Natural Occurrence of Entomopathogens in Pacific Northwest Nursery Soils and Their Virulence to the Black Vine Weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae)

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ABSTRACT The black vine weevil *Otiorhynchus sulcatus* (F.) is the primary insect pest of field and container-grown woody ornamentals in the Pacific Northwest (PNW). These studies were conducted to determine the natural occurrence of soil-borne entomopathogens in PNW nursery soils and determine their virulence to black vine weevil. Soil samples were collected July–September of 2002 from field-grown woody ornamental nursery stock in Oregon, Washington, and Idaho. Sample collection in each state took place in the major nursery production areas. A total of 280 samples was collected (Oregon, 170; Washington, 50; Idaho, 60). Entomopathogens were isolated using insect baiting (nematodes and fungi) as well as semiselective media (fungi). *Bacillus thuringiensis* Berliner was isolated through sodium acetate selection. Soil-borne entomopathogenic fungi occur widely throughout the major nursery production areas in the PNW. The entomopathogenic fungi *Metarhizium anisopliae* (Metchnikoff) Sorokin, *Beauveria bassiana* (Balsamo) Vuillemin, and *Paecilomyces tenuipes* (Peck) Samson were isolated. An entomopathogenic nematode (*Steinernema oregonense* Liu and Berry) and *B. thuringiensis* were also isolated. Of the 30 fungal isolates bioassayed, all but one was pathogenic to last-instar black vine weevil. None of the *B. thuringiensis* isolates collected were pathogenic to adult black vine weevil. The *S. oregonense* that were collected only infected a single black vine weevil larvae at 15 and 22°C. Pathogens collected from this soil survey will serve as a source of potential biological control agents for black vine weevil.

KEY WORDS *Beauveria bassiana*, *Metarhizium anisopliae*, *Bacillus thuringiensis*, *Steinernema oregonense*, *soil survey*

SOIL-INHABITING ENTOMOPATHOGENIC FUNGI (Harrison and Gardner 1991, Bing and Lewis 1993, Chandler et al. 1997, Bidochka et al. 1998, Klingen et al. 2002, Shapiro-Ilan et al. 2003), nematodes (Homminick 2002) and bacteria (Martin and Travers 1989) have been routinely isolated around the world. Isolating entomopathogens from soil provides insight into the naturally occurring pathogen biodiversity and provides a pool of potential biological control agents. Surveys of entomopathogen diversity commonly focus on a single pathogen group (entomopathogenic fungi, nematodes, or bacteria). However, all of these groups can occur simultaneously, each having their own potential impact on insect populations.

The black vine weevil, *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae), is a severe pest of field and container-grown ornamentals as well as small fruit crops worldwide (Moorhouse et al. 1992). The black vine weevil is a polyphagous and univoltine insect. Adults cause mainly esthetic damage to ornamentals by feeding on leaves. Eggs are deposited on the soil surface, and larvae cause severe damage by feeding on the roots of plants (Smith 1932). Weevils of the genus *Otiorhynchus* have been major pests of the nursery industry in the Pacific Northwest (PNW) for nearly 100 yr (Warner and Negley 1976). The major problem species are black vine weevil and the strawberry root weevil, *Otiorhynchus ovatus* L. (Coleoptera: Curculionidae). Current black vine weevil management programs center around the use of broad spectrum insecticide sprays targeted against adults. However, entomopathogenic nematodes and fungi have also been shown to be effective control agents of black vine weevil (Moorhouse et al. 1993a-c, 1994, Berry et al. 1997, Willmott et al. 2002).

Naturally occurring entomopathogens in the PNW are poorly characterized. The natural distribution of entomopathogenic nematodes in Oregon has been studied (Liu and Berry 1995, 1996), but no report has been made characterizing entomopathogenic fungi and bacteria occurrence in Oregon. There are no reports from Washington or Idaho characterizing entomopathogen occurrence. The fungi of primary inter-

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est in these studies were *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae) and *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae). *M. anisopliae* has been studied for black vine weevil control in Europe (Poprawski et al. 1985, Moorhouse et al. 1993a-c, 1994). *B. bassiana* is an ubiquitous fungus with a broad host range and has been studied extensively for control of a wide variety of insect pests (Hajek and St. Leger 1994). *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) has a long history as an effective biological control agent of a number of insect pests in several orders, but none of the strains tested to date are particularly effective against *Otiorhynchus* spp. (Anonymous 2002). While there are several nematode species currently being sold for black vine weevil larval control, strains with increased efficacy, persistence, and a more favorable range of operating temperatures are needed.

The objectives of these studies were to characterize nematode, fungal, and bacterial entomopathogen occurrence in field-grown nursery soils from the major nursery production areas in Oregon, Washington, and Idaho, as well as determine their virulence against black vine weevil.

Materials and Methods

Soil Sampling. Soil samples were collected July–September 2002 from commercial wholesale nursery fields in Oregon (20), Washington (7), and Idaho (8). Sample collection in each state took place in the major nursery production areas (Oregon—Benton, Clackamas, Lane, Marion, Polk, and Yamhill Counties; Washington—Cowlitz, King, Pierce, Snohomish, and Thurston Counties; Idaho—Canyon, Elmore, Gem, and Payette Counties). Nurseries surveyed meet two basic criteria: (1) they were located in the major nursery production areas in each state and (2) black vine weevil was known to occur in the area sampled. The production practices at each nursery were unique to each grower, field, and plant species, but all were typical of plant production practices throughout the industry. Plants in most nurseries sampled are harvested every 3–5 yr. A total of 280 samples were collected in all (Oregon, 170; Washington, 50; Idaho, 60). Samples were collected from a wide variety of field-grown nursery stock. An individual sample consisted of 10 soil cores (2 cm wide by 25 cm deep) taken randomly over an area of $\approx$20 m^2 that contained the same plant species. Because all samples were collected from commercial wholesale nurseries, field moisture throughout the growing season was maintained near field capacity to maximize plant growth. The 10 cores making up each sample were combined into a large (12 by 25 cm) sterile plastic bag (Nasco, Modesto, CA), mixed thoroughly, and placed in a cooler on ice until returned to the laboratory. Five to 10 samples were collected from each nursery. Soil samples from each location represented all areas of the nursery. The soil corer was disinfected with 95% ethanol between samples. Samples were stored up to 7 d at 4°C until processed.

Pathogen Isolation. Entomopathogenic nematode isolation was performed using the insect baiting method (Bedding and Akhurst 1975). Five *Galleria mellonella* L. (Lepidoptera: Pyralidae) larvae (obtained from Sunshine Mealworm, Silverton, OR) were placed on the soil surface of two petri dishes (15 by 100 mm) per sample. Petri dishes were nearly filled with soil, inverted, and incubated at 22°C. Larvae were removed from the petri dishes after 7–10 d. The assay was repeated using fresh *G. mellonella* in the same soil sample for another 7–10 d. Soil was kept moist (approximately field capacity) during isolation. Entomopathogenic nematodes were isolated from *G. mellonella* cadavers showing signs of infection (Kaya and Stock 1997). Larvae infected with nematodes were placed individually on White traps (White 1927) and held at 22°C, and infective juveniles (IJ) were collected and stored at 7°C. The entomopathogenic nematodes collected were exposed to fresh *G. mellonella* larvae to confirm pathogenicity. Nematodes were identified by Dr. Byron Adams (Department of Entomology and Nematology, University of Florida).

Fungal isolation was performed using insect baiting (Zimmermann 1986) as described above. Larvae infected with fungi were placed individually in snap-lock petri dishes (9 by 50 mm; Becton Dickinson, Franklin Lakes, NJ) with a piece of moist filter paper (Whatman no. 1). Infected larvae were held at 22°C and allowed to sporulate. In addition, semiselective media was used to isolate *B. bassiana* and *M. anisopliae* from each soil sample. Briefly, 10 g of soil from each sample were placed into 90 ml of a sterile 0.1% Tween 80 solution, shaken for 20 min at 200 rpm on a rotary shaker, and sonicated for 2 min. One hundred microliters of the suspension was spread onto media plates selective for *B. bassiana* (Doberski and Tribe 1980) and *M. anisopliae* (Veen and Ferron 1966). Plates were incubated at 28°C for 10 d. The fungi collected were identified based on macro- and microscopic characteristics (Humber 1997). Individual colonies of the respective fungi were removed from the selective media and cultured on potato dextrose agar (PDA).

After no more than two passes on PDA, single spore cultures of all fungal isolates were made, and each isolate was placed into long-term storage and cataloged. Fungi were prepared for long-term storage by growing each isolate on PDA plates (15 by 100 mm) at 28°C until sporulation. Using sterile technique, a plate was flooded with 10 ml of cryo-preservation solution (0.5% Tween 80 and 20% glycerol), and the surface of the plate was gently agitated with a sterile loop to place the spores into suspension. One milliliter of the conidial suspension was pipetted into cryo-vials (Nalgene Co., Rochester, NY). Once three vials were filled for each isolate, they were mixed with a vortex genie and placed directly into a freezer at −80°C.

The selective process for isolation of *B. thuringiensis* from soil was adapted from Travers et al. (1987) and Martin and Travers (1989), along with the modifications of Morris et al. (1998). Briefly, 1 g of soil was added to 20 ml of L-broth (tryptone 10 g, yeast extract 5 g, and NaCl 5 g in 1 liter of distilled water, pH 6.8),

supplemented with sodium acetate buffer (0.25 M, pH 6.8), in a 125-ml flask and shaken (250 rpm, 30°C) for 4 h. Samples of 500 μl were heat-treated at 80°C for 3 min. One hundred fifty microliters of each culture was spread onto LBA plates (tryptone 10 g, yeast extract 5 g, and NaCl 5 g in 1 liter of distilled water, pH 6.8, agar 15 g/liter, three plates/sample) and incubated at 30°C overnight. Using sterile toothpicks, 30 colonies were transferred onto three (10 colonies/plate) T3A plates (tryptone 3 g, tryptose 2 g, yeast extract 1.5 g, and $MnCl_2$ 0.005 g in 1 liter of 50 mM phosphate buffer, pH 6.8, agar 15 g/liter) and incubated for 40 h at 30°C. All colonies with typical *B. thuringiensis* colony morphology (fried egg) from the T3A plates were examined using phase contrast at 1,000× for spores and crystals indicative of *B. thuringiensis* (Tanada and Kaya 1993).

Long-term storage of the *B. thuringiensis* was performed using 48-h pure cultures grown on nutrient agar (NA). One milliliter of sterile nutrient broth (NB) with 17% glycerol was pipetted into a cryo-vial. A loop full of *B. thuringiensis* from a single cell culture was placed into the vial, mixed with a vortex genie, and immediately placed on ice until frozen at −80°C.

Laboratory Bioassays. Bioassays against the black vine weevil of the prominent pathogens from each group collected were performed. Five isolates of *B. bassiana* (Oregon—IP43, IP54, IP57, IP63, and IP237; Washington—IP113, IP135, IP325, IP350, and IP351; and Idaho—IP370, IP372, IP375, IP376, and IP377) and *M. anisopliae* (Oregon—IP37, IP38, IP39, IP272, and IP276; Washington—IP45, IP99, IP131, IP285, and IP326; and Idaho—IP79, IP80, IP86, IP368, and IP515) from each state were randomly selected and screened against last-instar black vine weevil. Each of the fungal isolates assayed originated from an individual soil sample collected from different nurseries. Isolates were selected in this way in an attempt to maintain their genetic uniqueness, because most nurseries were separated by several kilometers. Black vine weevil larvae were obtained from a laboratory colony maintained at the USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR. The isolates used in the bioassay were taken from pure cultures in long-term storage, grown on PDA at 28°C, and allowed to sporulate. Two plates (15 by 100 mm) were flooded with 10 ml of a sterile 0.1% Tween 80 solution, and the spores were removed by gentle agitation with a sterile loop. Hemocytometer counts of all suspensions were made, and the spore concentration was adjusted to 1×10^4 spores/ml. For the first run of the bioassay, a total of 20 last-instar black vine weevil were assayed for each isolate. Because of a limited number of insects, 10 larvae were used per isolate on the second run of the bioassay. The bioassays were performed on separate days, and fresh spore suspensions were made for the second run of the bioassay. Assays of *M. anisopliae* and *B. bassiana* assays were performed separately. Larvae were submerged, in groups of five, into 5 ml of spore suspension (used only once) for 1 min and placed on filter paper to remove excess solution. Larvae were then placed individually into 1-oz plastic cups (Sweetheart Cup Co., Owings Mills, MD) with artificial diet (Shanks and Finnigan 1973) and incubated at 22°C for 14 d, and larval mortality was recorded. Assays were performed at 22°C to simulate typical soil temperatures when last-instar black vine weevils are present in the field. A germination test of each isolate was performed for both runs of the bioassay to ensure a high level (90–100%) of viability (Goettel and Inglis 1997). All experiments included an untreated control (0.1% Tween 80) and were arranged in a completely randomized design.

The ability of the entomopathogenic nematode collected to infect last-instar black vine weevil at 10, 12, 15, and 22°C was determined. The nematode was reared on last-instar *G. mellonella* at 22°C (Kaya and Stock 1997) and was not subcultured more than twice in *G. mellonella* before use in experiments. Ten last-instar black vine weevil were held individually in snap-lock petri plates (9 by 50 mm; Becton Dickinson) containing filter paper (Whatman no. 1). An aliquot containing 400 IJ was added to each dish. Dishes were incubated at each temperature for 14 d, and larval mortality was recorded. The nematode bioassay was performed four times. All experiments included an untreated control (water only) and were arranged in a completely randomized design.

All *B. thuringiensis* isolates collected were screened for activity against black vine weevil adults. Isolates were taken from long-term storage and grown for 24 h on NA. Baffled flasks (250 ml) containing 100 ml of NB were inoculated with a loopful of 24-h cultures of *B. thuringiensis* and incubated on a rotary shaker (250 rpm, 30°C) for 5–6 d until a majority of the cells had lysed. The culture was centrifuged at 10,000 rpm for 5 min at 15°C, decanted, and resuspended in 100 ml dH_2O. The suspension was centrifuged as above, decanted, and resuspended in 25 ml dH_2O. With the use of a hemocytometer, the spore concentration was adjusted to 1×10^7 spores/ml. It is recommended when screening Lepidoptera with unknown *B. thuringiensis* isolates to do so with a mixed culture of spores and crystals (McGuire et al. 1997). The same approach has been taken here for screening unknown *B. thuringiensis* isolates for activity against black vine weevil. The bioassay was performed by spraying the suspension of each isolate onto one strawberry leaf until runoff. The treated leaves were placed in a laminar flow hood and allowed to dry. Once dry, the leaf petioles were placed into an aqua tube (Syndicate Sales, Kokomo, IN) filled with water to maintain freshness. A single leaf was placed into a large plastic deli container (C18–5032; Pactiv, Lake Forest, IL) along with five black vine weevil adults. Each treatment was replicated four times. The containers were held at 22°C and a 18:6 (L:D) cycle for 3 d. After 3 d, treated leaves were removed and replaced with untreated strawberry leaves. The weevils were allowed to continue feeding on untreated leaves for 7–10 d. At the conclusion of the experiment, all dead weevils were examined microscopically (1,000×) to determine if they were infected with *B. thuringiensis*. All experiments included a water

Table 1. Location of wholesale nurseries sampled and pathogens isolated

Location	Sand:Silt:Clay	*Metarhizium anisopliae*	*Beauveria bassiana*	*Paecilomyces tenuipes*	*Bacillus thuringiensis*	*Steinernema oregonense*
Caldwell, ID (1)[a]	33:45:22		√			
Caldwell, ID (2)	18:55:27	√	√			
Emmet, ID (1)	17:51:32	√	√			
Emmet, ID (2)	32:41:27	√	√			
Idaho City, ID	38:42:20	√	√	√		
Meridian, ID (1)	60:30:10	√	√			
Meridian, ID (2)	26:50:24					
Nampa, ID	23:62:15	√	√			
Aurora, OR (1)	34:49:17	√	√			
Aurora, OR (2)	31:55:14	√	√			
Aurora, OR (3)	26:55:19	√	√			
Aurora, OR (4)	71:25:4	√				
Aurora, OR (5)	21:57:22	√	√			
Canby, OR	44:40:16					
Corvallis, OR	44:34:22	√				
Gaston, OR	11:55:34	√				
Hubbard, OR	9:57:34	√	√			
Independence, OR (1)	11:53:36	√	√			
Independence, OR (2)	46:33:21	√	√		√	
Lowell, OR	38:37:25	√	√			
McMinnville, OR	17:52:31	√				
Monmouth, OR	46:28:26			√		
Mt. Angel, OR	7:58:35	√	√			
Salem, OR	72:14:14				√	
Silverton, OR	24:32:44	√				
St. Paul, OR	29:44:27					
Stayton, OR	49:29:22	√	√			
Woodburn, OR	18:52:30		√			
Burien, WA	39:45:16	√	√			
Des Moines, WA	24:56:20	√	√			
Kirkland, WA	67:16:17	√				
Longview, WA	35:43:22	√	√			
Olympia, WA	51:36:14	√	√	√		√
Puyallup, WA	55:33:12	√				
Seattle, WA	48:36:16	√	√	√		

[a] Notes multiple nurseries located in same city.

control and were arranged in a completely randomized design.

Data Analysis. Because of a lack of infection in the nematode and *B. thuringiensis* assays, no analysis were performed on these data. Data from the fungal bioassays were nonparametric and were analyzed using a χ^2 analysis (SAS Institute 1999). Data from the two bioassays were considered replicates, and a χ^2 analysis was performed on the combined data for each fungus to determine if there were any significant differences in larval mortality between isolates. A 2 by 2 Fisher exact test was used to determine if mortality differences between isolates of the same fungus were significantly different (SAS Institute 1999). Because of the large number of treatments in the nonparametric test, a reference probability of $P \leq 0.01$ was used throughout the data analysis.

Results and Discussion

Soil-borne entomopathogens occur widely throughout the PNW (Table 1). Entomopathogenic fungi were the most commonly isolated pathogen group, and their occurrence was typical of collections elsewhere (Harrison and Gardner 1991, Bing and Lewis 1993, Chandler et al. 1997, Klingen et al. 2002, Shapiro-Ilan et al. 2003). However, the occurrence of entomopathogenic nematodes and *B. thuringiensis* were lower than has been found in other areas (Akhurst and Brooks 1984, Martin and Travers 1989, Hara et al. 1991, Liu and Berry 1995, Shapiro-Ilan et al. 2003).

The frequency of nematode isolations from this survey was <1% (2/280). *Steinernema oregonense* (Rhabditidae: Steinernematidae) was collected from two soil samples from a single nursery in Olympia, WA. *S. oregonense* was originally isolated from the Oregon coast, and until now, had not been isolated from noncoastal areas (Liu and Berry 1995). Only considering *S. oregonense* occurrence in Washington, nematode frequency was still low at 4% (2/50). The insect bait method has been successfully used (Bedding and Akhurst 1975, Liu and Berry 1995, Shapiro-Ilan et al. 2003) for isolating entomopathogenic nematodes from soil. In this study, only nematodes infective to *G. mellonella* were isolated; thus, other species or strains of nematodes may have gone undetected. For example, *Steinernema scapterisci* Nguyen and Smart (Rhabditidae: Steinernematidae) is quite specific to *Scapteriscus* spp. (Orthoptera: Gryllotalpidae) (Nguyen and Smart 1990). Direct extraction of nematodes was not performed in this study and may have resulted in additional isolations. Also, had the insect baiting been performed at higher temperatures

Table 2. Occurrence of entomopathogenic fungi, nematodes, and bacteria collected from Pacific Northwest nursery soils

	State			Total mean[a]
	Oregon	Washington	Idaho	
Number of nurseries sampled	20	7	8	35
Number of soil samples	170	50	60	280
Number of nurseries with *M. anisopliae*	15	7	7	29
Percent soil samples with *M. anisopliae*	21	72	35	34
Number of nurseries with *B. bassiana*	11	5	6	22
Percent soil samples with *B. bassiana*	16	22	20	18
Number of nurseries with *P. tenuipes*	1	2	1	5
Percent soil samples with *P. tenuipes*	3	14	2	5
Number of nurseries with nematodes	0	1	0	1
Percent soil samples with nematodes	0	4	0	0.7
Number of nurseries with *B. thuringiensis*	2	0	0	2
Percent soil samples with *B. thuringiensis*	4	0	0	0.7

[a] Total number of nurseries or samples collected or the mean percentage of samples collected with each pathogen.

(25°C), additional nematode isolations may have been made. Therefore, these results should be considered as a conservative estimate of entomopathogenic nematode occurrence.

While *Heterorhabditis* and *Steinernema* spp. have previously been found to be widely distributed in Oregon, with the highest occurrence in costal soils, neither was found to occur in the Willamette Valley (the center of nursery production in Oregon and the location of samples in this study) (Liu and Berry 1995). The impact of agricultural activities such as tillage and the application of chemical pesticides and fertilizers have been found to have mixed results in relationship to nematode survival and persistence. Ornamental nursery plants are grown in intensively managed agricultural systems generally consisting of high levels of fertilizer and pesticide use. Organophosphate nematicides such as Mocap (ethoprop) is routinely used in the ornamental nursery industry. Broad spectrum biocides (methyl bromide and Vapam) are also used before planting by some growers. The long-term use of chemical fertilizers and pesticides in nursery production may be a potential cause for the lack of entomopathogenic nematodes. Tillage does not have a significant impact on the survival of *Heterorhabditis bacteriophora* (Rhabditidae: Heterorhabditidae), has a positive impact on *Steinernema riobrave* (Rhabditidae: Steinernematidae), and has a negative impact on *Steinernema carpocapsae* (Rhabditidae: Steinernematidae) (Millar and Barbercheck 2002). Entomopathogenic nematode persistence is enhanced in conservation tillage systems with increased levels of crop residue (Shapiro et al. 1999, Hummel et al. 2002). Even though ornamental nursery plants are perennially grown, intense tillage of the soil between plant rows is common. Prolonged exposure to inorganic fertilizers inhibit nematode infectivity, while short-term exposure generally is not detrimental (Bednarek and Gaugler 1997). Soil texture also effects nematode survival, dispersal, and persistence. As clay content increases, nematode survival (Kung et al. 1990) and dispersal (Georgis and Poinar 1983, Barbercheck and Kaya 1991) decrease. Because of the extremely low frequency of nematode isolation, it was not possible to perform meaningful correlation analyses to determine if there were any significant relationships between soil composition and nematode abundance. The soil composition of one sample from each location surveyed is presented for comparison (Table 1).

The strain of *S. oregonense* that was collected infected only a single black vine weevil larvae at 15 and 22°C over the four runs of the bioassay. Based on these data, this particular strain of *S. oregonense* would not be a good candidate for further development as a biological control agent for black vine weevil, particularly for use in the PNW where cool wet conditions prevail in the fall and spring months, when nematodes are applied for black vine weevil control.

Metarhizium anisopliae was isolated most frequently, followed by *B. bassiana* and *Paecilomyces tenuipes* (Peck) Samson (Eurotiales: Trichocomaceae) (Table 2). The occurrence of *M. anisopliae* in nursery soils in Washington and Idaho was particularly common, with at least one soil sample positive from every nursery surveyed in Washington and seven of eight nurseries in Idaho. *B. bassiana* was also widely distributed. *P. tenuipes* was not as common, occurring in only one nursery in Oregon, three in Washington, and one in Idaho. The use of both insect baiting and semiselective media proved important to obtain the most accurate estimate of entomopathogenic fungi occurrence in the soil. This was particularly true for *M. anisopliae* (Table 3).

It has been proposed that habitat, not insect selection, drives the population structure of *M. anisopliae* and *B. bassiana* (Bidochka et al. 1998, 2002). *M. anisopliae* occurs more frequently in agricultural habitats,

Table 3. Percent isolation of *Metarhizium anisopliae* and *Beauveria bassiana* via *Galleria* baiting[a] and semi-selective media[b]

	Metarhizium anisopliae	*Beauveria bassiana*
Insect Baiting	23%	17%
Selective Media	11%	1%
Total[c]	34%	18%

[a] (Bedding and Akhurst 1975, Zimmermann 1986).

[b] *Metarhizium anisopliae* (Veen and Ferron 1966); *Beauveria bassiana* (Doberski and Tribe 1980).

[c] Percent of the total samples collected from all three states positive for *M. anisopliae* (34%; 95/280) and *B. bassiana* (18%; 50/280).

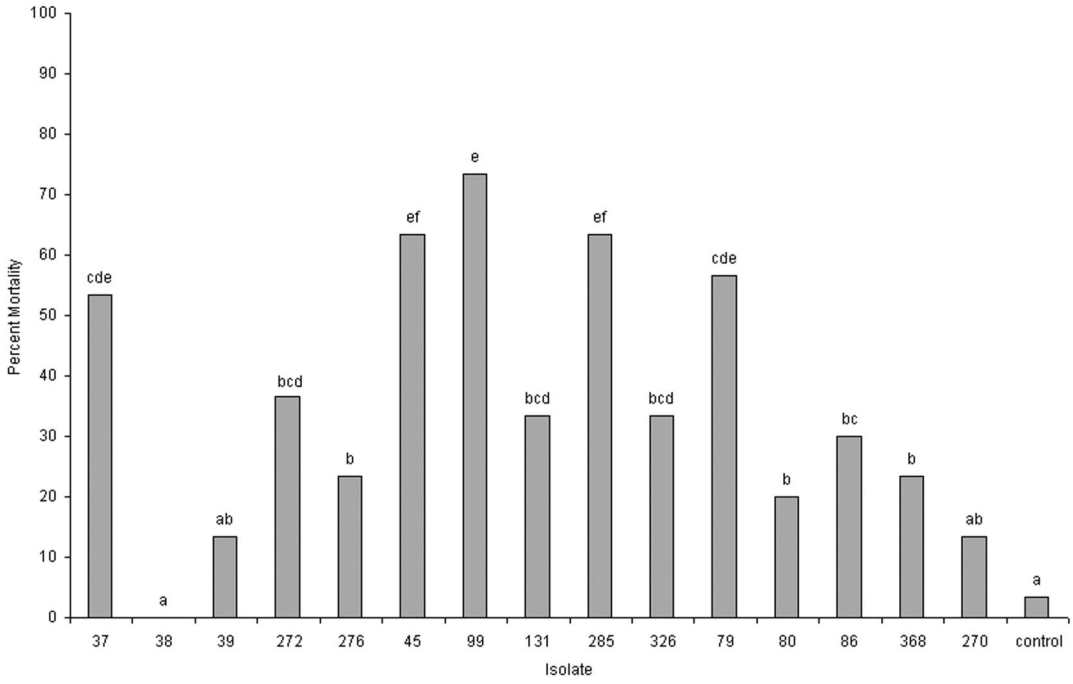

Fig. 1. Mean mortality from the bioassay ($N = 30$) of last-instar *O. sulcatus* after 14-d exposure to *M. anisopliae* isolates (1×10^4 spores/ml) collected from PNW nursery soils. Isolate means that share the same letter are not significantly different as determined by Fisher exact test (SAS Institute 1999).

whereas *B. bassiana* is more frequently isolated from forested habitats (Bidochka et al. 1998). While the number of nurseries with *M. anisopliae* and *B. bassiana* are similar (Table 2), there were nearly twice as many individual soil samples positive for *M. anisopliae* (95) as there were *B. bassiana* (50). The occurrence of *B. bassiana* and *M. anisopliae* have been shown to be negatively correlated with tillage intensity. Conservation and no-till systems are most compatible for the survival of soil-borne *B. bassiana* and *M. anisopliae* (Bing and Lewis 1993, Hummel et al. 2002). Pesticide usage (Hummel et al. 2002) and fresh cow manure (Rosin et al. 1996) also reduce entomopathogenic fungal survival and persistence. *B. bassiana* and *M. anisopliae* occurrence overall in soils collected from pecan orchards has been shown to be negatively correlated with manganese levels, whereas calcium and magnesium levels are positively correlated with *M. anisopliae* occurrence (Shapiro-Ilan et al. 2003). Because individual soil samples were not analyzed in this study, it is unknown if a similar relationship occurs between micronutrients. The amounts of sand, silt, or clay in the single soil sample characterized from each location and the occurrence of *M. anisopliae* or *B. bassiana* at that location were not significantly correlated (SAS Institute 1999).

Fungal bioassays revealed a wide range of virulence between isolates. All *M. anisopliae* isolates assayed but three (IP38, IP39, and IP270) caused substantially higher larval mortality than was found in the control ($P \leq 0.01$; Fig. 1). The *M. anisopliae* isolates IP37, IP45, IP79, IP99, and IP285 all caused at least 53% mortality, with IP99 causing 73% mortality (Fig. 1). All of the *B. bassiana* isolates tested caused infection (based on macro- and microscopic characteristics), but only isolates IP113, IP135, IP370, IP372, IP377, and IP375 caused significantly higher mortality than the control ($P \leq 0.01$; Fig. 2). Wide variability in weevil larval mortality between field-collected isolates of the same fungus has also been observed with the pecan weevil (Shapiro-Ilan et al. 2003). The most virulent isolates have been deposited in the USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY.

Bacillus thuringiensis was isolated from Oregon, but not Washington or Idaho soils (Tables 1 and 2). *B. thuringiensis* has a worldwide distribution and has been isolated from soils in a number of different environments (Martin and Travers 1989). While the presence of insects does not have any noticeable effect on *B. thuringiensis* occurrence, there are some areas that are more rich in *B. thuringiensis* than others (Bernhard et al. 1997). Eastern Asian soils are particularly high (78% of soil samples) in *B. thuringiensis*, whereas *B. thuringiensis* made up only 25% of the colonies examined in soils from the United States (Martin and Travers 1989). In agricultural fields in particular, the frequency of *B. thuringiensis* in the United States was only one-half that of the Republic of Korea (Martin and Travers 1989). The reason why *B. thuringiensis* was not isolated from nursery soils in Washington and Idaho is unclear. To test my isolation technique, a known culture of *B. thuringiensis* (HD-1)

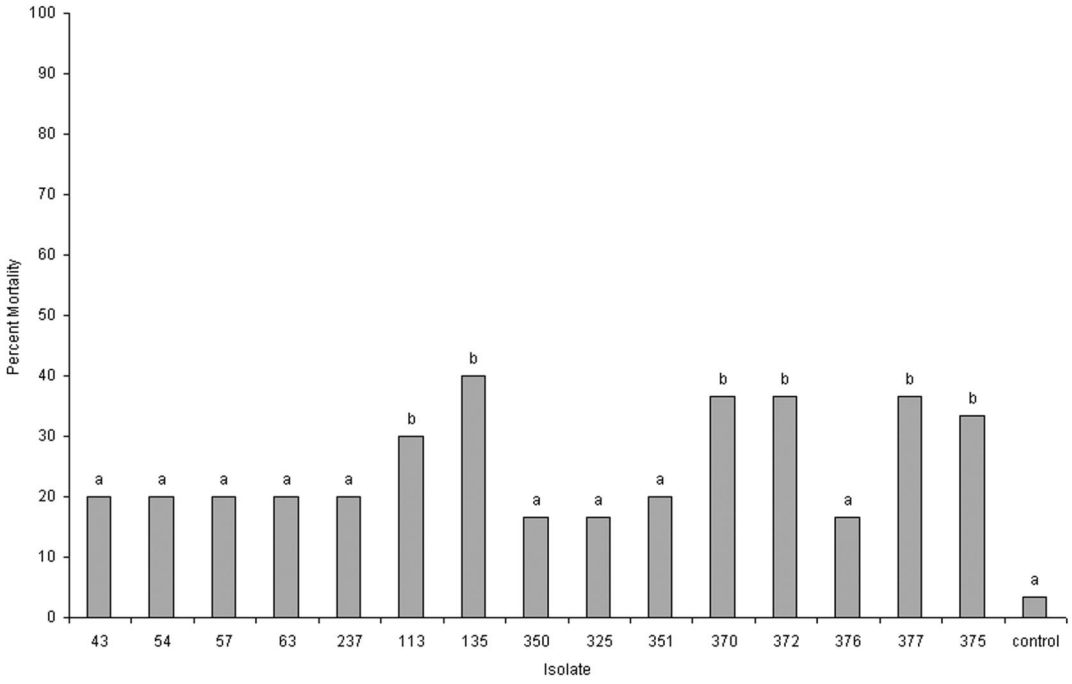

Fig. 2. Mean mortality from the bioassay ($N = 30$) of last-instar *O. sulcatus* after 14-d exposure to *B. bassiana* isolates (1×10^4 spores/ml) collected from PNW nursery soils. Isolate means that share the same letter are not significantly different as determined by Fisher exact test (SAS Institute 1999).

was subjected to the selection procedure, and all 30 colonies examined on the T3A plates were *B. thuringiensis.* None of the *B. thuringiensis* isolates collected and screened were found to be pathogenic to black vine weevil adults. Because of the complete lack of infectivity and a limited supply of adult weevils, the *B. thuringiensis* bioassay was not repeated.

Overall, the occurrence of entomopathogenic fungi from PNW nursery soils was typical of results from other surveys performed throughout the world. The occurrence of entomopathogenic nematodes and *B. thuringiensis* was less than was observed in other studies. The reason for the lack of nematode and bacteria isolations is unknown. The implementation of multiple isolation techniques may have resulted in the collection of additional nematode and bacteria isolates, as was the case for the fungi. It is also possible that the detection techniques for each pathogen group are not equal. For instance, had the insect baiting for entomopathogenic nematodes been performed at higher temperatures, additional isolations may have been made. In addition, soil samples in this study were collected only during the summer months, and the occurrence of entomopathogens in the soil may vary temporally throughout the year. Future studies will concentrate on determining the ability of the most efficacious fungal isolates (IP37, IP45, IP79, IP99, and IP285) collected in these studies, as well as potentially other yet to be determined isolates, to control black vine weevil.

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