Efficacy of *Metarhizium anisopliae* as a Curative Application for Black Vine Weevil (*Otiorhynchus sulcatus*) Infesting Container-Grown Nursery Crops[1]

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Abstract

The black vine weevil (BVW), *Otiorhynchus sulcatus* (F.) is a serious pest of nursery crops. The fungus, *Metarhizium anisopliae* (F52), is registered by the U.S. Environmental Protection Agency for BVW control. The objectives of this study were to determine the efficacy of a curative drench application of *M. anisopliae* for controlling BVW larval infestations in container-grown nursery plants and the effect of temperature on the rate of fungal growth and speed of kill. Trials evaluating the efficacy of *M. anisopliae* as a curative application were performed in the spring of 2004 and 2005 as well as the fall of 2006. Laboratory studies were performed to quantify the impact of temperature (10, 15, 20, 24 and 28C) on fungal growth and speed of kill. *Metarhizium anisopliae* applied in the greenhouse and outdoors in 2004 were 92 and 30% effective, respectively. Fungal applications to container-grown plant material maintained outdoors in the spring of 2005 were nearly 100% effective 28 days after application. Fall applications in 2006 provided statistically significant reductions in the number of live BVW larve per pot, but were not as effective as spring applications in 2005. The mean media temperature of containers maintained outdoors in the fall of 2006 dropped considerably (10–12C) over the course of the experiment and were likely the cause for the reduced efficacy. Laboratory experiments demonstrated that temperatures below 20C (68F) significantly slowed fungal growth and the speed at which *M. anisopliae* infected BVW larvae. In the field, drench applications of *M. anisopliae* were very effective at eliminating BVW larvae in container-grown nursery plants when media temperatures were adequate (> 15C (59F)). The use of *M. anisopliae* as a curative drench application has similar temperature-dependent limitations as the use of entomopathogenic nematodes for BVW control. Therefore, applications should occur as early in the fall as possible once egg laying has ended or in late spring just prior to pupation when media temperatures would be most conducive to fungal infection.

Index words: microbial control, biological control, pest management, drench.

Species used in this study: Black vine weevil, *Otiorhynchus sulcatus*, *Metarhizium anisopliae* (F52), *Pinus mugo*, *Taxus cuspidata*, *Picea mariana*.

Significance to the Nursery Industry

The black vine weevil (BVW), *Otiorhynchus sulcatus* (F.) is a serious pest of nursery crops. The BVW control program currently implemented by a majority of growers centers on the use of broad spectrum insecticides to target adults prior to oviposition. However, even when implementing an extensive insecticidal spray program, growers often discover plant material infested with larvae in the fall or in the spring prior to shipping. Infested plants cannot be sold and if infested plants are shipped, the grower risks refusal of the plants by the buyer and will incur the additional return shipping costs and potential loss of future sales. Many of the chemical insecticides currently available for curative applications (i.e. drenches) do not adequately control BVW larvae established in container-grown nursery stock. The prospect of new chemistries becoming available is slim as the chemical industry is reluctant to pursue registration of new compounds in what they perceive as a small potential market. Insect parasitic nematodes are commercially available for BVW larval control and can be quite effective, though limited by low soil temperatures. An alternative to chemical insecticides and nematodes is the use of the insect killing fungus, *Metarhizium anisopliae*. *Metarhizium anisopliae* (F52, Novozymes Biologicals, Inc., Salem, VA) has been recently registered for managing a number of pests including the BVW. The objectives of this study were to determine the efficacy of a curative drench application of *M. anisopliae* for BVW larval control in container-grown nursery plants and the effect of temperature on the rate of fungal growth and speed of kill. If effective, *M. anisopliae* (F52) would provide growers of container nursery stock another tool for curative control of BVW larvae.

Introduction

The black vine weevil (BVW), *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae) is a univoltine, polyphagous (over 140 larval and adult host species) insect that is a serious pest of field and container-grown ornamentals as well as small fruit crops worldwide (19). The BVW has a northern European origin and was first recorded in North America in 1835 (27). Larvae are the primary overwintering stage with adult weevils appearing in the spring. In regions of the country with mild winters, a small proportion of the previous year's weevil population can survive the winter as adults. Adults are awkward, move slowly when walking and often play dead when disturbed. The BVW has a preovipostion period of 20 to 40 days, during which it feeds on leaves before its repro-

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ductive system matures. Oviposition occurs at night with eggs either dropped on the soil surface or inserted into crevices or on plants (27). Early instars feed on small roots while the later instars feed on larger roots, especially on the phloem and cambium tissues near the soil surface (17). Adults are nocturnal and cause mainly cosmetic damage to plants by notching the leaves. Adults reproduce by thelytokous parthenogenesis, so a single individual left unchecked can result in the infestation of an entire nursery. Each adult can lay up to 900 eggs depending on the host plants that she has fed on (10).

The entomopathogenic fungus *Metarhizium anisopliae* (F52, Novozymes Biologicals, Inc., Salem, VA) (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) is a microbial pesticide registered with the US Environmental Protection Agency for use against BVW. *Metarhizium anisopliae* has been studied extensively for the biological control of a wide range of insect pests, including BVW (3, 5, 6, 7, 20, 21, 22) and various other soil-borne pests such as *Popillia japonica* (Coleoptera: Scarabaeidae) (29), *Ligyrus subtropicus* (Coleoptera: Scarabaeidae) (23), *Antitrogus parvulus* (Coleoptera: Scarabaeidae) (25) and *Adoryphorus couloni* (Coleoptera: Scarabaeidae) (24). While various isolates of the fungus have been evaluated against a range of pest insects, it was not until recently that an isolate of *M. anisopliae* registered for commercial use could be tested for BVW control. Different isolates of the same entomopathogenic fungus can have varying degrees of pathogenicity to a particular pest (5, 22). Each fungal isolate also responds uniquely to the biotic and abiotic conditions encountered in the field. These considerations make it important to focus research on an isolate that is commercially available to growers in the nursery industry and a potential management tool for BVW.

The objectives of this study were to determine the efficacy of a curative drench application of *M. anisopliae* for BVW larval control in container-grown nursery plants and the effect of temperature on the rate of fungal growth and speed of kill.

Materials and Methods

The application of *M. anisopliae* (F52) fungal spores as a curative treatment for BVW larval infestations in container-grown nursery plants was performed in the spring of 2004 and 2005 as well as the fall of 2006. The experimental design of each trial varied slightly (outlined below), but the method and rate of fungal application were consistent. Due to the spotty nature of BVW larval infestations, all pots (with the exception of the spring 2004 trial) were artificially infested with 10–15 last instar BVW obtained from a BVW colony maintained at USDA-ARS Horticultural Crops Research Laboratory, Corvallis OR (11). Larvae were allowed to establish in the containers for 5–10 days prior to fungal treatment. A granule formulation of *M. anisopliae* was produced in the laboratory by solid substrate fermentation. The granules produced consisted of *M. anisopliae* that had sporulated on rolled barley and was then dried. The concentration of spores on the dried grain was 1.0×10^9 spores/g. Spore suspensions were prepared by adding dried fungal granules to 50 ml sterile 0.05% Tween 80 and shaking at 250 rpm for 30 minutes. Hemocytometer counts were used to determine the concentration of each suspension. Spore viability was determined prior to each application (14) and the spore concentration adjusted. Each #1 container was treated with 1.5×10^9 fungal spores in 200 ml of water. Pots were drenched with a CO_2 sprayer (15 psi). After treatment, each container received an additional 200 ml of water to facilitate spore movement into the potting media. In the case of larger containers, the fungal rate and water volume were adjusted proportionately.

March 2004. Forty *Pinus mugo* in #5 containers heavily infested with last instar BVW were obtained from a commercial wholesale nursery located in the northern Willamette Valley, OR, and returned to the laboratory. Twenty plants were maintained outside in a container yard and twenty were placed in the greenhouse at a constant 21C (70F). Media temperatures were monitored from the center of containers maintained outdoors. Spore suspensions were prepared and applied as outlined above in 1,000 ml of water. Ten containers in the greenhouse and ten in the container yard were treated with fungal spores with the remaining containers receiving 1,000 ml of water only. All pots were searched 21 days later and the numbers of live and *M. anisopliae* infected larvae determined.

Spring 2005. In April of 2005, a total of 160 *Taxus cuspidata* in #1 containers were artificially infested with 10 last instar BVW. The plants were randomly divided into four equal groups of 40 plants. Each group of 40 plants was considered a separate run of the experiment. Twenty plants from each group were drenched with fungal spores (in 200 ml of water) and the remaining plants treated with water only (200 ml). After treatment, ten each of the fungal treated and control plants from each group were maintained in a greenhouse at a constant 21C (70F) or outdoors in a container yard. Media temperatures were monitored from containers maintained outdoors. A separate suspension of fungal spores was prepared for each run of the experiment. Suspension preparation, fungal rate and application were as described previously. The experiment was arranged in a randomized complete block design. Plants maintained in the greenhouse and outdoors were searched 14 and 28 days after treatment, respectively. The numbers of live and *M. anisopliae* infected larvae were determined.

Fall 2006. Experiments were performed in October 2006 to determine the efficacy of a *M. anisopliae* drench application applied early in the fall at the conclusion of egg laying. Experiments were performed using *Picea mariana* in #3 containers. Containers were artificially infested with ten last instar BVW. The experiment was performed in outdoor container yards at two locations. Media temperatures were monitored from containers at each location. Spore suspensions were prepared and applied as outlined above in 600 ml of water. Ten containers at each location were treated with fungal spores with the additional ten receiving 600 ml of water only. The experiment was arranged in a randomized complete block design. All containers were searched 30 days later and the numbers of live and *M. anisopliae* infected larvae determined.

Laboratory studies. Because of the apparent impact of temperature on the ability of *M. anisopliae* to infect BVW larvae infesting container-grown nursery stock, laboratory studies were performed to quantify the impact of temperature on the

Table 1. Mean (±SD) number of last instar black vine weevil out of 30 infected with *Metarhizium anisopliae* (F52) in laboratory bioassays at 10, 15, 20, 24 and 28C (50, 59, 68, 75 and 82F) after 14 and 28 days exposure.

Day	Temperature (C)	Number infected[z]
14	10	0(0)a
14	15	0(0)a
14	20	26.75(1.5)b
14	24	30(0)c
14	28	30(0)c
28	10	3.5(3.0)a
28	15	23.25(4.27)b
28	20	29.5(.58)c
28	24	30(0)c
28	28	30(0)c

[z]The number of live BVW larvae after 14 or 28 days exposure at various temperatures. Mean numbers of infected larvae with different letters from the same day are significantly different $P \leq 0.05$ (26).

rate of fungal growth and speed of kill. Growth was observed at constant temperatures of 10, 15, 20, 24 and 28C (50, 59, 68, 75 and 82F). Plates of Potato Dextrose Agar (PDA) were inoculated in their centers with the touch of sterilized needle that had been dipped into a suspension of 1×10^6 spores of *M. anisopliae*. Five replicate plates were maintained at each temperature in complete darkness. The diameter (mm) of the fungal colony on each plate was measured every 1–3 days up to 33 days after treatment, or until the colony covered the entire plate. Control plates were prepared at each temperature by touching the centers of PDA plates with a sterilized needle that had been dipped into sterile water. No fungal growth was observed from any of the control plates throughout the experiment. Simultaneously, parallel experiments were conducted to determine the impact of temperature on the speed at which BVW larvae succumbed to fungal infection. Four replicate containers of potting media (OBC Northwest Nursery Mix #1, OBC Northwest, Canby, OR) were inoculated with 1×10^6 spores of *M. anisopliae*/g dry potting media. Treatments were prepared following the protocol outlined in Bruck 2005 (6). Approximately 150 g of media were placed into each container along with 30 last instar BVW. Larvae were obtained from a colony maintained at the USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR (11). Four replicate containers containing untreated media and 30 last instar BVW were also prepared at each temperature. The fungal growth and larval infection experiments were arranged in a completely randomized design.

Data were analyzed using paired *t* tests of log-transformed counts to determine if there was a significant difference in the number of live larvae between fungal drenched and untreated control containers (26). Data from each application were analyzed and are presented separately. Data from laboratory experiments on the impact of temperature on fungal growth and infectivity were analyzed using ANOVA and means separated with Tukey's multiple range test (26). Abbot's formula was used to adjust for control mortality in all laboratory bioassays (1).

Results and Discussion

The elimination of BVW from container-grown stock prior to shipping is a high priority for nursery growers in all parts of the United States in which the BVW occurs. Established larval infestations are notoriously difficult for growers to manage. Chemical drenches such as Orthene can be applied and have been reported to be effective (8). However, there are concerns related to worker exposure when drenching containers with chemical insecticides as well as the potential for adverse environmental impacts (personal observations).

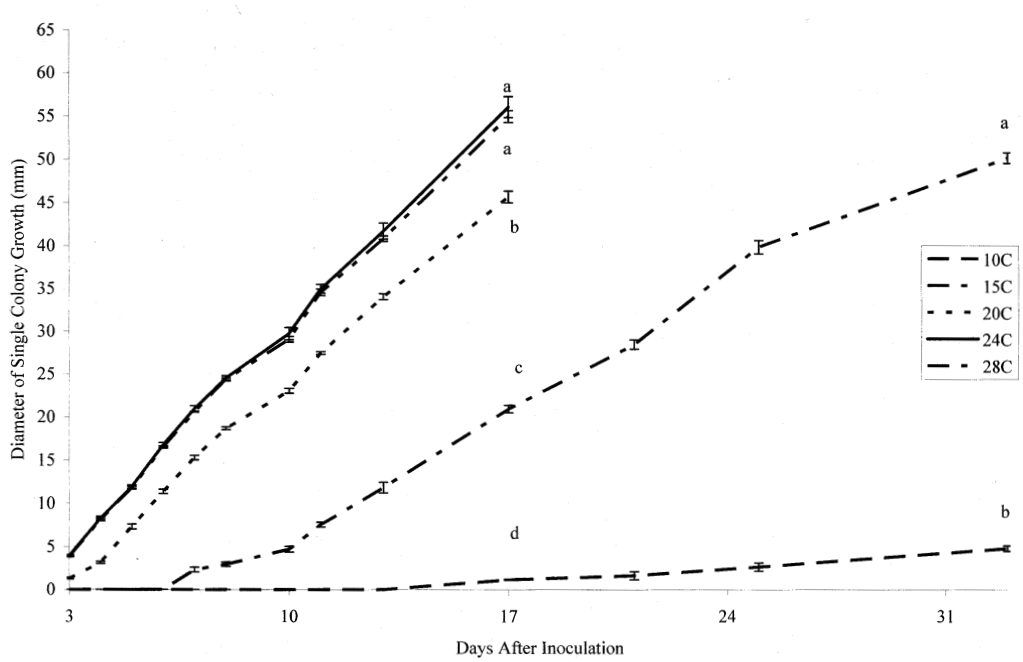

Fig 1. Mean diameter (mm ± SD) fungal growth at 10, 15, 20, 24 and 28C (50, 59, 68, 75 and 82F). Fungal measurements at 17 and 33 days with different letters are significantly different ($P < 0.05$) (26).

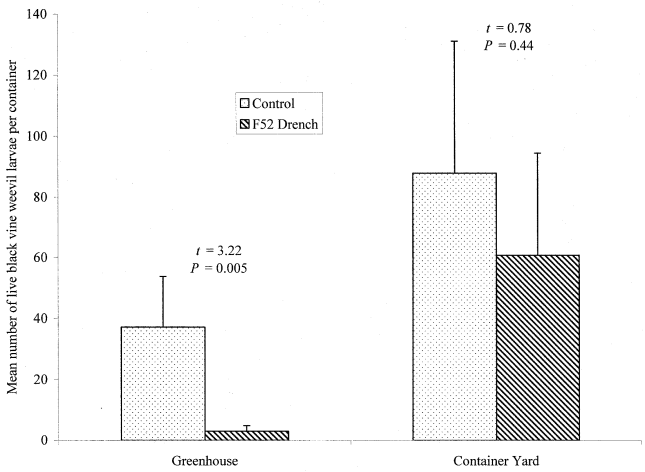

Fig. 2. Mean number (+SD) of live black vine weevil larvae per container receiving a fungal drench application or untreated controls in 2004. A paired *t* test of log-transformed counts was performed to determine if there was a significant difference in the number of live BVW larvae between treatments in greenhouse and container yard maintained plants (26).

Laboratory experiments to quantify the impact of temperature on fungal growth and infectivity against BVW larvae demonstrated that temperatures below 20C (68F) significantly retard fungal growth (Fig. 1) and the speed at which *M. anisopliae* infected BVW larvae (Table 1). There was no significant difference in fungal growth (Fig. 1) between 24 and 28C (75 and 82F) 17 days after inoculation. Fungal growth was significantly faster at 20C (68F) or above. Very little fungal growth occurred at 10C (50F) after 17 or 33 days. Statistical differences in the number of BVW larvae infected (Table 1) at each temperature tracked the differences in fungal growth. There was a significant temperature × day interaction for the number of infected larvae 14 or 28 days after treatment ($F = 241$; df = 9, 30; $P < 0.0001$; Table 1). There were significantly fewer larvae infected at 10C (50F) 14 and 28 days after treatment as well as 14 days post treatment at 15C (59F) than any of the other temperatures (Table 1). The 20C (68F) as well as the 15 and 20C (59 and 68F) temperature treatments after 14 and 28 days, respectively, had significantly fewer infected larvae than the higher temperature treatments. There were statistically significant differences in fungal growth and larval infection between 20, 24, and 28C (68, 75, and 82F). However, 89% of BVW larvae exposed to *M. anisopliae* at 20C (68F) were infected after 14 days. The infectivity of other *M. anisopliae* isolates are also limited at or below 15C (59F), while all isolates were highly infective (>95%) at 20C (68F) (28). The LT_{50} between *M. anisopliae* isolates ranged from 26.4 to 51.9 days (28). *Metarhizium anisopliae* isolates evaluated for the control of flower thrips also had limited growth at 15C (59F) with optimum growth and pathogenic activity between 25–30C (77–86F) (9).

In the spring of 2004 the efficacy of *M. anisopliae* drench applications was excellent when applied in the greenhouse (Fig. 2). In greenhouse experiments, fungal application reduced the number of live BVW larvae per container by 92%. Applications to the same plant material maintained outdoors resulted in only a 30% reduction in the number of live BVW larvae per container. Containers in the greenhouse were maintained at a constant 21C (70F). The mean daily temperature of media in the containers maintained outdoors from this experiment only exceeded 15C (60F) five days over the course of the experiment (Fig. 3).

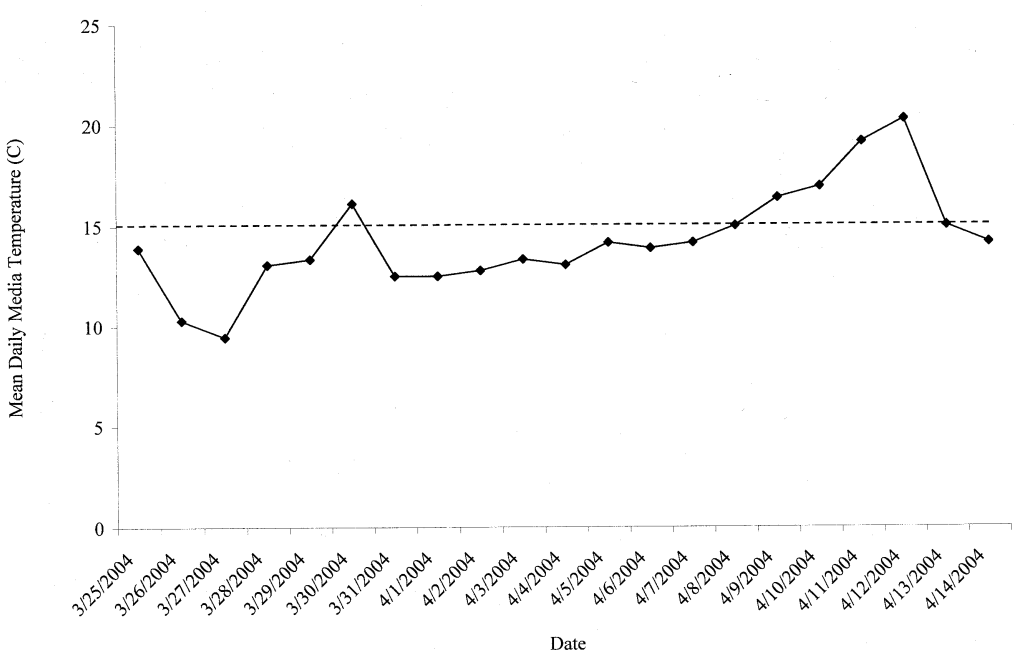

Fig 3. Mean daily media temperature (C) in containers maintained outdoors spring 2004.

Table 2. Mean (±SD) number of live black vine weevil larvae per container 28 days after receiving a treatment of *Metarhizium anisopliae* (F52) spores or an untreated control in the spring of 2005 either in the greenhouse or outdoors in a container yard.

Run[z]	F52 Application	Control	*t* statistic[y]	*P* > *t*
		Greenhouse		
1	0.2(.42)	8.2(1.61)	–15.12	<0.0001
2	0.1(.31)	8.6(0.69)	–35.03	<0.0001
3	0.1(.31)	8.4(0.96)	–25.82	<0.0001
4	0.1(.31)	8.6(1.26)	–20.62	<0.0001
		Container yard		
1	0.1(.31)	5.8(1.75)	–10.13	<0.0001
2	0.4(.51)	7.1(3.28)	–6.38	<0.0001
3	0.0(.00)	5.4(1.30)	–9.00	<0.0001
4	0.7(.82)	6.0(2.35)	–6.71	<0.0001

[z]The experiment was repeated four times with plants maintained in the greenhouse and container yard.

[y]A paired *t* tests of log-transformed counts was performed to determine if there was a significant difference in the number of live BVW larvae between fungal drench and untreated control pots from each run of the experiment (26).

Spring applications in 2005 significantly reduced the number of live BVW larvae per container in both the greenhouse and container yard (Table 2). All *M. anisopliae* applications in 2005 were equally effective at nearly eliminating BVW larvae from the containers. Mean media temperatures over the course of the experiment remained near or above 15C (60F) (Fig. 4). These relatively moderate media temperatures along with the extended incubation time (28 days) allowed *M. anisopliae* to be nearly 100% effective. The container yard applications in the spring 2005 mirrored the temperature infectivity results observed in the laboratory. Temperatures below 15C (60F) slow the growth and infectivity of *M. anisopliae*, but given 28 days of exposure larval infectivity nearly reached 100%. The spring 2005 fungal applications occurred immediately prior to pupation. These applications benefited from the warming media temperatures and may have also been more effective due to the larvae being predominately located near the media surface as they prepared to pupate. Larvae located near the surface would be unlikely to escape exposure to topically applied fungal spores.

There were significantly fewer live BVW larvae recovered from the fungal treated than untreated control containers at both container yard locations in the fall of 2006 (Fig. 5). The reduction in the number of live larvae, while statistically significant, was not as striking as those observed in 2005. The mean media temperature of containers maintained outdoors in the fall of 2006 dropped considerably (10–12C) (50–54F) over the course of the experiment (Fig. 6), while media temperature in the spring of 2004 (Fig. 2) and 2005 (Fig. 4) rose steadily.

Insect parasitic nematodes are the other commercially available biological control agent for BVW larval control. Nematodes can be purchased from a number of suppliers and have been used extensively for the biological control of a wide range of insect pests including the BVW (2, 4, 12, 13, 16, 30). Control of BVW larvae with nematodes is also limited by low soil temperatures when late instars are present in the field in north temperate regions such as the Pacific Northwest. Many species and strains of nematodes have been screened for their activity in cold conditions (15, 18). *Heterorhabditis marelatus* provided significantly greater BVW control (82.5% control) than *H. bacteriophora* (44.1% control) at low (14C) soil temperatures 12 days after application (2). The reportedly cold-active nematode *Steinernema kraussei* was effective at controlling overwintering BVW

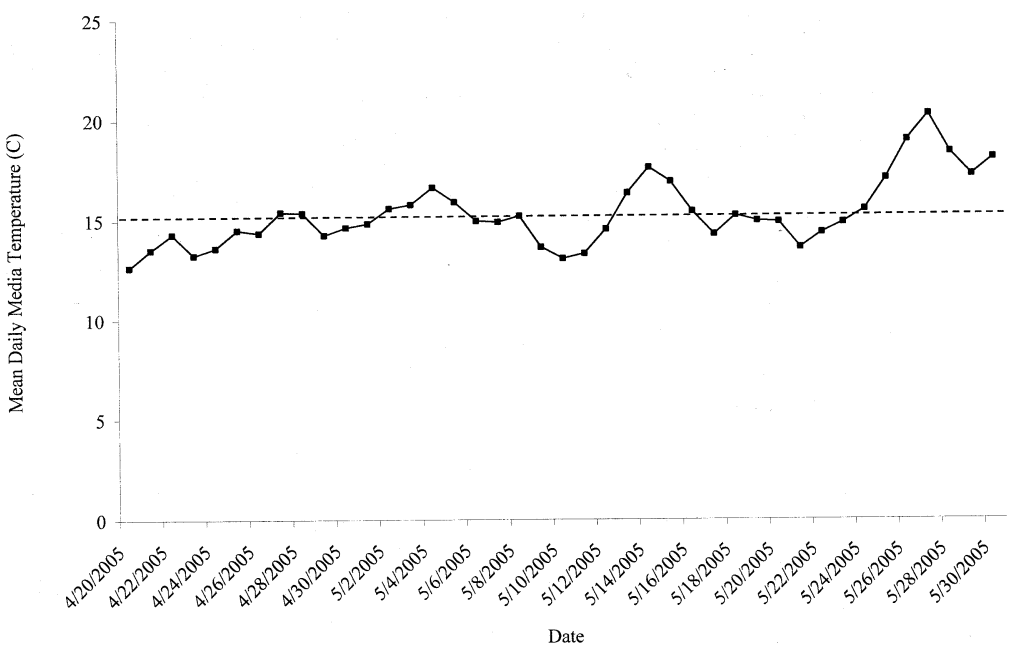

Fig 4. Mean daily media temperature (C) in containers maintained outdoors spring 2005.

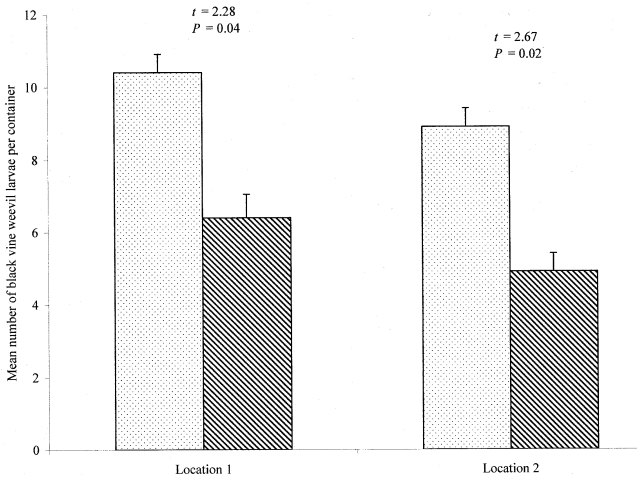

Fig 5. Mean number (+SD) of live black vine weevil larvae per container receiving a fungal drench application or untreated controls in fall 2006. A paired *t* test of log-transformed counts was performed to determine if there was a significant difference in the number of live BVW larvae between treatments (26).

larvae infesting outdoor strawberry plants in England (30). *Steinernema kraussei* was recently registered (2006) for commercial use in the United States and is currently undergoing field testing (Bruck, unpublished data).

Larval infestations of BVW can be prevented by the incorporation of *M. anisopliae* at potting. Field studies have shown that *M. anisopliae* (F52) persists well (up to 24 months) in the soilless potting media of container-grown plants maintained outdoors (Bruck, unpublished data), in peat and bark-based media for nearly one year (2) and is compatible with commonly used potting media components (7). Curative drench applications of *M. anisopliae* spores were effective at eliminating BVW larval infestations in container-grown nursery stock when media temperatures were adequate for fungal activity. The efficacy of *M. anisopliae* drench applications for BVW control was limited, as are entomopathogenic nematodes, by low media temperatures. Therefore, applications will have to be made as early in the fall as possible once egg laying has ended or in late spring just prior to pupation. An alternative may be to move infested plants to a location were media temperatures could be moderated, such as a hoop house or greenhouse. Media temperatures in excess of 15C (60F) for 28+ days, allowed *M. anisopliae* to be nearly 100% effective at eliminating BVW larvae from container-grown nursery plants. There are undoubtedly additional expenses associated with moving plants, but if the value of the plant material is high enough, growers may be willing to do so in certain situations.

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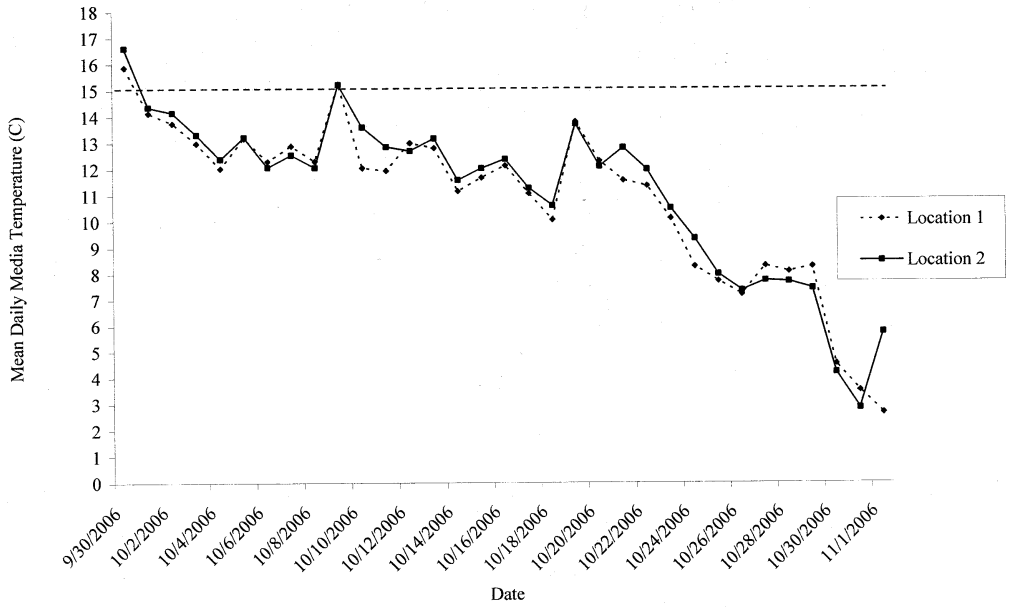

Fig 6. Mean daily media temperature (C) in containers maintained outdoors fall 2006.

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