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Article

(1*R*,2*S*,6*R*)-Papayanol, Aggregation Pheromone of the Guava Weevil, *Conotrachelus psidii*

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Comparative GC/MS analysis of airborne volatiles produced by males and females of the guava weevil *Conotrachelus psidii* (Coleoptera: Curculionidae: Molytinae) showed the presence of a male-specific electroantennographically active compound identified as (1*R*,2*S*,6*R*)-2-hydroxymethyl-2,6-dimethyl-3-oxabicyclo[4.2.0]octane (papayanol). Release of this compound was dependent on the photoperiod, taking place primarily during the scotophase, with a maximum release between 2 and 6 h after the onset of the scotophase. Y-tube olfactometer bioassays revealed a strong attractiveness of the papayanol to male and female weevils considering the presence of the plant volatiles.

Key words: GC/EAD, (1*R*,2*S*,6*R*)-2-hydroxymethyl-2,6-dimethyl-3-oxabicyclo[4.2.0]octane, pheromone release rate

Introduction

The guava weevil, *Conotrachelus psidii* (Coleoptera: Curculionidae: Molytinae), represents a problem for *Psidium guajava* L. orchards in some neo-tropical countries.[1-3] Both adults and larvae cause crop damages. Female adults lay eggs inside unripe fruits where feeding of the larvae causes rottenness, deformation and early ripening of fruits. Fully-grown larvae migrate to the soil and burrow down to pupate remaining there until weather conditions are favorable for the adult emergence. Adults feed on petioles, floral buds and peduncles of plants causing a decrease on guava production. In small orchard areas this insect is monitored and/or controlled by manual capture of adults, collection of infested fruits and by covering early developing fruits with bags. These actions are not practicable for large orchards being recommended insecticides application for adults control. However, larvae are not killed because its localization inside the fruits or soil.[4] Due to environmental and regulatory concerns, research on developing alternative control strategies is warranted. Since there has been a growing attention in understanding the chemical communication among weevils,[5] the identification of the aggregation pheromone of the *C. psidii* will help the farmers to minimize the damages caused by the presence of this specie on orchards as it has been studied for other curculio species.[6-8]

The identification of the pheromone of this guava weevil could be useful for the implementation of a pest management program to monitor or control the population as describe for others species of the curculionidae weevils.[6,7,9]

The goals of this study were to identify the pheromone component of *C. psidii* as well as to determine the emission pattern of the identified compound.

Experimental

Insects

Conotrachelus psidii adults of unknown age and mating status were collected in guava orchards in Linhares, Espirito Santo, Brazil, in June of 2011. Males and females were separated by sex based on the antenna insertion on the rostrum, hair on the abdomen surface[10] and structure of the tibiae of the fore legs. They were kept separately in plastic boxes (20 cm × 20 cm × 20 cm) in the laboratory under a under a photoperiod of 12 h light: 12 h darkness at at 25 ± 2 ºC and 60 ± 5% relative humidity. Adults were provided with fresh stems and fruits of guava.

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Collection of volatiles

Groups of 10 males and 10 females were maintained separately in 15 cm diameter × 33 cm long glass aeration chambers with a fresh stem of a guava tree (12 cm) and a small green guava fruit. A charcoal filtered humidified airstream was pushed through the aeration system at 1.5 L min^{-1}. Emitted volatiles were collected daily and trapped in glass tubes with 0.3 g of Altech HayeSep D 80-100 mesh (Lokeren, Belgium) and then eluted with 200 µL of distilled hexane.[11] Throughout this manuscript, this solution of natural volatiles in hexane is called "headspace extract". The headspace extracts were concentrated to one insect equivalent *per* 10 µL under an argon stream. Nine replicates were collected right after the pheromone emission was observed. The diel periodicity of release was measured by collecting the volatiles separately during both photophase and scotophase over a period of 3 days (n = 3 *per* treatment). To monitor the emission pattern of the target compound volatiles were collected during four successive scotophases each 2 h (n = 4 *per* treatment), period of higher release. Volatiles of guava plant were collected, without beetles being present, though.

Analytical procedures

Gas chromatographic analyses of extracts were carried out to detect sex-specific volatiles using a Shimadzu GC-2010 (Kyoto, Japan). The instrument was equipped with a J&W Scientific Inc. RTX-5 column (30 m × 0.25 mm i.d. and 0.25 µm film thickness) (Folsom, USA), and 1 µL of the extract was injected in the splitless mode with an injector temperature at 250 ºC. The program temperature of the column oven started at 100 ºC for 1 min, then it was raised to 250 ºC at a rate of 7 ºC min^{-1} and held for 7 min. Helium was used as the carrier gas at a column head pressure of 170 kPa. The Kovats Indices (KI) of the sex-specific component were calculated using a series of saturated C10-C26 hydrocarbons that were co-injected with the samples. Quantification of the sex-specific compound was based on an internal standard and an analytical curve using the synthetic pheromone at 10, 25, 50, 75 and 100 µg mL^{-1}.

For the analyses by gas chromatography with electro-antennographic detection (GC/EAD), the extracts were analyzed by GC/EAD using a Shimadzu GC-2010 and a Syntech electroantennography system (Hilversum, The Netherlands). The GC was operated with the same column as described above, using a temperature program starting at 70 ºC for 1 min, then raised to 250 ºC at a rate of 7 ºC min^{-1} and held for 7 min. One µL of the extract was injected in splitless mode at an injector temperature of 250 ºC. The column effluent was split 1:1, with one part going to the FID, and the other through a heated transfer line into a humidified airstream (280 mL min^{-1}) direct to a female antennal preparation. The antenna was fixed between two electrodes of stainless-steel using an electrically conductive Signa gel from Parker Labs (Fairfield, USA). GC/EAD recordings were analyzed with Syntech GC-EAD32 software (version 4.6).

Analyses by gas chromatography combined with mass spectrometry (GC/MS) of male extracts were carried out using a Shimadzu QP2010 PLUS MS system (Kyoto, Japan) equipped with a J&W Scientific Inc DB-5 capillary column (30 m × 0.25 mm i.d. and 0.25 µm film thickness (Folsom, USA) in electron impact mode at 70 eV. The injection was made in splitless mode for 1 min at 250 °C. The temperature program of the column started at 50 °C for 1 min, and then it was raised to 250 °C at a rate of 7 °C min^{-1} and held for 10 min. The transfer line was operated at 270 °C. Helium was used as the carrier gas.

Enantioselective gas chromatography was carried out on an Agilent 7890A equipment including a 5975C mass spectrometer (Santa Clara, USA), MSD ChemStation E.02.00.493 and a fused silica column (25 m × 250 µm i.d. and 0.25 µm film thickness) coated with heptakis (2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin (50 % in OV1701). Helium was used as the carrier gas at 110 ºC, isothermal. Signals were recorded as total ion current (TIC).

Chemicals

Racemic and optically active papayanol were obtained as previously described.[12] Tridecane used as internal standard was purchased from Aldrich Chemical (Milwaukee, USA).

Bioassays

Laboratory tests were performed to determine the attractiveness of the major compound during the period of higher release, between the 2nd and 6th hour of the scotophase. Bioassays were carried out using a dual choice horizontal Y-tube olfactometer of 2.5 cm diameter, 20 cm in length, 15 cm of arms and Y angle of 90° operated with a pre-humidified and charcoal filtered airflow of 2.5 L min^{-1}. After each test, the olfactometer was cleaned with a detergent solution, rinsed with alcohol and dried at 100 °C for 10 min to avoid contamination. The position of treatments was alternated after each replicate to avoid positional bias. The number of males and females evaluated varied from 26 to 44. One male or female was introduced into the base tube of the olfactometer, and the behavior was observed for 15 min. The choice was considered when the weevil moved 10 cm into one of the two arms. Individuals that did not make a choice during this time were excluded

from the statistical analysis. Age and mating status of the beetles were not controlled during the bioassays because of the difficulty of rearing these insects under laboratory conditions. The test consisted of an odor source to stimulate the insects described as follows: (*i*) natural male and host plant headspace obtained in the aeration (3 µL) versus host plant headspace extract (3 µL); (*ii*) synthetic pheromone component at 100 µg mL^{-1} (2 µL) plus host plant headspace extract (3 µL) *versus* host plant headspace extract (3 µL); (*iii*) synthetic pheromone component at 100 µg mL^{-1} (2 µL) versus hexane; and host plant headspace extract (3 µL) versus clean air. The synthetic pheromone is a mixture of isomers as showed in Figure 4c. For each replicate, we used a new piece of filter paper (1 cm × 1 cm) loaded with the various extracts or solutions of pure compounds. The evaporation time before starting the tests was 5 s.

Statistical analysis

Results of the diel periodicity of pheromone release during the photophase and scotophase as well as the emission during the scotophase were analyzed using one-way variance (ANOVA) followed by the Tukey test. Olfactometer bioassay data were compared by the X^2 test. The null hypothesis was considered 50:50 distribution. Statistical analyses were performed using BioStat 3.0[13] at a significance level of $p < 0.05$.

Results and Discussion

The comparison of gas chromatograms of the volatiles released by male and female weevils revealed the presence of one male-specific compound (Figure 1). Analysis of the headspace extracts of *C. psidii* males via GC/EAD repeatedly showed this male-specific component to elicit strong responses on the antennae of both male and female weevils (Figure 2).

Structure assignment of this male specific compound was carried out based on mass spectrometric data and also by comparison with an authentic standard. The mass spectrum (Figure 3) of the target compound (KI: 1266 on a DB-5 column) showed the following significant signals:

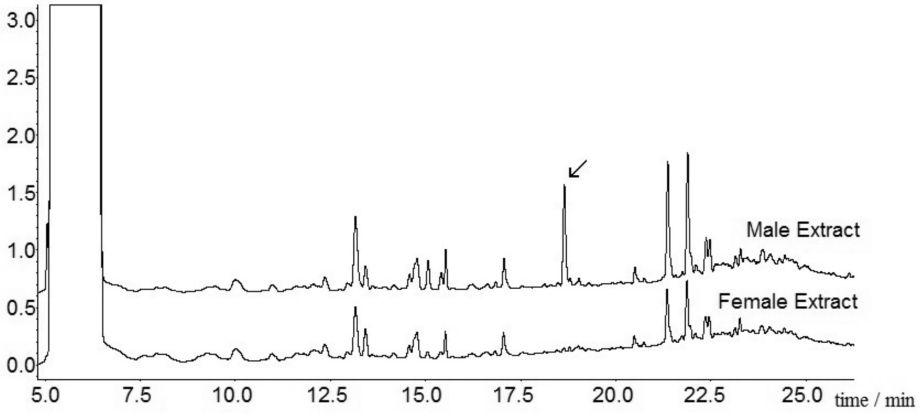

Figure 1. Gas chromatograms (FID) of headspace volatile extracts obtained from 24 h aeration of 10 males and females of *Conotrachelus psidii* containing host plant. The arrow indicates the male-specific compound.

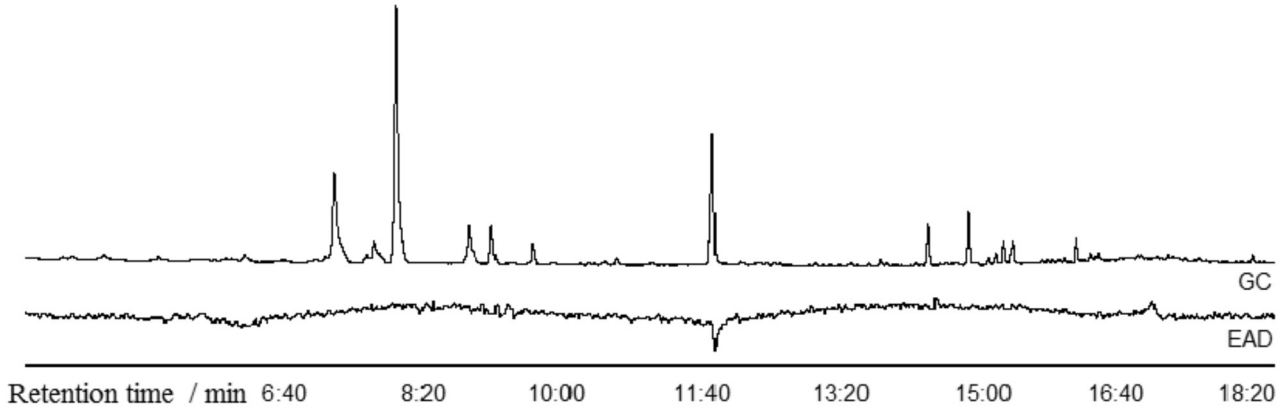

Figure 2. Simultaneously recorded GC/FID and EAD responses using a female *C. psidii* antenna stimulated by an extract of volatiles obtained during 24 h from 10 males.

m/z (%): 170(1) M^+, 139(100), 111(27), 81(30), 69(74), 43(50). The fragmentation pattern strongly resembled that of papayanol, a pheromone component of the papaya weevil, *Pseudopiazurus obesus.*[12] The identification of the compound was confirmed after co-injection with an authentic standard of papayanol.

The absolute configuration of the natural product was assigned according to results from the TIC-chromatogram employing a heptakis (2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin (50 % in OV1701) column. By comparison of the retention times of the natural and synthetic compounds, the male-specific compound of *C. psidii* was determined to be (1*R,*2*S,*6*R*)-2-hydroxymethyl-2,6-dimethyl-3-oxabicyclo[4.2.0]octane, accompanied by trace amounts of its (1*R*,2*R*,6*R*)-isomer (Figure 4). The same stereochemistry has been found for the papaya weevil, *P. obesus*.[12]

Analysis of the extracts obtained during three consecutive photophases and scotophases showed that *C. psidii* male release 85% of their pheromone during the scotophase ($p < 0.005$) (Figure 5a), with release rates gradually declining between onset and end of the scotophase (Figure 5b). Overall release rates ranged between 198 ± 27 to 496 ± 29 ng of pheromone *per* insect *per* day, and higher amounts of production were observed during the first six hours of the scotophase.

The presence of the host plant in the aeration chambers during the headspace extract collection could stimulate the pheromone release of male adults. This behavior has been documented occurring in some other curculionidae species such as *Anthonomus grandis,*[14] *Anthonomus musculus,*[15] *Rynchophorus phoencis,*[16] *Rynchophorus palmarum*,[17] *Pissodes strobe* and *P. approximates,*[18] among others.

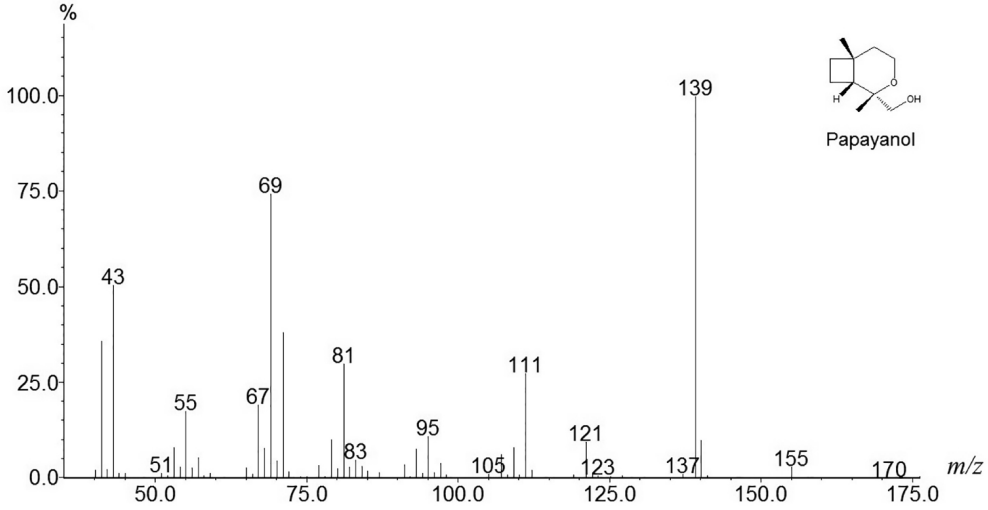

Figure 3. Mass spectrum and chemical structure of the natural male specific compound, papayanol.

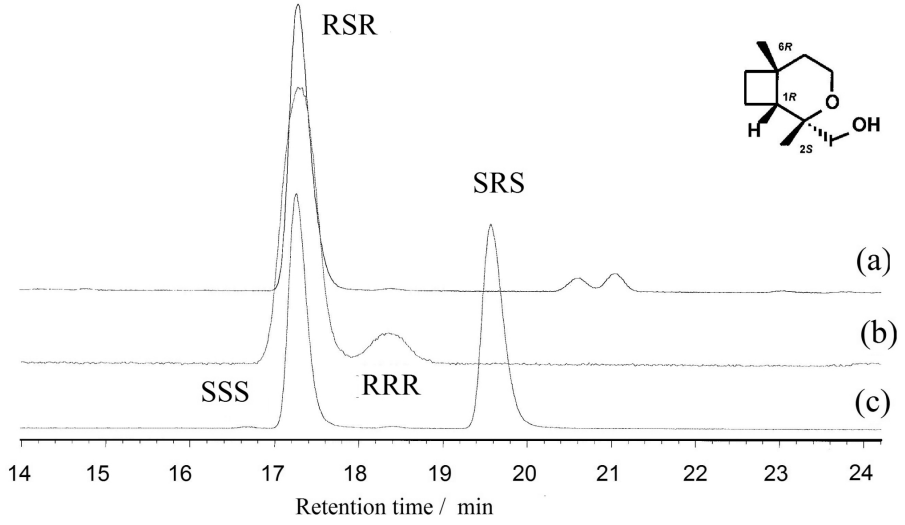

Figure 4. Total ion current (TIC) chromatogram of papayanol. (a): natural papayanol released by males; (b): synthetic (1*R*,2*S*,6*R*)-and (1*R*,2*R*,6*R*)-papayanol; (c): all four synthetic diastereomeres.

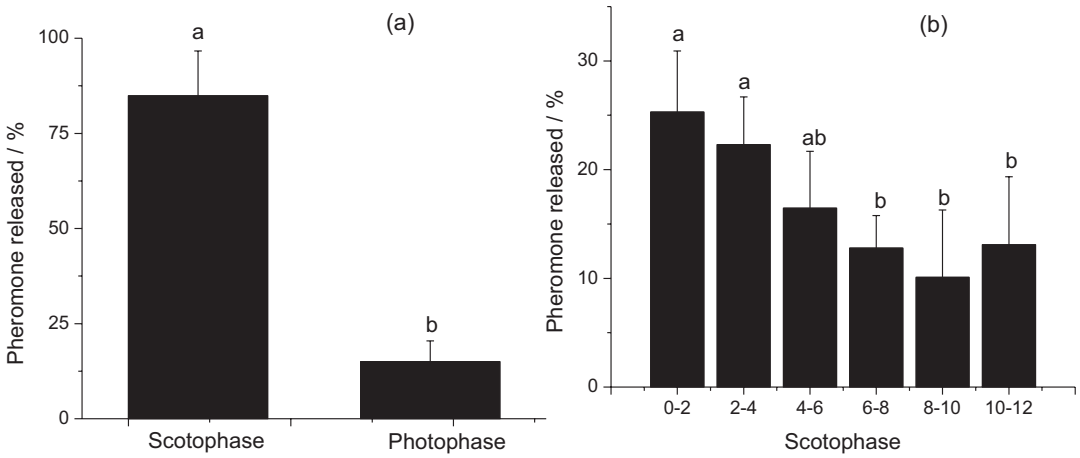

Figure 5. Pheromone released (%) of *C. psidii* males during photophase and scotophase in (a). Percentage of pheromone production (± SE) by *C. psidii* males during the scotophase. Means followed by the same letter are not significantly different using analysis of variance followed by Tukey test ($p < 0.05$) in (a).

Behavioral responses of *C. psidii* males and females in the Y-tube olfactometer to different odor sources are shown in Table 1. The attraction of males ($p = 0.018$; $X^2 = 6.43$) and females ($p < 0.0001$; $X^2 = 19.18$) to the natural headspace extract of males containing the host plant was significant as well as the attraction to the combination of host plant headspace extract with the synthetic pheromone component to males ($p = 0.010$; $X^2 = 7.41$) and females ($p = 0.0003$; $X^2 = 14.29$). On the other hand, neither the synthetic compound nor the host plant volatiles alone were attractive to males and females.

Male specific pheromone components have been identified in various curculionid species and have become useful for monitoring populations.[5,19] In the case of the subfamily Molytinae, *Conotrachelus nenuphar,*[6,7] *Sternechus subsignatus*[20,21] and weevils of the genus *Pissodes*[18,22] have been also described using male-specific pheromones as a part of their systems of chemical communication.

The positive response to the synthetic pheromone of *C. psidii* males and females was consistently synergized by the addition of host plant extract. The bioassays were carried out using the racemic pheromone in the ratio displayed in Figure 4c. The results of these bioassays revealed that the attraction of the racemic pheromone was very similar when compared with the attraction of the natural extract, that contains only one isomer, the (1*R,*2*S,*6*R*)-papayanol. This outcome let us to conclude that the biological activity is associated to the natural (1*R,*2*S,*6*R*)-isomer, and that the other isomers present in the synthetic sample did not show any antagonistic effect on the biological activity.

Generally the host plant volatiles are produced by plants as a result of oxidative degradation of surface lipids, including blends of six-carbon alcohols, aldehydes and esteres. These volatiles associated to pheromones have an ecological significance because they can influence the successful mate finding and consequently it is likely that they play an important role in reprodutive isolation.[23]

The positive influence of host plant volatiles in coleoptera was first reported in 1989.[14] Since then, this behavior has been observed in laboratory bioassays and in field tests for several weevils such as *Conotrachelus nenuphar,*[9] *Rhynchophorus* spp.[24-27] and *Dendroctonus ponderosae,*[28] among othes.

Based upon the strong attraction of male and female weevils observed in the behavioral bioassays and taking into

Table 1. Responses of individual male and female adults of *C. psidii* to treatments in a Y-tube olfactometer

Odor sources	Frequencies	
	Male	Female
Male extract *vs*. host plant		
Male extract	25*	22*
Host plant	10	1
Non-responders	2	3
Total	37	26
Synthetic compound + host plant *vs.* host plant		
Synthetic compound + host plant	28*	24*
Host plant	11	4
Non-responders	1	2
Total	40	30
Synthetic compound *vs.* Hexane		
Synthetic compound	8	7
Hexane	16	17
Non-responders	12	18
Total	36	42
Host plant *vs.* Air		
Host plant	18	9
Air	8	18
Non-responders	11	17
Total	37	44

*Statistically significant differences, using binomial test, $p < 0.05$

account the synergistic effect of the host plant volatiles we can suggest that males of *C. psidii* produce an aggregation pheromone.

Conclusions

In summary, our results demonstrate that, similar to the papaya weevil *Pseudopiazurus obesus,*[12,29] males of *Conotrachelus psidii* produce a male-specific compound, papayanol, which in the presence of plant volatiles is highly attractive to both sexes. Future trapping experiments in the field will be carried out to develop an environmentally safe method to monitor or control this insect in guava orchards using its aggregation pheromone in combination with plant volatiles.

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References

1. Bailez, O. E.; Viana-Bailez, A. M.; Lima, J. O. G. D.; Moreira, D. D. O.; *Neotrop. Entomol.* **2003**, *32*, 203.
2. Boscán, N.; Cásares, R.; *Agron. Trop.* **1980**, *30*, 77.
3. Vasquez, J.; Delgado, C.; Couturier, G.; Ferrero, D. M.; *Fruits* **2002**, *57*, 323.
4. Insuasty, B. O.; Monroy, R. R.; Dias, F. A.; Bautista, D. J.; *Manejo Fitosanitario del cultivo de la Guayaba en Santander*. ICA: Bogota, 2006.
5. Bartelt, R. J. In *Pheromones of non-lepidopteran insects associated with agricultural plants;* Hardie, J.; Minks, A. K., eds.; CABI Publishing: Wallingford, 1999, ch.4.
6. Eller, F. J.; Bartelt, R. J.; *J. Nat. Prod.* **1996**, *59*, 451.
7. Leskey, T. C.; Wright, S.; *J. Econom. Entomol.* **2004**, *97*, 79.
8. Piñero, J.; Aluja, M.; Vazquez, A.; Equihua, M.; Varon, J.; *J. Econom.Entomol.* **2003,** *96*, 334.
9. Piñero, J. C.; Prokopy, R. J.*; J. Chem. Ecol.* **2003**, *29*, 2735.
10. Silva-Filho, G.; Bailez, O. E.; Viana-Bailez, A. M.; *Neotrop. Entomol.* **2007**, *36*, 520.
11. Zarbin, P. H. G.; Ferreira, J. T. B.; Leal, W. S.; *Quim. Nova* **1999**, *22*, 263.
12. Zarbin, P. H. G.; Moreira, M. A. B.; Haftmann, J.; Tröger, A.; Franke, S.; Kopf, J.; Mori, K.; Francke, W.; *Org. Lett.* **2010**, *12*, 2447.
13. Ayres, M.; Ayres M. Jr.; Ayres, D.; Santos, A.; *BioEstat 3.0 Aplicações estatísticas nas áreas das ciências biológicas e médicas.* Sociedade Civil Mamirauá: Belém, 2003.
14. Dickens, J. C.; *Entomol. Exp. Appl.* **1989**, *52*, 191.
15. Szendrei, Z.; Averill, A.; Alborn, H.; Rodriguez-Saona, C.*; J. Chem. Ecol.* **2011**, *37*, 387.
16. Jaffé, K.; Sánchez, P.; Cerda, H.; Hernández, J. V.; Jaffé, R.; Urdaneta, N.; Guerra, G.; Martínez, R.; Miras, B.; *J. Chem. Ecol.* **1993**, *19*, 1703.
17. Oehlschlager, A. C.; Pierce, H. D. Jr.; Morgan, B.; Wimalaratne, P. D. C.; Slessor, K. N.; King, G. G. S.; Gries, G.; Gries, R.; Borden, J. H.; Jiron, L. F.; Chinchilla, C. M.; Mexzan, R. G.; *Naturwissenschaften* **1992**, *79*, 134.
18. Booth, D.; Phillips, T.; Claesson, A.; Silverstein, R.; Lanier, G.; West, J.; *J. Chem. Ecol.* **1983**, *9*, 1.
19. Mayer, M. S.; McLaughlin, J. R.; *Handbook of Insect Pheromones and Sex Attractants.* CRC Press: Boca Raton, 1991.
20. Ambrogi, B.; Palacio-Cortés, A.; Zarbin, P.; *J. Chem. Ecol.* **2012**, *38*, 272.
21. Ambrogi, B. G.; Zarbin, P. H. G.; *J. Appl. Entomol.* **2008**, *132*, 54.
22. Philips, T. W. W.; West, J. R.; Foltz, J. L.; Silverstein, R. M.; Lanier, G. N.; *J. Chem. Ecol.* **1984**, *10*, 1417.
23. Reddy, G. V. P., Guerrero, A. *Trends in Plant Sci.* **2008**, *9*, 253.
24. Gries, G.; Gries, R.; Perez, A. L.; Gonzales, L. M.; Pierce, H. D.; Oehlschlager, A. C.; Rhainds, M.; Zebeyou, M.; Kouame, B.; *J. Chem. Ecol.* **1994**, *20*, 889.
25. Rochat, D.; Meillour, P. N. L.; Esteban-Duran, J. R.; Malosse, C.; Perthuis, B.; Morin, J. P.; Descoins, C.; *J. Chem. Ecol.* **2000**, *26*, 155.
26. Weissling, T. J.; Giblin-Davis, R. M.; Gries, G.; Gries, R.; Perez, A. L.; Pierce, H. D.; Oehlschlager, A. C.; *J. Chem. Ecol.* **1994**, 20, 505.
27. Saïd, I.; Renou, M.; Morin, J.; Ferreira, J.; Rochat, D.; *J. Chem. Ecol.* **2005**, *31*, 1789.
28. Borden, J. H.; Pureswaran, D. S.; Lafontaine, J. P.; *J. Econ. Entomol.* **2008**, *101*, 1266.
29. Zarbin, P. H.; Moreira, M. A. B; Haftmann, J; Wittko, F.; Oliveira, A. R. M.; *J. Braz.Chem. Soc.* **2007**, *18*, 1048.

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