AN ABSTRACT OF THE THESIS OF

Joanna Fisher for the degree of Honors Baccalaureate of Science in BioResource Research presented on May 26, 2010. Title: Survey of rhizosphere associated entomopathogenic fungi on small fruits and Christmas trees in the Willamette Valley, OR

Abstract approved:

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Denny Bruck

The entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* are commercially available as microbial control agents for the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae), the key root-feeding insect pest in Pacific Northwest small fruits and ornamentals. Understanding habitat selection 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. in the rhizosphere of strawberry, blueberry, grape and Christmas tree crops in the Willamette Valley of Oregon. Entomopathogenic fungi were assigned to thirteen phylogenetic species based on molecular phylogenetic criteria. Four species of *Metarhizium* were isolated including *M. brunneum, M. guizhouense, M. robertsii,* and *M. pemphigi*. Nine *Beauveria* species were isolated including, *Beauveria brongniartii*, an undescribed species referred to as Clade C and seven morphologically cryptic phylogenetic species of *B. 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 *O. 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 pest control for *O. sulcatus*.

Key Words: microbial ecology, soil ecology, microbial control, *Otiorhynchus sulcatus*

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Survey of rhizosphere associated entomopathogenic fungi on small fruits and Christmas trees in the Willamette Valley, OR

by

Joanna J. Fisher

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Joanna J. Fisher, Author

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Dedication

This thesis is dedicated to my parents; Thank you for encouraging me to pursue my dreams even when they seemed far-fetched and for giving me the tools I needed to be successful.

Thanks!

Survey of rhizosphere associated entomopathogenic fungi on small fruits and Christmas trees in the Willamette Valley, OR

1. Introduction

The black vine weevil, *Otiorhynchus 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. Because of its parthenogenic lifecycle, a single weevil has the potential to start a new infestation. There is zero tolerance for *O. sulcatus* in shipped nursery stock because its parthenogenic nature and polyphagous diet make it a destructive pest on a broad variety of plants (Moorhouse et al., 1992). *O. sulcatus* has a host range of over 150 plant species (Moorhouse et al., 1992), primarily in the genera Ericaceae, Pinaceae, Primulaceae, Rosaceae, Saxifragaceae, Taxaceae and Vitaceae (Cowles, 1995). *O. sulcatus* originated in Northern Europe. It was first found in North America in 1835, and by 1871 was a notable pest in Missouri (Smith, 1932). *O. sulcatus* 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 lifecycle 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 used 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 potting media prior to planting. 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). Many of the failures in the field can be attributed to poor matching of the nematode with its correct insect host species and habitat (Gaugler, 1999). However, soil temperature may be the most critical factor influencing nematode's ability to provide effective control of *O. sulcatus* larvae. Cold-tolerant species such as *Heterorhabditis megidis* (Poinar) (Rhabditida; Heterorhabditidae) and *Steinernema feltiae* (Filipjev) (Rhabditida; Steinernematidae) remain active at temperatures as low as 12°C but summer applications have low persistence and efficacy because nematodes are highly susceptible to desiccation (Bruck, 2004b; Georgis et al., 2006; van Tol and Raupp, 2006).

Metarhizium anisopliae (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) has been commercially developed as a granular formulation (F52, Novozymes Biologicals Inc., Salem VA, USA) which is incorporated into potting media at planting for *O. sulcatus* control (Bruck and Donahue, 2007). *M. anisopliae* (F52) persists in soilless potting media for at least two years and provides 75-80% control during the fall and 50-60% control during winter months (Bruck and Donahue, 2007). In addition, *M.*

anisopliae (F52) is compatible with other management tools used in the nursery industry. In container-grown ornamentals, only three commonly used soil fungicides (etridiazole, propamocard and mafanoxam) have a significant detrimental impact on *M. anisopliae* germination or growth (Bruck, 2008). Experimentally, *M. anisopliae* (F52) has also been used as spore drench in curative applications to control existing *O. sulcatus* larval populations in container-grown nursery stock (Bruck, 2007). Despite their ability to kill insects, entomopathogenic fungi applied innundatively 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).

Since the discovery of *M. anisopliae* in the 1880s by the Russian microbiologist Metchnikoff, entomopathogenic fungi have been used to control a variety of agricultural, nursery and forest insect pests. *M. anisopliae* was first used commercially in 1888 when Krassilstschik mass produced *M. anisopliae* for controlling the grain beetle *Anisoplia austriac* (Coleoptera: Scarabaeidae) in Russian sugar beet fields (Lord, 2005). Since *M. anisopliae's* discovery, several other entomopathogenic fungi, most notably *Beauveria bassiana* (Balsamo) Vuillemin (Hypocreales: Clavicipitaceae), have been isolated from soil from around the world (Bidochka et al., 1998; Bing and Lewis 1993; Chandler et al., 1997; Harrison and Gardner 1991; Klingen et al., 2002; Shapiro-Ilan et al., 2003), including the United States Pacific Northwest (Bruck, 2004b). Soil surveys provide insight into the naturally-occurring entomopathogen biodiversity and provide a pool of novel fungal isolates for development into potentially new microbial control agents.

Historically, entomopathogens have been selected for release in the field based solely on their efficacy in laboratory bioassay tests, while largely ignoring their

microhabitat preferences and ecological constraints. It had been assumed that fungal population genetics are closely related to host insects (Bidochka et al., 2001). However, more recent research shows that *M. anisopliae* population structure may be driven by habitat selection, not insect host selection (Bidochka et al., 2002). Similarly, *B. bassiana* has evolved 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). Habitat (i.e. forested vs. agricultural areas) plays a large role in determining species distributions and thus community structure of *Metarhizium* and *Beauveria* (Bidochka et al., 2001, 2002; Meyling and Eilenberg 2006, 2007; Meyling et al., 2009). 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 competence wasn't 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 two weeks of exposure to inoculated roots (Bruck, 2005).

The behavioral response of an insect host to its fungal entomopathogen can also have a direct effect on the efficacy of the fungi as a microbial control and can contribute largely to the success or failure of the approach (Roy et al., 2010). *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 termites *Reticulitermes flavipes* (Kollar) and *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), mole crickets (Orthoptera: Gryllotalpidae) and wireworms *Agriotes obscures* L. (Coleoptera: Elateridae) all exhibited modified behavior when exposed to *M. anisopliae* (Engler and Gold, 2004; Kabaluk and Ericsson, 2007). Mole crickets also exhibited modified behavior when exposed to *B. bassiana* (Thompson and Brandenberg, 2005). Tritrophic interactions are not unique to soil-borne fungi. The fungal entomopathogen *Neozygites tanajoae* Delalibera J., Humber and Hajek (Zygomyces:

Entomophthorales) is induced to sporulate when Cassava is exposed to feeding by the cassava green mite *Mononychellus tanajoa* (Bondar) (Acari: Tetranychidae). In the absence of the mite, plant volatiles suppress *N. tanajoae* germination (Hountondjiet al., 2005).

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., 2009); 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 (Bishchoff 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 biological 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. 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 access the virulence 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 and blueberry fields using a standard golf hole corer (10.2 × 17.8cm, Pro II Hole Cutter, Markers Inc., Avon Lake, OH). Fields containing established plantings (>3 and 10 yrs for strawberries and blueberries, 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. 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. Root samples were randomly collected from plants distributed throughout the field or if the field was over five hectares, samples were taken from plants located in the first half of the field. Roots were placed in 3.78 L plastic reclosable bags in the field, and 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, 1 and 10 strawberry, blueberry, Christmas tree and grape fields were sampled throughout the Willamette Valley of Oregon resulting in the collection of 70, 109, 60 and 100 individual root samples from each crop, respectively.

2.2 Fungal Isolation

Entomopathogenic fungi were isolated from the roots of all plants using a modified version of the '*Galleria* bait method' (Zimmermann, 1986). Roots were shaken to remove non-rhizosphere soil and then placed into a large deep dish Petri plate (150 × 25mm, Thermo Scientific, Waltham, MA) that contained moistened filter paper (15cm, Grade p5, Fisher, Pittsburgh, PA). Live wax worms, *Galleria mellonella* (L.) (Lepidoptera: Pyralidae) were placed in the Petri plates and allowed to crawl and contact the roots. The Petri plates were sealed with Parafilm and placed in plastic reclosable bags in the incubator in complete darkness at 21°C. After a period of 1-2 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 occurred (any cadavers that didn't sporulate were discarded). After sporulation, fungi were isolated using the procedure outlined by Bruck (2004b). Conidia from the sporulated cadavers were streaked with a sterile loop onto media selective for *Beauveria* and *Metarhizium* spp. (Veen and Ferron, 1966) and incubated at 28°C for 10-14 days or until sporulation. Single spore colonies of the respective fungi were removed from the media and cultured on potato dextrose agar (PDA). After a clean culture was obtained, each isolate was cryo-preserved, placed into long-term storage and cataloged as described 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).

2.3 Isolate Lyophilization

One random isolate from an infected *G. mellonella* larva from each root sample was lyophilized in preparation for genetic analysis. If a sample contained larvae infected

with both *Metarhizium* and *Beauveria*, a random isolate of each was selected. Isolates were grown on PDA plates in complete darkness at 28°C until just prior to sporulation. Using sterile technique, the plates were flooded with 3 ml of sterile 80Ω water and scraped with a rubber policeman to suspend the mycelium. Five milliliters of spore solution were pipetted into a 125 ml Erlenmeyer flask filled with 35 ml of sterile Sabouraud dextrose + 0.5% yeast extract broth (SDY). The flasks were then covered and placed in a shaker incubator for 3-5 days at 100-125 rpm and 23-25°C. The broth and mycelium suspensions were then decanted into 50 ml sterile conical bottomed centrifugation tube. The tubes were filled to the 40 ml mark with 80Ω water, shaken and centrifuged at 3,500 rpm for 5 min. The supernatant was decanted and 25 ml of 80Ω water was added to the tubes. This procedure was repeated a total of three times until the broth was rinsed from the mycelium. Excess moisture was removed from the mycelium by pressing it between two pieces of sterile filter paper. A small amount of mycelium was scraped from the filter paper and placed in a 1.5 ml microcentrifuge tube. The tubes were covered with Parafilm which had numerous holes poked thru it with a sterile toothpick and the tubes were placed in the freezer at -80°C. After the tubes were frozen, they were placed in a lyophilizer (Free Zone6, Labconco, Kansas City, MO) and lyophilized at -50°C and 0.02 m Bar overnight.

2.4 DNA extraction

Approximately 25-50 mg of lyophilized mycelium was pulverized with glass-zirconia beads (Biospec, Bartlesville, OK) in a Fastprep (Q-Biogene, Solon, OH) sample grinder for 6 seconds at a speed setting of 4.5. The ground tissues were suspended in PrepMan (ABI) extraction reagent and boiled for 10 min. The boiled extracts were

cleared by centrifugation at 16,000 x g for 10 min and the supernatant transferred to a clean tube and stored at -20°C.

2.5 PCR

Metarhizium and *Beauveria* strains were sequence-characterized with nuclear loci including the 5' region of elongation factor-1 alpha (5'-*tef*1) and Bloc, which are informative for diagnosis of phyletic species in these genera (Bischoff et al., 2006, 2009; Rehner et al., 2006), respectively. The 5'-*tef*1 region in *Metarhizium* contains three sequence polymorphic introns and was amplified with primers EF1T (5'-ATGGGTAAGGA(A/G)GACAAGAC) and EF2T (5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT) (Rehner and Buckley, 2005). 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 included 1 µl genomic DNA extracts, 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 1X 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 sec, 56°C annealing for 30 seconds, 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.

2.6 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'-*tef*1, whereas an approximately 950 bp segment of Bloc was sequenced with internal primers B22U (5'-GTCGCAGCCAGAGCAACT) and B822L (5'-AGATTCGCAACGTCAACTT). 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.

2.7 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'-*tef*1 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'-*tef*1 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'-*tef*1 sequence matrix of 746 bp was created for *Metarhizium* that included 18 representative experimental strains and 29 authenticated *Metarhizium* strains identified to species or variety by Bischoff and colleagues (2009). Analysis of *Beauveria* Bloc

sequences included all field-collected strains and the aligned Bloc data matrix was 985 bp. Because phylogenetic species within *B. bassiana* have not been formally described an *ad hoc* system is used to distinguish well-resolved and supported terminal clades. Therefore, terminal taxa resolved within *B. bassiana* 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.

2.8 Larval Bioassay

Four isolates of *M. guizhouense, M. robertsii, M. brunneum* and three isolates of *M. pemphigi* (only three isolates were found throughout the study) were randomly selected and screened against 8-10 week (5^{th} 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 grown from single spore cultures 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 suspensions were made and the spore concentration adjusted to 5×10^5 spores/ml. Each fungal isolate was assayed against 5^{th} instar *O. sulcatus*. 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 for fungal sporulation. 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 found colonizing the rhizosphere of plants collected in the Willamette Valley, OR: *M. brunneum*, *M. robertsii*, *M. guizhouense* and *M. pemphigi* (Figure 1). Strawberries and Christmas trees had the greatest number of samples colonized and the greatest diversity of fungal species colonizing their roots (Figure 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* (11), *M. guizhouense* (24) and *M. pemphigi* (1). Strawberries were also colonized by all four species: *M. brunneum* (26), *M. robertsii* (1), *M. guizhouense* (1) and *M. pemphigi* (2) (Figure 2). Blueberries and grapes 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) (Figure 2).

Nine distinct *Beauveria* taxa were isolated during this study including seven cryptic phylogenetic of *B. bassiana*, *Bbas-16, Bbas-8, Bbas-4, Bbas-2, Bbas-18, Bbas-indet 1, Bbas-indet 2, B. brongniartii* and *Beauveria "Clade C"* (Figure 3). Two phylogenetic species are unique to this study, *Bbas-indet 1* and *Bbas-indet 2,* (Figure 3). Less than four root samples were colonized by any *Beauveria* species except for *Bbas-16*, which colonized 29 of the 100 grape root samples (Figure 4).

3.2 Larval Bioassay Test

All four species of *Metarhizium* bioassayed were pathogenic to *O. sulcatus* larvae and sporulated within two weeks of inoculation while none of the control larvae sporulated (Figure 5). Significantly more larvae sporulated when treated with *M. brunneum* and *M. robertsii* as compared to the control. However, *M. pemphigi* and *M. guizhouense* did not cause significantly more larval sporulation when compared to the control (Table 1)

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) but none have specifically targeted the rhizosphere. Our results suggest that certain species of *Metarhizium* and *Beauveria* are

more likely associated with the rhizospheres of strawberry, blueberry, grape and Christmas trees (Figure 2, 4). Also both *Metarhizium* and *Beauveria* are common in the rhizosphere and the level of observed diversity of *Beauveria* spp. parallels that observed in soils as a whole (Figure 2, 4; Meyling et al., 2009). Although we found that certain species of entomopathogenic fungi are associated with plant roots, 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 influences 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 each crop could enhance control of root-feeding insects.

Rhizosphere competence 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 had a significant association with *M. brunneum* and Christmas trees had a significant association with *M. guizhouense* and *M. robertsii* (Figure 2). All of the *Metarhizium* species isolated in this study were pathogenic to *O. sulcatus;* however, only *M. brunneum* and *M. robertsii* caused significantly greater sporulation when compared to the control (Table 1). Our results suggest that isolating fungal entomopathogens from the rhizosphere followed by bioassays against a target pest are potentially useful criteria for selecting fungal species for developing microbial controls for *O. sulcatus,* particularly when the goal is to protect the roots of plants from which these fungi are readily isolated. Bruck (2010) found that

the *Metarhizium* isolates F52 and IP99 were rhizosphere competent on the roots of *P. abies,* and their populations increased by nearly 10-fold, while IP285's population 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, the populations of all isolates decreased in the *Taxus baccata* L. (Taxales:Taxaceae) rhizosphere (Bruck, 2010). Our findings indicate that *Metarhizium* species exhibit differing abilities to colonize the rhizospheres of strawberry, blueberry and Christmas trees (Figure 2); therefore, the microhabitat preference of *Metarhizium* species should be considered prior to their development into microbial controls for use on specific plant types.

The rhizosphere associations of *Beauveria* have not been previously explored in any detail. However, our results indicate that *Beauveria* actively colonizes the rhizosphere of a diverse array of plant types. *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 the *Beauveria* colonizes the clover rhizosphere (Brownbridge et al., 2006). We found that *Bbas-16* was significantly associated with grapes (Figure 4), suggesting that this *Beauveria* clade is a common rhizosphere colonizer of grape. However, none of the other clades isolated in this study had a significant relationship with any of the other plants sampled (Figure 4). Interestingly, grapes were the only plant surveyed that were not significantly associated with at least one species of *Metarhizium* (Figure 2). It may be that *Beauveria* is able to outcompete

Metarhizium in the grape rhizosphere but not on the other plants sampled in this study. *B. bassiana* has been found to be more abundant in forest habitats, while *Metarhizium* is more abundant in agricultural habitats (Bidochka et al., 1998). Because grape vineyards are relatively undisturbed habitats compared to blueberry, strawberry and Christmas tree fields, vineyards may have soil and root microhabitats similar to those found in forest habitats that also experience little soil disturbance.

The effects of the field's microclimate and geographical location, soil type, soil moisture content, soil pH and other biotic and abiotic environmental factors were beyond the scope of the current study. However, soil texture, temperature and moisture have been found to affect the efficacy of soil fungal entomopathogens, while soil pH, cat ion exchange capacity and inorganic salts do not appear to have an impact on fungal entomopathogens (Jaronski, 2010). The efficacy of *Metarhizium* varied with different soil textures (sand, clay and organic soils) and soil moisture contents (Jaronski et al., 2005; Kabaluk et al., 2007). *Metarhizium* became inactive at water activity levels below 0.99 (-1.5 MPa) or at temperatures below 8-10°C (Kabaluk et al., 2007). Biotic factors in addition to plant-microbe interactions may also play a role in determining fungal entomopathogen diversity and prevalence. Microbes in unsterilized natural soils exhibit fungistasis for entomopathogenic fungi that may increase the fungal titers needed for successful fungal infection by several orders of magnitude (Pereira et al., 1993). Future studies are needed to determine the effects of soil moisture, type and temperature in addition to biotic influences on entomopathogenic fungi rhizosphere colonization.

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 (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, a fungal species that has been shown to be associated with a wide range of different target plant types could be selected for commercial development. 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 biology and significance of fungal entomopathogens in the rhizosphere is just beginning to take shape, 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 potential ramifications of this relationship are tremendous. An understanding of rhizosphere competence could lead to improved deployment of entomopathogens, improved formulation and efficacy and a better understanding of their ecological roles and niches (Vega et al., 2009).

Figure 1: Maximum parsimony phylogeny of *tef* 1 gene sequences of *Metarhizium* isolates. Bootstrap analysis was based on 1,000 pseudo-replicates. Scale bar represents five nucleotide changes.

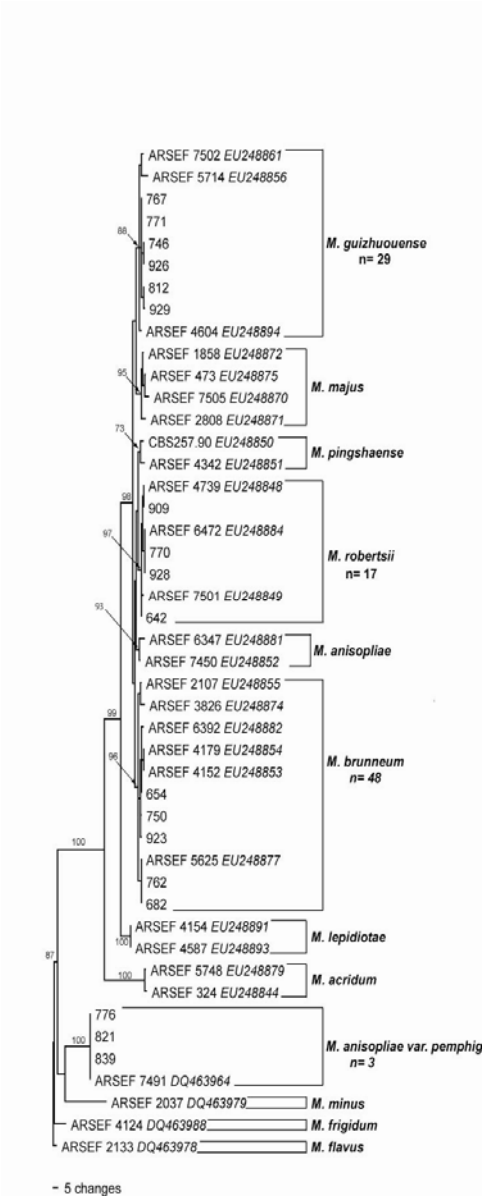

Figure 2: The number of blueberry, Christmas trees, grape and strawberries samples collected from fields throughout the Willamette Valley, OR colonized by *M. brunneum*, *M. robertsii*, *M. guizhouense* and *M. pemphigi*. Bars with different letters are significantly different ($P < 0.05$; SAS Institute,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

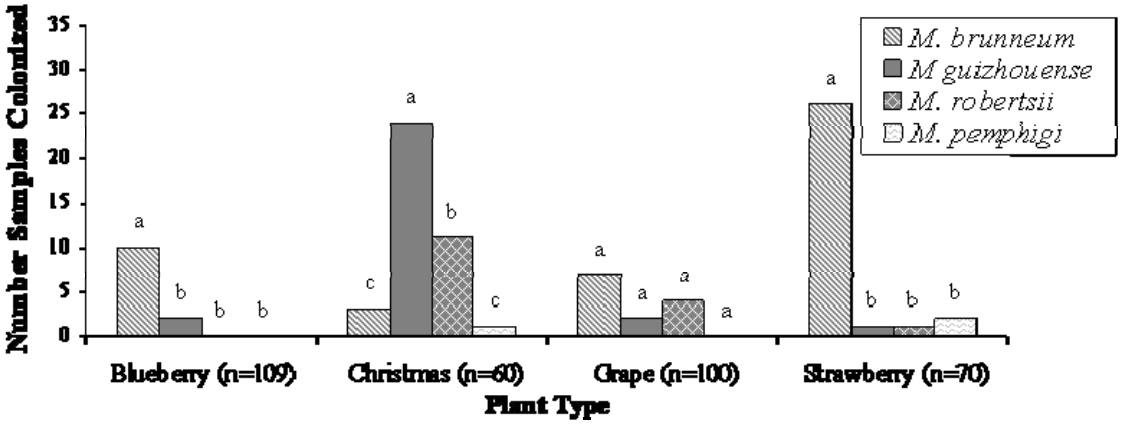

Figure 3: Maximum parsimony phylogeny of Bloc gene sequences of *Beauveria* isolates. Bootstrap analysis was based on 1,000 pseudo-replicates. Scale bar represents five nucleotide changes.

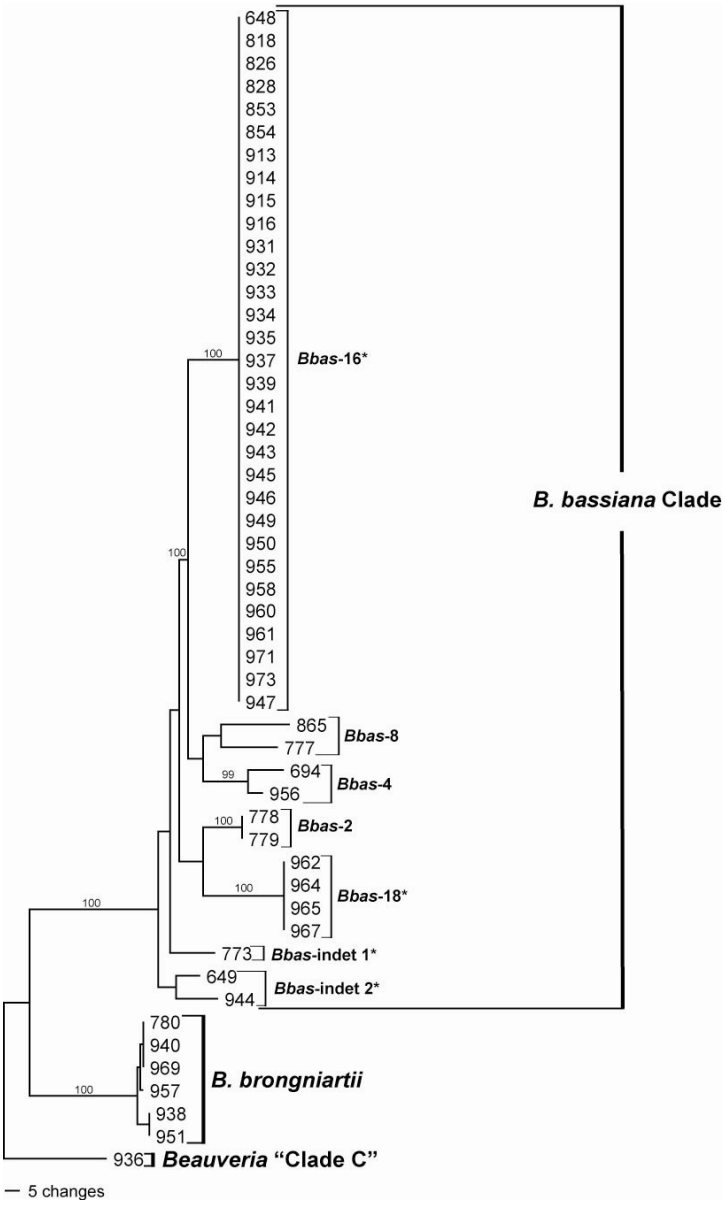

Figure 4: The number of blueberry, Christmas trees, grape and strawberries samples collected from fields throughout the Willamette Valley, OR colonized by nine *Beauveria* clades. Bars with different letters are significantly different ($P < 0.05$; SAS Institute, 1999).

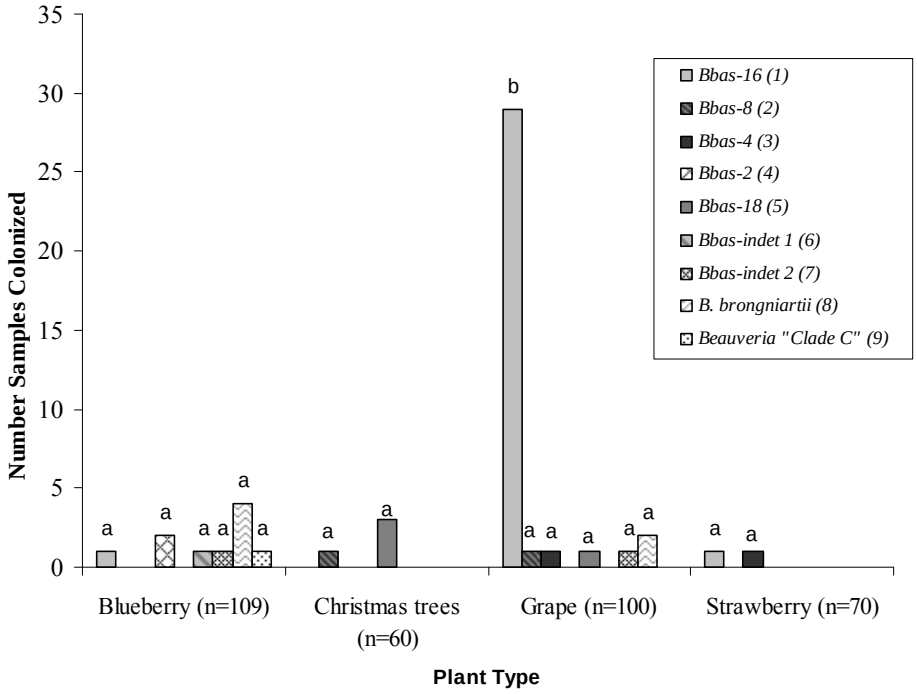

Figure 5: Percent larvae sporulated when treated with *M. robertsii*, *M. guizhoense*, *M. brunneum* and *M. pemphigi*. Bars with different letters are significantly different ($P < 0.05$; SAS Institute, 1999).

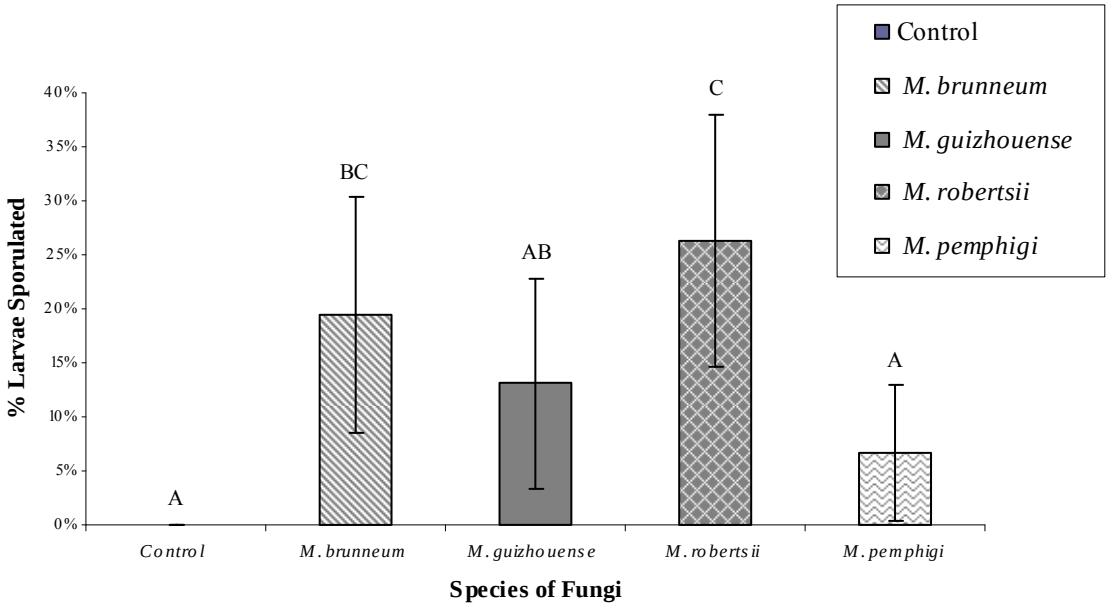

Table 1: Percentage of *O. sulcatus* infected with *Metarhizium* spp. in laboratory bioassays.

Treatment[a]	Percent Sporulation[b]
M. brunneum	19.4±21.8bc
M. guizhoense	13.1±19.4ac
M. robertsii	26.3±23.5b
M. pemphigi	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)

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