

Behavioral and Electroantennographic Responses of Adults of Guava Weevil, *Conotrachelus dimidiatus* (Coleoptera: Curculionidae), to Synthetic Host-Associated and Conspecific Volatiles

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Abstract

Immature guava fruits (*Psidium guajava* L.) of commercial orchards in Mexico are affected by adults of *Conotrachelus dimidiatus* (Champion) causing economic losses to producers. The aim of this study was to assess the behavioral and electrophysiological responses of the weevil to selected semiochemicals emitted by the host plant fruit and to conspecifics. Volatile organic compounds isolated from host plant as β -caryophyllene, (S)-(–)-limonene, hexanal, and nonanal significantly attracted adults of both sexes in behavioral bioassays. Electroantennogram recordings showed largest responses to papayanal and grandlure III + IV released by *C. dimidiatus* and *C. nenuphar*, respectively. Our results indicate that behavioral and electroantennographic assays using synthetic compounds from host plant volatiles and insect volatile pheromones produce olfactometry and electroantennographic responses in *C. dimidiatus*. This is the first report of intraspecific chemical communication in this weevil. We discuss the significance of these responses for the natural behavior in guava orchards and their potential use in a pest management strategy.

Key words: behavioral bioassay, electroantennography, *Psidium guajava*

The cultivation of the guava, *Psidium guajava* L., is of great importance in Mexico that is considered the fifth largest producer in the world with 311,594 Tons being United States the main customer. In United States, 94.5% of the consumed guava comes from Mexico (SAGARPA 2018). This fruit is characterized by its low caloric intake and high content of vitamin C, which is up to seven times higher than that of an orange. Among the main production factors that affect this crop are water scarcity, low use of fertilizers, and inadequate pruning. In addition, guava tree is affected by various pest organisms whose populations damage the fruits and cause losses in production (González-Gaona et al. 2002).

Although the genus *Conotrachelus* Dejean, 1835 (Coleoptera: Curculionidae: Molytinae) is considered one of the most numerous weevil genera (O'Brien and Couturier 1995), just three species have been reported attacking guava fruits in commercial orchards. Two species in North America: *Conotrachelus dimidiatus* (Champion) and *Conotrachelus copalensis* (Salas-Araiza and Romero-Nápoles 2012) and *Conotrachelus psidii* (Marshall 1922) in South America

(Romero-Frías et al. 2015) of which *C. dimidiatus* is the one that causes the greatest damage in Mexico (Tafoya et al. 2010).

Positive attraction responses of Coleopteran to compounds produced by other related species have been reported for *Carpophilus humeralis* (F.) (Zilkowski, B.W., and Bartelt 1999) and between cerambycid beetles *Cotyclytus curvatus* (Germar) and *Megacyllene acuta* (Germar) (Silva et al. 2018). Recently, components of aggregation pheromones of *C. psidii* were reported (Palacio-Cortés et al. 2015, Romero-Frías et al. 2016), and cross-attraction among species could also occur between *C. dimidiatus* and *C. psidii*. Moreover, recent qualitative analysis of insect emissions suggests that for guava weevil there are chemical compounds analogous to those of curculionids.

The objectives of this study were to test the hypothesis that synthetic volatiles isolated from guava fruit, other weevils, and conspecifics can elicit attracting response in adults of guava weevil measured by olfactometric and electroantennographic techniques under laboratory conditions.

Materials and Methods

Insects

Adults of *C. dimidiatus* were collected with a blanket on the floor and shaking the tree from guava orchards in Aguascalientes, Mexico, during 2016 for olfactometric and 2017 for electroantennographic experiments. Insects were transported to the laboratory in Puebla, Mexico, in polyethylene flasks and separated by sex after arrival using dichotomic keys according to Salas-Araiza and Romero-Nápoles (2012). They were kept separately in polyethylene flasks in the laboratory under a photoperiod of 12:12 (L:D) h at 23°C and 24% RH. They were fed with immature guava fruits and a source of water.

Chemicals

Chemicals with 98–99.7% purity from Sigma–Aldrich (St. Louis, MO) were used for standards preparation. Synthetic compounds assayed in the olfactometric and electroantennographic (EAG) tests correspond to defined isomers, whereas the compounds detected in fruit releases are referred generically in the manuscript. Terpenoid reference compounds were acquired from several sources: grandlure I (Chemos GmbH, Regenstauf, Germany), grandlure II (Bedoukian Research, Danbury, CT), and grandlure III + IV (Bedoukian Research). Grandisoic acid was extracted from pellets (Chemtica International, Costa Rica). Papayanol and papayanal were donated by colleagues (see Acknowledgments). Solutions were prepared by dissolving reference standards in hexane. Acetone (≥99.9%, Burdick & Jackson), Extran (Merck), and deionized water were used to clean the olfactometer.

Olfactometric Experiments

A Y-tube olfactometer with 15 mm ID and two arms 12 cm long at 75° was used to test the response of *C. dimidiatus* female and male to synthetic compounds. Humidified and purified air was passed from an air pump into each of the olfactometer arms at a rate of 1 liter/min and inside of a 1 m³ acrylic box. Illumination in the room was provided by an IR light bulb (50 W) at 1 m from the box. Assays were performed starting at 20 h in a dark room at 23°C and 24% RH. The nighttime activity of *C. dimidiatus* was previously observed in the field (Pérez-Torres et al. 2018), where the weevils flew and copulated since sunset, while daytime they kept hidden resting. Similar behavior was observed and considered in the olfactometric bioassays of *C. psidii* (Palacio-Cortés et al. 2015, Romero-Frías et al. 2015).

The concentration of 10 ng/μl was chosen as in Romero-Frías' (2015) work for *C. psidii* and *Scyphophorus acupunctatus* Gyllenhal (Altuzar et al. 2007). A test strip (12.5 × 6 mm) was loaded with 10 μl of the sample prepared in hexane, with the solvent being allowed to evaporate before the strip was placed into one arm of the Y-tube. A similar strip impregnated with 10 μl of hexane was placed in the second arm as a control. Female or male *C. dimidiatus* were individually released at the base of the central olfactometer arm and observed during 5 min and when remained 1 min into any of the lateral arms were recorded as positive or negative responses. Experimental weevils marched only once, and after each test, the olfactometer was rotated 180°, cleaned with acetone, Extran, and dried at 100°C for 2 h. The total number of replications was as follows: Individual females marched: 20 in hexanal, 25 in (S)-(-)-limonene, 20 in β-caryophyllene, 18 in grandlure III + IV, 23 in grandisoic acid, 20 in papayanol, and 20 in papayanal procedures.

Individual males marched: 20 in hexanal, 20 in (S)-(-)-limonene, 20 in β-caryophyllene, 25 in grandlure III + IV, 19 in grandisoic acid, 21 in papayanol, and 25 in papayanal tests. For the eight compounds chosen to test the olfactometric responses of *C. dimidiatus*, three of them come from immature guava: hexanal, β-caryophyllene, and (S)-(-)-limonene. Five other volatile compounds are released by *C. dimidiatus*: grandlure III + IV, grandisoic acid, papayanol, and papayanal.

Electroantennography Experiments

A pair of antennae of females of *C. dimidiatus* was cut off at the antennal scape using dissection tweezers under a stereomicroscope. We only tested the antennae of females because initially we aimed to clarify the preference of the females for the volatiles. The antennae were excised, mounted between the electrodes, and fixed with gel to guarantee good electrical contact. The signals were acquired with an EAG signal acquisition interface controller (model IDAC-2) and puffs with model CS-55 flow controller, both from SYNTech (Hilversum, The Netherlands). For the test, the stimulus air controller provided a humidified airflow toward the antenna (45 ml/min). The tested synthetic compounds were 10 μl of each tested solution (10 ng/μl) applied to a 12.5 × 6 mm test strip, solvent being allowed to evaporate, inserted into a Pasteur pipette, and placed into the hole of the glass tube oriented toward the antennae. The control stimulus was a strip impregnated only with 10 μl of hexane. The volatile stimuli were provided as puffs (2-s duration at 45 ml/min). Ten puffs of the same volatile were delivered 30 s apart from each other. The responses were recorded with Syntech software (EAG Pro 2000), and their amplitude was considered absolute. We tested ten puffs of each single compound with one pair of antennae. Then, the antennae were discarded. Next, a new pair of antennae was tested with a different compound. For each compound, the procedure was repeated twice with fresh antennae. Grandlure III + IV were measured in triplicate. The first and last differential response was discarded, and the mean value of eight puffs is reported.

Statistical Analysis

Statistical analysis was performed using a binomial error distribution, given that a two-path olfactometer-based assay has two possible responses. A 2 × 2 contingency table and the χ^2 test were used to compare the response of females and males to each terpenoid volatile. We compared the portion of females and males that chose the olfactometer arm with the attractant or the control, excluded the nonresponders. The null hypothesis was that the insect's sex and the olfactory response toward different terpenoid are independent of each other. The χ^2 equation used was as follows: $\chi^2 = \sum_{i=1}^r \sum_{j=1}^c \frac{(O_{ij} - E_{ij})^2}{E_{ij}}$ where O_{ij} is the observed value and E_{ij} the expected value of the nominal variables arranged in r rows and c columns. The critical value of χ^2 statistics $\chi^2_{\alpha, df}$ is determined by the level of significance $\alpha = 0.05$ and the degree of freedom (df) = $(r - 1)(c - 1)$. The normality test (Shapiro–Wilk test) was done for the EAG data for each compound, as well the descriptive statistics. Statistic W and P values were computed and compared with a significance level of 0.05. One-way ANOVA and Tukey's test were applied to observe a multiple comparison between the means of responses to volatile compounds. This analysis and all descriptive and nondescriptive statistical parameters were performed using OriginLab 8.5 SR1.

Results

Olfactometric Response of Female and Male *C. dimidiatus* to Single Volatiles

Grandisoic acid was preferred by the females more than by the males and was the only terpenoid that elicited a significantly different response between females and males ($\chi^2 = 4.49$, $df = 1$, $P = 0.03$). In contrast, the response of both sexes was not significantly different for any of the other compounds (Fig. 1). β -Caryophyllene was the most attractive volatile compound for males and the preference of the weevils for this terpenoid compound is similar for both sexes (80% males and 70% females). However, no significantly different compound attraction behavior was observed for females ($\chi^2 = 4.23$, $df = 6$, $P = 0.64$) and males ($\chi^2 = 12.13$, $df = 6$, $P = 0.06$) at $P < 0.05$.

EAG Response of Female *C. dimidiatus* to Single Volatiles

Synthetic volatiles tested in the olfactometry experiments were examined individually by EAG at a single concentration of 10 ng/ μ l (Fig. 2). They included the volatile organic compounds isolated from the insects as well as the most abundant chemical compounds emitted by immature fruit in the orchard daytime: limonene (21%), *cis*-3-hexenyl acetate (19%), hexanal (17%), nonanal (14%), and caryophyllene (2%). Overnight emissions of immature guavas contained mainly limonene (332.8 ± 5.6 μ g/g) and caryophyllene (33.9 ± 0.1 μ g/g; Elizalde-González and Segura-Rivera 2018).

EAG data for individual compounds showed a normally distributed population: β -caryophyllene: $W = 0.92$, $P = 0.44$; *cis*-3-hexenyl acetate: $W = 0.95$, $P = 0.75$; grandisoic acid: $W = 0.93$, $P = 0.56$; grandlure III + IV: $W = 0.85$, $P = 0.11$; hexanal: $W = 0.90$, $P = 0.32$; nonanal: $W = 0.97$, $P = 0.93$; (S)-(-)-limonene: $W = 0.96$, $P = 0.85$; papayanal: $W = 0.92$, $P = 0.43$; papayanol: $W = 0.91$, $P = 0.36$; hexane: $W = 0.91$, $P = 0.38$. Descriptive statistics such as mean and SD are shown in Table 1.

The level of significance in EAG responses of female *C. dimidiatus* to 10 volatile compounds and hexane as a control was statistically different (Table 1). Despite producing the lowest concentration

in the mentioned volatile's mixture, caryophyllene exhibits the highest antennal response (3.86 ± 0.08 mV) among the host plant volatiles, whereas, among the weevil volatiles, grandlure III + IV generates the second highest value (3.46 ± 0.16 mV; Fig. 2). Nonanal, present in the emissions of immature fruit, in comparable amounts (14%) to hexanal (17%), produced a signal of 3.32 ± 0.09 mV that was statistically similar than the signal of the grandlure III + IV mixture ($F = 4.58$; $df = 1$; $P = 0.05$). When comparing responses of EAG, papayanol (0.78 mV) was lower than the response toward papayanal (1.63 mV) for females ($F = 238.29$; $df = 1$; $P = 0.58 \times 10^{-6}$) and the increase in papayanal concentration did not rise the signal proportionally (Table 1).

Discussion

In the study performed by Pérez-Torres et al. (2018), behavioral field studies of *C. dimidiatus* were described. The adult weevils usually go toward the guava trees and live on them. The trees provide food and a niche for their eggs and larvae (Aragón García et al. 2015). In the laboratory, the olfactometric trials showed that both sexes of *C. dimidiatus* are attracted by volatile compounds identified in immature fruit. These responses of the weevils are probably mediated by kairomones emitted by the host. This statement is supported by the olfactometric and EAG results, where the response of the males of *C. dimidiatus* to caryophyllene was stronger than that of females. The response of males to β -caryophyllene was also stronger compared with other tested volatile compounds ($P = 0.44 \times 10^{-7}$). This result is similar to that obtained for raspberry weevil, *Aegorhinus superciliosus* (Guérin), to the host volatiles limonene and α -pinene. One interpretation of this would be the consistent search of host plants by male weevils because females are likely to look for oviposition and feeding sites, with the subsequent encounters and mating opportunities for males (Mutis et al. 2010). In the case of the females, their response was stronger to β -caryophyllene (3.86 mV) than that to (S)-(-)-limonene (1.59 mV; Table 1). The results of Romero-Frías et al. (2015), who found a lesser response of females and males of *C. psidii* to caryophyllene in comparison to limonene,

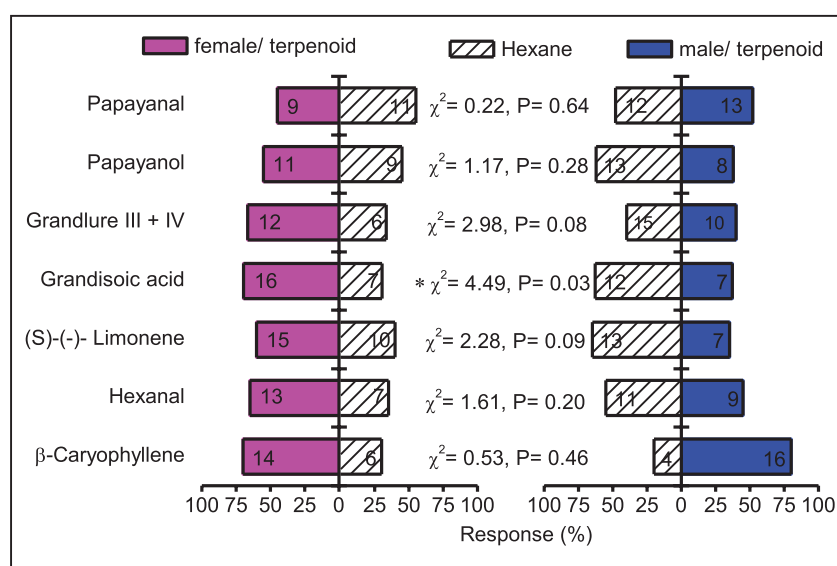


Fig. 1. *Conotrachelus dimidiatus* preference in the Y-tube olfactometer test. Depicted is the number of females ($n = 146$, left) and males ($n = 123$, right) choosing between seven different terpenoids and hexane as control. Chi-square test (χ^2): $P < 0.05$ values in the middle. Asterisk indicates a significant difference in the preference of females and males toward grandisoic acid.

could be explained by an evolutionary divergence of species with common ancestors but now occupying different habitats. All these data of the olfactometric and EAG responses of *C. dimidiatus* toward β -caryophyllene and (*S*)-(-)-limonene provide evidence for the potential function of these compounds as kairomones, to locate food or a possible host. Limonene has also been suggested as a kairomone for the weevils of *C. psidii* (Romero-Frías et al. 2015), and it has been used as an attractant in traps for monitoring *C. dimidiatus* (Tafoya et al. 2011).

Grandlure III + IV, the aggregation pheromones of *Anthonomus musculus* Say (Szendrei et al. 2011), produced the most intense signal (3.46 mV) among the identified C_{10} terpenoid volatiles in our EAG tests with *C. dimidiatus*. The olfactometric trials showed that besides grandlure III + IV, grandisoic acid also was more attractive for females (Fig. 1). These three compounds were identified in emissions of females and males of *C. dimidiatus* and are being reported in a paper in progress. Hence, males and females of *C. dimidiatus* showed similar response in the olfactometric trials to the tested immature fruit and conspecific volatiles, except grandisoic acid (see Fig. 1). It is probable that all three compounds are involved in the signalization of the insects.

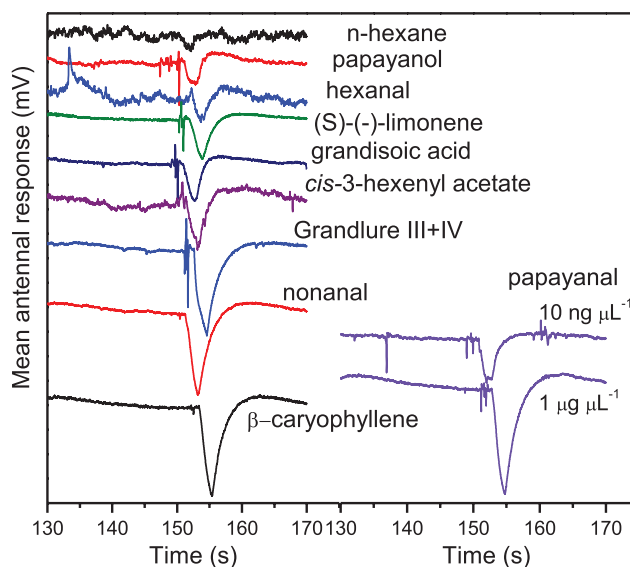


Fig. 2. Registration of the mean ($n = 8$) EAG response of female *C. dimidiatus* toward single volatiles (10 ng/ μ l), and control. Papayanol concentration 10 ng/ μ L and 1 μ g/ μ l.

The implication of this assumption is that the EAG study must also be accomplished with males. Male and female emissions of *C. dimidiatus* also presented grandisoic acid, a male-produced aggregation pheromone from the plum weevil, *C. nenuphar* (Eller and Bartelt 1996). Other authors demonstrated that grandisoic acid was important in the capture of *C. nenuphar* in combination with benzaldehyde (Altuzar et al. 2007, Leskey et al. 2014). For this reason, it was tempting to test the response of *C. dimidiatus* to benzaldehyde in bioassays even though this does not correspond to an emission from the insect. However, the olfactometric response of both sexes of *C. dimidiatus* to benzaldehyde was less than 40%, and no response was obtained by EAG performed with females. Therefore, we excluded these trials in Figs. 1 and 2, and Table 1. Although authors reported that grandisoic acid did not increase insect capture globally, more *C. dimidiatus* females than males were collected in the grandisoic acid-containing traps (González-Gaona et al. 2010). However, this result should be interpreted with care due to the small number of insects captured: five females versus three males and because the method of sexing in the field experiments is not clear.

In the EAG trials with *C. psidii* in Colombia, the male-specific volatile papayanol exhibited a slightly greater intensity (1.11 mV; Romero-Frías et al. 2015) in comparison to *C. dimidiatus*. In our study, a 100-fold increase in the papayanol concentration improved the signal intensity threefold. This led us to affirm that the concentration applied to *C. dimidiatus* study was enough to produce a reliable signal.

Conclusions

This study provides the first evidence of electroantennographic and behavioral responses of *C. dimidiatus* to synthetic host-associated and conspecific volatiles. Our result showed that the single volatile compounds such as caryophyllene, limonene, hexanal, and nonanal from the host plant were the most attractive substances for *C. dimidiatus*. These compounds could be used by the weevils as a kairomone to find sites for feeding and oviposition. On the other hand, the compounds grandisoic acid and grandlure III + IV released by the insect were the most attractive compounds for females. These results under laboratory conditions have implications for improving pest control in the orchard by using traps. From the current results, future work concerning the study of the behavioral response to blends of pheromones with selected host plant volatiles will be done with the potential to promote a restrictive effect and reduce feeding and oviposition of adult weevils in guava orchards.

Table 1. EAG responses of adult *Conotrachelus dimidiatus* females to selected synthetic single volatile compounds with concentration 10 ng/ μ l and statistical parameters

Volatile compounds released from		Mean \pm SD (mV)	F	df	P	SEM
Guava	Insect					
β -Caryophyllene		3.86 \pm 0.23	1067.74	1	0.44 $\times 10^{-7}$	0.08
	Grandlure III + IV	3.46 \pm 0.16	1226.45	1	0.31 $\times 10^{-8}$	0.06
Nonanal		3.32 \pm 0.09	1674.27	1	0.38 $\times 10^{-8}$	0.03
	<i>cis</i> -3-Hexenyl acetate	2.49 \pm 0.38	141.92	1	0.11 $\times 10^{-4}$	0.15
(S)-(-)-Limonene	Papayanol	1.63 \pm 0.08	238.29	1	0.58 $\times 10^{-6}$	0.03
	Papayanol, 1 μ g/ μ l	4.20 \pm 0.31	7.60 $\times 10^{-14}$	1	0.11 $\times 10^{-6}$	0.11
	Grandisoic acid	1.59 \pm 0.06	239.77	1	0.25 $\times 10^{-5}$	0.02
Hexanal		1.39 \pm 0.06	144.52	1	0.10 $\times 10^{-4}$	0.02
	Papayanol	0.78 \pm 0.19	2.02	1	0.18	0.07
		0.78 \pm 0.07	4.23	1	0.03	0.02

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