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Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees

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ABSTRACT

Understanding habitat selection of fungal entomopathogens is critical to improve the efficacy, persistence and cost of these fungi as microbial insecticides. This study sought to determine the prevalence of *Metarhizium* and *Beauveria* spp. isolated from the rhizosphere of strawberry, blueberry, grape and Christmas tree crops in the Willamette Valley of Oregon. Entomopathogenic fungi were assigned to thirteen species based on molecular phylogenetic criteria. Four species of *Metarhizium* were isolated including *Metarhizium brunneum*, *Metarhizium guizhouense*, *Metarhizium robertsii*, and *Metarhizium flavoviride* var. *pemphigi*. Nine *Beauveria* species were isolated including, *Beauveria brongniartii*, an undescribed species referred to as Clade C and seven phylogenetic species of *Beauveria bassiana*. Strawberries and blueberries were significantly associated with *M. brunneum* and Christmas trees with *M. guizhouense* and *M. robertsii*. Grapes were significantly associated with *B. bassiana* phylogenetic species *Bbas-16*. All of the *Metarhizium* isolates screened were pathogenic to *Otiorhynchus sulcatus* larvae in laboratory bioassays but only *M. brunneum* and *M. robertsii* caused significant levels of infection. The study results suggest that certain species of *Metarhizium* and *Beauveria* are significantly associated with the strawberry, blueberry and Christmas tree rhizosphere and could potentially provide better control of *O. sulcatus*.

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1. Introduction

Key to promoting epizootic development of entomopathogenic fungi is a thorough knowledge of their ecology and life history. The ecology and life history of entomopathogens can vary considerably among species (Pell et al., 2010); therefore it is imperative to have a reliable method of species identification. In the case of both *Metarhizium* and *Beauveria*, morphological crypsis occurs between both sister and non-sister taxa, probably as a result of heterogeneous morphological evolution, retention of symplesiomorphic morphologies and convergent morphological evolution due to occupation of similar ecological niches. As a result of these confounding evolutionary processes, morphological identification is not sufficient for distinguishing between species (Bischoff et al., 2009). With recent genetic analysis, it is now possible to identify members of both *Metarhizium* and *Beauveria* spp. (Bischoff et al., 2009; Rehner and Buckley, 2005; Rehner et al., 2006; Rehner, unpublished data).

According to Vega et al. (2009), a key question that needs to be addressed in the use of entomopathogenic fungi in insect microbial control is to determine the extent to which species engage in

unique associations with host plants. Thus, future research should integrate efforts to understand the capacity and significance of entomopathogenic fungi's role as endophytes, plant disease antagonists, plant growth promoters and rhizosphere colonizers in addition to their pathogenicity and virulence toward their intended insect hosts.

Metarhizium anisopliae (Metschn.) Sorokin (Hypocreales: Clavicipitaceae) has been commercially developed for the potted nursery industry as a granular formulation (F52, Novozymes Biologicals Inc., Salem VA, USA), which is incorporated into growing media at potting for *Otiorhynchus sulcatus* control (Bruck and Donahue, 2007). *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (Hypocreales: Cordycipitaceae) (GHA, BotaniGard ES™ Laverlam International Corporation, Butte, MT; ATCC 4040, Naturalis, Troy Biosciences Inc., Phoenix, AZ) is also commercially available and primarily used by growers as a foliar spray for the control of greenhouse pests. Despite their ability to kill insects, entomopathogenic fungi applied inundatively have performed inconsistently, due in large part to a lack of understanding of their ecology and biology and the expectation that they will perform similarly to synthetic pesticides (Roy et al., 2010). Historically, entomopathogens have been selected for release in the field based solely on their efficacy in laboratory bioassay tests, without consideration of their microhabitat preferences and ecological constraints. It had been assumed

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that fungal population genetics are closely related to host insects (Bidochka et al., 2001). However, recent research shows that *M. anisopliae* population structure may be driven by habitat selection, not insect host selection (Bidochka et al., 2001). Similarly, *B. bassiana* has adapted to selected habitats and any evidence of an insect-host-related population structure should be viewed primarily as coincidental and not as a result of co-evolution (Bidochka et al., 2002; Meyling and Eilenberg, 2006, 2007; Meyling et al., 2009). In temperate North American boreal forests, *B. bassiana* is more abundant in natural habitats while *M. anisopliae* is more abundant in agricultural habitats (Bidochka et al., 1998). A number of different factors have been studied in terms of their effect on the distribution of entomopathogenic fungi in the soil, including geographical location, habitat type, soil type and soil tillage (Rath et al., 1992; Sosa-Gomez and Moscardi, 1994; Vanninen, 1996). In addition to the important role that large landscape-scale habitat selection plays in the abundance and distribution of entomopathogenic fungi, it is becoming increasingly apparent that consideration of the microhabitats that entomopathogenic fungi occupy also plays an essential role when developing screening strategies for selecting strains as microbial control agents. *B. bassiana* was isolated from the wheat rhizosphere in Australia (Sivasithamparam et al., 1987), but the potential implications of this discovery on the microbial control of insects were not appreciated at the time. *M. anisopliae* has more recently been found in the cabbage rhizosphere; however, again the pest management implications of this phenomenon were not explored (Hu and St. Leger, 2002). The population of *M. anisopliae* in the inner cabbage rhizosphere remained at 10^5 propagules/g, while the populations in the non-rhizosphere soil decreased from 10^5 to 10^3 propagules/g after several months. Hu and St. Leger (2002) also noted that the carrying capacity of *M. anisopliae* (2575-GFP) in the cabbage rhizosphere (10^5 propagules/g) was higher than the LC50 value of the isolate against a number of insect pests. The pest management potential of rhizosphere colonization by entomopathogenic fungi was not determined directly until a study by Bruck (2005). Colonization of the *Picea abies* (L.) Karst. (Pinales: Pinaceae) rhizosphere by *M. anisopliae* (F52) provided nearly 80% control of *O. sulcatus* larvae after 2 weeks of exposure to inoculated roots (Bruck, 2005). Rhizosphere colonization can also directly affect the efficacy of the fungi as a microbial control and can contribute largely to its success or failure by modifying the behavioral response of an insect host to its fungal entomopathogen. *O. sulcatus* larvae are more attracted to *P. abies* plant roots in the presence of *M. anisopliae* spores than plants grown in the absence of fungal spores (Kepler and Bruck, 2006).

The black vine weevil, *O. sulcatus* (F.) (Coleoptera: Curculionidae), is a parthenogenic, polyphagous insect pest commonly found infesting container and field-grown ornamentals and small fruits throughout the Pacific Northwest. *O. sulcatus* has a host range of over 150 plant species (Moorhouse et al., 1992), primarily in the families Ericaceae, Pinaceae, Primulaceae, Rosaceae, Saxifragaceae, Taxaceae and Vitaceae (Cowles, 1995). *O. sulcatus* originated in Northern Europe and now occurs throughout the major nursery and small fruit growing regions of the United States, Canada, Australia, Japan, Chile and New Zealand. Movement throughout the world is associated with shipments of contaminated plants (Moorhouse et al., 1992). A wide host range coupled with a cryptic life cycle makes this insect a formidable pest of the nursery and small fruit industries.

Chemical, cultural, and biological controls can be used to manage *O. sulcatus*. Chemical controls are most effective in managing adult *O. sulcatus* if several applications are made to target preovipositional adults at night when the weevils are active (Moorhouse et al., 1992; Son and Lewis, 2005). The larval stage can be targeted with fall or spring soil drenches or by incorporating a pesticide into the growing media prior to potting. High volume sprays or heavy

irrigation after pesticide application is required for effective penetration of larvacides drenched on the soil surface (Moorhouse et al., 1992). Cultural control practices such as crop rotation; early season plowing and use of cover crops that are unattractive to weevils can slow the spread of infestation and reduce weevil populations but are not effective eradication methods (Moorhouse et al., 1992). Entomopathogenic nematodes can effectively control *O. sulcatus* larvae when applied in spring or fall (Bruck, 2004a) but their adoption has been limited due to their cost, short shelf life, unpredictable performance and low persistence (Georgis et al., 2006). Improved knowledge of the biology of rhizosphere associated fungi will allow for the development of novel control methods for root-feeding pests, including *O. sulcatus*.

In this study, the principal objective was to ascertain if there was an association between host plant and species of fungi by using phylogenetic analysis to determine the prevalence of naturally-occurring entomopathogenic fungi in the rhizosphere of *O. sulcatus* susceptible crops grown in the Willamette Valley of Oregon. The second objective of this study was to assess the pathogenicity of the *Metarhizium* spp. isolated to this key root-feeding insect.

2. Material and methods

2.1. Collection of root samples

Root samples were collected from strawberry, *Fragaria* (L.) (Rosales: Rosaceae), blueberry, *Vaccinium* (L.) (Ericales: Ericaceae), grape *Vitis* (L.) (Rhamnales: Vitaceae) and the following Christmas tree species: engelmann spruce, *Picea engelmannii* (Parry) ex engelm (Pinales: Pinaceae), noble fir, *Abies procera* (Rehder) (Pinales: Pinaceae) and douglas fir, *Pseudotsuga menziesii* (Mirb.) franco (Pinales: Pinaceae). Root samples were collected from strawberry and blueberry fields using a standard golf hole corer (10.2 × 17.8 cm, Pro II Hole Cutter, Markers Inc., Avon Lake, OH). Christmas tree and grape samples were collected using a shovel due to the difficulties associated with using a golf hole corer in the dry soils in those fields. Fields containing established plantings (>3 and 10 yrs for strawberries and other plants (blueberries, grapes, Christmas trees), respectively) were preferentially sampled over fields with younger plants so as to allow time for any entomopathogenic fungi present in the soil to colonize the rhizosphere. Root samples were randomly collected from plants distributed throughout the field, or, if the field was over five hectares, samples were taken from a five hectare section within the field. Roots were placed in 3.78 l plastic reclosable bags, placed in a cooler with ice until they were returned to the laboratory and refrigerated until use (0–4 days). A total of 7, 11, 6 and 10 strawberry, blueberry, Christmas tree and grape fields were sampled throughout the Willamette Valley of Oregon yielding collections of 70, 109, 60 and 100 root samples from each crop, respectively (refer to Table 1 for a list of field locations).

2.2. Fungal isolation and culturing

Entomopathogenic fungi were isolated from the rhizosphere of all plants using a modified version of the 'Galleria bait method' (Zimmermann, 1986). Individual roots were shaken in a standardized manner to remove non-rhizosphere soil and then placed into a deep dish Petri plate (150 × 25 mm, Thermo Scientific, Waltham, MA) that contained moistened filter paper (15 cm, Grade p5, Fisher, Pittsburgh, PA). The rhizosphere soil was defined as the soil still adhering to the roots after shaking. Live wax worms, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) were placed in the Petri plates and allowed to crawl and contact the roots and the rhizosphere soil. The Petri plates were sealed with Parafilm and placed in plastic reclosable bag and incubated in complete darkness at

Table 1

Location GPS coordinates and county of all field sites sampled.

Crop	Field	Coordinates	County	Crop	Field	Coordinates	County
Christmas	1	N44 26.663 W123 23.317	Benton	Strawberry	1	N44 33.655 W123 13.649	Linn
	2	N44 26.717 W123 23.244	Benton		2	N44 31.962 W123 13.887	Linn
	3	N44 26.804 W123 22.649	Benton		3	N45 09.613 W122 37.010	Clackamas
	4	N44 26.364 W123 24.514	Benton		4	N45 09.998 W122 37.169	Clackamas
	5	N44 26.728 W123 22.590	Benton		5	N44 31.642 W123 22.042	Benton
	6	N44 26.959 W123 22.912	Benton		6	N45 16.826 W122 44.892	Clackamas
Blueberry	1	N44 34.510 W123 11.859	Linn		7	N44 37.594 W123 11.825	Benton
	2	N45 00.799 W122 56.578	Marion	Grape	1	N45 01.979 W123 09.070	Polk
	3	N44 35.949 W123 13.410	Benton		2	N45 01.968 W123 09.064	Polk
	4	N44 35.951 W123 13.428	Benton		3	N45 06.923 W123 08.581	Yamhill
	5	N43 57.392 W122 48.979	Lane		4	N45 06.798 W123 08.654	Yamhill
	6	N43 57.391 W122 48.990	Lane		5	N44 52.330 W123 17.244	Polk
	7	N44 29.766 W123 16.222	Benton		6	N44 52.317 W123 17.137	Polk
	8	N44 29.663 W123 16.202	Benton		7	N44 52.354 W123 17.290	Polk
	9	N44 30.708 W123 15.787	Benton		8	N44 48.621 W123 15.420	Polk
	10	N44 30.631 W123 15.792	Benton		9	N44 48.706 W123 15.183	Polk
	11	N44 30.631 W123 15.792	Benton		10	N44 50.540 W123 18.876	Polk

21 °C. After 2 weeks, dead larvae were removed and placed in small snap-lock Petri dishes (9 × 50 mm; Becton Dickinson, Franklin Lakes, NJ) containing moistened filter paper (Whatman #1) and incubated in complete darkness at 21 °C until sporulation (cadavers that did not sporulate were discarded). After sporulation, fungi were isolated and cryo-preserved using the procedures outlined by Bruck (2004b). The prevalence of each fungal species/clade in the rhizosphere of each host plant was analyzed using a Fisher's exact test ($P \leq 0.05$) (SAS Institute, 1999).

Single-spore subcultures were established from a single infected *G. mellonella* larva per root sample. If a sample contained larvae infected with both *Metarhizium* and *Beauveria*, a random isolate of each was selected. Mycelia for DNA extraction were grown in 35 ml sterile 0.5% Sabouraud's dextrose broth (SDY) at 100–125 rpm at 25 °C. The mycelia were collected by centrifugation, washed 3× in sterile distilled water and excess moisture removed by blotting the mycelia between two layers of sterile filter paper. The mycelia were lyophilized overnight (Free Zone6, Labconco, Kansas City, MO) and stored at –20 °C.

2.3. DNA extraction

Approximately 25–50 mg of lyophilized mycelium was pulverized with glass-zirconia beads (Biospec, Bartlesville, OK) in a Fast-prep (Q-Biogene, Solon, OH) sample grinder for 6 s at a speed setting of 4.5. The ground tissues were suspended in 500 µl Prep-Man (ABI, Foster City, CA) extraction reagent and boiled for 10 min. The boiled extracts were cleared by centrifugation at 16,000g for 10 min and the supernatant transferred to a clean tube and stored at –20 °C.

2.4. PCR

Metarhizium and *Beauveria* strains were sequence-characterized with nuclear loci including the 5' region of elongation factor-1 alpha (*5'-tef1*) and Bloc, which are informative for diagnosis of phylogenetic species in these genera (Bischoff et al., 2006, 2009; Rehner et al., 2006), respectively. The *5'-tef1* region in *Metarhizium*, which contains three sequence polymorphic introns, was amplified with primers EF1T (5'-ATGGGTAAGGA(A/G)GACAAGAC) and EF2T (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT) (Rehner and Buckley, 2005). The marker Bloc is an intergenic region developed specifically for *Beauveria* and was amplified with primers B5.1F (5'-CGACCCGGCCAACTACTTTGA) and B3.1R (5'-GTCTTCCAGTACCACTACGCC) (Rehner et al., 2006). PCR reactions for each locus

included 1 µl genomic DNA extract, 200 µM dNTPs, 0.4 µM each of two locus-specific primers (Integrated DNA Technologies, Coralville, IA), and 1.0 unit of *Taq* DNA polymerase (Promega, Madison, WI) in a total reaction volume of 50 µl in 1× reaction buffer supplied by the manufacturer. Thermal cycling conditions included an initial template denaturation at 94 °C for 2 min, then 40 cycles of 94 °C for 30 s, 56 °C annealing for 30 s, 72 °C for 2 min; followed by a 72 °C extension for 15 min. PCR products were gel-purified in 1.5% NuSieve agarose gels (Cambrex, Walkersville, MD) and gel-slices of the amplicons were frozen at –80 °C and then extruded from the gel by centrifugation.

2.5. Sequencing and data preparation

Nucleotide sequencing was performed with BigDye Terminator Cycle Sequencing Kits on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence reaction volumes totaled 5 µl and included 1.5 µl DNA template, 0.25 µl sequencing primer (2.5 pmol), 0.5 µl BigDye Terminator, 1.0 µl BigDye sequencing buffer and 1.75 µl sterile distilled water and cycle sequenced according to the manufacturer's instructions. PCR primers were used to sequence *5'-tef1*, whereas an approximately 950 bp internal segment of Bloc was sequenced with primers B22U (5'-GTCCGA GCCAGAGCAACT) and B822L (5'-AGATTCCGCAACGTCAACT). Sequence data was edited and assembled with Sequencher 4.1 (GeneCodes, Ann Arbor, MI) and aligned in the Megalign module of DNASTAR 5 (Lasergene, Madison, WI) and output in the NEXUS file format.

2.6. Molecular phylogenetic identification

The phylogenetic diversity of *Metarhizium* and *Beauveria* strains recovered from the experimental field treatments was inferred by maximum parsimony (MP) and MP bootstrap analysis of *5'-tef1* and Bloc sequences, respectively. All MP searches for the shortest trees employed tree-bisection and reconnection branch swapping (TBR) and 1000 random sequence addition replicates. Nonparametric bootstrapping was conducted to assess clade support, and employed 1000 pseudo-replicates of the data, 10 random addition sequences per replicate and TBR branch swapping. An initial screen of the *Metarhizium 5'-tef1* sequences was undertaken to assess sequence haplotype diversity (not shown) and a subset of representative isolates was selected for further analysis. A 47-taxon *5'-tef1* sequence matrix of 746 bp was created for *Metarhizium* that included 18 representative experimental strains and 29 authenticated

Metarhizium strains identified to species by Bischoff et al. (2009). Analysis of *Beauveria* Bloc sequences included all field-collected strains and the aligned Bloc data matrix was 985 bp. Phylogenetic species within *B. bassiana* have not been formally described, hence an *ad hoc* system is used to distinguish well-supported terminal clades that likely represent discrete phylogenetic species. Accordingly, exclusive terminal clades and branches are referred to using an alphanumeric coding system that includes a four letter abbreviation of the Latin binomial followed by a hyphen and Arabic numerals as clade identifiers in order of their discovery (e.g., *Bbas-1*, *Bbas-2*...). BLAST searches either to published Bloc sequences (Meyling et al., 2009) or unpublished data (Rehner, unpublished data) were used to determine the status of the different *Beauveria* phylogenetic terminals.

2.7. Larval bioassay

Four isolates each of *Metarhizium guizhouense*, *Metarhizium robertsii*, *Metarhizium brunneum* and three isolates of *Metarhizium flavoviride* var. *pemphigi* (only three isolates were isolated in this study) were randomly selected and screened against 8–10 weeks (5th instar) *O. sulcatus* using a modified version of the procedure outlined by Bruck (2004b). *O. sulcatus* larvae were obtained from a laboratory colony maintained at the USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR (Fisher and Bruck, 2004). The isolates used in the bioassay were derived from single-spore subcultures and were grown on PDA at 28 °C in complete darkness until sporulation. One plate of each isolate (15 × 100 mm) was flooded with 10 ml of a sterile 0.1% Tween 80 solution and the spores removed by gentle agitation with a sterile loop. Hemocytometer counts of all spore suspensions were made and their concentrations were adjusted to 5×10^5 spores/ml. The experiment contained four replicates of each treatment each containing five larvae and arranged in a randomized complete block design. Larvae were individually submerged into 1 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 29 ml plastic cups (Sweetheart Cup Co., Owings Mills, MD) with artificial diet (Fisher and Bruck, 2004) and incubated at 21 °C for 14 d, at which time larvae were observed to determine mortality. Mortality was defined as cadavers that showed evidence of sporulation by 2 weeks. The bioassay was performed twice on separate days using fresh spore suspensions for each test. All experiments included an untreated control (0.1% Tween 80). The arc-sine transformations of the percentage of larvae infected with each species of *Metarhizium* (i.e. sporulating cadavers) in the larval bioassays were analyzed using the General Linear Models Procedure (SAS Institute, 1999). An arc-sine transformation of the square root of the percentage larval infection was performed to stabilize the variances and a *t*-test was used to separate means (Snedecor and Cochran, 1989).

3. Results

3.1. Fungal entomopathogen survey

Four species of *Metarhizium* were isolated from the rhizosphere of plants collected in the Willamette Valley, OR: *M. brunneum* (Petch), *M. robertsii* (J.F. Bisch., Rehner & Humber), *M. guizhouense* (Q.T. Chen & H.L. Guo) and *M. flavoviride* var. *pemphigi* (W. Gams & Rozsypa) (Fig. 1). Strawberries and Christmas trees had the greatest number of root samples colonized and the greatest species richness of fungal species colonizing their rhizosphere (Fig. 2). A total of 39 of the 60 Christmas trees samples and 30 of the 70 strawberry samples were colonized. Christmas trees were colonized by all four species of fungi: *M. brunneum* (3), *M. robertsii*

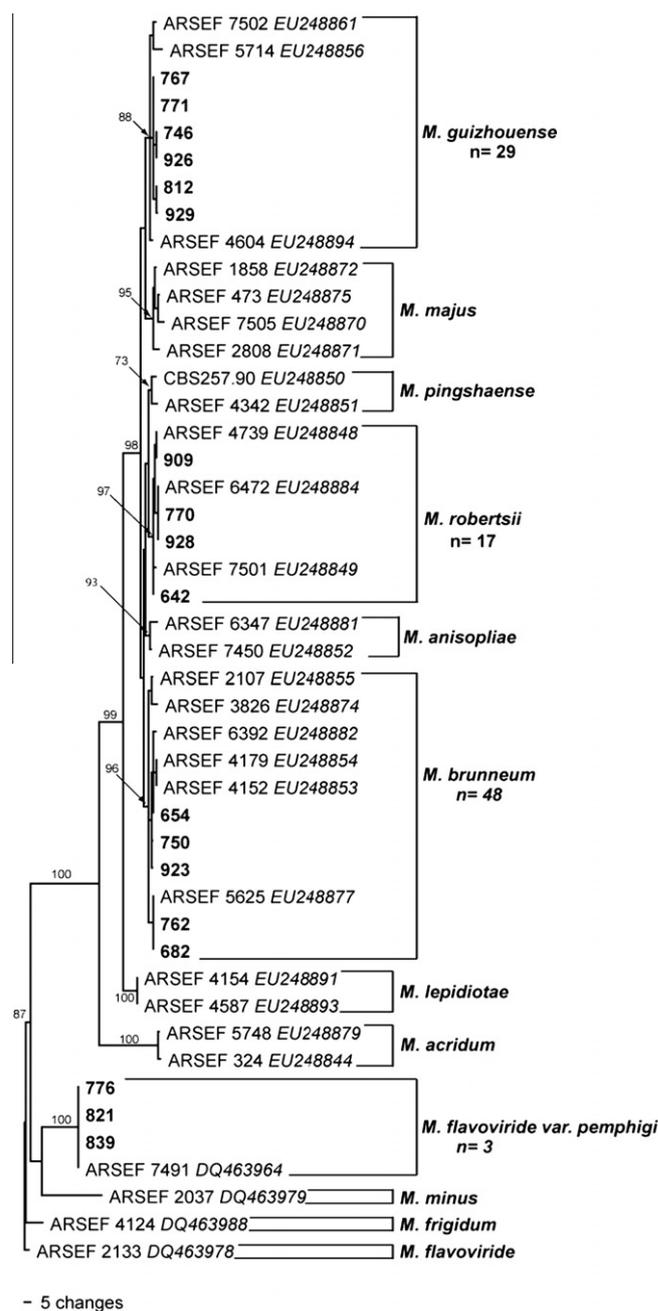


Fig. 1. Maximum parsimony phylogeny of *ref 1* sequences of *Metarhizium* isolates. Bootstrap analysis was based on 1000 pseudo-replicates; bootstrap values $\geq 70\%$ are listed above relevant internodes. Scale bar represents five nucleotide changes. Representative sequences are available from GenBank under accession HQ412787–HQ412795.

(11), *M. guizhouense* (24) and *M. flavoviride* var. *pemphigi* (1). Strawberries were also colonized by all four species: *M. brunneum* (26), *M. robertsii* (1), *M. guizhouense* (1) and *M. flavoviride* var. *pemphigi* (2) (Fig. 2). Blueberries ($n = 109$) and grapes ($n = 100$) had the lowest diversity and number of samples colonized of the four plant types. Only 12 of the blueberry samples and 13 of the grape samples were colonized. Blueberries were colonized by *M. brunneum* (10) and *M. guizhouense* (2). Grapes were colonized by *M. brunneum* (7), *M. robertsii* (4) and *M. guizhouense* (2) (Fig. 2).

Nine distinct *Beauveria* taxa were isolated including *Beauveria brongniartii* ($n = 6$) and *Beauveria* “Clade C” ($n = 1$) and seven phylogenetic species of *B. bassiana*: *Bbas-16* ($n = 31$), *Bbas-8*

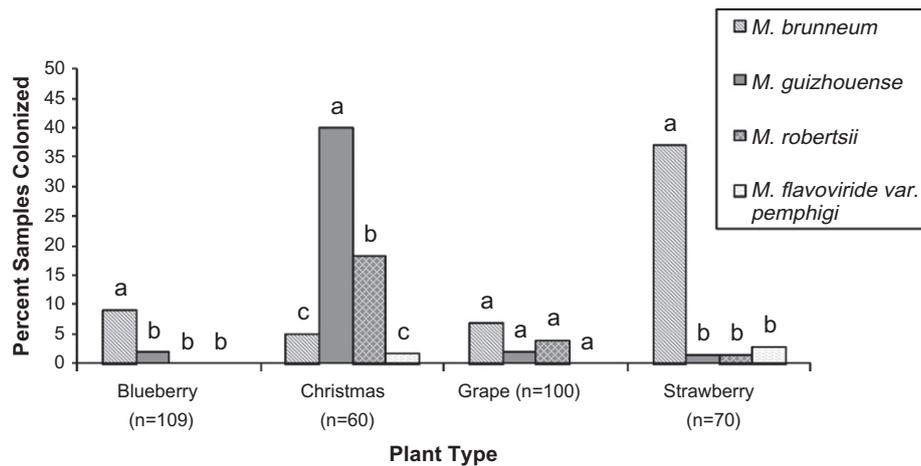


Fig. 2. The percent of blueberry, Christmas trees, grape and strawberries samples collected from fields throughout the Willamette Valley, OR colonized by *M. brunneum*, *M. robertsii*, *M. guizhouense* and *M. flavoviride* var. *pemphigi*. Bars with different letters are significantly different ($P < 0.05$; SAS Institute, 1999) for each plant type.

($n = 3$), *Bbas-4* ($n = 2$), *Bbas-2* ($n = 4$), *Bbas-18* ($n = 4$), *Bbas-indet 1* ($n = 1$), *Bbas-indet 2* ($n = 2$), (Fig. 3). Three *B. bassiana* phylogenetic species are unique to this study including *Bbas-16*, *Bbas-indet 1*, *Bbas-indet 2* (Fig. 3). Less than four rhizosphere samples were colonized by any *Beauveria* species except for *Bbas-16*, which colonized 29 of the 100 grape rhizosphere samples (Fig. 4).

3.2. Larval bioassays

All four species of *Metarhizium* bioassayed were pathogenic to *O. sulcatus* larvae and sporulated within 2 weeks of inoculation while none of the control larvae sporulated (Table 2). Excess mortality (dead larvae that did not sporulate) in the larval bioassay was never greater than 5.8%. Larval mortality was significantly greater in the larvae treated with *M. brunneum* and *M. robertsii* as compared to the control. However, *M. flavoviride* var. *pemphigi* and *M. guizhouense* did not cause significantly more larval sporulation when compared to the control (Table 2).

4. Discussion

This is the first study to specifically survey entomopathogenic fungi in the rhizosphere of crop plants under agricultural field conditions. Previous studies have sampled naturally-occurring entomopathogenic fungi in the soil (Bing and Lewis, 1993; Bidochka et al., 1998; Bruck, 2004b; Chandler et al., 1997; Harrison and Gardner, 1991; Klingen et al., 2002; Shapiro-Ilan et al., 2003; Meyling et al., 2009) but none have specifically targeted the rhizosphere. Our results demonstrate that multiple species of *Metarhizium* and *Beauveria* are frequently present in the rhizosphere and further suggest that certain species of *Metarhizium* and *Beauveria* may be significantly associated with the rhizospheres of particular plants as was observed in the case of strawberry, blueberry, grape and Christmas trees. *Metarhizium* and *Beauveria* are both common in the rhizosphere and the level of observed diversity of *Beauveria* spp. parallels that observed in soils as a whole (Meyling et al., 2009). Although we found that certain species of entomopathogenic fungi are associated with the rhizosphere, our study does not allow us to conclude whether these associations are a result of the plant type alone or are also influenced by other biotic and abiotic factors such as location in the field, availability of insect hosts, soil type, soil pH, soil moisture content and soil temperature (Jaronski, 2010). However, it seems plausible that matching of the prominent fungal species for use on a crop could enhance control of root-feeding insects.

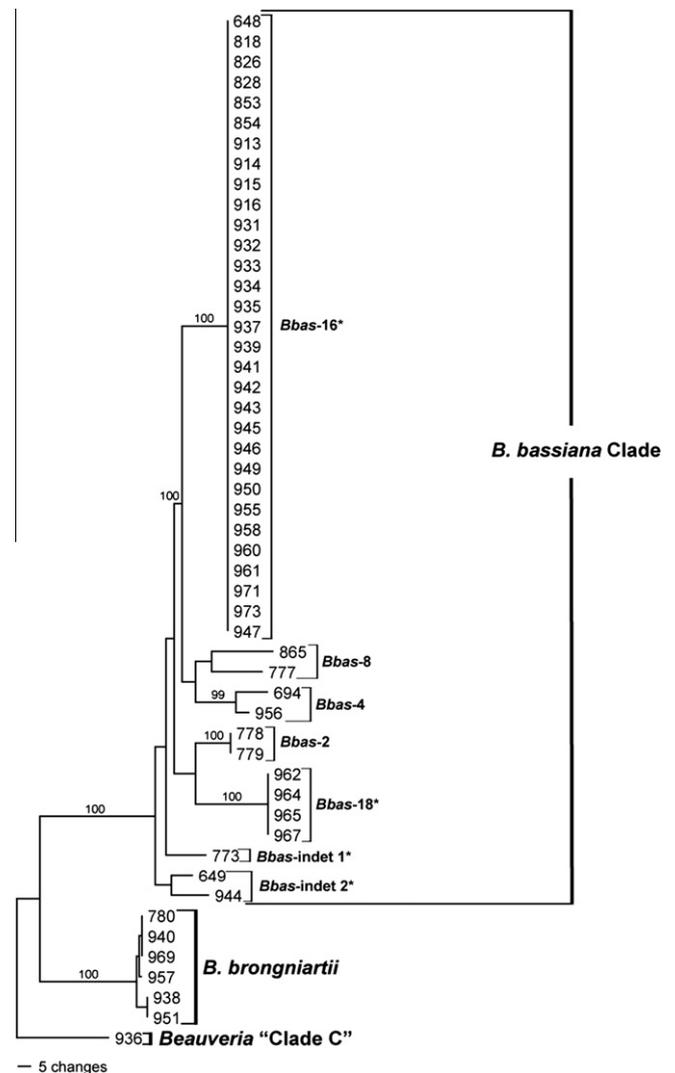


Fig. 3. Maximum parsimony phylogeny of Bloc intergenic sequences of *Beauveria* isolates. Bootstrap analysis was based on 1000 pseudo-replicates; bootstrap values $\geq 70\%$ are listed above relevant internodes. Scale bar represents five nucleotide changes. Asterisks denote novel lineages detected in this study. Representative sequences are available from GenBank under accession HQ412773–HQ412786.

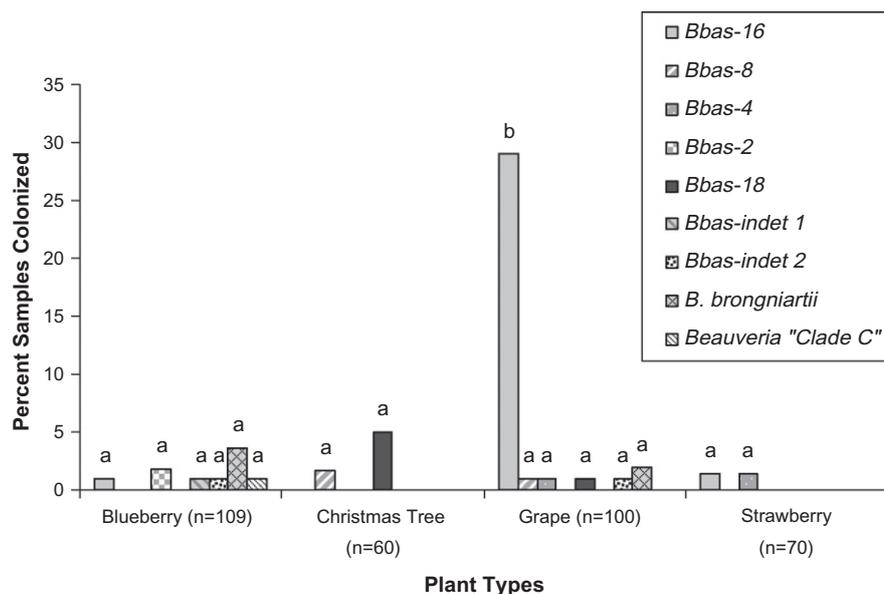


Fig. 4. The percent of blueberry, Christmas trees, grape and strawberries samples collected from fields throughout the Willamette Valley, OR colonized by nine *Beauveria* taxa. Bars with different letters are significantly different ($P < 0.05$; SAS Institute, 1999) for each plant type.

Table 2

Percentage of *O. sulcatus* infected with *Metarhizium* spp. in laboratory bioassays.

Treatment ^a	Percent mortality ^b
<i>M. brunneum</i>	19.4 ± 21.8bc
<i>M. guizhouense</i>	13.1 ± 19.4ac
<i>M. robertsii</i>	26.3 ± 23.5b
<i>M. flavoviride</i> var. <i>pemphigi</i>	6.7 ± 12.7a
Control	0 ± 0a

^a Bioassays performed at 5×10^5 spores/ml.

^b Means followed by the different letters are significantly different ($P < 0.05$; SAS Institute, 1999).

Rhizosphere competence, the ability of an organism to show enhanced growth in response to developing roots (Schmidt, 1979), has been documented several times for *Metarhizium*. The first case was documented by Hu and St. Leger (2002), who discovered that *M. anisopliae* persists in the cabbage rhizosphere. Further studies found that *M. anisopliae* persisted in the rhizosphere of a variety of coniferous ornamentals (Bruck, 2005). In the current study, strawberries and blueberries were significantly associated with *M. brunneum* and Christmas trees had a significant association with *M. guizhouense* and *M. robertsii*. All of the *Metarhizium* species isolated in this study were pathogenic to *O. sulcatus*; however, only *M. brunneum* and *M. robertsii* caused significantly greater mortality when compared to the control. Isolation of fungal entomopathogens from the rhizosphere followed by bioassays against a target pest are potentially useful criteria for selecting species and strains of entomopathogenic fungi as insect biocontrol agents, particularly when the goal is to protect the roots of plants from which these fungi are associated. Bruck (2010) found that the *Metarhizium* isolates F52 (*M. brunneum*) and IP99 actively colonized the rhizosphere of *P. abies*, and their populations increased by nearly 10-fold, while the population of IP285 remained constant over a 14-week period. Plant type also influenced the ability of these isolates to colonize the rhizosphere. All of the isolates tested in the study colonized the rhizosphere of *Picea glauca* (Moench) Voss (Pinales: Pinaceae) and *P. abies*; however, recovery rates of all three strains were lower in the *Taxus baccata* L. (Taxales: Taxaceae) rhizosphere treatment (Bruck, 2010). Our present findings indicate that *Metarhizium* species exhibit differing abilities to colonize the rhizosphere of strawberry, blueberry and Christmas trees.

The rhizosphere associations of *Beauveria* spp. have not been previously explored in detail. Bidochka et al. (1998) report that *B. bassiana* is more abundant in forest soils, while *Metarhizium* spp. predominate in agricultural habitats (Bidochka et al., 1998). In a separate study, *Beauveria* was isolated from the wheat rhizosphere in Australia (Sivasithamparam et al., 1987) and higher levels of inoculum were recovered from the first 6 cm of the soil profile containing clover roots than from the lower soil profile, suggesting *Beauveria* colonizes the clover rhizosphere (Brownbridge et al., 2006). Results of the present study demonstrate that *Beauveria* species occur frequently in the rhizosphere of diverse plant species. In particular, we found that *Bbas-16* was significantly associated with grape, indicating that this *Beauveria* clade is a common rhizosphere colonizer of grape. However, none of the other *Beauveria* taxa isolated in this study were significantly associated with any of the other plants sampled occurring at relatively low frequency. Interestingly, grapes were the only plant surveyed that were not significantly associated with at least one species of *Metarhizium*. Although it is not known whether entomopathogenic fungi interact competitively in the soil environment, this result suggests that *Bbas-16* may exclude *Metarhizium* in the grape rhizosphere but not on the other plants sampled in this study. Because grape vineyards are relatively undisturbed habitats as compared to blueberry, strawberry and Christmas tree fields, vineyards may have soil and rhizosphere microhabitats similar to those found in forest habitats that also experience little soil disturbance and favor the prevalence of *B. bassiana* (Bidochka et al., 1998). Further studies are planned to determine whether the *Beauveria* and grape association is due to the negative interaction of fungal entomopathogens or to a positive interaction between *Beauveria* and grapes.

The advantages associated with using fungal entomopathogens that are rhizosphere competent on the target crop plant could have significant economic impacts. Rhizosphere competent fungal entomopathogens incorporated into soil used during plant propagation would lead to a 10-fold reduction in the amount of fungal inoculum required to provide plant protection for container-grown nursery stock (Bruck, 2010). This reduction would drastically reduce the cost of using fungal entomopathogens to control root-feeding pests. The use of fungal entomopathogens that have a significant association with the target crop and are virulent against the target pest may lead to enhanced control due to

increased persistence and growth. However, it is probably not economically feasible to develop commercial isolates of unique entomopathogenic fungi isolates for use on specific plant types. Therefore, in order to increase their efficacy, persistence and performance, selection of new biocontrol strains to control soil-dwelling insect pests should target fungal species demonstrated to be naturally associated with a wide range of different target plants. In addition to aiding in the selection of entomopathogenic species for commercial development, this study sheds additional light on the specific microhabitat preferences and ecology of fungal entomopathogens. While our understanding of the ecology and biological significance of fungal entomopathogens in the rhizosphere is only beginning to take form, it is clear that an increased understanding of this relationship is likely to be an important piece in the puzzle of microbial control of soil borne insects. The ramifications of this relationship are potentially significant. Thus, improved knowledge of entomopathogen's ecology in niches within the rhizosphere could lead to improved deployment, formulation and efficacy against soil borne insect pests (Vega et al., 2009). Future research will focus on identifying entomopathogenic fungal isolates with broad ecological and pest host ranges that can be developed for the control of root-feeding insects.

References

- Bidochka, M.J., Kamp, A.M., Lavender, T.M., Dekoning, J., Amritha, De., Croos, J.N.A., 2001. Habitat association in two genetic groups of the insect-pathogenic fungus *Metarhizium anisopliae*: uncovering cryptic species? *Appl. Environ. Microbiol.* 67, 1335–1342.
- Bidochka, M.J., Kasperski, J.E., Wild, G.A.M., 1998. Occurrence of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* in soils from temperate and near-northern habitats. *Can. J. Bot.* 78, 1198–1204.
- Bidochka, M.J., Menzies, F.V., Kamp, A.K., 2002. Genetic groups of the insect-pathogenic fungus *Beauveria bassiana* are associated with habitat and thermal growth preferences. *Arch. Microbiol.* 178, 531–537.
- Bing, L.A., Lewis, L.C., 1993. Occurrence of the entomopathogen *Beauveria bassiana* (Balsamo) Vuillemin in different tillage regimes and in *Zea mays* L. and virulence towards *Ostrinia nubilalis* (Hübner). *Agric. Ecosys. Environ.* 45, 147–156.
- Bischoff, J.F., Rehner, S.A., Humber, R.A., 2006. *Metarhizium frigidum* sp. nov.: a cryptic species of *M. anisopliae* and a member of the *M. flavoviride* complex. *Mycologia* 98, 737–745.
- Bischoff, J.F., Rehner, S.A., Humber, R.A., 2009. A multilocus phylogeny of the *Metarhizium anisopliae* lineage. *Mycologia* 101, 508–528.
- Brownbridge, M., Nelson, T.L., Hackell, D.L., Eden, T.M., Wilson, D.J., Willoughby, B.E., Glare, T.R., 2006. Field application of biopolymer-coated *Beauveria bassiana* F418 for clover root weevil (*Sitona lepidus*) control in Waikato and Manawatu. *NZ Plant Protect.* 59, 304–311.
- Bruck, D.J., 2004a. Natural occurrence of entomopathogens in Pacific Northwest nursery soils and their virulence to the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae). *Environ. Entomol.* 33, 1335–1343.
- Bruck, D.J., 2004b. Stopping weevil deeds. *Am. Nurseryman*, 43–46.
- Bruck, D.J., 2005. Ecology of *Metarhizium anisopliae* in soilless potting media and the rhizosphere: implications for pest management. *Biol. Control* 32, 155–163.
- Bruck, D.J., 2010. Fungal entomopathogens in the rhizosphere. *BioControl* 55, 103–112.
- Bruck, D.J., Donahue, K.M., 2007. Persistence of *Metarhizium anisopliae* incorporated into soilless potting media for control of the black vine weevil, *Otiorhynchus sulcatus* in container-grown ornamentals. *J. Invertebr. Pathol.* 95, 146–150.
- Chandler, D., Hay, D., Reid, A.P., 1997. Sampling and occurrence of entomopathogenic fungi and nematodes in UK soils. *Appl. Soil Ecol.* 5, 133–141.
- Cowles, R.S., 1995. Black vine weevil biology and management. *J. Am. Rhododendron Sci.* 98, 83–85, 94–97.
- Fisher, J.R., Bruck, D.J., 2004. A technique for continuous mass rearing of the black vine weevil, *Otiorhynchus sulcatus*. *Entomol. Exp. Appl.* 113, 71–75.
- Georgis, R., Koppenhofer, A.M., Lacey, L.A., Belair, G., Duncan, L.W., Grewal, P.S., Samish, M., Tan, L., Torr, P., van Tol, R.W.H.M., 2006. Successes and failures in the use of parasitic nematodes for pest control. *Biol. Control* 38, 103–123.
- Harrison, R.D., Gardner, W.A., 1991. Occurrence of entomogenous fungus *Beauveria bassiana* in pecan orchard soils in Georgia. *J. Entomol. Sci.* 26, 360–366.
- Hu, G., St. Leger, R., 2002. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. *Appl. Environ. Microbiol.* 68, 6383–6387.
- Jaronski, S.T., 2010. Ecological factors in the inundative use of fungal entomopathogens. *BioControl* 55, 159–185.
- Kepler, R.M., Bruck, D.J., 2006. Examination of the interaction between the black vine weevil (Coleoptera: Curculionidae) and an entomopathogenic fungus reveals a new tritrophic interaction. *Environ. Entomol.* 35, 1021–1029.
- Klingen, I., Eilenberg, J., Meadow, R., 2002. Effects of farming system, field margins and bait insect on the occurrence of insect pathogenic fungi in soils. *Agric. Ecosys. Environ.* 91, 191–198.
- Meyling, N.V., Eilenberg, J., 2006. Occurrence and distribution of soil borne entomopathogenic fungi within a single organic agro-ecosystem. *Agric. Ecosys. Environ.* 113, 336–341.
- Meyling, N.V., Eilenberg, J., 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: potential for conservation biological control. *Biol. Control* 43, 145–155.
- Meyling, N.V., Lubeck, M., Buckley, E.P., Eilenberg, J., Rehner, S.A., 2009. Community composition, host range, and genetic structure of the fungal entomopathogen *Beauveria* in adjoining agricultural and semi-natural habitats. *Mol. Ecol.* 18, 1282–1293.
- Moorhouse, E.R., Charnely, A.K., Gillespie, A.T., 1992. Review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). *Ann. Appl. Biol.* 121, 431–454.
- Pell, J.K., Hannam, J.J., Steinkraus, D.C., 2010. Conservation biological control using fungal entomopathogens. *BioControl* 55, 187–198.
- Rath, A.C., Koen, T.B., Yip, H.Y., 1992. The influence of abiotic factors on the distribution and abundance of *Metarhizium anisopliae* in Tasmanian pasture soils. *Mycol. Res.* 98, 378–384.
- Rehner, S.A., Buckley, E.P., 2005. A *Beauveria* phylogeny inferred from nuclear ITS and EF1- α sequences: evidence of cryptic diversification and links to *Cordyceps teleomorpha*. *Mycologia* 97, 84–96.
- Rehner, S.A., Posada, F., Buckley, E.P., Infante, F., Castillo, A., Vega, F.E., 2006. Phylogenetic origins of African and Neotropical *Beauveria bassiana* pathogens of the coffee berry borer, *Hypothenemus hampei*. *J. Invertebr. Pathol.* 93, 11–21.
- Roy, H.E., Brodie, E.L., Chandler, D., Goettel, M.S., Pell, J.K., Wajnberg, E., Vega, F.E., 2010. Deep space and hidden depths: understanding the evolution and ecology of fungal entomopathogens. *BioControl* 44, 1–6.
- SAS Institute, 1999. The SAS Statistical System, Version 8. SAS Institute, Cary, NC.
- Schmidt, E.L., 1979. Initiation of plant root-microbe interactions. *Ann. Rev. Microbiol.* 33, 355–376.
- Shapiro-Ilan, D.I., Gardner, W.A., Fuxa, J.R., Wood, B.W., Nguyen, K.B., Adams, B.J., Humber, R.A., Hall, M.J., 2003. Survey of entomopathogenic nematodes and fungi endemic to pecan orchards of the Southeastern United States and their virulence to the pecan weevil (Coleoptera: Curculionidae). *Environ. Entomol.* 32, 187–195.
- Sivasithamparan, K., MacNish, G.C., Fang, C.S., Parker, C.A., 1987. Microflora of soil and wheat rhizosphere in a field following fumigation. *Aust. J. Soil Res.* 25, 491–498.
- Snedecor, G.W., Cochran, W.G., 1989. *Statistical Methods*, eighth ed. Iowa State University Press, Ames.
- Son, Y.S., Lewis, E.E., 2005. Effects of temperature on the reproductive life history of the black vine weevil, *Otiorhynchus sulcatus*. *Entomol. Exp. Appl.* 114, 15–24.
- Sosa-Gomez, D.R., Moscardi, F., 1994. Effect of till and no-till soybean cultivation on dynamics of entomopathogenic fungi in the soil. *Fla. Entomol.* 77, 284–287.
- Vanninen, I., 1996. Distribution and occurrence of four entomopathogenic fungi in Finland: effect of geographical location, habitat type and soil type. *Mycol. Res.* 100, 93–101.
- Vega, F.E., Goettel, M.S., Blackwell, M., Chandler, D., Jackson, M.A., Keller, S., Koike, M., Maniania, N.K., Monzon, A., Ownley, B.H., Pell, J.K., Rangel, E.E.N., Roy, H.E., 2009. Fungal entomopathogens: new insights on their ecology. *Fungal Ecol.* 2, 149–159.
- Zimmermann, G., 1986. The 'Galleria bait method' for detection of entomopathogenic fungi in soil. *J. Appl. Entomol.* 102, 213–215.