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Title: Isolation and Pathogenicity of Naturally Occurring Entomopathogenic Fungi to Clover Root Borer (Coleoptera: Curculionidae: Scolytinae), a Pest of Red Clover Seed Crops

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Entomopathogenic fungi are cosmopolitan microbial pathogens that cause fungous diseases in a wide range of insects via spore infection. Due to their natural enemy status, they have tremendous potential for use as microbial control agents against insect pests, especially subterranean pests for which few management options are available.

The clover root borer (*Hylastinus obscurus* Marsham) develops belowground, attacks the roots of red clover (*Trifolium pratense* L.) and causes a drastic reduction in seed yield. *Hylastinus obscurus* was controlled with organochlorine pesticides but their use was prohibited due to hazardous effects on the environment, and non-target organisms including humans. Subsequently, no alternative pest management strategy was developed for this pest. Meanwhile, little is known about impacts of entomopathogenic fungi on clover root borer.

The current study was conducted to isolate naturally occuring entomopathogenic fungi and assess their pathogenicity against the clover root borer. More than 150 isolates were collected from infected clover root borers and root zone soil samples from five red clover seed production fields in Western Oregon. Based on morphologic characters and phylogenetic analyses using ITS sequence data, these belonged to six species - *Beauveria bassiana*, *B. pseudobassiana*, *Metarhizium brunneum*, *Isaria fumosorosea*, *I. farinosa* and *Lecanicillium*

muscarium. A laboratory experiment showed that two species of field isolated fungi - *B. bassiana* and *I. fumosorosea* were as effective in killing clover root borer adults as commercial sources of *M. brunneum* and *I. fumosorosea* when exposed to these fungi in petri dishes with sterile white sand. Subsequently, *H. obscurus* adults were exposed to four spore concentrations of *B. bassiana* and *M. brunneum* separately in cups filled with field collected unsterilized and sterilized soil. There were no significant mortality differences on either sterilized and unsterilized soil. However, at the highest concentration, more than 80% of clover root borers died with *M. brunneum* while with *B. bassiana*, the mortality was not significantly different due to high mortality in the negative control.

Based on the study, at least six species of entomopathogenic fungi occur naturally in red clover seed production fields in western Oregon, and field isolated fungi are as virulent as commercial fungi against the clover root borer. Further research is needed for evaluation of the pathogenicity of these entomopathogenic fungi under field conditions.

Isolation and Pathogenicity of Naturally Occurring Entomopathogenic Fungi to Clover Root Borer (Coleoptera: Curculionidae: Scolytinae), a Pest of Red Clover Seed Crops

by
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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Anis S. Lestari, Author

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CONTRIBUTION OF AUTHORS

Dr. Sujaya Rao helped with experimental design, interpretation of data, and writing in Chapter 2 and 3. Dr. Joseph W. Spatafora assisted in data collection, data analysis, data interpretation, and fungi identification in Chapter 2.

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Chapter 1

General Introduction

The Clover Root Borer (CRB), *Hylastinus obscurus* Marsham (Coleoptera: Curculionidae: Scolytinae), is a soil-dwelling beetle pest that is the sole member of its genus in the US (Rockwood, 1926). It is a unique bark beetle pest that does not attack the stems and bark of trees like other bark beetles but instead infests woody parts of leguminous plants. The clover root borer injures a wide range of legume crops such as vetch, pea vines, Scotch broom, alsike, alfalfa, and sweet clover; however, it prefers mammoth and red clover (Rockwood, 1926; Considine, 1995).

Hylastinus obscurus is native to Europe. It was first noted as a pest in the US in 1878, and documented to be present throughout New York, Michigan, Ohio, and other northern parts of the USA (Rockwood, 1926). The clover root borer was reported for the first time in Oregon, in 1895 as a pest in commercial red clover seed production fields (Rockwood, 1926).

Hylastinus obscurus is difficult to detect in the field due to its minute size and subterranean presence for most of its life cycle. Adult beetles, 0.1 inch long and dark brown, are present above ground in spring when they emerge and then migrate from old clover roots. Beginning in late April and May, females lay eggs in niches in the walls of burrows made in clover roots. Each female lays approximately 20 eggs over her life span. Eggs hatch in 17-30 days. Larvae are creamy white and burrow within the roots of red clover. The four larval instar develop slowly over the summer, and pupate in pupal chambers at the end of the larval mines inside the clover root. The pupal period lasts 8-13 days. Adults are fully formed by late summer but remain below ground (Rockwood, 1926).

The pest overwinters primarily in the adult stage, although a few larvae are also present in the soil in winter and transform to adults the following spring. CRB is univoltine. The total development period from egg to adult is 90 days or more while the total life span of an individual may last a year or longer. As the egg laying period extends over a considerable length of time, there is great diversity in stages of development of the pest within a clover root at any given time from late spring to fall.

The earliest injury to new clover (first crop, seeded the previous year), is observed in the spring, one to two weeks after the first migratory flight (Rockwood, 1926). Damage by CRB results in a decrease in both forege yield and seed production in the subsequent year. Mining caused by the pest often becomes a site for infection with root rot fungus (*Fusarium solani*) and other pathogens that contribute to a decline of clover stands. Towards the end of July-August, when re-growth has occurred, severely injured fields show sickly and dead plants. Additionally, seed found on ripe heads is withered and light and color. Frequently, the stand is mostly dead by late summer of the second year unless there has been an opportunity for abundant self seeding (Rockwood, 1926).

Red clover is a perennial that is grown as an important forage legume in the US and worldwide (Taylor et al., 1996). It is also cultivated as a cover crop due to its nitrogen fixing abilities, and as a seed crop. Additionally, red clover is important for conserving pollinators. Its flowers provide a valuable food resource for bumble bees, which can in turn influence reproduction in red clover (Rao et al., 2009; Rundlöf et al., 2014).

The Willamette Valley in western Oregon is one of the few regions suitable for growing red clover for seed production due to the extensive rainfall during the growing season and dry

weather during harvest. In 2014, over 13,000 acres of red clover seed were harvested in the region, and the seed yield was 728 kg/ha (Oregon Agripedia, 2016).

A key factor affecting red clover seed production in western Oregon is damage resulting from feeding by both larvae and adults of the clover root borer (Rockwood, 1926). *Hylastinus obscurus* contributes to the root decline of red clover plants (Jin et al., 1992). An infestation of only 1.5 borers per plant decreases forage yield by 5.5% (Pruess et al., 1958). Moreover, Koehler et al. (1961) reported that when ~40% of first year plants are attacked then almost all plants are infested by the end of second year. Hence, a third year of seed production is generally not economical. In addition, attack by *H. obscurus* exacerbates red clover root rot caused by fungal infection of *Colletotrichum trifolii, Fusarium roseum, F. tricinctum,* and *Rhizoctonia solani* (Leath et al., 1973).

Hylastinus obscurus was controlled with organochlorine pesticides such as aldrin, chlordane, dieldrin, and heptachlor (Gyrisco et al., 1950; Gyrisco et al., 1954; Dickason et al., 1961) until their use was prohibited due to hazardous effects on the environment and non-target organisms, including humans (EPA, 2011; 2013). No new insecticides were labeled for the pest due to the challenge of getting insecticides to reach clover crown borer larvae and adults feeding within the roots. In a field trial conducted in 2011, four insecticides labeled for red clover seed production were evaluated but none caused significant mortality compared with the controls (Rao et al., 2012).

As part of an integrated pest management (IPM) program, other tactics have been proposed for management of *H. obscurus*. One of them is the use of semiochemicals, volatile compounds which attract and trap the pest in order to reduce their population in the field.

Individual and/or mixed root volatile compounds extracted from red clover roots were shown to attract adults in the laboratory, but none of those compounds were effective in the field (Kamm et al., 1984; Tapia et al., 2007). In Chile, researchers evaluated new varities of red clover and found 1 out of 6 cultivars was tolerant to *H. obscurus* (Alarcon et al., 2010). However, in the US, breeding studies focus mainly on developing cultivars that are resistant to root rot and not *H. obscurus* infestations (Steiner et al., 1999). Furthermore, breeders are faced with the challenge of developing cultivars that are not only resistant to the borer invasion, but also provide high flower density so that the new cultivars result in high yield and are well-accepted by the growers.

Entomopathogenic fungi are microbial control agents which infect their hosts and cause mortality. Some members of Hypocrealean fungi have been commercialized and are labeled for various insect pests such as gnats, root weevils, aphids, thrips, Lepidopteran and Coleopteran larvae. The field application of commercial fungal entomopathogens can be used as an augmentative control strategy which can be implemented by adding large numbers of their propagules for immediate pest control (inundative tactic) or by adding small numbers to build up population of natural enemy (inoculative tactic) in the agroecosystems. The other option is the conservation strategy which involves modified of management practices to favor the development and transmission of naturally occurring and commercial entomopathogenic fungi (Hajek, 1997).

Entomopathogenic fungi are present in many soil environments and can be effective against subterranean pests like the clover root borer. Researchers have isolated the entomopathogenic fungi *Beauveria* and *Metarhizium* from agricultural areas with a variety of crop plants, including pecans (Harrison et al., 1991), hazelnuts (Sevim et al., 2010), Christmas

trees, blueberries, strawberries, and grapes (Fisher et al., 2011). Aside from a study by Rockwood (1926) that reports the presence of the entomopathogenic fungus *Beauveria bassiana* (formerly known as *B. globulifera*) in western Oregon, little is known about the presence of other fungal entomopathogens in agroecosystems planted with red clover seed crops, and of their virulence against the clover root borer.

In this thesis I explore the potential for use of fungal entomopathogens as biological and/or microbial control agents for the clover root borer. In Chapter 2, I describe the field survey that I conducted for detection of naturally occurring entomopathogenic fungi in red clover seed production fields in western Oregon. The isolated fungi were initially identified based on morphology, and subsequently a phylogenetic analysis was used to determine their species identities.

Chapter 3 of this thesis describes a study conducted to determine the virulence of field isolated entomopathogenic fungi that were compared to commercial strains, with the goal of identifying fungal isolates with the greatest potential for suppressing clover root borer populations. A second bioassay was conducted with different concentrations of selected entomopathogenic fungi using unsterilized and sterilized field soil for assessing their virulence against the clover root borer in the presence of potential antagonistic microorganisms.

Chapter 4 synthesizes the results of both studies, provides an assessment of the potential of various entomopathogenic fungi for management of the clover root borer, and offers suggestions for future research.

Chapter 2

Isolation and characterization of naturally occurring entomopathogenic fungi isolated from red clover fields

Keywords: Entomopathogenic fungi, *Beauveria, Metarhizium, Isaria, Lecanicillium*, red clover

Introduction

Entomopathogenic fungi are microbial pathogens that cause fungous diseases in some invertebrates such as arachnids and insects (Robert et al., 2004; Rehner, 2005). They are widespread in most terrestrial ecosystems and provide valuable ecological services by regulating insect populations (Hajek, 1997). Since they are natural enemies, they have tremendous potential for use as microbial control agents against insect pests, especially subterranean pests for which few management options are available.

For development of entomopathogens as a microbial control strategy for pests in agroecosystems, information on their presence and pathogenicity to the target pests are needed (Meyling et al., 2007; Bruck, 2010). Additionally, by studying their species diversity, we can enhance our understanding of the relationship between entomopathogenic fungi and crop plants in agroecosystems.

Naturally occurring entomopathogenic fungi have been found in pecan orchards (Harrison et al., 1991), hazelnuts (Sevim et al., 2010) and landscapes with diverse cropping systems. They have been isolated from pasture land planted with various grasses and legumes (Baker et al., 1998), agricultural areas with organic and conventional farming systems (Klingen et al., 2002), a single organic agroecosystem with various plants (Meyling et al., 2006), nursery

field soils (Bruck, 2004), and in agricultural landscapes planted with maize, mixed vegetables and sorghum (Perez et al., 2014). Little is known about the presence of naturally-occurring entomopathogens in monocultures of crops raised conventionally.

Red clover (*Trifolium pratense* L.) is a commercial seed crop cultivated in over 13,000 acres in the Willamette Valley in western Oregon (Oregon Agripedia, 2016). A key factor affecting red clover seed production in western Oregon is damage resulting from feeding activity by both larvae and adults of the clover root borer, *Hylastinus obscurus* Marsham (Coleoptera: Curculionidae: Scolytinae) (Rockwood, 1926). The infestation of 1.5 individuals of *H. obscurus* reduces red clover yield by 5.5% (Pruess et al., 1958). Moreover, Koehler et al. (1961) reported that when ~40% of first year plants are attacked then almost all plants are infested by the end of second year. Hence, a third year of seed production is generally not economical. In addition, *H. obscurus* attack aggravates root rot caused by fungal infection of *Colletotrichum trifolii, Fusarium roseum, F. tricinctum,* and *Rhizoctonia solani* (Leath et al., 1973). Use of organochlorine insecticides which killed CRB infestation (Gyrisco et al., 1954; Dickason et al., 1961) was banned due to its negative impacts to the environment and non-target organisms. Hence, an alternative strategy for management of the clover root borer is required.

Naturally occurring entomopathogenic fungi can be beneficial for regulating populations of *H. obscurus* and other subterranean pests of red clover. *Beauveria bassiana* (formerly known as *B. globulifera*) was reported infecting *H. obscurus* by Rockwood (1926) and *Sitona lepidus* (Willoughby et al., 1998). Furthermore, in a study by Brownbridge et al. (2006), the abundance of *B. bassiana* propagules in the non-fungal treatment sites (control) is two times higher than treated pastures with mixed white clover and rye grass in Waikato and Manawatu, New Zealand

which suggests that this fungus is associated with clover crops. However, no information is available on fungal entomopathogens present in red clover fields.

The objective of this study was to isolate and identify naturally entomopathogenic fungi in conventional red clover fields using morphological characters and phylogenetic analyses.

Materials and Methods

Sampling sites

The study was conducted in the Willamette Valley in western Oregon. Five commercial conventional red clover seed production fields (Table 2.1) were sampled in October 2013 and in October-December 2014.

Detection of naturally occurring entomopathogenic fungi in red clover seed fields

In 2013, red clover roots collected from Field 1 (Table 2.1) were cut along the axis into four pieces and placed in Berlese funnels to extract clover root borers. Four of 30 adults that were collected were observed to be covered with fungal mycelia and spores on the external body surface. The spores and mycelia were inoculated on Potato Dextrose Agar (PDA, Difco) containing antibiotic chloramphenicol (0.2 % in 1 liter media). Pure cultures of fungi were maintained on Sabouraud Dextrose Agar (SDA, Difco) at room temperature (25-28^{o}C) for 5-7 days before being used for morphological identification.

In 2014, red clover roots surrounded by 1-2 cm of soil were collected from four fields (Field 2, 3, 4, 5, Table 2.1). In each field, three sampling locations were randomly selected using a 4x4 m^2 grid. Within each grid, 25 red clover plants were collected and bagged. In all, 12 bags

containing red clover plants and soil surrounding the roots were collected from the four fields. The bags were stored at 8^{o}C until used.

Isolation of entomopathogenic fungi from soil samples

The soil baiting method using larvae of the greater wax moth, *Galleria mellonella* (Lepidoptere: Pyralidae) as surrogate hosts was used to isolate entomopathogenic fungi from soil enveloping roots (Zimmerman, 1986). Four root-zone soil samples were taken from red clover plants collected from Field 1 in 2013. Soil samples were scraped from 4 randomly selected roots and transferred to sterile petri plates (85 mm x 15 mm). Five *Galleria* larvae were exposed to each soil sample and incubated at room temperature (25-28^{o}C). The larvae were monitored every two days for detection of dead *Galleria*. Dead larvae were rinsed with distilled water, placed on moistened filter paper (Whatman #1) and maintained at room temperature to observe fungal growth on the cadaver. From each cadaver that exhibited signs of mortality caused by the entomopathogenic fungus, the externally growing hyphae and spores were collected and inoculated on a sterile petri plate filled with PDA media containing antibiotic chloramphenicol (0.2 % in 1 liter media).

A similar procedure of soil baiting and isolation was used for the root zone-soil samples of red clover plants obtained from four fields in 2014. Out of the 25 plants collected from of each three locations within each field, roots from 10 plants were randomly selected. Thus, in 2014, 120 soil samples were collected.

Morphological identification

Genus level identification of isolated entomopathogenic fungi was determined based on macroscopic characteristics such as color of the spores, fungal colony growth on agar media,

morphology of conidia (spores) and conidiophores (spore bearing strutures) (LEICA DME) (Humber, 1992; Lacey et al., 2007).

DNA extraction

For DNA extraction, a subset of fungi belonging to genera *Beauveria* (45 isolates) and *Metarhizium* (22 isolates) were selected based on variations of morphological characteristics whereas for genera *Isaria* and *Lecanicillium*, all isolates were used. Spores and/or vegetative hyphae of each fungus were inoculated on SDA media covered with a piece of sterile Whatman filter paper (diameter: 42.5 mm) according to the protocol described by Kepler et al. (2014) with a slight modification (the whole filter paper was used), except for *Lecanicillium* isolates which were cultured on cellophane. The cultures were maintained at 28^{o}C for 5-7 days in a dark incubator, and isolates were harvested by transferring about 20-50 mg of fungal tissue to a sterile one ml microcentrifuge tube. The tissue was then frozen in liquid nitrogen, and ruptured using sterile blue micro pestles. The DNA extraction procedure was conducted using DNAeasy Plant Mini kits (50 preps), and the extracted DNA samples were stored at -20^{o}C until used.

PCR and sequencing

The Internal Transcribed Spacer region (ITS) of rRNA was amplified using GoTaq green mastermix (Promega). In the PCR amplifications, ITS-1F (5'-CTT GGT CAT TTA GAG GAA GTAA- 3') and ITS-4 (5'- TCC TCC GCT TAT TGA TAT GC-3') were used as the forward primer and reverse primer, respectively (White et al. 1990; Gardes et al., 1993). The PCR reaction was initiated with denaturation at 95^{o}C for two minutes followed by 35 cycles of 94^{o}C for 40 seconds, annealing at 52^{o}C for one minute, followed by 72^{o}C for one minute 30 seconds, and final extension at 72^{o}C for seven minutes. PCR products were purified using a Qiagen

QIAquick PCR purification kit and sequenced at the Center for Genome Research and Biocomputing, Oregon State University. Similar primers to those mentioned above were used for sequencing the ITS region. BLAST searches were performed on fungal sequences to confirm their identification by comparing with reference strains in NCBI GenBank to support phylogenetic analysis.

Phylogenetic Analysis

Sequences of ITS-1F and ITS-4 were assembled, edited and aligned using Bioedit (Hall, 1999). The sequences were also compared with the NCBI GenBank database using BLAST searches, which confirmed that the isolated fungi were entomopathogenic fungi members of the order Hypocreales. MEGA 6 was used to group and align the sequences with Clustal W followed by Maximum-Likelihood Analysis (Tamura et al., 2013). Phylogeny tests were performed with Nearest-Neighbor-Interchange as the ML-Heuristic method and General Time Reversible as the substitution model (Felsenstein, 2004). The reliability of the phylogram was tested with bootstrap analysis with 500 replicates.

Results

Detection of naturally occurring entomopathogenic fungi in red clover seed fields

In 2013, four isolates of entomopathogenic fungi obtained from four *H. obscurus* cadavers were identified as belonging to the genus *Beauveria* while seven isolates collected from 20 infected *Galleria* larvae were identified as belonging to the genera *Beauveria* (4) and *Isaria* (3). In 2014, from 600 *Galleria* larvae exposed to 120 root zone-soil samples, more than 120 isolates of entomopathogenic fungi were collected and identified as belonging to four genera -

Beauveria, Metarhizium, Isaria and *Lecanicilium.* Macroscopic and microscopic characteristics of four naturally occurring fungal genera are shown in Figure 2.1-2.5.

From five fields, three genera were detected in Field 2, while 2 genera were each detected from the other field. Of the four genera, *Beauveria* was the dominant genus detected in all fields, and was present in greater in abundance compared to the other genera. *Metarhizium* was the next most common genus; it was found in three fields but in much lower abundance than *Beauveria*. *Isaria* was detected in 2 fields while *Lecanicillium* was recorded from only 1 field (Table 2.2).

Phylogenetic placement of field isolated fungi

To confirm the identities of *Beauveria* isolates, 515-575 bp fragments of the ITS region were aligned after they were assembled and edited. Of the 45 isolates of field isolated *Beauveria*, 31 were identified as *B. bassiana* and 14 as *B. pseudobassiana*. As shown in the phylogram, field isolated genus *Beauveria* sequences were identical to those of *B. bassiana* (bootstrap value: 98%) and *B. pseudobassiana* (bootstrap value: 85%) previously characterized by Rehner et al. (2011) (Fig. 2.6). Based on the phylogenetic analysis and supported by similarity of morphological characteristics (Table. 2.5), all the remaining isolates of this genus were then identified as *B. bassiana* or *B. pseudobassiana*.

For *Metarhizium* strains, 530 – 560 bp fragments of ITS data were aligned along with similar gene regions of known species obtained from previous studies (Driver et al., 2000; Destafano et al., 2004; Bischoff et al., 2009; Schneider et al., 2011; Kepler et al., 2012). Based on the phylogram created using ITS data of isolates, sequences of seven isolates of field isolated *Metarhizium* were identical to *Metarhizium brunneum* (bootstrap value: 70%) (Fig. 2.7). Other *Metarhizium* isolates were closely related to *M. anisopliae, M. guizhouense, M. indigotica, M.*

pingshaense, and *M. robertsii,* which are all part of the *M. anisopliae* species complex (Kepler et al., 2014). The phylogenetic analysis is supported by the diverse of macro and microscopic characteristic data which suggests that there are more than one species belonging to genus *Metarhizium* collected from red clover fields (Table 2.4).

For *Isaria* isolates, after the sequences were edited, 550-600 bp fragment of the ITS region were aligned with sequence data from previous studies conducted by Luangsa-Ard et al. (2005), Inglis et al. (2006) and Rehner et al. (2011). According to the phylogram, sequences of 3 isolates belonging to genus *Isaria* collected from red clover fields are similar to *I. fumosorosea* and 1 isolate is identical to *I. farinosa* (bootstrap value: 99% for both clades) (Fig. 2.8). The phylogenetic analysis is supported by the characteristics and morphological data of their fungal colony, and spore color (Table 2.6).

For *Lecanicillium* isolates, after the sequences of the ITS region were edited, 535-590 bp fragments were aligned with ITS data from Zare et al. (2000), Kouvellis et al. (2008) and Sukarno et al. (2009). Based on the phylogram of the ITS region (Fig. 2.9), all sequences of field isolated *Lecanicillium* were identical to *Lecanicillium muscarium* (bootstrap value: 73%). This identification is supported by the characteristics and morphological data of their fungal colony, and spore color (Table 2.6).

Discussion

This is the first study to report the presence of naturally occurring entomopathogenic fungi in the root-zone soil from conventional fields with red clover as a single forage crop. Earlier studies on insect pathogenic fungi in agricultural landscapes were conducted by collecting soil samples from areas with a diversity of crop plants and farming systems (Harrison

et al., 1991; Klingen et al., 2002; Meyling et al., 2006; Meyling et al., 2009; Perez-Gonzalez et al., 2014), from rhizospheres of individual plants of nursery and orchard crops (Fisher et al., 2011), and from pasture lands (Barker et al., 1998).

The presence of entomopathogenic fungi are dependent on several factors such as the crop, production practices, abiotic factors such as soil moisture, pH, and temperature, and biotic factors including microbial communities (Meyling et al., 2007; Jaronski, 2010). The Pacific Northwest receives high rainfall and the resulting high soil moisture facilitates survivorship and germination of fungal spores. This could be the reason that we detected substantial diversity in entomopathogenic fungi in the red clover fields. In all, we detected at least six species of entomopathogenic fungi belonging to four genera from 124 root zone soil samples from five red clover fields collected over two years. The same four genera of naturally occurring entomopathogenic fungi were detected in a study by Meyling et al. (2006) conducted in Denmark in a single organic agroecosystem consecutively planted with different varieties of crop plants over 3 years. Two additional genera, *Hirsutella* and *Conidiobolus*, were detected though in very low abundance (1 and 7 isolates, respectively). The fewer fungal genera isolated in the current study is not surprising because fungicides were likely used in the field, which could have had negative impacts. Conventional farming systems are expected to provide a less suitable environment for development of entomopathogenic fungi (Klingen et al., 2002; Clifton et al., 2015).

This study revealed that *B. bassiana* was the major species found in red clover fields, which confirmed the hypothesis about the association of this fungus with clover crops (Brownbridge et al., 2006). However, a study conducted in pasture fields in New Zealand found

that *B. bassiana* was less common than *Metarhizium anisopliae* (Baker et al., 1998).This suggests that the prevalence of fungal entomopathogenic species may vary based on the agricultural soil environment and the region.

Our study revealed that some field collected adults of *H. obscurus* were infected with *B. bassiana.* This fungal species was also reported in association with the clover root borer in Oregon by Rockwood (1926) and suggests that *B. bassiana* may have potential as a microbial agent in controlling this pest, and perhaps other soil dwelling pests under western Oregon conditions. The application of commercial fungus *B. bassiana* strain GHA is possible because this fungus is labeled for subterranean pest such as borers and weevils (EPA[2], 2016) which is a pest in the Willamette Valley. However, the population dynamics of *B. bassiana* in red clover fields need to be studied to maximize its potential with augmentative application and also to develop conservation strategies for this important microbial control agent (Hajek, 1997).

This may be the first record of the presence of *B. pseudobassiana* in the Pacific Northwest. It was also found colonizing the root-zone soil samples of red clover, although there were fewer isolates compared to *B. bassiana*. In a study on naturally-occurring entomopathogens in the Pacific Northwest by Bruck (2004), *B. pseudobassiana* was not reported. *Beauveria pseudobassiana* was isolated from various infected insect cadavers in the subfamily Scolytidae in Canada (Rehner et al., 2011) while in Mexico, it was isolated from agricultural soil planted with various crop plants (Perez-Gonzalez et al., 2014) and from infecting white grub larvae (Carillo-Benitez et al., 2013). These suggest that *B. pseudobassiana* may also have potential as a microbial agent in red clover fields to control soil dwelling pests. It could be used as part of a conservation strategy because unlike *B. bassiana*, it is not produced commercially.

Metarhizium was the second most abundant fungal genus isolated from red clover field in this study after *Beauveria*. Phylogenetic analysis based on ITS sequence placed 7 isolates as belonging to species *M. brunneum.* Those isolates have 76% in bootstrap value of the ITS region to the commercially entomopathogenic fungus *M. brunneum*, strain F52 (Myrand et al., 2015) which indicates that they are similar species. This suggests that perhaps there were previous applications of commercial *M. brunneum* (F52) in the sampling sites or surrounding agroecosystem areas because this commercial fungus was an exotic species isolated in Austria. The application of commercial fungus *M. brunneum* strain F52 is possible because this fungus is labeled for subterranean pest such as root weevil (EPA[1], 2016) which is a pest in the Willamette Valley.

As shown in the phylogram of the genus *Metarhizium*, unresolved polytomy indicates 15 isolates of field isolated fungi genus *Metarhizium* are undetermined species. This provides evidence that the ITS region alone is not sufficient for identifying the species complex of *M. anisopliae* which is consistent with the report by Driver et al. (2000). Fisher et al. (2014) characterized the 5' region of the elongation factor-1 alpha (5'-*tef1*) of field isolated fungi in the genus *Metarhizium* isolated from orchard crops and Christmas trees, but further characterization and amplification of the *tef* region of the undetermined isolates of *Metarhizium* isolates are still necessary. Moreover, the morphology data of undetermined species are not definitive and it is difficult to differentiate the genus *Metarhizium* to species based on appearance of macro and microscopic characteristics (Bischoff et al., 2009). Despite this, it is evident more than one species of naturally occurring fungi in the genus *Metarizhium* were found in this study.

The genus *Lecanicillium* and the two species of *Isaria* were also not reported in the study conducted by Bruck (2004) and Fisher et al. (2014) in western Oregon. In the current study, *I. fumosorosea, I. farinosa,* and *L. muscarium* were detected, though in low abundance, across all the fields sampled. Our findings are similar to those of Meyling et al. (2006), who found both genera in a single agricultural field in a 3 year study. *I. fumosorosea* was found in agricultural soil planted with various crop plants in Turkey (Sevim et al., 2010), and *I. farinosa* but not *I. fumosorosea* was found in cultivated areas in Finland (Vaninnen, 1995). This suggests that perhaps the prevalence of *Isaria* is specific to certain agroecosystems and dependent on unique biotic and abiotic factors. *Lecanicillium muscarium* was identified in our study. Meyling et al. (2006) reported the presence of a similar fungus (it was formerly known as *L. lecanii*) in an organic cropping system in Denmark. This fungus is being commercially mass-produced and labeled for use against aerial pests (Faria et al., 2007) but is rarely isolated from soil. Hence, this study provides evidence that it can also survive in conventional cropping ecosystems.

Conclusions

Entomopathogenic fungi can be diverse in conventional fields of a single crop such as red clover. They have potential for use as biological control agents for soil dwelling pests, which may be of particular value for the clover root borer given the absence of any other control strategy. The naturally occurring fungi detected in this study can be integrated into conservation and augmentative microbial control strategies. Further research is needed to assess the virulence of naturally occurring entomopathogenic fungi in large scale field studies.

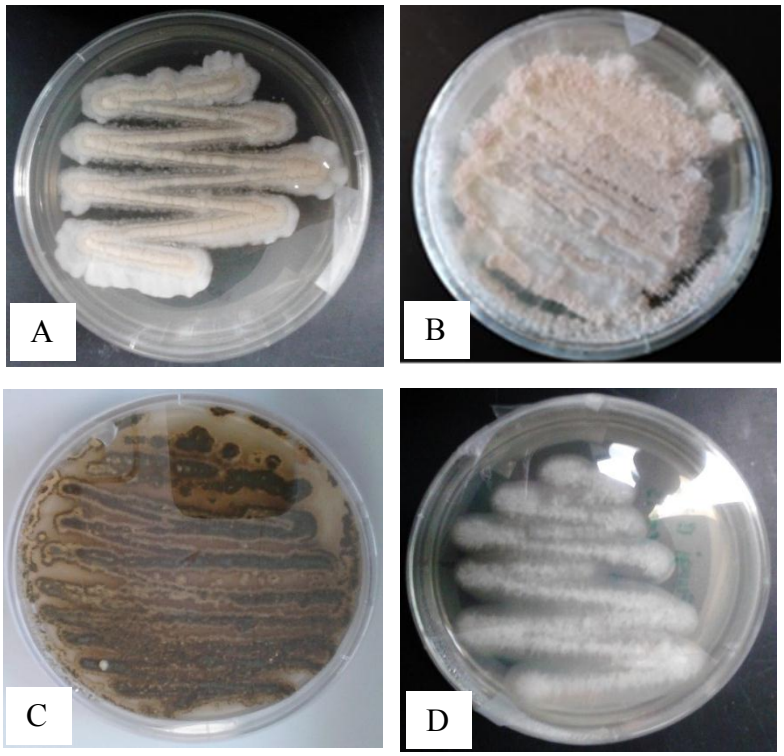

Fig. 2.1. Upper surface cultures of the naturally occurring fungi isolated from red clover fields in Willamette Valley, OR. *Beauveria* (A), *Isaria* (B), *Metarhizium* (C), and *Lecanicillium* (D)

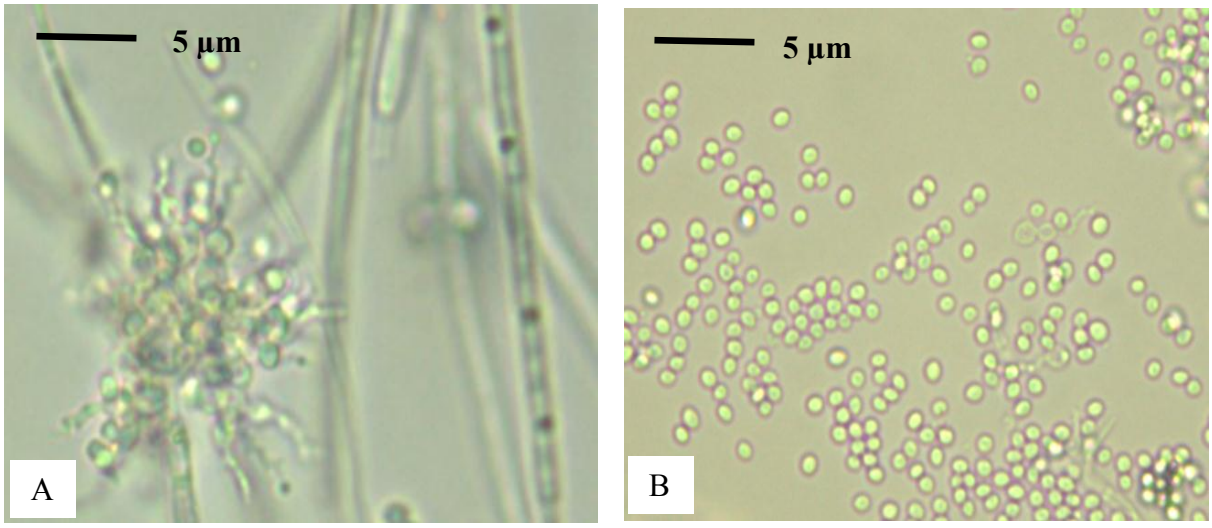

Fig. 2.2. Spore bearing structures (A) and spores (B) of the naturally occurring fungi in the genus *Beauveria* isolated from red clover fields in Willamette Valley, OR.

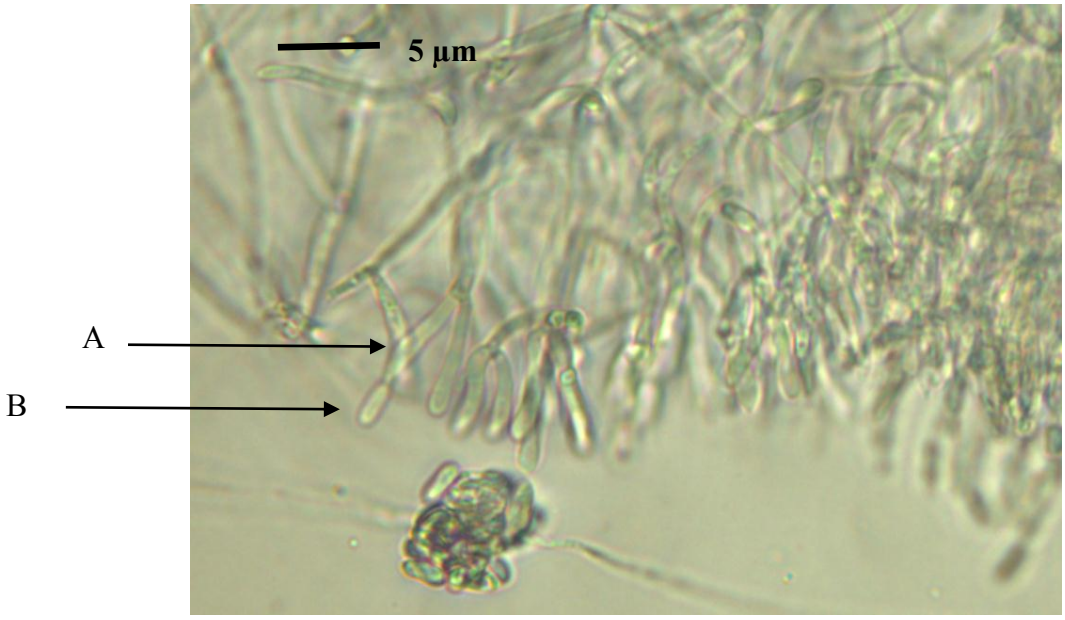

Fig. 2.3. Spore bearing structures (A) and spores (B) of the naturally occurring fungi in the genus *Metarhizium* isolated from red clover fields in the Willamette Valley, OR.

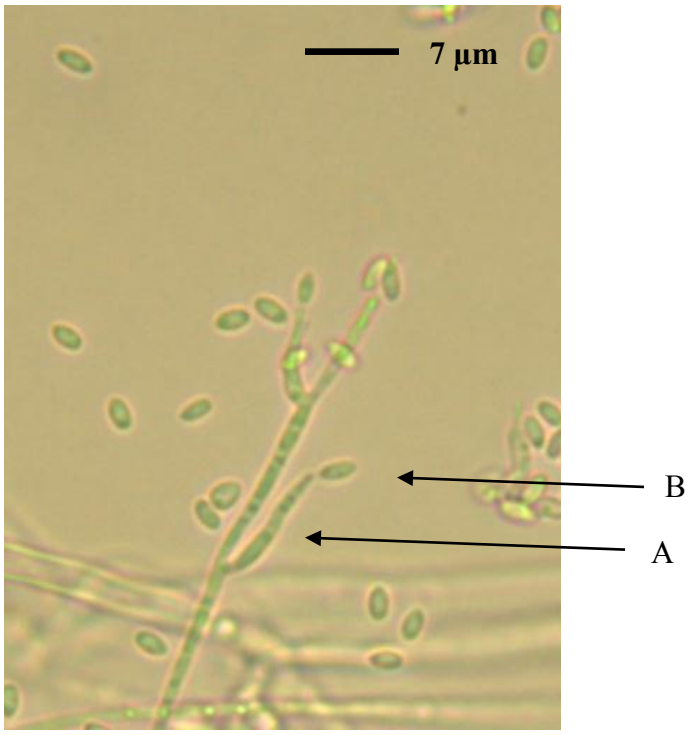

Fig. 2.4. Spore bearing structures (A) and spores (B) of the naturally occurring fungi genus *Isaria* isolated from red clover fields in the Willamette Valley, OR.

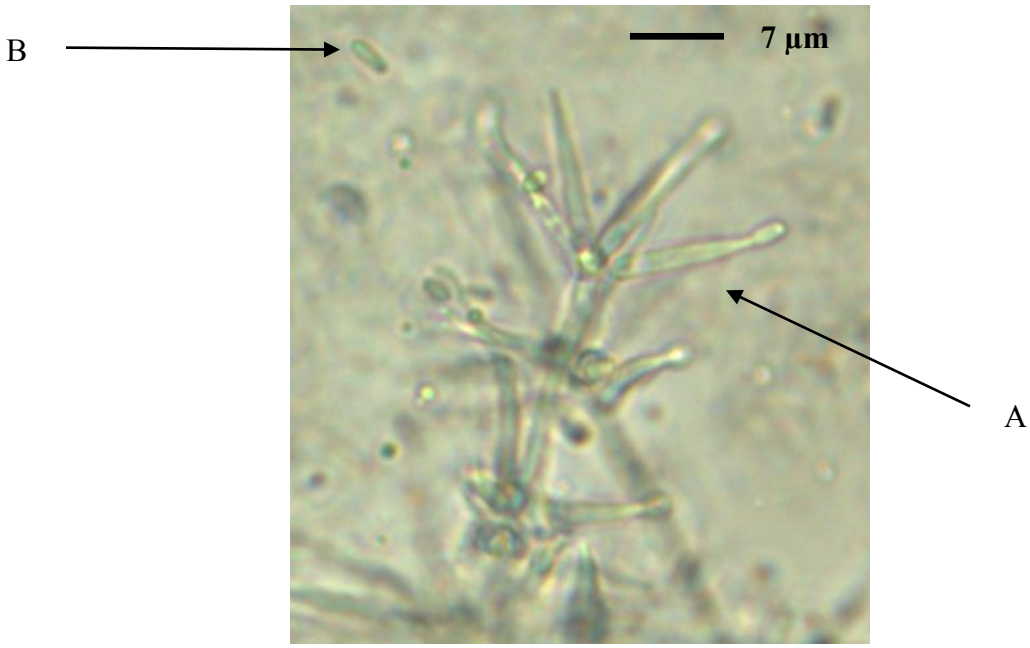

Fig. 2.5. Spore bearing structures (A) and spores (B) of the naturally occurring fungi genus *Lecanicillium* isolated from red clover fields in the Willamette Valley, OR.

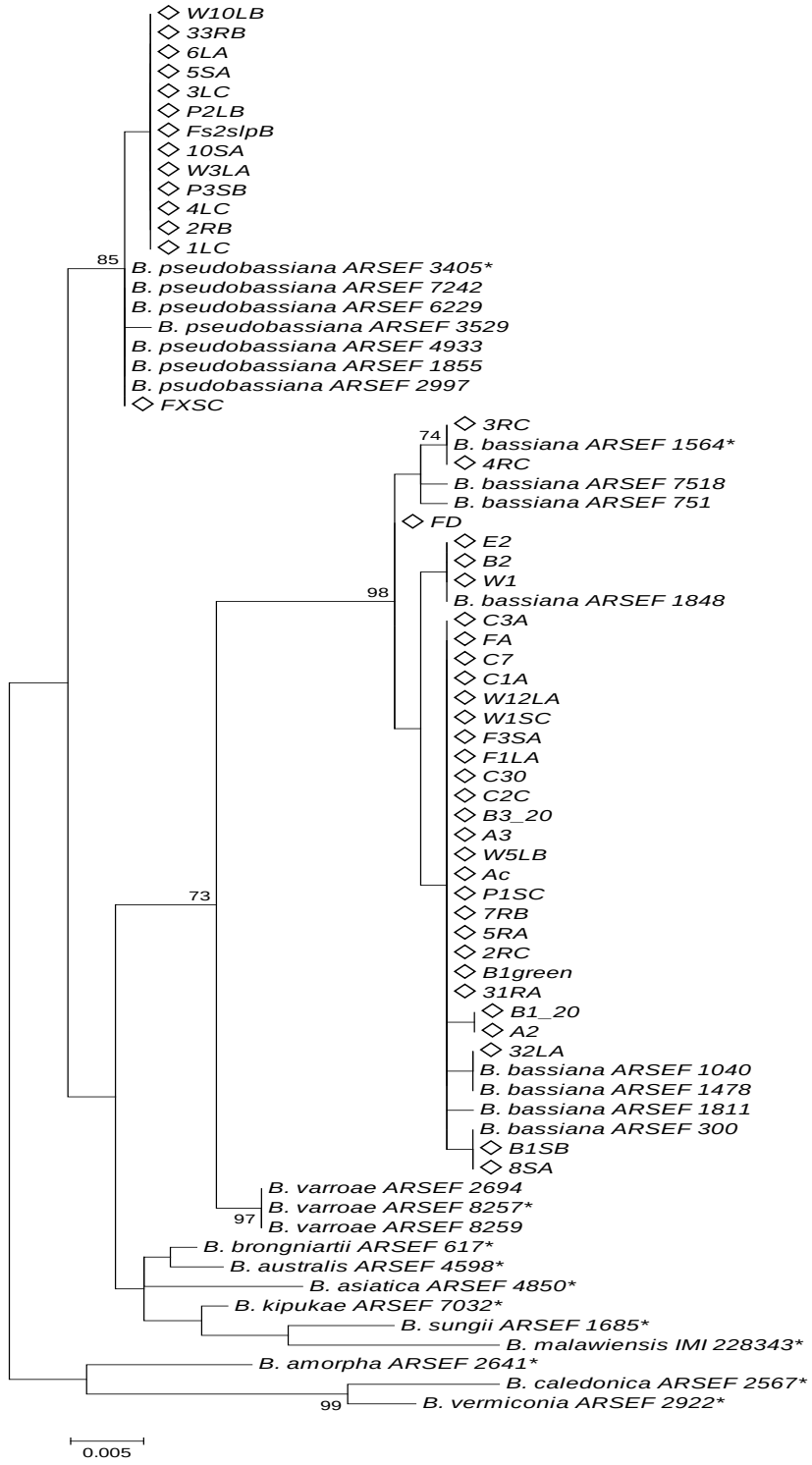

Fig. 2.6. Maximum likelihood phylogeny of 45 *Beauveria* isolates collected from five red clover fields based on ITS data. Bootstrap value ≥70 are labelled. * = type material. ◇ = field isolated fungus.

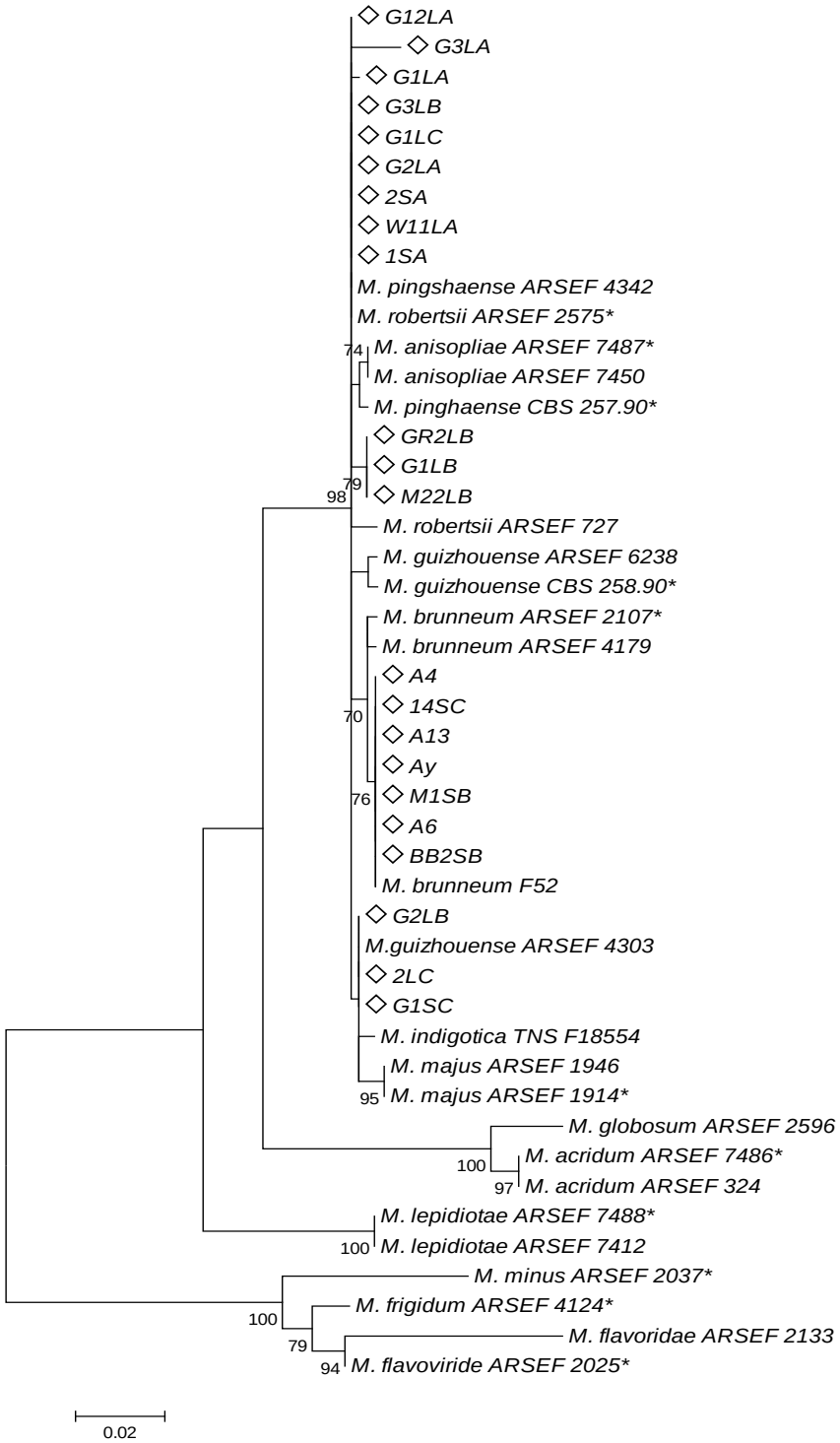

Fig. 2.7. Maximum likelihood phylogeny of 22 *Metarhizium* isolates collected from five red clover fields based on ITS data. Bootstrap value ≥70 are labeled. *= type material. ◇ = field isolated fungus.

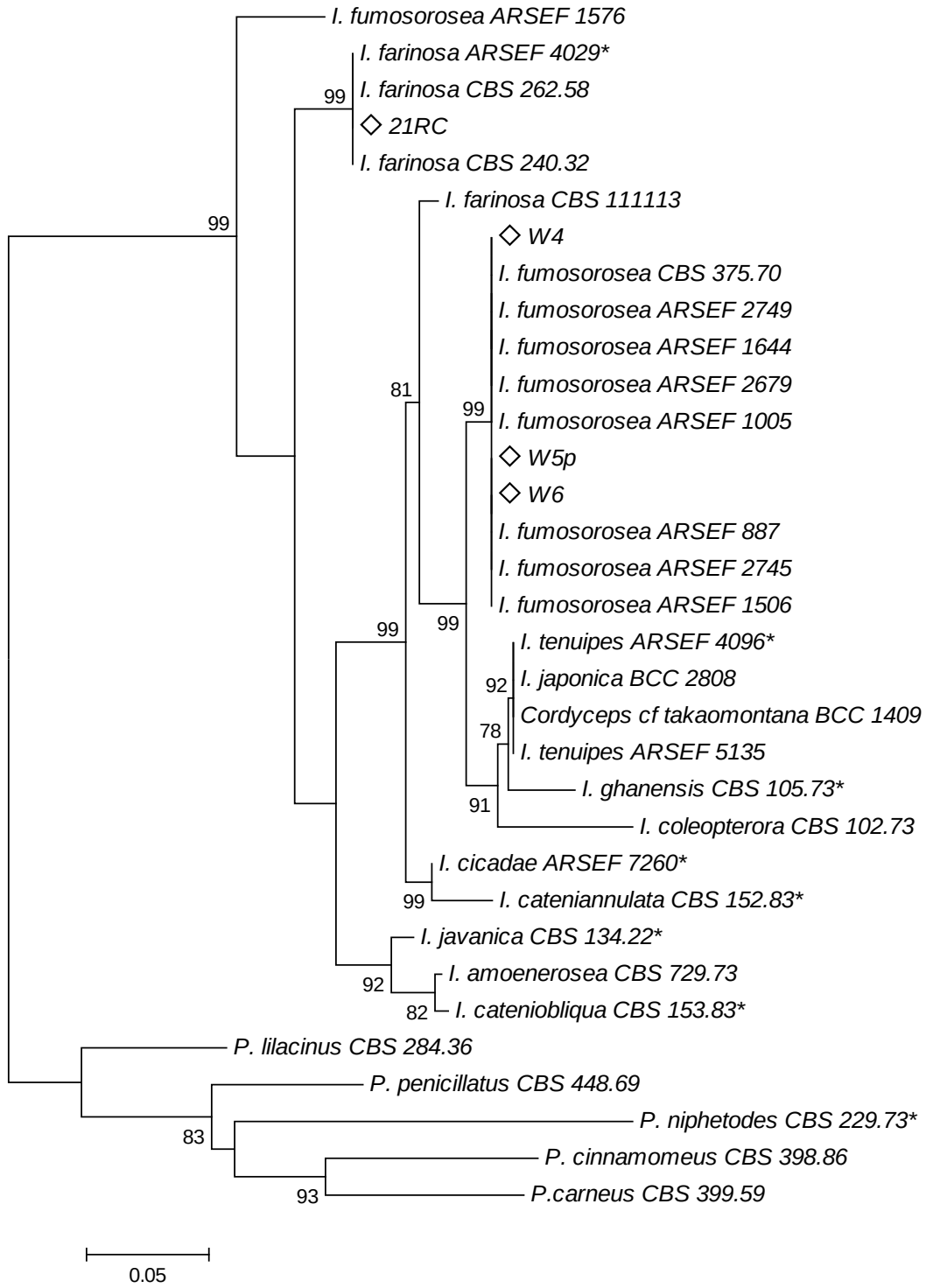

Fig.2.8. Maximum likelihood phylogeny of four *Isaria* isolates collected from five red clover fields based on ITS data. Bootstrap value ≥70 are labeled. * = type material. ◇ = field isolated fungus.

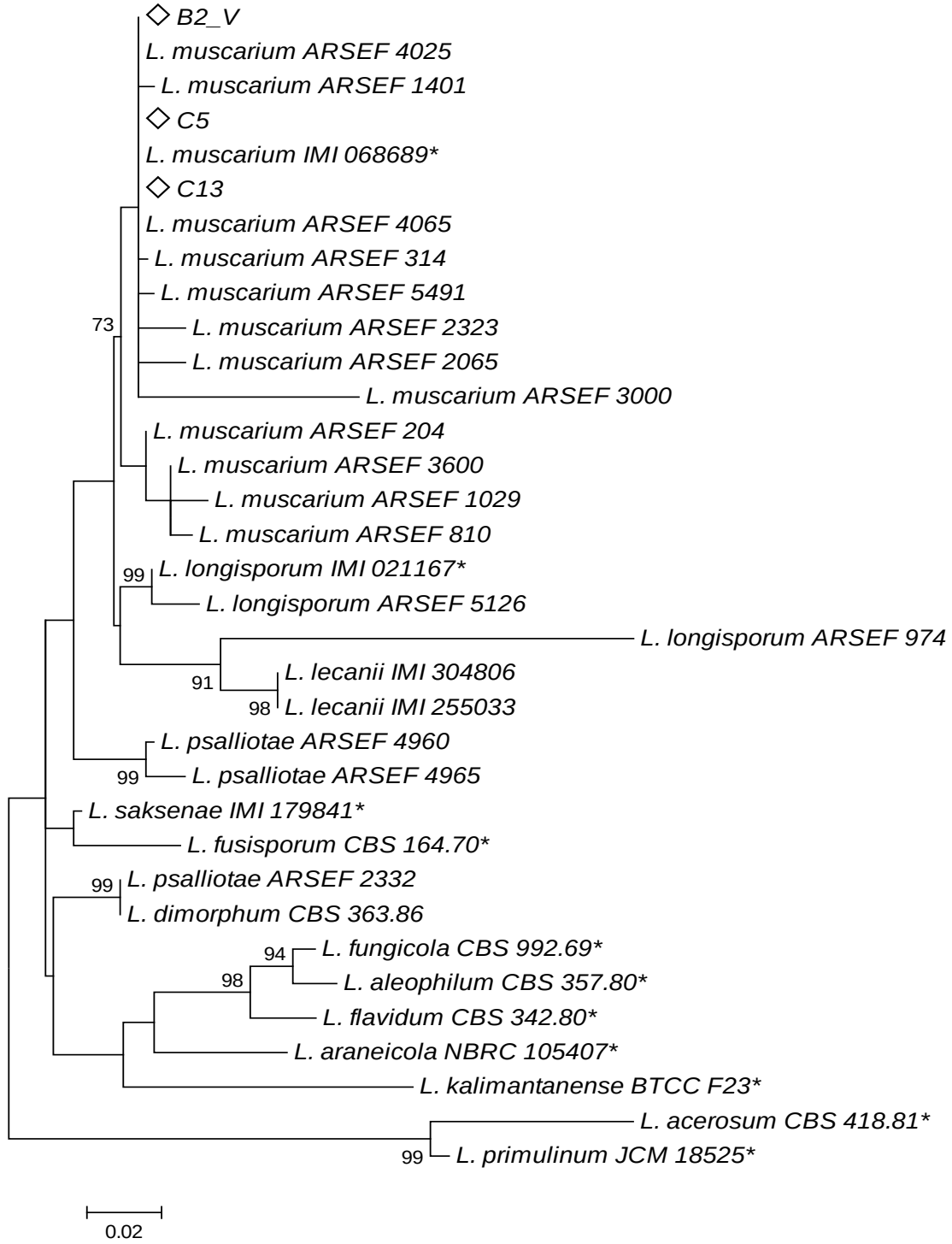

Fig. 2.9. Maximum likelihood phylogeny of three *Lecanicillium* isolates collected from five red clover fields based on ITS data. Bootstrap value ≥70 are labeled. * = type material. ◇ = field isolated fungus.

Table 2.1. GPS coordinates of five red clover fields sampled during the 2 year survey of entomopathogenic fungi in the Willamette Valley, OR.

Field	Year	Coordinates	County
1	2013	45° 00’ 49.4” N, 123° 12’ 29.1” W	Polk
2	2014	44° 55’ 01.1” N, 123° 13’ 48.6” W	Polk
3	2014	44° 39’ 41.9” N, 123° 09’ 45.7” W	Linn
4	2014	44° 28’ 27.1” N, 123° 14’ 25.0” W	Benton
5	2014	44° 30’ 05.8” N, 123° 14’ 27.5” W	Benton

Table 2.2. Diversity of entomopathogenic fungi that infected surrogate *Galleria* larvae exposed to soil samples collected from five red clover fields in the Willamette Valley, OR.

Location	n#	Genera of field isolated fungi				Total
		Beauveria	*Metarhizium*	*Isaria*	*Lecanicillium*	
Field 1	20	8*	-	3	-	11
Field 2	150	16	-	1	-	17
Field 3	150	40	6	-	3	49
Field 4	150	37	7	-	-	44
Field 5	150	30	14	-	-	44
	Total	131	27	4	3	165

= the number of *Galleria* larvae exposed

* = four isolates found from infected clover root borer

Table 2.3. The prevalence of fungal entomopathogens from the genus *Beauveria* observed infecting *Galleria* larvae exposed to soil samples collected from four red clover fields in the Willamette Valley, OR.

Location	n#	Species of field isolated fungus		Total
		B. bassiana	*B. pseudobassiana*	
Field 1	20	8*	-	8
Field 2	150	11	5	16
Field 3	150	40	-	40
Field 4	150	27	10	37
Field 5	150	15	15	30
	Total	101	30	131

= the number of *Galleria* larvae exposed

* = four isolates found from infected clover root borer

Table 2.4. The morphological characteristics of naturally occurring entomopathogenic fungi in the genus *Metarhizium* isolated from red clover fields in the Willamette Valley, OR.

No.	Field	Isolate codes	Species	Conidia/spores (µm)	Upper surface/ Spore color	Lower surface
1.	3	G1LB	*Metarhizium* sp.1	8.0-10.0 x 2.5-3.0	Green	yellow brown
2	3	M22LB	*Metarhizium* sp.2	8.0-10.0 x 2.5-3.0	Green	yellow brown
3	3	GR2LB	*Metarhizium* sp.3	8.0-10.0 x 2.5-3.0	Green	yellow brown
4	5	A4	*M. brunneum*	5.0-7.5 x 2.5-3.0	Green olive	dark orange
5	5	A6	*M. brunneum*	5.0-7.5 x 2.5-3.0	Green olive	dark orange
6	5	A13	*M. brunneum*	5.0-7.5 x 2.5-3.0	Green olive	dark orange
7	5	Ay	*M. brunneum*	5.0-7.5 x 2.5-3.0	Green olive	dark orange
8	2	BB2SB	*M. brunneum*	5.0-7.5 x 2.5-3.0	Green olive	dark orange
9	2	M1SB	*M. brunneum*	5.0-7.5 x 2.5-3.0	Green olive	dark orange
10	2	14SC	*M. brunneum*	5.0-7.5 x 2.5-3.0	Green olive	dark orange
11	2	G1SC	*Metarhizium* sp.4	8.0-12.5 x 2.5-3.0	Dark green-yellowish	gloomy yellow
12	3	G2LB	*Metarhizium* sp.5	8.5-10.0 x 3–3.5	Dark green-yellowish	white yellow
13	3	2LC	*Metarhizium* sp.6	10.0-12.5 x 2.5-3.0	Dark green-yellowish	bright cream
14	2	1SA	*Metarhizium* sp.7	6.0-7.5 x 2.5 – 3.0	Green	dark red
15	2	2SA	*Metarhizium* sp.8	6.0-7.5 x 2.5 – 3.0	Green	gloomy yellow

16	3	G2LA	*Metarhizium* sp.9	6.0-7.5 x 2.5 – 3.0	Green	gloomy yellow
17	3	G3LA	*Metarhizium* sp.10	6.0-7.5 x 2.5 – 3.0	Green	gloomy yellow
18	3	G3LB	*Metarhizium* sp.11	8.0 - 9.0 x 3 - 3.5	Green	gloomy yellow
19	3	G1LC	*Metarhizium* sp.12	6.0-7.5 x 2.5-3.0	Green	dark orange
20	3	G12LA	*Metarhizium* sp.13	6.0-10.0 x 2.5-3.0	Green	gloomy cream
21	3	W11LA	*Metarhizium* sp.14	6.0-7.5 x 2.5-3.0	Green	gloomy yellow
22	3	G1LA	*Metarhizium* sp.15	7.5-10.0 x 3.0-3.5	Green	white yellow
23	3	G13LA	*Metarhizium* sp.16	7.5-10.0 x 2.5-3.0	Green	gloomy cream
24	2	A1	*Metarhizium* sp.17	5.0-7.5 x 2.5-3.0	Green	dark orange
25	2	A11	*Metarhizium* sp.18	5.0-7.5 x 2.5-3.0	Green	dark orange
26	4	M2SB	*Metarhizium* sp.19	5.0-7.5 x 2.5-3.0	Green	dark orange
27	4	M3SB	*Metarhizium* sp.20	5.0-7.5 x 2.5-3.0	Green	dark orange

Table 2.5. Morphological characteristics of naturally occurring entomopathogenic fungi genus in the genus *Beauveria* isolated red clover fields in the Willamette Valley, OR.

No.	Field	Isolate codes	Species	Conidia/spores diameter (µm)	Spore color	Fungal culture characteristics		Isolation method
						Upper surface	Lower surface	
1.	1	FD	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white but red in the center	Isolated from dead *H. obscurus*
2	1	W1	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
3	1	W2	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
4	1	W3	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
5	1	W7	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
6	1	FA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Isolated from dead *H. obscurus*
7	1	FC	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Isolated from dead *H. obscurus*
8	1	E2	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Isolated from dead *H. obscurus*

9	2	4RA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
10	2	5RA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
11	2	31RA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
12	2	2RB	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
13	2	3RB	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
14	2	4RB	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
15	2	6RB	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
16	2	7RB	*B. bassiana*	2-3	white	white, powdery, thin mycelium layer	white but red in the center	Soil baiting
17	2	33RB	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
18	2	34RB	*B. bassiana*	2-3	white	white, powdery, thin mycelium layer	white but red in the center	Soil baiting
19	2	aRB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
20	2	1RC	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium	white	Soil baiting

						layer		
21	2	2RC	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
22	2	3RC	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
23	2	4RC	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
24	2	5RC	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
25	3	A2	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
26	3	A3	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
27	3	A5	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
28	3	A7	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
29	3	A8	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
30	3	A9	*B. bassiana*	2-3	Yellowish	white, powdery,	white	Soil baiting

					white	thin mycelium layer		
31	3	A10	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
32	3	A12	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
33	3	A13	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
34	3	A14	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
35	3	Ab	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
36	3	Ac	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
37	3	Ad	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
38	3	Af	*B. bassiana*	2-3	Yellowish white	white, powdery, this mycelium layer	white	Soil baiting
39	3	B1	*B. bassiana*	2-3	Yellowish white	white, powdery, this mycelium layer	white	Soil baiting

40	3	B2	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
41	3	B3_20	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
42	3	B4	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
43	3	B5	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
44	3	B6	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
45	3	B7	*B.bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
46	3	B1_20	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
47	3	B1green	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
48	3	C1	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
49	3	C2	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting

50	3	C3	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
51	3	C4	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
52	3	C6	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
53	3	C7	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
54	3	C8	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
55	3	C9	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
56	3	C10	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
57	3	C11	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
58	3	C12	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
59	3	C1A	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting

60	3	C2A	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
61	3	C2C	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
62	3	C1B	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
63	3	C3A	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
64	3	C30	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
65	4	3SA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
66	4	4SA	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
67	4	5SA	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
68	4	6SA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
69	4	7SA	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
70	4	8SA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting

71	4	9SA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
72	4	10SA	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
73	4	11SA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
74	4	12SA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
75	4	F1SA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
76	4	F2SA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
77	4	F3SA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
78	4	F4SA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
79	4	F5SA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
80	4	F6SA	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
81	4	3SB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium	white	Soil baiting

						layer		
82	4	4SB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
83	4	F1SB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
84	4	Fs2SlpB	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
85	4	F3SB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
86	4	P1SB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
87	4	P2SB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
88	4	P3SB	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
89	4	B1SB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
90	4	BCSB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
91	4	R1SB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting

92	4	11SC	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
93	4	12SC	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
94	4	13SC	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
95	4	F1SC	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
96	4	F2SC	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
97	4	F3SC	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
98	4	FXSC	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	pale brown	Soil baiting
99	4	P1SC	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
100	4	P2SC	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
101	4	W1SC	*B.bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
102	5	6LA	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting

103	5	32LA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
104	5	F1LA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
105	5	FLLA	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
106	5	W3LA	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
107	5	W12LA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
108	5	W13LA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
109	5	W14LA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
110	5	W15LA	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
111	5	P1LB	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
112	5	P2LB	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
113	5	W0LB	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
114	5	W1LB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium	white	Soil baiting

						layer		
115	5	W2LB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
116	5	W3LB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
117	5	W4LB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
118	5	W5LB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
119	5	W6LB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
120	5	W7LB	*B.bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
121	5	W8LB	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
122	5	W9LB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting
123	5	W10LB	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
124	5	W11LB	*B. bassiana*	2-3	Yellowish white	white, powdery, thin mycelium layer	white	Soil baiting

125	5	1LC	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
126	5	3LC	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
127	5	4LC	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
128	5	5LC	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
129	5	6LC	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
130	5	7LC	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting
131	5	8LC	*B. pseudobassiana*	2-3	Yellowish white	white, thick mycelium	rosy pink	Soil baiting

Table 2.6. Morphological characteristics of naturally occurring entomopathogenic fungi in the genera *Isaria* and *Lecanicillium* isolated red clover fields in the Willamette Valley, OR.

No.	Field	Isolate codes	Species	Conidia/ spores (µm)	Spore color	Fungal culture characteristics		Isolation method
						Upper surface	Lower surface	
1	1	W4	*I. fumosorosea*	3.5-5 x 2.5-3	pink	thick white mycelium with pink spores	white	Soil baiting
2	1	W5	*I. fumosorosea*	3.5-5 x 2.5-3	pink	thick white mycelium with pink spores	white	Soil baiting
3	1	W6	*I. fumosorosea*	3.5-5 x 2.5-3	pink	thick white mycelium with pink spores	white	Soil baiting
4	2	21RC	*I. farinosa*	2.5-5 x 2.5-3	yellow	white-yellowish mycelium	white	Soil baiting
5	3	B2-V	*L. muscarium*	3.5-6 x 1-1.5	white	white mycelium	yellow	Soil baiting
6	3	C5	*L. muscarium*	3.5-6 x 1-1.5	white	white mycelium	yellow	Soil baiting
7	3	C13	*L. muscarium*	3.5-6 x 1-1.5	white	white mycelium	yellow	Soil baiting

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Chapter 3

Pathogenicity of entomopathogenic fungi against the clover root borer (Coleoptera: Curculionidae: Scolytinae), a pest of red clover seed crops

Keywords: *Beauveria, Metarhizium,* microbial control, entomopathogenic fungi, red clover

Introduction

Entomopathogenic fungi are organisms that produce spores that infect and cause fungous diseases in insects. They are widespread in most terrestrial systems, and provide a valuable ecosystem service by regulating insect populations (Hajek, 1997). Because they are natural enemies and are environmentally safe when applied in terrestrial ecosystems (EPA[1], 2016), they have tremendous potential for integration into augmentative and conservation biological control strategies against insect pests, especially subterranean pests for which few management options are available. Fungi in the order Hypocreales *Beauveria bassiana* strain GHA and *Metarhizium brunneum* strain F52 have been isolated, and are being commercially propagated for microbial pesticides such as (Faria et al., 2007).

Hylastinus obscurus Marsham (Coleoptera: Curculionidae: Scolytinae), also known as the clover root borer (CRB), is a subterranean beetle pest of red clover (Rockwood, 1926; Considine, 2008). The larvae and adults mine and feed within the root tissues. At high pest density, little gallery-free tissue remains; consequently, nutrient and moisture transport from the root are hindered, and infested plants turn brown, wilt, and die (Rockwood, 1926). Additionally, the infested mining spots become sites for the growth of the root rot fungus (*Fusarium solani*) and other pathogens that also contribute to the decline of clover stands. In a study by Preuss et al.

(1958), infestation of an average of 1.5 borers per plant reduces 5.5 % of the seed yield. Moreover, Koehler et al. (1961) reported that when ~40% of first year plants are attacked, then almost all plants are infested by the end of second year. Hence, a third year of seed production is generally not economical. Thus, the impact of the clover root borer is considerable, and effective management is critical for economical red clover seed production.

Organochlorine pesticides were effective suppressants of infestations by the clover root borer (Gyrisco et al., 1950; Gyrisco et al., 1954) until their application was banned due to their negative effects on the environment, non-target organisms, and human health (EPA, 2011; 2013). Insecticides that are currently labeled for red clover are not effective against the clover root borer (Rao et al., 2012), and no alternative pest management strategy has been developed for the pest.

Entomopathogenic fungi belonging to the genera *Beauveria* and *Metarhizium* have been evaluated against subterranean insect pests such as the clover weevil, *Sitona lepidus* (Willoughby et al., 1998), larvae of the cabbage root fly, *Delia radicum* (Bruck et al., 2005), the western corn rootworm (Pilz et al., 2007), and click beetle larvae (Ansari et al., 2009). Rockwood (1926), observed the presence of *B. bassiana* (formerly known *as B. globulifera)* in association with *H. obscurus* in western Oregon. However, the virulence of *B.bassiana* and of other entomopathogenic fungi against the clover root borer are not known.

Typically, entomopathogenic fungi are initially evaluated against target pests under laboratory conditions prior to large scale field trials. Often the studies are conducted using sterilized soil or sand, which do not accurately replicate the conditions of the field environment with its many variables including the presence of additional microorganisms. In a study by Baker et al. (1974), biocontrol agents such as *Trichoderma viride, Peziza ostracoderma,* and *Pyronema*

confluens established swiftly and produced large numbers of spores in sterilized soil; however without sterilization these beneficial fungi failed to develop and sporulate. Keller et al. (1989) noted that the Hypocrealean fungi *Beauveria* and *Metarhizium* did not sporulate in unsterilized soil due to fungistatic effect from actinomycetes and other microorganisms. Since then, pathogenicity tests for entomopathogenic fungi have been conducted on sterilized soil to suppress unwanted effects from antagonistic microorganisms. Hence, there is lack of information on the infectivity of fungal entomopathogens against subterranean pests using unsterilized soil which better reflects their pathogenicity in the field.

The objectives of this study were to assess the virulence of entomopathogenic fungi against the clover root borer by comparing the following impacts: 1) use of naturally occurring and commercial sources of entomopathogenic fungi; and 2) use of unsterilized and sterilized soil.

Materials and Methods

Collection of clover root borer and naturally occurring entomopathogenic fungi

The clover root borer cannot be reared on an artificial diet in a laboratory, and hence, adults used in this experiment were obtained from infested roots collected from red clover seed crop fields in western Oregon. The roots (10-15 cm long, 2-3 cm in diameter) were rinsed with water and cut longitudinally into four pieces. The adults were extracted by placing cut roots in *Berlese* funnels overnight, then collected, rinsed in sterilized distilled water and stored in glass jars at 4°C until used in the bioassays.

Naturally occurring fungal entomopathogens were obtained from red clover seed production fields in the Willamette Valley in western Oregon as described in Chapter 2.

Objective 1. Comparison of virulence of naturally occurring and commercial sources of entomopathogenic fungi

For this study, the following entomopathogenic fungi were evaluated: 2 isolates of *Beauveria bassiana* (isolate FD and W1) and 2 isolates of *Isaria fumosorosea* (isolate W4 and W6) from red clover fields, and 2 commercial fungi, *Metarhizium brunneum* (F52) (formerly known as *M. anisopliae*) and *I. fumosorosea* (FE 9901). Commercial fungus *B. bassiana* (strain GHA) was considered for inclusion in the study, but was excluded due to low spore production. All commercial isolates were obtained from the USDA-ARS Horticultural Crops Research Laboratory in Corvallis, OR.

Entomopathogenic fungi were inoculated on Sabouraud Dextrose Agar (SDA) media and maintained at room temperature (25-28°C). After 10-14 days, each fungal culture was harvested by flooding its colony surface with 12 ml of a sterile 0.1% Tween 80 solution. Spores were gently scraped with a sterile inoculation needle to separate them from media, and each harvested spore isolate was passed through a double layer of sterile cloth to separate the spores from the mycelia. The harvested spores were counted using a hemocytometer, and the concentration of the solution was adjusted to 10^8 spores/ml.

The experiment was conducted twice and set up in a randomized block design with 3 replications. Ten clover root borer adults were immersed for 5-7 seconds in a spore suspension of each fungal strain, and a similar infection procedure was conducted with a sterile 0.1% solution of Tween 80 which was used as a negative control. The adults were then air dried and placed on sterile white sand in sterile petri dishes (Falcon, 15 x 45 mm). The petri dishes were placed in an incubator in the dark at a constant temperature of 22^{o}C and 70-75% humidity. The numbers of

dead clover root borer adults were recorded daily for 2 weeks. Each dead adult was incubated on wet filter paper for observations of fungal growth on the cadaver.

Objective 2. Comparison of impacts of naturally occurring entomopathogenic fungi in unsterilized and sterilized soil

For this study, *Beauveria bassiana* (isolate FD) and *Metarhizium brunneum* (isolate A4) were evaluated based on the virulence test result from the Objective 1 study. Spore suspensions were prepared as described above in Objective 1. The entomopathogenic fungi were evaluated at the following four concentrations: 10^4, 10^5, 10^6 and 10^7 spores/ml.

Field soil [Loam (Sand:Silt:Clay, 39.6: 39.85: 20.55)] was collected from a red clover field in Benton county, OR [GPS coordinate: 44^o 28' 27.1" N, 123^o 14' 25.0" W], and divided into two treatments: unsterilized and sterilized. For sterilization, the soil was autoclaved for 2 hours, left in the autoclave overnight and autoclaved for an additional hour. Both soil treatments were dried at room temperature (25-28^{o}C), ground with a porcelain mortar and were stored separately in Ziploc bags at room temperature until used.

Spore suspensions of each of four concentrations of each fungal isolate were poured into separate Ziploc bags filled with 175 grams of unsterilized soil until 27% (w/w) moisture was reached. The soil and spore suspensions in the Ziploc bags were mixed by hand, and 35 grams of inoculated soil was transferred to small cups (40 x 27 x 35) mm. Subsequently, five clover root borer adults were rinsed with distilled water and placed on the inoculated soil in each cup covered with lid. Each spore concentration x soil treatment combination was replicated 5 times. As a negative control, each soil treatment was inoculated with a sterile 0.1% of Tween 80 solution. The cups were maintained in an incubator with no light at a constant temperature of

22^{o}C and 70-75% humidity, and the numbers of dead adults were recorded after a two week exposure period. The experiment was arranged in a split-plot design combined with 3 factorial variables (2 soil treatments x 2 fungal species x 4 spore concentrations).

Data analysis

Data on numbers of dead clover root borers among the fungal treatments in each of the two trials in the Objective 1 study were subjected to a homogeneity test of variances (Bartlett test). The mortality data were transformed using an arc-sine of the square root to stabilize the variances (Snedecor et al., 1989). A Generalized Linear Model (GLM) and Tukey's studentized range test (HSD) were used to analyze the transformed data of adult clover root borers to compare the mean mortality between treatments and controls (SAS, 2002).

Data on adult mortality caused by each fungal strain (*B. bassiana* or *M. brunneum*) from the Objective 2 study were analyzed separately. The proportion of dead beetles exposed to *B. bassiana* and *M. brunneum* were modeled using logistic regression in PROC GENMOD with soil treatment and concentration as class variables. Additionally, overdispersion was tested and adjusted (SAS, 2002). Similar analyses and procedures were performed for data from the experiment with *M. brunneum* except for the exclusion of the zero response data related to the low concentrations and the controls. The number of dead CRB due to contaminant of *B. bassiana* was excluded in the analysis.

Results

Objective 1. Comparison of virulence of naturally occurring and commercial sources of entomopathogenic fungi

The mortality of clover root borer adults was not significantly different among entomopathogenic fungal treatments after 3 days of incubation ($F = 1.3$, $df = 6$, $p = 0.24$). However, on Day 5, 7 and 14, significant differences in the infection of all strains of entomopathogenic fungi were detected compared to the control (Day 5: $F = 13.34$, $df = 6$, $p < 0.0001$; Day 7: $F = 32.54$, $df = 6$, $p < 0.0001$; Day 14: $F = 34.04$, $df = 6$, $p<0.0001$). There was no significant difference in mortality of clover root borers when exposed to commercial and to naturally occurring fungal treatments on Day 14 (Tukey's HSD, $p>0.05$). Mortality in negative control was caused by infection with *B. bassiana*.

In clover root borers that became infected with fungal entomopathogens, fungal mycelial growth was observed from the thorax, mouth and anal regions within 2-3 days of incubation period. The color of spores that emerged from dead adults was used to confirm the genus level of their identity (Fig. 3.1 and 3.2).

Objective 2. Comparison of impacts of naturally occurring entomopathogenic fungi in unsterilized and sterilized soil

Assessment of Hylastinus mortality exposed to field soil inoculated with B. bassiana (isolate FD)

No significant difference in mortality of adult beetles exposed to soil treatments (with and without sterilization) was detected ($F= 2.32$, $df = 1$, $p = 0.1359$) though mortality was slightly higher (~ 5-10%) in the unsterilized treatments for all the concentrations tested (Fig. 3.3). There was also no interaction between soil treatments and concentrations ($F= 0.38$, $df = 4$, $p = 0.8231$). With all concentrations used with unsterilized soil, beetle mortality ranged from 40% to 60%. However, beetle mortality with all the treatments did not differ significantly from the controls ($F= 1.63$, $df= 4$, $p= 0.164$). Mortality of clover root borers caused by *B. bassiana* infection in the

negative control was relatively high, at 40% and 24% in unsterilized and sterilized soil, respectively.

Assessment of Hylastinus mortality exposed on field soil inoculated with M. brunneum (isolate A4)

There was no significant difference in the mortality of clover root borers to both soil treatments inoculated with spore suspensions of *M. brunneum* (F= 4.53, df= 1, p = 0.1103), although the percentage of dead beetles was slightly higher (~10%) when they were exposed to unsterilized soil (Fig. 3.4). There was no interaction between soil treatments and spore concentrations (F= 1.57, df= 4, p = 0.2394). However, mortality differed significantly between the two highest levels (10^6 and 10^7) of spore concentration of *M. brunneum* (F= 20.79, df= 1, p = 0.0003).

In the two lowest spore concentrations of *M. brunneum* (isolate A4), we observed clover root borers that were infected with *B. bassiana* (identified by spore color). The contaminant fungus caused up to 80% mortality.

Discussion

This is the first study to assess the virulence of entomopathogenic fungi against the clover root borer. Both commercial and naturally occurring entomopathogenic fungi isolated in red clover fields have potential for controlling the population of the clover root borer.

The current study confirms that two isolates *B. bassiana* that occur naturally in Oregon are effective against the clover root borer. Rockwood (1926) had observed the association of the fungus with *H. obscurus* in Oregon but no information was provided on its pathogenicity. The

other natural-occurring fungus in Chapter 2, *I. fumosorosea* (isolate W4) was also effective but caused slightly lower mortality (93%) compared with two isolates of *B. bassiana* (98%). With the commercial strain of *I. fumosorosea* (FE 9901), 98% mortality was observed within 14 days while with *B. bassiana*, this level of mortality occurred within 7 days. The second commercial fungus that was tested, *M. brunneum* (F52) also caused high mortality (98%) within 7 days. Thus, both *B. bassiana* and *M. brunneum* are the best options for suppressing clover root borers within the shortest period of time.

The high level of pathogenicity of *I. fumosorosea* contrasts with the results obtained by Hunter et al. (2011) which showed that *I. fumosorosea* infected Curculionid pest at low levels - 31-47% mortality of *Diaprepes abreviatus* was recorded after 9-15 days incubation period. They observed high levels of mortality (80-100%) with citrus aphids, leafhoppers and psyllids. The high mortality that was recorded in the current study against the beetle pest is noteworthy as the fungus is labeled for controlling white flies, aphids and thrips.

As indicated earlier, virulence studies with entomopathogenic fungi have often been conducted using sterilized soil or sand (Shapiro et al., 2003; Bruck et al., 2005) but these do not accurately reflect the conditions of the field environment with its many variables including the presence of additional microorganisms. In the current study, we assessed the virulence of *B. bassiana* and *M. brunneum* using both unsterilized and sterilized field soil. Our findings revealed that after 2 weeks, with both *B. bassiana* and *M. brunneum,* clover root borer mortality was higher on unsterilized compared to sterilized soil. These results contrast with those reported in the study by McCoy et al. (1988) in which conidial germination of *Beauveria* and *Metarhizium* was hindered in unsterilized soil, and hence, soil sterilization was performed to remove the

inhibitory activity from antagonistic microorganisms. Higher infectivity of *B. bassiana* and *M. brunneum* in the current study could be due to adaptation of these field-isolated fungi to micro-organisms in the field from which they were originally isolated.

In this pathogenicity study conducted with sterilized and unsterilized soil, beetles died in the controls as a result of *B. bassiana* infection. As clover root borers cannot be reared, beetles used in this study were collected from the field, and it is possible that some of them may have been infected with *B. bassiana* spores prior to the study. As indicated in Chapter 2, the dominant naturally-occurring entomopathogenic in red clover seed fields was *B. bassiana*. The expectation was that beetle mortality would be lower in the unsterilized treatment due to the presence of potential antagonistic influences by actinomycetes and other microorganisms. However, in the current study, mortality was higher with the unsterilized soil and this could be because of the additional infectivity from naturally occurring *B. bassiana* in the soil collected from the field. We were not able to do DNA molecular testing to distinguish between the fungal treatment of *B. bassiana* and the contaminant. However, morphological observation of spores and conidiophores using a microscope confirmed the identification of *B. bassiana*.

Mortality with *B. bassiana* was lower in this second study (especially with two high levels of spore concentration: 10^6 and 10^7 spores/ml) compared with the study conducted under Objective 1 using sterile white sand. One possibility is that a threshold level of *B. bassiana* spores had been reached and spore saturation in the soil probably led to intraspecific competition between endogenous *B. bassiana* and the extraneous one due to limited space, oxygen, and nutrients, thus, preventing fungus from colonizing the soil and sporulating (Baker et al., 1974; Brownbridge et al., 2006). Hence, adding more inoculum to the soil does not always result in

higher mortality.

In contrast to the study with *B. bassiana*, with *M. brunneum*, mortality of *H. obscurus* adults was positively correlated with the spore concentration, which is similar to the results observed when *L. lecanii* was evaluated against aphids (Hall, 1976) and *B. bassiana* against *Sitona lepidus* (Brownbridge et al., 2006).

In the current study, when CRB exposed to unsterilized and sterilized soil inoculated with *M. brunneum* (isolate A4), 80% and 70% mortality was observed respectively after 2 weeks. These results are similar to the study by Bruck et al. (2005) in which 85% of larvae of the cabbage maggot, *Delia radicum,* exposed to soil treated with 3.85 x 10^6 spores/g dry soil of *M. brunneum* (F52) were infected after two weeks. The response to *M. brunneum* exposure was faster in a study with adult ambrosia beetles, *Xylosandrus crassiusculus* (a beetle pest belonging Curculionidae) which were infected (78.9%) within 4 days after the beetles were sprayed (direct contact) with 600 spores/mm^2 ($\pm 0.6 \times 10^7$spores/ml) (Castrillo et al., 2013). The differences in virulence of *M. brunneum* across these studies could be due to infection method of the pathogen to the target host. Spray method causes direct mortality effect to the host whereas with soil inoculation, the colonization and development of fungal propagules in the soil is necessary before infecting the insect.

The higher level of infection with *M. brunneum* (isolate A4) in unsterilized soil observed in the current study is surprising since *Metarhizium* is known to require sterile soil conditions as it has a lesser ability to combat the fungistatic effects of the soil environment and other microbial communities compared to *B. bassiana* (Sharapov et al., 1984; Keller et al., 1989). It is possible that due to sterilization process, unknown changes happened which may hindered the

development of inoculated fungal spores. At low concentrations of *M. brunneum*, we observed considerable mortality due to contaminating *B. bassiana*, but the number of *B. bassiana* infections were gradually reduced as the spore concentration of *M. brunneum* in the soil increased. This indicates that high spore concentration is necessary for *M. brunneum* to compete against dominant microorganisms, sporulate and cause mortality against the target host.

Thus, pathogenicity of entomopathogenic fungi depends on several biotic factors including spore concentration, and the presence of other competing or antagonistic microorganisms.

Conclusions

Naturally occurring and commercially available entomopathogenic fungi have potential for suppressing populations of clover root borers. While the commercial strains can be used for augmentative biological or microbial control, naturally occurring strains can be integrated into a conservation biological control strategy such as reduction of use fungicides and providing adequate irrigation for their survivorship in the agroecosystems. Further research is needed to assess their virulence in large scale field studies for determining concentrations that are most effective under existing abiotic and biotic conditions including the presence of competing or antagonistic microorganisms.

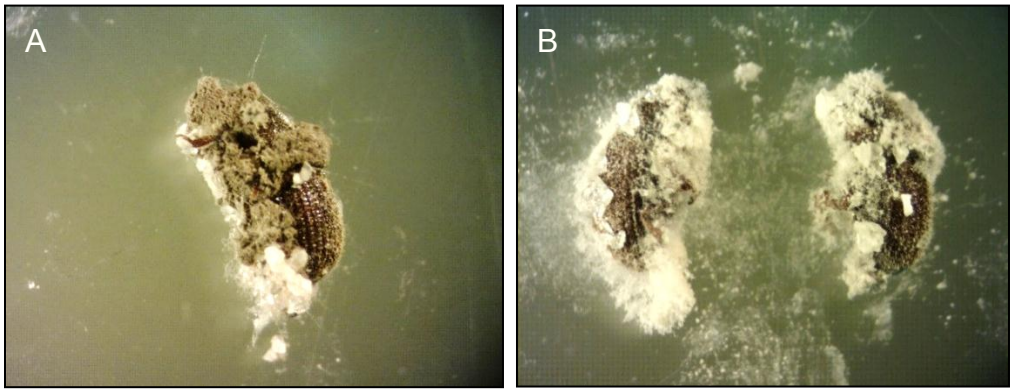

Fig. 3.1. Emergence of fungal propagules of different species of entomopathogenic fungi from CRB adults exposed to commercial entomopathogenic fungi. (A) *M. brunneum* (F52) and (B) *I. fumosorosea* (FE9901).

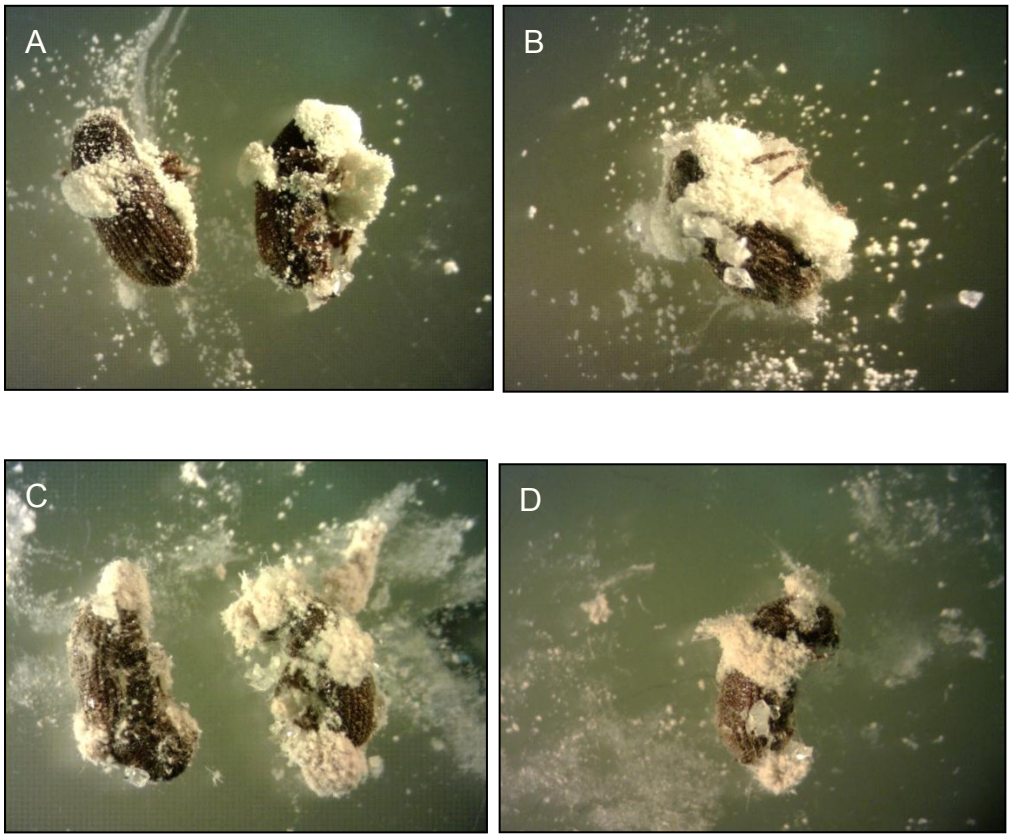

Fig. 3.2. Emergence of fungal propagules of different species of entomopathogenic fungi from CRB adults an after treated with field isolated entomopathogenic fungi. (A) *B. bassiana* isolate FD, isolate W1 (B); *I. fumosorosea* isolate W4 (C), isolate W6 (D).

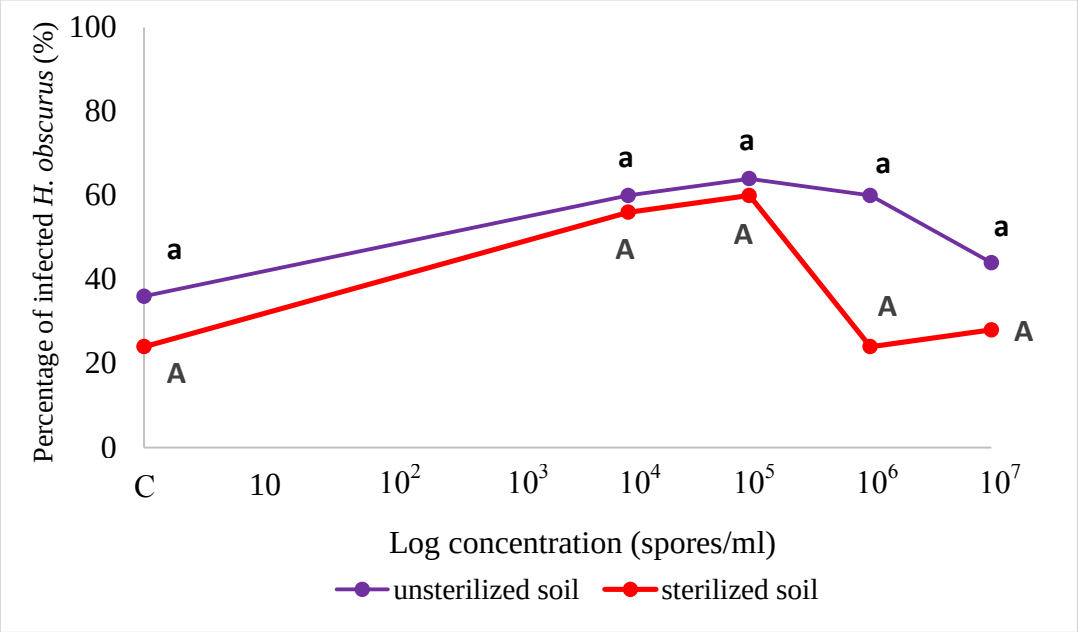

Fig 3.3. Mortality of CRB adult exposed to unsterilized and sterilized soil inoculated with difference concentration of *B. bassiana* isolate FD in the laboratory bioassay test. No difference between two soil treatments ($p > 0.05$). Percentages share similar letter were not significantly different ($\alpha = 0.05$). C : negative controls.

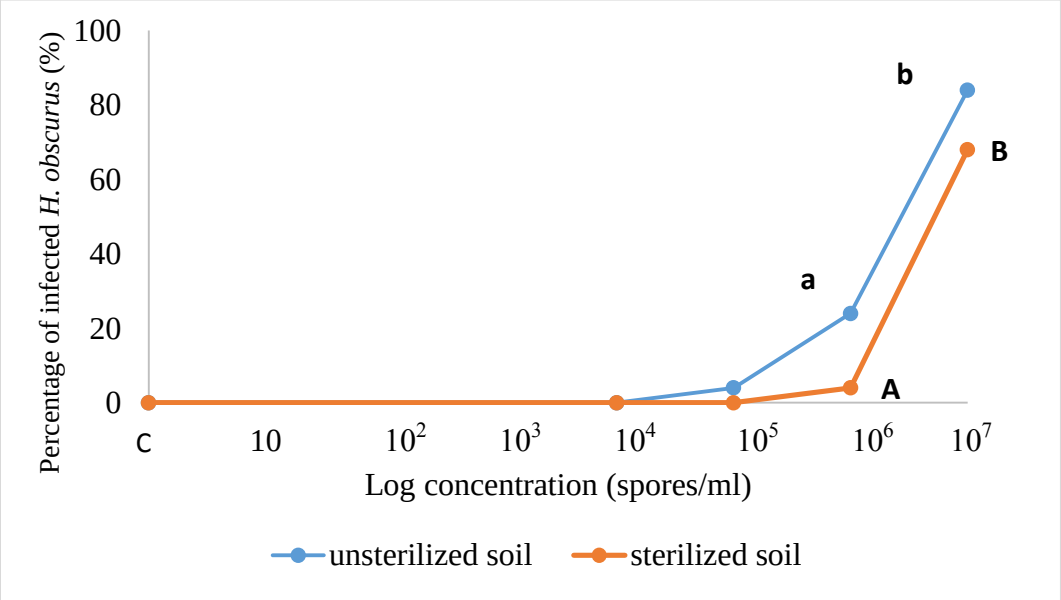

Fig. 3.4. Mortality of CRB adult exposed to unsterilized and sterilized soil inoculated with the field isolated fungus, *M. brunneum* isolate A4 in the laboratory bioassay test. No difference between two soil treatments ($p > 0.05$). Percentages with different letter (a-b) were significantly different ($\alpha = 0.05$). C: negative controls.

Table 3.1. Mortality (% ± SE) of *H. obscurus* adults treated with field isolated and commercial fungal entomopathogens at different post treatment periods

Fungal treatments[#]	**Mortality (%)**			
	3 days	**5 days**	**7 days**	**14 days**
Beauveria bassiana (FD)	0.29±0.17 a	39.61±0.19 c	95.46 ±0.21 cd	98.33±0.17 b
B. bassiana (W1)	2.57±0.21 a	39.53±0.22 c	97.71±0.23 d	98.33±0.17 b
Isaria fumosorosea (W6)	0.29±017 a	11.46±0.08 ab	55.96±0.38 b	90.78±0.41 b
I. fumosorosea (W4)	0.29±0.17 a	23.99 ±0.20 bc	69.12±0.18 bc	93.3±0.28 b
I. fumosorosea (FE9901)*	0±0 a	51.06±0.35 c	85.66±0.34 bcd	98.33±0.17 b
Metarhizium brunneum (F52)*	3.37±0.27 a	49.99±0.31 c	97.75±0.32 d	100±0 b
Control	0.29±0.17 a	0.29±0.17 a	0.29±0.17 a	11.06± 0.23 a
	$p > 0.05$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$

[#] spores concentration tested: 1×10^8 spores/ml
* commercial entomopathogenic fungus

Percentage mortality in the same column with different letters are significantly different (HSD Tukey test, $\alpha = 0.05$)

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Chapter 4

Summary

Review of findings

Entomopathogenic fungi are spore producing- pathogens that infect insects and some invertebrates and provide ecological services in regulating their populations. Since they are natural enemies and are environmentally safe in terrestrial ecosystems, they have tremendous potential for integration into augmentative and conservation biological control strategy against insect pests especially subterranean pests for which few management options are available. They can also be developed commercially and used for augmentative microbial control. Entomopathogenic fungi are of particular importance for subterranean pests for which few management options are available. Use of an entomopathogenic fungus for pest suppression requires an understanding of the occurrence of naturally fungal entomopathogens and their infectivity against the pest target.

Based on the studies conducted for this thesis, several entomopathogenic fungi have potential as biological control agents against the clover root borer, *Hylastinus obscurus*, a key subterranean pest that causes considerable economic damage to red clover seed crops in western Oregon. A survey of entomopathogenic fungi documented that root-zone soil of red clover plants are colonized by at least six species of fungal entomopathogens belonging to four genera - *Beauveri*a (the dominant genus), *Isaria, Lecanicillium* and *Metarhizium,* despite the use of pesticides in the fields. In this study, four species of entomopathogenic fungi – *Beauveria pseudobassiaana, Isaria fumosorosea, I. farinosa* and *Lecanicillium muscarium* were the first record in the Pacific Northwest.

Three species of entomopathogenic fungi, *Beauveria bassiana, Isaria fumosorosea* and *Metarhizium brunneum* were highly virulent against adults of *H. obscurus* in a laboratory study conducted with white sand as the substrate in petri dishes. Close to 100% *H. obscurus* by *B. bassiana* and *M. brunneum* occurred within 7 days while the same level of mortality occurred over 14 days with *I. fumosorosea*. In the laboratory bioassay conducted by exposing the adults on the unsterilized and sterilized soil treated with fungal treatments in cups, the field collected *M. brunneum* isolate A4 exhibited greater mortality (80%) than *B. bassiana* isolate FD (60%). Therefore, *M. brunneum* has greater potential for management strategy to the clover root borer.

Study limitations

Attempts were made to rear clover root borers in the lab but the beetles did not reproduce on any artificial diet. Hence the beetles used in the virulence tests were collected from red clover fields where naturally occurring *B. bassiana* was present. As a result, beetles used in the study conducted with sterilized and unsterilized soils appeared to have been infected with field isolated *B. bassiana* prior to the study as beetles in the controls and the treatments with various concentrations of *M. brunneum* were observed to be infected with *B. bassiana*. This affected interpretation of the results for experiments with both *B. bassiana* and *M. brunneum* in both the sterilized and unsterilized treatments.

Pathogenicity tests could not be evaluated on larval stages. Aside the fact that CRB cannot be reared on artificial diet, it is a challenge and tedious work to collect the larvae out from the roots. However, it is possible if the CRB rearing effort is successful.

The study was also limited by availability of adult *H. obscurus* adults which limited the number of replications and the numbers of questions that could be addressed. Beetles could not be collected in summer as the ground was too hard to dig plants. Beetles were collected from plants at other times in the year but could not be preserved for long periods.

Future research

Naturally occurring entomopathogenic fungi can be integrated into a conservation control strategy while the commercial strains can be used for augmentative biological or microbial control. Further research is needed to assess the virulence of entomopathogenic fungi in large scale field studies for determining concentrations that are most effective under existing abiotic and biotic conditions including the presence of competing or antagonistic microorganisms.

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