Response of the Beetle *Hylastinus obscurus* Marsham (Coleoptera: Scolytidae) to Red Clover (*Trifolium pratense* L.) Volatiles in a Laboratory Olfactometer

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ABSTRACT The response of field-collected *Hylastinus obscurus* Marsham (Coleoptera: Scolytidae) to volatiles from *Trifolium pratense* L. of different ages was studied in a four-arm olfactometer. Volatiles from 1.5-, 2-, and 2.5-yr-old plants were more attractive than volatiles from 1-, 3-, and 3.5-yr-old plants. Two-year-old plants were preferred during winter, spring, and summer. One-year-old plants were not preferred in their early stages, but preference increased as they aged. Volatiles from 3-yr-old plants were never preferred. The essential oils obtained from 2-yr-old *T. pratense* elicited an attraction response from *H. obscurus*. Beetle response is discussed in relation to host-locating behavior.

KEY WORDS Scolytidae, *Hylastinus obscurus*, volatiles, olfactometry, laboratory walking behavior

OLFACTORY RESPONSE TO PLANT volatiles is a critical step in the host-finding process of herbivorous insects, which usually perceive plant volatiles to choose between host and nonhost plants (Visser 1986, Bernays and Chapman 1994). In bark beetles (Coleoptera: Scolytidae), it has been reported as the most fundamental factor used to reject a tree or stand of an inappropriate host species (Borden 1997). Furthermore, odor perception may give relevant information to insects about the feeding suitability of the host, its quality as an oviposition site (Meiners and Hilker 2000, Hilker and Meiners 2002), pre-existing conspecific density (Quiroz et al. 1997), plant stress (Pickett et al. 2003), and the presence of natural enemies (Dicke and Grostal 2001). Plant volatiles have been shown to vary because of abiotic factors such as environmental variation (Takabayashi et al. 1994, Gouinguene and Turlings 2002), but little is known about how the olfactory response of the insect is affected by temporal variation of host plant volatiles.

Among beetles, those belonging to the family Scolytidae can select a host-plant by two general mechanisms: (1) individual random landing on host and nonhost plants or (2) individual discrimination between host/nonhost or healthy/weakened host plants (Wood 1982, Borden 1997). Results from a theoretical study carried out by Gries et al. (1989) show that, although beetles may find host trees at random, their success would increase greatly by searching upwind with short range olfactory attraction to susceptibles trees. Recent studies support the idea that host-finding and selection in many species of scolytids is based on detection of volatile compounds elicited from weakened host plants (Erasmus et al. 1994, Byers 1996, Bonello et al. 2001) or nonhost compounds (Borden 1997). However, most of these studies have been performed with scolytids that colonize the above ground portion of trees. Much less is known about host-finding mechanisms in scolytids that colonize the roots, let alone the roots of herbaceous plants.

The red clover root borer (*Hylastinus obscurus* Marsham) is a scolytid that feeds on red clover (*Trifolium pratense* L.). *H. obscurus* fly to new fields during the spring (Koehler et al. 1961); in Chile, therefore, its dispersed flight occurs between October and December. However, when weather conditions are unfavorable for flight, the insects disperse by crawling (Rockwood 1926). Newton and Graham (1960) have suggested that the increase of the population of *H. obscurus* after spring is related to the movements of the insects between plants. Another report has provided additional evidence that the crawling activity during summer is limited to short distances (Culik and Weaver 1994). The extent of localized crawling activity remains to be determined.

Fertilized females can lay eggs in roots of several red clover plants (Rockwood 1926). The adult females lay eggs in the crown of the plant, and the larvae mine out channels in the roots, which become sites for infection

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by root-rot fungi (Leath and Byers 1973). Although there are some reports indicating that *H. obscurus* is a pathogen vector (Kamm and Buttery 1984, Jin et al. 1992), the root borer is a primary causal agent of stand decline (Jin et al. 1992, Steiner and Alderman 2003), reducing herbage (Prues and Weaver 1958), and seed yield (Steiner and Alderman 1999). In Chile, *H. obscurus* is the main cause of *T. pratense* decline, and pesticides have not been successful in controlling this pest.

The interaction between this scolytid and its host has been associated with root volatile compounds from *T. pratense* (Buttery et al. 1984, Kamm and Buttery 1984). Several compounds isolated from roots, flowers, and leaves of *T. pratense* have been shown to attract *H. obscurus.* However, these results were obtained using both macerated roots and isolated aerial parts from the plant. Such approaches do not ensure that the compounds reported are released from nonmacerated tissues, the conditions under which *H. obscurus* select their host plants.

This study investigated the role of volatile compounds released from different aged *T. pratense* plants in eliciting different olfactory responses from adult *H. obscurus.* Seasonal variation of the olfactometric responses was also studied. This information will provide an understanding of the role of volatiles in mediating the host-selection process of this pest species and allow more specific studies into the use of volatiles in pest management. The exact chemicals involved in this relationship will be identified in a following study.

Materials and Methods

Insects. Adult *H. obscurus* were collected monthly from an experimental field at the Agricultural Experimental Station at Carillanca (Temuco, Chile) from June to January in the years 2001, 2002, and 2003. Once transferred to the laboratory, individuals were maintained in petri dishes (18–22°C and 18L:6D light regimen) on red clover roots (*T. pratense* cultivar Quiñequeli). Insects were used in the bioassays in the same month that they were collected and were only used once. To use individuals of appropriate physiological status, 2 h before each behavioral assay, adult *H. obscurus* were observed for 10 min; those that walked and were active in that time were selected for olfactory experiments. The sex of the tested insects was determined following the methodology described by Carrillo et al. (1978).

Plants. Noninfected individual 1-yr-old *T. pratense* cultivar Quiñequeli plants were extracted from a experimental crop at Carillanca in June 2001 ($n = 6$) established 1 yr before. A 40-cm PVC cylinder was introduced 30 cm underground to isolate one *T. pratense* plant from the field. Cylinders with plant and soil were transferred to the laboratory, but they were kept outdoors to keep the plants under natural environmental conditions. Only nonflowering *T. pratense* plants were used for experiments.

Olfactometric Bioassays General Procedure. Bioassays were performed in an olfactometer, as described by Pettersson (1970). Olfactometries were carried out between 1000 and 1800 hours (photophase) at 22 ± 1°C and 80 lux. An individual *H. obscurus* was enclosed in an arena, which was permeated at 250 ml/min by air coming from each of the four arms of the olfactometer and drawn out through a hole above the center. Two lines of air coming from a stimuli treatment (whether *T. pratense* plants or essential oil, see below) were connected in opposite corners of the arena; the other two lines of air came from a control treatment. The air was purified by passing it through glass tubes containing activated charcoal and molecular sieves (1 Å diameter). Thirty minutes before starting the experiment, the test specimen of *H. obscurus* was placed into the olfactomer for conditioning. Each insect was constantly observed for 30 min, and the time spent in each arm was noted. The ratio of cumulative time spent in treated versus control arms was calculated for each individual (response ratio). A ratio >1 indicated attractiveness and a ratio <1 indicated repellence.

Effect of Plant Age on Olfactory Response. To assess the insect response to *T. pratense* plants of different ages, individual *H. obscurus* ($n = 10$) were subjected to olfactometric bioassays as described above. Potted *T. pratense* plants enclosed in bell jar were used as stimuli treatments, whereas only a plastic pot and soil were used as control stimuli. Olfactory bioassays were performed with three plants every 6 mo, starting when they were 1 yr old and progressing until they were 3.5 yr old.

Seasonal Variation in Olfactory Response to Plants of Different Ages. To assess the insect response to *T. pratense* plants as the seasons changed, individual *H. obscurus* ($n = 10$) were subjected to olfactometric bioassays once a month from June through January with plants of different ages. One-year-old plants (three repetitions) were tested in June 2001 (winter) to assess their attractiveness to insects. These same plants were tested again in June 2002 and June 2003, when they were 2 and 3 yr old, respectively. Ten different *H. obscurus* individuals were tested for each plant in each month.

Effect of Essential Oil on Olfactory Response. Olfactometric assays were also conducted in response to *T. pratense* essential oil. The essential oil was obtained by 5 h of exhaustive steam distillation (0.20% *T. pratense* oil yield) using 100 g of 2-yr-old *T. pratense* leaves (Buchbauer et al. 1996). Sample and solvents (pentane) were applied to a piece of filter paper (6 by 0.5 cm; Whatman 10) and introduced into a 1-cm-diameter, 15-cm-long glass tube. This was the odor source for the treatment arms; another filter paper (6 by 0.5 cm) imbibed with only solvent was used as the odor source for the control arms. A total of 10 individual insects were studied.

Statistical Analysis. One-way analysis of variance (ANOVA) was performed to compare the response of *H. obscurus* to plant volatiles. Where necessary, data were $\log(x + 1)$ transformed to meet the assumptions

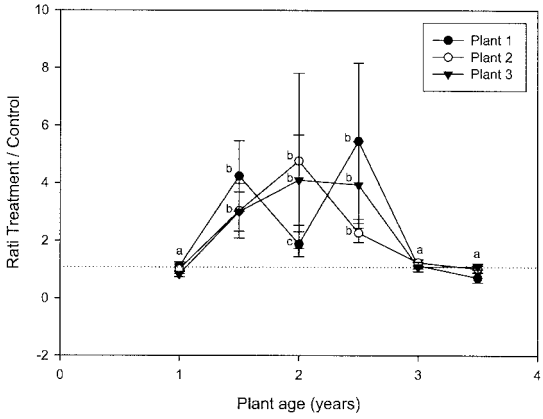

Fig. 1. Olfactory response of *H. obscurus* to volatiles from three plants of different ages of red clover, *T. pratense*, in olfactometer tests. Dotted line indicates equal response to treatment and control arm (ratio treatment/control = 1). Different letters within sets of bars mean that the differences between the values are significant at $P < 0.05$ (LSD multiple-comparison test). Bars indicate 1 SE.

of the ANOVA (Zar 1998). Multiple comparisons were performed with the least significant difference (LSD) test. The same analysis was applied to the data from the study of the seasonal variation in olfactory responses to plants of different ages. To evaluate the effect of sex on odor preference, the time spent in different treatments was compared between males and females using a nonparametric Mann-Whitney test for independent samples. Response of *H. obscurus* to essential oil was assessed with the nonparametric Wilcoxon matched pairs test. All statistical analyses were performed with the software STATISTICA 6.0 (StatSoft 2001).

Results

The response ratios for *H. obscurus* to differently aged plants varied significantly among the treatment groups in the three plants tested ($F_{(5,54)} = 6.61$, $P < 0.001$; $F_{(5,54)} = 3.39$, $P < 0.01$; and $F_{(5,54)} = 6.27$, $P < 0.01$). In the case of two plants (plants 2 and 3), the responses toward volatiles of 1.5, 2, and 2.5 yr olds were not significantly different, but were greater than the responses to volatiles from 1 and 3.5 yr olds, which did not elicit attraction (Fig. 1). Insect response to plant 1 showed a different behavior, with a reduced attraction as a 2 yr old (Fig. 1).

The effect of season on odor preference by *H. obscurus* was studied by conducting a series of olfactometer tests throughout the year. During winter (June to August), *H. obscurus* did not show a preference for the volatiles from 1-yr-old plants, which is apparent from the overlap between the 95% confidence interval and the ratio treatment/control of no-preference (Fig. 2A). Although from the end of spring (November) until midsummer (January), former 1-yr-old plants came to elicit an attraction response from

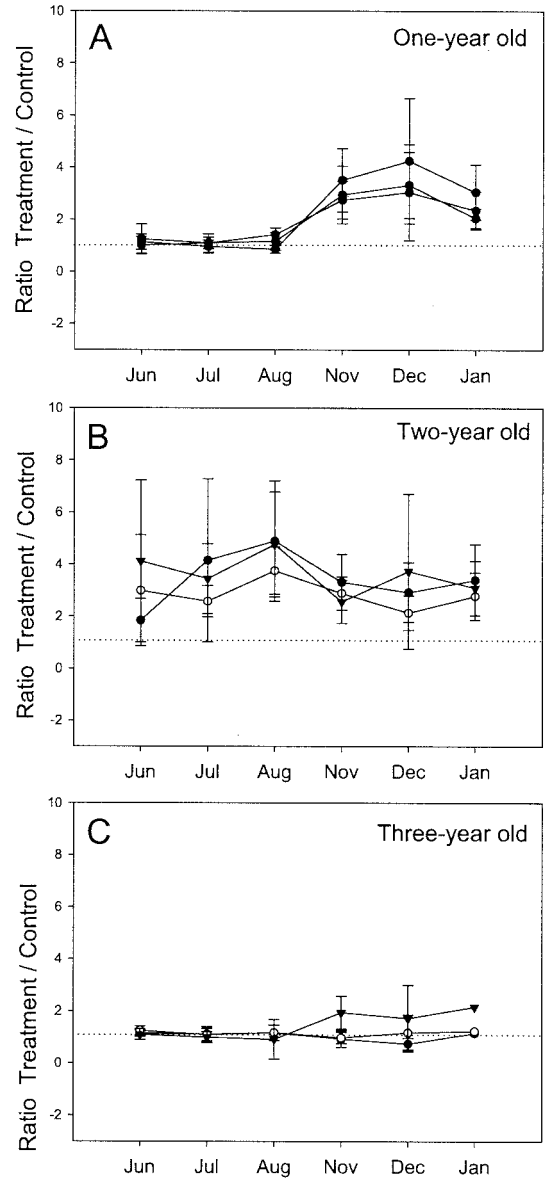

Fig. 2. Olfactory response of *H. obscurus* to volatiles from red clover plants (*T. pratense*) of (A) 1-yr-old, (B) 2-yr-old, and (C) 3-yr-old in olfactometer tests over 6 mo (three seasonal periods each year from 2001 to 2003). Three plants were used with 10 insects tested per plant each month. Dotted line indicates equal response to treatment and control arm (ratio treatment/control = 1). Bars indicate 95% confidence intervals.

H. obscurus (Fig. 2A). However, volatiles from 2-yr-old plants elicited an attraction response during the entire time period (winter to summer; with most of confidence interval of ratio treatment/control values above the no-preference line, Fig. 2B). Differently, 3-yr-old plants were never attractive (Fig. 2C).

There were no significant differences between male and female behavior responses to the different stimuli

Table 1. Response of *H. obscurus* to essential oil obtained from 2-yr-old red clover, *T. pratense*

Stimulus	Mean time spent in each arm (min)[a]	*n*	*P*[b]
Essential oil	16.51 ± 1.57	10	0.005
Control (pentane)	10.29 ± 1.52		

[a] Mean ± SE.
[b] Significantly different compared with $\alpha = 0.05$ following Wilcoxon matched-pairs test.

in the olfactometric bioassays ($Z = -0.850, P = 0.458$). However, volatiles produced by 2-yr-old *T. pratense* were isolated by steam distillation. A dilute extract of this essential oil (0.2% wt:vol) was tested in the olfactometer. The results showed that essential oils obtained from 2-yr-old *T. pratense* attracted *H. obscurus* relative to the pentane control (Table 1).

Discussion

Our results show that odors released from 1.5- to 2.5-yr-old *T. pratense* elicit attraction from *H. obscurus* (Fig. 1). This result agrees with field observations that show that the population peak of adults *H. obscurus* occurs when a plant is 2 yr old (70% plant damage) (Carrillo and Mundaca 1974). In this phenological state, the plant's radical system, the size of the main root, and the diameter of the crown are adequate for oviposition, and the larvae can mine out channels in the roots. In this way, both adult beetles and larvae can feed and live in these galleries during the winter. The difference in *H. obscurus* behavior when stimulated by volatiles from a 1- and 1.5-yr-old plant support the observations mentioned above; the plant elicited an attraction response only once it reached 1.5 yr (Fig. 2A), showing a correlation with the results seen in Fig. 1. A correlation between the two experiments can also be seen when comparing the study of volatile attractiveness from 1.5- and 2.5-yr-old plants to *H. obscurus* (Fig. 1) with the study of the attractiveness of the volatiles released starting with a 1-yr-old plant and progressing until it reached 1.5 yr old (Fig. 2A). This timing coincides with the dispersal flight of this insect, which starts in the first week on October and goes until the third week of December (Matamala 1976, Aguilera 1989). Our results did not show any significant behavioral differences between sexes in olfactometric bioassays. This fact could suggest that both males and females can recognized the host plant, and consequently their food source, with the same strength. Despite that other studies have reported differently responds to host volatiles from both adult female and male beetles (Zhu et al. 1999) and differences in food selection in phytophagous insects (Bernays and Chapman 1994), our results could be supported by the fact that red clover is the exclusive host plant for *H. obscurus* and the complete life cycle is developed around this legume. The fact that 3-yr-old plants did not elicit any response from *H. obscurus* (Fig. 2) can be explained by two possible mechanisms. (1) Insects had infested and may have strongly damaged the radical systems of these plants, which may change the semiochemistry of the plant-insect system (Schütz et al. 1997). Consequently, this damage would induce deter feeding responses from herbivores (Rosenthal and Berenbaum 1992). (2) It is likely that, regardless of insect infestation, 3-yr-old *T. pratense* plant volatiles do not elicit attractiveness per se. In relation to the former mechanism, although experimental plants used were initially uninfected, we cannot discard that natural infestation could have occurred during the study, and this possibility was not verified at the end of the experiment. Interestingly, whatever the underlying mechanism, these results suggest that *H. obscurus* could distinguish between suitable and unsuitable (weakened by the insect or by plant age itself) host plants by means of volatiles released from the aerial part of the plant. Because of the aerial part was not isolated from both soil and root part of the plants, volatiles that elicited an attractive response from *H. obscurus* also could be coming from roots of the clover plants. This result is according to Kamm and Buttery (1984) that showed that volatile from roots were more attractive to *H. obscurus* than volatiles from leaves, flowers, or pods of red clover.

The role of host physiology on volatiles and the corresponding response of *H. obscurus* has not been well understood. Previous studies have shown that *H. obscurus* adults were attracted to several chemical compounds isolated from diseased roots (Kamm and Buttery 1984). The results obtained by these authors could support the hypothesis that *H. obscurus* prefers diseased to healthy hosts. However, it is not clear whether they compared the response of *H. obscurus* against volatile extracts or pure compounds coming from healthy plants. In addition, the vegetal material was treated to strong mechanical and chemical stress; plant parts were cut or chopped, and afterward, the volatile components were isolated by direct solvent extraction. This procedure does not discriminate as to whether the chemical substances identified were produced by the plant-pathogen interaction or if they were artifacts generated by mechanical and/or chemical factors. In our study, odor stimuli were generated using intact *T. pratense.* Furthermore, from the results obtained by Kamm and Buttery (1984), it is not possible to separate the olfactory component from the tactile stimulus, because the bioassays used did not eliminate social interaction between test individuals (Ramírez et al. 2000). The olfactometer bioassays used in our investigation separated these variables. Despite these differences, the results of Kamm and Buttery (1984) suggesting that *H. obscurus* prefer diseased hosts to healthy hosts is supported by previous reports indicating that root pieces of *T. pratense* infected with different fungi were more attractive to borers than were healthy root pieces (Leath and Byers 1972, 1973). Most of the volatile compounds isolated from aerial parts, however, were not attractive to *H. obscurus;* only methyl salycilate, a compound commonly found in plants, elicited an attraction response (Kamm and Buttery 1984).

In summary, the results from this study suggest that *H. obscurus* show changes in attractiveness with plant age and also seasonal variation in attractiveness. The fact that *T. pratense* borers are attracted by odors coming from these plants suggests that volatiles may be participating in the host selection process of *H. obscurus* during its flight period. Results describe herein are confirming in intact plants previous results found by Kamm and Buttery (1984). However, the fact that essential oil elicits an attraction response from adult *H. obscurus* individuals suggests that this essential oil could be also used as an attractant in an integrated management program. Future studies will focus on the biological activities of the individual chemical constituents from the essential oil and of mixtures of the constituents in the field.

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