Examination of the Interaction Between the Black Vine Weevil (Coleoptera: Curculionidae) and an Entomopathogenic Fungus Reveals a New Tritrophic Interaction

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ABSTRACT The purpose of this study was to characterize the behavior of black vine weevil larvae, *Otiorhynchus sulcatus* (F.), in the presence of two possible control options: the synthetic pyrethroid bifenthrin and the entomopathogenic fungus *Metarhizium anisopliae* (Metch.) Sorokin. Five third-instar black vine weevil were placed in two-choice soil olfactometers that allowed larvae to infest one of two pots. Larvae were allowed to choose between *M. anisopliae* (1×10^6 spores/g dry media) and untreated media, bifenthrin (25 ppm) and untreated media, as well as *M. anisopliae*– and bifenthrin-treated media. For all comparisons, experiments were conducted without plants in the system to test for innate responses, as well as with plants to test host–plant influence. Larvae were significantly deterred by bifenthrin without plants present in the system. No significant effect on larval preference was observed when *M. anisopliae* was present in media for trials without plants. *M. anisopliae*–treated media was preferred by black vine weevil larvae over bifenthrin without plants present in the two-choice soil olfactometer. When plants were included, a significant attraction to *M. anisopliae*–treated media was observed over untreated media. Unlike comparisons without plants, larvae were not repelled by bifenthrin when plants were included in the two-choice soil olfactometer. The attraction of black vine weevil larvae to pots containing plants and fungus indicates the operation of a previously undescribed tritrophic interaction. This behavior may be useful in the development of more effective biological control programs.

KEY WORDS tritrophic interaction, *Metarhizium anisopliae*, bifenthrin, insect behavior, olfactometer

The black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae), is a highly polyphagous insect pest of economic importance in several parts of the United States, especially nursery and greenhouse operations in the Pacific Northwest (PNW) (Warner and Negley 1976). The black vine weevil typically overwinters as a prepupa or a last instar (Smith 1932). Overwintering larvae complete their development and adults eclose from early June to late July, with a peak eclosion near the middle of June. Adults may overwinter in the greenhouse and continue egg laying the following spring (Cowles et al. 1997). Oviposition begins in the middle of July for adults that emerge in June, after a preovipostion period of 28–50 d. Larval feeding on roots can cause high levels of plant damage. For some plant species (i.e., *Cyclamen*), one weevil larva is enough to kill the plant (Moorhouse 1990). Because the black vine weevil is parthenogenic, there is much emphasis put on keeping it out of nurseries before an infestation can begin. This has resulted in a zero tolerance level for the presence of black vine weevil in plants shipped between locations. To limit larval infestation, growers typically incorporate chemical insecticides in the potting media; the synthetic pyrethroid bifenthrin, sold under several trade names, including Talstar 0.2G, is the compound most commonly employed in the PNW (D. Hicks, personal communication).

The use of the entomopathogenic fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavacipitaceae) has also been shown to offer effective black vine weevil control (Moorhouse 1990, Moorhouse et al. 1993, Bruck 2005). *M. anisopliae* is a common soil-borne fungus that has a worldwide distribution (Zimmerman 1993), including the PNW (Bruck 2004). Recent uses of *M. anisopliae* include widespread applications in Brazil to control spittlebug *Mahanarva* spp. (Homoptera: Cercopidae) on sugarcane (McCoy et al. 1988) and in Africa to control locusts (Orthoptera: Acrididae) (Shah and Pell 2003). *M. anisopliae* is effective at controlling black vine wee-

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vil larvae when incorporated into potting media before potting plants (Moorhouse 1990). Additionally, *M. anisopliae* has recently been identified as a rhizosphere competent organism, able to persist in the rhizosphere in numbers significantly higher than those found in the surrounding bulk soil (Hu and St. Leger 2002, Bruck 2005). In the context of these studies, we use the definition of rhizosphere competence proposed by Schmidt (1979), who defined "rhizosphere competent" microorganisms as those showing enhanced growth in response to developing roots or a classical rhizosphere effect. Treatment of roots with a conidial suspension of *M. anisopliae* is also an effective means of controlling black vine weevil larvae (Bruck 2005).

Above-ground tritrophic interactions often involve a natural enemies response to plant volatiles released in response to herbivory (Turlings et al. 1995). Recent studies have revealed a tritrophic interaction playing a significant role in the ecology of a fungal entomopathogen used in the control of green mites on cassava, where green plant volatiles suppress the germination of the fungus *Neozygites tanajoe* Delalibera Jr., Humber and Hajek sp. nov. (Zygomycetes: Entomophthorales) in the absence of mite feeding (Hountondji et al. 2005). However, plant volatiles released in response to green mite feeding triggered conidiation, allowing the fungus to release infective spores when mites were present. Tritrophic interactions have also been shown to occur below ground in experiments examining entomopathogenic nematodes and their attraction to the conifer *Thuja occidentalis* L. (Pinales: Cupressaceae) after root herbivory by black vine weevil larvae (van Tol et al. 2001). The induction of natural enemy attractants in response to root herbivores has also been identified in turnips (Neveu et al. 2002), tulips (Aratchige et al. 2004), and corn (Rasmann et al. 2005).

The presence of synthetic insecticides can influence the behavior of the target pest. For example, female diamondback moths, *Plutella xylostella* L. (Lepidoptera: Plutellidae), avoided ovipositing on cabbage seedlings treated with the pyrethroid permethrin (Jallow and Hoy 2005). Biological control agents may also affect insect behavior. Young tobacco budworm larvae, *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), were deterred from feeding on discs treated with purified endotoxin of *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) (Gould et al. 1991). Mole crickets (Orthoptera: Gryllotalpidae) modify their behavior in response to *M. anisopliae* and *Beauveria bassiana* (Hypocreales: Clavacipitaceae) in a way that reduced their exposure to these fungi (Villani et al. 2002, Thompson and Brandenburg 2005). Over time, populations of insects subjected to intensive control measures for which they possess an innate, genetically heritable repulsion may develop behaviors that "reduce exposure to toxic compounds or that allow an insect to survive in what would otherwise be a toxic and fatal environment" a phenomena known as behavioral resistance (Sparks et al. 1989).

Consideration of broad ecological interactions is an important aspect of pest control operations. To date, there have been no studies to examine the impact of either *M. anisopliae* or bifenthrin on the behavior of black vine weevil larvae. Understanding the effect these management options have on black vine weevil larval behavior could provide insight into the basic below ground ecology and improve control of this insect. The objective of this experiment was to test for a behavioral response of black vine weevil larvae to soilless potting media incorporated with bifenthrin and *M. anisopliae.*

Materials and Methods

Source of Fungus, Insects, and Plants. We used a commercially produced formulation of *M. anisopliae* (strain F52; Earth BioSciences, New Haven, CT), which is registered with the U.S. Environmental Protection Agency for use against several insects, including black vine weevil. The granular product consisted of rice grains on which the fungus had sporulated and was subsequently dried. Granules were stored at 4°C until use. Black vine weevil larvae (third to fourth instar) were obtained from a colony maintained at the USDA–ARS Horticultural Crops Research Laboratory, Corvallis, OR (Fisher and Bruck 2004). Rooted cuttings of birds nest spruce *Picea abies* L. Karst. (Pinales: Pinaceae) variety *nidiformis* were obtained from a local nursery and used in all trials containing plants. Plants were maintained in a soilless media blend consisting of a 2:1 mixture of peat moss (Sunshine Mix #3; SunGro Horticulture, Bellevue, WA) and turkey grit (Cherry Stone #3; New Ulm Quartzite Quarry, New Ulm, MN). The turkey grit was used instead of perlite to provide drainage and aeration while not interfering with researchers locating the larvae at the end of the trials.

Design and Validation of the Two-choice Soil Olfactometer. To determine the ability of black vine weevil larvae to respond to different cues emanating from soilless potting media, two-choice soil olfactometers were constructed and used exclusively throughout the experiment. Plastic pots (12.7 by 8.9 by 8.9 cm) were modified by drilling a hole (3.2 cm in diameter) into the center of one side of each pot (7.62 cm from the top). Two pots were connected by the horizontal section (7.62 cm in length) of a T-shaped piece of PVC pipe (3.2 cm in diameter), and the vertical section (middle) of the T was plugged with a rubber stopper (Fig. 1).

To validate the effectiveness of the olfactometer, the attraction of black vine weevil larvae to actively growing plant roots was tested. To test for the effect of a live plant, rooted cuttings of *P. abies* variety *nidiformis* were potted into untreated media (in unaltered pots of the same type used for constructing the olfactometers) and maintained in the greenhouse (21–24°C) for at least 2 wk to allow them to recover from any potential shock of being repotted. Plants were transferred, media and all, to one side of the olfactometer. The other side of the olfactometer received

Fig. 1. Two-choice soil olfactometer used in experiments. Larvae were placed in the middle of the "T" tube (PVC pipe) connecting the two pots and allowed 24 h to move.

freshly prepared soilless media only. After 24 h, five third- to fourth-instar black vine weevils were placed in the middle of each T-tube connecting the two pots of the olfactometer. The T-tube did not contain media for these or subsequent comparisons. The absence of media in the T-tube allowed volatile compounds on either side of the olfactometer to move freely into the T-tube. However, volatile compounds in soil are detected by entomopathogenic nematodes over much longer distances (up to 50 cm) (Lewis et al. 1993, 1995, Grewal et al. 1994, Grewal et al. 1997, Boff et al. 2001, van Tol et al. 2001, Rasmann et al. 2005). After 24 h, during which time larvae were able to move freely, the olfactometers were thoroughly examined and the number of larvae on each side determined. Although possible, previous trials suggest that larvae do not move from one pot to another after their initial selection (unpublished data). All trials were performed under ambient light in the greenhouse (21–24°C). Experiments were arranged in a completely randomized design, with 10 replicate olfactometers of the validation trial performed six times. Larvae that remained in the T-tube connecting the pots were categorized as unresponsive. Plants and larvae were only used once, and pots were thoroughly washed before each use.

Experimental Design. To determine what effect various management techniques have on the behavior of black vine weevil larvae, the following design was used. The olfactometer described above was used to make pairwise comparisons of larval behavior when subjected to media incorporated with *M. anisopliae* and untreated media, media incorporated with bifenthrin and untreated media, and media incorporated with *M. anisopliae* and media incorporated with bifenthrin. Before incorporation into potting media, the viability of *M. anisopliae* was assessed by spreading 1 ml of 0.05% Tween solution containing 1×10^9 conidia of *M. anisopliae*/ml onto a petri dish of potato dextrose agar (PDA). The dish was placed in complete darkness (28°C) for 24 h, after which the percentage of spores producing germ tubes more than twice the length of the spore was determined. Potting media for use in experiments was incorporated with *M. anisopliae* granules at the rate of 1×10^6 viable spores/g dry soil by adding the appropriate quantity of granules to 1 liter of media and mixing in a 4-liter twinshell blender (Patterson-Kelley, East Stroudsburg, PA) for 5 min. This treated media were added to 9 liters of untreated potting media in a concrete mixer and again mixed for 5 min to ensure an even spore distribution. Bifenthrin (Talstar 0.2G; FMC, Philadelphia, PA) was incorporated into media as described above, at the recommended rate (25 ppm) for black vine weevil control (FMC 1999). To test for inherent effects of the treatments applied, all combinations were run without plants in either side of olfactometer. Trials were also run with plants in both sides of olfactometer to test host plant influence. After 24 h, the olfactometers were thoroughly examined and the number of larvae on each side determined. All trials were performed under ambient light in the greenhouse (21–24°C). Experiments were arranged in a completely randomized design, with 10 replicate olfactometers of each comparison performed six times. Larvae that remained in the T-tube connecting the pots were categorized as unresponsive. Plants and larvae were only used once, and pots were thoroughly washed before each use.

Trials Without Plants. The olfactometers containing media inoculated with *M. anisopliae* or bifentrhin were allowed to sit in a greenhouse for 5 d before receiving weevils. After 5 d, five third- to fourth-instar black vine weevils were placed in the middle of the T-tube connecting the two pots making up each olfactometer. After 24 h, the olfactometers were thoroughly examined, and the number of larvae on each side was determined.

Trials with Plants. To test for the effect of a live plant, rooted cuttings of *P. abies* variety 'nidiformis' were potted into media for all treatments (in unaltered pots of the same type used for constructing the olfactometers) and maintained in the greenhouse (21–24°C) for 21 d to allow them to recover from any potential shock of being repotted. Plants were transferred, media and all, to olfactometers as described previously. Twenty-four hours after transfer to the olfactometer, five third- to fourth-instar black vine weevils were placed in the middle of the T-tube of the olfactometer. After 24 h, the olfactometers were thoroughly examined, and the number of larvae on each side was determined.

Quantification of Fungal Populations. For all trials containing media incorporated with *M. anisopliae*, one pot from each trial was selected at random to estimate fungal population size. A sample of bulk potting media from the center of each pot was transferred to a sterile plastic bag (Nasco, Modesto, CA). Media samples were stored in a 4°C refrigerator until use. *M. anisopliae* will not grow at temperatures below 4°C

(Ekesi et al. 1999, Hallsworth and Magan 1999). Fungal populations were estimated in the following manner, adapted from Bruck (2005). Ten grams of bulk potting media was placed in a plastic 250-ml Erlenmeyer flask containing 90 ml of 0.05% Tween 80 solution, shaken (250 rpm) for 20 min at room temperature, and placed in an ultrasonic cleaner (model 5210; Branson Ultrasonic, Danbury, CT) for 2 min. Serial dilutions were plated using a spiral plater (iUL Instruments, Barcelona, Spain) onto two plates of media selective for *M. anisopliae* (Veen and Ferron 1966). Plates were incubated in complete darkness at 28°C for 4 d. The number of colony forming units (CFUs) per gram of dry bulk media was averaged across replicate plates for each sample. Colonies may have arisen from mycelial fragments or conidia. The quantification technique used does not allow colonies arising from mycelial fragments to be distinguished from those arising from conidia. To ensure that the colonies counted were *M. anisopliae*, a total of 20 colonies morphologically identical to those counted as *M. anisopliae* were randomly selected from a number of different plates from each sample and aseptically transferred to PDA and allowed to sporulate. The colonies transferred were identified based on macro- and microscopic characteristics (Humber 1997). All colonies transferred to PDA throughout the study were *M. anisopliae.* No effort was made to account for the *M. anisopliae* population in the rhizosphere soil from those treatments containing fungal inoculated media and plants.

Statistical Analyses. The validation experiment and each pairwise comparison were analyzed separately by counting the number of olfactometers scored as a one and analyzing that proportion out of the total number of olfactometers in the trial with an exact binomial test (null hypothesis = 0.5; Insightful 2003). For the validation experiment, olfactometers were scored a 1 if a simple majority of larvae were recovered from the pot containing a plant and a 0 if a majority were recovered from the pot without a plant. Olfactometers in trials containing a treatment with *M. anisopliae* were scored a 1 if a simple majority of larvae were recovered from the pot containing *M. anisopliae* and a 0 if the majority were recovered in the opposing pot. For trials with a bifenthrin treatment, if a simple majority of larvae were recovered from pots containing bifenthrin, they were scored as 0 and 1 if the majority were recovered from the opposing pot. The 95% confidence interval (CI) for the exact binomial test lies between 0 and 1. CIs containing 0.5 are unable to reject the null hypothesis (i.e., random movement). In the instance of a tie (because of dead or unresponsive larvae), the olfactometer was excluded from the analysis. The number of responsive larvae was compared with the number of unresponsive larvae for each paired test using a two-sample *t*-test (Insightful 2003). Differences between paired tests in the number of unresponsive larvae were compared using a Tukey's test (SAS Institute 1999).

The exact binomial test was chosen for data analysis because the level of independence was at the olfactometer and not individual larvae within each olfactometer (Ramírez et al. 2000). Because of this, analysis of whole numbers of larvae selecting one pot over the other was not appropriate. Second, the exact binomial test allows for the analysis of data where totals are determined beforehand, as was the case in these experiments (Conover 1999).

Results

The usefulness of the olfactometer designed for these experiments was validated by the ability of black vine weevil larvae to select media containing a plant over media without a plant. A significant portion of olfactometers had a majority of larvae recovered from media containing a plant ($P \leq 0.0001$; 95% CI 0.69, 0.90; Fig. 2). Larvae were recovered from throughout pots, indicating that contact with a root was not required to arrest movement after entering a pot.

The number of responsive larvae was significantly greater than unresponsive larvae (never less than 88% responsive) in all comparisons throughout the study ($P \leq 0.0001$; Table 1). There was a significant difference in the number of unresponsive larvae between paired comparisons (Table 1). The trial containing media incorporated with bifenthrin and *M. anisopliae* with plants had a significantly higher number of unresponsive larvae then all other trials except the comparison of bifenthrin-treated media and control media with plants (Table 1).

When larvae were allowed a choice between *M. anisopliae*–treated and –untreated media, without plants, larval movement was not significantly different from random ($P = 0.60$; 95% CI 0.41, 0.67; Fig. 3). When plants were present, a significant proportion of the olfactometers had a majority of larvae recovered from media incorporated with *M. anisopliae* than untreated media ($P = 0.02$; 95% CI 0.52, 0.79; Fig. 2). The mean fungal population in the potting media during trials with and without plants was 4.22×10^6 and 1.21×10^7 CFU/g dry media, respectively.

When larvae were given a choice between media incorporated with bifenthrin and untreated media, without plants, a significant proportion of olfactometers had a majority of larvae recovered from the untreated potting media ($P = 0.007$; 95% CI 0.55, 0.80; Fig. 3). When plants were present in the olfactometer, larval movement was not significantly different from random between the bifenthrin-treated and -untreated media ($P = 0.41$; 95% CI 0.42, 0.70; Fig. 2).

When larvae were allowed to choose between media incorporated with bifenthrin and media incorporated with *M. anisopliae*, without plants present, a significant proportion of olfactometers had a majority of larvae recovered from media incorporated with *M. anisopliae* than bifenthrin incorporated media ($P < 0.0001$; 95% CI 0.65, 0.87; Fig. 3). This deterrence to bifenthrin was not observed when plants were included in the olfactometer, with larval movement not significantly different from random ($P = 0.29$; 95% CI 0.44, 0.71; Fig. 2). The mean fungal population in the potting media during trials with and without plants

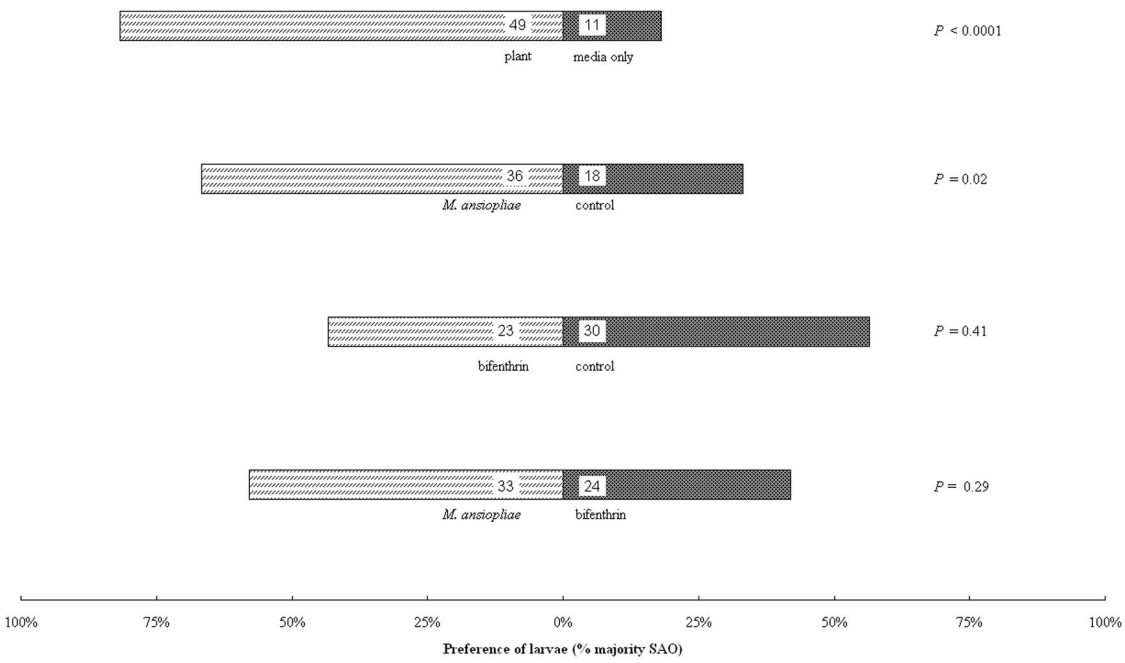

Fig. 2. Preference of black vine weevil larvae in trials with plants in either side of the two-choice soil olfactometer. Bars represent the percentage of olfactometers containing a majority of black vine weevil larvae. Numbers inside each bar represent the total number of olfactometers tallied for each treatment.

was 7.65×10^7 and 4.82×10^7 CFU/g dry media, respectively.

Discussion

Tritrophic interactions are well described for terrestrial systems (Sabelis and van de Baan 1983, Dicke et al. 1990, Turlings et al. 1990, Dicke et al. 1993, Turlings et al. 1995, Kessler and Baldwin 2001). In these systems, feeding by herbivores elicited the systemic production of secondary metabolites by the plant that serve as attractants to predators and parasitoids (Turlings and Tumlinson 1992, Dicke et al. 1993). More recently, tritrophic interactions have been found to operate below ground as well. The first evidence of this came from studies of the entomopathogenic nematode, *Heterorhabditis megidis* Poinar, Jackson and Klein (Rhabditidae: Heterorhabditidae) and its orientation to black vine weevil larvae feeding on plant roots. Boff et al. (2001) observed a positive attraction by *H. megidis* toward strawberry plants that had been fed on by black vine weevil larvae. However, they were unable to determine if the orientation was caused by chemical cues emitted from the plant. The attraction of *H. megidis* to cues released by the conifer *T. occidentalis* in response to black vine weevil larval feeding was confirmed by van Tol et al. (2001). Since these initial discoveries, the induction of natural enemy attractants in response to below ground herbivory has been identified in turnips (Neveu et al. 2002), tulips (Aratchige et al. 2004), and corn (Rasmann et al. 2005). In the case of corn, the primary chemical compound responsible for eliciting attraction was found to be (E)-β-caryophyllene and verified in a field setting (Rasmann et al. 2005). This dynamic serves to increase fitness for both the plant and the natural enemy of the herbivore by reducing the plants resource allocation to defense compounds and providing a food source or oviposition substrate for the natural enemy.

Our experiments revealed a tritrophic interaction that differed significantly from those currently reported. In our studies, it was not a natural enemy of the pest whose behavior was altered, but the behavior of the pest itself. From an evolutionary standpoint this makes logical sense, because *M. anisopliae* is not able to actively seek out its insect hosts. Currently, it is only possible to speculate on whether the fungus or plant is the origin of the attractive compound. The available evidence seems to indicate that the plant in association with the fungus may produce compounds attractive to black vine weevil larvae. However, it is also possible that when colonizing the rhizosphere, the fungus produces attractive compounds that are not produced in the absence of plant roots. Some isolates of *M. anisopliae* are known to be rhizosphere competent (Hu and St. Leger 2002, Bruck 2005, unpublished data), achieving a population difference that is a log greater in the rhizosphere than in bulk media (Bruck 2005). This tight association between plant and fungus can be interpreted in one of two ways: (1) the plant is maintaining the growth of the fungus through root exudates (actively or passively) or (2) that *M. anisopliae* is acting as a plant pathogen. The exact nature of the

Table 1. Results of two sample *t*-test of responsive and unresponsive larvae within paired comparisons and Tukey's test for differences in the no. unresponsive larvae between paired comparisons

	Paired comparison					
	M. a./control—no plants	*M. a.*/control—plants	Bifenthrin/control—no plants	Bifenthrin/control—plants	Bifenthrin/*M. a.*—plants	Bifenthrin/*M. a.*—no plants
Responsive[a]/unresponsive[b]	298/2	289/12	291/9	263/32	260/37	285/15
P value[c]	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Tukey's test[d]	c	bc	c	ab	a	bc

[a] No. larvae that responded to one of the two treatments.
[b] No. larvae remaining in the T-tube that did not respond to one of the two treatments.
[c] Results of each two sample *t*-tests performed to determine if there were significantly more responsive larvae than unresponsive larvae in each paired comparison.
[d] Results of the Tukey's test performed to determine if there was a significant difference in the no. unresponsive larvae between comparisons. Comparisons with the same letter are not significantly different ($P = 0.05$).

rhizosphere interaction has not been fully elucidated, but we have observed no measurable change in root growth of *P. abies* with *M. anisopliae* (strain F52) colonized rhizospheres from those that are not colonized suggesting a lack of pathogenicity (unpublished data).

Elliot et al. (2000) speculated on the existence of tritrophic interactions for fungi, bacteria, and viruses in association with plants and herbivores. They cite three separate mechanisms by which a plant might enable the formation of a tritrophic interaction: (1) plant maintenance of a population of pathogens; (2) plants facilitating an increase in contact rates between pathogen and host; and (3) plants increasing the susceptibility of an insect to pathogenic infection. The first mechanism is evident in the rhizosphere competent nature of *M. anisopliae.* The system studied in this work provides evidence that the second mechanism is also in operation. The increase in incidence of infection as a result of this interaction has not been tested directly, although high levels of infection do occur in black vine weevil larvae feeding on roots with *M. anisopliae* colonized rhizospheres (Bruck 2005). However, all three mechanisms need not be present for a tritrophic interaction to occur.

There is contradictory evidence in the literature concerning the ability of entomopathogenic fungi to affect insect behavior. Engler and Gold (2004) showed that the termites *Reticulitermes flavipes* (Kollar) and *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae) were attracted to both mycelial preparations and volatile extracts of *M. anisopliae.* Villani et al. (1994) observed that Japanese beetle females (Coleoptera: Scarabaeidae) oviposited preferentially on bare soil treated with mycelia, possibly in response to CO_2 released during mycelial growth. However, grubs where found to avoid regions of grass sod treated with *M. anisopliae.* Mole crickets (Orthoptera: Gryllotalpidae) in test arenas containing sod were also observed to modify their behavior in response to *M. anisopliae* and *B. bassiana* in a way that reduced their exposure to these fungi (Villani et al. 2002, Thompson and Brandenburg 2005). Thompson and Brandenburg (2005) showed that the cricket avoidance behavior was dependent on the isolate of fungi, which may in part account for these apparent contradictions. The proclivity of *M. anisopliae* to colonize the rhizosphere of growing plants is also isolate dependent (unpublished data). Consideration of an isolates biology is therefore important in future studies examining the effect of fungi on insect behavior in plant systems.

These data also indicate that the movement of black vine weevil larvae can be affected by the synthetic pyrethroid bifenthrin, which repelled black vine weevil larvae in all trials without plants when applied at the manufacturer's recommended rate for black vine weevil control. The presence of bifenthrin in soil has also been found to affect the behavior of late instar mole crickets in a way that reduces exposure to the chemical (Thompson and Brandenburg 2005). In these trials, a layer of an untreated soil was placed on top of a

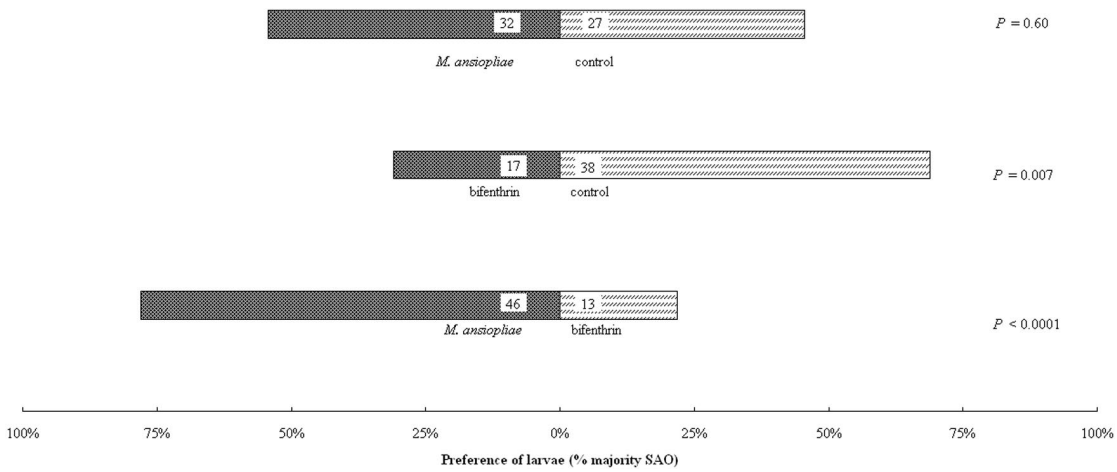

Fig. 3. Preference of black vine weevil larvae in trials without plants in either side of the two-choice soil olfactometer. Bars represent the percentage olfactometers containing a majority of black vine weevil larvae. Numbers inside each bar represent the total number of olfactometers tallied for each treatment.

bifenthrin-treated layer of soil. A significant proportion of crickets were located in the untreated top layer of soil. Tunneling was also restricted to the untreated layer, reducing the passage of crickets through treated soil.

The validation experiment showed that black vine weevil larvae exhibit a strong preference toward media containing a plant. The absence of deterrence to bifenthrin when plants were included in our study is likely caused by repellence and attraction cues emanating from the same side of the olfactometer leading to an outbalancing of attractiveness, thus resulting in a random distribution of larvae. This phenomenon provides an explanation for the lack of response when larvae were presented a choice between pots containing plants and media treated with either *M. anisopliae* or bifenthrin, as well as for the increased number of unresponsive larvae in bifenthrin trials with plants. Although not statistically significant, a larger proportion of olfactometers had a majority of larvae move to the side containing *M. anisopliae*, rather than bifenthrin treated media when plants were present (Fig. 2).

The repellent properties of bifenthrin could hamper black vine weevil management efforts in several ways. In the short term, the ability of larvae to move away from bifenthrin could result in larvae relocating to areas in a pot with low insecticide concentrations. These untreated areas may arise under several conditions during typical nursery operations. An uneven mixing of potting media may result in insecticide concentration gradients forming within pots. Bifenthrin adheres tightly to organic compounds present in potting media and has a low solubility in water (FMC 2003). Incorporation of 10–25 ppm is reported to provide "extended residual control" (FMC 2003). Refuges may also arise when container-grown plants are transferred to larger pots during the production process. If bifenthrin is used initially in the production process, but not for subsequent repottings (or vice versa), plants that have undergone several transfers may be growing in both treated and untreated soils. The length of time that a plant is grown before it is transferred to a larger pot varies considerably between nursery operations and plant species. It is not unusual for plants to be transferred several times to larger containers over a 3- to 5-yr period in a wholesale nursery application (unpublished data). The efficacy of media incorporated with bifenthrin drops precipitously after 2 yr (unpublished data). The development of insecticide refuges under similar production practices has been implicated in other studies where bifenthrin failed to control black vine weevil (Swier et al. 1998).

These data advance the understanding of tritrophic interactions by revealing a novel association between *P. abies*, black vine weevil larvae, and *M. anisopliae.* Although the attraction of black vine weevil larvae to the plant-fungus system is evident, there is a need to clarify which compounds are responsible for the attraction, as well as whether they arise from the plant or the fungus. These avenues will be the focus of future research. Concomitant with this, research is needed to clarify the ecology of *M. anisopliae* in the rhizosphere, particularly which plants will support growth and what effect this has on plant fitness, as well as the extent to which colonization occurs in wild plants. This information will not only increase the ecological understanding of the new tritrophic system and its effect on plant communities, but will also aid in developing effective pest management programs using *M. anisopliae* as a rhizosphere inoculant by identifying those isolates most effective at attracting and infecting larvae.

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