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ENTOMOPATHOGENIC FUNGI AND NEMATODES FOR MICHIGAN TREE FRUIT MANAGEMENT TARGETING PLUM CURCULIO (*CONOTRACHELUS NENUPHAR*)

presented by

Renee Jean Pereault

has been accepted towards fulfillment
of the requirements for the

Masters degree in Entomology

Mark E. Whalon
Major Professor's Signature

12/10/2008
Date

DATE DUE	DATE DUE	DATE DUE

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By

Renee Jean Pereault

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTERS OF SCIENCE

Entomology

2008

ABSTRACT

ENTOMOPATHOGENIC FUNGI AND NEMATODES FOR MICHIGAN TREE FRUIT MANAGEMENT TARGETING PLUM CURCULIO (*CONOTRACHELUS NENUPHAR*)

By

Renee Jean Pereault

Tree fruit growers are seeking effective alternatives to US EPA-mitigated insecticides for managing plum curculio, *Conotrachelus nenuphar* Herbst (Coleoptera: Curculionidae). Formulations and novel delivery mechanisms of entomopathogenic nematodes (*Steinernema riobrave, Steinernema carpocapsae* and *Heterorhabditis bacteriophora*) and fungi (*Beauveria bassiana* and *Metarhizium anisopliae*) were investigated in the laboratory and in Michigan orchards. Mortality of fungus-exposed adults ranged from 38-100% and occurred 17-23 d post treatment in the laboratory. Fall granular fungus formulation applications failed to reduce overwintering survival of adults. Larvae were introduced to orchard soils -10, -5, 0, 5, 10, 15, or 20 d from entomopathogen application. *Steinernema riobrave* consistently reduced adult emergence most effectively in sandy sites. *Beauveria bassiana* exhibited limited effectiveness and sensitivity to environmental and site factors. Both nematodes and fungi were promising tactics against plum curculio, but more investigation is needed to determine if oviposition damage and the second generation can be reduced to economic levels.

Dedicated to Michigan Tree Fruit Farmers.

ACKNOWLEDGEMENTS

I thank my major advisor, Mark Whalon for his encouragement and passion. Thank you to my committee members for reviewing my thesis and in particular Leah Bauer for guiding me at the Society for Invertebrate Pathology meeting, John Biernbaum for engaging me in organic horticulture, and David Smitley for additional pest biocontrol expertise. Special thanks to Diane Alston for technical assistance and review. I thank Willye Bryan for her dedication to rearing plum curculio in "the cave." I thank Dan Nortman, Alex Johnson, Peter Nelson, Soo-hoon Kim, Ki Kim, Zach Koan, Karlyn Page, Michael Hosking, Jeannette Wilson, Christopher Archangeli, Saunté Sutton, Abbra Puvalowski, Jennifer Silveri, Andrew Skwiercz, Robert Brown, and Lisa Losievsky for technical assistance. I thank Richard Humber for entomopathogenic fungus identification and accession and Fred Warner for nematode diagnostics. We thank the Michigan Agricultural Experiment Station for providing orchard sites and support (Jerry Skeltis, Gayle "Peach" Byler, and Denise Ruwersma at Clarksville Horticulture Experiment Station and the staff at Northwest Horticulture Research Station). A special thanks to farmers for sharing their perceptions, advice, and orchards: Gene Garthe, Bruce Walton, Sheryl and Alan Kobernik, Jim Laubach, Donald Smeltzer, Merle Brown, Cal Lutz, Dennis Mackey, Jim Koan, and Steve Tennes. Funding contributions were provided by Project GREEEN, Michigan Apple Research Committee, Cherry Marketing Institute, Michigan State Horticultural Society, IR-4, and SARE.

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CHAPTER 1

LITERATURE REVIEW

I. *Conotrachelus nenuphar*

A. Geographic Distribution and Hosts

Conotrachelus nenuphar (Herbst) (Coleoptera: Curculionidae), plum curculio, is native to North America. Populations are distributed east of the Rocky Mountains (Chapman 1938). One known exception is a localized infestation in eastern Box Elder County, Utah, first detected in 1980 (Alston et al. 2005).

Hosts are limited to the plant family Rosaceae, and are reviewed and surveyed in Connecticut by Maier (1990) and in Georgia by Jenkins et al. (2006a). Native hosts include plum, choke cherry, and pin cherry (*Prunus* spp.), hawthorn (*Crataegus* spp.), serviceberry (*Amelanchier* spp.), and highbush blueberry (*Vaccinium* spp.) (Antonelli et al. 1992). Important exotic hosts include apple (*Malus* spp.), pear (*Pyrus* spp.), peach, apricot, and nectarine (*Prunus* spp.).

B. Characteristics and Life History

The adult is dark gray and brown in color. The rough, deeply ridged elytra and hunched appearance prompted the nickname "hunchback of the orchard" (Quaintance and Jenne 1912). The sexes can be separated based on a groove between the 2nd and 3rd pair of legs on the male's ventral side, which is absent in females (Thomson 1932).

Univoltine (single brood) populations, such as those in Michigan, undergo an obligate winter reproductive diapause as adults. Females begin oogenesis and mating only after spring emergence from overwintering (Smith and Salkeld 1964). Although geographic strain distributions were illustrated by Chapman (1938), voltinism in the

Midatlantic US remains unclear. Evidence of bivoltine populations has been documented as far north as West Virginia (Leskey and Wright 2004) and southern Delaware (Stearns et al. 1935), where historic records assert only univoltine populations occur. Northern and southern populations have been compared on the basis of molecular markers, fertility of cross-population matings, and *Wolbachia* gut symbionts (Zhang 2008).

The majority of adults experimentally labeled with ^{65}Zn in Quebec overwintered 3-5 m into woodlots with a thick layer of fallen deciduous leaves, adjacent to orchards. Overwintering mortality is greater for adults in leaves than for adults remaining in such substrates as orchard turf (Lafleur et al. 1987), grass litter (Bobb 1949), and bare soil (Smith and Flessel 1968). Some adults may penetrate loose soil down to 8 cm (Armstrong 1958, Smith and Flessel 1968, Lafleur et al. 1987). They are not gregarious in the fall and winter, as are some other species of beetles (Lafleur et al. 1987, Smith and Flessel 1968).

The first appearance of adults in spring is associated with temperature and host plant phenology (Quaintance and Jenne 1929, Whitcomb 1929, Cox 1951, Smith and Flessel 1968). In spring, adults may congregate in the groundcover at the base of the perimeter row of fruit trees (Lafleur and Hill 1987, Racette et al. 1991, Chouinard et al. 1993, 1994). The aggregations are possibly caused by sounds produced by both sexes (Mampe and Neunzig 1966). Males may mate with as many as 3 females per day (Yonce and Jacklin 1978), or an average of 10.4 females per 30 days (Johnson and Hays 1969).

Adults feed on leaves and buds before feeding on newly developing fruit (Garman and Zappe 1929). Adults have depleted fat levels at the time of emergence from hibernation and require feeding for ovary maturation (Smith and Salkeld 1964).

The female oviposits into fruit. With the proboscis, she cuts a characteristic crescent-shaped flap in the fruit skin and chews a small cavity underneath, reducing chances of the egg and developing larva being crushed under the pressure of the growing fruit (Quaintance and Jenne 1912). A portion of the characteristic oviposition marks never receive an egg. The pearly white egg hatches within 3-12 d (Paradis 1956). The legless larva tunnels and feeds throughout the fruit interior for four instars. Instars are classified by head capsule width ranges as quantified by Garman and Zappe (1929) and Chapman (1938). The last instar exits fruit and enters the ground where it pupates 1-8 cm below the surface (Quaintance and Jenne 1912).

Garman and Zappe (1929) in Connecticut observed larvae in soil for an average of 11.6 d after entering soil, the pupal stage lasted 11 d, and the adult remained in the soil an additional 9.8 d. Intensive fruit feeding by this "summer generation" has been observed in Michigan cherries (Whalon unpublished) and in apples before adults seek overwintering habitats (Smith and Salkeld 1964, Racette et al. 1992). The average fall dispersal rate was 3.3 m/d (Lafleur et al. 1987), with a maximum recorded total distance of 142 m (Lafleur and Hill 1987).

C. Pest Status & Management Techniques

Oviposition scarring and the infestation by feeding larvae diminish the marketability of fresh produce. Oviposition and feeding can result in mangled, misshapen fruit if it does not abscise prior to harvest. Fruit abscission caused exclusively by plum curculio damage is difficult to assess, and is not considered a negative economic consequence in apples; the abscission may contribute to the process of fruit thinning (Levine and Hall 1977, Racette et al. 1992).

In the southern US, peaches tend to be the most adversely impacted crop, whereas, in Michigan damage is most economically important in apples and cherries. Processor compliance with a national zero-tolerance standard for infested tart cherries (USDA-AMS 1941) can result in the rejection of an entire fruit load, resulting in serious economic consequences for the grower, who must then pay to clean the processing line and dispose of the rejected load. The zero-tolerance is not likely to change due to consumer expectations for worm-free fruit, and consumer demand for unblemished fresh produce necessitates an alternative, lower price use of cosmetically damaged fruit, such as juice or cider making.

In the upper Midwest, organophosphate insecticides have been used for over 50 years as the primary control for plum curculio; however, US use will be terminated in 2012 in compliance with the Food Quality Protection Act (1996). Newer chemistries, such as the neonicotinoids, oxadiazines, spinosyns, and insect growth regulators, are being evaluated as alternatives (Wise et al. 2006a). To reduce the number of synthetic chemical applications, recent advancements were made in plum curculio monitoring including traps and lures (Coombs 2001, Leskey and Wright 2004).

Prior to the introduction of synthetic chemical insecticides, cultural and mechanical controls were practiced by orchardists (Racette et al. 1992). According to studies of plum curculio movement, overwintering habitat should be managed both within and up to 300 m from the orchard (Lafleur et al. 1987). Habitat management can consist of a spring burning of overwintering habitat (Stearns et al. 1935) or removal of neglected or wild hosts (Maier 1990). Alternatively, early-flowering hosts or wild plums could be kept in borders as refuge or a "trap crop," (Leskey et al. 2008), and that

concentrated area of plum curculio could be treated with an insecticide (Shapiro-Ilan et al. 2008, Prokopy et al. 2003, Lafleur et al. 1987). Some organic Michigan orchardists use a "push-pull" strategy, similar to the "trap crop" strategy, wherein lures are placed in border rows that remain free of kaolin clay, and the concentrated adult plum curculios are targeted with Pyganic® (Whalon communication). Kaolin clay coverage is widely adopted by fruit growers for preventing fruit damage from plum curculio and a number of other pests, but keeping coverage during high precipitation levels in springtime increases labor costs and fuel consumption for tractors. With no effective control strategies for plum curculio available for organic producers, organic apple plots may be a source of infestation into nearby IPM and conventional plots (Lafleur et al. 2007).

Removal of adults from trees can be accomplished by limb jarring, but reduction of oviposition damage has varied from 1-36% (Chapman 1938). Jarring trees and collecting adults with hand labor was historically prescribed (Cook 1890), but may now be prohibitively expensive in large orchards. Removal and destruction of infested fruit could be accomplished by hand labor, livestock, or with a modified golf ball collecting machine (Stedman 1904, Quaintance and Jenne 1912, Chapman 1938). Cultivating the orchard by disking has been described as another effort to disrupt the soil-dwelling pupal stage (Garman and Zappe 1929, Stearns et al. 1935, Stedman 1904).

II. Biological Control Agents

A. Natural Enemies

Observations of natural enemies killing plum curculio are few, perhaps because 1) adults are cryptic in appearance and behavior, 2) the egg and instar development takes place inside the protection of the fruit host, and 3) the 4th instar emerges from fruit and

quickly burrows into soil, another cryptic habitat. A list consisting of several parasitoids and generalist insect predators is summarized by Racette et al. (1992). The most important natural enemies of plum curculio in a more recent natural enemy survey were ants (*Formicidae* spp.) carrying away soil-burrowing larvae (Jenkins et al. 2006b).

Vertebrates can remove adult plum curculios or wormy fruit in orchards. Chouinard et al. (1992) found evidence that toads fed on ^{65}Zn-labeled adults. Free range foul failed to control plum curculio in a Michigan orchard (Clark and Gage 1996). Quantitative measures for hogs feeding on infested June drop apples in Michigan showed some early success (Epstein submitted).

Entomopathogenic fungi and a bacterium have been observed as natural enemies of the plum curculio. These entomopathogens are known for their inconsistent natural epizootics and performance as augmentative applications against pest insect species. Garman and Zappe (1929) report observations of two diseases of plum curculio: the green muscardine fungus, *Metarhizium anisopliae* (Metchnikoff) Sorokin, and an unidentified white fungus, "*Isaria* sp.", perhaps *Beauveria bassiana* (Balsamo) Vuillimen. In North Carolina, where plum curculio were collected as larvae in blueberry fields, 90% of adults that died in the lab were infected by the entomopathogenic fungus *B. bassiana* (McGiffen and Meyer 1986); the contamination source was not identified. Adults infected with *B. bassiana* were collected from spring rearing boxes in Quebec within mixed deciduous forests (Lafleur et al. 1987). The bacterial pathogen *Bacillus thuringiensis* (var. entomocidus or subtoxicus Heimpel) was also isolated from the same group of adults.

No records of natural infections of entomopathogenic nematodes were found, although nematodes occurring in plum curculio-infested soil, trapped by waxworms (*Galleria mellonella* L.), caused high mortality when applied to plum curculio larvae (Alston et al. 2005).

B. Entomopathogenic nematodes

Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are microbivorous, not directly entomophagous (Kaya and Gaugler 1993). They are mutualistically associated with the bacteria *Xenorhabdus* and *Photorhabdus*, respectively, which are carried in the nematode's intestine. There, bacteria reside in a quiescent stage inside the nematode intestine (Boemare 2002), where the bacteria remain without food. The bacterium is released when an infective juvenile enters the haemocoel of an insect host (Forst et al. 1997). The infective juvenile gains entry into the haemocoel by entering a host spiracle, mouth, or anus. Following multiplication of the bacterium, the host dies from septicemia within 24-48 h, at which time the nematode feeds on the bacteria and produce one to several generations before new infective juveniles leave the cadaver.

Sometimes these bacteria are pathogenic to insects when experimentally injected alone (without the nematode) into insect haemocoel, or ingested by an insect. Reversely, sometimes nematodes are pathogenic to insects when injected alone into insect haemocoel; for instance, when axenic (grown without bacteria) *Steinernema carpocapsae* are injected into *Galleria mellonella*, they produce infective juveniles, completing their life cycle (Forst and Clarke 2002). However, *Xenorhabdus poinarii* and *Steinernema scapterisci*, when injected into a *Galleria mellonella* larva body cavity without each other, are not pathogenic; they are only pathogenic when combined (Bonifassi et al. 1999).

While some non-symbiotic bacteria are able to provision some of these nematodes in the absence of the naturally occurring symbiotic bacteria, the natural bacteria provide nutrition for the most efficient development.

The complex microbial ecology involved in keeping the natural bacteria associated with its nematode counterpart complicates the act of mass culturing these nematodes over many generations in laboratories for subsequent use in augmentative applications. There is inadequate understanding of the specially required signals, nutritional substances, and hormonal substances secreted between these symbionts and of the physiological significance of bacterial phase variants which may occur during mass culture (Grewal et al. 1997).

To eliminate microbial competitors present in the nematode gut or in the insect haemocoel, these bacteria produce antimicrobial barriers, such as molecules with high levels of antibiotic activity both *in vitro* and *in vivo* (Maxwell et al. 1994). The specific antibiotics produced by different *Xenorhabdus* and *Photorhabdus* spp. vary (Forst and Clarke 1992). Each nematode thus contains only one bacterial species. These nematodes are proposed to live in a biologically unique state of "natural monoxeny;" the state occurs inside of the free-living (soil-dwelling) nematode's intestine, as well as within the body cavity of the insect cadaver (Bonifassi et al. 1999).

The insect immune system protects haemolymph with cellular and humoral responses, and is reviewed with respect to invading nematodes by Dowds and Peters (2002). The cellular haemocytes can phagocytize small microorganisms (bacteria) or encapsulate large invaders (nematodes) by nodulation. Haemocytes can trigger deposition of a layer of melanin around the invader by converting prophenoloxidase to

phenoloxidase. Induced cecropins, constitutive lysozymes, and other antibacterial peptides may be released as other humoral (cell-free) responses to bacterial invasion, but these can be overcome by enzymes produced by the bacteria.

Nematodes may resist encapsulation by evasion (where nematode surface lipids protect against recognition or the nematode molts), tolerance (where nematode numbers overwhelm the haemocoel), or immune suppression (nematode surface coat proteins). Either way, nematodes may release bacteria before they are encapsulated, which adhere to and kill haemocytes, and may cause insect death with or without nematode reproduction.

C. Entomopathogenic fungi

The deuteromycete, hyphomycete fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin are facultative pathogens. They are the causal agents of white and green muscardine disease in insects, respectively. Although these fungi may enter the host via mouth, anus, or spiracles, direct penetration of the insect cuticle after attachment of a germinating spore (conidium) is the standard approach of fungal entry into the insect body. These fungi form an appresorium and penetration peg, structures also produced by some plant pathogens. Once inside the haemocoel, the mycelium moves through the body and forms blastospores (hyphal bodies). Host death is caused by a combination of fungal toxin, physical obstruction of blood circulation, nutrient depletion, and invasion of organs (Goettel and Inglis 1997). The hyphae then emerge from the cadaver and produce conidia under appropriate temperature and humidity conditions, which may then infect new hosts.

In common with entomopathogenic nematodes, host susceptibility to the fungal pathogen is influenced by fungal strain, host physiological state, nutrition, defense mechanisms, cuticular and epicuticular microorganisms, and the environmental conditions (Goettel and Inglis 1997). Also common to both fungi and nematodes are the cellular and humoral responses described above.

St. Leger (1993) illustrates the barriers to cuticle penetration by fungi. Preformed barriers in insect epicuticles include surface structures, a hydrophobic barrier (important for dry, nonsticky conidia), electrostatic charges, low relative humidity, low nutrient levels, competing microbial flora, toxic cuticular lipids and phenols, and tanned proteins. Preformed barriers to the procuticle include protease inhibitors and tanned proteins in combination with crystalline chitin which results in a stiff and desiccated cuticle. Barriers in the cuticle that are induced upon recognition of the spore include attachment and activation of prophenoloxidase, resulting in melanization.

Mucus formed by *M. anisopliae* may act as both an adhesive and a protectant from dessication and host polyphenols (Boucias and Penland 1991). The restricted host range of fungal strains may be a result of specific requirements for germination, involving nutrients such as cuticular lipids like hydrocarbons (Charnley 1984). Most strains within both of these species tend to have a broad host range and little specificity for infection site; so appresorium formation occurs immediately upon germination on a flat cuticular surface, but hyphal growth may be directed away from heavily sclerotized regions, on the path of least resistance (Pekrul and Grula, 1979). Therefore, sclerotization, cuticle thickness, and tensile strength of the cuticle formed by chitin lamellae may affect

growth behavior of the pathogens during penetration (Zacharuk 1970, Hassan and Charnley 1989).

Metarhizium anisopliae has low endogenous reserves, and so the host cuticle must provide nutrients required for penetration, which are freed by the secretion of extracellular cuticle-degrading enzymes produced by the fungus. Cuticle-degrading endoproteases contribute more significantly to the virulence of pathogens than do other host specificity factors. Host recognition mechanisms are keyed to nutrient levels available on appropriate host cuticles (St. Leger et. al 1992). Only when these extracellular nutrients are depleted does the pathogen form structures for penetration. Protein, chitin, and lipids are the main polymers in insect cuticle; a range of cuticle-degrading enzymes produced by pathogens correspond to these polymers (Charnley and St. Leger 1991). Using a labeled antibody against a pathogen protease, Goettel et al. (1989) showed that procuticle penetration involves both enzymatic degradation and mechanical separation of the lamellae, whereas epicuticle penetration is primarily by enzymatic degradation only.

When tested, the impact of the cellular host defenses on *M. anisopliae* var. *acridum* appeared to be minimal since the haemocytes remained unattached to fungal particles and nodules did not incorporate fungal particles (Gillepsie et al. 2000). The pathogen itself secretes toxins. Destruxins are cytotoxic fungal metabolites produced by *M. anisopliae* var. *anisopliae* that are toxic to haemocytes (Huxham et al. 1989). One counter to a host resistance mechanism is the detoxification of the insect's polyphenoloxydase. The destruxins prevent phenoloxydase production by locust

haemocytes by destroying the cells that produce it (Cerenius et al. 1990). The metabolites beauvericin and oosporein produced by *Beauveria* sp. exhibit antibacterial action.

Inundative releases of *M. anisopliae* and *B. bassiana* typically involve application of aqueous or granular formulations to soils, where these species reside as facultative saprophytes. A granular formulation of *Beauveria brongniartii* is commercially available for European cockchafer biocontrol (Kessler et al. 2004). A simple maize-based *B. bassiana* formulation developed for subsistence farmers was effective against banana root weevil (Nankinga and Moore 2000) and granular *M. anisopliae* increased yield in fields with sugarbeet root maggot (Campbell et al. 2006). A granular formulation of *B. bassiana* on rice established in pasture better than a biopolymer when considered for clover root weevil control (Nelson et al. 2004).

D. Insect responses to entomopathogenic nematodes and fungi

The effectiveness of entomopathogens for insects may be influenced by behavioral responses of insects, both by affecting infection rates of insects exposed to inocula and dispersal of inocula to other insects. Such behaviors may not be limited to grooming, secretion of antibiotics, nest hygiene in social insects, avoidance, and dispersal.

Horizontal infection has been documented in many insect/entomopathogen systems. Confoundingly, both insect avoidance of and attraction to entomopathogens has been described in some systems. In the laboratory, termites were repelled by the nematode *Heterorhabditis indica* for up to 17 days (Wang et al. 2002). Colorado Potato Beetle was not repelled by sporulating cadavers infected with *B. bassiana* (Klinger et al. 2006). Japanese beetle grubs actively avoided areas where *M. anisopliae* was added (Villani et al. 1994), but black vine weevil grubs were attracted to *M. anisopliae* only

when applied in conjunction with plants. This finding suggests a complex tritrophic interaction only in the early stages of exploration (Kepler and Bruck 2006). The authors hypothesize that the fungus may produce volatile compounds attractive to the pest only in the presence of a plant rhizosphere, but this has yet to be tested.

In the presence of nematodes, white grubs exhibit aggressive grooming behaviors such as rubbing with an abrasive raster on the abdomen, brushing with legs, or scraping or chewing with mandibles (Koppenhöfer et al. 2000). The spiracles of white grubs are covered with sieve plates, making them inaccessible to nematodes. In some ants, grooming, including the use of tibial combs, rubbing of legs, and licking, does not result in the ingestion of inocula since they are collected in an infrabuccal pocket (Eisner and Happ 1962). Dissemination of inocula between individuals by grooming was seen in termites (Kramm et al. 1982). Secretions of venom alkaloids, phenyl acetic acid, or myrmicacin seemingly reduce pathogenicity of fungi in ants (Oi and Pereira 1993). Removal of *B. bassiana*- infected cadavers from ant nests and isolation of infected nestmates by burial have been observed (Oi and Pereira 1993).

Since insects are "cold-blooded," they thermoregulate themselves behaviorally. A febrile response, or so-called "behavioral fever," is associated with infection of locusts by *M. anisopliae*. Here the individual insect moves to an area of elevated temperature, and this is thought to suppress pathogen growth and enhance the immune system.

Behavioral modulation of insects by parasites may enhance the parasite's transmission potential. For instance, ants and flies infected with the entomopathogenic fungus *Entomopthora* have a propensity to disperse to and die at elevated locations, such

as clinging (or fastened by fungal rhizoids) to the tops of grass blades, facilitating dispersal of the pathogen spores (Humber 1981).

In conclusion, the physical, biochemical, and behavioral barriers to entomopathogen infection of an insect host are abundant, poorly understood, and vary greatly between entomopathogen strains and insect species. Concepts related to volatile cues and toxins are only in the early period of scientific understanding. As conventional chemical control agents for use in agriculture and forestry are cancelled by the Environmental Protection Agency, control tactics that are perceived as more natural, such as entomopathogens, are becoming more significant as exhibited by the recent literature. Forthcoming knowledge of the mechanisms of insect resistance to entomopathogens may soon prove to increase the efficacy of augmentative applications of entomopathogens as replacements for conventional insecticides. Further integration of new physiological, ecological, and behavioral understandings may result in a conservation approach, where landscapes are manipulated ecologically to enhance crop protection by natural or naturalized populations of entomopathogens.

E. Entomopathogen experiments against plum curculio

In laboratory assays, entomopathogenic nematodes, particularly strains of *Steinernema riobrave* (Cabanillas, Poinar, and Raulston), *Steinernema feltiae* (Filipjev), *Steinernema carpocapsae* (Weiser), and *Heterorhabditis bacteriophora* (Poinar), have demonstrated efficacy against last instar plum curculio larvae (Brossard et al. 1989, Shapiro-Ilan et al. 2002, Alston et al. 2005). Olthof & Hagley (1993) reported 73-90% mortality for *S. feltiae* Biosys27 strain and *S. carpocapsae* All strain against last instar plum curculio; however, other authors found low efficacy of both the *S. carpocapsae* All

strain as well as a strain of *S. carpocapsae* collected in Mississippi (Tedders et al. 1982, Shapiro-Ilan et al. 2002). *Steinernema riobrave* 355 strain reduced adult emergence by 77-97% when applied to soil in field cages containing plum curculio larvae in Georgia (Shapiro-Ilan et al. 2004a). *S. feltiae* was not effective in the same trial, and demonstrated lower mortality (22-39%) in another caged trial in a drier climate in Utah (Shapiro-Ilan et al. 2004a, Alston et al. 2005).

Cold tolerance is generally exhibited by *Steinernema* spp. while heat tolerance is generally exhibited by *Heterorhabditis* spp. (Molyneux 1986, Grewal et al. 1994). A strain of *H. bacteriophora* from Utah was overall more virulent than *S. feltiae* to all plum curculio life stages, and adults and pupae were more susceptible than larvae (Kim and Alston 2008). On the surrogate insect species *Galleria mellonella*, virulence and reproductive potential of *H. bacteriophora* was superior to *S. feltiae* at 30°C, and inferior at 10°C (Kim and Alston 2008).

Soil applications of *S. riobrave* 355 strain targeting plum curculio larvae in a wild plum thicket caused 100% control in two years, while 3-8b strain caused > 87.9% control (Shapiro-Ilan et al. 2008). Late-term *S. riobrave* and *S. carpocapsae* applications targeting plum curculio after onset of adult emergence failed to result in significant control (Shapiro-Ilan et al. 2008). Foliar applications of *S. carpocapsae* in June did not reduce plum curculio damage at harvest in apples, but border-row treatments reduced damage by 75% and 30%, with economically acceptable levels of damage in only one of the two years (Bélair et al. 1998).

In a laboratory bioassay, a South Carolina strain of the entomopathogenic fungus *B. bassiana* caused slower mortality of last instar plum curculio larvae, compared to a

South Carolina strain of the fungus *M. anisopliae* (Tedders et al. 1982). A California strain of *M. anisopliae* was not effective in this same study. Strains of *M. anisopliae* tested against larvae in small cups of soil in the laboratory showed high variability in LC_{50} values between strains (Alston et al. 2005), but *M. anisopliae* was not evaluated in the field. *Beauveria bassiana* GHA strain did not significantly reduce numbers of emerging adults when applied to soil containing larvae in Georgia (Jenkins et al. 2006b). While efficacy of nematodes for suppression of plum curculio adults has been evaluated in the laboratory (Shapiro-Ilan et al. 2002), neither *M. anisopliae* nor *B. bassiana* have been evaluated against plum curculio adults.

Field efficacy of entomopathogens to consistently control plum curculio at levels greater than 40% has only been demonstrated for *S. riobrave* 355 strain in the southern US (Shapiro-Ilan et al. 2004a, 2008) and with *H. bacteriophora* in residential fruit trees in Utah (Kim 2007). Kim (2007) demonstrated 56, 69, and 73% control of plum curculio after one, two, and three years of multiple *H. bacteriophora* applications. Due to high costs associated with augmentative applications of entomopathogens and historically variable field results, region-specific and production system-specific field efficacy evaluations of candidate strains are necessary. Results could provide growers with the information needed to weigh these benefits against the higher cost of treatment.

IV. Thesis Research

The goal of the research herein is to demonstrate the efficacy of augmentative releases of entomopathogenic fungi and nematodes for control of plum curculio in Michigan orchards. Adults were targeted with entomopathogenic fungi and larvae were targeted with both entomopathogenic fungi and nematodes. Field efficacy experiments

against larvae were conducted for three years in a total of ten different orchards. Alternative means of exposing adult plum curculios to fungi were investigated, including aqueous and granular formulation applications to soils and surfaces that could be adapted to an autodissemination trap. This research is a stepping stone for farmscale implementation of entomopathogens for plum curculio management.

The next phases of this research will be to: 1) develop a phenology model that predicts larval emergence from fruit to narrow recommended entomopathogen application timings for tree fruit growers in different regions, 2) improve the understanding of short-term inundative entomopathogen efficacy persistence in different formulations and in different soil types under microhabitat moisture manipulation, and 3) investigate the efficacy of fungus formulations and delivery methods against adults in orchards.

CHAPTER 2

LABORATORY EVALUATIONS OF ENTOMOPATHOGENIC FUNGUS EFFICACY AGAINST LAST INSTAR PLUM CURCULIO

1. Introduction

Plum curculio, *Conotrachelus nenuphar* (Herbst), is a key pest of tree fruit and is susceptible to disease caused by entomopathogenic fungi. Although initial damage to fruit would not be prevented within-season, mortality at the last-instar stage would reduce the emergence of second generation adults that cause damage the following season. Currently the fungus *Beauveria bassiana* (Balsamo) Vuillemin strain GHA is registered with EPA for food crops and labeled for plum curculio. *Metarhizium anisopliae* (Metschnikoff) Sorokin strain F52 is registered as a control for greenhouse and nursery crops, but not currently for food crops. Both have a relatively broad host range.

In a laboratory soil bioassay, a South Carolina strain of *B. bassiana* caused mortality of last-instar plum curculio, but caused mortality at a slower rate than a South Carolina strain of the fungus *M. anisopliae* (Tedders et al. 1982). Yet both isolates caused high levels of mortality after 14 d. In a similar laboratory bioassay against last-instars, 17 *M. anisopliae* isolates resulted in varying LT_{50} (4-22.8 d), indicating differing virulence between strains (Alston et al. 2005). In Georgia, a field application of *B. bassiana* strain GHA in Mycotrol-O formulation to orchard soils with plum curculio-infested fruit overlaid, failed to reduce adult emergence (Jenkins et al. 2006b). The researchers used naturally plum curculio-infested fruit, with a single *B. bassiana* application, so the time between *B. bassiana* application and larval contact was unknown. Further, there was no mention of soil type or other edaphic factors that could have influenced mortality.

Another factor that could have contributed to limited field efficacy is that *B. bassiana* GHA strain may not be as virulent as other strains of entomopathogenic fungi.

Laboratory-based virulence is not the only factor that contributes to successful field efficacy; for instance, a strain collected from an orchard might be expected to persist better in the field following augmentative application if it is locally adapted to both biotic and abiotic conditions characteristic to that environment. Alternatively, the 'new associations' hypothesis in biological control asserts that the introduction of natural enemies from the prey species native range may be less effective than novel combinations of natural enemies and prey (Hokkanen and Pimental 1984). Other factors such as dose, timing, coverage, pH, and pest age and condition contribute to efficacy (Tanada and Kaya 1993).

The objective of this study was to test the efficacy of the commercial *B. bassiana* and *M. anisopliae* strains, as well as a native strain against pupation-bound last-instar plum curculio in the laboratory. Bioassays were carried out by standard immersion exposure methods.

2. Methods

2.1 Larvae and Entomopathogens

Newly emerged (<24 h) last-instar plum curculios were obtained from a colony maintained by the Pesticide Alternatives Laboratory, Michigan State University, East Lansing, MI. The colony was reinitiated annually from adults and infested cherries and apples collected in six orchards in Benzie, Ionia, Leelanau, and Manistee Co., MI. Adults were collected using circle traps (Mulder et al. 1997) and "Whalon pyramid traps" (1.6 m x 0.8 m) made from recyclable black corrugated plastic with high-contrast reflective tape

(Great Lakes IPM, Vestaburg, MI). Both trapping systems were equipped with plum essence lures and stabilized benzaldehyde vials (Coombs 2001, Leskey and Wright 2004, Leskey et al. 2005). In order to break diapause and induce mating and oviposition, adults were exposed to thinning apples that had been dipped in a solution of 750 mg pyriproxifen (Esteem® 35 WP) per L water (Hoffmann et al. 2007).

We used commercial strains of *B. bassiana* (GHA strain received as Mycotrol-O® from Laverlam International, Butte, MT) and *M. anisopliae* (F52 strain received as Taenure® granular formulation, Novozymes Biologicals, Inc., Salem, VA). A strain of *M. anisopliae* referred to hereafter as strain 8270 was isolated on a selective medium from a highly infested organic tart cherry orchard in Leelanau Co., MI in June 2006 (identified by Richard Humber, accession #8270 at the USDA Agricultural Research Service Entomopathogenic Fungus Culture Collection (ARSEF), Ithaca, NY). Fungal inoculum viability was assessed according to Goettel & Inglis (1997) by pouring 50 ul of each suspension within 30 min of application onto three dishes of potato dextrose agar and checking 200 spores/dish for the presence of germ tubes after 20 h with an Olympus BX40 research microscope.

2.2 Immersion Bioassay

Aqueous suspensions of *M. anisopliae* F52, *M. anisopliae* 8270, and *B. bassiana* GHA were prepared in 10 ml plastic centrifuge tubes. Conidia concentrations were adjusted to 1 x 10^5 and 1 x 10^6 conidia/ml (8270 was tested at 1 x 10^6 only). Last-instars were immersed individually for 10 s and placed on sterile Whatman #1 filter paper to remove excess liquid. The tubes were vortexed for 15 s between larvae. The larvae were placed individually in 90 x 10 mm Petri dishes lined with two pieces of sterile filter paper

with 1.5 ml sterile water, sealed with parafilm. Petri dishes were incubated in the dark at 25C for 14 d, after which mortality and signs of sporulation visible under a Nikon SMZ1000 (Mager Scientific, Inc., Dexter, MI) stereo dissecting microscope were recorded.

There were 12 larvae per treatment and the experiment was repeated on five dates. The *M. anisopliae* 8270 treatment was tested on only two of the five dates. Percent mortality and percent sporulation were both arcsine square root transformed to meet normality assumptions and pairwise comparisons were made among all treatments using Tukey's HSD, α=0.05 (SAS Institute 2004).

3. Results

3.1 Immersion Bioassay

Spore viability was always greater than 95% (Table 2.1). The F52 10^6 treatment resulted in mortality and sporulation (98 and 93%) significantly higher than all other treatments ($p<0.05$) (Figure 2.2). Mortality and sporulation in the 8207 10^6 treatment (58 and 50%) was comparable to F52 10^5 and significantly lower than the F52 10^6, but significantly higher than control. Mortality and sporulation in GHA 10^5 and GHA 10^6 were not different from control. Mortality in GHA 10^6 was not different from F52 10^5 and 8207 10^6.

Table 2.1. Percent viability of conidia in immersion suspensions on each test date.

	Date				
Isolate	1	2	3	4	5
Bb GHA	98.83	99	99.33	99.67	98.83
Ma F52	99.83	95.33	99.88	100	99.33
Ma 8270	-	-	99.88	-	98.83

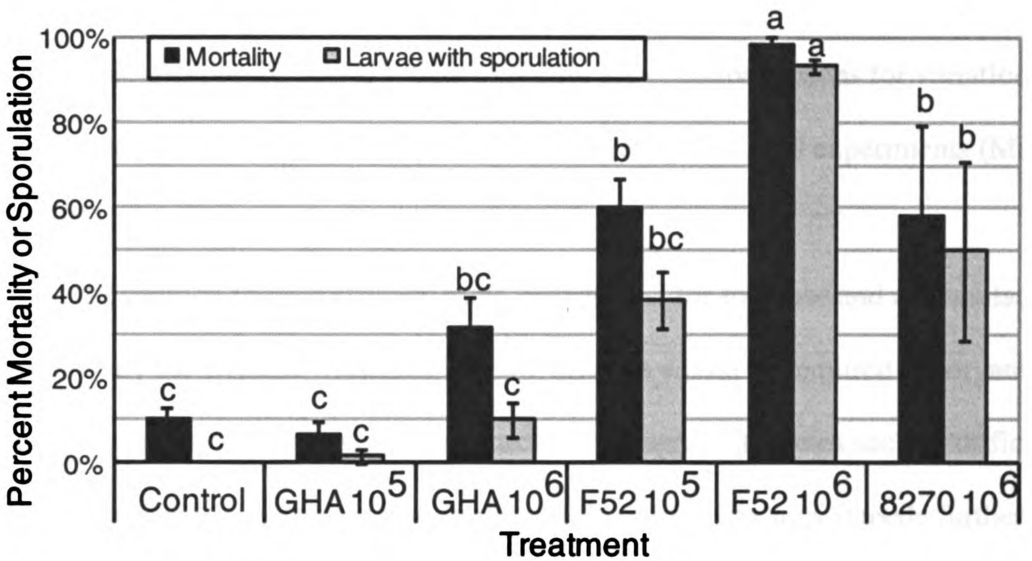


Figure 2.1. Mean (± SE) percent mortality and percent of larvae with sporulation 14 d after a 10 s immersion in $1x10^5$ or $1x10^6$ conidia/ml suspensions of *M. anisopliae* F52, *B. bassiana* strain GHA, *M. anisopliae* 8270, or water control. Bars with different letters indicate significant difference among treatments within mortality or within sporulation (Tukey's HSD, α=0.05).

4. Discussion

In the immersion bioassay at optimal conditions, the commercial *M. anisopliae* strain F52 resulted in higher mortality and sporulation on plum curculio larvae cadavers than the commercial *B. bassiana* strain GHA labeled for plum curculio. More replicates of the *M. anisopliae* Michigan soil isolate 8270 may reduce variability and show more clearly whether 8270 causes higher mortality than GHA.

Laboratory virulence assays, especially those using an immersion method, may not be indicative of field efficacy. For instance, one physical aspect that this immersion assay does not account for is the difference in hydrophobicity between fungus species which may interfere with conidia adhesion to the insect cuticle; consequently, dose levels cannot be measured precisely (Goettel and Inglis 1997). Consecutive immersion of larvae into a single conidia suspension may have reduced the conidia concentration between

immersions. Field conditions such as soil texture, temperature, water activity, agrochemical exposure, and UV light exposure are common reasons for variation in entomopathogenic fungus efficacy between laboratory and field experiments (McCoy et al. 1992).

In addition to field efficacy trials, registration for food use and a formulation approved by the Organic Materials Review Institute would be required for organic grower use of *M. anisopliae* for plum curculio management. Issues such as difficulty sourcing organic materials and the small organic market size may impede further investments by the companies that hold rights to the EPA-registered strains and formulations. The cost of registration processes for entomopathogenic fungi is an impediment to the registration of other strains (Butt and Coping 2000), so had the 8270 strain been significantly more virulent to plum curculio, the time and a high cost to register the strain would likely inhibit registration.

CHAPTER 3

ENTOMOPATHOGENIC NEMATODE AND FUNGUS FIELD EFFICACY AGAINST LAST INSTAR PLUM CURCULIO

1. Introduction

Plum curculio, *Conotrachelus nenuphar* (Herbst), has adapted well to cultivated tree fruits throughout its range in eastern North America (Maier 1990) and is a key pest in cherry, apple, and peach production. In Michigan, overwintering adults emerge from obligate diapause in overwintering habitats, migrate into orchards, feed, mate, and oviposit in fruits (Smith 1957). Larval feeding occurs exclusively inside fruit throughout development. Fourth instars exit fruits, burrow into the soil where they pupate at a depth of 1-8 cm (Quaintance and Jenne 1912), and emerge as summer adults. If early season control is incomplete, summer adults can contribute significantly to late-season damage (Racette et al. 1992) and become the next year's ovipositing population.

In the upper Midwest, organophosphate insecticides have been used for over 50 years as the primary control for plum curculio particularly in cherry, but also in apples, plums, and peaches. However, US use will be terminated in 2012 in compliance with the Food Quality Protection Act (1996). Newer chemistries, such as the neonicotinoids, oxadiazines, spinosyns, and insect growth regulators, are being evaluated as alternatives to the organophosphates (Wise et al. 2006a). The over-reliance on one class of insecticides in agricultural systems raises concerns about the potential for insecticide resistance (Denholm and Rowland 1992, Nauen and Denholm 2005, Whalon et al. 2008) and negative effects on non-target insects and other invertebrates (Croft and Whalon 1982, Theiling and Croft 1988, Desneux et al. 2007). Incorporation of control agents such

as entomopathogenic fungi and nematodes into orchard integrated pest management systems has been proposed as a way to mitigate some of these concerns (Bathon 1996, Roy and Pell 2000, Lacey and Shapiro-Ilan 2008).

For producers in the wet and humid Upper Midwest where plum curculio is problematic, the higher humidity and rainfall may enhance effective augmentative releases of natural enemies, presenting an opportunity to produce quality fruit that may approach the cosmetic standards of fruit produced in more arid regions where plum curculio is not present. Such improvements in pest management will contribute to the feasibility of increasing fruit produced for local consumption in this region. If production was maintained and expanded in the Upper Midwest, significant reduction in energy consumption from long-distance western shipping would result.

Historically, only active adult plum curculio were targeted with chemical insecticide sprays because the organophosphates yielded excellent control, including "curative" activity on life stages within fruit (Wise et al. 2007). Augmentative entomopathogen application for control of soil-dwelling life stages offers an opportunity to suppress the pest at a previously untargeted life stage - a major goal of higher level IPM programs (Kogan 1988). The plum curculio life cycle includes four duff- and soil-dwelling stages that may be vulnerable to soil-borne entomopathogens: the soil-burrowing last-instar larva, the pupa, the emerging adult, and the overwintering adult.

In laboratory assays, entomopathogenic nematodes, particularly strains of *Steinernema riobrave* (Cabanillas, Poinar, and Raulston), *Steinernema feltiae* (Filipjev), *Steinernema carpocapsae* (Weiser), and *Heterorhabditis bacteriophora* (Poinar), have demonstrated efficacy on last-instar plum curculio, (Shapiro-Ilan et al. 2002, Alston et al.

2005). Olthof & Hagley (1993) reported 73-90% mortality for *S. feltiae* Biosys27 strain and *S. carpocapsae* All strain against last-instar plum curculio; however, other authors found low efficacy of both the *S. carpocapsae* All strain as well as a Mississippi-collected *S. carpocapsae* strain (Tedders et al. 1982, Shapiro-Ilan et al. 2002). *Steinernema riobrave* 355 strain reduced adult emergence by 77-97% when applied to soil in field cages containing plum curculio larvae in Georgia (Shapiro-Ilan et al. 2004a). *S. feltiae* was not effective in the same trial, and demonstrated lower mortality (22-39%) in another caged trial in a drier climate in Utah (Shapiro-Ilan et al. 2004a, Alston et al. 2005).

In a laboratory bioassay, a South Carolina strain of the entomopathogenic fungus *Beauveria bassiana* (Balsamo) Vuillemin caused slower mortality of last-instar plum curculio, compared to a South Carolina strain of the fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin (Tedders et al. 1982). A California strain of *M. anisopliae* was not effective in this same study. Strains of *M. anisopliae* tested against larvae in small cups of soil in the laboratory showed high variability in LC_{50} values between strains (Alston et al. 2005), but *M. anisopliae* was not evaluated in the field. In Georgia, *B. bassiana* GHA strain did not significantly reduce numbers of emerging adults when applied to soil containing larvae (Jenkins et al. 2006b). The researchers used naturally plum curculio-infested fruit, with a single application, so the time between *B. bassiana* application and larval contact was unknown. Further, there was no mention of the soil type or other edaphic factors that could have influenced mortality.

Field efficacy of entomopathogens to consistently control plum curculio at levels greater than 40% has only been demonstrated for *S. riobrave* 355 strain in the southern

US (Shapiro-Ilan et al. 2004a, 2008) and with *H. bacteriophora* in residential fruit trees in Utah (Kim 2007). Kim (2007) demonstrated 56, 69, and 73% control of plum curculio after one, two, and three years of multiple *H. bacteriophora* applications. Due to high costs associated with augmentative applications of entomopathogens and historically variable results, region-specific and production system-specific field efficacy evaluations of candidate strains are necessary. Efficacy results would provide growers with the information needed to weigh these benefits against the higher cost of treatment in the context of reduced environmental and ecosystem services impacts of entomopathogens (Goettel and Hajek 2000, Barbercheck and Millar 2000).

The overall goal of this study was to evaluate efficacy of commercially available entomopathogens against plum curculio in Michigan. Our objective was to evaluate field efficacy of five soil-applied entomopathogens (*M. anisopliae, B. bassiana, H. bacteriophora, S. carpocapsae, S. riobrave,* and *H. bacteriophora*) timed precisely against emerged last-instars or against last-instars as they emerged from fruit over time.

2. Materials & Methods

2.1 Entomopathogens

We used commercial strains of *B. bassiana* (GHA strain Mycotrol-O® liquid formulation, Laverlam International, Butte, MT) and *M. anisopliae* (F52 strain Taenure® granular formulation, Novozymes Biologicals, Inc., Salem, VA). The commercial strains of nematodes included *S. carpocapsae* (All strain in a vermiculite-based formulation) and *S. riobrave* (355 strain in a petroleum-based gel formulation) were supplied by Becker Underwood (Ames, IA). The *H. bacteriophora* strain isolated from Utah (Alston et al. 2005) was not formulated.

Aqueous suspensions of fungi were prepared by scraping *M. anisopliae* or *B. bassiana* conidia from 2-wk old potato dextrose agar (PDA) plate cultures into sterile water with 0.01% Tween-80. A granular formulation of *B. bassiana* was produced in the laboratory by colonizing broken rice in autoclavable bags following methods of Nelson et al. (2004). The bags were inoculated with conidia on PDA isolated from a last-instar plum curculio 8 d after immersion in a Mycotrol-O suspension. The number of conidia per gram of granular material was approximated by placing a 5 g sample into a flask containing 100 ml of 0.01% Tween-80. Flasks were placed on a rotating shaker at 180 RPM for 5 min, and the number of spores per ml of solution was determined using a hemacytometer (4 counts per flask). The mean (±SE) count of 3 flasks was 4.75±0.25 x 10^8 conidia/g.

Fungal inoculum viability was assessed according to Goettel & Inglis (1997) by pouring 50 ul of each suspension within 30 min of application onto three dishes of PDA and checking 200 spores/dish for the presence of germ tubes after 20 h, with an Olympus BX40 compound microscope. Viability of nematode infective juveniles (IJs) was determined within 30 min of application by viewing 3 samples of 100 IJs under a Nikon SMZ1000 (Mager Scientific, Inc., Dexter, MI) stereo dissecting microscope. Straight, still IJs not responding to a probe and without a characteristic 'J' shape were considered dead (Goettel & Inglis 1997). Nematode viability was >95% for every application.

2.2 Field Sites

Soil samples to a depth of 8 cm from field sites were analyzed by Michigan State University Soil and Plant Nutrient Lab (Table 3.1). A nematode community analysis was conducted on samples from the 2008 sites at the Michigan State University Nematode

Diagnostics Lab, East Lansing, MI (Table 3.2). Percent soil moisture values were obtained using the gravimetric method by weighing oven-dried soil samples (Table 3.3).

In 2006, the trial was conducted among two organic tart cherry orchards and two organic apple orchards. In 2007, multiple trials were conducted in seven 0.21 ha apple orchard blocks at the Michigan State University Clarksville Horticulture Experiment Station, Ionia County, Michigan. Apple trees were planted in 1994 and were mixed cultivars as described by Salazar et al. (2007). Tree spacing was 1.5 m within tree-row and row spacing was 4.5 m. The drive row and drip line were mowed 14±2 d prior to treatment applications. The orchard understory was primarily composed of orchard grass and bare soil. Experimental plots received water from drip irrigation tubing placed along tree trunks throughout the experiment. Experimental plots received no chemical herbicides or insecticides; however, sulfur and other agents were applied in experimental plots to control apple scab (Wise et al. 2006b) as indicated in the spray record (Table 3.2).

In 2008, the experiment was conducted among three conventional tart cherry and two organic apple orchards.

2.3 Efficacy and Timing with Emerged Larvae

Trials were conducted in 2006, 2007, and 2008 using newly emerged last-instars to evaluate the effectiveness of various entomopathogen formulations in pots in orchards. Among all trials, the experimental unit was ten last-instars per pot. The number of adults emerging from each pot was recorded every 5-10 d until day 60, starting 25 d post-treatment (adults were collected less frequently in 2008).

The round plastic pots (25 cm tall x 10 cm diam.) were fitted with a screen bottom for rain water drainage and a conical boll weevil trap top (USDA Boll Weevil Eradication

Foundation, Abilene, TX). The trap top was modified to fit tightly in the pot's upper rim to collect emerging plum curculio adults. Pots were installed under the canopy of trees within 1 m of the trunk by removing soil cores with a 10 cm diam. golf-hole cutter. Soil from the cutter was carefully transferred into pots without disturbing the interface between the duff and the A horizon. Filled pots were placed into the cutter holes, with pot rims uniformly level with the soil surface.

Oviposition-marked fruits were held in the laboratory on metal screen racks over aluminum trays lined with moist paper towel. Emerged larvae were held in groups of ten in 90 mm Petri dishes at 25°C in the dark. The dishes were lined with two pieces Whatman #1 filter paper moistened with 1.25 ml sterile water and sealed with Parafilm®. Larvae were added to the soil surface in pots within 36 h of emergence from fruits.

2006: Granular & sprayed B. bassiana with a single timing

Beauveria bassiana was tested in both Mycotrol-O and granular (rice) formulation. In two tart cherry and one apple orchard, treatments included three rates of rice (2×10^{13}, 2×10^{14}, and 2×10^{15} conidia/ha for low, medium, and high rates, respectively) and a single rate of Mycotrol-O spray (2×10^{15} conidia/ha). Mycotrol-O was delivered to soil with a 7 L Solo backpack sprayer in 50 ml water per m^2. Four pots with the same treatment were installed under each of three trees per orchard, randomly selected within one row. In the remaining apple orchard, treatments included the high rate of rice and Mycotrol-O spray applied on 14 July. Six pots with the same treatment were installed under each of three randomly selected trees in a row.

Water was delivered to plots at a rate of 500 ml/m^2 following applications. A wood and window screen ground cage was installed over the top of pots to prevent

interference by wild predators. Ground cages were not used in 2007 and 2008, to reduce chances of causing microhabitat changes in humidity, temperature, and soil moisture from normal orchard conditions. A furrower was passed over the plots at 6 cm depth prior to pot installation, only in 2006.

Larvae were placed on the soil surface in pots within 1 h of pathogen application. Larvae were collected from tart cherries collected from Leelanau Co. in the northernmost orchard.

2007: B. bassiana, M. anisopliae, S. carpocapsae, & S. riobrave with three timings

The 2007 trial expanded upon the 2006 trial by testing several aqueous pathogen formulations, with the addition of larvae at one of three intervals: 0, 3, or 5 d from pathogen applications.

An entomopathogen (*B. bassiana, M. anisopliae, S. carpocapsae,* or *S. riobrave*) or the "no" treatment control (50 ml sterile water with 0.01% Tween-80) was applied by evenly pouring 50 ml of aqueous suspension onto the soil surface within each pot. Fungal suspensions were adjusted to a concentration of 5×10^{13} conidia/ha. The two nematode species in their respective commercial formulations were adjusted to a concentration of 4×10^{9} IJs/ha. Viability was 98.3%, 95.3%, and 100% for *B. bassiana, M. anisopliae,* and both nematodes, respectively.

There were four pathogen treatments and one untreated control, each with three larva-placement timings (post-treatment intervals). This resulted in 12 "pathogen x larva-timing" treatments and 3 "untreated control x larva-timing treatments". Ten pots per tree were installed and treatments were randomly assigned to pots in each of six tree rows. There were 30 pots of each entomopathogen and control for each of the no delay larva-

timings and 15 pots of each entomopathogen and control for each of the 3-d and 5-d larva-timings in one orchard block. The experiment was repeated on 30 July, but reps were reduced to 25 and 12 pots per treatment, respectively.

Larvae were reared from on-site apples held in cages with adults for oviposition. Adults had been collected using circle traps (Mulder et al. 1997) and black corrugated plastic "Whalon pyramid traps" (1.6 m x 0.8 m) with high-contrast reflective tape (Great Lakes IPM, Vestaburg, MI), equipped with plum essence lures and stabilized benzaldehyde vials (Coombs 2001, Leskey and Wright 2004, Leskey et al. 2005) in April, May, and June in cherry and apple orchards in Benzie and Manistee Co., MI.

2008: B. bassiana, S. riobrave, and H. bacteriophora with seven timings

The 2008 trial expanded upon the 2007 trial by testing aqueous pathogen applications with the addition of larvae at one of seven intervals: -10, -5, 0, 5, 10, 15, or 20 d from pathogen application.

This experiment was repeated in July 2008 in five orchards with several modifications as follows. The nematodes *S. riobrave* and *H. bacteriophora* were tested at a low and high rate (1×10^9 and 4×10^9 conidia/ha). *Beauveria bassiana* was tested at the same rate as in 2007 (5×10^{13} conidia/ha), but in Mycotrol-O® formulation instead of a Tween-80 conidia suspension. There were six pathogen treatments (*B. bassiana*, *H. bacteriophora* low, *H. bacteriophora* high, *S. riobrave* low, *S. riobrave* high, and control). Larvae were added to pots -10, -5, 0, 5, 10, 15, or 20 d from pathogen application. Larvae were obtained using the same method as in 2007. There were eight reps of each of the "pathogen x timing" combinations per orchard, and these were randomly assigned within

3-tree plots containing 36 pots each. *H. bacteriophora* was not tested for the -10, 10, and 20 d timings.

Statistical Analysis

The adult emergence data was analyzed with SAS (SAS Institute 2004) with α=0.05. Emergence data from 2006 were analyzed for each orchard individually using proc GLM and mean separations were conducted using Student-Newman-Keuls procedure. Emergence data for the two 2007 orchard blocks were combined and analyzed using proc GLM and Student-Newman-Keuls procedure. In 2008, mean separations for adult emergence were conducted proc GLM and Student-Newman-Keuls procedure, for all orchards combined and then separately by orchard.

2.4 Efficacy with Adult Trapping

In 2007 only, the efficacy of a single application of entomopathogens to soil for suppression of a natural population of plum curculio was studied. Each of the seven orchard blocks was divided into four plots of 60 trees each (5 trees wide by 12 trees long), with a two-tree buffer strip between plots.

Each of the four plots within each block was randomly selected for treatment with commercially formulated *S. carpocapsae, S. riobrave, M. anisopliae*, or water alone (control). We did not test *B. bassiana* due to limited space and based on laboratory trials where the *M. anisopliae* strain was more virulent to larvae than the *B. bassiana* strain (Whalon, unpublished data).

Plots were irrigated with 45,000 L water/ha 1-3 h prior to treatment applications using a 1,900 L tractor-drawn tank and side-mounted PVC pipe with holes drilled every 5 cm. The entire area of groundcover under the tree canopy (approximately 0.5 m from the

trunk) received water and entomopathogen treatments. Nematodes in their respective formulations were applied with a hand-pump backpack sprayer without a filter screen to the designated area (223 m^2 per plot) in 15 L of water (a rate equivalent to 673 L/ha). The sprayer tank was continuously shaken during application. The *M. anisopliae* granules were applied by hand, followed by a 15 liter spray of water. Control plots received a 15 liter spray of water. Conidia viability was 93%; nematode viability was 100% for both *S. carpocapsae* and *S. riobrave*. Applications were made on 9 and 10 July (687 Degree Days base 10°C).

Four circle traps were placed in each plot and secured on uniformly spaced tree trunks. Plum curculio adult catch was recorded weekly for 6 wk starting 3 d after entomopathogen application. The total number of adults caught per trap was analyzed using proc GLM (SAS Institute 2004).

2.5 Efficacy with Caged Fruit

In 2007 only, another field experiment was conducted to evaluate adult emergence from the plots described above, except that cages were used, since screen trap catch may be a poor indicator of adult suppression in small plots due to adult behavior and movement in the fall (Johnson et al. 2002, Leskey and Wright 2004). One cage was installed over each of the four soil treatment plots in each of the seven orchard blocks, within the dripline of two trees along the center row of the plots.

The cage base (0.5 x 1.8 m) was constructed of 1.3 cm diam PVC pipe. Netting (0.8 mm mesh) was tightly affixed to the pipe base and tented over the interior at a height of 30 cm at the apex. A 5 cm deep trench was dug to fit the PVC base. Cages were

secured with metal L-shaped landscaping stakes and moist topsoil was mounded around the entire base to prevent escape of new adults after emergence.

Infested apples were hand-picked from naturally infested trees in the Clarksville orchard blocks between 5 and 17 June and held in the laboratory in mesh bags until field deployment. We evenly distributed 300 apples per cage over 0.8 m^2 of ground on 7 July.

In the laboratory, degree days were calculated by averaging the daily maximum and minimum temperature, and subtracting the base temperature of 10°C. Temperatures were recorded with a Watchdog datalogger (Spectrum Technologies, Inc, Plainfield, IL). In the field, degree days were calculated using numerical integration (hourly data) from an onsite Michigan Automated Weather Network weather station (Michigan Climatological Resources Program, East Lansing, MI). A subsample of 3,000 of these picked apples was held in the lab suspended over moist paper towel in trays in order to determine the daily number of larvae emerging from the picked fruits. Approximately 71% of larvae had emerged before the entomopathogen application date. Therefore, the single entomopathogen applications targeted only the last 29% of natural larval emergence from fruit at the site. This serves also as a proximal measure for the adult trapping experiment, since the fruit were collected from the same orchards.

Soil and organic material from the area underneath cages were sampled for plum curculio on three dates (26, 34, and 54 d post treatment) to collect all adults present. Cages were lifted, and any adults present on the interior net surface were collected. Simultaneously, the groundcover under each cage was systematically vacuumed for 2 min with a 10 HP leaf blower modified for suction, with a 15 cm diam. hose. Debris was collected into bags (0.8 mm mesh) that were secured with rubber bands to the end of the

vacuum hose. Cages were put back into place within 3 min of sampling. Debris was hand-separated in the laboratory and adults were recovered.

The number of adults collected was pooled over the three dates for each cage, and results were analyzed by proc GLM (SAS Institute 2004).

3. Results

3.1 Efficacy and Timing with Emerged Larvae

2006 Results

In 2006, adult emergence was significantly reduced by at least one *B. bassiana* treatment in one of two apple orchards and one of two cherry orchards (Figure 3.1). In the Genesee Co. Sandy Loam apple orchard, adult emergence was reduced by 32% and 48% by rice and Mycotrol-O treatments, respectively, but only the Mycotrol-O spray treatment was significant. Only the high rice treatment reduced adult emergence significantly from controls (by 77%) in the Benzie Co. Clay Loam tart cherry orchard. The contents of all pots were sifted through after day 60, and the only finding was one dead adult.

2007 Results

In 2007, only the *S. riobrave* entomopathogen treatment resulted in significantly reduced adult emergence compared to untreated controls for all three larval application timings: immediately, 3 d, and 5 d after entomopathogen application (Figure 3.2). Reduction from control was 88%, 89%, and 80% for 0, 3, and 5 d timings, respectively. The *S. carpocapsae* treatment significantly reduced adult emergence (56% from control) only for the 3 d timing. All other entomopathogen x larva-timing combinations were not significantly different from their respective control x larva-timing combinations.

2008 Results

In 2008, when data from the 5 sites was combined, the high rate of nematode *S. riobrave* significantly reduced adult emergence when larvae were added to pots -5, 0, and 10 d from nematode application, but not 5 d (Figure 3.3). When data from each individual site was analyzed separately, the ANOVA was significant ($p<0.05$) at the conventional tart cherry sites only. Within those sites, mean separations (SNK) revealed that only the *S. riobrave,* high rate, -5 d or 0 d treatments were significant (Table 3.4).

Soil moisture values in 2008 are presented in Table 3.3. In 2007, the soil moisture in pots at day of entomopathogen application was 9.4%. In 2007, rainfall from 21 June to 25 July was less than 4.5 cm total. Rainfall was greater than 0.13 cm per day on 21 and 27 June; 4, 10, 14 July, and 25 July (0.38, 0.56, 0.49, 0.84, 0.33, and 1.57 cm, respectively).

3.2 Efficacy with Trapping

There were no significant differences in screen trap catch of adult plum curculio between treatments ($F = 0.76$, $p = 0.5199$, $df = 165$). Mean ± S.E. adults caught per trap was 1.3 ± 0.2, 1.1 ± 0.3, 1.1 ± 0.2, and 1.4 ± 0.3 adults per cage for control, *M. anisopliae*, *S. carpocapsae*, and *S. riobrave* treatments, respectively.

3.3 Efficacy with Caged Fruit

There were no significant differences in adult plum curculio recovery per cage between treatments ($F = 0.68$, $p = 0.5721$, $df = 27$). Mean ± S.E. recovery per cage was 11.1 ± 1.5, 12.4 ± 2.6, 11.1 ± 2.4, and 9.2 ± 3.1 adults per cage for control, *M. anisopliae*, *S. carpocapsae*, and *S. riobrave* treatments, respectively. An average of 0.50 (1,499 total) larvae emerged per fruit from the 3,000 fruits held in the laboratory.

4. Discussion

Variability year-to-year, site-to-site, and from lab-to-field is characteristic of augmentative entomopathogen studies (McCoy et al. 2000). Such variations within this study and their probable causes are discussed.

Beauveria bassiana was not effective against plum curculio as a conidia suspension or formulated in an oil carrier in 2007 and 2008 respectively. However, *B. bassiana* applications targeting last-instar plum curculio significantly reduced adult emergence from controls by 48% (granular) in one of four orchards and 77% (spray) in one of four orchards in 2006. These two orchards had lower sand content than the other two orchards (Table 3.2). Although immersion method laboratory bioassays indicated higher virulence of *M. anisopliae* than *B. bassiana* to last-instar plum curculio, *M. anisopliae* was not effective when applied as a conidial suspension or as a granular formulation in trials conducted in 2007.

In 2007 when larvae were added to pots of soil, *S. riobrave* at 0, 3, and 5 d and *S. carpocapsae* at 3 d only reduced emergence of subsequent adults. Shapiro-Ilan et al. (2002) found that *S. carpocapsae* was less virulent than *S. riobrave* to plum curculio larvae in laboratory assays. *Steinernema riobrave* also has higher optimum temperature range than *S. carpocapsae* (Grewal et al. 1994), which the infective juveniles may have experienced in the soil during the time when infection was most likely. Foraging strategy may also affect successful infection. Plum curculios tend to pupate in the upper 5 cm of soil if sufficient moisture is present. The "sit-and-wait" forager *S. carpocapsae* outcompeted the active forager *H. bacteriophora* for *Galleria mellonella* larvae at the soil surface, and vice versa for 5 cm below the surface (Alatorre-Rosas and Kaya 1990). The

Steinernema riobrave foraging strategy seems to be an intermediate of the two (Lewis et al. 2006).

Besides differences between the two nematode species such as virulence, foraging method, or optimal temperature ranges, formulation is a major factor that could have contributed to the observed difference in efficacy. *Steinernema riobrave* was only available in a gel carrier, while *S. carpocapsae* was available in a vermiculite carrier, specifically developed for approval by the Organic Materials Review Institute (OMRI). Likewise, unformulated *H. bacteriophora* was ineffective while gel-formulated *S. riobrave* was partially effective in 2008 trials.

To estimate needs for re-application over the duration of larva soil residence, it was necessary to determine the reduction in efficacy of entomopathogens over a time period longer than from 0 to 5 d post-application. In 2008 *S. riobrave* significantly reduced adult emergence when larvae were added to pots -5, 0, and 10 days from nematode application. Mean percent reduction from control was 25%, 66%, 50%, 27%, 36%, and 8% for the -10, -5, 0, 5, 10, and 15 day timings, respectively. This is in contrast to the 2007 experiment, in which mortality from *S. riobrave* was greater than 80% for the 0 and 5 d timings. Abbott's corrected mortality means (Abbott 1925) revealed greater than 50% mortality at the sandiest three sites for -5 and 0 d *S. riobrave* high rate treatments. Variation between pots within a treatment was so high that not all of these means were significantly lower than the control; -5 d was significant for all three sites and 0 d was significant for one site.

These entomopathogens may have caused additional mortality to other soil-contacting life stages of plum curculio. For instance, adults are susceptible to

entomopathogenic nematodes (Brossard et al. 1989, Bélair et al. 1998, Shapiro-Ilan et al. 2002). Observations of natural infections of *B. bassiana* and *M. anisopliae* in adults have been made (Lafleur et al. 1987), but efficacy of augmentative applications has not been reported. The pupa is susceptible to nematodes (Kim and Alston 2008) and may have been attacked by the entomopathogens in our study. We found no studies on the efficacy of entomopathogenic fungi against pupae and adults.

The experimental methods introduced several unrealistic conditions. In order to develop a spatially contained three-dimensional plot that could be effectively evaluated in the field, we chose plastic pots as field soil "cages" for these experiments. Therefore, efficacy levels observed in the potted experiments may be higher than would be expected in a normal orchard setting. Larvae were introduced to treated soil within 1 h of entomopathogen application on all 0 d timings; however, the population of larvae dropping naturally from fruit into soils would be making first contact with an entomopathogen applied several days before to several days after, depending on the interval of days between applications. Also, nematodes reproduce inside insect cadavers and their offspring exit the cadaver in search of a new insect host. This "nematode cycling" may have had an increased potential for reducing plum curculio adult emergence in our experiment, since we used a higher density of larvae (10 larvae per 77 cm^2) than would likely occur in an orchard with moderate to high plum curculio infestation. These densities may however be achieved in some organic orchards where growers attempt to rake or broom dropped fruit into the drive row or into piles.

Another factor of variability introduced by the experiment methods was detection of high densities of larvae and subsequent predation by ant colonies. In 2008 the pots

were observed up to 2 h after larva application, until all larvae had burrowed, and ant predation was noted. All pots with ant predation were excluded from the analysis. Observation of ant predation was highest at the sandy tart cherry sites, reaching 17% of pots in two sites, but was under 3% for the other three sites. Ant predation was not observed in 2006 or 2007, but likely contributed to experimental error. In our experience experiments using infested fruit containing larvae rather than directly placing high densities of larvae on the soil surface could reduce detection and predation by ants. A bifenthrin ring treatment to prevent entry of fire ants in a similar situation did not affect mortality of plum curculio (Jenkins et al. 2006b), and should be considered in this type of experiment.

When infested fruit rather than free larvae were placed in field plots and adults were trapped on trees in plots or more directly in cages, no effects of entomopathogens on reducing adult densities were observed. Late entomopathogen application timing was probably a contributing factor to this, since 71% of larvae were expected to have dropped from fruit prior to applications.

Other than entomopathogen-application timing, there are many reasons why the open trunk traps may not have accurately reflected the populations in plots. Differences may not have been detected due to a low overall trap catch. Other possible reasons are small plot size, dispersal of adults between and outside of plots, poor attraction of adults to traps and thus inaccurate representation of adult populations in plots, low emergence of larvae from fruit and thus low population pressure in plots, etc. However, one would expect to see differences in the cages for treatments that were effective in the pots. High variability between plots suggests that more replication is needed per treatment.

Entomopathogens are generally sensitive to water stress and maintaining soil moisture constant across our treatments over time was not economically or practically possible (Table 3.3). Therefore, our results reflect a reasonable expectation of what conditions would be in Michigan during the time of larval drop, since very few Michigan orchards are irrigated at this time. Although some orchardists use drip tube irrigation, this delivery system is not designed to deliver moisture to the entire drip-line under the tree, where plum curculios pupate. A way to ameliorate variable moisture conditions would be to use an under-tree micro-jet sprinkler irrigation system to maintain moisture, as is used to control citrus root weevil (McCoy et al. 2002). This may be cost-effective for tree fruit growers that maintain drip irrigation lines, as the sprinklers are easily substituted into the holes created by the drip emitters. Larva suppression by nematodes irrigated with sprinklers lasts no longer than 1 or 2 weeks (McCoy et al. 2000, 2002), but efficacy within that time presumably would be higher than comparable sites without irrigation, and pathogen cycling in higher moisture conditions may add to the efficacy of these entomopathogen tools.

The physical properties of soils may contribute greatly to efficacy of entomopathogens (Barbercheck 1992, Georgis and Gaugler 1991). Larval mortality of citrus root weevil caused by *S. riobrave* was positively correlated with the proportion of sand in soils (Shapiro-Ilan et al. 2000, Duncan et al. 2001, McCoy et al. 2002). (2002) and Shapiro-Ilan et al. (2000). Generally, nematode motility decreases with decreasing soil pore size even though the soil type may hold less water (Molyneux and Bedding 1984, Kaya 1990, Kung et al. 1990, Roy Kaspi personal communication). Efficacy among largely different soil textures was not considered in prior plum curculio studies,

where the Utah study residential sites soils had a smaller percent sand range than the current study (54-73%) (Kim 2007) and the southern sites were loamy sand and sandy loam (Shapiro-Ilan et al. 2004a, 2008).

Alternatively, persistence of *B. bassiana* near the soil surface (0-5 cm depth) may be higher in soils with small pore sizes (Vänninen et al. 2000). A clay coating on *Beauveria bassiana* blastospores reduces biodegradation (Fargues et al. 1983). In our 2008 study, sites highest in sand resulted in marginally higher reduction of plum curculio adult emergence. In 2006, *B. bassiana* efficacy was observed in the clay loam and loamy sand sites, and none in the two sand sites.

These entomopathogens are particularly sensitive to the UV-B component of light; therefore, adjuvants with UV-radiation protecting properties are sometimes added to formulations of entomopathogens (Inglis et al. 1995). The rice in 2006 and the formulations used in the 2007 experiments contained no such adjuvants. Since the entomopathogens were applied on sunny days in 2006 and 2007, some reduction in efficacy may have occurred due to UV-radiation damage. In the 2008 trials, UV exposure was avoided by applying entomopathogens at most 2.5 h before sunset.

Compounds such as copper and sulfur, along with their surfactants, are often sprayed in Michigan organic orchards (Table 3.5) to manage plant pathogens such as apple scab, sometimes to the detriment of beneficial organisms such as fungi and nematodes (Krishnayyaand and Grewal 2002). In a study of six fungicides used in commercial pecan production, the growth of *B. bassiana* and *M. anisopliae in vitro* were least affected by sulfur (Tedders 1981). Growth of *B. bassiana* mycelium was not effected by sulfur in other laboratory tests, but the effect on spores was not tested (Sterk

et al. 2002). The effect of sulfur on entomopathogen activity has yet to be tested in the field. Such testing is needed if augmentative entomopathogens are to be adopted in orchards where toxic compounds may accumulate in soils after rainfall, especially in organic orchards or other production systems that need to rely on sulfur, copper, and other OMRI-approved fungicides. One feature of cherry orchards in the Upper Midwest that would be significantly different from other areas is that associated fungicides cease at least 10-21 d before harvest and therefore as much as 17-28 d before entomopathogen application under our timing schemes.

Nematode diversity and abundance displayed in Table 3.2 may be an indicator of high competition or suitable habitat for the augmented nematode species. However, since differences in efficacy between sites are marginal we cannot conclude an interaction between endemic nematodes and augmentative nematodes, but work should be done in this area.

More research is needed to validate the assumption that the reduction in summer-generation adult emergence caused by nematode applications in small or large areas targeting larvae will reduce damage the following year. Plum curculios immigrate to orchards from overwintering sites in spring (Racette et al. 1992), so adults surviving in a refuge such as nearby unsprayed habitats may immigrate into the orchard of concern. It is a longstanding recommendation to remove refuge habitats such as alternate hosts around orchards to prevent plum curculio damage (Racette et al. 1992). This practice would likely be essential to reduce the chances of immigration of spring adults within the immigration range of an orchard under a management program using nematodes against last-instars. Shapiro-Ilan et al. (2008) found that *S. riobrave* applications targeting soil-

dwelling larvae in a wild plum thicket plot adjacent to an orchard caused 87.9-98.6% control. In terms of anticipated longevity of *S. riobrave* in the soil, our results suggest that multiple applications of *S. riobrave* at the high rate (4×10^9 IJs/ha) in summer would be necessary to target the majority of plum curculio larvae exiting fruit, since larvae exit fruit for several weeks. Applications may need to be applied every 10-15 d during larval drop from fruit, but more studies considering nematode efficacy longevity under controlled soil moisture management should be conducted to support this tentative recommendation.

A strategy that employs *S. riobrave* targeting larvae may be feasible for adoption by Michigan tree fruit growers with a need for environmentally-friendly plum curculio management tools. However, the growers that may need these tools the most may be organic certified growers, and *S. riobrave* in OMRI approved formulation such as the vermiculite formulation is not yet available and has not been tested against the gel formulation. This strategy, although more expensive, may be used in home yards, where people prefer to avoid use of conventional insecticides (Kim 2007). To reduce costs, nematodes might be applied to areas only where oviposition is concentrated, and where irrigation is possible. Plum curculio oviposition marks are typically concentrated in border rows, and are easy to detect visually. Other strategies that aggregate infested fruit in limited regions below the trees could also allow growers to more effectively target emerging larvae.

The replacement of chemical insecticides with "softer" tactics in tree fruit may depend on the practical management of other pests that typically resurge with reductions

of chemical inputs, such as codling moth and oriental fruit moth; however, some of these pests may be controlled with microbials as well (Lacey and Shapiro-Ilan 2003, 2008).

In conjunction with quantifying residual activity of an augmentative entomopathogen application, a measure of the duration of a plum curculio population's soil residence will help growers make application decisions. The timing of larval emergence from fruit has been studied in tree fruits in West Virginia (Brown 2005) and Utah (Kim 2007), and in blueberries in New Jersey (Polavarapu et al. 2004). However, calendar or phenology models for plum curculio larval emergence from fruit remain undeveloped for many regions. More research is needed to develop more precise entomopathogen-application timing recommendations according to the phenology of larval emergence from fruit crops and effective and practical application strategies.

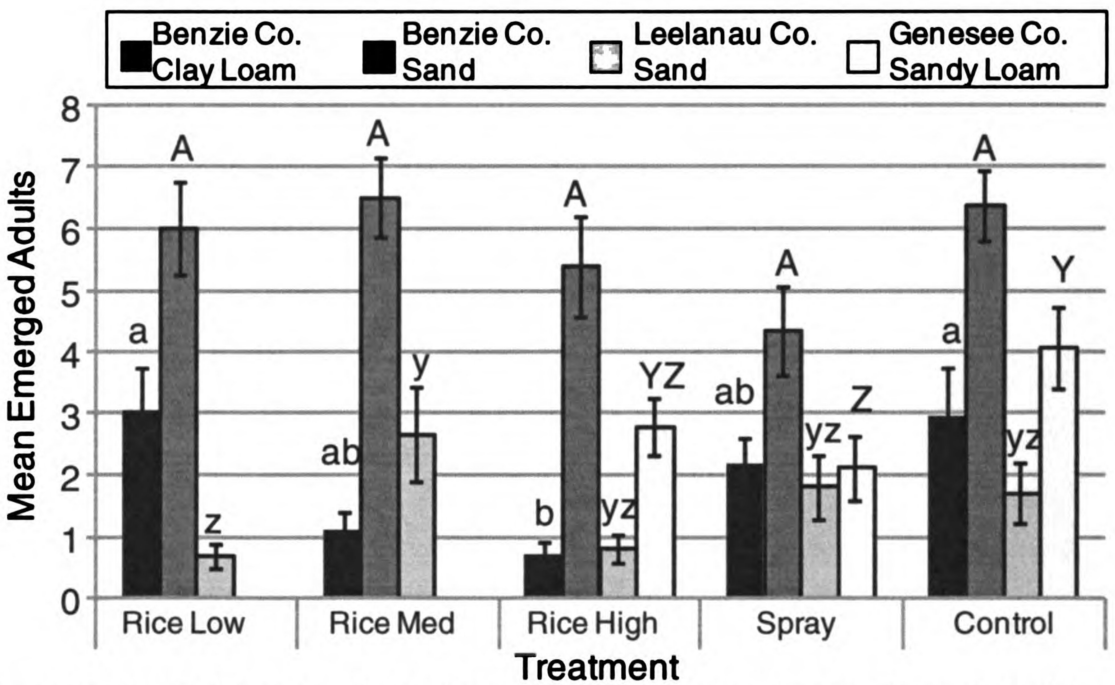


Figure 3.1. Mean (± SE) number of adult plum curculio emerged from *B. bassiana* GHA-treated soil in pots (10 larvae/pot) over a 50-d period in 2006 at four orchards. Rice treatments (*B. bassiana* grown on rice) were not conducted in Genesee Co. Bars with the same letter within an orchard are not significantly different (SNK, alpha=0.05).

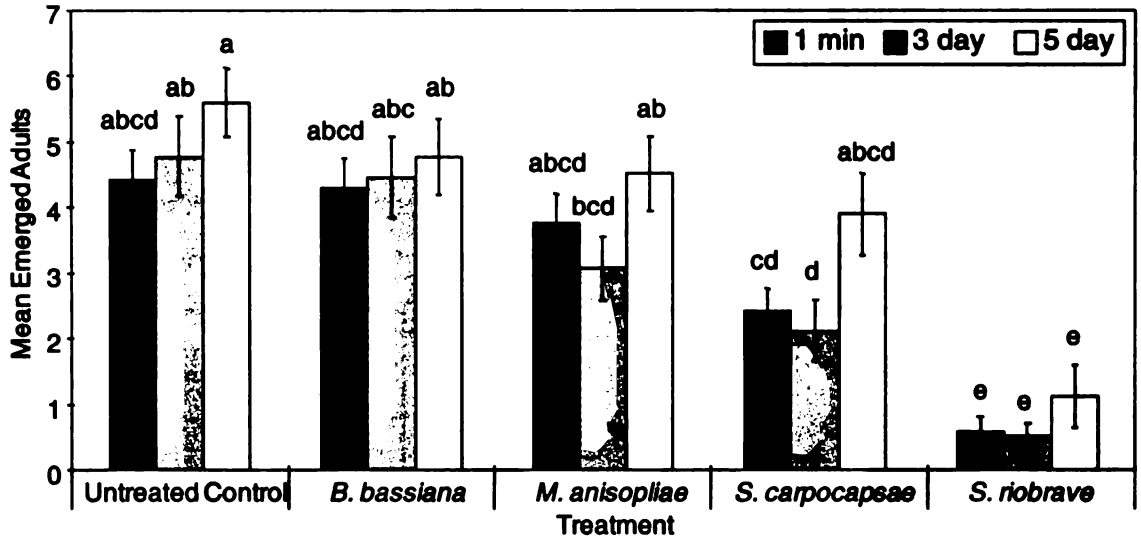


Figure 3.2. Mean (± SE) number of adult plum curculio emerged from entomopathogen-treated soil in pots over a 50-d period in 2007 at a single orchard. Ten larvae were added to each pot either immediately (1 min), 3 d, or 5 d after entomopathogen application. Bars with the same letter are not significantly different (SNK, alpha=0.05).

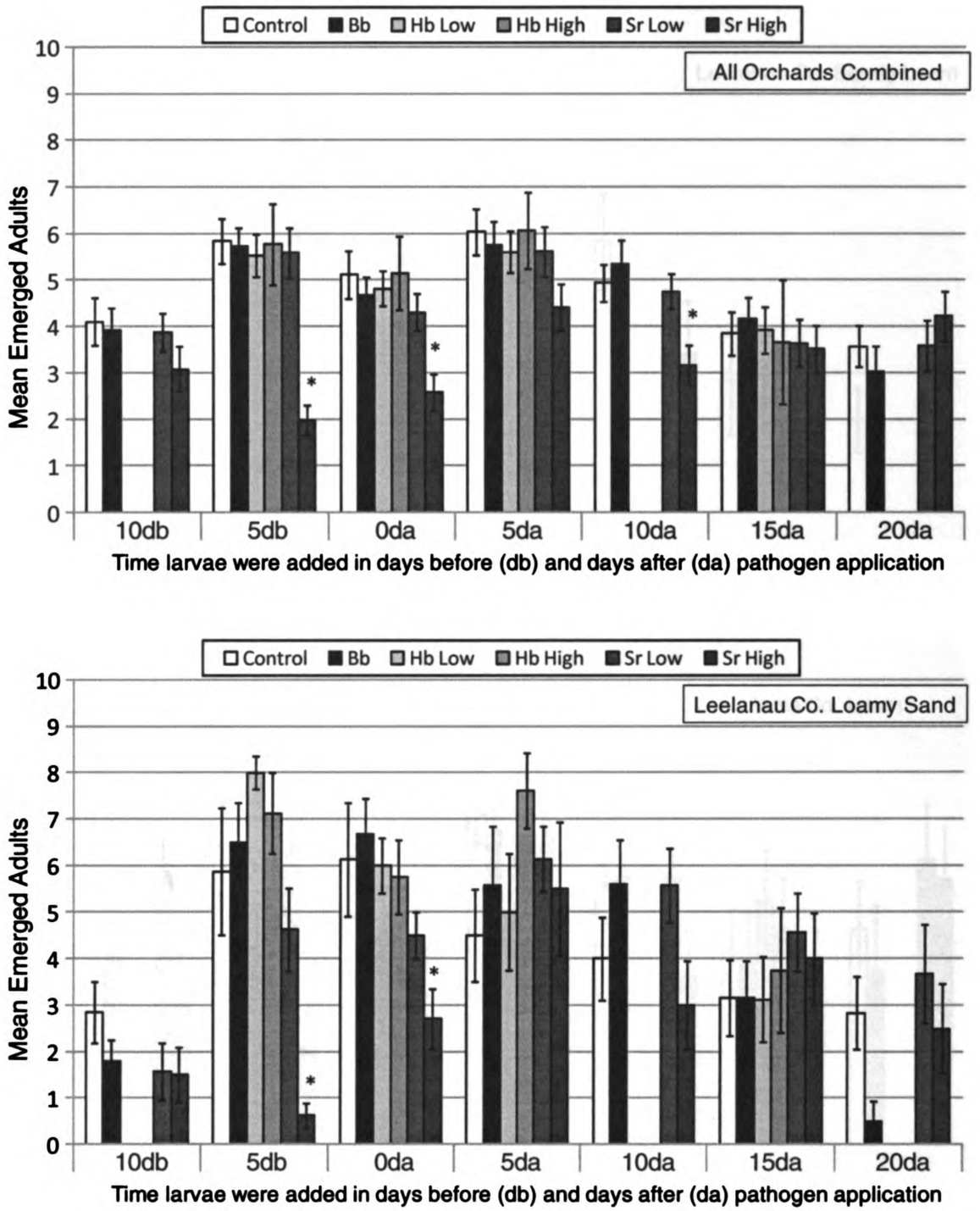
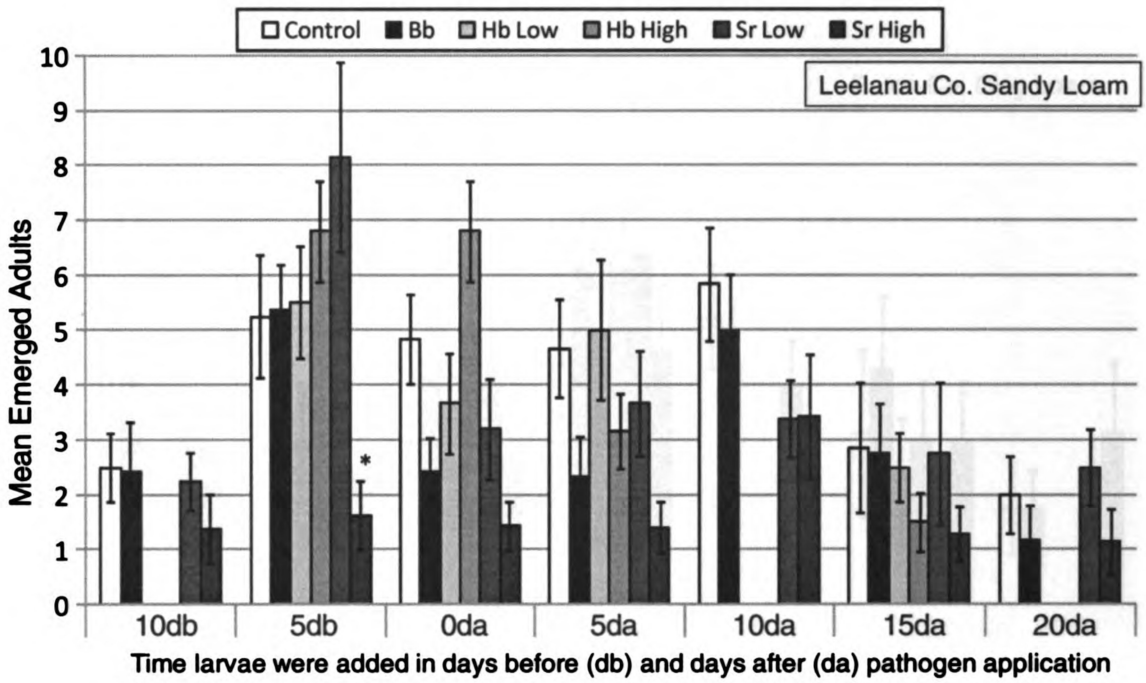


Figure 3.3. Mean (± SE) number of adult plum curculio emerged from entomopathogen-treated soil in pots over a 50-d period in 2008. Ten larvae were added to each pot either -10, -5, 0, 5, 10, 15, or 20 d from entomopathogen application. Note Hb treatments were not conducted on -10, 10, and 20 d. Bars with an asterisk are significantly different from the control within the same larva addition timing (SNK, α=0.05). All orchards combined and Leelanau Co. Sandy Loam site.



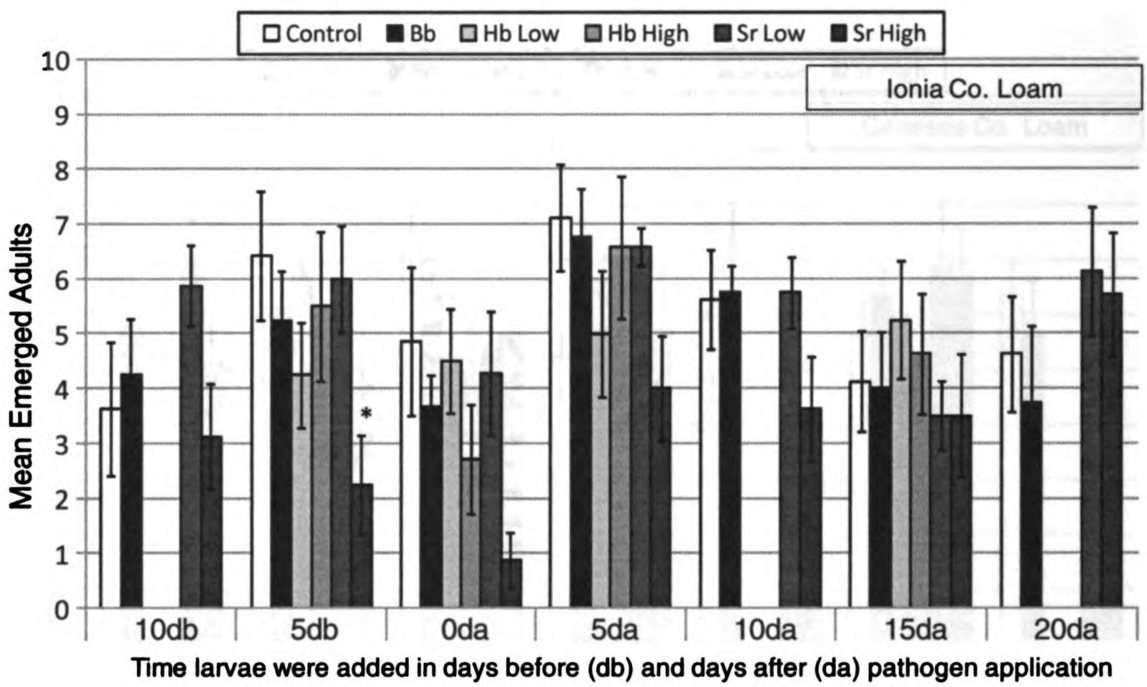
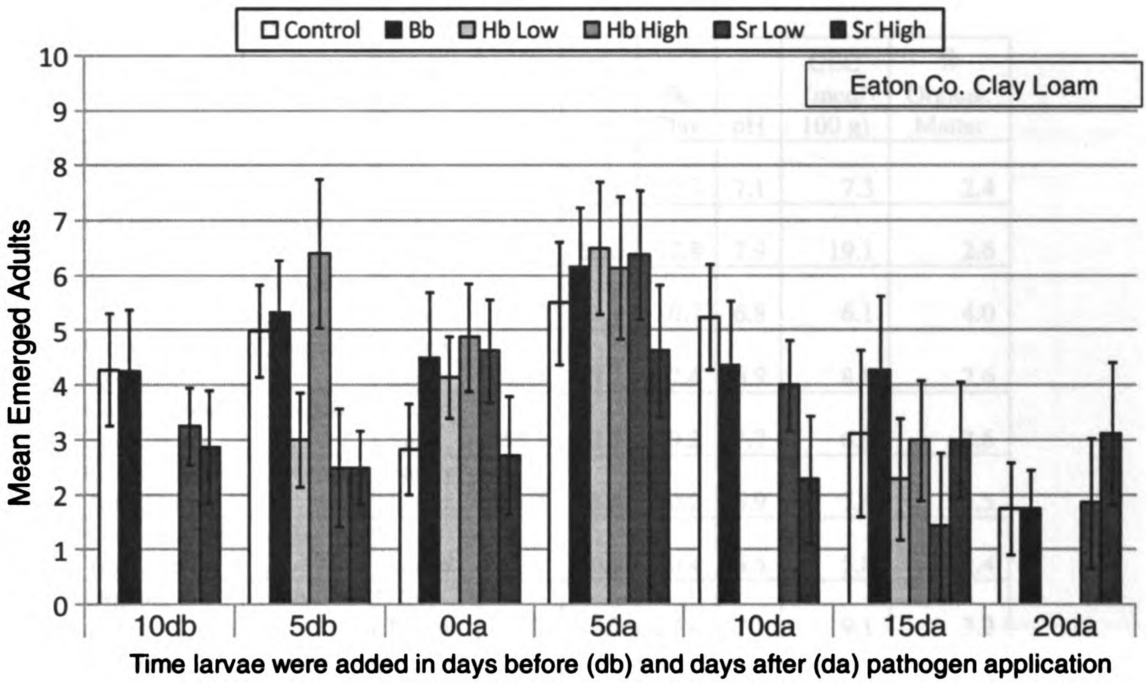


Figure 3.4. Mean (± SE) number of adult plum curculio emerged from entomopathogen-treated soil in pots over a 50-d period in 2008. Ten larvae were added to each pot either -10, -5, 0, 5, 10, 15, or 20 d from entomopathogen application. Note Hb treatments were not conducted on -10, 10, and 20 d. Bars with an asterisk are significantly different from the control within the same larva addition timing (SNK, α=0.05). Leelanau Co. Sandy Loam site and Ionia Co. Loam site.



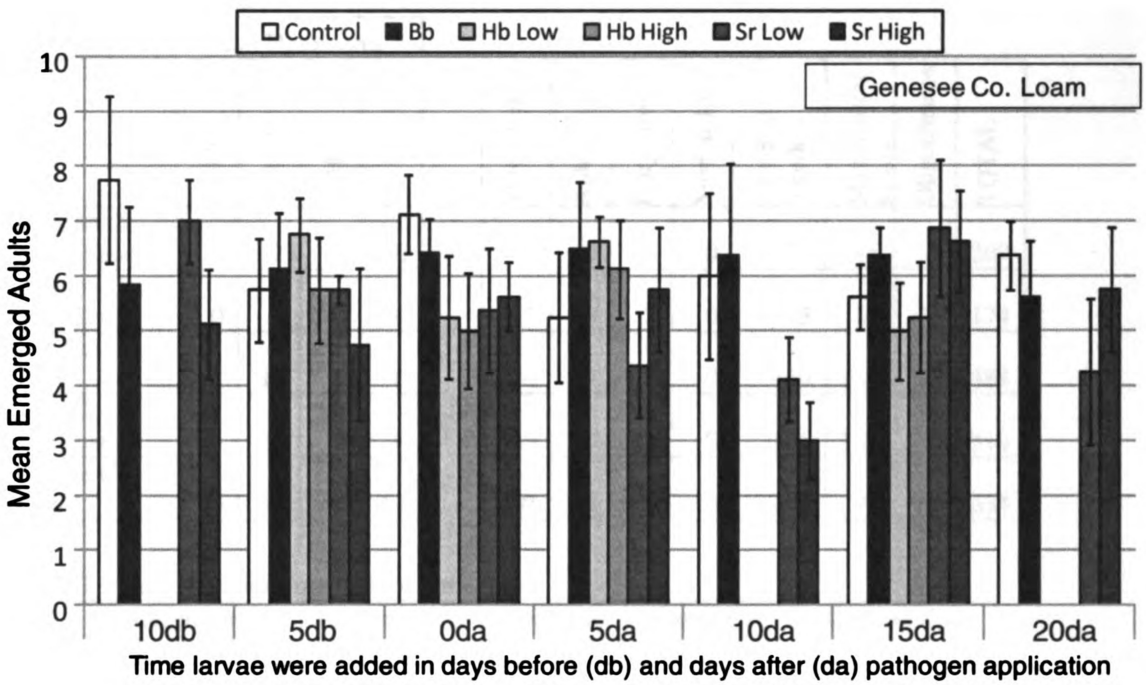


Figure 3.5. Mean (± SE) number of adult plum curculio emerged from entomopathogen-treated soil in pots over a 50-d period in 2008. Ten larvae were added to each pot either -10, -5, 0, 5, 10, 15, or 20 d from entomopathogen application. Note Hb treatments were not conducted on -10, 10, and 20 d. Bars with an asterisk are significantly different from the control within the same larva addition timing (SNK, α=0.05). Eaton Co. Clay Loam site and Genesee Co. Loam site.

Table 3.1. Soil Analysis for 2006 and 2008 sites.

Year	Crop	Site	Soil Type	% Sand	% Silt	% Clay	pH	CEC (meq/ 100 g)	% Organic Matter
2006	Organic Tart Cherry	Leelanau Co.	Sand	88.2	9.1	2.7	7.1	7.3	2.4
2006	Organic Tart Cherry	Benzie Co.	Clay Loam	32.4	29.8	37.8	7.9	19.1	2.6
2006	Organic Apple	Benzie Co.	Sand	89.2	10.1	0.7	6.8	6.1	4.0
2006	Organic Apple	Genesee Co.	Sandy Loam	63.4	29.2	7.4	6.9	8.8	2.6
2008	Conventional Tart Cherry	Leelanau Co.	Loamy Sand	88.4	2.5	9.2	7.7	6.8	2.6
2008	Conventional Tart Cherry	Leelanau Co.	Sandy Loam	81.4	8.5	10.2	6.9	7.4	3.5
2008	Conventional Tart Cherry	Ionia Co.	Loam	36.7	40.9	20.4	6.5	5.8	2.4
2008	Organic Apple	Eaton Co.	Clay Loam	40.7	31.9	27.4	7.1	9.1	3.3
2008	Organic Apple	Genesee Co.	Loam	36.7	38.9	24.4	6.7	12.5	6.9

Table 3.2. Nematode Community Analysis, Michigan State University Diagnostic Service Laboratory, from pre-application soils, 2008.

	Soil Herbivores											
	Lesion	Dagger	Ring	Pin	Tylenchus	Aphelencus	Dorylamis	Mononahs	Bacterial Feeders	Mycorrhizal Fungi	Oligochaetes	TOTAL
Leelanau Co. Loamy Sand	0	2	2	0	0	0	35	0	70	95	5	209
Leelanau Co. Sandy Loam	0	4	0	0	5	10	5	0	95	10	45	174
Ionia Co. Loam	1	19	0	0	70	15	0	0	160	110	10	385
Eaton Co. Clay Loam	4	61	0	0	90	30	15	0	165	145	0	510
Genesee Co. Loam	20	23	44	17	160	20	30	10	245	35	30	634

Table 3.3. Percent soil moisture in 2008 sites.

Day	Leelanau Co. Loamy Sand	Leelanau Co. Sandy Loam	Ionia Co. Loam	Eaton Co. Clay Loam	Genesee Co. Loam
-10	3.0%	2.7%	20.1%	15.7%	48.0%
-5	3.8%	3.5%	21.3%	15.5%	15.1%
0	7.0%	13.8%	20.3%		38.6%
5	5.0%	6.8%	7.5%	11.4%	28.1%
10	1.6%	2.7%	11.3%	4.0%	25.7%
15	3.5%	6.4%	10.0%	5.3%	14.4%

Table 3.4. Mean percent reduction from control treatments (Abbott's Corrected Mortalities) for plum curculio in *S. riobrave* high rate treatment. Larvae were applied -10, -5, 0, 5, 10, 15, or 20 d from *S. riobrave* application to pots of soil. Percentages with an asterisk (*) indicate that the corresponding mean adult counts were significantly lower than control within a larva timing (SNK, α=0.05).

	Larva Timing						
Site	-10 d	-5 d	0 d	5 d	10 d	15 d	20 d
All Orchards	25%	66%*	50%*	27%	36%*	8%	-18%
Leelanau Co. Loamy Sand	48%	89%*	56%*	-22%	25%	-26%	12%
Leelanau Co. Sandy Loam	45%	69%*	70%	70%	41%	55%	43%
Ionia Co. Loam	14%	65%*	82%	44%	36%	15%	-24%
Eaton Co. Clay Loam	33%	50%	4%	16%	56%	4%	-79%
Genessee Co. Loam	34%	17%	21%	-10%	50%	-18%	10%

Table 3.5. Spray record for Clarksville orchard blocks in 2007.

Date	Active Ingredient
3/30/2007	Copper hydroxide for fireblight scab
4/20/2007	Sulfur
4/24/2007	Sulfur
4/27/2007	Calcium polysulfate (lime sulfur)
5/7/2007	Sulfur
5/14/2007	Sulfur
5/14/2007	*Bacillus subtillis*
5/24/2007	Sulfur
5/24/2007	Rosemary, clove, thyme oil
5/29/2007	Sulfur
5/29/2007	Rosemary, clove, thyme oil
5/29/2007	Kaolin clay
6/2/2007	Calcium polysulfate (lime sulfur)
6/2/2007	Streptomycin sulfate
6/8/2007	Streptomycin sulfate
6/18/2007	Sulfur
6/18/2007	Rosemary, clove, thyme oil
7/3/2007	Sulfur
7/3/2007	Rosemary, clove, thyme oil

CHAPTER 4

LABORATORY AND FIELD EVALUATIONS OF ENTOMOPATHOGENIC FUNGUS EFFICACY AGAINST ADULT PLUM CURCULIO

1. Introduction

Plum curculio, *Conotrachelus nenuphar* (Herbst), is a serious pest of tree fruits in eastern North America. Changes in insecticides available to growers have prompted the development of alternative management tactics (Wise et al. 2006a). Adult plum curculio cadavers have been found infected with entomopathogenic fungi (Garman and Zappe 1929, McGiffen and Meyer 1986, Lafleur et al. 1987). This susceptibility to muscardine disease could be used in management of plum curculio. The plum curculio life cycle includes three duff- and soil-dwelling stages that may be vulnerable to soil-borne entomopathogens: the soil-burrowing last-instar, the pupa, and adult.

Several strains of the entomopathogenic fungi *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin are currently registered by the US EPA. While efficacy of entomopathogenic nematodes for suppression of plum curculio adults has been evaluated in the laboratory (Shapiro-Ilan et al. 2002), neither *M. anisopliae* nor *B. bassiana* have been evaluated against plum curculio adults. Only last-instars have been targeted in fungus efficacy evaluations in the lab (Tedders et al. 1982, Alston et al. 2005) and field (Jenkins et al. 2006b). However, a trunk perimeter spray of *B. bassiana* controlled pecan weevil adults with effects up to 1 wk post-application, with best results in plots receiving irrigation (Shapiro-Ilan et al. 2004b).

Inundative releases of *M. anisopliae* and *B. bassiana* typically involve application to soils, where these species reside as facultative saprophytes. A granular formulation of

Beauveria brongniartii is commercially available for European cockchafer biocontrol (Kessler et al. 2004). A simple maize-based *B. bassiana* formulation developed for subsistence farmers was effective against banana root weevil (Nankinga and Moore 2000) and granular *M. anisopliae* increased yield in fields with sugarbeet root maggot (Campbell et al. 2006). A granular formulation of *B. bassiana* on rice established in pasture better than a biopolymer when considered for clover root weevil control (Nelson et al. 2004).

A surface other than soil that an insect is likely to contact may serve as an alternative substrate for targeting certain insect life stages. A fabric trunk band was effective against the Asian longhorned beetle (Dubois et al. 2004, Hajek et al. 2006). If there is a sufficiently long enough period of time between inoculation and mortality, perhaps inoculated adults could transfer conidia to mates, serving as an autodisssemination management tactic (Vega et al. 1995). Several studies utilizing "autodissemination traps," demonstrated that this approach was effective for the sweet potato weevil (Yasuda 1999), diamondback moth (Furlong et al. 1995, Furlong and Pell 2001), Japanese beetle (Klein and Lacey 1999), and brown-winged bug (Tsutsumi et al. 2003).

Adult plum curculios aggregate underneath trees in the perimeter rows of orchards in the spring (Lafleur and Hill 1987, Racette et al. 1991, Chouinard et al. 1993, 1994). Males may mate with as many as 3 females per day (Yonce and Jacklin 1978), or an average of 10.4 females per 30 days (Johnson and Hays 1969). Mobile adults can be trapped by walking or flying to and climbing onto Whalon Lab high contrast pyramid traps (Great Lakes IPM, Vestaburg, MI) or on tree trunks fitted with screen traps (Mulder

et al. 1997). Trap catch is highest before petal fall (Coombs 2001, Leskey and Wright 2004).

The goal of this study was to evaluate efficacy of commercially available entomopathogenic fungi against plum curculio adults. Our objectives were to: 1) determine the mortality of adults after exposure to surfaces with *B. bassiana* and *M. anisopliae*, and 2) determine the mortality of unexposed adults caged with exposed adults.

The exposure surfaces included: 1) soil or peat with surface-applied aqueous suspensions of conidia; 2) walking arenas with colonies of conidia on Petri dishes; 3) a moist cotton wick that could be positioned inside an "autodissemination" trap top; and 4) conidia-covered granules applied to orchard groundcover.

2. Materials & Methods

2.1 Insects and Entomopathogenic Fungi

Northern strain adult plum curculios were obtained from a colony maintained by the Pesticide Alternatives Laboratory, Michigan State University, East Lansing, MI. The colony was reinitiated annually from adults and infested cherries and apples collected in six orchards in Benzie, Ionia, Leelanau, and Manistee Co., MI. Adults were collected using circle traps (Mulder et al. 1997) and "Whalon pyramid traps" (1.6 m x 0.8 m) made from recyclable black corrugated plastic with high-contrast reflective tape (Great Lakes IPM, Vestaburg, MI). Both trapping systems were equipped with plum essence lures and stabilized benzaldehyde vials (Coombs 2001, Leskey and Wright 2004, Leskey et al. 2005). In order to break diapause and induce mating and oviposition, adults were exposed to thinning apples that had been dipped in a solution of 750 mg pyriproxifen (Esteem® 35

WP) per L water (Hoffman et al. 2007). Adults used in the field trial were not exposed to pyriproxifen.

Commercial strains of *B. bassiana* (GHA strain obtained from the USDA Agricultural Research Service Entomopathogenic Fungus Culture Collection, Ithaca, NY) and *M. anisopliae* (F52 strain received as Met52® granular formulation, Novozymes Biologicals, Inc., Salem, VA) were used in this study.

Aqueous suspensions of fungi were prepared by scraping *M. anisopliae* or *B. bassiana* conidia from 2-wk old potato dextrose agar plate cultures into sterile water with 0.01% Tween-80. Fungal inoculum viability was assessed according to Goettel & Inglis (1997) by pouring 50 ul of each suspension within 30 min of application onto three dishes of potato dextrose agar and checking 200 conidia/dish for the presence of germ tube formation after 20 h with an Olympus BX40 research microscope.

The field experiment took place within the dripline of trees in an unsprayed and neglected apple orchard at the Michigan State University Entomology Farm, East Lansing, MI. Soil for the laboratory assays was gathered from the same location to a depth of 8 cm. The soil was mixed and a sample was tested at the Michigan State University Soil and Plant Nutrient Laboratory, East Lansing, MI. The soil was loamy sand (79.3% sand, 15.7% silt, 5.0% clay; 1.8% organic matter, pH=7.2).

2.2 Laboratory Assay: Aqueous Suspensions on Soil

Field soil and peat were passed through a #18 sieve and each was autoclaved for 45 min and dried at 110°C. The soil was brought up to 14% moisture and the same amount of water was added to the peat, resulting in 60% moisture calculated

gravimetrically. The soil or peat was placed in 30 ml autoclaved medicine cups (Premium Plastics, Chicago, IL).

Conidia suspensions were prepared by scraping conidia from 2-wk old cultures on PDA into 0.01% Tween-80. Suspensions were adjusted such that 1 ml was delivered by pipette to each cup at a rate of 8×10^{14} conidia/ha. Control cups received 1 ml of 0.01% Tween-80. Conidia viability was 96% and 97% for *B. bassiana* and *M. anisopliae*, respectively.

Adult plum curculios were placed individually in the cups and fed with a 5 g piece of thinning apple. Cups were sealed with three layers of cheesecloth secured with a rubber band, and were incubated in large glass jars at 25°C. The jars were sealed with perforated parafilm and contained saturated sterile filter paper to maintain humidity. Mortality was checked and apple pieces were replaced every 3 d for 15 d. There were five replicated cups per soil type x entomopathogen treatment and the experiment was repeated once.

In a second assay, treatments included only loamy sand soil with two rates: 1×10^{13} or 5×10^{13} conidia/ha of either *M. anisopliae* or *B. bassiana*. There were three replicate cups per entomopathogen x rate treatment, and the experiment was repeated four times.

Mortality data passed assumptions of normality and homogeneity of variance and were analyzed by one-way ANOVA. Means were separated using Student Neuman-Keuls procedure and Dunnett's test for the first and second assays, respectively (SAS Institute 2004).

2.3 Laboratory Assay: Petri Dish and Wick

Newly emerged adults were held for 3-6 wk before the study on thinning apples in vented plastic containers under a 16:8 L:D photoperiod, at 25°C. Adults were separated by sex the day before the experiment began according to Thompson (1932). Males were marked with a small dot of yellow enamel paint on the left elytron for identification, while females were unpainted.

Two surfaces were tested: 1) a two-week old fungus colony on SDAY (Sabouraud Dextrose Agar + Yeast) in a 90 mm Petri dish and 2) a two-week old colony on the surface of a moist dental wick with SDY (no agar). The wicks were prepared by autoclaving and then dipping them individually in a suspension of *B. bassiana* or *M. anisopliae* conidia at a rate of 1×10^7 conidia/ml of SDY solution. Each wick soaked up approximately 10 ml. The suspensions were prepared by scraping conidia off the surface of two-week old cultures on SDAY held at 25±1°C in the dark. Control adults were exposed to either sterile SDAY Petri dishes or to a wick dipped in sterile water. The dipped wicks were suspended inside sterile glass mason jars from hardware cloth. The mason jars were lined with a moist, sterile Whatman no.1 filter paper, and topped with a Petri dish sealed with Parafilm®.

A single adult was forced to walk across the fungus-colonized agar or wick surface for 5-10 s. These exposed adults were then placed individually into 59 ml soufflé cups (Solo Cup, Urbana IL) containing 20 ml of field soil prepared as in the previous assay. An autoclaved square galvanized wire screen (0.5 cm diameter mesh) was suspended 4 cm above the surface of the soil, and a thinning apple was placed on the screen. Cups were incubated at 25°C, 16:8 L:D, and approximately 70% RH. An

unexposed adult of the opposite sex was introduced into the plastic soufflé cup 4 h later.

There were five pairs of adults in each of four repetitions for a total of 20 pairs per pathogen x surface x exposed sex combination (Table 4.1).

Table 4.1. Dish/Wick assay treatments.

Pathogen	surface	exposed sex	introduced mate	PC pairs per rep	reps	n (# of pairs)
Bb	Dish	M	F	5	4	20
Bb	Dish	F	M	5	4	20
Bb	Wick	M	F	5	4	20
Bb	Wick	F	M	5	4	20
Ma	Dish	M	F	5	4	20
Ma	Dish	F	M	5	4	20
Ma	Wick	M	F	5	4	20
Ma	Wick	F	M	5	4	20
Control	Dish	M	F	5	4	20
Control	Dish	F	M	5	4	20
Control	Wick	M	F	5	4	20
Control	Wick	F	M	5	4	20

Adult pairs were checked daily for mortality for 28 d. Copulation and mortality dates were recorded throughout. All cadavers were removed to individual moist chambers and viewed under a Nikon SMZ1000 (Mager Scientific, Inc., Dexter, MI) stereo dissecting microscope for signs of sporulation 3 wk post mortality.

One-way ANOVAs using arcsine square root transformed data were run to compare mating occurrences, time until death, and percent mortality between treatments, followed by Tukey's HSD a posteriori tests, $\alpha=0.05$ (SAS Institute 2004).

2.4 Field Study: Granular Formulation against Overwintering Adults

A granular formulation of *B. bassiana* was produced in the laboratory by colonizing ground barley (1-3 mm) in autoclavable bags following methods of Nelson et al. (2004). The Met52 formulation of *M. anisopliae* consisted of conidia bound to unbroken rice. The number of conidia per gram of granular material was approximated by

placing a 5 g sample into a flask containing 100 ml of 0.01% Tween-80. Flasks were placed on a rotating shaker at 180 RPM for 5 min, and the number of conidia per ml of solution was determined using a hemacytometer (4 counts per flask). The mean of 3 flasks was calculated: 3.09×10^9 and 2.09×10^9 conidia/g for *B. bassiana* and *M. anisopliae*, respectively. Viability was 96.7% and 90.5% for *B. bassiana* and *M. anisopliae*, respectively.

A wire cone-shaped ground cage (Mulder et al. 1997) with a 0.3 m^2 base was installed 1 m from the trunk of each of 24 apple trees at the East Lansing orchard. Cage bottoms were fitted into a 4 cm deep trench, staked, and soil was mounded over the base to prevent plum curculio escape. The groundcover consisted of thick orchard grass, cut to 6 cm. Each treatment (*B. bassiana, M. anisopliae,* or control) was assigned to 8 cages in a completely randomized design. Prior to cage installation, granules were distributed uniformly onto the groundcover of each cage at a rate of 5×10^{13} conidia/ha. Control cages did not receive any grains. Immediately following treatments, the groundcover of every cage was sprayed with 100 ml of water from a backpack sprayer. Adults (24 per cage) and a ripe apple picked from a tree on-site were introduced to cages on 17-October; 24 h after treatments were applied.

Ambient air temperatures at ground level were recorded hourly with a Watchdog Datalogger Model 425 (Spectrum Technologies, Inc) and rainfall was recorded at an on-site weather station (Michigan Automated Weather Network, East Lansing, MI) (Table 4.2). Temperatures inside the cages averaged 1.1°C cooler than outside. Boll weevil trap tops (Great Lakes IPM, Vestaburg, MI) were installed on cage tops on 14 March and adults were collected from the tops until 9 June. Data analysis was conducted using

ANOVA after passing normality and homogeneity of variance assumptions (SAS Institute 2004).

3. Results

3.1 Laboratory Assay: Aqueous Suspensions on Soil

Conidia suspensions of *M. anisopliae* and *B. bassiana* pipetted onto field soil at $8x10^{14}$ conidia/ha resulted in 100% and 90% mortality of plum curculio adults following constant exposure of adults over 15 d (Fig. 4.1). Applications of the fungi to peat soil caused 10% and 20% mortality, levels much lower than the control.

Comparison of low (1×10^{13}) and high rates (5×10^{13} conidia/ha) of *M. anisopliae* and *B. bassiana* applied to field soil indicated that only the high rate of *B. bassiana* caused significantly higher adult mortality (70% higher) than controls (Fig. 2.2).

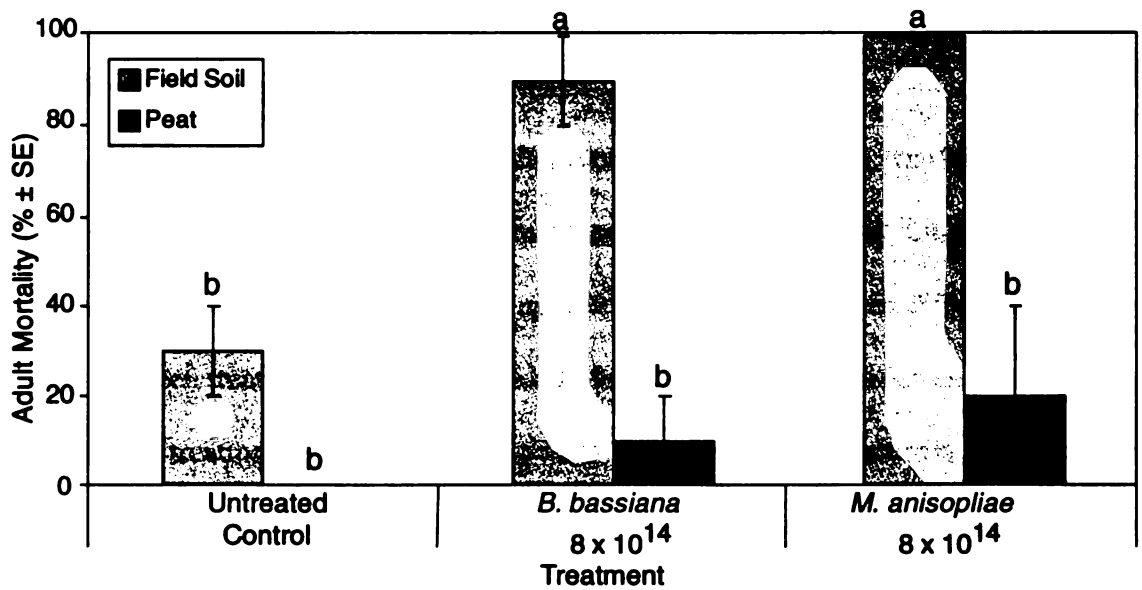


Figure 4.1. Mortality of adult plum curculio exposed to entomopathogen-treated soil or peat at a rate of 8×10^{14} conidia/ha after 15 d. Bars with the same letter are not significantly different (SNK, α=0.05).

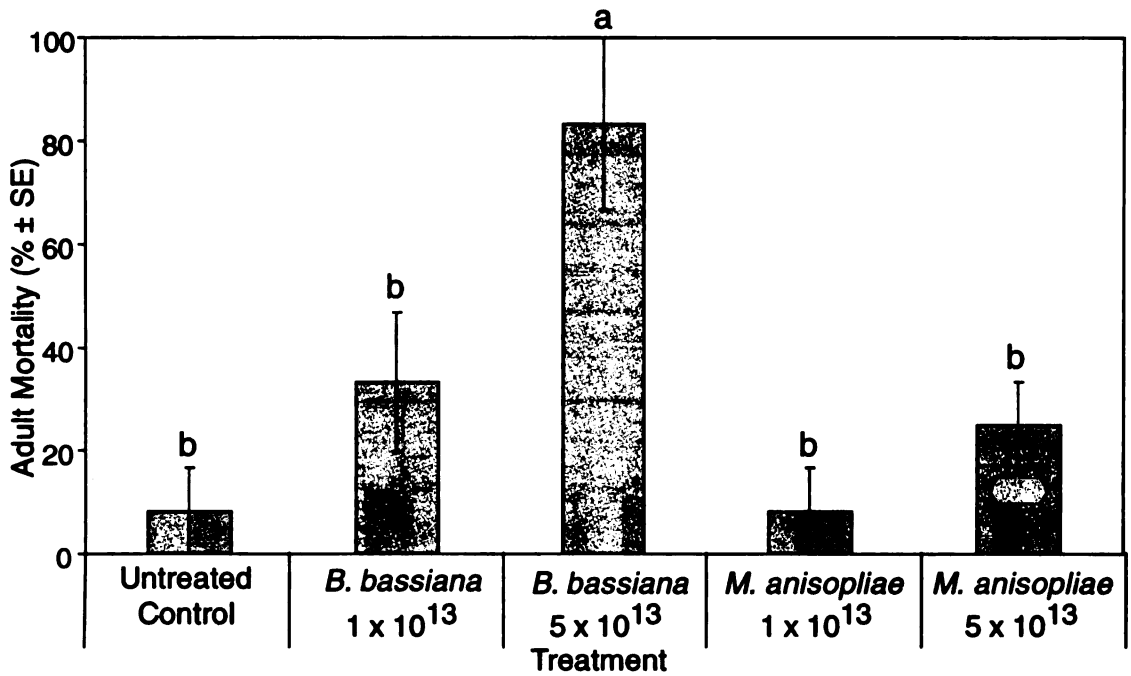


Figure 4.2. Mortality of adult plum curculio exposed to entomopathogen-treated soil (low rate = 1 x 10^{13} conidia/ ha; high rate = 5 x 10^{13} conidia/ha) after 12 d. Bars with the same letter are not significantly different (Dunnett's procedure, α=0.05).

3.2 Laboratory Assay: Petri Dish and Wick

Conidia viability, assessed 20 h after plum curculio exposure, was 93% and 0% for *B. bassiana* dish and wick and 96% for *M. anisopliae*, respectively. Of the paired plum curculios, 47% were observed mating at least once. There was no difference in mating between treatments (F=0.78, df=11, p=0.6595), and no difference in time until death among treatments (F=1.40, df=20, p=0.1284) (Figure 4.3). There was a quantitative, but not significant mortality in females exposed to the control wick and females held with males exposed to the control wick, as well as those in the Bb wick treatments, which may indicate a higher background mortality of females as compared to males. Mortality was significantly higher than control in all fungus treatments except for Bb wick and Ma wick (males paired with exposed females). There was no significant difference among the

treatments with significantly higher mortality than the control. Sporulation was quantitatively highest among the Bb dish treatments, but only significantly higher than the Bb wick and Ma wick males paired with exposed females.

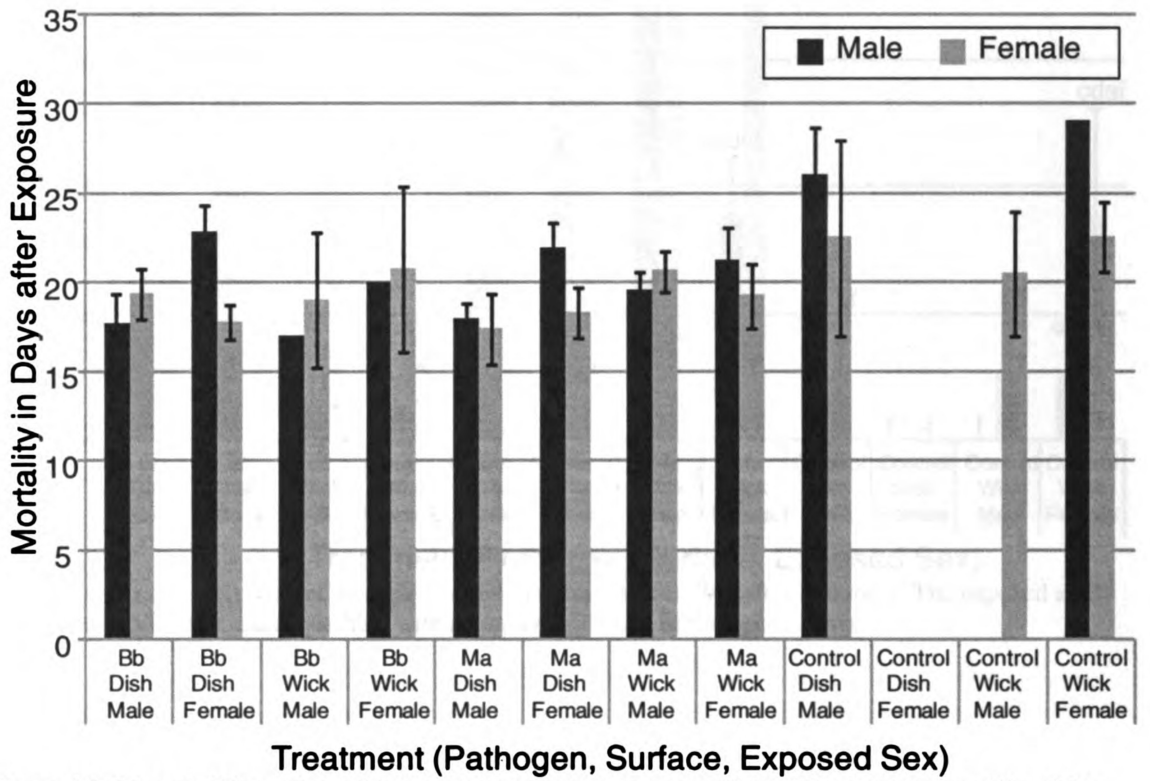


Figure 4.3. Mean (±SE) number of days after treatment exposure until mortality of plum curculio adults. The exposed adult (sex indicated in the x-axis) was held with an unexposed mate of the opposite sex.

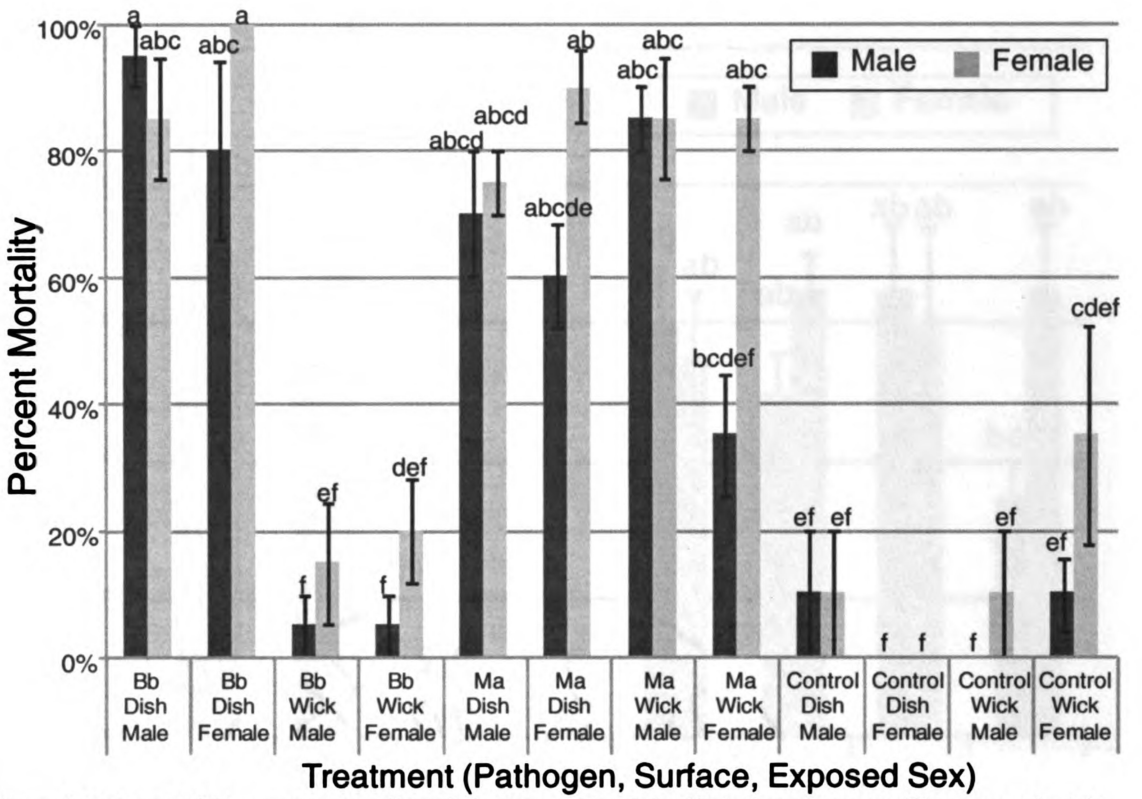


Figure 4.4 Mean (±SE) percent mortality of plum curculio adults 29 d after treatment. The exposed adult (sex indicated in the x-axis) was held with an unexposed mate of the opposite sex.

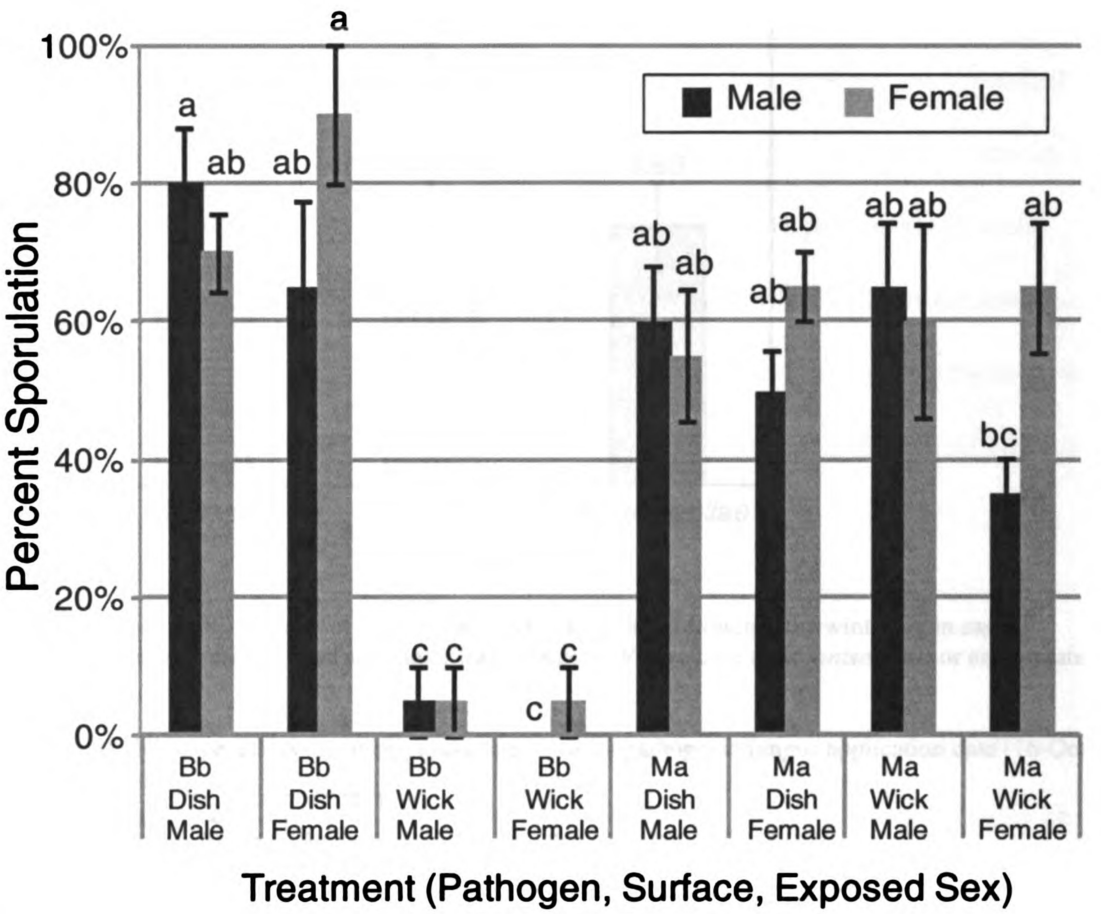


Figure 4.5. Mean (±SE) percent sporulation of plum curculio adults 37 d after treatment exposure. The exposed adult (sex indicated in the x-axis) was held with an unexposed mate of the opposite sex. Bars with same letter indicate that the corresponding means are not significantly different (Tukey, α=0.05).

3.3 Field Study: Granular Formulation against Overwintering Adults

Although fewer total adults emerged from the *B. bassiana* (77) and *M. anisopliae* (69) than control (86) cages, mean emergence was not significantly different (F=0.11, df=2, p=0.896). Abbott's corrected mortalities (Abbott 1925) were 10% and 8% for *M. anisopliae* and *B. bassiana*, respectively. The standard error for *B. bassiana* (6.8) was much higher than for *M. anisopliae* (1.7) or control (1.2) treatments. Abbott's Corrected Mortalities were 8% and 10% for *B. bassiana* and *M. anisopliae*, respectively (Abbott 1925).

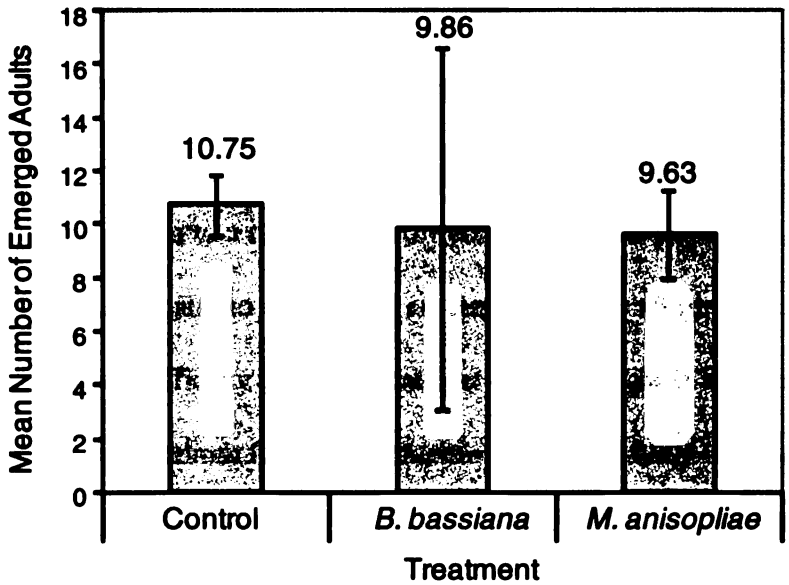


Figure 4.6. Mean (±SE) number of emerged adults in the spring following overwintering in cages. Groundcover treatments included a granular formulation of *B. bassiana* or *M. anisopliae*, or an untreated control.

Table 4.2. Daily temperatures and precipitation near entomopathogenic fungus application date (16-Oct) in overwintering trial.

Date	High Temp (C)	Low Temp (C)	Precip (cm)
14-Oct	11.7	0.6	0.53
15-Oct	21.7	7.8	0.00
16-Oct	**16.7**	**13.9**	**0.38**
17-Oct	21.7	11.1	0.08
18-Oct	24.4	16.1	2.69
19-Oct	17.2	11.1	1.45
20-Oct	18.9	10.0	0.03
21-Oct	25.6	13.9	0.00
22-Oct	22.2	10.0	0.41
23-Oct	10.6	6.7	0.86
24-Oct	11.7	3.9	0.00
25-Oct	13.9	1.1	0.00
26-Oct	17.2	6.1	0.00
27-Oct	13.3	1.1	0.71
28-Oct	11.7	-1.7	0.00
29-Oct	14.4	-1.1	0.00
30-Oct	17.8	5.0	0.00

4. Discussion

Applications of the two fungi to field soil in the lab showed promising reductions in plum curculio adult survival, especially at higher rates. The *B. bassiana* treatment was effective down to a rate of 5×10^{13} conidia/ha. The physical properties of soils may contribute greatly to efficacy of entomopathogens (Barbercheck 1992), as demonstrated by the poor efficacy on peat. Peat was tested alongside a loamy sand soil because peat is a common medium for testing entomopathogenic fungi against greenhouse and nursery pests, such as the black vine weevil (Moorhouse et al. 1993, Shah et al. 2007, Bruck 2005). In these situations the absence of a rhizosphere may contribute to poor control (Bruck 2005).

Adults experienced high mortality to fungi, but this was under conditions of constant exposure over 15 d to the conidia on a soil surface in a confined area, which moving adults in the field would not necessarily experience. A shorter fungus exposure period for any assay method should be tested to simulate realistic field conditions. The duration of contact with the soil or peat substrate for individuals during the assay remains unknown, but many adults were observed to be attached to the apple when checked rather than on the substrate surface. To reduce this behavior in future assays, exposure to apples could be reduced. Further testing could conclude whether plum curculio adults exhibit behaviors indicating attraction or repulsion to fungus-treated substrates.

In the assays with Petri dishes and wicks, since the exposed mate was placed directly into the cup on the soil surface following exposure to the fungus, the entire cup environment was likely contaminated with conidia dislodged from the adult. It cannot be determined whether infection of introduced mates was due to direct physical contact with

the exposed mate, or to exposure to contaminated soil, cup, and apple surfaces. Adults exposed to the fungal surface would likely spend hours if not days losing unattached conidia on tree or soil surfaces before encountering a mate in an orchard. We cannot be certain whether adult pairs actually mated or how much time they spent in physical contact, since they were only observed once daily. In future assays, exposed adults should be held over soil to shed conidia for increasing time periods, and subsequently introduced into an uncontaminated arena with a mate for a known time period. The time period of physical contact could be held more constant or be reviewed on a camera recording.

For unknown reasons, conidia viability of *B. bassiana* on the wick was 0%, while that of *M. anisopliae* on the wick was 98%. It was not surprising then that the mortality of beetles in the *B. bassiana* wick treatments was comparable to the controls. However, *B. bassiana* dish treatments caused 65-100% mortality. The method of culturing *B. bassiana* on the wick surface should be altered to bring conidia viability near 100% and the viability over time should be tested under field conditions.

An emerging pattern was revealed in the fungus treatments. Mates of exposed females seem to suffer less mortality than the female, whereas mates of exposed males seemed to suffer comparable mortality to the male. The differences were not statistically significant, but a possible interpretation is that males transmit the fungus to females better than females transmit the fungus to males. The experiment should be repeated with the introduction of unexposed mates several days after exposed mate exposure (a "conidia dislodgement" period), within an environment that hasn't been contaminated by the exposed mate before this can be concluded, and with a known period of contact between mates.

The time until death for treated adults ranged from an average of 17 to 22.7 days after exposure, and these means were comparable across all treatments, whether the adults were directly exposed or indirectly exposed by an exposure to an infected mate mate. If the first lab assays were carried out up to 23 days, then perhaps mortality would have increased. The goal of this application in orchards would be to reduce oviposition scarring and larval emergence, not necessarily accelerate mortality. From infection until death, adults could be mating, potentially transferring conidia, making oviposition scars in fruits, and laying viable eggs. Reduced number of eggs laid and percent egg hatch were demonstrated for yellowish elongate cockchafer infected with *Beauveria brongniartii*, confirming suppression of subsequent generations before the onset of mortality (Yaginuma 2006). Further exploration of the behavioral and reproductive consequences in the weeks following exposure or infection is warranted to determine whether such tactics could reduce damage in orchards.

The laboratory assays represent optimum temperature and moisture conditions. In the field, the entomopathogens are likely to succumb to sensitivities to moisture and temperature stress. The values in Table 4.2 indicate surprisingly optimal conditions for infection in late October, with temperature minimums between 10 and 16°C, and maximums between 17 and 26°C for six days post-application. The application date was also surrounded by precipitation events, which kept the soil uncommonly moist. Neither fungus controlled the adults. Poor or "patchy" coverage of the treated areas may have been the result of the large sizes of the granular formulated material. A liquid or wettable powder formulation could avoid distribution and host contact problems caused by large (2.5 mm) granular formulations under field conditions (Gaugler et al. 1989, Campbell et

al. 2006). Only spring-tilled application of granular *B. bassiana* gave control of Colorado potato beetle, but authors cite low temperatures, insufficient time between application and emergence, and the inactive state of overwintering adults as reasons for poor overall efficacy (Gaugler et al. 1989).

Appendix 1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 2008-10

Title of thesis or dissertation (or other research projects):

ENTOMOPATHOGENIC FUNGI AND NEMATODES FOR MICHIGAN TREE FRUIT MANAGEMENT TARGETING PLUM CURCULIO (*CONOTRACHELUS NENUPHAR*)

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name(s) (typed)
Renee Pereault

Date December 9, 2008

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America. Bull. Entomol. Soc. Amer. 24: 141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.

Copies: Include as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

Appendix 1.1

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Conotrachelus nenuphar (Herbst)	USA MICH Ingham CO. Michigan State University Plum curculio colony, orig. Manistee & Benzie CO. 13 August 2008 Renee Pereault coll.					10	10		

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Renee Pereault

Date December 9, 2008

Voucher No. 2008-10

Received the above listed specimens for deposit in the Michigan State University Entomology Museum

Curator

1 Dec 2008

Date

REFERENCES

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