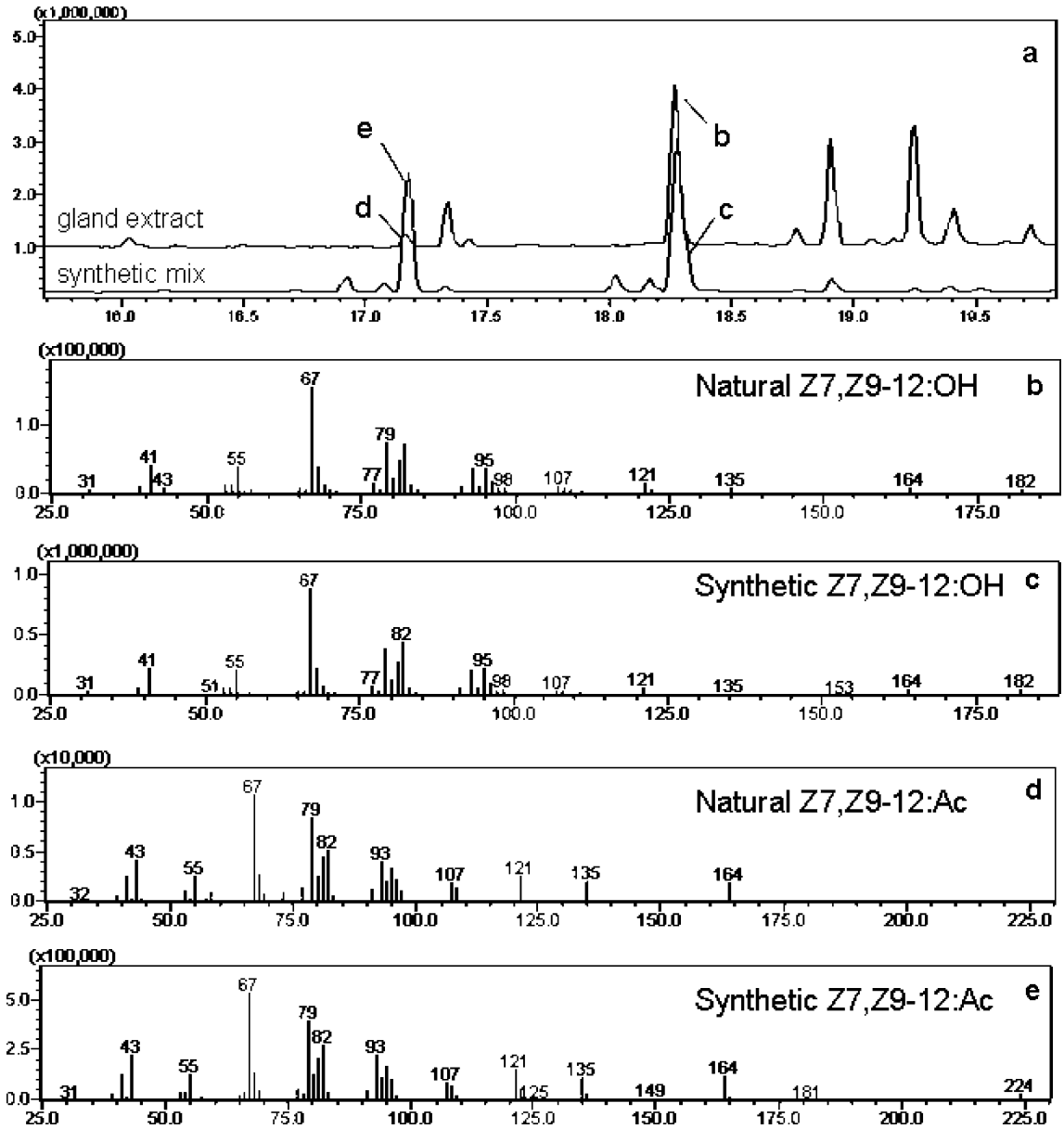

Fig. 2 Comparison of GC-MS analyses of ten female equivalent extracts of *E. aporema* pheromone glands (**a**, *upper trace*) and a synthetic mixture of (*Z*,*Z*)-7,9-dodecadienyl acetate (*Z7,Z9-12:Ac*) and (*Z*,*Z*)-7,9-dodecadienol (*Z7,Z9-12:OH*) (**a**, *lower trace*). The mass spectra of natural (**b**, **d**) and synthetic (**c**, **e**) compounds are shown. Runs were performed consecutively on an AT-WAX column, using identical temperature programs and splitless injection

nonconjugated dodecadienyl alcohol; El-Sayed 2008), indicated a conjugated dienyl system (Fig. 2b, c) (Ando et al. 2004). The natural and synthetic acetates showed almost identical spectra (Fig. 2d, e). The fragments characteristic of aliphatic dienes were also present with an additional fragment at *m*/*z* 43 (CH_3CO^+) and a molecular ion at *m*/*z* 224 in the synthetic sample. The absence of the molecular ion in the spectrum of the natural acetate is attributed to the low amount of the compound in the sample. The total ion chromatogram of gland extracts showed that the alcohol and the acetate were present in a 15:1 ratio.

A second set of comparative GC-MS analyses was performed with the pheromone gland extract and mixtures of geometric isomers of 7,9-dodecadienol and 7,9-dodecadienyl acetate. The retention time of the natural 7,9-dodecadienol matched that of synthetic Z7,Z9-12:OH (see Supplementary Figure S1), while the retention time of the natural Z7,Z9-12:Ac (Fig. 2d) matched that of synthetic Z7,Z9-12:Ac. There was an unknown compound in the gland extract that eluted at the same time as the E7, E9-12:Ac, but its mass spectrum (not shown) indicated that it was not a 7,9-12:Ac isomer.

These two compounds are produced by the European congener, *E. tedella*, in a 1:1 ratio (Priesner et al. 1989). In fact, most genera within the tribe Eucosmini (subfamily: Olethreutinae) include species that are attracted to Δ7 and/or Δ9 dodecenyl/dodecadienyl alcohols and acetates (El-Sayed 2008). Therefore, this Neotropical tortricid produces compounds closely related chemically to its closest relatives.

Wind Tunnel Experiments The time for male flight initiation ranged between a few seconds up to 3 min. The shortest time corresponded to the responses to virgin females, which was significantly shorter than the flight initiation time to the control. The tests with the synthetic pheromone blends resulted in intermediate flight initiation times, which were not significantly different from times to either virgin females or the control (Fig. 3a). In terms of response to the pheromone (including flying within the plume and/or wing fluttering), the synthetic pheromone blend with the same proportion as found in the female glands (15:1), the pure alcohol (1:0), and virgin females elicited the greatest responses with 70%, 60%, and 50% of males, respectively, responding to these treatments. However, only the 15:1 synthetic blend elicited a significantly greater percentage of males to respond than did the control and 1:1 (i.e., high proportion of acetate) treatments (Fig. 3b).

With respect to the arrival at the pheromone source, the 15:1 synthetic blend was the most attractive with 60% of males arriving at the septum within 10 min. In most cases, males arrived, left, and flew back to the source several times. The percentage of males arriving at the source for this treatment was significantly greater than for all other treatments. Less than 15% of males arrived at the calling females or the septa loaded with other blends, and no males arrived at the control septa (Fig. 3c).

Although the 15:1 proportion of the two pheromone components in the gland extracts may not reflect the actual proportion emitted by the female, the synthetic treatment with this ratio elicited the strongest responses from males, even at the low dose at which it was tested (1.6 μg). Although males responded to the alcohol-only treatment, indicating that this compound is probably crucial for male responses, the lower rates of males arriving at the source (compared to the 15:1 blend) indicates that the acetate is also an important component of the blend. The relatively low responses (arriving, especially) of males to the virgin females may be due either to an atypical or discontinuous pheromone emission because of the stressful conditions of the wind tunnel tests or simply to the low amount of pheromone females emit compared to that from the septa.

Pheromone-based monitoring of *E. aporema* could become an important tool for the integrated management of this pest in soybean crops in southern South America.

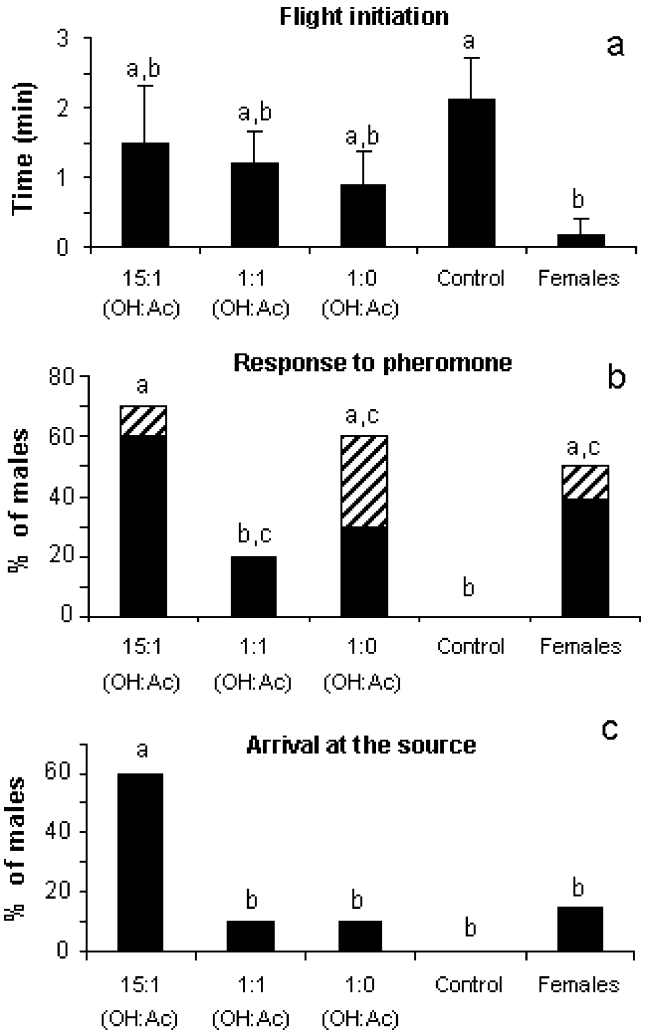

Fig. 3 Behavioral responses of *E. aporema* males to various treatments including virgin calling females and rubber septa loaded with different blends of (*Z*,*Z*)-7,9-dodecadienyl acetate (*Ac*) and (*Z*,*Z*)-7,9-dodecadienol (*OH*). **a** Time for flight initiation (mean minutes±SEM). **b** Percentage of males responding to the pheromone (*solid bars* flight within the plume, *striped bars* wing fluttering only). **c** Percentage of males arriving at the pheromone source. *Different letters above treatments* indicate significant differences at $P<0.05$

Control of this pest is complicated by the fact that the larvae are inside buds. Hence, monitoring of adults could be used to schedule insecticide applications more precisely, when larvae are exposed. The deficiency of a monitoring system not only results in overuse of insecticide, but also hinders the development of alternative control measures, such as use of insect hormone mimics or biological control. These alternatives need to be used within a specific time window during larval development, which is only possible after accurate detection of adult flight. Moreover, insecticides for the control of *E. aporema* are usually employed early in the growing season, causing a decline in populations of natural enemies that would otherwise control later-appearing noctuid moths. This, in turn, results in more

insecticides being applied later in the season; a trend that, combined with the expansion of soybean production, is threatening the sustainability of soybean and neighboring agroecosystems. To evaluate the potential of pheromone-based monitoring of *E. aporema*, field tests with various blends and pheromone doses are planned for the upcoming austral summer season.

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