Behavioral Responses of Clover Root Borer to Long-Chain Fatty Acids from Young Red Clover (*Trifolium pratense*) Roots

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ABSTRACT The olfactory and contact behavioral responses of clover root borer, *Hylastinus obscurus* (Marsham) (Coleoptera: Curculionidae), against fatty acid components present in 9-mo-old red clover, *Trifolium pratense* L., roots were investigated by using an automated behavioral observation system. From GC-MS analysis of dichloromethane extracts of *T. pratense* roots, of 15 compounds identified in total, four long-chain free fatty acids lauric, palmitic, oleic, and stearic acids were found to be main components in the extracts. In a four-arm olfactometer the clover root extract significantly attracted both male and female *H. obscurus*. When solutions of the four individual synthetic fatty acids and their blend at the ratio found in the root extract (10 μg/ml) were assayed with *H. obscurus*, lauric, palmitic, and oleic acid elicited an attractant behavioral response from females, whereas all substances tested did not elicit a response from males. In contact bioassays, wood dummies coated with root extract, the four fatty acids, or their respective blend of the same composition found in the root extract at 100 μg/ml, were significantly preferred by female *H. obscurus*. In contrast, males showed preference only for dummies treated with root extract, or palmitic or oleic acid. The behavioral evidences suggest that long-chain free fatty acids present in 9-mo-old red clover roots may play a role as close-range olfactory, tactile cues, or both in the host-finding process of *H. obscurus*.

KEY WORDS Scolytidae, *Hylastinus obscurus*, fatty acids, red clover, *Trifolium pratense*

Red clover, *Trifolium pratense* L., is a legume that has the ability to produce large quantities of high-quality forage. This species is one of the most important legume crops in the temperate climates of the world (Steiner et al. 1997). In Chile, the cultivated area with red clover represent ≈20% (120,000 ha) of the cultivated grassland area in total (Torres and Sierra 1991). Botanically considered a perennial, red clover does not persist indefinitely in permanent pasture because of stands deterioration caused by fungal pathogens and insect infestations (Carrillo and Mundaca 1974).

The red clover root borer, *Hylastinus obscurus* (Marsham), is one of the most serious pests of red clover in the world (Steiner and Alderman 2003). *H. obscurus* is native to Africa and Europe, and has been introduced into North America and Chile, but appears to be absent from Asia and Australasia (Wood and Bright 1992). Both larvae and adults of this scolytid bore and feed into the roots, causing a significant reduction in production levels and persistence of red clover stands within 2 yr after sowing (Cuevas and Balocchi 1983, Steiner and Alderman 2003). Pesticides have not been successful in controlling borer infestations and at present there is no control for clover root borers other than crop rotation (Aguilera et al. 1996). Alternative strategies for control of this pest are a high priority for clover producers, and we are investigating the role of plant chemistry in the life cycle of *H. obscurus* with the aim of developing management tactics that can be integrated into clover production.

In a previous study performed with root extracts from 1.5- and 2.5-yr-old red clover plants collected before flowering, we determined that *H. obscurus* was attracted to root volatiles of 1.5-yr-old clover plant extracts but not to those volatiles from 2.5-yr-old plants (Tapia et al. 2007). In both root extracts, we identified methyl benzoate and *E*-2-hexenal that attracted *H. obscurus* and limonene that was repellent toward them. Quantification of the active compounds in root materials of red clover revealed a significant reduction of the *E*-2-hexenal concentration from 2.12 to 0.76 μg/ml and an increase in the limonene concentration from 0.26 to 0.68 μg/ml in 1.5 versus 2.5-yr-old plants, respectively. This finding explained the lower response of the borer for older plants.

Carrillo and Mundaca (1974) have reported that *H. obscurus* colonizes red clover stands at about a year after sowing. We hypothesized that during colonization of red clover plantings, adult *H. obscurus* are

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Table 1. Composition of volatiles and free fatty acids in dichloromethane extracts of 9-mo-old red clover roots

Compound	Retention time (min)	Kovats indices		Reliability of identification[a]	Relative area (%)
		DBP-5 (experimental)	DBP-5 (reference)		
1-Nonene	4.19	–	891	2	5.9
3-Octanol	4.41	–	991	2	4.0
2-Pentyl furan	7.9	931	1,001	2	4.7
Benzyl alcohol	8.9	962	1,032	1	1.4
Limonene	9.5	969	1,036	1	4.3
Maltol	10.86	970	1,108	2	2.8
Benzoic acid	13.52	1,076	1,210	1	7.9
o-Acetyl *p*-cresol	16.47	1,137	1,316	2	1.2
Eugenol	19.95	1,276	1,551	1	1.6
Lauric acid	23.25	1,397	1,568	1	8.0
Caryophyllene oxide	24.38	1,543	1,573	1	6.4
Pentadecanal	26.34	1,591	1,710	2	2.0
Palmitic acid	31.73	1,679	1,984	1	33.2
Oleic acid	34.81	1,933	2,144	1	12.1
Stearic acid	35.19	2,132	2,200	1	4.5

[a] The reliability of the identification is indicated by the following letters: 1) mass spectrum (MS), Kovats indices and matching with standard; and 2) mass spectrum and Kovats indices agree with corresponding data in the literature.

arrested at close-range by chemicals occurring in the roots of young red clover plants. Consequently, we attempted a chemical analysis of root materials obtained by solvent extraction from 9-mo-old red clover by coupled gas chromatography-mass spectrometry, indicating the presence in the extract of a variety of compounds including long-chain free fatty acids, which are low volatile substances that might act as semiochemicals at close-range after arrival of the insect at the plant.

Some research on legumes has investigated the presence and function of fatty acids in the epicuticular seed waxes. These fatty acids are common components of their structure and are important mediators of host acceptance by bruchid beetles (Coleoptera: Bruchidae) (Piergiovanni et al. 1990, Parr et al. 1998). Fatty acids and other components found in the essential oil from roots of *Cirsium japonicum* (Fisch. ex DC.) elicited oviposition activity from female *Ostrinia zealis* (Miyasawa et al. 2003). For this reason, we hypothesized that the presence of long-chain free fatty acids in the root extract of red clover acts as close-range cues in host finding by *H. obscurus.*

Thus, the objectives of this study were to: 1) analyze, qualitatively and quantitatively, by GC-MS the long-chain free fatty acid content in root extracts of 9-mo-old red clover obtained by solvent extraction, and 2) evaluate the behavioral response of *H. obscurus* toward root extracts and to fatty acids identified in olfactory and contact bioassays by using an automated system for recording insect behavior.

Materials and Methods

Plant Samples. Nine-month-old red clover (*T. pratense*, 'Quiñequeli' plants) were sampled from INIA-Experimental Station at Carillanca, La Araucanía, Chile. Whole plants were sampled in January 2008 with a sufficient amount of soil to avoid root damage. Only flowering plants were used for experiments.

Insects. Red clover roots infested with adult *H. obscurus* were collected every 7 d from an experimental red clover field at the Agricultural Experimental Station at Carillanca (La Araucanía, Chile) from March to June 2008. Once we transferred the clover roots to the laboratory, the beetles were removed from the roots with Berlese funnels and maintained in petri dishes at 18–22°C and a photoperiod of 18:6 (L:D) h on red clover roots. Insects were used in the bioassays in the same week they were collected and were used only once. To use individuals of appropriate physiological status, 10 h before each behavioral assay adult *H. obscurus* were placed individually in petri dishes and those insects that were able to walk were used for olfactory and contact experiments. The sex of the tested insects was determined after bioassays according the methodology described by Carrillo et al. (1978).

Root Extracts. The root extracts were prepared according to the methodology reported by Tapia et al. (2005) with some modifications. Fresh roots (2.0 g) of red clover plants were extracted with 130 ml of distilled dichloromethane (CH_2Cl_2, 99%) for 12 h at 20°C. The solvent was removed under reduced pressure on a rotary evaporator to afford a crude extract, which was redissolved in 3 ml of distilled dichloromethane. An aliquot of 500 μl of the extract solution was concentrated under nitrogen flow to a 100 μl solution, which was maintained at 5°C for used as stock solution.

Chemicals. Pure synthetic compounds, lauric, palmitic, oleic, and stearic acid were obtained commercially (>99%, Cayman Chemical Co., Ann Arbor, MI). Hexane solutions of the fatty acids were prepared for GC-MS analysis and behavioral assays. An artificial blend containing the four synthetic fatty acids at the ratios that they occurred in the root extract (Table 1) was also prepared for testing in the bioassays.

Olfactometer Bioassays. Olfactometer bioassays were performed by using an octagonally shaped olfactometer (6.5 × 6.5 × 1.6 cm) as described by Pet-

tersson (1970) and the experimental conditions have been reported previously (Quiroz et al. 2005, Tapia et al. 2007). The olfactometer arena was divided into four arm zones and one zone in the center was designated as the decision zone. Olfactometer bioassays were carried out between 1000 and 1800 hours (photophase) at 22 ± 1°C and 80 lux. An individual *H. obscurus* was placed in the central part of the arena permeated by charcoal filtered air (600 ml/min) coming from each of the four arms of the olfactometer and drawn out through a hole above the center of the arena. Two lines of air coming from each stimulus treatment were connected in opposite corners of the arena; the other two lines released air from control treatments. The behavior of each insect was recorded for 20 min, and the time spent in each arm was registered and evaluated by using EthoVision 3.1 software (Noldus et al. 2002). Olfactory experiments were performed with root extract solutions formulated in distilled dichloromethane at 10 μg/ml, and with individual hexane solutions of synthetic fatty acids at 10 μg/ml or with a solution of their blend formulated in hexane at 10 μg/ml (1.5 μg of lauric acid, 5.7 μg of palmitic acid, 2.0 μg of oleic acid, and 0.8 μg of stearic acid), according to Tapia et al. (2007). In the experiments, each treatment was replicated 15 times with root extract and 20 times with synthetic fatty acids.

Gas chromatography-mass Spectrometry (GC-MS). An aliquot of 1 μl of the root extract was analyzed using a FOCUS-GC and a DSQ mass spectrometer detector (Thermo Electron, Austin, TX) with electron impact ionization (70 eV) detection by using a DBP-5 capillary column (30 m × 0.22 mm × 0.25 μm film thickness) with helium as the carrier gas. The GC oven was programmed to ramp from 40 to 280°C at 5°C/min and then to hold for 10 min. The injector and transfer line temperatures were set at 250°C. The fatty acids present in the root extract were detected by monitoring the characteristic single ions at *m/z* 60 and *m/z* 83 for saturated and monoenoic fatty acids as well as *m/z* 67, *m/z* 79 and *m/z* 93 for fatty acids with more than one double bond (Silverstein and Webster 1998). Fatty acids were identified by calculation of the Kovats indices and comparing the mass spectra of compounds with those from commercial standards and by means of a library searching in the NIST mass spectral database (NIST ver. 2.0, Thermo). The fatty acid composition was calculated with the relative area of the peaks on the chromatogram.

Contact Bioassays. To assay for close-range responses, wood dummies (5 mm in diameter × 15 mm in length), simulating a piece of red clover root, were washed with dichloromethane in a Soxhlet apparatus and air dried before use. The wood dummies were coated separately with different solutions: 1) root extract formulated in distilled dichloromethane at 100 μg/ml; 2) individual synthetic fatty acids formulated in hexane at 100 μg/ml; and 3) a blend of synthetic fatty acids formulated in hexane at 100 μg/ml (15 μg of lauric acid, 57 μg of palmitic acid, 20 μg of oleic acid, and 8 μg stearic acid), according to the methodology reported by Mutis et al. (2009). Control treatments consisted of distilled dichloromethane or hexane when root extracts or synthetic compounds were tested, respectively. The treated wood dummy was placed in the center of a petri dish (5 cm in diameter), and centered in a rectangular area (22 mm × 30.8 mm). The time spent on the dummy and in the rectangular area were both recorded and pooled for the statistical analysis. One insect was placed on the dummy and its behavior was recorded for 10 min by using EthoVision 3.1 software (Noldus et al. 2002). Each treatment was replicated 20 times.

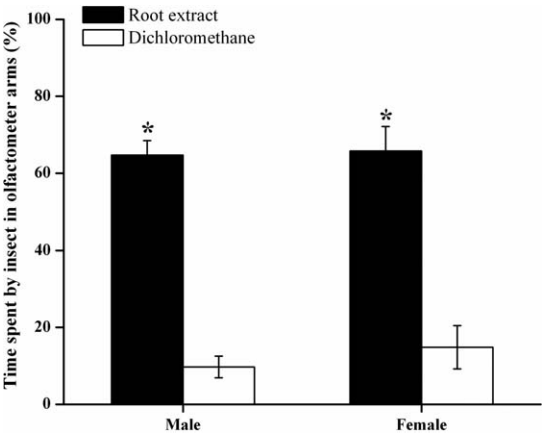

Fig. 1. Olfactory response of male and female *H. obscurus* to 9-mo-old red clover root extract and to dichloromethane control. * Significantly different from control based on the Wilcoxon test ($P < 0.05$). Male, $n = 9$; Female, $n = 6$.

Statistical Analysis. In the olfactometer bioassays, the time spent by the insects in stimulus or control arms of the olfactometer was compared by using a Wilcoxon test ($\alpha = P < 0.05$). In the contact bioassays, the time spent on the wood dummy and in the rectangle were analyzed by using a Kruskal–Wallis test, and groups were separated by using the Conover–Inman test ($\alpha = P < 0.05$) (Conover 1999).

Results and Discussion

Olfactory Response of *H. obscurus* Toward Red Clover Root Extracts. In the olfactometer bioassays, the extract (10 μg/ml) from 9-mo-old red clover roots was attractive to both sexes of the borers (Fig. 1), males, ($P = 0.002$); females ($P = 0.015$). The response of both male and female *H. obscurus* to the root extracts from 9-mo-old red clover plants suggests that substances therein could play an active role in the host recognition process by these borers.

GC-MS Analyses of Red Clover Root Extracts. With the aim to identify the potential chemical compounds responsible of the biological activity, we analyzed the root extract without chemical transformation. In the GC-MS analyses of the root extracts, 15 compounds in total were identified, including four fatty acids: lauric (C12:0), palmitic (C16:0), oleic (C18:1), and stearic acid (C18:0), whose identifications were corroborated with the calculation of the Kovats indices. Palmitic

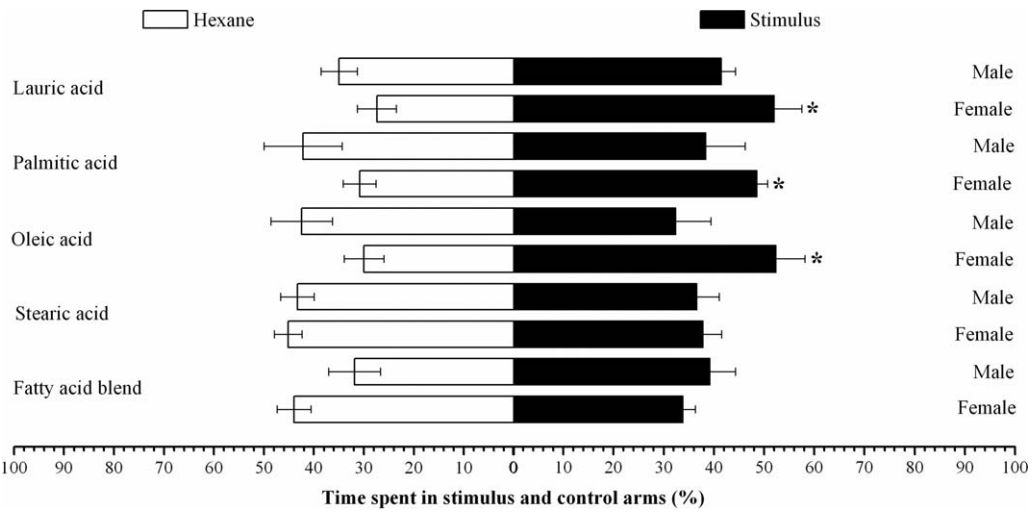

Fig. 2. Olfactory response of male and female *H. obscurus* to four pure fatty acids and their blend in the ratio found in the root extract at 10 μg/ml compared with their response to hexane control. * Significantly different from control based on the Wilcoxon test ($P < 0.05$). Male, $n = 8$; Female, $n = 12$.

acid (C16:0) was the major fatty acid present in root extracts (33.2%) (Table 1).

Characterization of long-chain free fatty acids from young red clover roots has not been reported before. These findings indicated that the main fatty acids present in red clover roots were of a similar nature to those fatty acids present in most plant species (Dewick 2002). The relative amounts of palmitic, oleic, and stearic acids found in clover root extracts were in accordance with their relative concentration levels reported in fatty acid profiles from leaves of red clover (Murata et al. 1982).

Olfactory Response of *H. obscurus* Toward Pure Fatty Acids. Because of the low volatility of long-chain fatty acids, we were interested in whether these compounds could play a role in olfaction with *H. obscurus.* Thus, we assayed the four indentified fatty acids and a synthetic blend that mimicked their proportions in root extracts. Hexane solutions of lauric acid ($P = 0.008$), palmitic acid ($P = 0.001$), and oleic acid ($P = 0.03$) significantly attracted female *H. obscurus* (Fig. 2). Females showed no preference to either stearic acid or to a blend of fatty acids when compared with the response to the control. In contrast, no significant differences in male responses were observed in the attraction to any of the substances, when compared with the control. Thus, despite their low volatility, lauric, palmitic, and oleic acid elicited a significant attractive olfactory response from female *H. obscurus.* Therefore, fatty acids that occur in young red clover roots could be involved in the attractive activity in root materials toward female *H. obscurus.*

Contact Bioassays. Previous studies on the behavior of flies (Cole et al. 1989) revealed that carboxylic acids of low volatility are probably perceived by contact receptors rather than by olfactory receptors. Thus, we performed contact bioassays by using wooden dummies treated with fatty acids and extracts for evaluating the behavior of *H. obscurus.* The treatments included samples of root extract, individual pure fatty acids (100 μg/ml), and a blend of the four pure fatty acids (100 μg/ml) in ratios found in the root extract. The results showed that the extract was preferred significantly by male ($P = 0.0034$) and female ($P = 0.0124$) *H. obscurus* when compared with the control (Fig. 3). However, both the synthetic pure fatty acids and the blend showed significant ($P < 0.05$) effects on female *H. obscurus* behavior (Fig. 4). The palmitic and oleic acid treatments also had similar significant ($P < 0.05$) effects on the preference of male *H. obscurus*, whereas lauric or stearic acid as well as the blend of

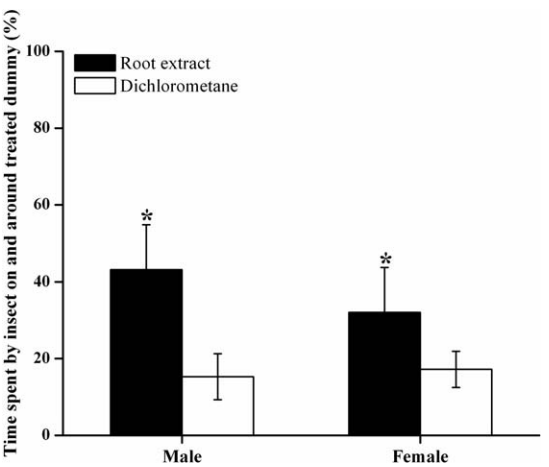

Fig. 3. Mean ± SE (%) time spent by male and female *H. obscurus* in the observation area containing a dummy treated separately with 9-month-old red clover root extract sample at 100 μg/ml and with dichloromethane control. * Significantly different from control based on the Wilcoxon test ($P \leq 0.05$). Male, $n = 10$; Female, $n = 10$.

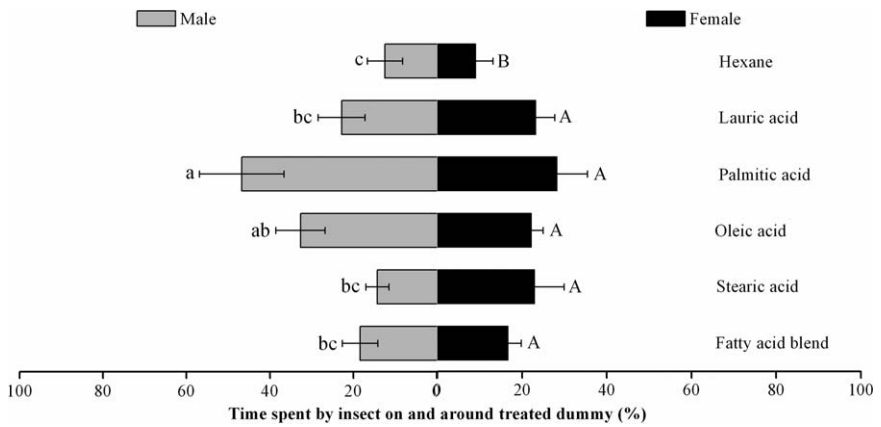

Fig. 4. Mean ± SE (%) time spent by male and female *H. obscurus* in the observation area containing a dummy treated separately with samples of the test substances at 100 μg/ml. Histogram bars within a sex with the same letter are not significantly different based on the Kruskal–Wallis test followed by the Conover–Inman test ($P < 0.05$). Male, $n = 10$; Female, $n = 10$.

fatty acids showed no significant effects on male behavior (Fig. 4). Because female *H. obscurus* responded to the fatty acid blend in the contact bioassay (Fig. 4), but did not respond to this stimulus in the olfactometer (Fig. 2), they may be able to detect both the individual fatty acids and the blend whit contact receptor. However, only a subset of the individual fatty acids may be detected by olfactory receptors (Fig. 2). It is interesting to note that the dummy coated with the fatty acid blend (Fig. 4) mimicked the preference response of female *H. obscurus* to the dummy treated with clover root extract (Fig. 3). In contrast, in olfactometer bioassays the fatty acid blend (Fig. 2) did not mimic the attractant response of females to clover root extract (Fig. 1).

The importance of long-chain free fatty acids as semiochemicals has been demonstrated for females of various insect species. Thus, both oleic and linoleic acids were found to be components of ovipositional host-finding cues for the navel orangeworm, *Amyelois transitella* (Walker) females (Phelan et al. 1991). Fatty acids from C14–C24 present in the epicuticular waxes of seeds of the mung bean, *Vigna radiata* (L.) R. Wilczek; and chickpea, *Cicer arietinum* L.; stimulated or deterred oviposition in the bruchid beetle, *Callosobruchus maculatus* (F.), depending on the ratio of fatty acids in mixtures assayed (Parr et al. 1998). Also, C14–C28 acids elicited significant oviposition responses in the spruce budworm, *Choristoneura fumiferana* (Clemens), only at doses that exceeded their levels in foliage waxes of the host trees, *Picea* and *Abies* spp. (Grant et al. 2000). Furthermore, fatty acids in the frass of cotton bollworm, *Helicoverpa armigera* (Hübner), significantly deterred oviposition of conspecifics (Xu et al. 2006). Finally, Dong et al. (2005) have shown that calling behavior and mating rate were reduced significantly when female cotton bollworm, *Helicoverpa armigera*, were exposed to Z6-octadecenoic, Z9-octadecenoic, 9-octadecenoic, or Z11-eicosenoic acid. However, free fatty acids such as linoleic, oleic, and stearic acid, identified in *Zea mays* L. seedling, have been reported as semiochemicals for host location by larvae of the western corn rootworm *Diabrotica virgifera* Leconte (Hibbard et al. 1994).

The behavioral evidence reports in this work, suggest that female *H. obscurus* could perceive some individual long-chain fatty acids by both olfactory and contact receptors. Thus, long-chain free fatty acids that are present in 9-mo-old red clover roots might function as close-range olfactory and/or tactile cues for both host recognition and acceptance process by female *H. obscurus* as they search for early growth stages of red clover.

Further studies with the identified fatty acids, such as electroantennogram and oviposition experiments, are needed to gain a more detailed understanding of the effects of these compounds on the behavior of female *H. obscurus.*

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