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Behavioural and electrophysiological responses of the small avocado seed borer, *Conotrachelus perseae* to avocado leaf, flower and fruit volatile extracts

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Abstract

The small avocado seed borer, *Conotrachelus perseae* Barber (Coleoptera Curculionidae), is an important insect pest of avocado (*Persea americana* Miller, Lauraceae) in Mexico. In this study, we showed that both sexes of *C. perseae* were attracted to flower, fruit, and leaf avocado (cv. Hass) volatile extracts, compared to a solvent control, in a Y-tube olfactometer. Among extracts, females were more attracted to flower and fruit extracts than to leaf extract, while males did not show preference for any of the extracts. In addition, females also showed stronger electroantennogram (EAG) responses to flower than to leaf extracts, while their EAG responses to fruit extracts were intermediate between their responses to flower and leaf extracts. In contrast, males did not show any differences in their EAG responses to the three extracts. Gas chromatography-mass spectrometry analyses showed that flower extracts contained a mixture of compounds including hydrocarbons, monoterpenes (hydrocarbons and oxygenated), and sesquiterpenes. Monoterpenes were the main components in flower extract, while sesquiterpenes were the main components in fruit and leaf extracts. We demonstrated that *C. perseae* females and males responded behaviourally and electrophysically to leaf, flower and fruit volatiles of *P. americana* (cv. Hass). These results can help to formulate an attractant for *C. perseae*. However, further work is needed to elucidate the crucial compounds involved in weevil attraction.

Key words: kairomones, *Persea americana*, avocado flower volatiles, Coleoptera Curculionidae.

Introduction

The small avocado seed borer, *Conotrachelus perseae* Barber (Coleoptera Curculionidae), is a major pest of avocado fruits, *Persea americana* Miller (Laureceae). This weevil species is distributed in several avocado-growing regions of Mexico, causing severe damage to production (Llanderal and Ortega, 1990; Téliz *et al*., 2000; CESAVEEM, 2010; Vazquez *et al*., 2015). The female perforates with her rostrum and lays eggs directly in the fruit pulp, preferring the basal part of the fruit for oviposition. Upon hatching, larvae begin to bore through the fruit pulp, making an increasingly larger gallery on their way to the seed. The larvae enter the seed and make galleries in all directions, sometimes destroying the seed completely (Diaz, 1972). On average, 5.4 larvae can develop per fruit (SENASICA, 2016). Later, mature larvae leave the fruit and drop into the soil to pupate. Adults feed on leaves, flowers and young fruits (Coria, 1999; Francia, 2008).

Control of *C. perseae* in all avocado-growing regions of the country is based on applications of the chemical insecticide malathion and permethrin (SENASICA, 2010). Because of the cryptic habits of *C. perseae*, chemical control has little effect, spurring more insecticide applications, which increase environmental and health risks (Damalas and Eleftherohorinos, 2011). Under this scenario, it is necessary to investigate other strategies for management of *C. perseae* populations.

A number of studies have documented that insect are attracted to host plant volatiles (Agelopoulos *et al*., 1999, Bruce *et al*., 2005, Derstine *et al*., 2020). Host plant volatiles may be useful in the development of novel strategies for insect pest management (Alpizar *et al*., 2002). Several studies have reported that host plant volatiles attract weevil species, including the plum curculio *Conotrachelus nenuphar* (Herbst) (Leskey and Prokopy, 2000; Leskey *et al*., 2001), the cabbage seed weevil *Ceutorhynchus assimilis* (Paykull) (Bartlet *et al*., 1997), the black vine weevil *Otiorhynchus sulcatus* (F.) (Van Tol *et al*., 2002), and the agave weevil *Scyphophorus acupunctatus* (Gyllenhaal) (Altuzar *et al*., 2007), among others. Host volatile compounds can function as attractants themselves, as pheromone synergists, or both.

Two studies have reported that, upon adult emergence, both sexes of *C. perseae* feed on leaves, shoots, flowers and developing fruits, and later gravid females oviposit into the fruits (Coria, 1999; Francia, 2008). A recent study showed that both sexes of *C. perseae* were attracted to avocado fruits (Cruz-López *et al*., 2020). However, whether the host-finding behaviour of *C. perseae* is mediated by volatile compounds released by the different parts of the avocado plant is not yet known. Therefore, in order to gain insight into the chemical ecology of this weevil species, we investigated the attraction of *C. perseae* to avocado (cv. Hass) volatile extracts. First, we collected avocado leaf, flower, and fruit volatiles, and then we evaluated the behavioural and electrophysiological responses of *C. perseae* males and females to host extracts. The volatile compounds from the extracts were identified by gas chromatography-mass spectrometry. Finally, the attraction responses of *C. perseae* to a selected 12-component blend of synthetic chemical compounds was evaluated in the laboratory.

Materials and methods

Biological material

Avocado fruits (cv. Hass) at different stages of ripeness exhibiting external damage by *C. perseae* were collected from orchards located in the municipalities of Comitán de Domínguez (16°18'1"N 91°57'18"W) and Las Margaritas (16°18'36.82"N 92°7'48"W), Chiapas, Mexico. The fruits were taken to the insectary of El Colegio de la Frontera Sur in Tapachula, Chiapas, Mexico, and placed in plastic containers (5 L) covered with a fine mesh and kept at room temperature (25-26 °C). Daily observations were made to record larval emergence from the fruits. Once larvae left the fruit, they were transferred into plastic containers (1 L) with 10 cm soil and kept in a rearing chamber at 25 ± 2 °C, 70 ± 10% RH, and L12:D12 photoperiod. Upon emergence, adults were sexed as described by Dominguez *et al.* (2015) and Francia (2008) and fed avocado fruits. In addition, healthy fruits, about 4 cm in diameter, leaves, and flowers were also cut from the avocado trees for volatile collection. None of these plant parts had symptoms of damage or disease that might alter the volatile profiles. After harvesting, all plant parts were transported on ice to the laboratory where they were used immediately. The samples were evaluated within 24 hours of harvest.

Volatile collection by dynamic aeration

Healthy green avocado fruits (550 g circa) and leaves (200 g circa) were placed separately in 1 L glass containers and 50 g of flowers were placed in a 250 mL container. The container covers had two orifices, one for air entry and the other for air exit. A tube containing charcoal was inserted into the entry orifice to filter the air. In the exit orifice, a Super Q tube (30 mg; ARS, Gainesville, FL, USA) was inserted. The volatiles were collected with a vacuum pump (Cole-Parmer, IL, USA) that delivered air at approximately 1 L minutes^{-1} for 24 hours. The volatiles were eluted from the adsorbent with 400 μL dichloromethane (HPCL grade, Sigma-Aldrich, St. Louis, MO, USA), deposited in 2 mL glass vials and stored at −20 °C until use. In total, 20 collections of each plant tissue were performed.

Chemical analyses

Plant extract volatile compounds were analysed by gas chromatography coupled with mass spectrometry (GC-MS). The equipment used was a GC Varian model Star 3400 CX GC coupled with a MS (Varian Saturn 4D, Palo Alto, CA, USA) equipped with a non-polar DB5 column (30 m long × 0.25 mm in diameter, 0.5 μm thick, J. & W Science Folsom, CA, USA). The oven temperature was programmed for 2 minutes at 50 °C, and 15 °C minute^{-1} increments up to 280 °C and held for 10 minutes. Helium was the carrier gas with a flow of 1 mL minute^{-1}. Ionization was carried out by electron impact at 70 eV and 250 °C. The compounds were identified by mass spectral matches to the NIST 14 database (version 2.0), comparing the retention indices with those from the literature and confirming identification with injections of synthetic standards. The relative amount of each compound was calculated from the peak area. Calibration curves using known concentrations were obtained for each of the 12 compounds of interest to determine the concentrations based on the peak area proportions of each compound. Calibration curves were obtained using the same GC-MS analysis conditions above described. The experimental retention indices were obtained through injection of a homologous series of alkanes (*n*C7–*n*C30, Sigma-Aldrich, Toluca, Mexico).

Chemicals

Authentic chemical standards of α-pinene, β-pinene, 6-methyl-5-hepten-2-one, β-myrcene, limonene, cis-ocimene, linalool, methyl salicylate, eugenol, α-copaene and β-caryophyllene were purchased from Sigma-Aldrich Inc. (Toluca, Mexico) and trans-ocimene from Alfa Chemistry (NY, USA). Purity of all compounds, according to the supplier, was 95-99%.

Behavioural responses to volatile extracts and synthetic blend

Male and female *C. perseae* responses to the volatile extracts from different parts of the avocado plant and to a mixture of synthetic compounds were evaluated using an olfactometer similar to that described by Ruiz-Montiel *et al.* (2008). Briefly, the olfactometer consisted of a glass Y-shaped tube 1.5 cm in diameter and 15 cm long. Each arm of the olfactometer was connected to the odour source, a glass container (4.5 cm in diameter and 15 cm long), and both recipients were connected to a flowmeter, which was coupled to a vacuum pump (Cole-Parmer, IL, USA) that emitted an airflow of 0.5 L minute^{-1}. The air entering the chambers was filtered with activated carbon. Filter paper (Whatman no. 1, Whatman, Maidstone, England) was impregnated with 5 μL plant volatile extracts. A blend of 12 synthetic compounds common in the extracts was prepared using 1 mL dichloromethane and concentrations of each compound in accord with the proportions listed in table 1: α-pinene (1.5 ng μL^{-1}), β-pinene (2.6 ng μL^{-1}), 6-methyl-5-hepten-2-one 3.3 ng μL^{-1}), β-myrcene (4.5 ng μL^{-1}), limonene (9.8 ng μL^{-1}), cis-ocimene (12 ng μL^{-1}), trans-ocimene (55 ng μL^{-1}), linalool (20.8 ng μL^{-1}), methyl salicylate (5 ng μL^{-1}), eugenol (18.5 ng μL^{-1}), α-copaene (24.6 ng μL^{-1}) and β-caryophyllene (42 ng μL^{-1}). Five μL (1 μg) of this blend was placed in one arm of the olfactometer, and 5 μL dichloromethane (control) in the other arm.

C. perseae adults were individually placed at the stem of the Y-tube, and response was recorded when the insect passed over the decision line (7 cm past the intersection of two arms) and stayed there for at least 20 seconds. Each test lasted 5 minutes (Ruiz-Montiel *et al.*, 2008; Mutis *et al.*, 2010; Silva-Filho *et al.*, 2012). After each trial, the position of the odour sources in the olfactometer was changed; the Y-tube was replaced with a clean Y-tube and a new insect was used. The assays were performed between 13:00 and 20:00 hours. Forty replicates per sex for each extract or mixture of synthetic compounds were performed. After each trial, the olfactometer was disassembled, washed with water and neutral soap, soaked with acetone and dried in the oven at 120 °C for 30 minutes to avoid contamination. The environmental conditions were 25 ± 2 °C, 70 ± 10 RH in the dark. Four- to 10-day-old males and females starved for 12 hours prior the bioassays were used.

Table 1. Relative amount (mean proportion ± SE) of volatiles emitted from avocado (*P. americana*) leaves, flowers, and fruits.

	RT	RI	Leaves	Flowers	Fruits
1 α-Pinene(1,2,3)	5.47	914	t	0.66 ± 0.78	0.10 ± 0.03
2 β-Pinene(1,2,3)	6.05	968	t	1.2 ± 0.038	0.07 ± 0.08
3 6-Methyl-5-hepten-2-one(1,2,3)	6.10	980	t	1.5 ± 0.049	0.168 ± 0.63
4 β-Myrcene(1,2,3)	6.40	990	t	2.1 ± 0.05	0.25 ± 0.05
5 Limonene(1,2,3)	6.65	1036	t	4.5 ± 0.98	4.2 ± 0.26
6 Eucalyptol(1,2,3)	6.74	1044	Nf	7.6 ± 0.14	t
7 Cis-ocimene(1,2,3)	7.79	1046	t	5.5 ± 0.38	0.168 ± 0.83
8 Trans-ocimene(1,2,3)	6.82	1050	7.84 ± 0.01	25.2 ± 0.092	8.1 ± 0.01
9 Linalool oxide (*E*)-furanoid(1,2,3)	7.16	1080	Nf	5.6 ± 0.69	Nf
10 Linalool oxide (*Z*)-furanoid(1,2,3)	7.34	1095	Nf	9.3 ± 0.33	Nf
11 Linalool(1,2,3)	7.44	1105	t	9.8 ± 0.49	0.39 ± 0.04
12 Perillene (isomer)(1,2)	7.57	1117	Nf	64.26 ± 0.42	Nf
13 Benzyl nitrile(1,2,3)	7.68	1130	Nf	20.4 ± 0.03	Nf
14 Linalool oxide (*E*)-piranoide(1,2)	8.18	1174	Nf	0.6 ± 0.19	Nf
15 Linalool oxide (*Z*)-piranoide(1,2)	8.29	1185	Nf	3.1 ± 0.03	Nf
16 Methyl Salicylate(1,2,3)	8.53	1207	1.6 ± 0.22	9.8 ± 0.88	5.6 ± 0.48
17 Eugenol(1,2,3)	8.65	1349	t	2.6 ± 0.37	0.39 ± 0.081
18 α-Cubebene(1,2)	9.10	1355	3.4 ± 0.35	8.6±0.35	4.4 ± 0.033
19 α-Copaene(1,2,3)	9.99	1358	5.4 ± 0.03	11.3 ± 062	7.4 ± 0.92
20 β-Cubebene(1,2,3)	10.28	1393	17.76 ± 0.65	11.1 ± 0.70	13.9 ± 0.29
21 β-Caryophyllene(1,2,3)	10.71	1443	20.7 ± 0.21	19.3 ± 0.059	5.7 ± 0.77
22 α-Caryophyllene(1,2,3)	11.01	1448	3.2 ± 0.29	3.7 ± 0.50	1.1 ± 0.32
23 γ-Cadinene(1,2)	11.23	1503	8.5 ± 0.10	7.2 ± 0.92	0.95 ± 0.59
24 α-Farnesene(1,2)	11.26	1560	4.4 ± 0.05	2.7±0.27	Nf
25 Dendrasaline(1,2)	11.82	1580	2.0±0.09	4.7 ± 0.17	17.5 ± 0.071
26 Hydrocarbon C17:2(1,2)	12.56	1672	Nf	5.2 ± 0.06	Nf
27 Hydrocarbon C17:1(1,2)	12.61	1678	Nf	14.3 ± 0.50	Nf
28 Hydrocarbon C17(1,2)	12.77	1700	2.5 ± 0.87	39.8 ± 0.01	4.4 ± 0.41
29 Hydrocarbon C19(1,2)	14.18	1900	Nf	11.9 ± 0.03	Nf

Identification by [1]mass spectrometry, [2]retention index and [3]commercial standards. RT = retention time, RI = retention index, Nf = not found, t = traces (peak area < 0.5), N = 20.

Electroantennogram (EAG) response of *C. perseae* to volatile extracts

An antenna was carefully detached from the insect head. Both ends of the antenna were then fixed to the stainless-steel electrodes with a conducting gel (Spectra 360, electrode gel Parker), and the electrodes were connected to an amplifier (Syntech IDAC-02). Immediately, 5 µL of each volatile extract was placed on a piece of filter paper (5 × 10 mm). After 20 seconds, the filter paper was placed in a Pasteur pipette, which in turn was connected to a hose from the airflow controller (1 L minute^{-1}). The tip of the pipette was introduced into one orifice located at one end of the metal tube through which moist air flowed constantly (1 L minute^{-1}) in direction of the insect antenna. A l-second stimulus generated by the controller (Syntech Cs-05) was then applied to puff over the compounds contained in the filter paper toward the insect antenna. As the control, 5 µL dichloromethane was used. The signal generated by the antennae was recorded in a PC equipped with software to analyse the signal (EAG 2000, Syntech 1200 BM). Dichloromethane (control) was presented at the beginning and end of each trial, and extracts of avocado plant parts were tested in random order. Fifteen replicates were performed for each sex, and for each insect antenna exposed to leaf, flower and fruit extracts, and the control (dichloromethane). Four- to 10-day-old males and females starved 12 hours prior EAG tests were used.

Statistical analyses

Behavioural responses in the Y-tube olfactometer were analysed with a G-test. The null hypothesis was that response distribution would be 50:50. Data analyses were performed in WinSTAT® for Excel 2007 (V. 2012.1.0.84). EAG data were transformed using the Box-Cox transformation for analysis of variance (ANOVA). When significant treatment effects were found, means were compared using the Tukey test. Analyses were performed using the software R Core Team (2016).

Results

Chemical analyses

Chemical analyses of the extracts from avocado flowers, fruits and leaves (cv. Hass) showed that the flower extract contains a larger number of volatiles than fruit and leaf extracts. Flower extract includes hydrocarbons, monoterpenes (hydrocarbons and oxygenated) and sesquiterpenes. The most abundant component is an isomer of the monoterpene perillene followed by heptadecane and linalool oxide (*E*)-furanoid. The fruit extract contains the sesquiterpenes dendrasaline, β-cubebene, α-copaene and β-caryophyllene as the major components, and the leaf extract contains the sesquiterpenes β-cubebene and β-caryophyllene and γ-cadinene as major components followed by linalool oxide (*E*)-furanoid (table 1).

Behavioural responses to avocado extracts

Females were more attracted to the flower ($G = 24.45$, d.f. = 1, $P = 0.0001$), fruit ($G = 19.93$, d.f. = 1, $P = 0.0002$) and leaf volatile extracts ($G = 3.58$, d.f. = 1, $P = 0.05$) than to the solvent control. In addition, females were more attracted to the flower extracts than to the leaf extracts ($G = 5.21$, d.f. = 1, $P = 0.0023$) and to fruit extracts more than to leaf extracts ($G = 5.23$, d.f. = 1, $P = 0.0024$), but they did not show any preference for flower over fruit extracts ($G = 4.021$, d.f. = 1, $P = 0.1049$) (figure 1A). Similarly, males were more attracted to leaf ($G = 7.45$, d.f. = 1, $P = 0.0063$), flower ($G = 12.31$, d.f. = 1, $P = 0.0004$) and fruit ($G = 14.01$, d.f. = 1, $P = 0.0008$) extracts than to the solvent control. Furthermore, males did not show preference between flower or leaf extracts ($G = 2.18$, d.f. = 1, $P = 0.1396$), between fruit or leaf extracts ($G = 2.44$, d.f. = 1, $P = 0.1176$) nor between flower or fruit extracts ($G = 0.668$, d.f. = 1, $P = 0.4135$) (figure 1B).

EAG responses of *C. perseae* to avocado extracts

The ANOVA showed that the avocado extracts affected EAG responses of *C. perseae* females and males ($F_{3,64} = 71.68$, $P = 0.001$). Flower extracts elicited stronger antennal responses than leaf extracts and dichloromethane. No interaction was observed between males and females ($F_{3,64} = 0.956$, $P > 0.05$) (figure 2).

Behavioural responses to synthetic blend

Females ($G = 4.2$, d.f. = 1, $P = 0.04$) (figure 3A) and males ($G = 4.5$, d.f. = 1, $P = 0.03$) (figure 3B) were more attracted to the 12-component blend than to the control.

Discussion

In this study, we showed that both sexes of *C. perseae* preferred avocado (cv. Hass) flower, fruit, and leaf volatile extracts over the solvent control in a Y-tube olfactometer. Similarly, Cruz-López *et al.* (2021) have recently shown that both sexes of *C. perseae* were attracted to host avocado fruit. We also showed that there were sexual differences in the responses of *C. perseae* to host volatiles because females were able to discriminate among the three types of extracts, preferring those from flowers over leaves, while males were not. This result is

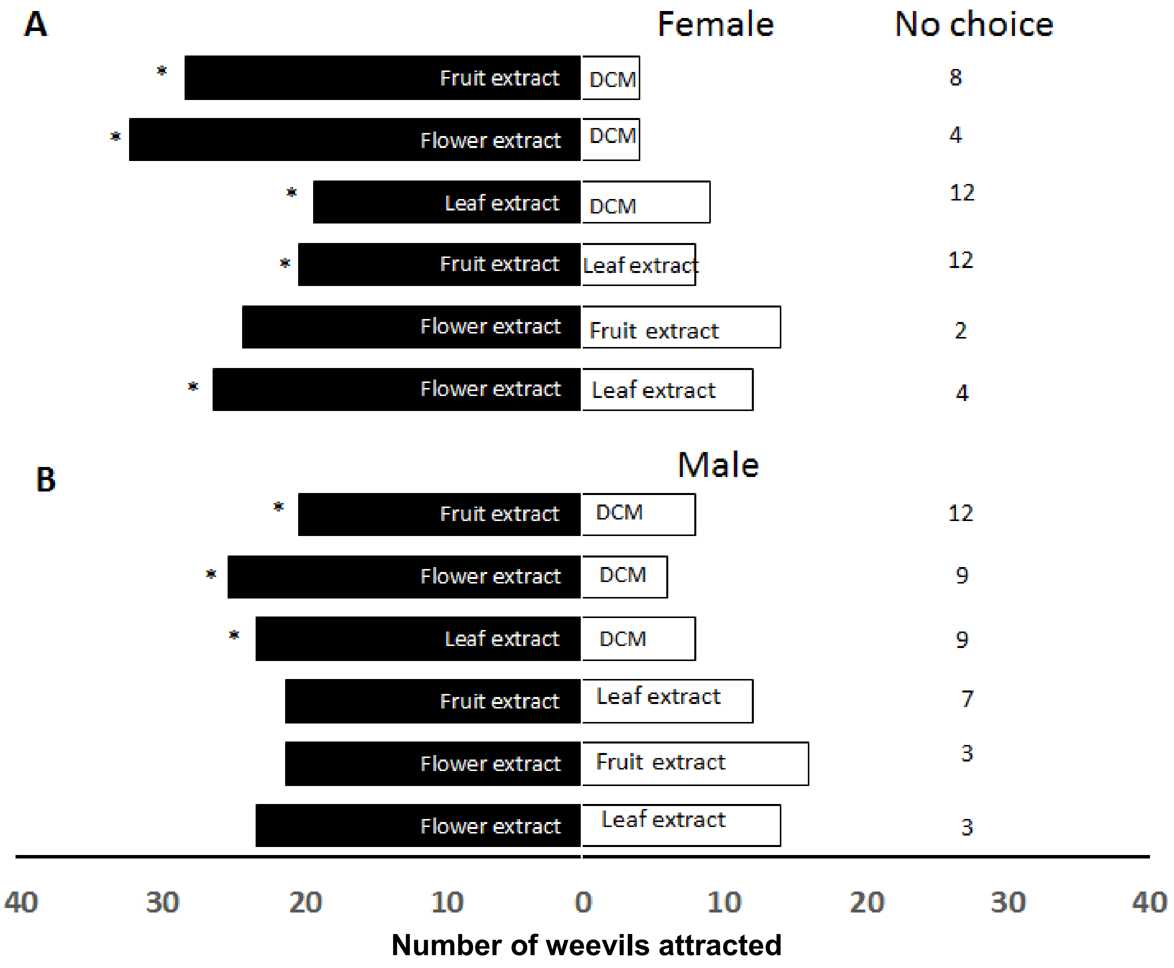

Figure 1. Behavioural responses of *C. perseae* females (**A**) and males (**B**) to volatile extracts from avocado leaves, flowers and fruits. *Indicates significant differences between choices ($P \leq 0.05$). DCM = dichloromethane. N = 40.

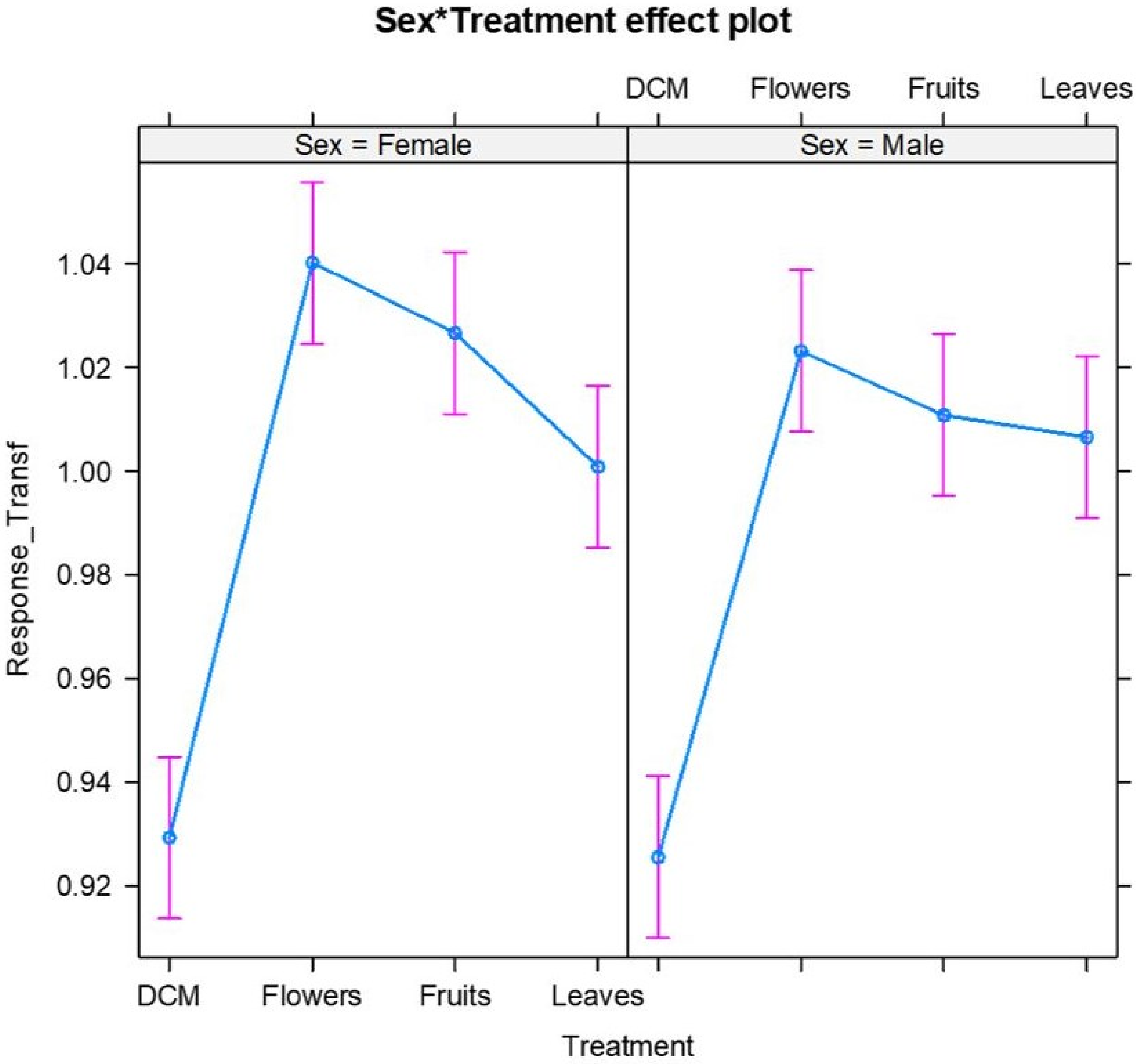

Figure 2. EAG responses of *C. perseae* females and males to different phenological stages of the avocado plant. Means with the same letter are not significantly different (Tukey; $P < 0.05$). DCM = dichloromethane.

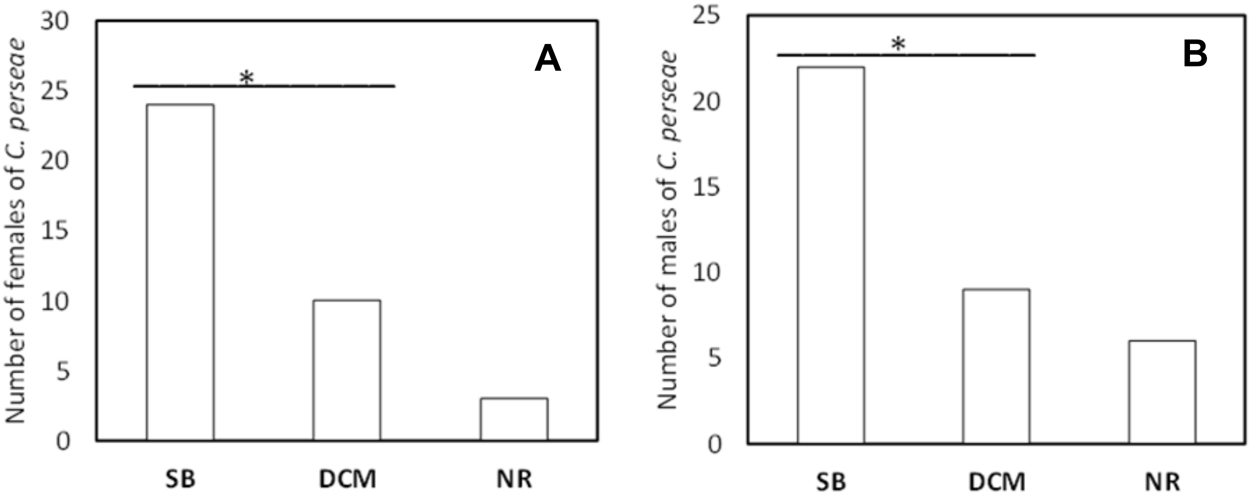

Figure 3. Behavioural responses of *C. perseae* females (**A**) and males (**B**) to the synthetic blend vs control. * = significantly different (P ≤ 0.05). SB = synthetic blend, NR = no response. DCM = dichloromethane. N = 10.

difficult to explain in light of the biology of *C. perseae* because both sexes feed on leaves, flowers and developing fruits after leaving the ground (Coria, 1999; Francia, 2008). However, females may show stronger response to flowers than to leaves for reasons other than searching for food. For example, they may use flower volatiles as cues for searching oviposition sites. Weevils seem to discern specific volatiles of their host at different phenological stages. For instance, *Callosobruchus maculatus* (F.) females were highly attracted to cowpea bean (*Vigna unguiculata* L.) seed extracts (Adhikari *et al.*, 2002) and *Bruchus pisorum* (L.) males responded more strongly to pea flower and pea pod extracts than to vegetative part extracts (Ceballos *et al.*, 2015). Males of *Conotrachelus psidii* Marshall were attracted to guava flower buds rather than to fruits (Silva-Filho *et al.*, 2012) but females were not. It is likely that these weevils use guava flower volatiles as a kairomonal cue to find the plant.

Previous studies have reported that several weevil species are attracted to host volatiles, including the pepper weevil *Anthonomus eugenii* Cano (Addesso and McAuslane, 2009), the plum curculio *C. nenuphar* (Leskey and Prokopy, 2000), the raspberry weevil *Aegorhinus superciliosus* (Guerin-Meneville) (Parra *et al.*, 2009), and the guava weevil, *C. psidii* (Silva-Filho *et al.*, 2012), among other species. Therefore, the use of host volatiles by members of this family seems to be common. Knowledge of host volatiles is important because several studies have shown that those volatiles synergize the weevil's response to its aggregation pheromone (Reddy and Guerrero, 2004; Dickens, 2006; Wibe *et al.*, 2014) or they induce the production/release of the pheromone (Rochat *et al.*, 1991; Ruiz-Montiel *et al.*, 2009).

In addition, EAG responses support the behavioural bioassays since when avocado volatile extracts were puffed over to the weevil's antenna, we found differences in EAG responses between volatiles from different phenological stages of avocado and sexes. *C. perseae* females showed stronger antennal responses to flower and fruit volatiles than to leaf volatiles, while male antennal responses to volatiles in fruit, flower, and leaf extracts were not different.

We found that more volatiles are emitted by flowers than by fruit and leaves. Flower extract includes hydrocarbons, monoterpenes (hydrocarbons and oxygenated) and sesquiterpenes. The most abundant component is an isomer of the monoterpene perillene followed by heptadecane and linalool oxide (*E*)-furanoid, while the fruit extract contains the sesquiterpenes dendrasaline, β-cubebene, α-copaene and β-caryophyllene as the major components and the leaf extract contains the sesquiterpenes β-cubebene and β-caryophyllene and γ-cadinene as the major component followed by linalool oxide (*E*)-furanoid. Similar avocado flower volatile composition has been reported by Campuzano-Granados and Cruz-López (2021). Volatiles of avocado fruits and leaves have been analysed using different techniques (Sagrero-Nieves and Bartley, 1995; Pino *et al.*, 2000; 2004; Ogunbinu *et al.*, 2007; Torres-Gurrola *et al.*, 2009). Our results coincide in that volatiles from avocado fruits and leaves are rich in terpenoid compounds, α-pinene, β-caryophyllene and α-copaene being the most common volatiles. Wu *et al.* (2014), in addition to terpene compounds, found estragole, chavicol and trans-anethole in volatiles from leaves of Mexican, Guatemalan and west-Indian avocado races, but we did not detect those compounds in cv. Hass avocado volatiles.

Both sexes of *C. perseae* showed similar attraction to the 12-component blend derived from the flower, leaf and fruit extracts. However, future studies will investigate whether all compounds are crucial in insect attraction or if some of them are redundant. For example, Magalhães *et al.* (2018) found that the cotton boll weevil is attracted to a 10-component blend from cotton plant volatiles. However, they found that of this blend only six compounds were crucial for attraction.

In conclusion, we have demonstrated that *C. perseae* females and males are attracted to leaf, flower and fruit volatiles of *P. americana* (cv. Hass). The behavioural responses of *C. perseae* also matched antennal responses to the extracts. However, females were more attracted to flower extract than to leaf extract, while males showed no preference for any extract. We also showed that females and males were attracted to a 12-component blend derived from flowers, leaves and fruit volatiles. Further research will identify the crucial compounds of the synthetic blend to enable their use in monitoring this insect pest. Because we used virgin weevils in our study, it would be interesting to investigate the response of mated weevils to host volatiles.

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