

AN ABSTRACT OF THE THESIS OF

Ryan M. Kepler for the degree of Master of Science in Entomology presented on July 13, 2005.

Title: An Evaluation of Factors Influencing the Management of *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae)

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The black vine weevil (BVW), *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae) is a persistent pest of nursery operations in the United States, feeding on over 140 species of plants. The goals of this research were to 1) assess the behavioral response of BVW to a commonly employed insecticide bifenthrin (Talstar 0.2G®) and the entomopathogenic fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae), 2) determine what effect application of *M. anisopliae* has on root growth when applied as a root dip and quantify differences in BVW larval herbivory between plants treated with *M. anisopliae* and controls.

The behavioral response of BVW larvae to bifenthrin and *M. anisopliae* were assessed using a still air olfactometer developed for the purposes of this experiment. Larvae were allowed to choose between *M. anisopliae* (1×10^6 spores per g dry media) and untreated media, bifenthrin (25 ppm) and untreated media, as well as *M. anisopliae* and bifenthrin treated media. For all comparisons, experiments were conducted without plants in the system to test for innate

responses, as well as with plants to test typical nursery conditions. Larvae were significantly deterred from potting media incorporated with bifenthrin in all situations tested. Larvae exhibited no such deterrence to media incorporated with *M. anisopliae*. In trials without plants, larvae exhibited no discernable pattern of movement in response to *M. anisopliae*. With plants in the system larvae showed a significant attraction to plants growing in soil treated with *M. anisopliae*.

The effect of treatment of plant roots with *M. anisopliae* on root growth and herbivory by BVW larvae was assessed in the following manner. Plants were randomly allocated to one of three treatments: immersion in 1×10^6 spores mL⁻¹ of *M. anisopliae* up to the root crown, immersion in 0.05% Tween 80 (spore surfactant) or an untreated control. Each treatment was performed with and without BVW larval infestation. Measurements of root length for each plant used in these experiments were obtained prior to treatment, and two and six weeks after BVW larval infestation. Results suggest that there is no inherent reduction in root growth as a result of treatment with *M. anisopliae*. The effects of *M. anisopliae* on BVW larval feeding are less clear, but there is an indication that some level of protection may be afforded.

An Evaluation of Factors Influencing the Management of *Otiorhynchus sulcatus*
(F.) (Coleoptera: Curculionidae)

by
Ryan M. Kepler

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An Evaluation of Factors Influencing the Management of *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae)

CHAPTER 1. Introduction

The black vine weevil (BVW), *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae), is a pest of increasing importance for agricultural and horticultural crops worldwide in both field and glasshouse settings. The weevil is endemic to temperate regions in Europe (Moorhouse et al. 1992). Movement of infested plant material allowed the BVW to become established in North America, Chile, Japan, Australia and New Zealand (Moorhouse et al. 1992). The first record of its presence in the USA is from 1835, but recorded under the name *Curculio apiculatus*. The first record of economic damage for the USA is in 1871, with European records as early as 1834 (Smith 1932). Early records for this species often use the genus name *Brachyrhinus*. In North America, *O. sulcatus* is commonly found on both coasts. On the east coast it extends as far west as the Mississippi River and as far south as parts of North Carolina. The northern extent reaches into Canada. The habitat occupied in western North America begins on the eastern edge of Idaho and reaches the coast, extending to southern California and north to British Columbia. The distribution is described as spotty due to the tendency of populations to occur in clumps (Warner and Negley 1976).

The BVW typically overwinters as a prepupae or last instar. A long, warm fall season in association with relatively benign winter conditions may allow for adults to overwinter and emerge the following spring and begin laying eggs (Smith 1932). Overwintering larvae complete their development and adults eclose from

early June to late July, with a peak eclosion near the middle of June. Oviposition begins in the middle of July for adults that emerge in June, after a preoviposition period of 28 - 50 days. Some eggs laid very early may produce individuals capable of developing into adults before winter (Smith 1932). In the greenhouse setting, the life cycle may be advanced by two months (Moorhouse et al. 1992). Adults may also remain active in the greenhouse through December and January, all the while continuing to oviposit (Smith 1932).

The BVW is a highly polyphagous species. Smith (1932) listed 77 plant species that were fed upon. An additional 70 plant species were listed by the USDA Plant Pest Survey Files (Warner and Negley 1976). Particular plant groups of importance fed upon by BVW include *Rhododendron spp.*, *Cyclamen spp.*, *Taxus spp.* and *Fragraria spp.* Evidence of feeding by adults is a notching on the edges of leaves. Unless the population is extremely large, adult feeding rarely causes plant mortality and the worst of the damage is cosmetic. Feeding by larvae on roots can cause high levels of damage to plants. For certain species (e.g. *Cyclamen*) one weevil is enough to kill the plant (Moorhouse 1990). Larvae have been seen to girdle the root system of a plant and in some cases have been found in the stem of a plant above ground level. It is for these reasons that the larval stage is the part of the life cycle that is targeted in pest management strategies.

Management practices for BVW include cultural methods such as maintenance of proper nursery sanitation and the removal of potential source habitats in the surrounding environment. Most growers initiate spray programs against adults in the spring before the beginning of oviposition. However mild

winters may permit survival of adults from the previous year to begin egg-laying much sooner than current year adults, thereby hampering control efforts (Bruck 2004a). The failure of spray programs against adults has resulted in growers impregnating potting media with synthetic insecticides in an effort to reduce the deleterious impacts of larval feeding. For horticultural operations in Oregon, one of the most commonly used insecticides is the synthetic pyrethroid bifenthrin, marketed under the trade name Talstar® 0.2G. The control obtained by use of this product can be inconsistent, depending upon the plant under protection, type of potting media used, and the application rate of the pesticide (Cowles 2001).

The degradation of bifenthrin is dependent upon soil characteristics. The potential of movement in soil is limited by its attraction to organic matter. This pesticide is known to be acutely toxic to stream invertebrates and fish (FMC 1999). Additionally, it was found to negatively impact populations of beneficial insects in British strawberry fields (Easterbrook 1997). Given the potential non-target effects of bifenthrin, as well as increasing public concern of the use of synthetic pesticides in plant production, there is a need to develop management practices for this pest that address these pressures. The wide host range of BVW places serious constraints on the ability to breed resistant varieties.

The entomopathogenic fungus *Metarhizium anisopliae* (Metchnikoff.) Sorokin (Hypocreales: Clavicipitaceae) has been successfully employed as a microbial control agent against BVW (Moorhouse 1990, Moorhouse et al. 1993a, Bruck 2005) and presents itself as a candidate for adoption into integrated farming practices. A commercial formulation of *M. anisopliae* (strain F52) (Earth

BioSciences, New Haven, CT) is currently registered with the US Environmental Protection Agency (EPA) for use against several insects, including BVW.

Infection occurs when conidia come into contact with the cuticle of an insect, producing a germ tube which penetrates the hemocoel through the production of cuticle degrading enzymes and mechanical pressure (Charnley 1984). The fungus then multiplies as yeast, switching to a mycelial growth phase before sporulating on the external surface of the insect.

Development of successful insect integrated pest management programs are dependent upon consideration of the full array of factors that can prevent management objectives from being realized. The role of behavior in pest management settings is poorly understood, but advances are being made. Behavior can play a role in insect resistance to a chemical or pathogen. By understanding the stimuli that influence resistance behavior, as well as host selection by a pest, more informed decisions can be made about a management program and result in more effective control. Behavioral resistance can be defined as “evolved behaviors that reduce an insect’s exposure to toxic compounds or that allow an insect to survive in what would otherwise be a toxic and fatal environment” (Sparks et al. 1989). Behavioral resistance need not function outside of biochemical or physiological resistance and may sometimes be directly linked (Sparks et al. 1989). Some aspects of an insect’s biology may contribute to the development of behavioral resistance, such as mobility, an untreated area to escape to and insecticides that possess some level of irritancy or repellence (Sparks et al. 1985).

To date there have been no studies to assess the behavioral response of BVW larvae to either bifenthrin or *M. anisopliae*. Thompson and Brandenburg (2005) found that the presence of bifenthrin in soil affects the behavior of late instar mole crickets (Orthoptera: Gryllotalpidae) in a way that reduces exposure to the chemical. Their trials consisted of a bifenthrin-treated layer of soil beneath a layer of an untreated soil. A significant proportion of crickets were located in the untreated top layer of soil. Tunneling was also restricted to the untreated layer, reducing the passage of crickets through treated soil. The Japanese beetle *Popillia japonica* Newman (Coleoptera: Scarabaeidae) was found to avoid soil that was inoculated with the fungus *M. anisopliae* (Villani et al. 1994). It is therefore germane to test for the same trend of effects on the behavior of BVW.

In Chapter 3 we developed a still air olfactometer (SAO) capable of accurate larval behavior assessment. This SAO was then employed to test the behavioral response of BVW larvae to potting media incorporated with bifenthrin or *M. anisopliae*. Trials were conducted to test for innate treatment responses, as well as responses under simulated field conditions. Knowledge of whether or not BVW is able to make a behavioral response to currently available control measures can help pest managers make a better informed decision when implementing a pest control program, thereby increasing their effectiveness.

It has recently been shown that the entomopathogenic fungus *M. anisopliae* is rhizosphere competent (Hu and St. Leger 2002, Bruck 2005). This capacity to associate closely with plants is not surprising, given the placement of this fungus in the family Clavicipitaceae (Sung et al. 2001), a group containing many known

plant pathogens. *Beauveria bassiana* Vuillemin (Hypocreales: Clavicipitaceae) is another entomopathogen from the same family capable of close association with plants. *Beauveria bassiana* is an endophyte of corn (Bing and Lewis 1991), and has recently been shown to be rhizosphere competent as well (Bruck, unpublished data). This ability of certain entomopathogenic fungi to enter into such an intimate relationship with plants presents a novel method to protect plants from insect herbivory.

In the case of *M. anisopliae*, plant roots appear to provide a refuge capable of preventing the same degree of population decline observed in the bulk soil (Hu and St. Leger 2002, Bruck 2005). When applied as a broadcast treatment to field grown cabbage, the population in the bulk soil declined from a mean of 2.45×10^5 to 2.96×10^4 propagules g^{-1} dry soil after four months, whereas the population at the root base was near that observed after application. There was a four fold increase observed between populations in the inner and outer rhizosphere, indicating that plant root exudates may contribute to the persistence of *M. anisopliae* in the soil. Additionally, the detection of *M. anisopliae* on roots as far as 10 cm away from the stem of the plant indicates that the fungus is capable of vertical movement in the soil profile; however the mechanism is currently unclear (Hu and St. Leger 2002). Trials examining the difference in fungal populations between bulk and rhizosphere soil in pots containing rooted cuttings of *Picea abies* var. *nidiformis* planted in soilless potting media found rhizosphere populations to again be resistant to the decline observed for the bulk soil (Bruck 2005).

Bruck (2005) tested the ability of *M. anisopliae* to infect BVW larvae when applied as a root dip to *P. abies* at a rate of 2×10^6 spores ml⁻¹. Used in this manner, 76% of larvae became infected. Infected larvae were located near the roots, and in some cases had begun feeding before succumbing to infection. It was determined that the level of infection resulting from this manner of treatment warrants further investigation into development of a system that can be used by commercial nursery operations to protect against BVW and possibly other nursery pests.

In Chapter 4 we examine the effects of root inoculation with *M. anisopliae* alone and in the presence of BVW larvae. The metric measured in this experiment was the overall change in root length, as determined by comparisons of images obtained before treatment and at two sampling dates post treatment. Images were obtained through the use of the WinRhizo2001 (Regent, Quebec, Canada) scanning software package. Trials conducted without the presence of BVW larvae in pots with treated plants allowed for an assessment of changes in root length due to the inoculation of *M. anisopliae* alone. Effects on plant growth in the absence of pests can be an important consideration when selecting microbial control organisms to be used in the rhizosphere (Kozdroj et al. 2004). Trials conducted in the presence of larvae allowed for the level of root protection afforded by treatment with *M. anisopliae* to be assessed. Although larval mortality as a result of rhizosphere inoculation with *M. anisopliae* has been achieved (Bruck 2005), the degree to which this actually reduces losses from herbivory are unknown.

CHAPTER 2. Literature Review

Ryan M. Kepler

Metarhizium anisopliae

Taxonomy and basic biology. The entomopathogenic fungus

Metarhizium anisopliae was first described by Metschnikoff in 1879, although he assigned it to the genus *Entomophthora*. A revision of this classification was made by Sorokin, who changed the genus to *Metarhizium*. No further revisions at the genus or species level have been made and the correct classification is *M. anisopliae* (Metschnikoff) Sorokin (Zimmermann 1993). A review of the genus by Tulloch (1976) recognized two species, *M. flavoviride* and *M. anisopliae*, the later being divided into the long-spored variety *major* and a short-spored variety, the type form, *anisopliae*. The size of conidia, specifically length, has traditionally been used to distinguish between the long-spored and short-spored forms. The long-spored form has conidia lengths of 9.0 - 18.0 μm , usually 10.0 - 14.0 μm and the short-spored form has lengths between 3.5 - 9.0 μm , usually between 5.0 - 8.0 μm (Tulloch 1976).

The advent of molecular techniques has allowed classification schemes to expand beyond morphological characteristics and examine the genetic material of an organism to determine its similarity (or dissimilarity) to other organisms. Utilizing techniques to examine rDNA, Driver et al. (2000) found 10 clades within the genus *Metarhizium*, based on available material. This includes the species *M. album*, regarded by Tulloch to be an immature form of *M. anisopliae*, five varieties of *M. flavoviride*, and four varieties of *M. anisopliae* (*acridum*, *lepidiotum*, *anisopliae*, and *majus*). An in-depth examination of rDNA in *Metarhizium* by Pantou et al. (2003) supports the findings for *M. anisopliae*,

however, they were unable to show support for some of the varieties of *M. flavoviride*.

There is no known sexually reproductive stage for *M. anisopliae*.

Although recombinant genotypes have been found, they are considered a result of parasexuality (Bidochka et al. 2001). This lack of a true sexual component earns *M. anisopliae* a place in the Deuteromycota (Fungi Imperfecti) (McCoy et al. 1988). It is also considered a hyphomycete due to the production of free conidia. Recent genetic work indicates that the genus belongs in the order Hypocreales and the family Clavicipitaceae (Sung et al. 2001). The Clavicipitaceae is a family containing many pathogens of both plants and insects.

Distribution. *Metarhizium anisopliae* is a common fungus that has a worldwide distribution (Zimmerman 1993), including the Pacific Northwest (Bruck 2004b). With such a large distribution, many different strains have developed, most likely in response to different host species and the habitats associated with them (Bidochka et al. 2001). The habitat preferences of *M. anisopliae* remain unclear. A study performed in Sweden found that geographic location was the only significant factor that influenced the occurrence of the fungus (Vänninen 1995). However, in Canada, a clear preference for agricultural sites was observed (Bidochka et al. 1998).

Growth parameters. Different strains of *M. anisopliae* have the ability to tolerate a wide breadth of environmental conditions. Optimal growth occurs at pHs between 5 - 8 (Hallsworth and Magan 1996). Strains capable of producing colonies at temperatures as low as 8°C are known (Amritha De Croos and

Bidochka 1999). Different tolerances to UVB have also been observed (Braga et al. 2001).

Metarhizium anisopliae grows optimally at temperatures between 25 - 35°C (Walstad et al. 1970, Hallsworth and Magan 1996), a pH of about 6 (Hallsworth and Magan 1996) and a relative humidity of 100% (Walstad et al. 1970) in artificial media. Temperatures above 35°C prevent spore germination and above 49°C cause death of the fungus (Walstad et al. 1970). Temperatures below 4°C inhibit growth (Hallsworth and Magan 1996, Ekesi et al. 1999).

When *M. anisopliae* is present in soil, the degree of soil saturation can be a serious limitation to fungal persistence. Li and Holdom (1993) found that conidial survival declined most rapidly in very wet soils. Some isolates in saturated soils may also be more sensitive to temperature differences than those in drier soils (Ekesi et al. 2003). Laboratory trials by Zimmerman (1982) showed that viability of conidia at high temperatures was enhanced as relative humidity decreased, indicating a relationship between desiccation and the thermal death point.

Mode of Infection. The production of chitinases is widespread in fungi. Because chitin is an important structural component of the rigid fungal cell wall, chitinases play an important role in allowing the hyphae to elongate (Gooday et al. 1992). In addition, chitinases in association with a suite of other enzymes such as lipases, esterases and proteases, as well as mechanical pressure aid in the penetration of host cuticle by entomopathogenic fungi (Charnley 1984). When a spore becomes attached to the insect cuticle by nonspecific, hydrophobic interactions (Boucias et al. 1988), proteases and esterases are produced first,

allowing the chitin fibers to be exposed (St. Leger et al. 1986a), the presence of which induces the elevated production of chitinases (St. Leger et al. 1986b) and allows further degradation of the insect cuticle.

Variability in the production of cuticle degrading enzymes has been observed between isolates (Gupta et al. 1991, St. Leger et al. 1993). This variability may affect the efficiency of different strains to penetrate the host cuticle, therefore potentially influencing the virulence of a given strain (Gupta et al. 1991). Variability across strains has also been observed for the production of destruxins, compounds that possibly aid in mortality for some species (Kershaw et al. 1999).

Historical and Current Usage. *Metarhizium anisopliae* has a long history as an insecticide. Shortly after the possibility of using a microorganism to control pest species was postulated, Metchnikoff attempted to use *M. anisopliae* to control the wheat cockchafer, *Anisoplia austriaca* Herbst (Coleoptera: Scarabaeidae) and the sugar beet curculio, *Cleonus puctivenris* Germ (Coleoptera: Curculionidae) in 1879. Initial results were favorable, and large scale production attempted, however lack of knowledge about the nature of epizootics lead to the eventual cessation of the program (McCoy et al. 1988).

Recent uses of *M. anisopliae* include: widespread applications in Brazil to control spittlebug on sugarcane; *Mahanarva* spp. (Homoptera: Cercopidae); Australia to control pasture cockchafer (Coleoptera: Scarabaeidae); China to control *Colasposoma* spp. (Coleoptera: Chrysomelidae); regions of the South Pacific to control the rhinoceros beetle *Oryctes rhinoceros* (L.) (Coleoptera:

Scarabaeidae); United States to control the pecan weevil (McCoy et al. 1988) and Africa to control locusts (Orthoptera: Acrididae) (Shah and Pell 2003). Positive results using *M. anisopliae* to control the black vine weevil (BV W) *Otiorynchus sulcatus* (F.) (Coleoptera: Curculionidae) (Moorhouse 1990, Moorhouse et al. 1993a,) and corn rootworm *Diabrotica* spp. (Coleoptera: Chrysomelidae) (Krueger and Roberts 1997) amongst others have been obtained.

Safety Concerns. *Metarhizium anisopliae* appears to be relatively benign to non-arthropod organisms. Although *M. anisopliae* as a species displays pathogenicity to a wide array of arthropods, individual strains show a more limited host range (Zimmerman 1993). This accounts for the limited non-target arthropod effects observed by some researchers (e.g. Rath et al. 1995). A review of non-target effects showed no adverse affects to fish, mice, rats, guinea pigs, quail or rabbits (Zimmerman 1993). Based on these results, Zimmerman (1993) determined the fungus to be safe and advocated its use as a biological control agent. A review by Strasser et al. (2000) concluded the fungi currently used in biocontrol, including *M. anisopliae* were safe to use due to the low persistence in the soil and the high level of host specificity usually displayed by the various strains, however they make a call for increased research into ways to monitor the organisms and their metabolites to ensure that safety is maintained. It should be noted that cases of human infection by *M. anisopliae* in both immunocompetent (Revankar et al. 1999) and immunocompromised (Burgner et al. 1998) individuals have been reported. There is no indication that any of these cases were related to the commercial use of the fungus as a biocontrol agent.

Application Methods and Effectiveness. *Metarhizium anisopliae* is typically applied to plants as a topical foliar spray or incorporated into the soil. When applied to foliage, the exposed substrate of a leaf surface can create problems including desiccation, exposure to UV light and removal by rain water (Inyang et al. 2000). Oils in the spraying mixture may help to limit the detrimental effects of UV light, therefore increasing the efficacy of this process (Ibrahim et al. 1999, Malsam et al. 2002). Field trials using different oil carriers have shown that control of foliar insects (i.e. mustard beetle) can be obtained. However, high concentrations and application rates are required, likely making use uneconomical (Inyang et al. 2000). Given the worldwide distribution of *M. anisopliae* in soil, it seems pertinent to utilize this fungus as a biocontrol agent of soil insects. The soil environment lacks many of the problems associated with foliar applications, such as UV light exposure and desiccation. The soil environment is also more insulated against rapid temperature fluctuations. However, moisture and temperature may interact to reduce the level of infectivity seen in an isolate (Ekesei et al. 2003).

Metarhizium anisopliae can be applied to the soil in several different ways including as conidia, blastospores, fungus-killed cadavers and mycelial fragments (Krueger et al. 1992). In laboratory trials against scarab grubs, Krueger et al. (1992) found that mortality was quicker with mycelial rather than conidial applications. Total mortality from both applications was similar. In a field trial of *M. anisopliae* against the scarab beetle *Adoryphorus couloni* (Burmeister) (Coleoptera: Scarabaeidae) Rath et al. (1995) found that larvae and adults could be reduced by 45 - 79%. Up to 63% of the larvae were reduced before their most

damaging stage. This reduction carried over into an increase in the biomass produced by perennial grasses during the autumn months. This study also showed the long-term effects of *M. anisopliae*. The initial application of spores was 5.1×10^4 spores g^{-1} soil. A 5 to 10 fold increase in the number of spores in the soil was found after the first round of killed larvae began to sporulate. After the initial sporulation, the concentration of spores reached equilibrium at $0.8 - 1.0 \times 10^5$ spores g^{-1} soil, a two-fold increase above the initial application level, into the fourth year after the project was began. Rath et al. (1995) also demonstrated the low rate of migration of *M. anisopliae*. The presence of the fungus was not detected in the soil adjoining experimental plots used as a control for the experiment until the last year of the study, and this was at a concentration twice as low as the initial application. The effect on non-target organisms was also low, with no difference between treated and untreated plots. Movement was believed to be the result of infected *A. couloni* adults migrating to the control plot. Collembola have also been implicated in the dispersal of *M. anisopliae* (Dromph 2001). A study by Milner et al. (2003) showed similar results to the Rath et al. (1995) study. They found that certain isolates of *M. anisopliae* could persist in the soils of sugar cane fields for up to 3.5 years, although concentration declined over time. Persistence was the only parameter evaluated and the amount of fungal movement or impact on non-target organisms is unknown. They indicate that sporulation from cadavers could serve to augment the level of conidia and extend the duration of crop protection.

***Metarhizium anisopliae* in the Rhizosphere.** Microbial interactions occurring below ground are notoriously hard to examine (Rovira 1991). Soil properties such as moisture content, dissolved oxygen and pH can exert a large influence on microbial relations, often on a microhabitat scale. The ability to accurately determine population dynamics of soil microbes is further hampered by the inability of many species to grow on available media preparations. As a result, advances in understanding of the soil environment have been slow in coming.

Plant roots also exert a strong influence over the below ground dynamics of microbial populations. As a root makes its way through the soil, photosynthate is exuded, as well as lubricant (mucilage), to aid in soil passage (Hawes et al. 2003). Changes in pH relative to the bulk soil can result from the selective uptake of different ions (Marschner et al. 1987). These factors, as well as cells sloughed off in the process of growth, provide a substrate capable of supporting the growth of various microbes. However, this is not to imply that the plant is a passive participant in these interactions. Plants are capable of modifying the environment in order to foster the growth of certain organisms at the exclusion of others in a relationship that is largely determined by the genetics of both plants and microbes (Hawes et al. 2003). From these observations on the below ground dynamics around plant roots the term “rhizosphere” has arisen, defined here as “that narrow zone of soil subject to the influence of living roots, as manifested by the leakage or exudation of substances that affect microbial activity” (Curl and Truelove 1986).

Rhizosphere dynamics can vary depending upon plant species and the type of soil they are grown in (Bachman and Kinzel 1992), location along the growing

root, and the age of the roots examined (Foster 1986). Much of the nature of rhizosphere interactions are determined by the growing root cap. The root cap secretes mucilage to assist passage of the root through the soil, and also releases border cells into the soil environment. Border cells modify the soil habitat around the root through their secretion of exudates that aid in the uptake of nutrients and influence the microbial community that develops around the root (Hawes et al. 2003). However, the root cap is not the only site of exudation and the profile of exudates changes significantly across the length of the root (Foster 1986).

In spite of the somewhat limited overall understanding of rhizosphere interactions, attempts to modify this environment for biological control purposes have been undertaken (Whipps 2001). Virtually all of these attempts have involved the use of organisms antagonistic towards plant pathogens. Various species of *Trichoderma* have been found to protect against root pathogens. In laboratory trials, the use of *Trichoderma koningii* Rifai. as a seed coating proved beneficial to tomato plants grown in the presence of the pathogen *Sclerotium rolfsii* Sacc., the causative organism of damping off. When treated with *T. koningii*, plants showed improved emergence, as well as increases in height and fresh weight. Increases in height and fresh weight were observed for treated plants grown in both pathogen infested and control soils (Tsahouridou and Thanassoulopoulos 2002). A commercially available strain of *Trichoderma harizianum* 1295 - 22 has been shown in field trials to significantly reduce the incidence and spread of dollar spot on turfgrasses (Lo et al. 1996). Another strain, *T. harizianum* T - 35, has been shown to reduce the colonization of *Fusarium* spp.

in cotton and melon rhizosphere soil, possibly as a direct result of competition for plant resources (Sivan and Chet 1989).

When considering possible biological control agents, care must be taken in the extrapolation of laboratory results to field uses. For example, several microbes were identified as potential control agents of wheat seedling blight, *Bipolaris sorokiniana* (Sacc.) Shoem., in the laboratory, but failed to provide adequate control in field settings (Dal Bello et al. 2003). Another concern is the possibility of negative effects of the control agent on the protected host when the pathogen is not present. This situation is seen in the application of two species of bacteria, *Pseudomonas putida* and *P. chlororaphis*, examined as biological control agents for maize root diseases. Both species were shown to reduce overall plant growth when no pathogen was present. This effect is possibly due to competition between the plant and bacteria (Kozdroj et al. 2004).

It has recently been shown that the entomopathogenic fungus *M. anisopliae* is rhizosphere competent (Hu and St. Leger 2002, Bruck 2005). This capacity to associate closely with plants is not surprising, given the placement of this fungus in the family Clavicipitaceae (Sung et al. 2001), a group containing many known plant pathogens. *Beauveria bassiana* Vuillemin (Hypocreales: Clavicipitaceae) is another entomopathogen from the same family capable of close association with plants. *Beauveria bassiana* is an endophyte of corn (Bing and Lewis 1991), and has recently been shown to be rhizosphere competent as well (Bruck, unpublished data). This ability of certain entomopathogenic fungi to enter into such an intimate

relationship with plants presents a novel method to protect plants from insect herbivory.

In the case of *M. anisopliae*, plant roots appear to provide a refuge capable of preventing the same degree of population decline observed in the bulk soil (Hu and St. Leger 2002, Bruck 2005). When applied as a broadcast treatment to field grown cabbage, the population in the bulk soil declined from a mean of 2.45×10^5 to 2.96×10^4 propagules g^{-1} dry soil after four months, where as the population at the root base was near that observed after application. There was a four fold increase observed between populations in the inner and outer rhizosphere, indicating that plant root exudates may contribute to the persistence of *M. anisopliae* in the soil. Additionally, the detection of *M. anisopliae* on roots as far as 10 cm away from the stem of the plant indicates that the fungus is capable of vertical movement in the soil profile; however the mechanism is currently unclear (Hu and St. Leger 2002). Trials examining the difference in fungal populations between bulk and rhizosphere soil in pots containing rooted cuttings of *Picea abies* (L.) Karst. (Pinales: Pinaceae) var. *nidiformis* planted in soilless potting media, rhizosphere populations again proved to be resistant to the decline observed for the bulk soil (Bruck 2005).

Bruck (2005) tested the ability of *M. anisopliae* to infect larvae of the Black Vine Weevil when applied as a root dip to *P. abies* at a rate of 2×10^6 spores ml^{-1} . Used in this manner, 76% of larvae became infected. Infected larvae were located near the roots, and in some cases had begun feeding before succumbing to infection. It was determined that the level of infection resulting

from this manner of treatment warrants further investigation into development of a system that can be used by commercial nursery operations to protect against BWV and possibly other nursery pests.

Biological control

The Pesticide Paradigm. In the United States and around the world, public concern is growing about the effects of pesticides used in agricultural production. In the year 2000, the U.S. applied at least 247.3 million kg of active pesticide ingredient to agricultural crops alone, for a total of 1 kg per acre (U.S. Census Bureau 2002). The cost of these pesticides for the same year was over \$8.5 billion, which amounts to approximately 4% of the total production expenses (Anonymous 2003, Agriculture Statistics). In 1999, the total U.S. expenditures on pesticides were over \$11.1 billion. This amounted to about a third of the entire world market of \$33.5 billion (Anonymous 2003, <http://www.epa.gov>). Pest control is a major investment for the American farmer.

Pesticide usage of in the U.S. is estimated to cause \$8.1 billion in social and environmental damages (Pimentel et al. 1993). The use of herbicides in the United Kingdom and North America has been shown to have negative impacts on beneficial invertebrate and bird populations indirectly through changing plant communities (Freemark and Boutin 1995). Birds are particularly sensitive to the effects of pesticides because they lack the physiological detoxification systems necessary to deal with the presence of the toxin (Walker 1983). The drift from agricultural operations in the central valley of California have been shown to lead

to declines in populations of several endangered species of amphibians in the Sierra Nevada Mountains (Davidson et al. 2002).

Pimentel et al. (1993) provide evidence that changes in farming practices for several major crops could feasibly reduce the need for pesticides by 50%, at a cost of approximately \$1 billion. This would translate to a 0.6% increase in the cost of food to the consumer. Although costs are associated with the reduction of pesticide usage, the amount of money spent repairing environmental and public health damages would be much reduced. In addition, consumers may spend a little more if they could be assured that the food they purchased had the lowest level of pesticide residues possible.

A reduction in the use of pesticides does not necessarily have to mean negative economic ramifications, manifest in price increases for consumers and society in general. The case of wheat production in the United Kingdom bears this point out. If farmers were to switch from a conventional system, to a low input system, their total profit margin would remain largely unchanged, in spite of a reduced yield. Profit would remain stable due to the reduction in input costs. A reduction in pesticide inputs was also deemed to benefit the society in general, due to lowered production levels and thus a reduced need for price support mechanisms that maintain a high price for farmers (Webster et al. 1999). In Ontario Canada, overall pesticide usage declined approximately 40% between the years 1983 and 1998 due to government policies designed with that aim in mind. An analysis of the economic benefit derived from reduced environmental risks was determined to be approximately US\$ 188 per household per year. Savings for the

province were determined to be US\$ 771 million. These environmental gains were obtained primarily through the reduced usage of high risk pesticides, and their replacement with more benign chemistries (Brethour and Weersink 2001).

Biological Control. Biological control provides a method whereby the use of pesticides can be reduced. Biological control can be defined in many ways. However the definition most useful to this discussion is “the use of parasitoid, predator, pathogen, antagonist, or competitor populations to suppress a pest population, making it less abundant and thus less damaging than it would otherwise be” (Van Driesche 1996). The manner in which this process is applied can take many forms, using a wide breadth of the forces in nature that influence the size, structure and stability of an organism’s population.

There are many advantages to biological control, including the reduction in pesticide use, potential for long-term control, and a management option when a pest becomes resistant to pesticides. Biological control can also be more economical than the use of pesticides in certain situations (Vail et al. 2001). For pests that become widespread in remote locations or in a manner where standard practices can not be used, biological control can be a viable option. For instance, in parts of California the plant known as Klamath weed, or St. John’s wort, became widely established occupying vast expanses of pasture and grass land. Two species of leaf beetle (*Chrysolina* sp. (Coleoptera: Chrysomelidae)) were found that could effectively control populations in one area, and then disperse to others. It was then able to establish long-term control (Vail et al. 2001).

Development of biological control products could also benefit agrochemical companies by allowing them to expand into new markets, and achieve faster market entry due to less regulatory requirements relative to chemicals (Froyd 1997). In spite of the many benefits, investment in biological control has been low and the market remains a small part of the pest control economy (Richards and Rodgers 1990, Dent and Waage 2000). Concerns about efficacy and consistency on the part of the consumers, and uncertainty about marketability on the part of producers hamper the expansion of these products.

History. The history of biological control in the USA dates back to early efforts by the United States Department of Agriculture (USDA) (Vail et al. 2001). The most famous of these efforts is the introduction of the ladybird beetle *Rodolia cardinalis* (Mulsant) (Coleoptera: Coccinellidae) to control cottony cushion scale, *Icerya purchasi* (Maskell) (Hemiptera: Margarodidae) on oranges in California in 1888 (Caltagirone and Doult 1989, Van Driesche 1996, Vail et al. 2001). *Rodalia*, in combination with the parasite *Cryptochetum iceryae* (Diptera: Cryptochetidae) has provided long-lasting, effective control of the cottony cushion scale in California, as well as other areas around the world (Caltagirone and Doult 1989).

Approaches to Biological Control. The importation of *R. cardinalis* sparked the development of what has subsequently become known as “classical biological control”. This is understood to be the importation of one species to reduce the negative effects of another imported species. It is ultimately hoped that the introduction will lead to permanent establishment of the agent to achieve long-term control.

The classical biological control method has subsequently given rise to what has been termed the “neoclassical” or “new association” method of biological control (Hokkanen and Pimentel 1984, 1989). Neoclassical control seeks to use an introduced control agent to control a naïve host. It is hypothesized that because the two species have not coevolved together, the effect of the control agent will be greater due to a lack of avoidance or defense mechanisms in the target pest (Hokkanen and Pimentel 1984). The approach was formulated from an examination of previously documented introductions and the success rate reported for each. Great care must be utilized in this approach because it is the nonspecificity of the control agent that is being exploited. This could potentially lead to damaging effects on non-target species.

Augmentative biological control is implemented when the population of a native control agent is supplemented by individuals reared artificially to increase the effects of the agent. The main drawback to this type of control is that formulation of an appropriate diet may be difficult and the rearing process may be cost prohibitive at a large scale (Hopper 2003).

The inundative release of biocontrol agents is also sometimes referred to as the biological pesticide method because there is generally no expectation that the introduction will result in establishment of the biocontrol agent. Rather, large numbers of the control agent are released at once, in hopes that the result will be a rapid decline in the negative effects of the pest of interest, much like a chemical pesticide would provide. This approach can be implemented when dealing with many types of control agents; native, exotic or microbial.

Microbial Control. The use of insect pathogens to reduce pest problems falls under the umbrella of microbial control. Pathogens that have been used in biological control include bacteria, fungi, viruses, protozoans and nematodes. The history of microbial pest control begins in Russia. In 1879 Elias Metchnikoff successfully used *M. anisopliae* to control pests of wheat and sugar beets (McCoy et al. 1988). Since this first foray into microbial control, progress has been slow, with the development of chemical pesticides taking precedence over other methods. However, microbial control holds much promise as an effective, economical and safe means of pest control.

Because they are living organisms (except viruses), microbial control agents are subject to limitations not encountered with chemical pesticides. Improper handling and storage conditions, such as extreme temperatures, can result in reduced viability. Short storage life is also regarded as a drawback. Environmental conditions in the field can adversely affect the outcome of a microbial treatment. Many microbial agents are sensitive to relative humidity and UV exposure, which can reduce the concentration of organisms able to infect the target pest, the end result being insufficient control. Some agents, such as viruses and protozoans, require *in vivo* production. This can make production costs too high for widespread usage to be economically feasible. The narrow range of host specificity for some isolates, although attractive from an environmental perspective, is considered a drawback by companies that must have a product they can sell to a wide array of consumers.

Because of these reasons, microbial insecticides have been slow to reach the world market. However, in spite of these limitations, successful pest control can be obtained. Further investment into production methods, as well as exploration and discovery of hardy, reliable strains of organisms will allow this method of pest control to become a viable management option.

Protozoans. Protozoans are a diverse group of single celled organisms, many of which associate with insects in capacities ranging from mutualistic to pathogenic. It is the pathogenic species, mostly microsporidians, which have attracted the most attention as biocontrol agents (Solter and Becnel 2000). The infective stage in the life-cycle of a microsporidian is an environmentally resistant spore (Solter and Becnel 2000) with ingestion, or sometimes association with eggs, necessary to instigate infection (Brooks 1988). Symptoms are fairly nondescript including sluggishness, irregular growth and loss of appetite, as well as reduced adult survival and fecundity. It is sometimes difficult to attribute death to the infective agent. Evidence for the activity of toxins is lacking. Competition for resources with the host may play a role in mortality.

Microsporidia show potential as control agents for aquatic dipterans, as well as terrestrial Hymenoptera, Lepidoptera and Orthoptera. Host ranges vary between species, but increasing evidence suggests that it is usually fairly narrow. The spore concentration used during host range testing can influence the apparent host range because high concentrations may overwhelm the defenses of an otherwise non-host. Concentration can also be important in the ability of a microsporidian to infect a particular life stage of the host (Solter and Becnel 2000).

Successful use of microsporidia is contingent upon several factors.

Tolerance of some level of the pest is required to ensure the pathogen is able to be maintained without additional applications. Application of microsporidia has the greatest potential when the possibility exists for long-term persistence of the pathogen (Solter and Becnel 2000). They fit well as a supplement in an integrated pest management approach by causing a pest population to crash sooner than normal.

Bacteria. Insect pathogenic bacteria occur in five families; however most commercially available bacterial pesticides are derived from the Bacillaceae. Species in two genera, *Bacillus* and *Paenibacillus*, as well as the species *Serratia entomophila*, represent the bacterial pesticides most readily available today. Formulations usually consist of bacterial spores or toxins produced by the bacteria (Siegel 2000).

Bacterial insect pathogens are probably the most commonly used form of microbial control. This is due to the widespread use of *Bacillus thuringiensis* (*Bt*) (Lacey et al. 2001). In 1995, 73% of all microbial insecticides registered with the EPA contained products from *Bt* (Siegel 2000). This bacterium can be used in a manner similar to chemical pesticides and yield similar results. *Bt* enjoys a cosmopolitan distribution around the globe and is a common component of many natural soil communities. It is a Gram-positive, motile, aerobic endospore forming bacteria.

Several metabolic products contribute to the pathogenicity of *Bt*. Of primary importance are the Insecticidal Crystal Proteins (ICPs). Many different

strains or subspecies of this bacterium are now known and they can exhibit specificity to different groups of organisms (Charnley 1991, Lacey et al. 2001) depending on the protein composition of the ICP (Siegel 2000). The mode of action for ICPs is by disrupting the osmotic balance of gut epithelial cells, ultimately leading to cell lysis (Siegel 2000, Lacey et al. 2001). In order for them to be effective, they must be consumed by the insect. Several strains of *Bt* also possess the capacity to produce exotoxins. Some of these exotoxins can potentially be very toxic to a wide array of insects as well as some vertebrates. The presence of these compounds in commercial products is prohibited in the USA (Siegel 2000).

There are other bacteria currently used as pest control agents, however they are less frequently utilized, relative to *Bt* (Lacey et al. 2001). *Bacillus sphaericus* is employed against mosquito larvae. Low level, long-term control of scarab beetles can be achieved with *Paenibacillus popilliae*.

Viruses. Viruses comprise a large, variable group of agents capable of providing control of pest populations. The group of viruses most commonly researched and employed in biocontrol are the baculoviruses (rod-shaped viruses). This group forms an occlusion body consisting of a proteinaceous matrix around the viral particles. This structure conveys a degree of environmental stability and resilience to the virus, thereby aiding its persistence and likelihood of infecting a host. There are two groups within the *baculoviridae* that receive attention as biocontrol agents: the nucleopolyhedroviruses and the granuloviruses.

In order for the infection process to begin, the virus must enter the pest through the mouth. Once the infection process begins, the speed of kill is usually slow, relative to chemical alternatives. However, immediate death is not essential for positive effects to be witnessed (Evans 2000). Viruses that develop within the gut may cause feeding to cease or slow, thereby ending damage before the death of the pest, which is ultimately the desired effect. The host range for most viruses is typically narrow, allowing them to be selective and permitting beneficial insects to thrive. Application of the virus is usually achieved using spray technology. However, it is possible for viruses to cycle and spread in the population of a pest species through ecological forces such as autodissemination, allowing for control to extend beyond the initial application. Dispersal is often aided by behavioral changes that arise in the host, causing them to move to elevated locations that make viral particles more likely to contact new hosts. This process can be further aided by the action of rainwater (Evans 2000).

Nematodes. Entomopathogenic nematodes occur in 23 different families, seven of which contain species of interest as potential insect biocontrol agents. There are only two families of nematodes whose members currently find use in microbial control, the Steinernematidae and the Heterorhabditidae. Steinernematids and heterorhabdids are both obligate parasites of insects, requiring an appropriate host to complete their life cycles. Their pathogenicity is derived from the action of symbiotic bacteria residing within the gut. Steinernematids form symbioses with bacteria of the genus *Xenorhabdus* and Heterorhabdids with the genus *Photorhabdus*. The association with bacteria evolved independently for

both families, and current evidence does not support a common ancestor between the two (Adams and Nguyen 2002). They are naturally found in the soil and their use against soil insects provides the greatest efficacy. However, they may also be used as a foliar applicant.

Nematodes have been employed in the control of a wide range of pest species. Orders of insects where successful control has been achieved include: Diptera, Lepidoptera, Coleoptera, Orthoptera, Blattoidea and Siphonaptera. Despite high levels of efficacy for some pests (e.g. pink bollworm of cotton), economic conditions often prohibit widespread use when less expensive and equally effective means can be employed (Shapiro-Ilan et al. 2002)

Before infection can occur, a nematode must first find its host. Host searching occurs only during the infective juvenile stage of development. This is the only free living stage in the life cycle. Host location varies from species to species and can have effects on the efficacy of nematodes as a control agent. A continuum between active searching and sit and wait behavior can be seen for entomopathogenic nematodes. In order for infection to occur, the nematode must enter the body through some kind of opening, such as the mouth, anus or spiracle, or thin sections of the insect cuticle. Once within the hemoceal, mutualistic bacteria are released from the gut of the nematode. These bacteria produce toxins that kill the host through septicemia. The cadaver of the host provides an environment where the nematodes feed on the mutualistic bacteria, reproduce for several generations, and produce more infective juveniles.

Nematodes are commonly applied inundatively, as one would apply a chemical pesticide. Application can typically be achieved with conventional spray equipment. If environmental and ecological conditions are appropriate, the nematode population may persist and recycle in the soil after the initial treatment. This has been seen in the classical biocontrol agent *Steinernema scapterisci* employed against mole crickets in Florida (Koppenhofer 2000).

Fungi. There are approximately 700 species of fungi with known pathogenicity to insects. Of these, only 10 have been or are currently employed for pest control (Hajek and St. Leger 1994). Some of the major genera currently being used are *Beauveria*, *Metarhizium*, and *Verticillium*. For some of the species in these genera, wide ranges of virulence, host specificity and tolerance to environmental conditions can be seen.

Fungi can be used alone, or they can be incorporated into an integrated pest management (IPM) program. For example, when larvae of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), were exposed to a sub-lethal dose of the pesticide imidacloprid, high levels of mortality were observed after treatment with *Beauveria bassiana* (Furlong and Groden 2001). This type of integration can decrease the chances evolved resistance in a pest species.

Precautions to Biological Control. There have been calls to alarm raised by some that biological control is capable of causing great amounts of ecological damage (Howarth 1991, Simberloff and Stiling 1996, Samways 1997, Michaud 2002). These fears often focus on the lack of knowledge about the non-target

species affected by the introduced agents, as well as the damages witnessed by historical failed introductions. For instance, the introduction of the *Myxoma* virus in Great Britain to control rabbits resulted in drastic, unforeseen changes in ecosystem functioning that led to the extinction of the large blue butterfly, *Maculinea arion* (L.) (Lepidoptera: Lycaenidae). It must be cautioned that some of the data used to make claims about the negative aspects of classical biological control (e.g. Howarth 1991), stem from largely anecdotal sources, or those with poor quality control (Follett et al. 2000).

There remains a definite need for greater levels of post-release monitoring in order to assess exactly what happens after an introduction is deemed “successful” (Follett et al. 2000, McEvoy and Coombs 2000). Without post-release monitoring, it is impossible to know if an introduction is causing damage to native or non-target species. It is unlikely that if a problem with an introduced control agent occurs, much could be done to remedy the problem. However, insights might be gained that could prevent future introductions from reaching the same fate. As with other types of biological control, concern for the effects on non-target species in inundative control is driving the call for increased regulation and monitoring of introductions (van Lenteren et al. 2003).

Black Vine Weevil (*Otiorhynchus sulcatus*)

Introduction. Weevils (Coleoptera: Curculionidae) are notorious worldwide as important pests of a wide array of agricultural crops. At least 39 species of weevil have been introduced into North America, north of Mexico (Warner and Negley 1976). The greatest numbers of these are from the genus

Otiorhynchus (Lindroth 1957). The natural distribution of the genus is Palearctic. Many species of *Otiorhynchus* are important pests (Warner and Negley 1976).

The black vine weevil (BVW), *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae), is a pest of increasing importance for agricultural and horticultural crops worldwide in both field and glasshouse settings. The weevil is endemic to temperate regions in Europe (Moorhouse et al. 1992). Movement of infested plant material allowed the BVW to become established in North America, Chile, Japan, Australia and New Zealand (Moorhouse et al. 1992). The first record of its presence in the USA is from 1835, but recorded under the name *Curculio apiculatus*. The first record of economic damage for the USA is in 1871, with European records as early as 1834 (Smith 1932). Early records for this species often use the genus name *Brachyrhinus*. In North America, *O. sulcatus* is commonly found on both coasts. On the east coast it extends as far west as the Mississippi River and as far south as parts of North Carolina. The northern extent reaches into Canada. The habitat occupied in western North America begins on the eastern edge of Idaho and reaches the coast, extending to southern California and north to British Columbia. The distribution is described as spotty due to the tendency of populations to occur in clumps (Warner and Negley 1976).

Life Cycle. The BVW typically overwinters as a prepupae or a last instar. A long, warm fall season in association with relatively benign winter conditions may allow for adults to overwinter and emerge the following spring and begin laying eggs (Smith 1932). Overwintering larvae complete their development and adults eclose from early June to late July, with a peak eclosion near the middle of

June. Oviposition begins in the middle of July for adults that emerge in June, after a preoviposition period of 28 - 50 days. Some eggs laid very early may produce individuals capable of developing into adults before winter (Smith 1932). In the greenhouse setting, the life cycle may be advanced by two months (Moorhouse et al. 1992). Adults may also remain active in the greenhouse through December and January, all the while continuing to oviposit (Smith 1932).

Eggs and Egg Laying. The BVW is a parthenogenic, univoltine insect. Female weevils are indiscriminate in their oviposition behavior. Smith (1932) observed egg-laying on the ground, in plant debris on the soil surface, and between yew needles up to three feet off the ground. Eggs are also placed within crevices and gaps in the ground. The egg produced is at first a shiny, crème colored subspherical globe, with a diameter not exceeding 0.8 mm (Smith 1932). When placed in a crevice it may become disfigured, however this does not appear to affect the viability of the developing embryo. Between 20 - 80 hours after oviposition the egg surface becomes darkened. This is apparently an important step in the developmental process because if melanisation does not take place no larvae will emerge (Moorhouse et al. 1992). Temperature is important for the proper development of eggs. At 27°C the time to hatching is 8.4 days. However at 9°C hatching took 56 days (Stenseth 1979). Temperatures between 5 - 30°C are suitable for development, however outside of this range no hatching occurs (Moorhouse 1990).

Larvae. Black vine weevil larvae are legless and typically a whitish color with a chestnut brown head capsule. The body is transparent and the stomach

contents can be seen through the integument. Size of the head capsule is 0.318 mm in the first instar and attains a size of 1.5 mm by the sixth. As development proceeds, the body assumes a crescent shape as a result of the thickening thoracic segments. In order for the larvae to grow, fresh plant roots must be present (Smith 1932).

The number of instars before pupation appears to be variable. Smith (1932) reported one group attaining six instars, and another attaining a seventh. La Lone and Clark (1981) found only six. Temperature is a strong determinant of the duration of the larval stage. Larvae developing at lower temperatures will take longer to pupate than those at high temperatures (Stenseth 1979).

Pupae. The pupae of BVW are typically white in the early stages of development. Subsequent darkening, beginning with the eyes, ensues as the change into adulthood progresses (Smith 1932). The amount of time spent in the pupal stage varies with temperature. At 24°C adult eclosion occurred in 10 days, and at 15°C, 50 days were required (Stenseth 1979).

Adults. After eclosion, the adult weevil is a milky white, except the eyes and snout which are black, and the antennae, coxae, femoral tips, tibiae and tarsi which have a rusty color. Three days after eclosion the body is entirely black, with a shiny appearance. Yellowish hairs appear after four or five days and at this point the adult emerges from the soil and begins to feed. Adults range in size from 10.5 - 11.5 mm. They are flightless and the striated elytra are fused (Smith 1932). Adults are nocturnal, typically hiding during the daytime under litter or within grooves of bark.

Host Range and Plant Damage. Black vine weevil is a highly polyphagous species. Smith (1932) listed 77 plant species that were fed upon. An additional 70 plant species were listed by the USDA Plant Pest Survey Files (Warner and Negley 1976). Particular plant groups of importance fed upon by BVW include *Rhododendron spp.*, *Cyclamen spp.*, *Taxus spp.* and *Fragraria spp.* Evidence of feeding by adults is a notching on the edges of leaves. Unless the population is extremely large, adult feeding rarely causes plant mortality and the worst of the damage is cosmetic. Feeding by larvae on roots is capable of causing high levels of damage to plants. For certain species (e.g. *Cyclamen*) one weevil is enough to kill the plant (Moorhouse 1990). Larvae have been seen to girdle the root system of a plant and in some cases have been found in the stem of a plant above ground level. It is for these reasons that the larval stage is the part of the life cycle that is targeted in pest management strategies.

Analyses run on the olfactory response of BVW to certain chemicals released by plant leaves give some idea of the factors that aid in host plant location and selection. High responses were seen to compounds known as green leaf volatiles, compounds produced in varying quantities by all plants. High responses were also seen to defense chemicals produced by members of the Rosaceae and Taxaceae in response to herbivore attack, families that contain important host plants of BVW (van Tol and Visser 2002). Weevil damaged leaves of *Taxus* and *Euonymus* were found to be more attractive than damaged leaves of *Rhododendron* and *Fragraria* olfactometer choice tests (van Tol et al. 2002). Although BVW has a broad host range, not all plants are equal in terms of a food source. Nielsen and

Dunlap (1981) found that weevils fed *Taxus spp* survived longer and had higher fecundity when compared with weevils fed other plants. Some plants were potentially toxic to weevils, killing most of those fed the plant with only few surviving to oviposition.

Control Options. Black vine weevil is attacked by a wide range of natural enemies. These include moles, hedgehogs, starlings, carabid beetles, toads and spiders (Smith 1932, Moorhouse et al. 1992). Naturally-occurring insect pathogens, such as fungi, nematodes and bacteria have also been found to infect weevils. However, there are no reports of mortality as a result of viral infection (Moorhouse et al. 1992). Although subject to a wide range of predators and diseases, these factors are usually insufficient to keep populations below a threshold that is acceptable for farmers and horticulturalists. As a result, management practices must be undertaken in order to limit damage by weevils to plants.

Plant resistance. Planting of resistant cultivars and strains is one manner by which weevil populations can be managed. *Rhododendron smirnowii* Trautvetter has been found to reduce weevil survival (Nielsen and Dunlap 1981). Shanks and Doss (1986) found that wild beach strawberry, *Fragraria chiloensis* (L.), was eaten less and lowered reproductive success relative to a commonly grown cultivar.

Cultural Control. The use of physical barriers, such as sticky tape placed around the base of a plant (Smith 1932) may work on a small scale basis, but is not practical for large-scale operations or situations where the foliage of plants is in

contact with one another. Cultural control methods include removal of plant leaf material after harvest (Garth and Shanks 1978) and flooding of infested pots or areas (Smith 1932). It is also recommended that all infested material in the surrounding hedgerows be removed and destroyed, along with any infested pot plants (Smith 1932). Although a level of protection is conveyed by these measures, they may not be applicable in all situations.

Synthetic Chemicals. Because of their effectiveness, chemical pesticides have long been advocated as a means of control. Early control efforts centered on the use of lead arsenate (Smith 1932). This was later replaced by more effective chemicals, such as organochlorines (Moorhouse et al. 1992). Subsequent concerns over the ecological and health impacts of many of these chemicals have resulted in their removal from the market and are now no longer available as means of control. Replacements offering a comparable level of control, without the negative side effects, have been slow in coming, and effective, economical control by pesticides is unavailable.

One of the most commonly used insecticides is the synthetic pyrethroid bifenthrin, marketed under the trade name Talstar 0.2G[®]. The control obtained by use of this product can be inconsistent, depending upon the plant under protection, type of potting media used, and the application rate of the pesticide (Cowles 2001). The degradation of bifenthrin is dependent upon soil characteristics. The potential of movement in soil is limited by its attraction to organic matter. This pesticide is known to be acutely toxic to stream invertebrates and fish (FMC 1999).

Additionally, it was found to negatively impact populations of beneficial insects in British strawberry fields (Easterbrook 1997).

Biological Control. In response to the environmental concerns associated with chemical pesticides, focus has shifted to biological control. Of particular interest are entomopathogenic nematodes and fungi. If used properly levels of control similar to those obtained by chemicals can be obtained.

Entomopathogenic nematodes have been found by a number of workers to provide effective control in a variety of settings. Gill et al. (2001) demonstrated that *Heterorhabditis bacteriophora* (Poiner) was able to significantly reduce BVW larvae populations in potted plants. The level of control with nematodes was equal to that conveyed by the synthetic pesticides acephate and imidacloprid.

Nematodes caused 90 - 100% mortality in all trials. In field trials, the nematode species *H. megidis* and *Steinernema carpocapsae* delivered satisfactory control. The nematodes were delivered via the irrigation system into raised strawberry beds. *Steinernema carpocapsae* reduced early instars by nearly half and was also able to provide a higher level of control than *H. megidis* for late instars. *Steinernema carpocapsae* also persisted in the soil longer and experienced virtually no reduction in viability as a result of being passed through the irrigation system, relative to *H. megidis* (Kakouli-Duarte et al. 1997).

Berry et al. (1997) demonstrated the ability of local entomopathogenic nematodes to effectively control BVW in the Pacific Northwest. The indigenous nematode *H. marelatius* was found to have greater cold activity than the nonindigenous strain of *H. bacteriophora*. This translated into an eight-fold

increase in mortality to BVW at 14°C. In trials at 10°C, *Steinernema kraussei*, a nematode isolated from a cold weather climate, successfully infected *Galleria mellonella*, while a commercial formulation of *S. carpocapsae* showed no activity. *Steinernema kraussei* also significantly lowered the number of weevils recovered from strawberry plants grown outdoors over winter (Willmott et al. 2002). Development of commercially available products based on cold active nematodes could increase the range of successful control and reduce dependence on chemical methods.

In natural systems, a wide range of saprophytic and entomopathogenic fungi have been observed infecting BVW at all stages of its lifecycle (Smith 1932, Moorhouse et al. 1992). This relationship can be used to the advantage of farmers and horticulturalists looking for an environmentally sound and effective means of reducing weevil populations. Of the entomopathogenic fungi that infect BVW, *M. anisopliae* has received the most attention as a potential microbial control agent. Natural epizootics of *M. anisopliae* have been known to occur in BVW populations (Soares et al. 1983). *Metarhizium anisopliae* is commercially available under the trade name Taenure (Earth BioSciences, New Haven, CT).

Metarhizium anisopliae is a highly variable fungus, with strains capable of growing under a wide range of conditions and exhibiting different levels of pathogenicity (see previous section on *M. anisopliae* for details). Temperature is often the most important environmental determinant of fungal activity in soil environments; therefore, strains able to infect and kill weevil larvae at suboptimal temperatures are desirable. As a result of this, bioassays have been conducted to

identify the most efficacious strains under field conditions (Soares et al. 1983, Moorhouse et al. 1993b). Greenhouse trials indicate that *M. anisopliae* is most effective when applied as a prophylactic measure, with total control achieved in some cases. When used as a curative treatment, control is usually reduced (Moorhouse et al. 1993b, 1993c, 1993d). Field trials in strawberries have provided over 85% control (Moorhouse 1990). However, field trials using a dried rice mycelium formulation of an indigenous *M. anisopliae* strain in the Pacific Northwest on cranberries provided only minimal, statistically insignificant, results (Booth et al. 2000). It is possible that in this experiment, problems with the formulation of the product contributed to the poor results. Field trials on hardy ornamental species also produced variable results, possibly as a result of host plant effects (Moorhouse 1990).

All stages of the life cycle of BVW are susceptible to infection by *M. anisopliae*. The eggs appear to be the most resistant stage of the life cycle (Moorhouse 1990). Poprawski et al. (1985) found that infection of eggs and neonates by *M. anisopliae*, although not statistically significant, was lower than most other fungi tested. As weevils progress through successive instars, rate of infection rises. The pupal stage is highly susceptible to fungal infection. The adult stage is less susceptible to infection than the pupal or larval stages. The LC_{95} for the adults was calculated to be 1.12×10^9 , whereas that for sixth instar larvae was 5.41×10^6 (Moorhouse 1990).

Insect Behavior

The term behavior covers a vast array of activities witnessed during the life of an organism. When dealing specifically with insects, it is useful to divide behavior into two broad categories: maintenance and communicatory.

Maintenance behaviors are those behaviors that benefit the individual, such as grooming, without any real consequence to others. Communicatory behaviors are “other directed”, seeking to convey a message or information of some sort to another organism. An example of this type of behavior is the ritualistic motions performed during courtship in some species (Matthews and Matthews 1978).

In insects, there is good evidence for the existence of innate, genetically controlled behavioral responses to specific stimuli or conditions, sometimes equated with instinct. This type of behavior is commonly referred to as a fixed action pattern. The stimuli that initiate a fixed action pattern are referred to as releasers or sign stimuli (Matthews and Matthews 1978). Insects are also capable of learning as a result of memory. There are several broad categories within this type of learning. Habituation results when an insect is continually exposed to a stimulus that is not harmful or is unavoidable. Over time, the behavioral response to this stimulus is lessened. This type of learning reduces the unnecessary triggering of innate responses. Associative learning is the product of two previously meaningless stimuli becoming linked as a result of some form of reinforcement, either reward or punishment. Latent learning occurs without any obvious reinforcement, such as bees and wasps learning the location of the nest through exploratory flights (Matthews and Matthews 1978).

The role of behavior in pest management settings is poorly understood, but advances are being made. Behavior can play a role in insect resistance to a chemical or pathogen. By understanding the stimuli that influence resistance behavior, as well as host selection by a pest, more informed decisions can be made about a management program and hopefully result in more effective control. Behavioral resistance can be defined as “evolved behaviors that reduce an insect’s exposure to toxic compounds or that allow an insect to survive in what would otherwise be a toxic and fatal environment” (Sparks et al. 1989). Behavioral resistance need not function outside of biochemical or physiological resistance and may sometimes be directly linked (Sparks et al. 1989). Some aspects of an insect’s biology may contribute to the development of behavioral resistance, such as mobility, an untreated area to escape to and insecticides that possess some level of irritancy or repellence (Sparks et al. 1985).

In a study on cockroaches, *Blattella germanica* L. (Dictyoptera: Blattellidae) Jones and Raubenheimer (2002) found that the addition of toxicants to the food source elicited changes in the number of meals eaten and the duration of each meal within a five hour period. As the feeding session progressed the rate of ingestion declined. No directed movement away from the poisoned food was observed, indicating that the only mechanisms regulating ingestion were affected. These effects on feeding behavior, while not limiting the amount of food eaten during the period examined, could lead to the ingestion of a sublethal dose of the toxin over the life of the insect.

Integrated pest management systems depend upon multiple control methods to achieve adequate levels of control. The ability of parasitoids to distinguish between healthy and unhealthy hosts can be important in systems when used in conjunction with pathogens. Lord (2001) found that the parasitic wasp *Cephalonomia tarsalis* (Ashmead) (Hymenoptera: Bethyridae) was unable to distinguish between hosts that had been infected by *Beauveria bassiana* and those which were uninfected. Oviposition occurred on infected hosts, and those eggs failed to develop. The adults of *C. tarsalis* were found to be susceptible to the fungus as well. However, in another system, the parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) was able to discern and reject infected whiteflies, *Trialeurodes vaporariorum* (Westwood) (Homoptera: Aleyrodidae), four days after exposure to the fungus *Aschersonia aleyrodinis* Webber. Rejection occurred after the ovipositor was inserted into the host, and some transfer of the fungus from infected to uninfected hosts was seen to occur, however this was minor (Fransen and van Lenteren 1993). These examples show that the behavioral response of a parasitoid to infected hosts can be varied and must be taken into consideration when developing a pest management program.

Examples exist where avoidance of a food source or substrate by a pest has been observed. The moth *Spodoptera exigua* (Hubner) (Lepidoptera: Noctuidae) has been found to actively avoid artificial diets and leaves treated with the bacteria *Bacillus thuringiensis* (*Bt*) as well as leaves of plants genetically modified to produce the *Bt* toxin (Stapel et al. 1998). The Japanese beetle *Popillia japonica* Newman (Coleoptera: Scarabaeidae) was found to avoid soil that was inoculated

with *M. anisopliae* (Villani et al. 1994). Mole crickets (Orthoptera: Gryllotoalpidae) have been observed to alter their behavior in such a way that exposure to both *M. anisopliae* and *B. bassiana* was reduced (Villani et al. 2002). Gange et al. (1994) speculated that the observed increase in percent of root infected by vesicular-arbuscular mycorrhizae in treatments with high larval density resulted from selection of uninfected root as a food source by BVW. It is apparent that insect behavior can affect the outcome of pest management strategies. Increased understanding of the role played by insect behavior in resistance and resource selection will make pest control efforts more effective and efficient.

CHAPTER 3

The Behavioral Response of *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae) Larvae to *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) and the Synthetic Pyrethroid Bifenthrin

Ryan M. Kepler and Denny J. Bruck

ABSTRACT

The black vine weevil (BVW) *Otiorhynchus sulcatus* (F.) is a serious pest of horticultural operations in the Pacific Northwest. In order to understand what effect management options have on BVW behavior, larval response to the synthetic pyrethroid bifenthrin (Talstar 0.2G[®]), and the entomopathogenic fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin was assessed. Five 3rd instar BVW were placed in still air olfactometers (SAO) which allowed larvae to infest one of two pots. Larvae were allowed to choose between *M. anisopliae* (1×10^6 spores g⁻¹ dry media) and untreated media, bifenthrin (25 ppm) and untreated media, as well as *M. anisopliae* and bifenthrin treated media. For all comparisons, experiments were conducted without plants in the system to test for innate responses, as well as with plants to test typical nursery conditions. Results indicate that larvae are significantly deterred by bifenthrin both with and without plants present in the system. No significant effect on larval movement was observed when *M. anisopliae* was present in the media without plants in the SAO. When plants were included, a significant attraction to *M. anisopliae* treated media was observed. *Metarhizium anisopliae* treated media was preferred by BVW larvae over bifenthrin both with and without plants present in the SAO. Avoidance of BVW larvae to bifenthrin could result in reduced control. Conversely, the use of *M. anisopliae* in planted systems could result in BVW larvae entering a lethal environment and increasing the likelihood of mortality.

Key words: *Metarhizium anisopliae*, *Otiorhynchus sulcatus*, microbial control, insect behavior, olfactometer

Introduction

The black vine weevil (BVW), *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae), is a highly polyphagous insect pest of economical importance in several parts of the United States, especially nursery and greenhouse operations in the Pacific Northwest (PNW) (Warner and Negley 1976). The BVW typically overwinters as a prepupae or a last instar (Smith 1932). Overwintering larvae complete their development and adults eclose from early June to late July, with a peak eclosion near the middle of June. Oviposition begins in the middle of July for adults that emerge in June, after a preoviposition period of 28 - 50 days. Some eggs laid very early may produce individuals capable of developing into adults before winter (Smith 1932). In the greenhouse setting, the life cycle may be advanced by two months (Moorhouse et al. 1992). Adults may also remain active in the greenhouse through December and January, all the while continuing to oviposit (Smith 1932). Adults notch leaves, however the extent of the damage is usually cosmetic. Larval feeding on roots causes high levels of plant damage. For some plant species (i.e. *Cyclamen*) one weevil larvae is enough to kill the plant (Moorhouse 1990). Larvae can girdle the root system of a plant and in some cases have been found in the stem of a plant above ground level. It is therefore desirable to reduce the larval population in order to limit plant damage. Because BVW is parthenogenic, there is much emphasis put on keeping BVW out of nurseries before an infestation can begin. This has resulted in a zero tolerance level for the presence of BVW in plants shipped between locations. To prevent larval infestation growers typically incorporate chemical insecticides; the synthetic

pyrethroid bifenthrin, sold under the trade name Talstar 0.2G[®], is the compound most commonly employed in the PNW (D. Hicks, personal communication).

The use of the entomopathogenic fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae), has also been shown to offer effective BVW control (Moorhouse 1990, Moorhouse et. al. 1993a, Bruck 2005). *Metarhizium anisopliae* is a common soil-borne fungus that has a worldwide distribution (Zimmerman 1993), including the PNW (Bruck 2004b). Recent uses of *M. anisopliae* include: widespread applications in Brazil to control spittlebug *Mahanarva* spp. (Homoptera: Cercopidae) on sugarcane; Australia to control pasture cockchafer (Coleoptera: Scarabaeidae); China to control *Colasposoma* spp. (Coleoptera: Chrysomelidae); regions of the South Pacific to control the rhinoceros beetle *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae); United States to control the pecan weevil (McCoy et al. 1988); and Africa to control locusts (Orthoptera: Acrididae) (Shah and Pell 2003).

There is a growing body of work indicating that pest management efforts can be significantly affected by the behavioral response of an insect to the applied method. This phenomenon can be termed behavioral resistance, defined here as “evolved behaviors that reduce an insect’s exposure to toxic compounds or that allow an insect to survive in what would otherwise be a toxic and fatal environment” (Sparks et al. 1989). To date, there have been no studies to examine the impact of either bifenthrin or *M. anisopliae* on the behavior of the BVW larvae. Understanding the potential effect these management options have on BVW behavior of could improve their effectiveness at controlling this pest. The

objectives of these experiments were to test for a behavioral response of BVW larvae to soilless potting media incorporated with bifenthrin or *M. anisopliae* at rates recommended for BVW larval control.

Materials and Methods

Source of Fungus, Insects and Plants. A commercial formulation of *M. anisopliae* (strain F52) (Earth BioSciences, New Haven, CT) registered with the U.S. Environmental Protection Agency (EPA) for use against several insects, including BVW was used. The formulated product consisted of *M. anisopliae* that had sporulated on rice grains and was then dried. Granules were stored at 4°C until use. Black vine weevils (3rd – 4th instar) were obtained from the colony maintained at the USDA-ARS Horticultural Crops Research Laboratory, Corvallis, OR (Fisher and Bruck 2004). Rooted cuttings of birds nest spruce *Picea abies* (L.) Karst. (Pinales: Pinaceae) var. *nidiformis* were obtained from a local grower.

Design and Validation of the Still Air Olfactometer. To determine the ability of BVW larvae to respond to different cues emanating from soilless potting media, still air olfactometers (SAO) were constructed in the following manner. Plastic pots (8.9 × 8.9 × 8.9 cm) were modified by drilling a hole (3.2 cm in diameter) into one side of the pot (7.62 cm from the top and 3.5 cm from the side). Two pots were connected by the horizontal section of a “T” shaped piece of PVC (3.2 cm in diameter), and the middle (vertical section) of the PVC was plugged with a rubber stopper (Fig. 3.1).

To validate the effectiveness of the SAO, the attraction of BVW larvae to actively growing plant roots was tested in the following manner. Rooted cuttings

of *P. abies* var. *nidiformis* that had been growing in unaltered pots of the same size used for constructing the SAO for at least two weeks were placed, media (a 2:1 mixture of peat moss (Sunshine Mix #3 SunGro Horticulture, Bellevue, WA) and turkey grit (Cherry Stone #3 New Ulm, MN)) and all, in one half of the SAO described above. The turkey grit was used in place of perlite to provide drainage and aeration while not interfering with researchers locating the larvae and the end of the trials. The other side of the SAO received freshly prepared potting media only. After assembly, the SAO was allowed to sit for 24 hours in the greenhouse (ambient light and 21 - 24°C). Greenhouse conditions were used in order to maintain a constant environment over the course of the experiments. After 24 hours, five 3rd - 4th instar BVW were placed in the middle of each “T” tube connecting the two pots making up the SAO. The SAO sat overnight to allow the larvae time to move. The following day, the SAO was thoroughly examined and the number of larvae on each side determined.

Experimental Design. In order to determine what effect various management options have on the movement of BVW larvae, the following design was utilized. The SAO described above was used to make pairwise comparisons of larval behavior when subjected to media incorporated with *M. anisopliae* and untreated media, media incorporated with bifenthrin and untreated media, as well as media incorporated with *M. anisopliae* and media incorporated with bifenthrin. Before incorporation into potting media, the viability of *M. anisopliae* was assessed by spreading 1 ml of 0.05% Tween solution

Fig. 3.1: Still-air olfactometer used in experiments. Larvae were placed in the middle of the PVC tube connecting the two pots and collected from media inside the pots.



containing 1×10^9 conidia of *M. anisopliae* per ml onto a Petri dish of Potato Dextrose Agar (PDA). The dish was placed in complete darkness (28°C) for 24 hours, after which the percentage of spores producing germ tubes more than twice the length spore was determined. Potting media for use in experiments was incorporated with *M. anisopliae* at the rate of 1×10^6 viable spores g^{-1} dry soil by adding the appropriate quantity of granules to 1L of media and mixing in a 4L twinshell blender (Patterson-Kelley East Stroudsburg, PA) for 5 minutes. This treated media was then added to 9L of untreated potting media in a concrete mixer and again mixed for 5 minutes in order to ensure an even distribution. Bifenthrin (Talstar 0.2G[®], FMC Corporation, Philadelphia, PA) was incorporated into media as described above, at the recommended rate for BVW control of 25 ppm (FMC 1999). Treated media was added to the pot on one side of the SAO while the other pot received untreated media, except in the case where *M. anisopliae* and bifenthrin were directly compared to one another. In order to test for inherent effects of the treatments applied, all combinations were run without plants being potted in either side of the SAO. Trials were also run with plants (*P. abies* var. *nidiformis*) potted in the media on both sides of the SAO to more closely mimic field conditions. Experiments were arranged in a completely randomized design, with 10 replicates and repeated six times. Plants and larvae were only used once, and pots were thoroughly washed before each use.

Trials Without Plants. The SAO containing media inoculated with *M. anisopliae* were allowed to sit in a greenhouse for five days to allow the fungus to germinate. Olfactometers containing media incorporated with bifenthrin were also

allowed to sit for five days before receiving weevils. After five days, five 3rd - 4th instar BVW were placed in the middle of the "T" tube. The SAO stood overnight to allow the larvae time to move. The next day, SAO were thoroughly examined and the number of larvae on each side counted.

Trials With Plants. To test for the effect of a live plant in the system, rooted cuttings of *P. abies* var. *nidiformis* were potted into media incorporated with *M. anisopliae*, bifenthrin or untreated media as described above. Plants were then transferred to a greenhouse (21 - 24°C) for 21 days to allow them to recover from any potential shock of being repotted. Plants were then transferred, media and all, to the SAO. Twenty four hours later, five 3rd - 4th instar BVW were placed in the middle of the "T" tube. The SAO stood overnight to allow the larvae time to move. The next day, the SAO were thoroughly examined and the number of larvae on each side counted.

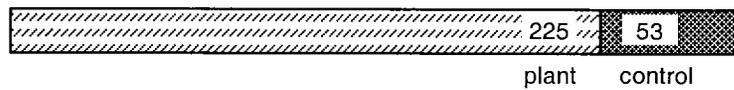
Quantification of Fungal Populations. For all trials containing media incorporated with *M. anisopliae*, one pot from each trial was selected at random to verify fungal growth and serve as an estimate of fungal population size. A sample of bulk potting media from the center of each pot was transferred to sterile plastic bags (Nasco, Modesto, CA). Media samples were stored in a 4°C refrigerator until use. *Metarhizium anisopliae* will not grow at temperatures below 4°C (Eskesi et al. 1999, Hallsworth and Magan 1999). Fungal populations were estimated in the following manner, adapted from Bruck (2005). Ten g of bulk potting media were placed in a plastic 250 ml Erlenmeyer flask containing 90 ml of 0.05% Tween 80 solution, shaken (250 rpm) for 20 min at room temperature, then placed in an

ultrasonic cleaner (Model 5210, Branson Ultrasonic Corp., Danbury, CT) for 2 min. Serial dilutions were plated using a spiral plater (iUL Instruments, Barcelona, Spain) onto two plates of media selective for *M. anisopliae* (Veen and Ferron 1966). Plates were then incubated in complete darkness at 28°C for 4 days. The number of colony forming units (CFU) g⁻¹ dry bulk media were then averaged across replicate plates for each sample. To ensure that the colonies counted were *M. anisopliae*, a total of 20 colonies morphologically identical to those counted as *M. anisopliae* were randomly selected from a number of different plates from each sample and aseptically transferred to PDA and allowed to sporulate. The colonies transferred were identified based on macro and microscopic characteristics (Humber 1997). All colonies transferred to PDA throughout the study were *M. anisopliae*.

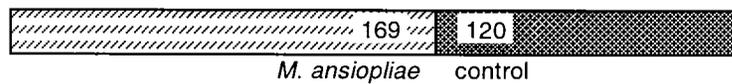
Statistical Analysis. Paired *t*-tests were used to compare weevil movement for each trial. Any weevil still remaining in the center of the PVC tube and not associated with any media was scored as having not moved and excluded from the analysis. Analyses were performed using SPlus version 6.2 for Windows (Insightful Corp. 2003).

Results

The usefulness of the SAO designed for these experiments was validated by BVW larvae effectively selecting media containing a plant over media without a plant. A significant portion of larvae moved towards media containing a plant (Fig. 3.2). In addition, the ability of the larvae to move preferentially to media



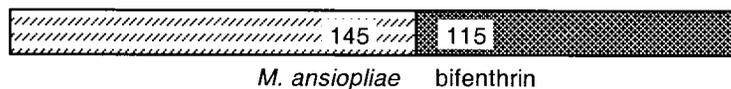
$t = 13.91$
 $df = 118$
 $P < 0.0001$



$t = 4.06$
 $df = 118$
 $P = 0.0001$



$t = 3.75$
 $df = 118$
 $P = 0.0003$



$t = 2.33$
 $df = 118$
 $P = 0.02$

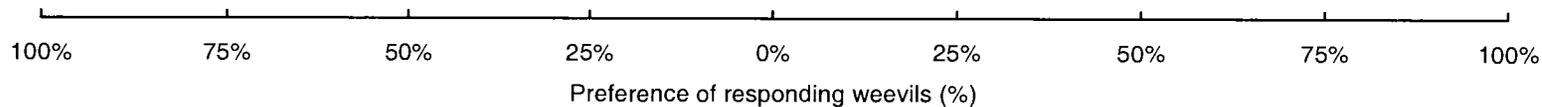


Fig. 3.2

Fig. 3.2: Preference of *O. sulcatus* larvae in still-air olfactometers for validation and trials with plants. Bars represent the percentage larvae responding to the treatment given beneath. Numbers inside each bar represent the total number of weevil larvae responding to each treatment from all trials.

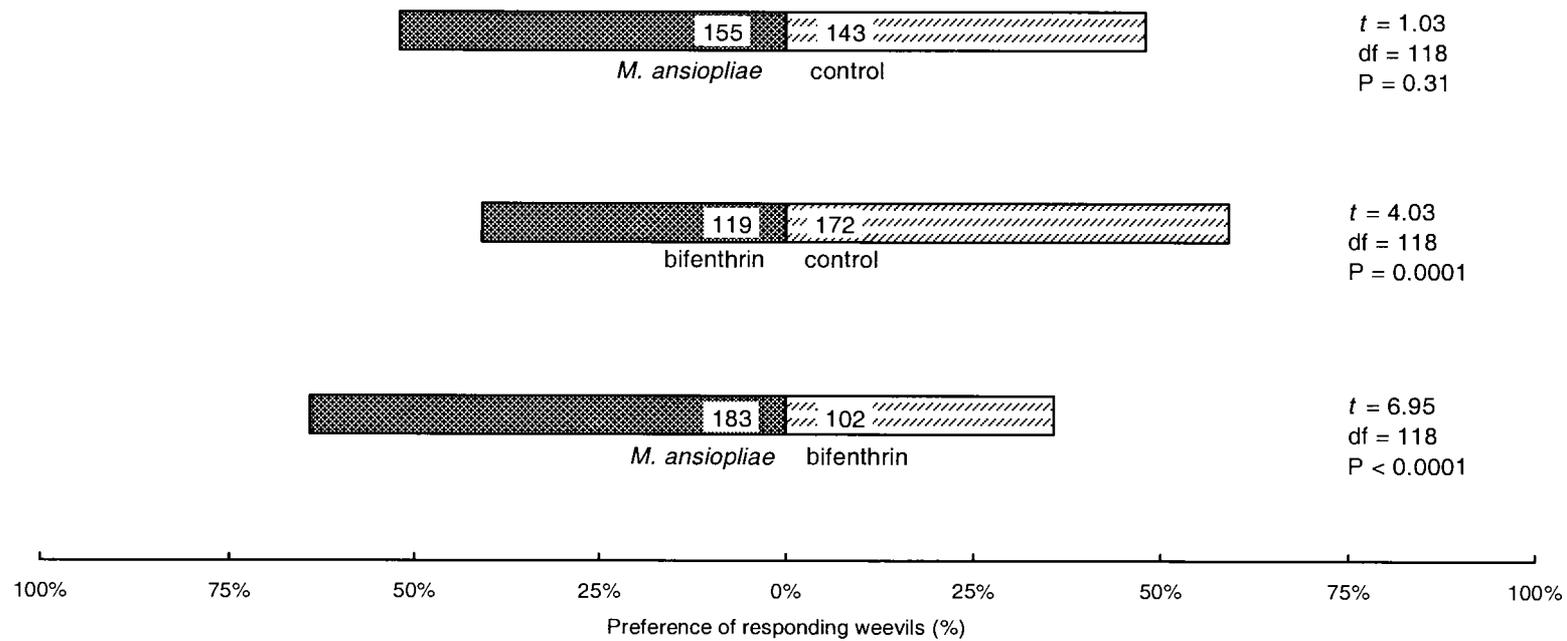


Fig. 3.3: Preference of *O. sulcatus* larvae in still-air olfactometers for trials without plants. Bars represent the percentage larvae responding to the treatment indicated below them. Numbers inside each bar represent the total number of weevil larvae responding to each treatment from all trials.

containing a plant demonstrates their ability to select an environment that was more favorable for their survival. The percentage of responding weevils was never less than 88% in any trial throughout the study.

When larvae were allowed a choice between *M. anisopliae* treated and untreated media, without plants in the SAO, there were no significant differences between treatments (Fig. 3.3). When plants were present in the SAO, larvae were found to be attracted to media incorporated with *M. anisopliae* (Fig. 3.2). The mean fungal population in the potting media during trials with and without plants was 4.22×10^6 and 1.21×10^7 CFU g⁻¹ dry media, respectively.

For trials where BVW larvae were given a choice between media incorporated with bifenthrin and untreated media, without plants present in the SAO, significantly more larvae were recovered from the untreated potting media (Fig. 3.3). When plants were included in the SAO, larval movement was again directed away from media incorporated with bifenthrin (Fig. 3.2)

When BVW larvae were allowed to choose between media incorporated with bifenthrin and media incorporated with *M. anisopliae*, without plants present in the SAO, significantly more larvae were present in the media incorporated with *M. anisopliae* than media incorporated with bifenthrin (Fig. 3.3). This positive response to *M. anisopliae* was also observed when plants were included in the SAO (Fig. 3.2). The mean fungal population in the potting media during trials with and without plants was 7.65×10^7 and 4.82×10^7 CFU g⁻¹ dry media, respectively.

Discussion

This work demonstrates the first usage of a still air olfactometer known of by these authors able to effectively allow larval movement below ground to be assessed. Previous studies have relied on the use of radiographic methods (Krueger et al. 1992) or tunnel castings (Villani et al. 2002, Thompson and Brandenburg 2005). The ease of construction and inexpensive nature of this test arena allow for rapid assessment of pairwise comparisons for larval response to treatment.

The results of these experiments indicate that the behavior and movement of BVW larvae can be affected by the management option employed. This is especially true for the synthetic pyrethroid bifenthrin, which repelled BVW larvae in all scenarios tested when applied at the manufacturer's recommended rate for BVW control. The presence of bifenthrin in soil has also been found to affect the behavior of late instar mole crickets (Orthoptera: Gryllotalpidae) in a way that reduces exposure to the chemical (Thompson and Brandenburg 2005). Their trials consisted of a bifenthrin-treated layer of soil beneath a layer of an untreated soil. A significant proportion of crickets were located in the untreated top layer of soil. Tunneling was also restricted to the untreated layer, reducing the passage of crickets through treated soil.

The repellent properties of bifenthrin could hamper BVW management efforts in several ways. In the short-term, the ability of larvae to direct movement away from this chemical could result in the larvae relocating to areas in a pot with lower concentrations of the pesticide. Untreated areas may arise under several conditions during typical nursery operations. An uneven mixing of potting media

may result in insecticide concentration gradients forming within pots. Refuges may also arise when plants are transferred to larger pots during the production process. If pesticides are not used consistently throughout the production process, plants that have undergone several transfers may be growing in both treated and untreated soils. Such refuges have been implicated in other studies where bifenthrin failed to provide control of BVW (Swier et al. 1998). Furthermore, given the highly polyphagous nature of BVW, treatment of only one crop in a nursery setting may lead to the displacement of pest problems, rather than actually controlling the population, especially if avoidance to bifenthrin is observed in egg-laying adults. Sparks et al. (1985) lists a predisposition to detect and avoid potentially toxic substances as a prerequisite for the evolution of resistance in insect populations. This study indicates that such a predisposition may exist for the BVW. Although it is unlikely that the use of bifenthrin in controlling this pest is currently widespread or intensive enough to actually lead to the evolution of resistance, prudence would dictate that such factors be considered when making management decisions.

In contrast, the entomopathogenic fungus *M. anisopliae* did not have repellent properties in any scenario tested, and in fact appeared to attract BVW larvae when incorporated into media containing a plant. Entomopathogenic fungi are capable of causing an avoidance response in insects. Krueger et al. (1992) observed that Japanese beetle grubs (Coleoptera: Scarabaeidae) could avoid regions of soil treated with *M. anisopliae*. Mole crickets (Orthoptera: Gryllotalpidae) were observed to modify their behavior in response to *M.*

anisopliae in a way that reduced their exposure to the fungus (Villani et al. 2002). Thompson and Brandenburg (2005) observed mole crickets to direct their movement away from soil treated with the entomopathogenic fungus *Beauveria bassiana* (Hypocreales: Clavicipitaceae). This work also showed that the cricket avoidance behavior was strain dependent, indicating insect behavior may be an important factor to consider when developing microbial products for commercial use.

Metarhizium anisopliae is known to be an effective biological control agent against BVW (Moorhouse 1990, Moorhouse et al. 1993a, Bruck 2005). This experiment provides support for its use in the nursery setting. By not negatively affecting the movement of BVW larvae, they are more likely to remain in a hostile environment, increasing their exposure to the pathogen and the likelihood of infection. The ability of *M. anisopliae* to attract BVW larvae when plants were grown in the SAO is curious. Several explanations arise as to why this may occur. The relationship between host and pathogen is sometimes considered as an “evolutionary arms race” where the host evolves novel means of reducing the instance of infection, and the pathogen evolves novel means of increasing infection that are in sync with one another. It may be the case that *M. anisopliae* in some way directly attracts BVW larvae; however, this seems unlikely given the absence of such a response when only fungus treated media alone was present in the SAO. Another more plausible hypothesis is that *M. anisopliae* directly or indirectly interacts with the plant and its environment to produce a more favorable habitat to BVW larvae than a plant alone. *Metarhizium anisopliae* is rhizosphere

competent (Hu and St. Leger 2002, Bruck 2005). It is possible that this interaction results in changes in the microbial community in the rhizosphere, and these changes in turn present themselves as more favorable to BVW larvae. Another possibility is that the presence of *M. anisopliae* in the rhizosphere causes changes in the exudates released by the roots that are more appealing to BVW larvae than plants without the fungus present.

Today's social climate is putting pressure on agricultural and horticultural operations to reduce the quantity of synthetic chemicals used in their operations due to environmental and health concerns surrounding the use of such products. In response to this demand, the need for a wider variety of ecologically sensitive pest control methods must be addressed. This is especially true in the case of bifenthrin where complete control of BVW is achieved at 32 ppm (Cowles 2001), a rate in excess of that labeled for use against BVW (FMC 1999). At the population levels obtained in this study, *M. anisopliae* is capable of causing 93 - 97% BVW mortality (Bruck 2005). The isolate of *M. anisopliae* used in these experiments is registered with the EPA and is scheduled to be made available for commercial use. The data presented here are relevant from a management perspective and can be used to inform pest control decisions in a nursery setting. Adequate field application will depend on an consideration of factors such as temperature effects and the effect of host plant on behavior under various management tactics.

Acknowledgements

We would like to thank Amanda Griffith for rearing and supplying the larvae used in these experiments; Kelly Donahue for assistance in plating fungal soil samples; Jim Fisher contributed useful information on the biology and natural history of the Black Vine Weevil.

CHAPTER 4

Rhizosphere Inoculation with *Metarhizium anisopliae*: Impact on Root Growth and Larval Feeding by *Otiorhynchus sulcatus* (Coleoptera: Curculionidae)

Ryan M. Kepler and Denny J. Bruck

ABSTRACT

The black vine weevil (BVW) *Otiorhynchus sulcatus* (F.) is a serious pest of horticultural operations in the Pacific Northwest. A potential candidate for inclusion into an integrated pest management program for the BVW is the entomopathogenic fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin. The objectives of these experiments were to determine the effect of rhizosphere inoculation of bird's nest spruce, *Picea abies* var. *nidiformis* with *M. anisopliae* on root growth and BVW larval feeding. Plants were randomly allocated to one of three treatments: immersion in 1×10^6 viable spores mL⁻¹ of *M. anisopliae* up to the root crown, immersion in 0.05% Tween 80 (spore surfactant) or an untreated control. Each treatment was performed with and without BVW larval infestation. Measurements of root length for each plant used in these experiments were obtained prior to treatment, and two and six weeks after BVW larval infestation. The results of this study did not show a significant effect on plant growth when plants are treated with *M. anisopliae* relative to Tween or control treatments in the absence of larvae. *Metarhizium anisopliae* does not show plant pathogenic tendencies. In the presence of larvae the data suggest that *M. anisopliae* is able to provide gains in root length relative to Tween or control treatments. This work provides grounds for further development of *M. anisopliae* as a root dip against BVW.

Keywords: *Metarhizium anisopliae*, *Otiorhynchus sulcatus*, microbial control, rhizosphere, plant growth

Introduction

The black vine weevil (BVW) *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae) is a persistent pest of nursery operations in many parts of the United States, including the Pacific Northwest (PNW) (Warner and Negley 1976). The BVW is a polyphagous (over 140 species) and univoltine insect. Adults reproduce by parthenogenesis, so a single individual left unchecked can infest an entire nursery. Adults are nocturnal feeders and cause mainly cosmetic damage to leaves, while the larval stage, developing in the soil and feeding on roots, is the most damaging. The BVW typically overwinters as a prepupae or last instar (Smith 1932). Overwintering larvae complete their development in the spring and adults eclose from early June to late July, with peak eclosion near the middle of June. Oviposition begins in the middle of July for adults that emerge in June, after a preoviposition period of 28 - 50 days. Some eggs laid very early may produce individuals capable of developing into adults before winter (Smith 1932). In the greenhouse setting, the life cycle may be advanced by two months (Moorhouse et al. 1992). Adults may also remain active in the greenhouse through December and January, all the while continuing to oviposit (Smith 1932). For some species of plant (e.g. *Cyclamen sp.*) one larva is enough to cause death (Moorhouse 1990). Larvae have been observed to girdle the root system or bore into the above ground portions of larger stems. There is a zero-tolerance for BVW in nursery stock. Infested plants can not be sold and if infested plants are shipped, the grower risks refusal of the plants by the buyer and will incur the additional return shipping costs and potential loss of future sales.

Although cultural management practices, such as the removal of natural reservoirs of infested plant material surrounding the nursery and the removal of infested plant material that can not be effectively or economically treated can limit the possibility and extent of an infestation (Bruck 2004a), most growers still rely on the use of broad spectrum insecticides that target adults in the spring in an attempt to prevent oviposition as the primary means of managing BVW populations. However, even after implementing an extensive insecticidal spray program against adults, nursery growers often discover plant material infested with last instars in the winter or in the spring prior to shipping. In a further attempt to protect their crop, many growers have begun to incorporate insecticides into the soil at potting. In the PNW, the synthetic pyrethroid bifenthrin (Talstar[®] 0.2G), is currently the most commonly employed soil insecticide (D. Hicks, personal communication).

The entomopathogenic fungus *Metarhizium anisopliae* (Metchnikoff.) Sorokin (Hypocreales: Clavicipitaceae) has been successfully employed as a microbial control agent against BVW (Moorhouse 1990, Moorhouse et al. 1993a, Bruck 2005) and presents itself as a candidate for adoption into integrated farming practices. A commercial formulation of *M. anisopliae* (strain F52) (Earth BioSciences, New Haven, CT) is currently registered with the US Environmental Protection Agency (EPA) for use against several insects, including BVW. Infection occurs when conidia come into contact with the cuticle of an insect, producing a germ tube which penetrates the hemocoel through the production of cuticle degrading enzymes and mechanical pressure (Chamley 1984). The fungus

then multiplies as yeast, switching to a mycelial growth phase before sporulating on the external surface of the insect.

In addition to its use as a microbial insecticide, *M. anisopliae* has been shown to be rhizosphere competent, capable of forming populations an order of magnitude higher in rhizosphere soil than in the surrounding bulk soil (Hu and St. Leger 2002, Bruck 2005). Employing *M. anisopliae* as a rhizosphere colonizer is a novel means of achieving plant protection; when roots were inoculated with *M. anisopliae*, larval mortality as high as 76% was achieved (Bruck 2005). Although a reduction in weevil population has been observed, it is currently unknown how this relates to gains realized by the plant. Control of plant pathogens with rhizosphere competent microbes has been achieved (Whipps 2001); for example, *Trichoderma koningii* Rifai has been successful in reducing damage by *Sclerotium rolfsii* Sacc., the causative organism of damping off in tomatoes (Tsayhouridou and Thanassouloupoulos 2002). The addition of a rhizosphere competent microbial control agent can have varying direct effects on plant growth. *Pseudomonas putida* and *P. chlororaphis*, bacteria examined as biological control agents for maize root diseases, have been shown to reduce overall plant growth in the absence of pathogens (Kozdroj et al. 2004). Conversely, improvements in growth were observed from treatment with *T. koningii* in the absence of *S. rolfsii* (Tsayhouridou and Thanassouloupoulos 2002). The objectives of these experiments were to quantify the effect of rhizosphere colonization of *Picea abies* (L.) Karst. (Pinales: Pinaceae) var. *nidiformis* with *M. anisopliae* on root growth in the presence and absence of BVW larval feeding pressure.

Materials and Methods

Experimental Design. Experiments were arranged in a three by two factorial design, with three levels of root treatment, and two levels of weevil presence. The three root treatments were as follows: an untreated control, immersion in 0.05% Tween 80 (spore surfactant) up to the root crown, or immersion in 1×10^6 viable spores mL^{-1} of a rhizosphere competent isolate of *M. anisopliae* (strain F52) (Earth BioSciences, Fairfield, CT). In the context of these studies, we use the definition of rhizosphere competence proposed by Schmidt (1979) who defined “rhizosphere competent” microorganisms as those showing enhanced growth in response to developing roots or a classical rhizosphere effect. Each treatment was performed with and without BVW larval infestation. Measurements of root length for each plant (determined using WinRhizo2001, Regent, Quebec, Canada) used in these experiments were obtained prior to treatment, and two and six weeks after BVW larval infestation (Fig. 4.1). Samples were taken two and six weeks after weevil introduction to coincide with the earliest anticipated signs of infection and the final stadium before pupation, respectively. At the time of sampling, the numbers of live, dead and infected larvae were enumerated, and the rhizosphere fungal population determined. The experiments were replicated five times with two plants randomly assigned to each treatment. One plant from each treatment was randomly selected for evaluation at two weeks post larval infestation with the remaining plant evaluated at 6 weeks. The entire experiment was repeated three times. A treatment of untreated roots with larvae present was not performed during the first trial. Plants for trial one were potted on

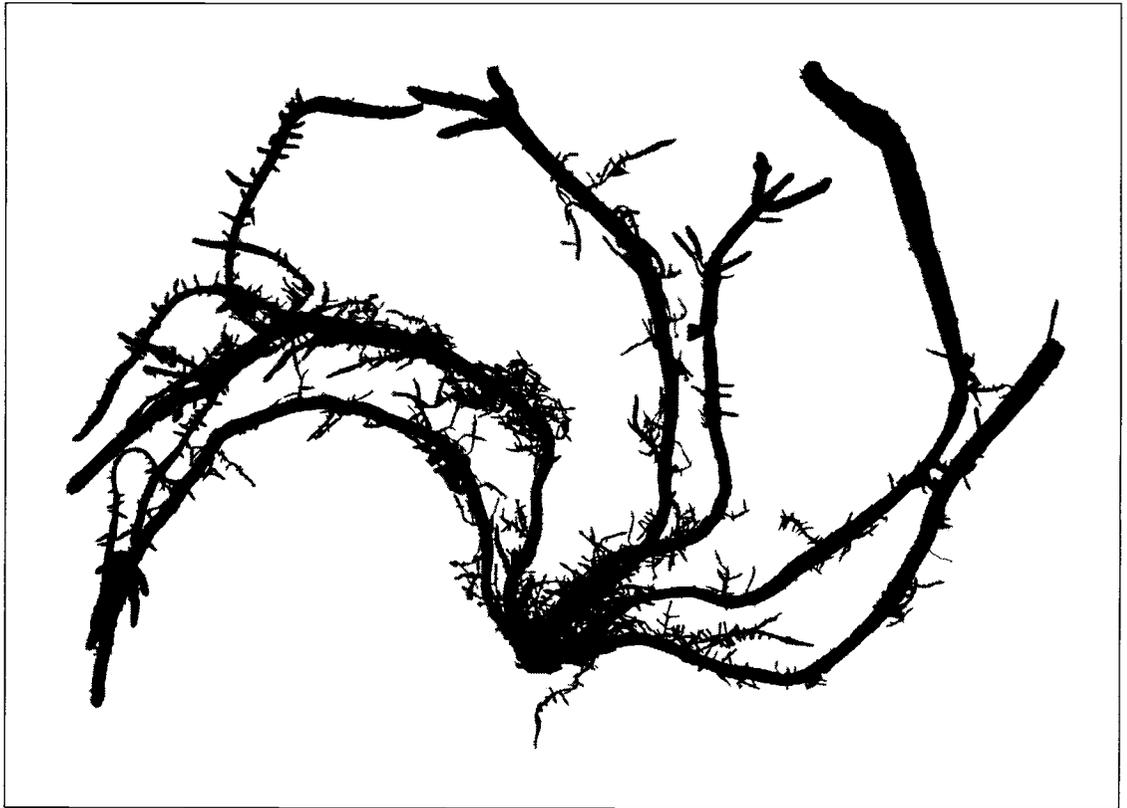


Fig 4.1: Example of image obtained using WinRhizo2001 after editing in Photoshop.

3 February 2005 and the final sample taken on 23 March 2005. Plants for trial two were potted on 7 April 2005 and the final sample taken on 24 May 2005. Plants for trial three were potted on 11 May 2005 and the final sample taken on 28 June 2005.

Preparation of the Plants. Rooted cuttings of *Picea abies* var (L.) Karst. (Pinales: Pinaceae) var. *nidiformis* obtained from a local nursery grower were used in the experiments. Plants used in the first two runs of the experiment were maintained in perlite (Supreme Perlite Co., Portland, OR) and kept in a mist bed until use. Plants used in the third run of the experiment were maintained in a bark-based media before use. For each experiment, the roots of 60 *P. abies* were washed under running tap water to remove the perlite or potting media clinging to the roots. Plants were then randomly distributed among the six treatments, assigned a unique code to maintain their identity throughout the course of the experiment, and scanned. Root images were obtained by floating plants in water in a transparent tray positioned on a digital scanner. Because particles of potting material and organic matter may become dislodged during the scanning process, all images obtained were edited using Adobe Photoshop Version 8.0 (Adobe Systems Inc. 2003) in order to eliminate any debris that could interfere with later analysis. After scanning, plants were stored overnight at 4°C before root treatment and potting the following day.

Prior to root treatment, the viability of *M. anisopliae*, was assessed by spreading 1 ml of 0.05% Tween 80 solution containing 1×10^9 conidia *M. anisopliae* ml⁻¹ onto a Petri dish of Potato Dextrose Agar (PDA). The dish was

placed in complete darkness at 28°C for 24 hours, after which the percentage of spores producing germ tubes more than twice the length of a spore was determined. Spores were then added to 1L of 0.05% Tween 80 solution at a rate of 1×10^6 viable spores mL⁻¹. Plants receiving this treatment were then dipped into the solution up to the top of the root crown and then placed on paper towels to air dry before potting. Plants that received the Tween 80 only treatment were prepared in the above manner. Plants not receiving any root treatment were potted directly. For all trials, a 2:1 mixture of peat moss (Sunshine #3 mix, SunGro Horticulture, Bellevue, WA) and turkey grit (Cherry Stone Grit #3, New Ulm, MN) was the potting media used. Turkey grit was used in place of perlite to provide drainage and aeration while not interfering with researchers locating the larvae and the end of the trials. After root treatment, all pots were moved to a climate controlled greenhouse (ambient light, 22 - 24°C) and arranged in a Latin Square design.

Addition and Sampling of Larvae. After the plants were allowed to acclimate in the pots for one week, three 3rd - 4th instar BVW were added to the appropriate pot by placing them on the soil surface. Larvae quickly burrowed into the soil (5 - 10 minutes). Any larvae that had not burrowed into the potting media within 15 minutes were replaced. Larvae were obtained from the colony maintained at the USDA-ARS Horticultural Crops Research Laboratory in Corvallis, OR (Fisher and Bruck 2004).

Two and six weeks after larval infestation, five pots (one from each replicate), selected at random, were chosen for analysis. Pots that had received

larvae had the total number of live, dead and infected individuals counted. An individual was counted as infected only if there were visible signs of fungal presence (i.e. sporulation). All plants were gently removed from the pots and shaken to remove any media loosely adhering to the roots. Root masses were then severed from the stem at the root crown, placed in a sterile bag (Nasco, Modesto, CA), and stored at 4°C until analysis. *Metarhizium anisopliae* will not grow at temperatures below 4°C (Eskesi et al. 1999, Hallsworth and Magan 1999).

Quantification of Rhizosphere Fungal Population. To quantify the *M. anisopliae* population in the rhizosphere, the entire root system from each plant was placed into a plastic 250 ml Erlenmeyer flask containing 90 ml of 0.05% Tween 80 solution. Trial one was placed in a shaker for 20 minutes at 220 rpm. All subsequent trials were shaken for 20 minutes at 175 rpm to reduce damage to the roots prior to scanning. After shaking, roots were placed in an ultrasonic cleaner (Model 5210, Branson Ultrasonic Corp., Danbury, CT) for 2 min. Serial dilutions were plated using a spiral plater (iUL Instruments, Barcelona, Spain) onto two plates of media selective for *M. anisopliae* (Veen and Ferron 1966). Plates were incubated in complete darkness at 28°C for 4 d. The number of colony forming units (CFU) g⁻¹ dry bulk media was averaged across replicate plates for each sample. To ensure that the colonies counted were *M. anisopliae*, a total of 20 colonies morphologically identical to those counted as *M. anisopliae* were randomly selected from a number of different plates from each sample and aseptically transferred to PDA and allowed to sporulate. The colonies transferred

were identified based on macro and microscopic characteristics (Humber 1997).

All colonies transferred to PDA throughout the study were *M. anisopliae*.

To quantify the amount of rhizosphere media on the root system of each plant sampled, the suspension remaining in each flask (once the roots are removed) was poured into a pre-weighed aluminum pan. Each flask was carefully flushed with distilled water to remove all soil particles. Pans containing the suspension were placed in a 38°C drying oven until dry (approximately 24 h) and weighed. After the procedures to assess the rhizosphere fungal population were complete, each root system was scanned, using the methods described above to determine changes in root length.

Statistical Analysis. Data analysis was performed using the General Linear Models Procedure (GLM) with Tukey's multiple range test used to separate means (SAS Institute 1999). The data from each run of the experiment were analyzed separately. Although samples were collected from each treatment over time, a repeated measures analysis was not required because plants were maintained individually, destructively sampled and the change in root length and the percentage of surviving weevils in each pot quantified only once. In order to normalize the data, the initial and final root length measurements were log transformed and the difference in the log root length used in the GLM analysis. The values and standard deviations reported in the tables are the untransformed values. A linear regression analysis was also performed to determine the relationship between the number of live BVW larvae and the change in log root length.

Results

There was no significant relationship between the number of live BVW larvae and the difference in log root length (Fig.4.1). The effect of sample date was not found to be significant for any trial (Tables 4.1). The effect of treatment was found to be significant for trial one, with *M. anisopliae* showing a net increase in root length and Tween showing a net decrease. A significant increase was also seen for control plants not receiving larvae in trial one, however, this trial did not include a control with larvae treatment (Tables 4.1 and 4.3). Treatment was also found to be significant for trial two, with *M. anisopliae* showing a significant increase in root length over control plants (Tables 4.1 and 4.3). The effect of treatment on plants treated with Tween was not significantly different from *M. anisopliae* or control plants for trial two (Tables 4.1 and 4.3). The presence of weevils was found to significantly decrease root length for all trials (Table 4.1 and 4.4).

The interaction of sample date with treatment level was found to be significant for trial three only. In this trial, the increase in root length for plants treated with *M. anisopliae* at week six was found to be significantly greater than gains seen for Tween or control treated plants at six weeks (Tables 4.1 and 4.5). The interaction of sample date with presence of larvae was found to be significant for trials one and three; however, the ability to discern the nature of the interaction for trial one is hampered by the lack of a control with larvae treatment. For trial three, the presence of larvae significantly decreased root length compared to the increases in root length seen without larvae at both sample dates (Tables 4.1 and

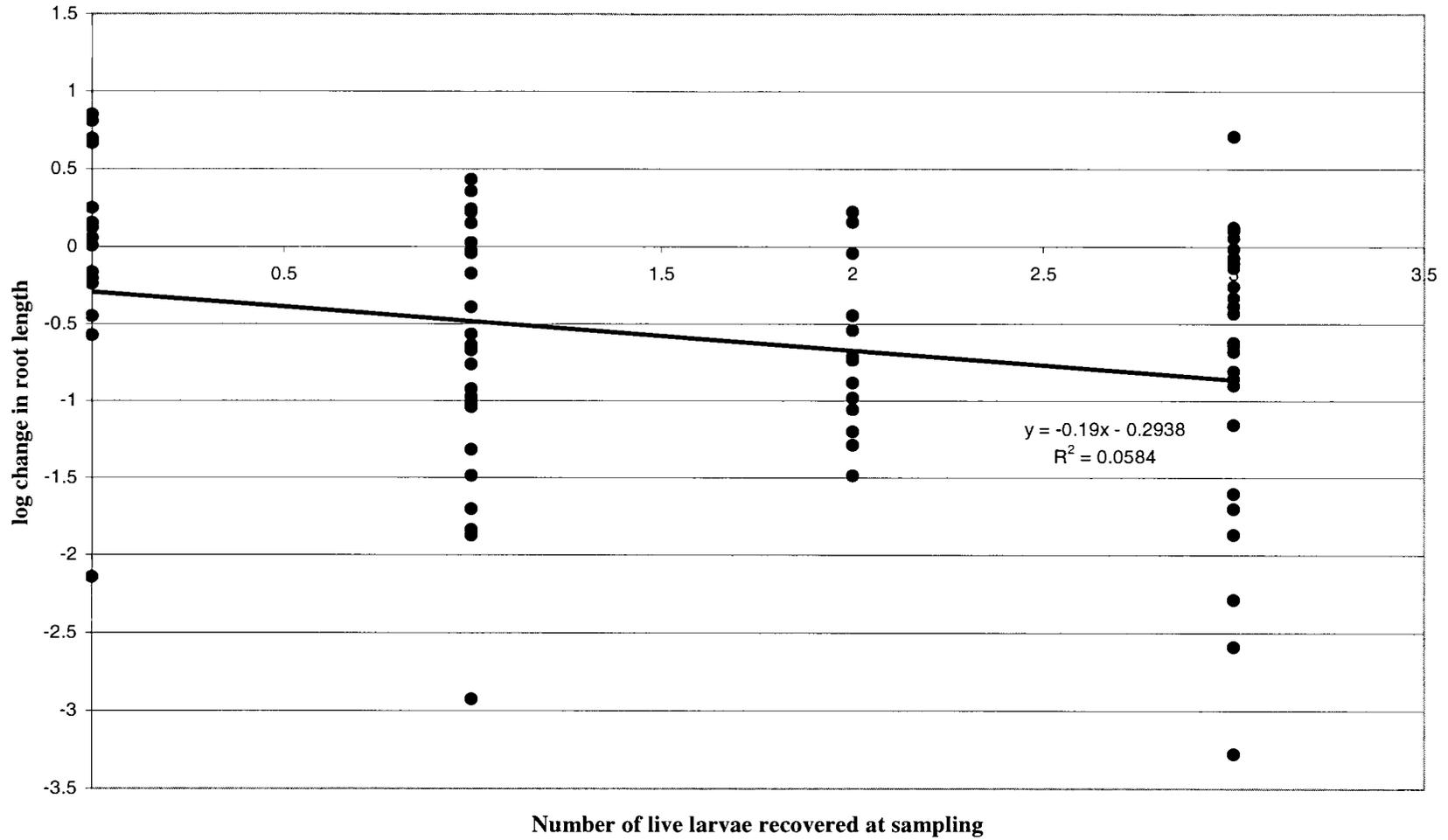


Fig. 4.2: Linear regression of log change in root length on the number of live larvae recovered at time of sampling

Table 4.1: ANOVA table from the difference in log root length analysis

	Trial 1			Trial 2			Trial 3		
	F	df	<i>P</i>	F	df	<i>P</i>	F	df	<i>P</i>
<u>Main effects</u>									
Sample Date	3.18	1	0.082	1.40	1	0.24	0.03	1	0.87
Root Treatment	4.72	2	0.014	3.21	2	0.049	0.59	2	0.56
Larvae (Presence-absence)	108.03	1	<.0001	38.39	1	<.0001	43.19	1	<.0001
<u>Two-way interactions</u>									
Sample by treatment	0.99	2	0.38	1.32	2	0.28	3.19	2	0.05
Sample by larvae	16.46	1	0.0002	1.93	1	0.17	6.31	1	0.015
Treatment by larvae	13.53	1	0.0007	3.54	2	0.037	0.68	2	0.51
<u>Three-way interaction</u>									
Sample by treatment by larvae	2.56	1	0.12	2.53	2	0.090	3.23	2	0.049

Table 4.2: Mean (\pm SD) root length (cm) differences for samples taken at two and six weeks past larval infestation

	2 weeks ^a	6 weeks ^b
Trial 1	-19.4 (35.3) ^a ^c	36.7 (85.1) ^b
Trial 2	18.5 (56.7) ^a	76.2 (107) ^a
Trial 3	11.5 (77.7) ^a	50.4 (157.6) ^a

^a Sample taken two weeks after addition of larvae to those treatments receiving them.

^b Sample taken six weeks after addition of larvae to those treatments receiving them.

^c Means followed by the same letter within a row are not significantly different ($P < 0.05$) (SAS Institute 1999).

Table 4.3: Mean (\pm SD) root length (cm) differences due to treatment with *M. anisopliae*, Tween or the untreated control

	<i>M.a.</i> ^a	Tween ^b	Control
Trial 1	3.3 (58.3) ^a ^c	-4.7 (87.3) ^c	46.0 (41.8) ^b
Trial 2	79.1 (83.4) ^a	31.9 (95.52) ^{ba}	31.0 (82.4) ^b
Trial 3	49.2 (126.4) ^a	22.5 (127.8) ^a	21.1 (124.4) ^a

^a Roots treated with an aqueous solution of *M. anisopliae* at 1×10^6 viable spores mL⁻¹ in 0.05% Tween 80.

^b Roots treated with an aqueous solution of 0.05% Tween 80.

^c Means followed by the same letter within a row are not significantly different ($P < 0.05$) (SAS Institute 1999).

Table 4.4: Mean (\pm SD) root length (cm) differences due to treatments with and without larvae

	Without larvae	With larvae ^a
Trial 1	51.0 (53.4) ^a ^b	-54.9 (36.8) ^b
Trial 2	103.0 (53.1) ^a	-7.3 (83.9) ^b
Trial 3	115.6 (69.8) ^a	-53.7 (109.3) ^b

^a Addition of three 3rd - 4th instar BVW one week after planting.

^b Means followed by the same letter within a row are not significantly different ($P < 0.05$) (SAS Institute 1999).

Table 4.5: Mean (\pm SD) root length (cm) differences due to the interaction between sample date and treatment

	2wk^a/<i>M.a.</i>^b	2wk/Tween^c	2wk/Control	6wk^d/<i>M.a.</i>	6wk/Tween	6wk/Control
Trial 1^e	-22.0 (34.2) ^{a^f}	-32.6 (35.1) ^a	12.3 (19.4)	28.6 (67.7) ^b	23.2 (114.5) ^a	79.8 (26.5)
Trial 2	30.6 (62.8) ^{ab}	17.3 (42.4) ^{ab}	7.8 (57.0) ^b	127.7 (74.2) ^a	46.5 (130.4) ^b	54.3 (99.5) ^b
Trial 3	-10.5 (96.3) ^b	32.9 (53.9) ^{ab}	12.13 (78.8) ^{ab}	108.9 (128.5) ^a	12.1 (177.07) ^b	30.0 (162.1) ^b

^a Sample taken two weeks after addition of larvae to those treatments receiving them.

^b Roots treated with an aqueous solution of *M. anisopliae* at 1×10^6 viable spores mL⁻¹ in 0.05% Tween 80.

^c Solution of 0.05% Tween 80.

^d Sample taken six weeks after addition of larvae to those treatments receiving them.

^e Trial does not include control with weevil treatment, therefore comparisons of control means not valid.

^f Means followed by the same letter within a row are not significantly different ($P < 0.05$) (SAS Institute 1999).

Table 4.6: Mean (\pm SD) root length (cm) differences due to the interaction between sample date and larval presence

	2wk^a/no larvae	2wk/larvae	6wk^b/no larvae	6wk/larvae
Trial 1^c	43.8 (45.1)	-54.4 (25.3)	98.0 (30.3)	-55.3 (47.1)
Trial 2	61.4 (29.6)a ^d	-24.4 (33.8)b	142.6 (37.8)a	9.7 (113.2)b
Trial 3	56.2 (18.4)a	-33.2 (88.8)b	175.0 (47.0)a	-74.2 (126.4)b

^a Sample taken two weeks after addition of larvae to those treatments receiving them.

^b Sample taken six weeks after addition of larvae to those treatments receiving them.

^c Trial does not include control with weevil treatment, therefore comparisons of means not valid.

^d Means followed by the same letter within a row are not significantly different ($P < 0.05$) (SAS Institute 1999).

Table 4.7: Mean (\pm SD) root length (cm) differences due to the interaction between treatment and larval presence

	<i>M.a.</i> ^a /no larvae	<i>M.a.</i> /larvae	Tween ^b /no larvae	Tween/larvae	Control/no larvae	Control/larvae
Trial 1	43.8 (45.1)a ^c	-37.2 (38.6)b	63.2 (71.9)a	-72.5 (25.9)c	46.0 (41.8)a	na ^d
Trial 2	107.5 (54.0)a	50.8 (100.0)a	102.7 (60.7)a	-38.9 (66.7)b	95.9 (49.0)a	-33.8 (66.7)b
Trial 3	129.4 (83.9)a	-31.0 (111.4)b	105.1 (61.5)a	-60.2 (124.6)b	112.3 (67.4)a	-70.1 (92.2)b

^a Roots treated with an aqueous solution of *M. anisopliae* at 1×10^6 viable spores mL⁻¹ in 0.05% Tween 80.

^b Solution of 0.05% Tween 80.

^c Means followed by the same letter within a row are not significantly different ($P < 0.05$) (SAS Institute 1999).

^d Experimental combination not tested for this trial.

4.6). There was a significant interaction between treatment and larval presence in trials one and two (Tables 4.1 and 4.7). For trials one and two, the mean change in root length for plants receiving *M. anisopliae*, Tween and control without larvae were not significantly different. The mean change in root length for *M. anisopliae* and Tween with larvae were significantly different for trial one, with Tween showing a greater reduction in root length than the *M. anisopliae* (Table 4.7). For trial two, the increase in root length for plants treated with *M. anisopliae* and larvae was not significantly different from any of the without larvae treatments, but was significantly different from the losses sustained by both Tween and control treatments in the presence of larvae.

The three-way interaction between sample date, treatment level and presence of larvae was found to be significant only for trial three (Tables 4.1 and 4.8). Since this trial also showed a significant effect of sample date and treatment level, an analysis of the treatment by larval presence interaction was performed for each separate sample date. This revealed a trend of interaction between treatment level and presence of larvae at the six week sampling date for all trials. This analysis showed a significant interaction between treatment level and larval presence in trial one ($F = 8.75$, $df = 1$, $P = 0.0078$) and trial two ($F = 3.72$, $df = 1$, $P = 0.039$) (Table 4.9). For both trials one and two, no significant differences between treatment levels without larvae were observed (Table 4.9). For trial one, the reduction in root length for *M. anisopliae* treated plants was significantly less than the loss realized by plants treated with Tween in the presence of larvae. For trial two, plants treated with *M. anisopliae* gained root

Table 4.8: Mean (\pm SD) root length (cm) differences due to the interaction of treatment and larvae presence at two and six weeks post larval infestation

	Sample date	<i>M. a</i> / no Larvae ^a	<i>M.a.</i> / Larvae ^b	Tween / no Larvae ^c	Tween / Larvae ^d	Control / no Larvae ^e	Control / Larvae ^f
Trial 1	Two week	1.7 (11.1)a ^g	-45.7 (33.2)b	-2.0 (17.2)a	-63.1 (11.9)c	12.3 (19.4)a	na ^h
	Six week	85.9 (5.8)a	-28.7 (45.5)b	128.4 (26.8)a	-82.0 (33.9)c	79.8 (19.4)a	na
Trial 2	Two week	80.7 (46.3)a	-19.6 (21.2)a	49.6 (6.2)a	-15.0 (37.4)a	54.0 (13.6)a	-38.5 (42.1)b
	Six week	134.3 (51.2)a	121.2 (98.3)a	155.7 (34.8)a	-62.8 (84.7)c	137.8 (28.9)a	-29.2 (63.2)b
Trial 3	Two week	62.1 (22.3)a	-83.1 (84.8)b	51.7 (18.9)a	14.0 (72.7)c	54.7 (16.2)a	-30.5 (72.7)ab
	Six week	196.6 (63.6)a	21.2 (117.9)a	158.6 (32.2)a	-134.3 (126.2)b	169.8 (41.2)a	-109.7 (92.8)b

^a Roots treated with an aqueous solution of *M. anisopliae* at 1×10^6 viable spores mL⁻¹ in 0.05% Tween 80 without addition of three 3rd - 4th instar BVW.

^b Roots treated with an aqueous solution of *M. anisopliae* at 1×10^6 viable spores mL⁻¹ in 0.05% Tween 80 with addition of three 3rd - 4th instar BVW.

^c Roots treated with an aqueous solution of 0.05% Tween 80 without addition of three 3rd - 4th instar BVW.

^d Roots treated with an aqueous solution of 0.05% Tween 80 with addition of three 3rd - 4th instar BVW.

^e no BVW larvae added.

^f Three 3rd - 4th instar BVW added.

^g Means followed by the same letter within a row are not significantly different ($P < 0.05$) (SAS Institute 1999).

^h Experimental combination not performed this trial.

Table 4.9: Mean (\pm SD) root length (cm) differences due to the interaction of treatment and larvae presence for six weeks post infestation with larvae

	<i>M. a</i> ^a / no Larvae	<i>M.a.</i> / Larvae ^b	Tween ^c / no Larvae	Tween / Larvae	Control / no Larvae	Control / Larvae
Trial 1	85.9 (5.8) ^a ^d	-28.7 (45.5) ^b	128.4 (26.8) ^a	-82.0 (33.9) ^c	79.8 (19.4) ^a	na ^e
Trial 2	134.3 (51.2) ^a	121.2 (98.3) ^a	155.7 (34.8) ^a	-62.8 (84.7) ^b	137.8 (28.9) ^a	-29.2 (63.2) ^b
Trial 3	196.6 (63.6) ^a	21.2 (117.9) ^a	158.6 (32.2) ^a	-134.3 (126.2) ^b	169.8 (41.2) ^a	-109.7 (92.8) ^b

^a Roots treated with an aqueous solution of *M. anisopliae* at 1×10^6 viable spores mL⁻¹ in 0.05% Tween 80 without larvae.

^b Addition of three 3rd - 4th instar BVW.

^c 0.05% Tween 80 without larvae.

^d Means followed by the same letter within a row are not significantly different ($P < 0.05$) (SAS Institute 1999).

^e Experimental combination not performed this trial.

Table 4.10: Mean population (\pm SD) of *M. anisopliae* obtained from rhizosphere of inoculated *P. abies* roots

	Sample date ^a	<i>M.a.</i> ^b / no larvae (CFU/g dry soil ^c)	<i>M.a.</i> / larvae (CFU/g dry soil)
Trial 1	Two week	5.8×10^7 (5.0×10^7)	1.0×10^7 (1.0×10^7)
	Six week	2.0×10^7 (1.3×10^7)	1.5×10^7 (2.7×10^7)
Trial 2	Two week	3.6×10^8 (4.4×10^8)	2.2×10^8 (1.4×10^8)
	Six week	7.6×10^6 (1.3×10^7)	1.0×10^8 (1.9×10^8)
Trial 3	Two week	5.0×10^7 (4.4×10^7)	2.4×10^7 (1.1×10^7)
	Six week	1.3×10^7 (4.0×10^6)	6.5×10^7 (9.1×10^7)

^a Sample taken two or six weeks after addition of larvae to those treatments receiving them.

^b Roots treated with an aqueous solution of *M. anisopliae* at 1×10^6 viable spores mL⁻¹ in 0.05% Tween 80 without addition of three 3rd - 4th instar BVW.

^c Mean number of colony forming unit (CFU) from all replicates per treatment at each sample date.

length, which was significantly different from the loss incurred by plants treated with Tween or control in the presence of larvae.

Potting material obtained from the rhizosphere and plated on media selective for *M. anisopliae* showed the fungus successfully colonized and persisted in the rhizosphere of all plants receiving fungal spores throughout the course of the experiment (Table 4.10). For trial two, the colony forming unit (CFU) counts for the *M. anisopliae*-no larvae treatment at six weeks showed a two log drop relative to the two week sample. This was most likely due to the overnight storage of serial dilutions used for plating due to the spiral plater malfunctioning. All other trials were plated immediately after making serial dilutions. *Metarhizium anisopliae* was not detected from the roots of plants in the Tween-only or control treatments.

Discussion

A high degree of variance in changes to root length was observed in these studies. Feeding behavior of BVW larvae may be one factor influencing this result. During the course of the experiment, larvae were observed feeding at various locations along the length of the root. This feeding behavior often severed a significant portion of the roots, and caused the effect of the larvae to be greater than what is actually due to herbivory. An accurate assessment of larval feeding using the techniques of this experiment depends upon the linear removal of root material from the ends of the tips upward. This effect may be increased by the pattern of colonization often exhibited by introduced rhizosphere microbes. Populations are often centered on the growing root tip and root crown where exudates and resources are abundant, resulting in a "C" shaped pattern of distribution (Ahmad and Baker 1987, Harman 1992). Hu and St. Leger (2002) showed that *M.*

anisopliae has an uneven distribution in the rhizosphere of cabbage roots, forming a decreasing population gradient from the root crown to root tips. A larva encountering a root with this distribution pattern of *M. anisopliae* might avoid a lethal dose of the fungus if feeding commences in the middle of the root, thus allowing more extensive feeding by the larvae.

The physiological state of the plants may have added to variance in the results. Trials were conducted from February to June 2005, as *P. abies* was entering the spring growing season. Alternately, it may be that one week after potting is not enough time for the plants to recover from the stress of the scanning and treatment process before infestation with larvae. This could have put the plants in a weakened state of low growth when larvae were added to the pots, and thus made them more susceptible to the effects of herbivory. Rhizosphere colonization by *M. anisopliae* does not appear to have been affected by these factors, since populations counted from both with and without larvae plants are higher than the initial inoculation rate (Table 4.10).

In spite of the high degree of variance observed in these studies, several trends of treatment effect were indicated. There is no evidence that treatment with *M. anisopliae* has any inherent negative effects on plant growth, as measured by changes in root length. Mean differences in root length for *M. anisopliae* treated plants in the absence of BVW larvae were not significantly different from Tween treated or untreated control plants without larvae for any trial (Table 4.7). Rhizosphere organisms introduced as control agents can affect plant growth in the absence of the target organism ranging from increasing plant height and fresh weight (Tsahouridou and Thanassouloupoulos 2002) to a reduction in overall plant growth (Kozdroj et al. 2004). Kozdroj et al. (2004) view this as

an important consideration during the selection process for microbial control agents of root pathogens. Extending this criteria to microbial control agents against insects seems reasonable.

Although not statistically significant, data from samples taken six weeks following larval infestation suggest *M. anisopliae* is able to limit reduction in root length as a result of BVW larval herbivory (Table 4.9). The reduction in root length for *M. anisopliae* treated plants was less than that of Tween when exposed to larvae. For trials two and three, plants treated with *M. anisopliae* and exposed to larvae realized gains in root length that were not different from any of the no larvae treatments. However, the Tween and control treatments receiving larvae showed losses (Table 4.9). *Metarhizium anisopliae* rhizosphere population levels achieved in these trials are sufficient to infect 76% of BVW larvae (Bruck 2005) (Table 4.10). Significant BVW mortality was not seen in this trial (Fig. 1), however the manner in which larvae were applied to the system differed from that of Bruck (2005), where larvae were placed in direct proximity to treated plant roots. In this study larvae were placed on top of the media surface and allowed to burrow.

The roots of a plant are the primary means by which water and mineral nutrients are obtained. The effects of below ground herbivory can significantly influence the relation of a plant to surrounding vegetation and above ground herbivores (Blossey and Hunt-Joshi 2003). Below ground herbivory has been linked to a reduction in plant fitness (Preus and Morrow 1999), increases in drought susceptibility (Brown and Gange 1990) and reduction of reproductive potential (Matter 2001). Protection of roots is therefore a serious consideration when seeking to maximize plant production.

The formation of a successful integrated pest management program against BVW that is able to simultaneously meet society's desires for reductions in the use of synthetic chemicals and grower needs to minimize input costs will depend upon accurate knowledge of the system under consideration. Because biological and microbial control agents are living organisms, whose growth and presence may have effects reaching beyond those immediately apparent, it is important to explore many levels of their autecologies. The results of this work begin to describe the below ground dynamics of *M. anisopliae* in a management setting and potential implications of its use as a microbial control agent. This research did not show *M. anisopliae* to limit the growth of plant roots when applied as a root dip. Further testing on other plant fitness parameters will yield a more complete picture of the interaction occurring between *M. anisopliae* and treated plants. The lack of any inherent deleterious effects from inoculation of plant roots with *M. anisopliae* in the absence of herbivory pressure would provide grounds for further development of its use in this manner. The effects of *M. anisopliae* on herbivory by BVW were less clear, however, the data did suggest that some level of protection is achieved relative to controls. Application of the methods used in this experiment to plants having a more diffuse root system might be able to limit the variance in response of root length to feeding. Distributing root biomass between a greater numbers of roots could limit the amount of length removal not directly associated with feeding. This would allow the formation of a more complete picture of BVW feeding ecology in the presence of *M. anisopliae*.

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CHAPTER 5. Conclusions

The intent of this research was two fold: to provide insight into the behavioral dynamics BVW and the management tactics employed against it in order to improve control of this pest in horticultural operations, and examine the specific situation of *M. anisopliae* as a root dip of *P. abies* and its impact on root growth and herbivory due to BVW larval feeding.

The development of a SAO able to accurately represent the below ground movement of BVW larvae in response to soil treatments will help to further future research in this area. Use of this SAO in these experiments was able to show an inherent deterrence of BVW larvae to the synthetic pyrethroid bifenthrin. Deterrence was also shown under conditions similar to those encountered in the field. This trend helps to explain behavior noted by previous researchers, and could account for ineffective control of BVW populations under certain conditions.

The ability to detect and avoid bifenthrin impregnated media should be taken into consideration for the long-term control of BVW. Without attending to this issue, resistance may develop in the pest. When resistance evolves to management methods, development of new approaches can often be difficult and expensive, leaving growers with reduced efficacy in controlling pests and potential decreases in profit. *Metarhizium anisopliae* was shown to have no such deterrent effect on the behavior of BVW larvae. Furthermore, the ability of this fungus to attract larvae when plants are present increases the likelihood of larvae entering a lethal environment. This observation further bolsters the argument for inclusion of *M. anisopliae* into integrated pest management practices. Future work in this area should seek to determine if the same trends observed in these

experiments hold in the nursery setting. Examination of the effects of bifenthrin on adult movement and egg-laying behavior is another area that could reveal important aspects of BVW ecology that can influence pest management.

This research also provided the first insights into how *M. anisopliae* affects plant growth when applied as a root dip. There is evidence to suggest that no inherent reduction of plant root growth occurs when applied in this manner. However, the range of effects that *M. anisopliae* can have on parameters of plant health not accounted for in this study are great. Questions about the physiological response of the plant to root treatment should be addressed. It would also be desirable to know if there is any affect on the plant in its ability to respond to or withstand changes in environmental conditions.

There was no indication that root inoculation with *M. anisopliae* was able to reduce the observed effects of herbivory from BVW feeding. However, a trend of decreased herbivory was noticed. The feeding ecology of BVW may have affected this response. By severing roots at varying lengths without actually feeding on the entire root, higher losses than are attributable to direct herbivory could be realized. This is an important consideration to bear in mind when conducting future studies.

Overall, this work increases the body of knowledge concerning BVW and its interaction with management tactics. It also provides information on the ecology of *M. anisopliae* as a participant in rhizosphere interactions. These topics are germane to the pursuit of improving control efficacy against BVW.

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