

Electrophysiological and behavioral responses of pea weevil *Bruchus pisorum* L. (Coleoptera: Bruchidae) to volatiles collected from its host *Pisum sativum* L.

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The pea weevil (*Bruchus pisorum* L.) (Coleoptera: Bruchidae) is one of the most damaging pests of pea (*Pisum sativum* L.) We investigated the role of pea volatiles on the electrophysiological and behavioral response of *B. pisorum* using electroantennography (EAG) and olfactometry bioassays. Plant volatiles emitted at different phenological stages were collected *in situ* by headspace on Porapak Q traps and analyzed through gas chromatography coupled to mass spectrometry (GC-MS). Most abundant volatiles identified in all phenological stages were terpenes and green leaf volatiles. All tested volatile extracts elicited significant EAG responses in both male and female *B. pisorum*, with females exhibiting a greater response (1.35 mV) than males (1.02 mV) to pea-pod volatiles. Volatiles from each phenological stage stimulated an attractant behavioral response of both males and females *B. pisorum* in olfactometer bioassay. A larger attraction of *B. pisorum* females was observed to volatiles from pods over other phenological stages ($P < 0.001$). These results suggest the relative importance of volatiles cues from plant mediating host location by *B. pisorum*. This work showed that plant volatiles elicited electrophysiological and behavioral responses and that *B. pisorum* female can discern between phenological stages of *P. sativum* based on those chemical cues.

Key words: Bruchids, EAG, IPM, pest monitoring, plant volatiles.

INTRODUCTION

Plants synthesize hundreds of metabolites; some of them are volatiles that confer a characteristic blend of odor to the plants. These volatile organic compounds released by plants can act as semiochemicals playing an important role in enabling insects to recognize host plants from a distance (Holopainen, 2004; Bruce et al., 2005; Bleeker et al., 2009). Find and selecting a host plant is crucial for a phytophagous insect to fulfill its nutritional requirements and to find suitable oviposition sites (Bruce et al., 2005). However, the plant volatiles emission is known to vary depending on both internal and external factors. Besides the climatic and geographic factors, high phenological variation in volatiles emission occurs (Casado et al., 2006; Dudareva et al., 2006) and compounds have different systematic distributions (Kundsen et al., 1993). The perception of these compounds by insects depends on its olfactory receptor neurons, mostly in the antennal-sensillae (Sato and Touhara, 2009; Carey and Carlson, 2011; Hansson and Stensmyr, 2011). The perception

of blends of plant volatiles plays a pivotal role in host recognition, non-host avoidance and ensuing behavioral responses as different responses can occur to a whole blend compared to individual components (Bruce and Pickett, 2011).

The pea weevil, *Bruchus pisorum* L. (Coleoptera: Bruchidae), is a cosmopolitan and most destructive insect pest of the pea (*Pisum sativum* L.) cultivars (Hardie and Clement, 2001; Clement et al., 2009). This strictly monophagous pest completes its univoltine life cycle only on *P. sativum*. The larvae, once hatched, burrow through the pod wall into maturing seeds to consuming them and complete its development, resulting in loss of yield and quality of pea crops (Clement, 1992; Michael et al., 1993). Emerging males after winter hibernation are sexually mature, but females must feed on pollen before they can lay eggs only on green pea pods (Clement, 1992; Hardie and Clement, 2001). Adults emerge from seed leaving an exit hole about 2-3 mm, this behavior cause a direct damage by consumption of the seed and reduction of its capacity of germination, and infestations levels reported worldwide ranging from 10% to 90% (Pesho et al., 1977; Gerding et al., 1987; Horne and Bailey, 1991). This newly emerged weevil seeks sheltered sites to spend the winter and remain there until the following spring (Hardie et al., 1995).

Evidences about nature and identity of stimuli used in host finding by *B. pisorum* are scarce. Annis and O'Keeffe (1987) indicate that the monophagy of *B. pisorum* may be due to the response of adult females to deterrents. Pajni

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(1986) suggested that host specificity in bruchids that affects crops in field, including *B. pisorum*, is determined by the obligatory feeding of the adults on the pollen and nectar of their hosts for the maturation of the gonads and subsequent initiation of copulation and oviposition. Other bruchids pests of legumes respond to its host volatiles, *Vicia faba* (L.) flower-volatiles were electrophysiological active on the antennae of *B. rufimanus* (Bruce et al., 2011). *Callosobruchus maculatus* F. (Coleoptera: Bruchidae) in an electroantennogram experiment showed a dose-dependent response to a dichloromethane extract of its host-plant *Vigna unguiculata* (L.) Walp., the extract stimulated both males and females but females showed a greater response than males (Adhikari et al., 2002). In the same study authors find a similar dose-dependent response of *C. maculatus* in an olfactometer bioassay. Non-host volatiles, neem (*Azadirachta indica* A. Juss.) elicited a repellent behavior on *C. maculatus* in olfactometer indicating the suitability of neem leaf-volatiles as botanical repellent in the control of this pest (Paranagama et al., 2003).

There are not experimental evidences of chemical cues used by *B. pisorum* in host finding. In the present study we investigate the activity of the odor extracts from different phenological stages of *P. sativum* on the antennal response of *B. pisorum*. In olfactometric bioassay our objective was evaluate the attraction of odor extracts on both males and females of *B. pisorum*. We were able to identify volatiles from *P. sativum* that act as chemical cues eliciting an attractant behavior of the pest and obtain an initial approach to elucidate the role of pea volatile in host finding by *B. pisorum*.

MATERIAL AND METHODS

Male and female *B. pisorum*

Adults were collected from infested pea seeds harvested on an experimental pea stand at Instituto de Investigaciones Agropecuarias INIA, Chillán, Chile, between September 2009 and February 2010. Once transferred to the laboratory, male and female adults emerging from peas were maintained separately at 5 °C in plastic boxes. One hour before each behavioral assay, *B. pisorum* adults were observed for 30 min, and those that were active in that time were selected for the experiments.

Collection of volatiles from host plant

Headspace collection of naturally released plant volatiles were made in the experimental pea stand at INIA, Chillán, for a 24 h period (at 1 L min⁻¹) by enclosing aerial part of an individual plant of *P. sativum* 'Ambassador' (15-30 cm tall) in a 900 mL Pyrex glass chamber (6 cm ID and 30 cm high). Depending of the phenological stage, the aerial part of each plant included leaves, flowers, and pods. Volatiles were absorbed on 100 mg Porapak Q columns (80-100 mesh, Waters Associates, Milford, Massachusetts, USA),

previously cleaned with 1 mL of redistilled diethyl ether (Chromatographic grade; Merck, Darmstadt, Germany), and conditioned at 150 °C for 2 h in a stream of nitrogen at 70 mL min⁻¹. The entrainment was performed by using a positive/negative pressure air system (Agelopoulos et al., 1999). The air was dried and purified by passage through activated 5-Å molecular sieves and then charcoal, and finally drawn through glass chamber. Volatiles were extracted from the Porapak Q by elution with 1 mL of redistilled hexane (Chromatographic grade, Optima Scientific, USA) which was then concentrated to 100 µL under a gently flow of nitrogen. Odor sources included three phenological stages of pea: vegetative, flowering period, and pod formation.

Identification and semi-quantification of plant volatiles

An aliquot (1.0 µL) of the volatile extracts was analyzed using a gas chromatograph coupled to a mass spectrometer (GCMS QP2010 Plus; Shimadzu, Tokyo, Japan) equipped with a Restek capillary column (Rxi-5ms: 5% diphenyl-95% dimethylpolysiloxane; 30 m × 0.25 mm ID × 1.0 µm; Restek Corporation, Bellefonte, Pennsylvania USA). Helium gas was used as the gas carrier; with a flow rate of 1.4 mL min⁻¹. Mass spectrum acquisition was performed in the mass range from 35 to 500 *m/z*. The ionization was performed by electron impact at 70 eV with an ion source at 200 °C. The GC oven was programmed to remain at 40 °C for 5 min and increase at 5 °C min⁻¹ to 230 °C and held for 10 min. The interface temperature was programmed at 250 °C. The volatiles collected were identified by comparing gas-liquid chromatography (GLC) Kovats indices (KIs) and mass spectra (MS) with the corresponding commercial standards (Sigma Aldrich, St. Louis, Missouri, USA) and library database spectra using the National Institute of Standards and Technology (NIST) mass spectral search program (version 2.0; Standard Reference Data, NIST, Gaithersburg, Maryland, USA), and the experimental KIs were compared with theoretical KIs from synthetic standards compounds and reported in the Pherobase (El-Sayed, 2014) and NIST webbook (NIST, 2014). Calibration curves based on peak area ratio were constructed using standards for quantification of each volatile compound identified from the samples of *P. sativum*.

Electrophysiological recognition and behavioral bioassay of plant volatiles

Olfactory sensitivity of *B. pisorum* to volatile compounds from its host *P. sativum* was studied with electroantennographic (EAG) recordings, the apparatus and general procedures are described in Palma et al. (2012). For each recording, a glass pipette Ag/AgCl reference electrode (containing KCl and 0.1% polyvinylpyrrolidone) was inserted into the foramen of a weevil's excised head. The tip of a similar recording electrode was cut to match the diameter of the tip of

antenna, and positioned in air stream receiving effluent from the EAG apparatus. The analog signal was detected through a probe (EAG Kombi-Probe, Syntech, Hilversum, The Netherlands), and processed with a data acquisition controller (IDAC-4, Syntech), which was linked to a personal computer running the EAG software to analyze the recordings. The tested plant volatile-extracts were applied to a piece of 8 × 50 mm filter paper strip (Whatman nr 1) using 35 µL of each extract. After allowing for solvent evaporation, the paper strips were placed inside a glass Pasteur pipette (Fisher Scientific, Pittsburgh, Pennsylvania, USA). A stimulus controller (CS-05, Syntech) was set to provide a continuous flow of 0.6 L min⁻¹, and the stimuli were provide as 2 s puffs. At least 1 min was allowed between successive stimulations for antennal recovery. Ten replicates were performed per sex and volatile extract, using hexane as control.

The behavioral response of *B. pisorum* to pea volatile extracts was evaluated in a four-arm airflow olfactometer adapted from Pettersson (1970). The observation area was divided into four zones and one indifferent zone in the center was designated as the decision zone. One *B. pisorum* was enclosed in the observation arena permeated by charcoal filtered air (800 mL⁻¹) coming from each of its four stretched-out corners and drawn out through a hole above its center. Olfactometric bioassays were carried out between 10:00 and 18:00 h (photophase) at 22 ± 1 °C and 80 lux. Two lines of air coming from each stimulus treatment were connected in opposite corners of the arena; the other two lines released hexane as a control treatment (Parra et al., 2009). The chemical stimuli (odors of plant extracts) were applied onto a paper strip (8 mm × 60 mm, Whatman nr 1 filter paper) and placed into a glass tube (10.0 cm high; 1.0 cm ID), connected to each olfactometer arm. The time spent in each arm of the olfactometer was recorded during 10 min with the olfactometer being rotated (90° clockwise) every minute to minimize directional bias. Five minutes before starting the experiment, the test specimen of *B. pisorum* was placed in the olfactometer for acclimation. The insects were tested only once, and 10 replicates per sex were performed for each extract using a different clean-olfactometer.

Data analysis

The spent time by insects in different zones of the olfactometer was submitted to a non-parametric one-way analysis based on ranks trough Kruskal-Wallis test, and groups were separated using the Conover-Inman test ($P \leq 0.05$) (Derryberry et al., 2010). An olfactometric-preference index (OPI), where $OPI = 2T/(T + C)$ (Kogan and Goeden, 1970; Kogan, 1972), was calculated to reflect the effect of the stimulus on the behavior of the insect. The OPI values ranged from 0 to 2, with OPI = 1 indicating no olfactometric preference for the control or treatment odor source, OPI > 1 indicating preference for treatment odor source, and OPI < 1 indicating preference

for control odor source. Furthermore, to ensure that insects were not biased in their preferences, a complete set of experiments for each sex was done using hexane in all arms. According to Kogan (1972) if no factor other than random distribution of the insect in the arena accounted for time-spent in each zone of the olfactometer, the mean value of OPI for all replicates control using hexane (OPI_H) should be approximate 1. These values were used to correct OPI within each series of test following an adaptation of Abbott's formula: $OPI_K = OPI_H (1 + K)$, where IPO_K is the corrected value of OPI (Kogan, 1972). Values of OPI_K were analyzed with procedures describe above, with non-parametric one-way analyses based on ranks trough Kruskal-Wallis test followed by Conover-Inman test ($P \leq 0.05$) (Derryberry et al., 2010). The EAG data between sexes were analyzed by *t*-test ($P \leq 0.05$), and data within a same sex were submitted to ANOVA and groups were separated by Tukey's test ($P \leq 0.05$).

RESULTS AND DISCUSSION

Identification of plant volatile compounds

The literature concerning qualitative and quantitative data of the volatile content in *P. sativum* is scarce. To our knowledge, the identification and quantification of the headspace composition of volatiles from live plants of *P. sativum* have not been carried out previously. The GC-MS analysis of plant-emitted volatiles showed qualitative and quantitative differences between analyzed phenological stages (Table 1). Terpenes were the most abundant compounds in all collections, and terpinene and 1-*S*-verbenone were detecting only at flower stage at 19.92 and 2.02 ng µL⁻¹, respectively (Table 1). Flowers emitted large amounts of all compounds, except for myrcene (0.87 ng µL⁻¹) and *n*-dodecane (3.82 ng µL⁻¹). Pea pods emitted small quantities of (*Z*)-2-hexen-1-ol, 2,4-hexadienal,

Table 1. Compounds identified in a headspace sample of pea (*Pisum sativum*) at vegetative, flower, and pod phenological stages.

Compound ^a	Rt	Linear retention indices		Concentration at phenological stages		
		Exp.	Lib.	Veg.	Flower	Pod
	min			ng µL ⁻¹		
Hexanal ^(1,2,3)	7.14	772	773	3.50	5.41	nd
(<i>Z</i>)-2-Hexen-1-ol ^(1,2)	10.08	863	865	2.03	6.48	0.51
2,4-Hexadienal ^(1,2)	11.10	885	884	2.08	2.52	1.49
α-Pinene ^(1,2,3)	13.04	933	934	5.42	9.57	1.37
Camphene ^(1,2,3)	13.55	946	952	0.12	0.55	nd
β-Pinene ^(1,2,3)	14.57	971	978	6.78	11.78	1.66
Myrcene ^(1,2)	15.21	986	986	3.16	0.87	0.54
3-Carene ^(1,2,3)	15.84	1000	1001	1.42	3.65	nd
Limonene ^(1,2)	16.56	1021	1017	7.11	5.56	0.07
Terpinene ^(1,2)	17.05	1035	1055	nd	19.92	nd
Terpinolene ^(1,2)	18.85	1084	1084	3.36	12.20	nd
1-Methylbutyl-benzene ^(1,2,3)	19.45	1099	1082	2.03	5.00	nd
1-(<i>S</i>)-Verbenone ^(1,2)	22.12	1181	1191	nd	2.02	nd
<i>n</i> -Dodecane ^(1,2,3)	22.74	1199	1199	2.34	3.82	6.77

^aReliability of the identification is indicated by: ¹mass spectrum; ²linear retention indices; ³commercial standard compound. Rt. Retention time; Exp.: experimental; Lib.: library; Veg.: vegetative; nd: not detected.

α -pinene, β -pinene, myrcene, and limonene, except for *n*-dodecane that was the dominant compound at this stage with $6.77 \text{ ng } \mu\text{L}^{-1}$.

The highest compound concentration was found in vegetative and flower stages, which coincides with the report of Dudareva et al. (2004). They indicated that the emission of volatiles increases when leaves are young and flowers are prepared to be pollinated. The quantitative differences in volatile compounds emitted by flowers may be especially important to attract pollinators (Custodio et al., 2006). Monoterpenes, such as myrcene and α - and β -pinene, have been well studied in many species of bark beetles and in their host plants. The results showed that they were attractive to *Hylastes ater* and *Hylurgus ligniperda* (Reay and Walsh, 2002). Limonene was attractive to species of the Curculionidae family, such as *Hylobius pales* (Siegfried, 1987) and *Smicronyx fulvus* (Roseland et al., 1992). According to Garcia (2010), α - and β -pinene, 3-carene, (*S*)-*cis*-verbenol, and (*1S*)-(-)-verbenone are attractive to males and females of *H. ligniperda* (Scolitidae). It has been demonstrated that *trans*-2-hexenal is the most abundant compound in flower volatiles of alfalfa (*Medicago sativa* L.) and it may be involved in mechanisms of pest and disease resistance (Tava and Picetti, 1997).

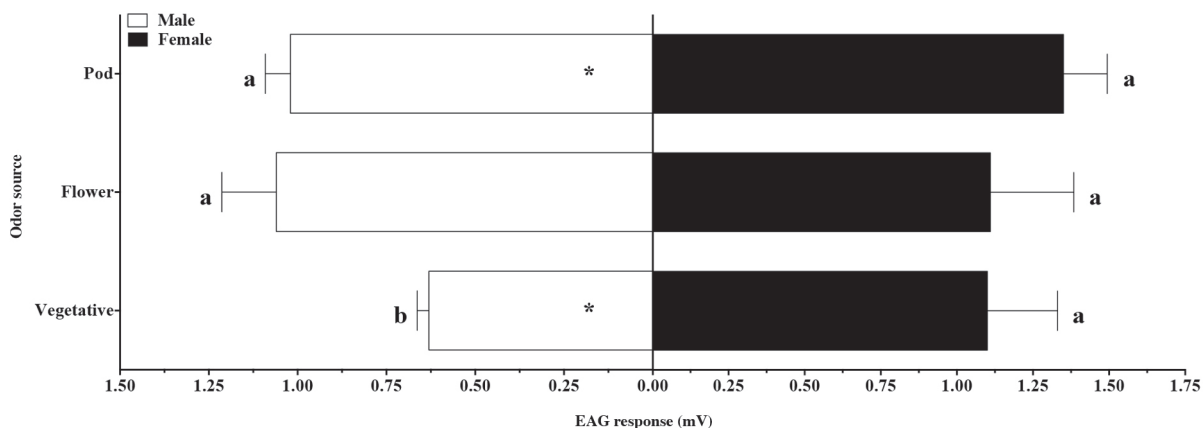
Electroantennogram (EAG) responses

The electrophysiological response of *B. pisorum* has not been carried out previously, and these recordings from insect antennae revealed responses to chemical stimuli from its host *P. sativum*. The pea-volatiles from all phenological stages elicit a significant EAG response in both male and female, and the amplitude in response to those stimuli was in the range of 0.63 to 1.35 mV (Figure 1). In general female showed greater EAG response than males, but this was not always significant. Opposite to our results, Adhikari et al. (2002) found higher EAG responses

in males of *C. maculatus* than females toward lemongrass (*Cymbopogon citratus* (DC.) Stapf), neem and curry (*Murraya koenigii*). However, a greater respond of females of *C. macullatus* was observed when the stimulus was a seed-extract of cowpea (*Vigna unguiculata* (L.) Walp.) (Adhikari et al., 2002). This results could be explain by the fact of the *C. macullatus* lay their eggs on the external surface of a dry seed of Fabaceae species. The statistical analysis showed that pea-volatiles from vegetative and pod elicit greater response in female 1.35 and 1.10 mV than males 1.02 and 0.63 mV, respectively (Figure 1). The EAG response to volatile extract from flower showed a not significant difference between sexes. In males the EAG response to extracts from flower and pod were 76% and 68% greater and significant than the vegetative stage, respectively. In contrast, female EAG responses between phenological stages of pea were not significant each other. This behavior may be founded on the insect female's need to find a suitable host that provides food and safety for the progeny (Paukku and Kotiaho, 2008). Our results permit to establish the olfactory perception of *B. pisorum* for its host, and several studies have support the hypothesis that the characteristic blend of volatile compounds produced by host plants are fundamental for species-specific attraction of herbivorous insects (Webster et al., 2010; Hare, 2011; Scala et al., 2013).

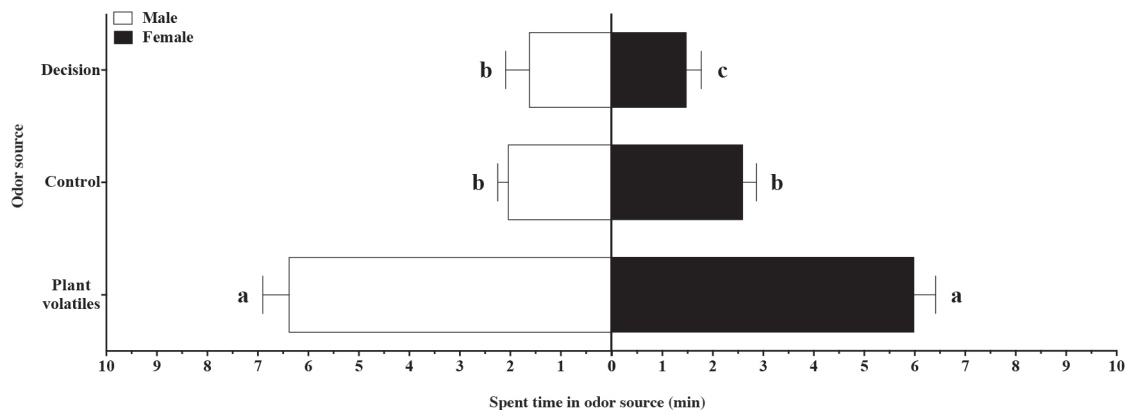
Olfactometer bioassay of volatiles from *P. sativum*

Olfactometer bioassay showed that both male and female were attracted to pea-volatiles from vegetative, flower, and pod vegetative phenological stages. Males and females of *B. pisorum* showed significant attraction to vegetative pea-volatiles spending 60% of the bioassay time, but no significant differences were found between sexes (Figure 2). The bruchids were significantly attracted to pea-volatiles from flower (Figure 3). Similarly to pea-volatiles from vegetative stage, females spent 60% of the



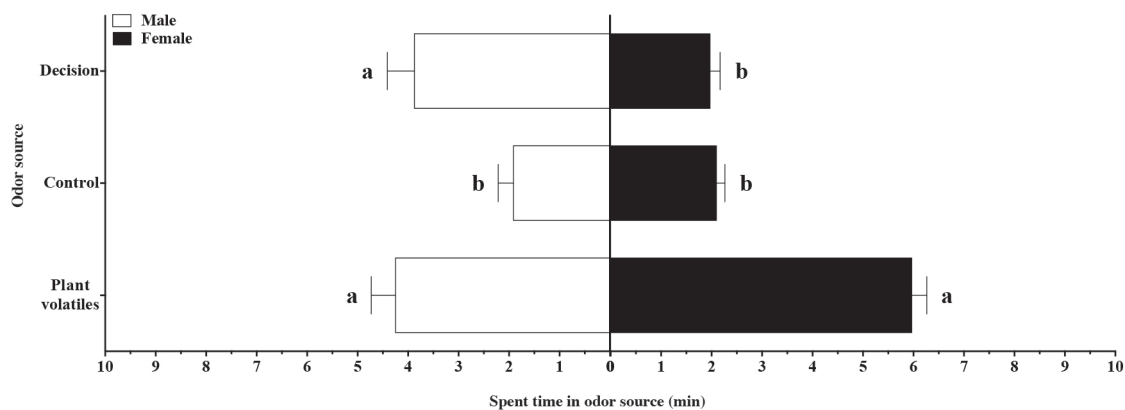
*Indicate differences between sexes, for each odor source, according to Student's *t*-test ($P < 0.05$). Means sharing a letter, for each sex, do not differ significantly according to Tukey's test ($P < 0.05$). EAG: Electroantennography.

Figure 1. Electrophysiological response (mV \pm SE) of male and female *Bruchus pisorum* to *Pisum sativum* volatiles emitted at different phenological stages.



Means sharing same letter, for each sex, do not differ significantly according to Conover-Inman test ($P < 0.05$).

Figure 2. Behavioral response of *Bruchus pisorum* to *Pisum sativum* volatiles from vegetative phenological stage. Average time spent (\pm SE) by *B. pisorum* in an olfactometric bioassay ($n = 10$).

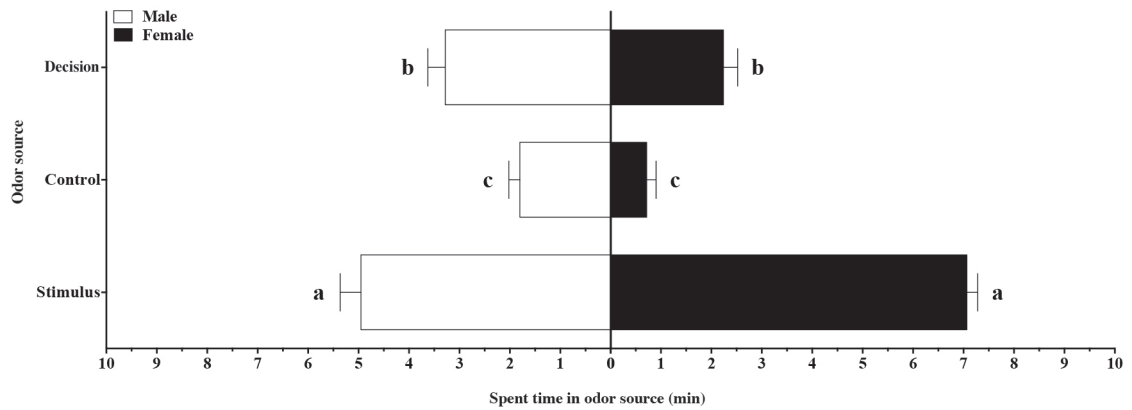


Means sharing same letter, for each sex, do not differ significantly according to Conover-Inman test ($P < 0.05$).

Figure 3. Behavioral response of *Bruchus pisorum* to *Pisum sativum* volatiles from flower phenological stage. Average time spent (\pm SE) by *B. pisorum* in an olfactometric bioassay ($n = 10$).

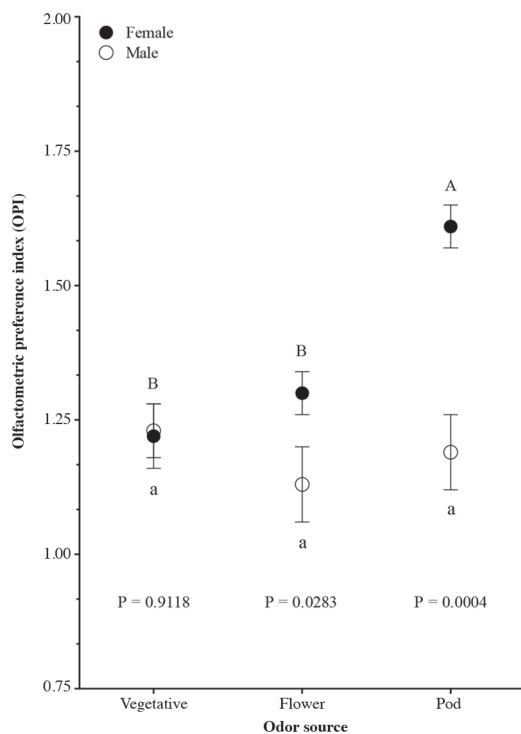
bioassay time visiting pea-volatiles from flower zone in the olfactometer. However, males spent 42% of the bioassay time to visit the stimuli zone (Figure 3). Pea-volatiles from pod elicit a strong significant attraction to females of *B. pisorum*, who spent 71% of the bioassay time visiting the stimuli zone in the olfactometer (Figure 4). On the other hand, males spent 49% to visit the zone stimulated with pea pod-volatiles (Figure 4). The attractive behavior exhibited by the insect could be explained by the presence of the terpenes such as α - and β -pinene; the predominant compounds in volatile extracts. That agrees with studies that have been shown how beetles are highly attracted to the monoterpenes of their host plant. For example, *Tomicus piniperda* is attracted to α -pinene, terpinolene, and 3-carene (Schroeder and Weslien, 1994). According to Hofstetter et al. (2008), α -pinene and myrcene are attractive to *Dendroctonus brevicomis* (Curculionidae: Scolytinae). Rodríguez et al. (2008) pointed out that limonene is considered to be toxic to insects and α - and β -pinene have been reported to be attractive to beetles.

α -Pinene has a kairomonal activity for beetles, especially for bark beetles, which means that the process involves individuals from different species, and the adaptive benefit is more favorable to the organism that receives the message than to the one that emits it (García, 2010). Miller and Lindgren (2000) indicated that α -pinene and myrcene are attractive to *Dendroctonus ponderosae* (Coleoptera: Scolitydae) and they are used in traps. These results from olfactometric bioassays were transformed to an olfactometric-preference index (OPI), to reflect the effect of the plant-volatile stimuli on the behavior of the insect (Figure 5). In general, OPI indicated that insects have preference by pea-volatiles and females showed greater preference than males. Statistical analysis revealed difference in OPI between males and females by pea-volatiles from flower ($P = 0.0283$) and pod ($P = 0.0004$). No discrimination between pea-volatiles from different phenological stages was found in *B. pisorum* males. According to OPI results, females showed a significant attraction to pea-volatiles from pod, and no



Means sharing same letter, for each sex, do not differ significantly according to Conover-Inman test ($P < 0.05$).

Figure 4. Behavioral response of *Bruchus pisorum* to *Pisum sativum* volatiles from pod phenological stage. Average time spent (\pm SE) by *B. pisorum* in an olfactometric bioassay ($n = 10$).



OPI = 1 indicates no olfactometric preference for the control or treatment odor source, OPI > 1 indicates preference for treatment odor source, and OPI < 1 indicates preference for control odor source. Means with same letter, lower-case males and uppercase females, do not differ according to Conover-Inman test ($P < 0.05$). P-value from *U* Mann-Whitney test ($P < 0.05$) indicates differences between males and females for each phenological stage.

Figure 5. Average olfactometric preference index (OPI, \pm SE) of *Bruchus pisorum* in olfactometer zones in response to plant volatiles from different stages of *Pisum sativum*.

discrimination by pea-volatiles from vegetative or flower (Figure 5). The results may be explained as males already have spermatozoa when they emerge from the grain, therefore, they do not need pollen or any other plant part, unlike females, which need it to become sexually mature

(Medina, 1990). Nevertheless, female adults of *B. pisorum* did show a significant preference for the volatile extract of pea pod, this behavior maybe is due to the female's need to provide food for her offspring (Clement, 1992).

The results of the present study demonstrate that volatiles released by *P. sativum* at different phenological stages have qualitative and quantitative differences. Furthermore, these compounds elicited electrophysiological and behavioral responses from both males and females *B. pisorum*, in such a way that the female could be able to discriminate between volatiles from each phenological stage. The identification of these chemical cues from host plant of *B. pisorum* could be used to development of a monitoring system or to lure them into traps in the field. More comprehensive studies are needed to evaluate the effect of the individual compounds or combinations of them at different dose on the behavior of host selection by *B. pisorum*. Our study is a starting point to provide the basic and essential information to develop efficient kairomones to be used in monitoring and Integrated Pest Management (IPM) programs for pea weevil.

CONCLUSIONS

Volatiles released by *Pisum sativum* at different phenological stages have qualitative and quantitative differences in the mixtures of chemicals. More volatiles are releasing at flower phenological stage and most abundant chemical constituents are terpenes. Our study confirmed the antennal response and behavioral importance of the pea-volatiles from different phenological stages. Both males and females of *Bruchus pisorum* are attracted to pea-volatiles, but these chemical compounds elicited large electrophysiological and behavioral responses in females than males. These naturally occurring volatiles emitted by flowers and pods of pea-plants are used as innate cues in host finding by *B. pisorum*.

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