ENTOMOPATHOGEN EFFICIENCY AGAINST COLORADO POTATO BEETLE IN DIVERSE MANAGEMENT SYSTEMS

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of RICARDO A. RAMIREZ II find it satisfactory and recommend that it be accepted.

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Chair

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ENTOMOPATHOGEN EFFICIENCY AGAINST COLORADO POTATO BEETLE IN DIVERSE MANAGEMENT SYSTEMS

Abstract

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Cropping systems are faced with a multitude of pest problems and as a result must implement a diverse suite of pest management tactics. In the potato cropping system, the Colorado potato beetle is one of many serious potato pests. Traditionally, insecticides have been the primary control method, but because of the beetle's rapid development of resistance to most chemicals, alternative control measures are being considered. While the use of entomopathogens seems promising for potato beetle control their effectiveness under field conditions has been quite variable. In chapter 2, I examined the impact of mustard green manures, a common practice for plant parasitic nematode control, on entomopathogen infection. I found that the use of mustard green manures negatively impacted entomopathogen infection in both field surveys and laboratory studies. In chapter 3, I focused on the impact of animal manure and synthetic fertilizer on the inundative release of entomopathogenic nematodes (EPNs). I found that host infection by EPNs was negatively impacted by animal manure compared to synthetic fertilizer. Furthermore, these data indicate that increased microbial activity, consisting of EPN competitors and antagonists, in soil amended with animal manure may decrease EPN effectiveness. Finally, in chapter 4, I tested the hypothesis that through conservation biological control, herbivore

suppression strengthens with increasing natural enemy biodiversity. Colorado potato beetle eggs and small larvae occur in plant foliage where they are attacked by a guild of generalist predators, whereas later-stage larvae burrow into the soil to pupate and are attacked by a guild of entomopathogens. I found that potato beetle densities decreased, and plant biomass increased, with greater enemy biodiversity. However, beetle suppression strengthened only for predator-pathogen pairs, and not for pairings within the same natural enemy guild. This body of research shows that crop health and pest management strategies may interfere with biological control using EPNs; in addition, it shows that conservation of entomopathogens is important to improving biological control.

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Dedication

This dissertation is dedicated to my love, Christine.

CHAPTER ONE

Introduction

In many cropping systems growers are faced with multiple pest pressures including arthropods, plant pathogens, and weeds. As a result growers are implementing multiple pest management strategies to combat these different pest types. In addition to pest management, growers are also concerned with other crop health issues such as soil fertility. While research has traditionally focused on the control of a specific pest, this dissertation examines the interactions between crop and pest management and biological control. In the potato cropping system growers are dealing with pests such as the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say, early and late blight of potato, several species of plant parasitic nematodes such as the root-knot nematode, and various broadleaf weed species. Traditional, chemical control for these agricultural pests has grown problematic because of increasing pesticide resistance, environmental concerns and human toxicity. Consequently, there has been an increase in the adoption of alternatives to the application of synthetic chemical insecticides for pest control, including the use of entomopathogens to control pest arthropods and mustard bio-fumigants to control plant pathogens and early season weeds. In addition to the adoption of these alternative control measures there has been an increase in organic production throughout the USA. Moving from conventional potato production to organic potato production there are two distinct differences, the adoption of animal manure to increase soil fertility and a reliance on biological control to manage insects and other pests. This chapter provides a literature review highlighting the Colorado potato beetle, entomopathogens (specifically entomopathogenic

nematodes and *Beauveria bassiana*), mustard bio-fumigation, and interactions between soil organisms and animal-manure fertilizers. Chapter two examines the interactions between mustard bio-fumigation and entomopathogens. It is formatted for submission to the peer-reviewed journal, Biological Control, and is entitled "Harmful effects of mustard bio-fumigants on entomopathogenic nematodes." Chapter three examines the interactions between manure and entomopathogens. It is formatted for submission to Biological Control, and is entitled "Animal manure harms entomopathogenic nematodes." Chapter four examines the interactions between aboveground insect predators and belowground entomopathogens of the Colorado potato beetle. It is formatted for submission to the peer-reviewed journal, Ecology Letters, and is entitled "Scared sick? Cascading effects of predator-pathogen complementarity." The first author, Ricardo A. Ramirez, carried out all field and laboratory experiments, statistical analyses, and writing of each manuscript, while co-authors contributed to various aspects of research and manuscript preparation. D. Henderson provided greenhouse and laboratory assistance; E. Riga and L. Lacey, contributed to laboratory experiments and editing the manuscripts; L. Carpenter-Boggs provided assistance with specialized soil techniques; and W.E. Snyder, advisor, contributed to the experimental design, statistics, and editing of all manuscripts.

Literature review

Colorado potato beetle

The Colorado potato beetle (CPB, *Leptinotarsa decemlineata* Say) is a serious pest of potatoes worldwide (Hare 1990). Unchecked, potato beetles reproduce rapidly and can entirely defoliate the crop, with catastrophic consequences for yields (Hare 1980b). The Colorado potato beetle is presumed to be a native of Central America that was not an agricultural pest in the USA until the mid 1800's (Hare, 1990).

Adult Colorado potato beetles are recognized by ten distinct black stripes running the length of the yellow to orange elytra (hardened forewings of coleopterans) and measuring 9.5 mm long and 6.4 mm wide (Pedigo, 2002). Colorado potato beetles overwinter as adults in soil at a depth of 7.6 - 12.7 cm (Lashomb et al., 1984; Hare, 1990). In the spring adults crawl up to the soil surface and walk or fly to host plants and start to feed (Voss and Ferro, 1990a; Voss and Ferro, 1990b). Adult potato beetles mate multiple times and females oviposit eggs in clusters of 20 - 60 eggs per cluster on the undersides of solanaceous plant leaves (Hare, 1990). Eggs of the potato beetle range from bright yellow to deep orange in color. Four to nine days after eggs are deposited larvae emerge and begin feeding immediately. There are four larval instars or stages that vary in size; all larval stages feed on plant leaves and stems. Larvae are orange/red in color with a single row of black spots running along the lateral sides of the body (Pedigo, 2002). Within a population early stage potato beetle larvae, in the 1st and 2nd instars, make up approximately 10% of total leaf consumption, while later stages, in the 3rd and 4th instars, make up greater than 90% of total leaf consumption (Ferro et al., 1985; Logan et al., 1985; Tamaki and

Butt, 1978). The last instar will stop feeding, drop to the soil surface and burrow into the soil to pupate (Hare, 1990). The duration of the potato beetle life cycle from egg to adult is approximately 33 days, with 4.5 days spent as an egg, 4 days for each of the first, second and third instars, 5.5 days as a fourth instar, and 10.5 days as a pupa (Hare, 1990; Yaşar and Güngör, 2005). Development and movement of the potato beetle is dependent on a number of factors including temperature, photoperiod, and host-plant quality (Ferro et al., 1985; Tauber et al., 1988a; Tauber et al., 1988b; Tauber et al., 1988c; Voss et al., 1988; Voss and Ferro, 1990a; Voss and Ferro, 1990b). Several generations occur each growing season but the number depends on latitude and the overall length of the growing season (Hurst, 1975).

Control strategies

Traditionally, frequent sprays of broad-spectrum insecticides have been used to control Colorado potato beetle on potatoes in the USA, encouraging the beetle's rapid development of resistance to most chemical insecticides (Hare, 1980a; Forgash, 1981; Harris and Svec, 1981; Gauthier et al., 1981; Forgash, 1985; Johnston and Sandovol, 1986; Casagrande, 1987; Kennedy and Farrar, 1987; Argentine et al., 1989). The neo-nicotinoids (imidacloprid, thiamethoxam, and clothianidin), pyrethroids, and carbamates are the primary classes of insecticides used against the Colorado potato beetle (Alyokhin et al., 2007). The search for and development of insecticides that can be included in a pesticide rotation is an important part of resistance management. Spinosad, a mixture of bacterial fermentation products, is an insecticide that has been evaluated for cross-resistance with neo-nicotinoids and shows promise in a resistance management program (Mota-Sanchez et al., 2006). While aldicarb has been effective against the Colorado

potato beetle, combinations of bio-insecticides (*Beauveria bassiana* [Balsamo] Vuillemin and *Bacillus thuringiensis* Berliner) provide low numbers of overwintering adult beetles (Lacey et al., 1999). In addition, potato varieties genetically engineered to contain insecticidal genes from *B. thuringiensis* have been effective (Reed et al., 2001), but their marketability, availability, and quality is a concern (Mota-Sanchez et al., 2006).

Crop rotation is a cultural control tactic utilized to delay the colonization of potato fields from the emerging overwintering CPB adults that walk and fly in search of their host (Ng and Lashomb, 1983). This cultural control tactic drastically reduces the need for early season insecticide applications (Lashomb and Ng, 1984; Wright, 1984). In addition, early planting of short-season potato varieties has been used to generate a similar effect (Boiteau, 1986; Casagrande, 1987; Wright et al., 1987b). Interestingly, copper- and tin-based fungicides used for control of plant pathogens in potato also have anti-feedant properties that harm Colorado potato beetle (Hare, 1984; Hare et al., 1983; Hare and Moore, 1988; Wright et al., 1987b).

Colorado potato beetles are attacked by a diverse community of arthropod and pathogen natural enemies, which together exert biological control (Chang and Snyder, 2004; Hough-Goldstein et al., 1993; Lacey et al., 1999). These natural enemy communities often are devastated following the application of broad-spectrum insecticides (Root and Gowan, 1978; Croft, 1990; Hardin et al., 1995; Ruffle and Miller, 2002). However, recent years have seen the development of selective insecticides less-harmful to Colorado potato beetle natural enemies, and also growing interest in organic pest management in potato production, reviving interest in the biological control of Colorado potato beetle and other potato pests (Koss and Snyder, 2005). As mentioned above, Colorado potato beetles exhibit a complex life cycle, with eggs, adults and earlier-stage larvae feeding in potato foliage, while fourth (final) instar larvae burrow into the

soil to pupate (Hare, 1990). In potato fields in the Columbia Basin region of eastern Washington State and adjacent Oregon, as Colorado potato beetle move between these two habitats they transition between two distinct communities of natural enemies. Aboveground, generalist predators dominate, including primarily predatory nabid and geocorid bugs, coccinellid beetles, spiders, and carabid beetles (Reed et al., 2001; Chang and Snyder, 2004; Koss et al., 2005). Belowground, Colorado potato beetle prepupae and pupae are attacked by entomopathogenic nematodes in the genera *Heterorhabditis* and *Steinernema* (MacVean et al., 1982; Wright et al., 1987a; Nickel et al., 1994; Liu and Berry, 1995; Berry et al., 1997; Stewart et al., 1998; Armer et al., 2004a), and also fungal pathogens in the genus *Beauveria* (Fargues, 1972; Anderson et al., 1988; Tanada and Kaya, 1993; Drummond and Groden, 1996; Lacey et al., 1999).

Entomopathogens

Entomopathogenic nematodes

Either through conservation or through augmentative releases, entomopathogenic nematodes have the potential to increase potato beetle mortality (Nickel et al., 1994; Lacey et al., 1999; Liu and Berry, 1995; Berry et al., 1997; Reed et al., 2001; Armer et al., 2004a). Locally, as elsewhere, *Heterorhabditis* spp. and *Steinernema* spp., two genera of entomopathogenic nematode, have been particularly well-studied as Colorado potato beetle control agents (MacVean et al., 1982; Wright et al., 1987a; Nickel et al., 1994; Liu and Berry, 1995; Berry et al., 1997; Stewart et al., 1998; Armer et al., 2004a). Entomopathogenic nematodes enter host insects through the mouth, anus or other natural openings; some species can also penetrate

directly through the host cuticle via intersegmental membranes using an anterior tooth (Bedding and Molyneux, 1982; Peters and Ehlers, 1994). Upon entering the hemocoel, the nematodes release a symbiotic bacterium that reproduces rapidly and kills the insect (Boemare et al., 1996). The nematodes then develop and multiply by feeding on digested host tissues and these bacteria (Griffin et al., 2005). Mortality of the host insect occurs relatively quickly, from septicemia, between 24-48 hours. *Steinernema* spp. is associated with *Xenorhabdus* spp. bacteria, while *Heterorhabditis* is associated with *Photorhabdus* spp. bacteria (Boemare et al., 1993). Both entomopathogenic nematode genera have a single free-living stage, known as the infective juvenile that carries the bacterium in its body. Each bacterial symbiont resides in the intestinal tract of its respective host nematode.

Heterorhabditis spp. are hermaphroditic but can also engage in sexual reproduction in subsequent generations (Dix et al., 1992), while *Steinernema* spp. requires both a male and female for reproduction. Several thousand infective juveniles are produced within a single host insect with multiple generations occurring within a single host. Eventually these entomopathogenic nematodes emerge from the host as resources deplete, and search for a new host.

Entomopathogenic nematodes have a very broad host range (Kaya and Gaugler, 1993; Poinar, 1986). However, factors such as dispersal ability, foraging strategy, host discrimination, and infection behavior can narrow their effective host range (Griffin et al., 2005). The infective entomopathogenic nematode stages of different species can occur within different areas of the soil profile, both horizontally and vertically (Lewis, 2002). Some species such as *S. carpocapsae* are found at upper levels of the soil profile (Georgis and Poinar, 1983; Schroeder and Beavers, 1987), while *S. glaseri* and *H. bacteriophora* are found moving deeper in the soil profile

(Campbell et al., 1995; Lewis, 2002). In addition, studies have shown that *Heterorhabditis* species generally disperse farther than do *Steinernema* species (Westerman, 1995; Downes and Griffin, 1996). Entomopathogenic nematodes are associated with various foraging strategies within a continuum from ambushers to cruisers (Lewis et al., 1992; Grewal et al., 1994; Campbell and Gaugler, 1997). Ambushing nematodes are usually found at the soil surface where they are more likely to infect mobile insects. These entomopathogenic nematodes nictate or "stand" and attach to their host, sometimes with a leaping action, when the host comes near (Campbell and Gaugler, 1993; Ishibashi, 2002). Cruisers, on the other hand, search for their hosts by moving through the soil profile and are often associated with sedentary hosts (Lewis, 2002). Differences among nematodes in arthropod host choice (Grewal et al., 1993) and in the ability to enter into the host have also been considered as factors influencing host specificity (Wang and Gaugler, 1999). Their close association with the soil makes entomopathogenic nematodes suitable biological control agents against many species of soil inhabiting insects (Lacey et al., 2001).

Several entomopathogenic nematode species have been examined against the Colorado potato beetle, with varied success. *Steinernema carpocapsae*, formulated as a Pesta-pellet, was found to control 94% of Colorado potato beetle prepupae in the laboratory (Nickel et al., 1994). Stewart et al. (1998) found that 79% of potato beetle prepupae were infected one day after exposure to field application of *S. carpocapsae*. *Heterorhabditis bacteriophora* killed 40-60% of fourth instar potato beetle larvae one day after field application (Wright et al., 1987a). More recently, *H. marelatus*, a species of entomopathogenic nematode discovered in the Pacific Northwest, showed promise against the Colorado potato beetle (Armer et al., 2004a). Armer et al. (2004a) found that potatoes receiving higher levels of nitrogen displayed an increase in

alkaloids, but this did not interfere with the performance of *H. marelatus* in controlling the Colorado potato beetle. *Heterorhabditis marelatus* provided approximately 50% potato beetle control under field conditions and did not interfere with a resident parasitoid fly (*Myiopharus doryphorae*) that also attacks the potato beetle. Reproduction in the target pest would be a favorable property for these biological control agents because recycling allows new infective juveniles to persist in the soil. Unfortunately, entomopathogenic nematodes have had little success developing in the Colorado potato beetle (Armer et al., 2004b). Interestingly, potato beetle mortality was 98% in the presence of *H. marelatus*; however, reproduction by *H. marelatus* within the potato beetle host occurred less than 6% of the time. Armer et al. (2004b) attributed this to a heat labile factor in the hemolymph that suppresses antibody production by the bacterium and results in a decrease in nutrient availability to the entomopathogenic nematode. While the heat labile factor does not directly impact the nematode, limiting nutrients available to the symbiotic bacterium decreases the size of entomopathogenic nematodes and their reproductive fitness.

Plant parasitic nematodes pose a second major pest threat to potato production. Interestingly, inundative releases of EPNs also have shown success in dampening populations of plant parasitic nematodes (Bird and Bird, 1986; Ishibashi and Kondo, 1986; Smitley et al., 1992; Grewal et al., 1997; Lewis et al., 2001; Somasekhar et al., 2002; Henderson, 2008). The specific mechanism through which this occurs is not known, although the attraction of entomopathogenic nematodes to CO_2 from plant roots repelling plant-parasitic nematodes (Bird and Bird, 1986), the application of large quantities of EPNs increasing nematode antagonists (Ishibashi and Kondo, 1986) and allelopathy from entomopathogenic nematode symbiotic bacteria is suspected (Grewal et al., 1997). Thus, entomopathogenic nematode applications for the control of insect pests might have

the additional benefit of improving control of plant parasitic nematodes (Lewis and Grewal, 2005).

Beauveria bassiana

Beauveria bassiana (Balsamo) Vuillemin is a widely distributed entomopathogenic fungus that occurs naturally in Pacific Northwest soils (Bruck, 2004) and has been formulated as a bio-pesticide against several major economic pests, including the Colorado potato beetle (Fargues, 1972; Campbell et al., 1985; Anderson et al., 1989; Tanada and Kaya, 1993; Drummond and Groden, 1996; Lacey et al., 1999). This entomopathogen is recognized by white mycelia covering the host insect body from which spores develop (Tanada and Kaya, 1993), termed white muscardine disease. Upon closer examination, species in the genus *Beauveria* are characterized by a zigzag or denticulate rachis from which conidia are singly borne (Tanada and Kaya, 1993; Humber, 1997). The infection process starts with the attachment of the conidial spore to the host insect cuticle through high hydrophobic forces (Boucias et al., 1988). Once attached, the conidial spore germinates using enzymes to break down the insect integument and providing the hyphal growth an opportunity to gather nutrients and penetrate farther into the host (Boucias and Pendland, 1991; Tanada and Kaya, 1993). While infection has been observed through the insect gut and oral cavity (Broom et al., 1976), infection usually occurs through the integument (Boucias and Pendland, 1991). In cases where *B. bassiana* attaches to heavily sclerotized regions of a host insect a single hyphal structure, known as a germ tube, will grow from the spore over the surface of the insect cuticle until an area that is easily penetrated is located (Pekrul and Grula, 1979). *Beauveria bassiana* penetrate into the insect hemocoel using physical force and a suite of chitinases (Ferron, 1981). Penetration by *B. bassiana*, by breaking down the insect's first line of

defense, can also lead to secondary infection from opportunistic microorganisms (Vey and Fargues, 1977). Within the insect hemocoel, *B. bassiana* produces hyphal bodies that multiply by budding (Tanada and Kaya 1993). These hyphal bodies overwhelm the hemocytes in the hemolymph and take up nutrients from muscles and other tissues (Tanada and Kaya, 1993). Death of the host insect can occur within days (Farges et al., 1994; Fernandez et al., 2001; Furlong and Groden, 2003) and results from a decrease in hemocytes and nutrients, and fungal toxicity or septicemia from invading opportunistic microorganisms (Tanada and Kaya, 1993). After the insect dies mycelia cover the insect body through saprophytic growth. When the environment is conducive the fungus sporulates, enabling *B. bassiana* to disseminate and infect new hosts (Roberts et al., 1981).

The potential of *B. bassiana* to control the Colorado potato beetle has been known for several years (Ferron, 1981), and shows considerable potential in potato crops (Drummond and Groden, 1996). *Beauveria bassiana* can be foliar or soil applied as a bio-pesticide (Gaugler, et al., 1989; Lacey et al., 1999; Wraight and Ramos, 2002). In addition, resident *B. bassiana* in the soil show low levels of mycosis of potato beetle life stages. Colorado potato beetle eggs, which are laid on the undersides of leaves, show no susceptibility to *B. bassiana* (Long et al., 1998). On the other hand, the larvae have varied levels of susceptibility to *B. bassiana* (Ignoffo, 1983; Fernandez et al., 2001; Joergensen, 2000). The variability has been attributed to several factors including differences in susceptibility to varied amounts of inoculum, differing immune response between larval stages, increases in hemocyte volume with each successive larval stage, and differences in feeding rate and nutrition (Seryczynska and Bajan, 1974; Logan et al., 1985; Bauer et al., 1998; Fernandez et al., 2001). It has been well documented that ecdysis can remove fungal conidia from the insect body decreasing the susceptibility to pathogen attack (Vandenberg et al., 1998;

Vey and Fargues, 1977). Soil applications of *B. bassiana* provide control of pupae, reducing the emerging potato beetle adult population by up to 74% (Watt and LeBrun, 1984). Although adult potato beetles are susceptible to *B. bassiana*, their susceptibility is generally lower than that seen in early life stages (Fargues, 1972; Fargues, 1991). Factors such as temperature (Watanabe, 1987), humidity (Walstead et al., 1970) and UV light (Daoust and Pereira, 1986; Joergensen, 2000) can influence *B. bassiana* infectivity. While *B. bassiana* and other entomopathogens have potential as biological control agents their effectiveness in the field is highly variable (Brower et al., 1995).

Soil management

Mustard bio-fumigation

Soil borne pests such as plant diseases also threaten potato production. Traditionally, synthetic soil fumigants such as methyl bromide, metam sodium, 1, 3-dichloropropene, and chloropicrin have been used against soil pests (Matthiessen and Kirkegaard, 2006). These broad-spectrum fumigants diffuse and penetrate rapidly into the soil by using high vapor pressure and plastic tarps to retain the gas (Matthiessen and Kirkegaard, 2006). Specifically, methyl bromide has come under scrutiny due to environmental and human health concerns and is currently being phased out (Martin, 2003; Cox, 2006). In the 1950's an alternative less costly broad-spectrum synthetic soil fumigant, metam sodium, was available for use against soil borne pests of potato (van Berkum and Hoestra, 1979). Upon contact with water metam sodium produces methyl isothiocyanate (Turner and Corden, 1963; Munnecke, 1967; Smelt and Leistra, 1974) that is

biologically active against nematodes, plant diseases, insects, and weeds (Munnecke et al., 1962; Richardson and Thorn, 1969). Because many of these synthetic pesticides are continually being targeted and may be taken off the market by changing federal regulation, alternatives are being examined. Brassicaceous plants have the ability to produce isothiocyanate compounds naturally, as breakdown products derived from secondary plant compounds known as glucosinolates (Rosa et al., 1997; Fahey et al., 2001). These compounds form natural "bio-fumigants" useful for soil pest management in potato and other crops (Blank et al., 1982; Chan and Close, 1987; Muehlchen et al., 1990; Mojtahedi et al., 1993; Davis et al., 1996; Akiew and Trevorrow, 1999; McLeod and Steele, 1999).

Glucosinolates are sulfur compounds of secondary plant products commonly found in widely cultivated brassicaceous crops (Fenwick et al., 1983). Glucosinolates themselves have no biological activity but upon reacting with an enzyme found in the plant known as myrosinase create a variety of active products, an interaction termed the "glucosinolate-myrosinase system" (Bones and Rossiter, 1996; Rask et al., 2000). Throughout the plant, glucosinolate and myrosinase exist separately. Glucosinolate is a very stable water-soluble compound that resides in the cytosol, while the enzyme myrosinase is sequestered in vacuoles (Rosa et al., 1997; Fahey et al., 2001) of plant cells. Upon maceration of plant cells, glucosinolate and myrosinase mix, triggering hydrolysis reactions in the presence of water, leading to the production of biologically active products such as isothiocyanates, organic cyanides, oxazolidinethiones, and ionic thiocyanates (Brown and Morra, 1997). Among these, isothiocyanates have been reported to be the most biologically active (Brown and Morra, 1997; Rosa and Rodrigues, 1999).

Potato growers in Washington have increasingly adopted the use of mustard (*Brassica* and *Sinapis* spp.) bio-fumigants, tilled into the soil preceding the planting of potato crops, as an

alternative to synthetic soil fumigants (McGuire, 2003). Several studies have examined the effectiveness of these mustard bio-fumigants for the control of plant-parasitic nematodes. Lazzeri et al. (2004) found that *Meloidogyne javanica*, a root-knot nematode, was negatively impacted by several hydrolysis products of glucosinolates including 2-phenylethyl, benzyl, 4-methylthiobutyl, and prop-2-enyl isothiocyanate. These plant parasitic nematodes were significantly reduced by the soil integration of *Brassica hirta* cultivars in laboratory experiments, leading to greater than 65% nematode mortality (Zasada and Ferris, 2004). In separate studies, Mojtahedi et al. (1991) and Riga et al. (2004) found that using mustard bio-fumigants also negatively impacted another plant parasitic nematode species, *M. chitwoodi*. While the broad-spectrum activity of mustard bio-fumigants shows promise against soil borne pests, negative non-target impacts on beneficial soil flora and fauna are unknown.

Interactions with manure

Organic potato acreage has been increasing in eastern Washington State with more than a 10-fold increase in organic production in the last decade (Koss, 2003). Organic production removes synthetic chemical inputs for crop and pest management, instead emphasizing beneficial biotic processes (National Research Council 1989; EEC Council Regulation 2092/9, Annex II B). Nutrients from organic matter are used to replace synthetic chemical inputs (Lampkin, 1990). In particular, animal manure provides one alternative to synthetic fertilizer. While the application of animal manure may provide agronomic and environmental benefits by improving soil quality (Stockdale et al., 2001), it is unclear whether this translates to improving other beneficial ecosystem services such as natural pest control. There is a weak practical understanding of soil

as a living system and the implications of soil management for sustainable agriculture (Watson et al., 2002).

The addition of synthetic fertilizer or animal manure can change soil properties, including chemistry (Bulluck et al., 2002). The incorporation of animal manure adds organic matter to the soil, increasing total carbon and cation exchange capacity, and decreasing bulk density (Bulluck et al., 2002). These properties improve soil quality by providing greater water infiltration rates (Jackson, 1988), carbon storage (Drinkwater et al., 1995; Peacock et al., 2001; Nahar et al., 2006), soil aggregation, and water holding capacity (Hafez, 1974), and reducing nutrient leaching (Drinkwater et al., 1995; Peacock et al., 2001) and soil erosion (Reganold et al., 1987). These factors can influence the structure of the soil community. With the application of synthetic fertilizer there is no addition of organic matter but there are various changes to soil chemistry including acidification, a change in pH, and an increase in concentrations of ammoniacal and anhydrous ammonia (Stamatiadis et al., 1999; Rodriguez-Kabana, 1986). However, the addition of manure or any organic matter to the soil, by increasing soil biodiversity, seems to more strongly influence interactions within the soil community than synthetic fertilizers (Mäder et al., 2002). Disturbances caused by manipulating organic matter, soil pH, nitrogen, and soil moisture can change the dynamics of these soil interactions (Schnürer et al., 1986; Korthals et al., 1996; Wardle, 2006).

There is a lot of complexity to food webs in the rhizosphere because of the diversity of species supported by plant roots, their byproducts (Moore and Hunt, 1988), and soil characteristics. Moore et al. (1988) categorized the decomposer species assemblages in soil into root, bacterial, and fungal based energy channels. The root energy channel focuses on living root tissue and consists of root-feeding insects, nematodes, and microbes (that may have a symbiotic

relationship with plant roots). The bacterial and fungal energy channels have a stronger relationship with decomposition of organic matter. The difference between these two energy channels is the composition of organisms and the rates of turnover for available nutrients to plants and other organisms. The bacterial energy channel includes organisms such as bacteria, protozoa, rotifers, nematodes, and some arthropods. The fungal energy channel is composed of saprophytic fungi, nematodes, and arthropods. Specifically, fungi are better able to breakdown more resistant plant cells (e.g. tissue high in tannins) than bacteria that are more efficient at using more labile root exudates and organic matter (Curl and Truelove, 1986). The bacterial energy channel has a relatively faster rate of decomposition and resulting nutrient availability than the fungal energy channel.

Species assemblages of the root, bacterial, and fungal energy channels interact with predaceous bacteria, fungi, nematodes, and arthropods that may impact biological control. Manure enhances microbial biomass (Fauci and Dick, 1994; Joergensen et al., 1995; Villenave et al., 2003; Nahar et al., 2006), activity (Doran et al., 1987), and biodiversity (Mäder et al., 2002). Studies have found that improving resource quantity and quality had no effect on bacterial biomass overall but did increase the biomass at higher trophic levels (Wardle and Yeates, 1993; Santos et al., 1981). With added organic matter there was an initial flux in bacterial biomass resulting in an increase in bacterial feeding nematodes, eventually leading to a decline in bacterial biomass. Manure adds both organic matter and microbes to the soil providing a food source (Andrén and Lagerlöf, 1983; Weiss and Larink, 1991) that benefit these bacterivorous and fungivorous nematodes (Bohlen and Edwards, 1994; Freckman, 1988; Griffiths et al., 1994; Bulluck et al., 2002; Villenave et al., 2003; Forge et al., 2005; Nahar et al., 2006). Plant-parasitic nematodes were suppressed in soils treated with manure because of enhanced densities of

competitive, predaceous, or antagonistic microorganisms (Linford et al., 1938; Clark et al., 1998; Akhtar and Malik, 2000). On the contrary, a nematode trapping fungus, *Hirsutella rossiliensis*, parasitized fewer plant parasitic nematodes when in the presence of soil incorporating composted chicken manure (Jaffee et al., 1994). Manure has been reported to suppress exotic entomopathogenic nematodes (Duncan et al., 2007) by increasing antagonistic interactions from *Arthrobotrys oligospora*, the nematode trapping fungus (Jaffee and Strong, 2005), nematophagous mites, and collembolans (Epsky et al., 1988). Resident entomopathogenic fungi (Klingen et al., 2002) and nematodes benefit from (Bednarek and Gaugler, 1997; Duncan et al., 2007) soil amended with animal manure; however, the factors encouraging fungal establishment were unknown. Entomopathogenic nematodes are thought to benefit because certain animal manures such as chicken manure can negatively impact nematode trapping fungi allowing the nematodes to persist (Bednarek and Gaugler, 1997). There is a lot of complexity in how these different soil communities may interact with changing soil management in agricultural systems.

This dissertation describes a systems approach to examine the interactions between soil management and biological control. First, by testing how green manures used for the control of plant pathogens impact entomopathogens; second, by testing how animal manure used as a method of soil fertility impacts entomopathogenic nematodes; and third, by testing how entomopathogens interact with aboveground natural enemies.

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CHAPTER TWO

Harmful effects of mustard bio-fumigants on entomopathogenic nematodes

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Abstract

Mustard (*Brassica* and *Sinapis* spp.) green manures tilled into the soil preceding potato crops act as bio-fumigants that are toxic to plant parasitic nematodes, providing an alternative to synthetic soil fumigants. However, it is not known whether mustard green manures also kill beneficial entomopathogenic nematodes (EPNs) that contribute to the control of pest insects. We used sentinel insect prey (*Galleria mellonella* larvae) to measure entomopathogen activity in Washington State (USA) potato fields that did or did not utilize mustard green manures. We found that rates of entomopathogen infection on average were lower in fields where mustard bio-fumigants were applied, compared to those not receiving this cultural control method. In laboratory bioassays we then tested whether extracts from two mustard (*Brassica juncea*) cultivars differing in glucosinolate levels disrupted the abilities of a diverse group of EPN species to infect insect hosts. In these trials we used *G. mellonella* larvae as hosts, and included multiple EPN spp. in the genera *Steinernema* (*S. carpocapsae*, *S. feltiae*, *S. glaseri*, and *S. riobrave*) and *Heterorhabditis* (*H. bacteriophora*, *H. marelatus*, and *H. megidis*). EPN infection rates were lower in laboratory arenas receiving mustard extracts than the control (water), and lower still when EPNs were exposed to extracts from plants with high versus low glucosinolate levels. However, one EPN species, *S. feltiae*, appeared relatively unaffected by exposure to mustard extracts. *Steinernema* species generally exhibited higher infection rates than did *Heterorhabditis* species. However, there was no evidence that susceptibility to the negative effects of mustard extracts differed on average between the two EPN genera. Together, our results suggest that the use of mustard bio-fumigants for the control of plant parasitic nematodes has the potential to interfere with the biocontrol of insect pests using entomopathogens. Thus, it may be difficult to combine these two approaches in integrated pest management programs.

Keywords: *Beauveria*; Bio-fumigant; *Galleria mellonella*; Colorado potato beetle; *Leptinotarsa decemlineata*; *Brassica juncea*; Entomopathogen; *Heterorhabditis*; Potato; *Steinernema*

1. Introduction

Plant parasitic nematodes pose a major pest threat to potato production in many parts of the world (Santos et al., 1995; Marks and Rojancovski, 1998; Turner and Evans, 1998; Sun and Miller, 2007), including the irrigated potato growing region in the Columbia Basin of Washington State and adjacent Oregon, USA (Ingham et al., 2005; Riga and Neilson, 2005). Particularly damaging in the Columbia Basin are Columbia root-knot nematode (*Meloidogyne chitwoodi* Golden, O'Bannon, Santo, and Finley), northern root-knot nematode (*M. hapla* Chitwood), root lesion nematode (*Pratylenchus* spp.), and stubby root nematode (*Paratrichodorus* spp.) (Santo et al., 1987; Ingham et al., 1991; Riga and Neilson, 2005; Ingham et al., 2005). These nematodes stunt plant growth and reduce yields, and/or reduce marketability of tubers by causing galls on the tuber surface, forming necrotic lesions in tuber flesh, or causing necrosis of tuber flesh due to transmission of fungal and viral pathogens (Jensen and Allen, 1964; Rowe and Powelson, 2002). Until recently, almost universally these harmful nematodes have been controlled using applications of broad-spectrum, synthetic soil fumigants (i.e., methyl bromide, metam sodium, 1, 3-dichloropropene). These synthetic soil fumigants are highly toxic to pests, but also to many beneficial soil organisms (de Jong et al., 1995; Schreiner et al., 2001; Cox, 2006). In addition, many of these conventional soil fumigants exhibit vertebrate toxicity

and other damaging environmental effects (Cox, 2006). Together, these negative environmental and human health concerns have driven a search for more benign alternatives (Martin, 2003).

In recent years, potato growers in Washington have increasingly adopted the use of mustard (*Brassica* and *Sinapis* spp.) green manures, tilled into the soil preceding the planting of potato crops, as an alternative to synthetic soil fumigants (McGuire, 2003a). Mustard foliage and seeds contain glucosinolate compounds that upon hydrolysis produce isothiocyanates, which act as natural bio-fumigants (Halbrendt, 1996; Brown and Morra, 1997). These bio-fumigant compounds suppress plant parasitic nematodes (Mojtahedi et al., 1991; Riga et al., 2004; Zasada and Ferris, 2004), weeds (Brown and Morra, 1995), pathogenic fungi (Kirkegaard et al., 1996), and other soil-borne pests. Mustard bio-fumigants have many advantages over synthetic soil fumigants, including the relative lack of regulatory issues and their contribution to reducing soil erosion and building soil organic matter (McGuire, 2003a). However, benefits to soil biodiversity are less clear. The relatively broad-spectrum activity of mustard bio-fumigants could lead to negative non-target impacts on beneficial soil flora and fauna, although this has not been previously investigated.

Included in the beneficial soil biota in regional potato fields are a diverse group of entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* (Liu and Berry, 1995; Berry et al., 1997). These nematodes enter host insects through natural openings (e.g., mouth, anus), then release a symbiotic bacterium that reproduces rapidly and kills the host (Boemare et al., 1996); the nematodes multiply by feeding on digested host tissues and these bacteria (Griffin et al., 2005). Death of the host insect comes relatively quickly, in 2-5 days following initial infection (Grewal and Peters, 2005). Through conservation of endemic species, or application as bio-insecticides, entomopathogens have been shown to contribute to the

biological control of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Berry et al., 1997; Jaros-Su et al., 1999; Lacey et al., 1999; Fernandez et al., 2001; Armer et al., 2004), a key pest of potato in the Columbia Basin and many other potato-growing regions (Hare, 1990). Thus, any negative effects of soil fumigants on these beneficial entomopathogens could disrupt biological control of pest insects in potato and other crops. Here, for the first time we report an examination of the non-target impacts of mustard bio-fumigants on entomopathogen activity, both in production potato fields and under controlled conditions in the laboratory.

2. Materials and methods

Our project had two objectives. First, using sentinel waxworm (*Galleria mellonella* L.) hosts we measured entomopathogen activity in production potato fields in the Columbia Basin of Washington State, USA, that differed in pest management regime (certified organic versus conventional) and in the use of mustard bio-fumigation. These data suggested that mustard bio-fumigants harm endemic entomopathogens. Therefore, we designed laboratory based assays to examine the impacts of extracts from two *Brassica juncea* (L.) cultivars, differing in glucosinolate concentrations, on *G. mellonella* infection by several *Steinernema* and *Heterorhabditis* EPN species commercially available for use as bio-insecticides.

2. 1. Entomopathogen activity in production potato fields

Using waxworm (*G. mellonella*) larvae as sentinel hosts (these insects are highly susceptible to pathogen infection [Kaya and Stock, 1997]), we surveyed 23 production potato fields in the

Columbia Basin of Washington State for entomopathogen activity. The fields varied both in pest management regime, certified organic versus conventional, and in mustard treatment, mustard green manure crop soil-amended in the fall preceding the potato crop or no mustard bio-fumigant used (Table 1). The soil composition of the Columbia Basin is predominately Quincy fine sand, Quincy loamy sand, and Shano silt loam (Lenfesty, 1967; Rasmussen, 1971; Gentry, 1984). All fields were irrigated using a center pivot irrigation system. Fertility of organic potato fields was enhanced using composted animal manure (approximately 10 tons/acre of cow manure and 6 tons/acre of chicken manure), whereas the conventionally managed potato fields received synthetic fertilizers (approximately 600 - 1500 lbs/acre dependent on soil fertility needs) equivalent to rates in Lang et al. (1999). The organic growers refrained from using insecticides but utilized foliar applied copper fungicides (per. comm. Brad Bailey, Lenwood Farms; and Stacy Kniveton, Johnson Agriprises). The conventional potato growers utilized several synthetic pesticides including seed coat fungicides (e.g. flutolanil), copper fungicides, lateblight and white mold control (e.g. boscalid, fluazinam, azoxystrobin), and occasionally insecticides (including some or all of the following: methamidophos, pymetrozine, and esfenvalerate) (personal comm. Stacy Kniveton, Johnson Agriprises; Gilbert Hintz, Ephrata Farms; and Troy Grimes, Watts Brothers Farms). Organic fields not receiving mustard bio-fumigation were virgin ground that received no soil fumigant, whereas conventional no-mustard fields were treated with soil applications of aldicarb and metam potassium at approximately 39 gal/acre and 18 lbs/acre, respectively. Fields utilizing mustard bio-fumigation planted Caliente brand mustard, a blend of *B. juncea* and *S. alba,* while conventional fields also incorporated soil applications of aldicarb (Table 1).

Groups of five *G. mellonella* larvae (purchased from Sunshine mealworms, Silverton, OR) were placed in mesh bags made from fiberglass window screen, which were then closed and sealed with a twist tie. Ten of these bags were placed in each field in a single linear transect, with bags spaced 9.14 meters apart. Sentinel hosts were buried 10-15 cm under the soil, reproducing the depth at which CPB pupate in the soil (Hare, 1990), for 48 hrs, after which the sentinel hosts were retrieved and returned to the laboratory. Once in the laboratory waxworms were placed, individually, onto modified White Traps (filter paper that lay on transparency film sat atop a sponge, moistened with 5 ml distilled water, and housed within a 9 cm diameter x 1.5 cm high Petri dish [White, 1927]). These larvae were then monitored daily, for one week, for infection by entomopathogens. EPNs infecting a larva were identified to genus using the distinctive color of infected hosts. Insects infected by *Heterorhabditis* species turn a red-brown color indicative of infection by the symbiotic *Photorhabdus* bacteria associated with EPNs in this genus (Han et al., 1990). Insects infected by *Steinernema* species assume a tan to grey appearance indicative of infection by the symbiotic *Xenorhabdus* bacteria associated with EPNs in that genus (Millar and Barbercheck, 2001). Our sampling regime was designed to measure EPN activity, but fortuitously we also found evidence of infection by fungal pathogens. Entomopathogenic fungi formed distinctive hyphal masses in infected hosts, and larvae infected by fungi were placed into Petri dishes (9 cm diameter x 1.5 cm high) lined with filter paper and taken to Dr. L.M. Carris (Department of Plant Pathology, Washington State University, Pullman, WA, USA) for identification to genus.

2. 2. Effects of mustard extracts on entomopathogenic nematodes in the laboratory

Our field measurements of entomopathogen activity suggested that mustard bio-fumigant use might have negative effects on endemic entomopathogens (see "Results"). However, in these field measurements, a broad range of management practices and environmental factors differed among potato fields irrespective of mustard bio-fumigant treatment (Table 1). Thus, we conducted assays using Petri dish arenas in the laboratory wherein we compared the effects of extracts from two *B. juncea* cultivars that differed in their glucosinolate concentrations, on a diverse group of *Heterorhabditis* and *Steinernema* EPN species. Our goals were to determine the impacts of mustards on *G. mellonella* infection by the EPN species, whether impacts differed between members of the two EPN genera, and whether impacts differed between the two mustard cultivars.

We examined two cultivars of *B. juncea*, 'Arid' and 'Pacific Gold'. 'Arid' has lower glucosinolate levels, ca. 10 μmol g^{-1} (Malhi et al., 2007), than 'Pacific Gold' which has higher glucosinolate levels, ca. 300 μmol g^{-1} (Brown et al., 2004). We planted 10 plants of each of the two mustard cultivars in the greenhouse (16:8 L:D, 27º C) and 30 days later, just prior to flowering, chopped the plants. This reproduced the typical practice in production potato fields, wherein mustard plants are chopped and tilled into the soil just before flowering to maximize bio-fumigation (McGuire, 2003b). We then prepared extracts from each mustard variety by blending 10 g of fresh plant material (stems and leaves) with 100 ml of de-ionized water. The resulting slurry was then sieved to create an aliquot of extract solution (Tyagi, 2002; Matthiessen and Shackleton, 2005). Solutions prepared in this way capture biologically active plant extracts because maceration of plant tissues releases glucosinolate and myrosinase compounds, which are water-soluble (Brown and Morra, 1997). Biofumigant effects may not derive from the glucosinolates directly but from the enzymatic degradation of glucosinolates by myrosinase in

the presence of water (Brown and Morra, 1995). These solutions were then immediately used in our EPN bioassays, described next.

The first experiment included three *Steinernema* species: *S. carpocapsae*, *S. glaseri*, and *S. feltiae*. The second experiment included the above species, an additional *Steinernema* species (*S. riobrave*), and three *Heterorhabditis* species (*H. bacteriophora*, *H. marelatus*, and *H. megidis*). These EPN species were selected because they are available commercially, or have been examined for use, as biopesticides (Grewal, 2002), and/or because all but *H. megidis* have been investigated for use in Colorado potato beetle biological control (MacVean et al., 1982; Toba et al., 1983; Wright et al., 1987; Cantelo and Nickle, 1992; Nickle et al., 1994; Thurston et al., 1994; Berry et al., 1997; Stewart et al., 1998; Armer et al., 2004). *Steinernema glaseri*, *H. bacteriophora*, and *H. megidis* were purchased from a commercial supplier (Integrated Fertility Management, Wenatchee, WA), whereas our *S. feltiae*, *S. riobrave*, *S. carpocapsae* and *H. marelatus* came from laboratory colonies maintained by the authors using *G. mellonella* larvae as hosts (Riga et al., 2006).

Each EPN species was subjected to each of the three mustard extract treatments, with 10 replicates of each EPN species-mustard treatment combination: CONTROL, no mustard extract; ARID (extract from *B. juncea* cv. 'Arid'), and GOLD (extract from *B. juncea* cv. 'Pacific Gold'). Our experimental arenas were 9-cm-diameter Petri dishes lined with filter paper. GOLD and ARID treatments received 1 ml of aqueous solution of *B. juncea* extract, freshly blended and sieved as described above, from the appropriate *B. juncea* cultivar; CONTROL replicates received 1 ml of de-ionized water. Thereafter, we immediately applied EPNs at a rate of 250 infective EPN juveniles per dish. Petri dishes were then left undisturbed for 24 hours (20º C-24º C), allowing time for mustard extracts to impact the nematodes, before five *G. mellonella* larvae

were placed into each arena. These larvae were left in the dishes for 1 week, after which each larva was scored for infection by EPNs.

2. 3. Analyses

The field data were analyzed within a 2 X 2 factorial design in ANOVA, with two levels of pest management (Organic, Conventional) and two levels of mustard treatment (Applied, Not applied).

Infection data from the first laboratory experiment were analyzed within a two-way ANOVA with three levels of mustard treatment (CONTROL, ARID, GOLD) fully crossed with the three nematode species (*S. carpocapsae*, *S. glaseri*, *S. feltiae*). For the second experiment, mustard and species effects were nested within the two genera (*Heterorhabditis*, *Steinernema*). For both laboratory experiments, within the full experimental design, mustard treatment main effects (or interactions between the mustard treatments and EPN species or genus) could be due to differing EPN activity in the presence versus absence of mustard extracts, differing EPN activity in the presence of extracts from the two mustard cultivars, or both. Thus, we followed our initial analysis with two planned, additional tests. First, within the complete multi-factorial design we pooled data from the two mustard extract addition treatments, to yield two levels of mustard manipulation (MUSTARD+, MUSTARD-). Second, we compared the impacts of the two mustard varieties and their interactions with EPN taxa by dropping the CONTROL treatment from the model, again yielding two levels of mustard manipulation (ARID, GOLD).

All proportion infection data were arcsine square-root transformed prior to analysis. Analyses of data from the field study and the first laboratory experiment were analyzed using SYSTAT

(version 11.0; SPSS, Chicago, IL) software. Data from Experiment 2 were analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3. 1. Entomopathogen activity in production potato fields

There was a trend for overall rates of entomopathogen infection on *G. mellonella* to be lower in production potato fields receiving mustard bio-fumigation, an effect that approached statistical significance (mustard main effect, $P = 0.061$; Table 2; Fig. 1A). However, effects of pest management regime (organic vs. conventional), and the interaction between mustard bio-fumigation and pest management regime, were clearly not significant (Table 2). Sentinel *G. mellonella* larvae were infected by members of the EPN genera *Heterorhabditis* and *Steinernema*, and the entomopathogenic fungus *Beauveria* species. Across all field types the majority of *G. mellonella* infections were by *Heterorhabditis* species, representing 48% of all *G. mellonella* infections. Among the three pathogen genera, activity of *Heterorhabditis* species exhibited the greatest magnitude of difference among field types (Fig. 1B). Mustard treatment and pest management regime main and interactive effects were not statistically significant for *Heterorhabditis*, *Steinernema* or *Beauveria* species when these entomopathogen taxa were analyzed separately (Table 2; Fig. 1B-D).

3. 2. Effects of mustard extracts on entomopathogenic nematodes in the laboratory

In the first laboratory experiment we examined the impact of two *B. juncea* cultivars (the low glucosinolate cultivar 'Arid' and the high glucosinolate cultivar 'Pacific Gold'), as compared to a water control, on infection of *G. mellonella* larvae by three *Steinernema* species (*S. carpocapsae*, *S. feltiae* and *S. glaseri*). Across the complete experimental design, we found a significant mustard treatment x EPN species interaction (Table 3). To further investigate the nature of this interaction we conducted two additional tests. When lumping treatments receiving mustard extract from the two cultivars (pooled ARID + GOLD), we again found a significant mustard treatment x EPN species interaction ($P = 0.003$; Table 3; Fig. 2A). This interaction appeared to be driven by the high infectivity of *S. feltiae* regardless of mustard extract addition (Fig. 2A); EPN activity generally declined in the presence of mustard extract (mustard main effect, $P = 0.004$; Table 3; Fig. 2A). When dropping the CONTROL treatment from the analysis to highlight comparison between the two *B. juncea* cultivars, the mustard x EPN species interaction remained statistically significant ($P = 0.048$; Table 3; Fig. 2A), an effect again apparently driven by the consistently strong performance of *S. feltiae* across extracts of the two *B. juncea* cultivars (Fig. 2A). EPN species generally exhibited poorer *G. mellonella* infectivity in extract from the high-glucosinolate cultivar 'Pacific Gold' than in extract from the low-glucosinolate cultivar 'Arid' (mustard main effect, $P = 0.04$), and species significantly differed in overall *G. mellonella* infectivity (EPN species main effect, $P = 0.001$) (Table 3; Fig. 2A).

Upon measuring the impact of *B. juncea* extracts on four *Steinernema* (*S. carpocapsae*, *S. feltiae*, *S. glaseri* and *S. riobrave*) and three *Heterorhabditis* (*H. bacteriophora*, *H. marelatus* and *H. megidis*) species, within the complete experimental design, we found that mustard treatment and EPN genus exerted statistically significant impacts on EPN infectivity ($P < 0.001$ for both main effects; Table 4; Fig. 2B); all other main and interactive effects were not

statistically significant (Table 4). Pooling the two mustard extract treatments and comparing them to the water control revealed a significant mustard extract x EPN species interaction (P = 0.043), indicating that species differed in their response to mustard extracts. Again, this interaction appeared to be influenced by the relatively robust performance of *S. feltiae* in both the presence and absence of mustard extract, and by the consistently poor performance of *H. megidis* across treatments (Fig. 2B). Results for these two species run counter to the general trend of lower EPN infection rates with the addition of mustard extracts across all EPN species (mustard main effect, $P < 0.001$; Table 4; Fig. 2B). *Steinernema* species exhibited generally higher infection rates than did *Heterorhabditis* species (genus main effect, $P < 0.001$; Table 4), an effect not influenced by mustard addition (genus x mustard interaction, $P = 0.28$; Table 4). We next compared ARID to GOLD by dropping the water-only CONTROL treatment. This test revealed that EPN species generally performed more poorly when exposed to extracts from the high glucosinolate mustard cultivar 'Pacific Gold' than the low glucosinolate cultivar 'Arid' (mustard main effect, $P = 0.012$), and that *Steinernema* species again outperformed *Heterorhabditis* species (genus main effect, $P < 0.001$) (Table 4; Fig. 2B). However, interactions between mustard cultivar and EPN species and genus were not statistically significant (Table 4), suggesting that on average EPN species were similarly further harmed by the high glucosinolate *B. juncea* cultivar.

4. Discussion

While somewhat ambiguous, our field measurements of entomopathogen activity suggested that entomopathogens were less likely to infect sentinel prey in potato fields treated with mustard

bio-fumigants. In production potato fields, entomopathogen infection of *G. mellonella* on average was ca. 20% less common in fields receiving mustard bio-fumigants (Fig. 1A), representing the only effect that approached statistical significance ($P = 0.061$; Table 2) in our field sampling. EPNs in the genus *Heterorhabditis* were the most commonly collected entomopathogens in our field sampling, representing 48% of all *G. mellonella* infections. *Heterorhabditis* species infections on *G. mellonella* were on average 35% less common in mustard treated fields, with the most dramatic reduction apparent for organic fields (Fig. 1B); however, the data were highly variable and this reduction was not statistically significant (Table 2). Interestingly, infections on *G. mellonella* by the other two entomopathogen taxa, *Steinernema* species nematodes (27% of all infections) and *Beauveria* species fungi (25% of all infections), on average barely differed among treatments (Fig. 1C-D; Table 2). Mechanisms behind the resiliency of these two taxa with changing soil management merit further investigation.

We made no attempt to identify the entomopathogens that we collected to species, but earlier work in regional potato fields reported members of both EPN genera used in our study attack CPB (Liu and Berry, 1995; Berry et al., 1997). Indeed, in subsequent laboratory trials we found that at least one of the *Heterorhabditis* species and *Steinernema* species that we collected was capable of infecting CPB larvae (Ramirez, 2008). Thus, at least some of the EPN species in local potato fields may be playing a role in CPB control. *Beauveria* species fortuitously collected within our sentinel prey have not been examined for infectivity towards potato beetles, and there is no previous work examining endemic entomopathogenic fungi in regional potato fields. However, in a survey of endemic entomopathogens in Pacific Northwest nursery soil *Beauveria bassiana* was among the most common fungal pathogens collected (Bruck, 2004). Clearly, these beneficial fungi warrant more attention in the future.

In laboratory arenas, exposure to *B. juncea* extracts generally reduced the likelihood that EPNs would infect *G. mellonella* larvae (Fig 2A-B). Furthermore, exposure to extract from the high-glucosinolate *B. juncea* cv. 'Pacific Gold' had a greater negative effect toward EPN infectivity than extract from the low-glucosinolate content cultivar 'Arid' (Fig. 2A-B). In these trials *Heterorhabditis* species generally performed more poorly than did *Steinernema* species. However, the members of these two genera were similarly susceptible to the negative effects of mustard extracts, as the genus by mustard interaction terms were not statistically significant in any of our comparisons (Table 4). However, individual species did not always adhere to the overall trend of weakened infectivity in the presence of mustard extracts. In both laboratory experiments, *S. feltiae* exerted relatively high infection rates in both the presence and absence of mustard extracts, and when exposed to extracts from either *B. juncea* cultivar. This species may warrant attention for use as a bio-pesticide in potato or other cropping fields where mustard bio-fumigants are also used. Currently the mode of action of mustard bio-fumigants on nematodes is unknown (Sipes and Schmitt, 1998). However, it is possible that *S. feltiae* possesses biological traits that render this species inherently more resistant to the toxins released during mustard degradation. Similarly, different species of plant-parasitic nematodes vary in their susceptibility to mustard bio-fumigants (Zasada and Ferris, 2004).

Synthetic soil fumigants such as methyl bromide, metam sodium, and 1, 3-dichloropropene carry substantial environmental and human-health risks, leading to government restrictions on their use, which has lead to an ongoing search for effective alternatives. In addition to *Brassica* and *Sinapis* mustard species, sudan grass (Mojtahedi et al., 1993; Viaene and Abawi, 1998), oat and rye (Faulkner and McElroy, 1964), and forage millet and marigolds (Ball-Coelho et al., 2003; Riga et al., 2005) have been examined as rotational or green manure crops as a tactic to

reduce buildup of plant-parasitic nematode populations. Additional advantages of bio-fumigants include little or no risk to the environment or to humans, reduced soil erosion, improved soil fertility, and sometimes additional income for growers (Matthiessen and Kirkegaard, 2006). Mustards have been particularly attractive bio-fumigant candidates because of the broad activity of their toxic breakdown products against a range of soil pests (Brown and Morra, 1995; Kirkegaard et al., 1996; Riga et al., 2004; Zasada and Ferris, 2004). Furthermore, biologically-active compounds are retained in waste-products following conversion of mustard seed to biofuels, forming an inexpensive and likely growing source of these soil amendments (Cohen and Mazzola, 2004). However, our work suggests that this same broad toxicity may carry tradeoffs. Beneficial bacteria, fungi, and entomopathogenic nematodes form an important component of good soil health that could also be harmed by the use of mustard bio-fumigants. Our work suggests a particular conflict between mustard bio-fumigation for the control of plant-parasitic nematodes, and the conservation and/or augmentation of entomopathogenic nematodes for biological control. However, increasing the period of time between bio-fumigation and the application of EPN bio-pesticides could reduce negative effects on EPNs, if toxicity of bio-fumigants is relatively short-lived.

Entomopathogenic nematodes and fungi have a long record of showing effective pest control in laboratory arenas, but often limited efficacy in the field (Gaugler, 1988; Klein, 1990; Smith, 1999). Rotational and green manure crops (this study), soil fertility practices (Duncan et al., 2007), biotic resistance by other soil organisms (Kaya and Koppenhöffer, 1996), and tillage intensity (Millar and Barbercheck, 2001) all have impacts on entomopathogen communities in the soil. Thus, maximizing the effectiveness of beneficial entomopathogens through their

conservation and augmentation likely requires a systems-based approach, balancing tradeoffs among the full range of soil-management tactics.

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Table 1. Potato fields where entomopathogen activity was measured.

Regime	Soil treatment	Acres	County	Grower	Year
Conventional	metam sodium	120	Grant	Ephrata	2004
Conventional	aldicarb and metam potassium	120	Adams	Johnson	2004
Conventional	metam sodium	120	Grant	Ephrata	2005
Conventional	aldicarb and metam potassium	120	Adams	Johnson	2005
Conventional	aldicarb and metam potassium	60	Adams	Johnson	2005
Conventional	aldicarb and metam potassium	120	Adams	Johnson	2005
Conventional	aldicarb and metam potassium	120	Adams	Johnson	2005
Conventional	metam sodium, 1, 3-dichloropropene	120	Benton	Watts Bros.	2005
Conventional	Caliente mustard blend and aldicarb	60	Adams	Johnson	2004
Conventional	Caliente mustard blend and aldicarb	120	Adams	Johnson	2004
Conventional	Caliente mustard blend	45	Benton	Paterson	2004
Conventional	Caliente mustard blend	45	Benton	Paterson	2004
Conventional	Caliente mustard blend and metam sodium	120	Benton	Watts Bros.	2004
Conventional	Caliente mustard blend and aldicarb	60	Adams	Johnson	2005
Conventional	Caliente mustard blend and aldicarb	60	Adams	Johnson	2005
Conventional	Caliente mustard blend and aldicarb	120	Adams	Johnson	2005
Conventional	Caliente mustard blend	45	Benton	Paterson	2005
Organic	None	60	Franklin	Lenwood	2004
Organic	None	60	Adams	Johnson	2005
Organic	None	60	Franklin	Lenwood	2005
Organic	None	60	Franklin	Lenwood	2005
Organic	Caliente mustard blend	60	Adams	Johnson	2004
Organic	Caliente mustard blend	60	Adams	Johnson	2005

Table 2. Statistical analyses of field measurements of entomopathogen activity for all pathogens combined, *Heterorhabditis* spp. nematodes, *Steinernema* spp. nematodes, and *Beauveria* spp. fungi.

Effect	df	*F*	*P*
All entomopathogens			
Mustard	1, 19	3.97	0.061
Management regime	1, 19	0.20	0.663
Mustard * Management	1, 19	2.01	0.173
Heterorhabditis spp.			
Mustard	1, 19	1.66	0.214
Management regime	1, 19	0.20	0.660
Mustard * Management	1, 19	1.72	0.205
Steinernema spp.			
Mustard	1, 19	0.38	0.548
Management regime	1, 19	1.63	0.217
Mustard * Management	1, 19	0.13	0.720
Beauveria spp.			
Mustard	1, 19	0.01	0.906
Management regime	1, 19	0.67	0.423
Mustard * Management	1, 19	0.02	0.896

Table 3. For the first laboratory experiment, statistical analyses of *Galleria mellonella* infection by three nematode species (*S. carpocapsae*, *S. glaseri* and *S. feltiae*) fully crossed with three levels of mustard treatment (CONTROL, ARID, and GOLD).

Effect	df	*F*	*P*
Full design			
EPN species	2, 81	1. 76	0.178
Mustard cultivar	2, 81	6.63	0.002
EPN species * Mustard cultivar	4, 81	4.87	0.001
Control versus Mustard			
EPN species	2, 84	0.14	0.867
Mustard cultivar	1, 84	8.70	0.004
EPN species * Mustard cultivar	2, 84	6.41	0.003
Arid versus *Pacific Gold*			
EPN species	2, 54	7.71	0.001
Mustard cultivar	1, 54	4.43	0.040
EPN species * Mustard cultivar	2, 54	3.22	0.048

Table 4. For the second laboratory experiment, statistical analyses of *Galleria mellonella* infection by seven nematode species from two genera (*Heterorhabditis* and *Steinernema*) fully crossed with three levels of mustard treatment (CONTROL, ARID, and GOLD).

Bioassay 2

Design	Source	SS	df	MS	F	P-value
Full						
	Model	9.452	20	0.473	4.100	0.0001
	Error	21.797	189	0.115		
	Corrected total	31.249	209			
	Genus	3.503	1	3.503	30.37	0.0001
	Species (Genus)	0.742	5	0.148	1.29	0.2713
	Mustard cultivar	3.496	2	1.748	15.16	0.0001
	Genus*Mustard cultivar	0.205	2	0.102	0.89	0.4134
	Species*Mustard cultivar (Genus)	1.506	10	0.151	1.31	0.2300
Contrast 1 (Control versus Mustard)						
	Model	8.523	13	0.656	5.650	0.0001
	Error	22.726	196	0.116		
	Corrected total	31.249	209			
	Genus	3.503	1	3.503	30.210	0.0001
	Species (Genus)	0.742	5	0.148	1.280	0.2741
	Contrast 1	2.778	1	2.778	23.960	0.0001
	Genus*Contrast 1	0.139	1	0.139	1.200	0.2756
	Species*Contrast1 (Genus)	1.361	5	0.272	2.350	0.0425
Contrast 2 (*Arid* versus *Pacific Gold*)						
	Model	4.462	13	0.343	3.12	0.0005
	Error	13.850	126	0.110		
	Corrected total	18.313	139			
	Genus	3.038	1	3.038	27.64	0.0001
	Species (Genus)	0.495	5	0.099	0.900	0.4825
	Contrast 2	0.718	1	0.718	6.540	0.0118
	Genus* Contrast 2	0.066	1	0.066	0.600	0.4396
	Species*Contrast 2 (Genus)	0.144	5	0.029	0.260	0.9329

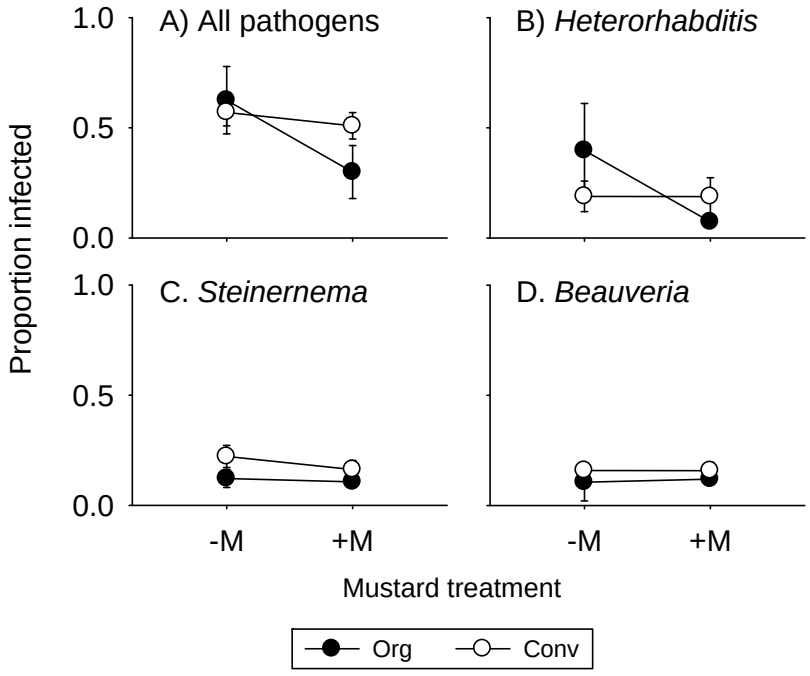

Figure 1. Proportion of sentinel *Galleria mellonella* hosts infected by a) all entomopathogens, b) *Heterorhabditis* spp. nematodes, c) *Steinernema* spp. nematodes, and d) *Beauveria* spp. fungi. Mustard treatments: mustard bio-fumigant not used (M-), mustard bio-fumigant used (M+). Pest management regime: conventional pest management (○), certified organic (●). Means are ± 1 S.E.

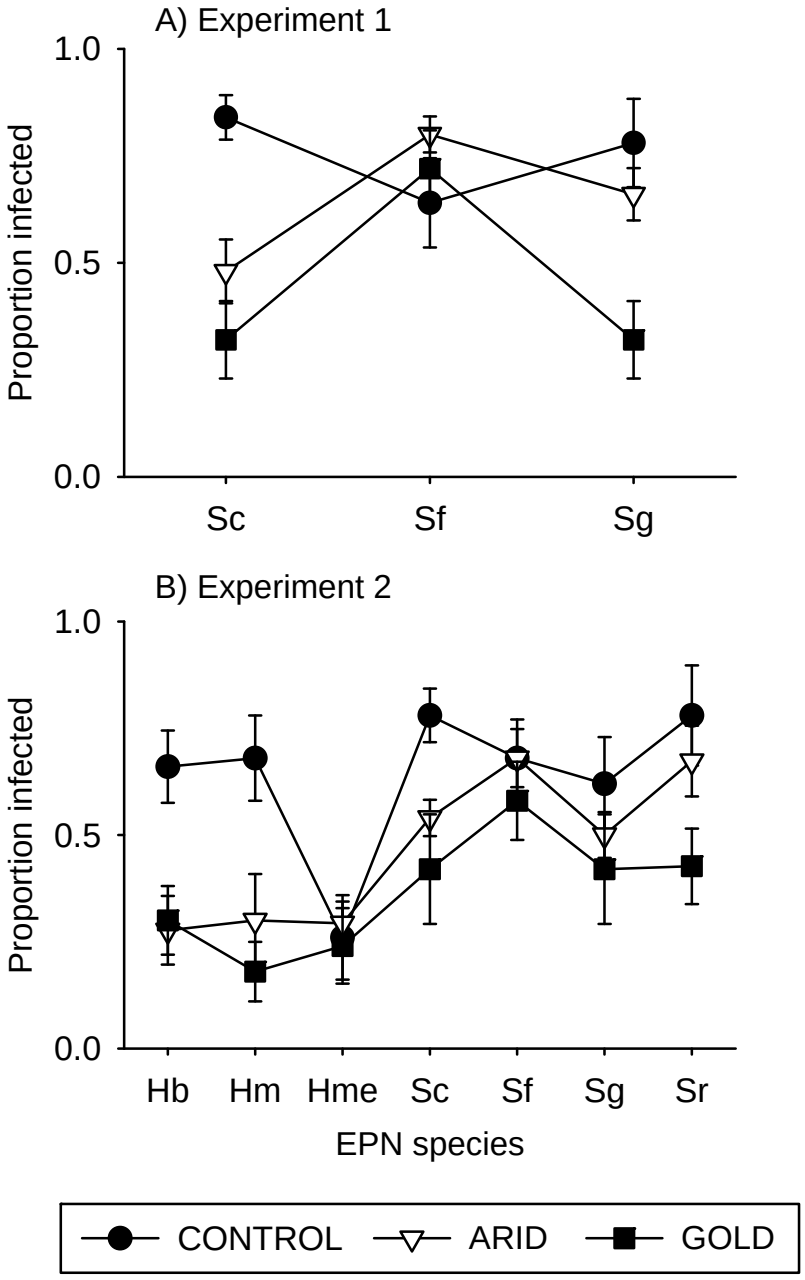

Figure 2. For a) laboratory experiment 1 and b) laboratory experiment 2, proportion of *Galleria mellonella* hosts infected by entomopathogenic nematodes. EPN species treatments: *H. bacteriophora* (Hb), *H. marelatus* (Hm), *H. megidis* (Hme), *S. carpocapsae* (Sc), *S. feltiae* (Sf), *S. glaseri* (Sg), and *S. riobrave* (Sr). Mustard treatments: water (CONTROL), extract from *B. juncea* cv. 'Arid' (ARID), and extract from *B. juncea* cv. 'Pacific Gold' (GOLD). Means are $\pm$ 1 S. E.

CHAPTER THREE

Animal manure harms entomopathogenic nematodes

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Abstract

Animal manure is an alternative to synthetic fertilizer that provides the additional benefits of reducing nutrient leaching and soil erosion, and promoting greater soil biodiversity. Studies show that animal manures can suppress plant parasitic nematodes by increasing densities of antagonistic microbial species or increasing concentrations of anhydrous ammonia. However, animal manure adds organic matter to the soil, providing additional resources for free-living nematode populations. With these opposing effects on nematodes, it is unclear how animal manure impacts entomopathogenic nematodes (EPNs), beneficial nematodes that are important biocontrol agents of pest insects, and how these effects might differ from those of chemical fertilizers. In field experiments the effects of a mixture of chicken and cow manure, versus those of the synthetic dry formulated complete fertilizer on *Heterorhabditis marelatus* and *Steinernema carpocapsae* EPNs, were compared. Fertilizer was applied to field plots of potato (*Solanum tuberosum*) and EPNs were released, and then tracked for persistence using sentinel *Galleria mellonella* and *Leptinotarsa decemlineata* larvae. In two different years, EPN infection rates were lower and persistence shorter in plots receiving animal manure than in those receiving synthetic fertilizer. The microbial activity, measured using a dehydrogenase enzyme activity measurement, was greater in manure than synthetic fertilizer plots. Endemic pathogens, primarily *Beauveria bassiana*, were also recovered, but densities of these species were not altered by our fertilizer or EPN release treatments. There was no evidence that competition by resident entomopathogens contributed to the negative effect of animal manure on EPNs. Soil pH and soil moisture were not different between the two fertilizer types. These results suggest that biotic resistance from increased microbial activity with animal manure, and not competition by resident entomopathogens or altered moisture levels or soil chemistry, reduced EPN effectiveness. The

results suggest that there may be a conflict between using animal manures to manage soil fertility and EPNs as insect biocontrol agents.

Keywords: Entomopathogenic nematodes; *Steinernema carpocapsae*; *Heterorhabditis marelatus*; manure; synthetic fertilizer; biotic resistance; potato

1. Introduction

Animal manure provides many advantages over chemical fertilizers for soil health, including greater water infiltration rates (Jackson, 1988), carbon storage (Drinkwater et al., 1995; Peacock et al., 2001; Nahar et al., 2006), soil aggregation, and water holding capacity (Hafez, 1974), reduced nutrient leaching (Drinkwater et al. 1995; Peacock et al., 2001) and soil erosion (Reganold et al., 1987). In addition to improving physical soil properties, animal manure also enhances microbial biomass (Fauci and Dick, 1994; Joergensen et al., 1995; Villenave et al., 2003; Nahar et al., 2006), activity (Doran et al., 1987), and soil biodiversity (Mäder et al. 2002).

Plant parasitic nematodes pose a major pest threat to potato production in many parts of the world (Santos et al., 1995; Marks and Rojancovski, 1998; Turner and Evans, 1998; Sun and Miller, 2007), including the irrigated potato growing region in the Columbia Basin of Washington State and adjacent Oregon, USA (Ingham et al., 2005; Riga and Neilson, 2005). Studies have shown that these harmful plant-parasitic nematodes can be suppressed by the application of animal manures (Badra et al., 1979; Derrico and Maio, 1980; Siddiqui, 2004; Nahar et al., 2006). The reduction of plant-parasitic nematodes in soils treated with animal manures may result from enhanced densities of competitive, predaceous, or antagonistic

microorganisms (Linford et al., 1938; Clark et al 1998; Akhtar and Malik, 2000; Duncan et al., 2007) or increased concentrations of ammoniacal or anhydrous nitrogen that animal manures provide (Rodriguez-Kabana, 1986; Oka and Yermiyahu, 2002). However, the suppression of plant-parasitic nematodes by animal manures does not seem to translate into a negative effect on free-living nematodes (Akhtar and Mahmood, 1996). Animal manure adds both organic matter and microbes to the soil providing a food source (Andrén and Lagerlöf, 1983; Weiss and Larink, 1991) that benefit bacterivorous and fungivorous nematodes (Freckman, 1988; Bohlen and Edwards, 1994; Griffiths et al., 1994; Bulluck et al., 2002; Villenave et al., 2003; Forge et al., 2005; Nahar et al., 2006).

A diverse group of entomopathogenic nematodes (EPNs) in the genera *Steinernema* and *Heterorhabditis* are a part of the beneficial nematode community in potato fields in the northwestern USA (Liu and Berry, 1995; Berry et al., 1997; Ramirez et al., in review). These nematodes enter host insects through mouthparts, spiracles, and the anus, then release a symbiotic bacterium that kills the host (Boemare et al., 1996). The nematodes then develop and reproduce by feeding on digested host tissues and these rapidly reproducing bacteria (Griffin et al., 2005). Mortality of the host insect occurs within 2-5 days following initial infection (Grewal and Peters, 2005). Through conservation of endemic species, or application as bio-insecticides, entomopathogenic nematodes have been shown to contribute to the biological control of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Toba et al., 1983; Wright et al., 1987; Nickel et al., 1994; Berry et al., 1997; Stewart et al., 1998; Armer et al., 2004), a key pest of potato in the Columbia Basin and many other potato-growing regions (Hare, 1990). Thus, any negative effects of animal manure on these beneficial nematodes could disrupt biological control of pest insects in potato and other crops. In previous studies animal manure has been variously

shown to suppress exotic EPNs (Duncan et al., 2007), have no effect on EPN releases (Ellers-Kirk et al., 2000), and enhance resident EPN populations by encouraging EPN establishment and recycling (Bednarek and Gaugler, 1997; Duncan et al., 2007). Here, we report on an examination of the effects of animal manure and synthetic fertilizer on the persistence of EPNs following their release as a bio-pesticide in potato crops in Washington State, USA.

2. Materials and Methods

The field experiment was conducted at Washington State University's Research and Education Center in Othello, WA. In our experiments we used a mix of two EPN species, *Steinernema carpocapsae* "All" strain and *Heterorhabditis marelatus*. These species were selected because they have shown potential as control agents against the Colorado potato beetle (e.g., *H. marelatus* [Armer et al., 2004] and *S. carpocapsae* [Nickle et al., 1994; Stewart et al., 1998]). *Steinernema carpocapsae* and *H. marelatus* came from laboratory colonies, maintained by the authors using waxworm larvae as hosts following methods described by Kaya and Stock (1997).

We conducted two field experiments, wherein we examined the persistence of EPNs in plots receiving either animal manure (ORG) or synthetic fertilizer (CON). The plots measured 4.57 x 4.57 m, encompassing 5 rows of potatoes. In the first field experiment conducted in the summer of 2005, we applied EPNs at three rates: Control (no EPNs), Low rate (5.2×10^5 EPNs per plot), and High rate (10.4×10^6 EPNs per plot), with 8 replicates of each EPN-fertilizer treatment combination for a total of 48 plots. In the summer of 2006 we conducted a second field experiment wherein two EPN rate treatments were applied: Control (no EPNs) and High rate

(10.4 x 10^6 EPNs per plot), with 6 replicates of each EPN-fertilizer treatment combination for a total of 24 plots. EPNs were applied using a backpack sprayer (Field King 18.93 L Deluxe Sprayer, Forestry Suppliers Inc., Jackson, MS) with all filters removed. In the first experiment EPNs were applied twice during the season (June 18 and July 4, 2005), while in 2006 a single application was made (June 19, 2006).

Animal manure was applied using a pre-plant shovel application of composted chicken (1.35 kg/m^2) and cow manure (2.24 kg/m^2) mixture (gathered from Johnson Agriprises, Othello, WA and Lenwood Farms, Connell, WA, respectively). The synthetic fertilizer was pre-plant applied using dry formulated synthetic fertilizer at a rate of NPK lbs/acre 100-230-200 (Simplot, Othello, WA). Only one application of fertilizer was made before the start of each experiment; after two weeks the fertilizer was incorporated into the soil. Non-treated potato (cv. Russet Ranger) seed pieces (purchased from Skone and Connors, Warden, WA) were straight-planted into approximately 1012 m^2 of land. Two fallow rows were left as a buffer to adjacent plots, with 4.6 m of unfertilized potatoes separating plots within rows. Throughout the season plots were sprinkler irrigated three hours per day three days a week.

EPNs were applied in the morning, between 0800 and 0900 hours. All plots were sprinkler irrigated one hour prior to application and then at least one hour after application. All EPN sampling was conducted *in situ* using sentinel *G. mellonella* (purchased from Sunshine mealworms, Silverton, OR) and *L. decemlineata* 4th instar larvae collected from an adjacent potato field. Groups of five larvae were placed in mesh bags made from fiberglass window screen (a single 10 cm x 10 cm square), which were then closed and sealed with a twist tie. Within each plot 5 mesh bags were buried 10-15 underneath the soil for 48 hrs, after which bags were retrieved, larvae were supplied with water, and larvae monitored for infection. *Leptinotarsa*

decemlineata larvae were used once (July 6, 2005) during the first experiment and were introduced into the soil in a similar fashion as *G. mellonella*. Dissections for most of the beetle larvae were done to determine mortality caused by nematodes.

Soil moisture and microbial activity using a dehydrogenase enzyme activity measurement (Tabatabai, 1994) was gathered in the 2005 field season. In 2007 three animal manure and three synthetic fertilizer plots were set up as described above to test soil pH, soil moisture, and microbial activity. Soil moisture was gathered by taking ten soil core samples from each plot, 10-15 cm deep, using a soil probe (83.8 cm length, 2.2 cm diameter, Forestry Suppliers Inc., Jackson, MS). The soil from each plot was mixed well and sieved to create a soil aliquot for each plot. Five grams of soil per plot was weighed and placed into aluminum containers (3 sub-samples per plot) and placed into an oven at 21ºC for 4 days, after which soil moisture was calculated (Black, 1965). The remainder of the soil aliquots was then used to determine microbial activity. All methods followed those described by Tabatabai (1994). Soil pH was examined in 2006 and 2007. First 5 soil cores were taken from each plot and mixed to create an aliquot as previously described. For each plot 20 grams of soil was placed into a plastic cup (Dixie®, 5.5 oz. soufflé cup) and 20 ml of de-ionized water was added. The soil slurry was mixed and pH was measured using a pH meter (ExStick pH meter, Forestry Suppliers Inc., Jackson, MS).

2.1. Analyses

Galleria mellonella infection data from the 2005 field experiment were analyzed within a two-way ANOVA with repeated measures with two levels of fertilizer treatment (ORG and CON) fully crossed with three EPN rates (CONTROL, LOW, and HIGH), with 5 sample dates.

Leptinotarsa decemlineata infection data were analyzed using two-way ANOVA with the same fertilizer and EPN rate factors. In 2006 infection data were analyzed within a two-way ANOVA with repeated measures with two levels of fertilizer treatment (ORG and CON) fully crossed with two EPN rates (CONTROL and HIGH), sampled at 4 dates. The zero time step, before EPN application, in both the 2005 and 2006 field experiments was used to verify that there were no significant differences between treatments before EPN application, and were not included in the repeated measures analyses. All proportion infection data were arcsine-square root transformed prior to analysis.

Soil pH data from the 2006 field season were analyzed within a two-way ANOVA with two levels of fertilizer treatment and two time steps (June and August). In 2007 soil pH was analyzed within a two-way ANOVA with repeated measures accounting for three time steps (June, July, August). Soil moisture was analyzed differently in 2005 than in 2007; in 2005 we utilized one-way ANOVA with two levels of fertilizer treatment (ORG and CON) within a single sample date, whereas in 2007 we used one-way ANOVA with repeated measures across three time steps (June, July, August). Microbial activity data in 2005 were analyzed within a two-way ANOVA with two levels of fertilizer treatment and three levels of EPN application rate, while one-way ANOVA with two levels of fertilizer treatment was used in 2007. All data were analyzed using SYSTAT (version 11.0; SPSS, Chicago, IL) software.

3. Results

In the 2005 field experiment there were significant main effects of fertilizer and EPN rate treatments on infection of sentinel waxworms by EPNs (Table 1; $F_{1, 42} = 10.05$, $P = 0.003$ and $F_{2,}$

$_{42}$ = 385.45, $P < 0.001$, respectively). Infection of *G. mellonella* was lower in the presence of animal manure compared to the synthetic fertilizer treatment (Fig. 1A), and with increasing rate of EPN application there was an increase in infection of sentinel insects. The significant time by EPN rate interaction (Table 1; $F_{8, 168} = 5.06$, $P < 0.001$) indicated no change with infection for the control (no EPN) whereas infection of *G. mellonella* at Low and High EPN rate decreased through time (Fig 1A). The significant main effect of time revealed an overall decrease in infection of *G. mellonella* following the initial EPN application ($F_{4, 168} = 15.90$, $P < 0.001$). Similarly, for sentinel CPB larvae infection rates were higher when more EPNs were applied to plots ($F_{2, 42} = 146.46$, $P < 0.001$), but reduced when animal manures were applied ($F_{1, 42} = 6.54$, $P = 0.014$) (Fig. 2). There was no significant interaction between EPN rate and fertilizer treatment ($F_{2, 42}$=1.93, P=0.158) for EPN infection of *L. decemlineata*.

In 2006 *G. mellonella* infection by EPNs showed several statistically-significant two-way interactions (Table 2), complicating interpretation of these data. The significant time by EPN rate interaction ($F_{3, 51} = 10.88$, $P < 0.001$) indicated no change in infection of *G. mellonella* by EPNs in control (no EPN) plots through time, while infection in the high EPN application rate decreased through time (Fig. 1B). A time by soil fertility interaction ($F_{3, 51} = 3.54$, $P < 0.021$) appeared to be driven by a decrease in EPN infection in the synthetic fertilizer treatment through time, whereas infection in animal manure plots was little changed through time (Fig. 1B).

Animal manure decreased EPN infection of *G. mellonella*, leading to a significant fertilizer by EPN rate interaction (Fig. 3; $F_{1, 17} = 7.57$, $P = 0.014$). *Galleria mellonella* infection by entomopathogens other than EPNs revealed an interaction between soil fertility and EPN rate that approached significance (Table 2; $F_{1, 17} = 3.82$, $P = 0.067$). Here resident entomopathogens were negatively impacted by the combination of synthetic fertilizer and EPN release, while there

was no difference between the combination of animal manure with EPNs and the CONTROL treatment (Fig. 4). All other interactions were not significant (Table 2).

Soil pH measurements revealed no significant interaction between fertilizer treatment and time during the 2005 ($F_{1, 33} = 1.02$, $P = 0.319$) and 2006 ($F_{2, 8} = 0.69$, $P = 0.528$) field seasons. In 2005 soil pH did not differ between animal manure and synthetic fertilizer treatments ($F_{1, 33} = 0.09$, $P = 0.767$). Similarly in 2006 fertilizer treatments did not significantly impact soil pH ($F_{1, 4} = 1.75$, $P = 0.257$); the Time main effect ($F_{2, 8} = 5.84$, $P = 0.027$) indicated an increase in soil pH later in the season (Fig. 5A). Soil moisture measurements both years showed no significant interaction between fertilizer treatment and time ($F_{2, 8} = 0.65$, $P = 0.547$) and no significant main effect of fertilizer treatment ($F_{1, 4} = 0.02$, $P = 0.902$). There was however, a significant main effect of time ($F_{2, 8} = 28.07$, $P < 0.001$) indicating a decrease in soil moisture through the field season (Fig. 5B).

Microbial activity increased with animal manure compared to synthetic fertilizer ($F_{1, 41} = 6.30$, $P = 0.016$; Fig. 6A). There was no significant interaction between fertilizer treatment and EPN rate ($F_{2, 41} = 0.19$, $P = 0.832$). In 2007 we found a positive trend towards increasing microbial activity with animal manure, but there was no statistically significant difference between the two fertilizer treatments (Fig. 6B; $F_{1, 4} = 3.52$, $P = 0.134$).

4. Discussion

Results from both field experiments revealed that EPN infection of sentinel hosts was reduced in potato plots treated with animal manure, compared to those receiving synthetic fertilizer. The negative effect of animal manure was seen for infection of both *G. mellonella* and

L. decemlineata larvae. While organic matter may encourage the establishment and recycling of resident entomopathogens (Klingen et al., 2002; Meyling and Eilenberg, 2006), including EPNs (Bednarek and Gaugler, 1997; Duncan et al., 2007), it may also promote populations of antagonistic biota or induce changes in soil chemistry that negatively impact EPNs (Georgis et al., 1987; Shapiro et al., 1996; Duncan et al., 2007). The harmful effect of animal manure diminished by the end of each experiment, with infection in both manure and synthetic fertilizer treatments converging to relatively low levels (Fig. 1A and B).

While relatively high rates of infection of sentinel hosts by EPNs was sustained only for several weeks, EPN-treated plots continued to produce low levels of EPN infection until the end of each field season (Fig. 1A and 1B). This suggests that EPNs recycled through hosts, because field longevity of inundative applications of EPNs in the absence of recycling generally decline below detectable levels within 4 weeks (Georgis, 1992; Smits, 1996), far shorter than the duration of our experiments. Previous studies have shown EPN efficacy to decline by half within 10 days (Armer et al., 2004) and 70% over 3 weeks (Ellers-Kirk et al., 2000) with low levels of persistence thereafter. EPN infection in our study was detectible six (in 2005) to ten (in 2006) weeks after EPN application, suggesting *in situ* EPN reproduction.

Biotic interference may occur through competition for hosts between applied versus endemic entomopathogens (Kaya and Koppenhöfer, 1996), or via predation by antagonists (Epsky et al., 1988; Kaya and Koppenhöfer, 1996; Jaffee and Strong, 2005; Duncan et al., 2007). We noticed some infection of sentinel waxworms by non-EPN entomopathogens in the first year's study, and so in the second year we carefully cultured and measured infection by these resident entomopathogens. *Beauveria bassiana* was the primary resident entomopathogen. One possible explanation for lower EPN activity in manure-treated plots could have been that populations of

resident entomopathogens were encouraged in these plots, such that these species were out-competing the EPNs that we released. But, infection of sentinel hosts by endemic, non-EPN entomopathogens was unaffected by our fertilizer or EPN-release treatments (Fig. 4), and so it appears that this possibility could be excluded. However, animal manure plots exhibited higher microbial activity than was recorded in synthetic fertilizer plots (Fig. 6), suggesting that competition with native entomopathogens was not a key factor leading to poorer EPN performance in plots treated with animal manure. Biotic interference from other soil organisms has been shown in several studies to negatively impact nematode communities (e.g., Linford et al., 1938; Clark et al., 1998; Akhtar and Malik, 2000) including EPNs (Kaya and Koppenhöffer, 1996; Duncan et al., 2007). We did not have a direct measure of biotic interference, but we did observe nematode-trapping fungi and nematophagous mites that may have decreased EPN effectiveness in animal manure plots. These antagonists were reported in other studies to reduce EPN densities (Epsky et al., 1988; Jaffee and Strong, 2005; Duncan et al., 2007).

Soil moisture and pH are environmental factors altered by animal manure applications that have been shown to impact nematode populations (Schnürer et al., 1986; Korthals et al., 1996; Wardle, 2006). However, in our study soil moisture and pH did not significantly differ between fertilizer treatments (Fig. 5), further suggesting that biotic and not environmental factors may be decreasing EPN effectiveness in plots treated with animal manures.

We found that field application of EPNs led to infection and eventual death of sentinel host insects. We used a low EPN rate in an attempt to test a cost-effective rate; our high and low rates were calculated at $65/ha and $3.30/ha, respectively, using a price of $100/ha for commercial *S. carpocapsae* (Millenium®). High rates were more effective (Fig. 1A and 2). In general, reported EPN effectiveness against *L. decemlineata* in the field has been variable, ranging from 38% to

79% mortality (Toba et al., 1983; Wright et al., 1987; Stewart et al., 1998; Armer et al., 2004). Our results are within the range of EPN efficacy found in these other studies, high rates (4.9 x 10^5 EPNs/m^2) and repeated EPN applications may have contributed to extended EPN success in this study. Because multiple EPN species, and multiple applications of these species, may be required for suitable pest control in the field, focus should be on reducing EPN production cost to make these high application rates affordable.

To maximize the effectiveness of beneficial nematodes through their conservation and augmentation, EPN efficacy must be measured under different field conditions (Shapiro and McCoy, 2000). Soil fertility management (this study), the planting of rotational and green manure crops (Ramirez et al., submitted), biotic resistance by other soil organisms (Kaya and Koppenhöffer, 1996; Duncan et al., 2007) and the intensity of tillage (Millar and Barbercheck, 2001) all have impacts on EPNs in the soil. The high cost of entomopathogenic nematodes makes it imperative to determine the compatability between soil management practices and the use of EPNs as bio-pesticides.

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Table 1. For the 2005 field season, statistical analysis of *Galleria mellonella* infection by entomopathogenic nematodes applied at each of three rates (CONTROL, LOW, and HIGH) fully crossed with two levels of fertilizer treatment (ORG and CON) across five sample dates.

2005 Field season

Source	SS	df	MS	F	P-value
Between subjects					
Soil fertility	0.177	1	0.177	10.045	0.003
EPN rate	13.615	2	6.808	385.451	0.0001
Soil fertility*EPN rate	0.049	2	0.025	1.389	0.261
Error	0.742	42	0.018		
Within subjects					
Time	1.146	4	0.286	15.904	0.0001
Time*Soil fertility	0.050	4	0.013	0.696	0.596
Time*EPN rate	0.729	8	0.091	5.061	0.0001
Time*Soil fertility* EPN rate	0.103	8	0.013	0.718	0.676
Error	3.025	168	0.018		

Table 2. For the 2006 field season, statistical analyses of *Galleria mellonella* infection across four sample dates by (a) entomopathogenic nematodes and (b) resident entomopathogens (NON-EPNs) in potato plots receiving entomopathogenic nematodes applied at each of two rates (CONTROL and HIGH), across two fertilizer treatments, synthetic fertilizer (CON) and animal manure (ORG).

(a) 2006 Field season EPN infection

Source	SS	df	MS	F	P-value
Between subjects					
Soil fertility	0.010	1	0.010	0.320	0.579
EPN	3.062	1	3.062	100.956	0.000
Soil fertility*EPN	0.230	1	0.230	7.572	0.014
Error	0.516	17	0.030		
Within subjects					
Time	0.292	3	0.097	5.146	0.003
Time*Soil fertility	0.201	3	0.067	3.541	0.021
Time*EPN	0.617	3	0.206	10.879	0.0001
Time*Soil fertility*EPN	0.045	3	0.015	0.789	0.506
Error	0.965	51	0.019		

(b) 2006 Field season NON-EPN infection

Source	SS	df	MS	F	P-value
Between subjects					
Soil fertility	0.053	1	0.053	2.551	0.129
EPN	0.347	1	0.347	16.773	0.001
Soil fertility x EPN	0.079	1	0.079	3.818	0.067
Error	0.178	17	0.020		
Within subjects					
Time	0.085	3	0.028	1.304	0.284
Time*Soil fertility	0.118	3	0.039	1.809	0.157
Time*EPN	0.086	3	0.029	1.316	0.279
Time*Soil fertility*EPN	0.060	3	0.020	0.923	0.436
Error	1.114	51	0.022		

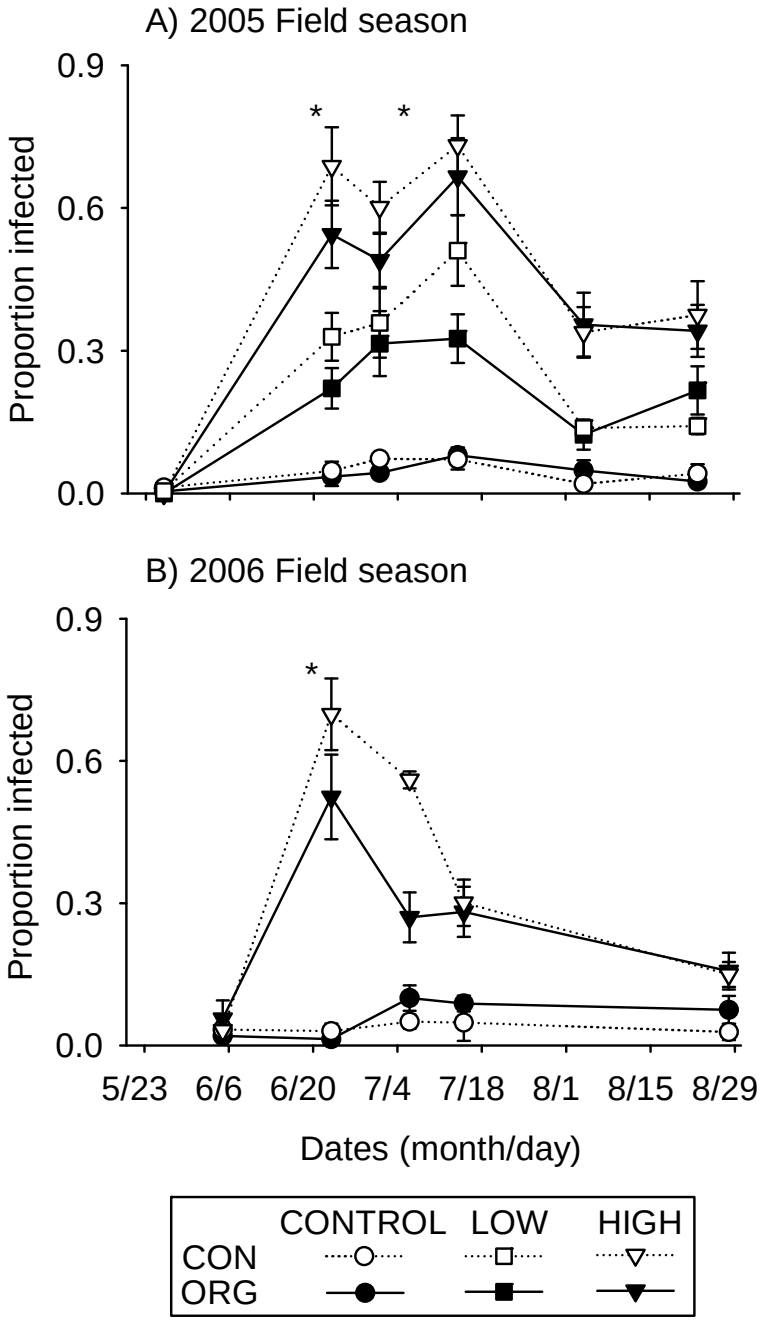

Figure 1. Proportion of sentinel *Galleria mellonella* hosts infected by entomopathogenic nematodes applied at each of three rates (CONTROL, LOW, and HIGH) during the a) 2005 field season and each of two rates (CONTROL and HIGH) during the b) 2006 field season across two fertilizer treatments, synthetic fertilizer (CON) and animal manure (ORG). Treatments: CON with CONTROL (○), LOW (□), and HIGH (▽) rates of EPNs applied, ORG with CONTROL (●), LOW (■), and HIGH (▼) rates of EPNs applied. Date of EPN application indicated by *. Means are ± 1 S. E.

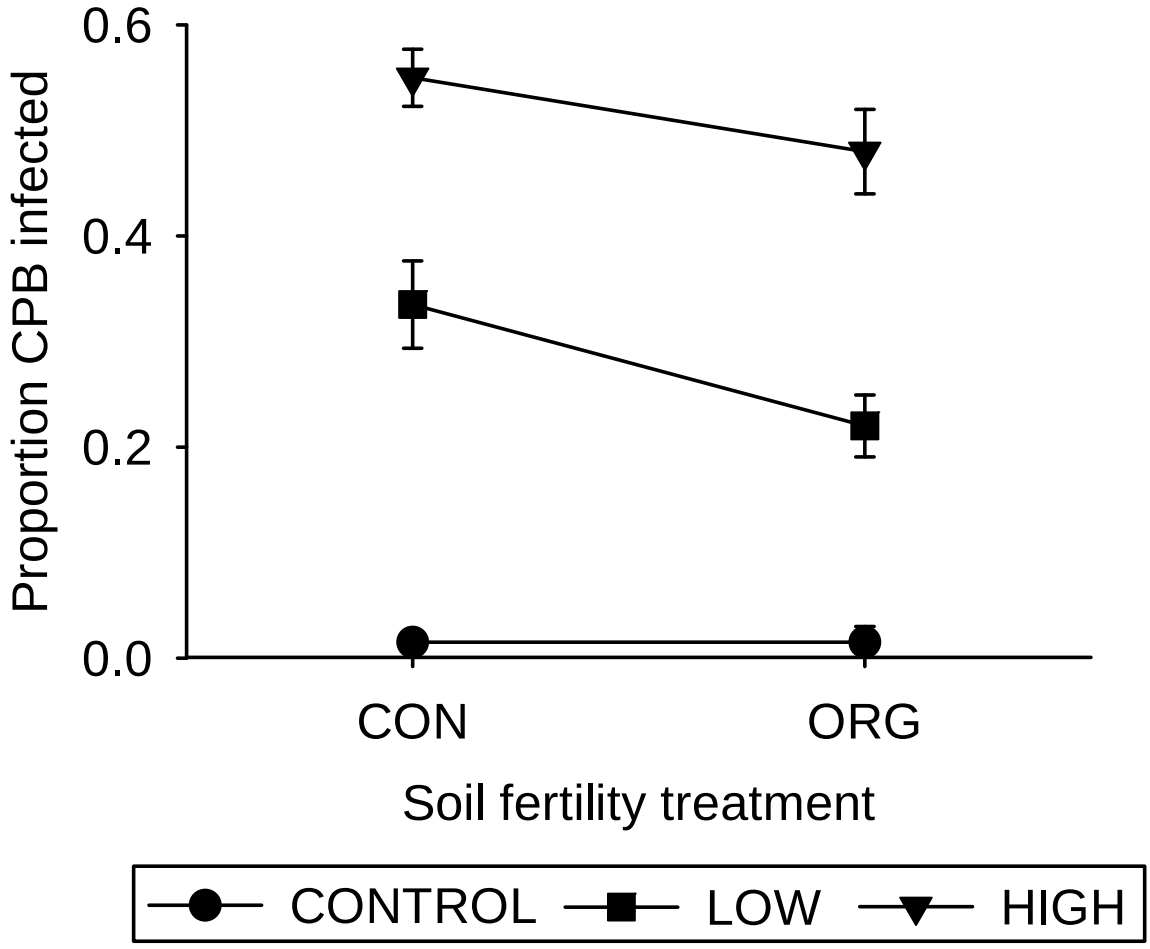

Figure 2. From the 2005 field experiment, proportion of sentinel Colorado potato beetle (CPB) larvae infected by entomopathogenic nematodes applied at each of three rates, CONTROL (●), LOW (■), and HIGH (▼), across two fertilizer treatments, synthetic fertilizer (CON) and animal manure (ORG). Means are ± 1 S. E.

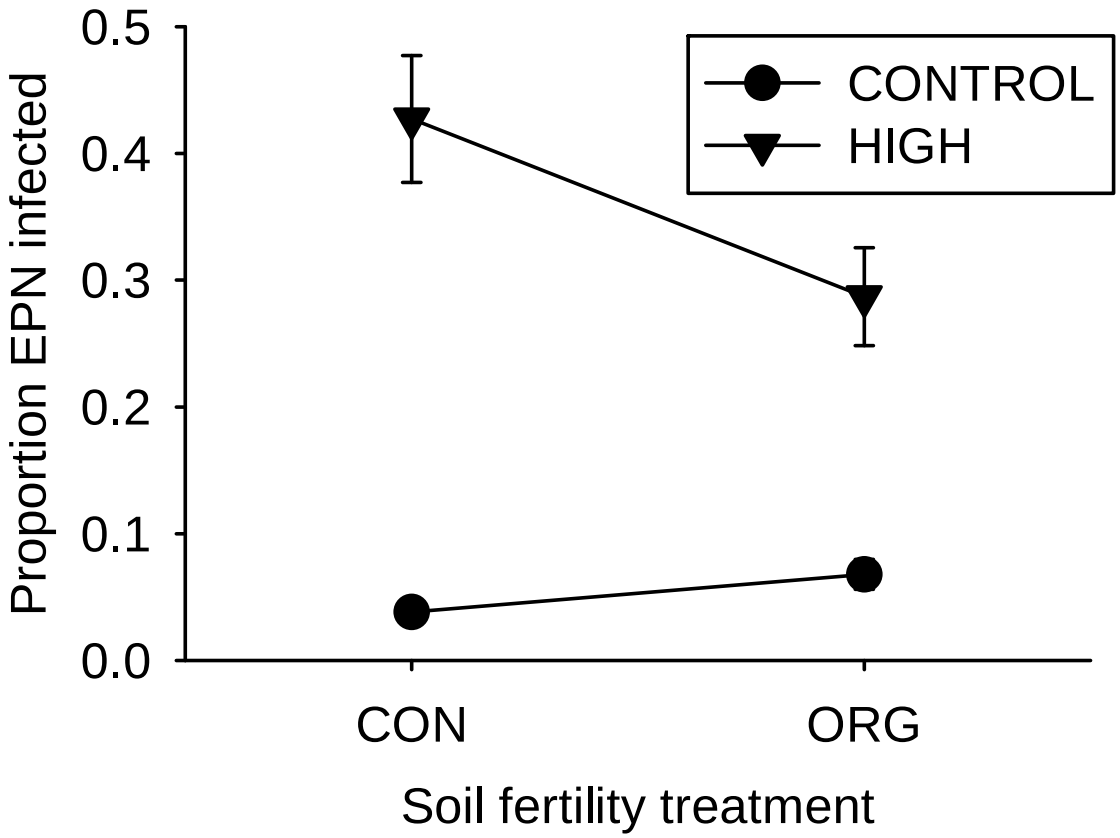

Figure 3. From the 2006 field experiment, proportion of sentinel *Galleria mellonella* hosts infected by entomopathogenic nematodes applied at each of two rates, CONTROL (●) and HIGH (▼), across two fertilizer treatments, synthetic fertilizer (CON) and animal manure (ORG). Means are ± 1 S. E.

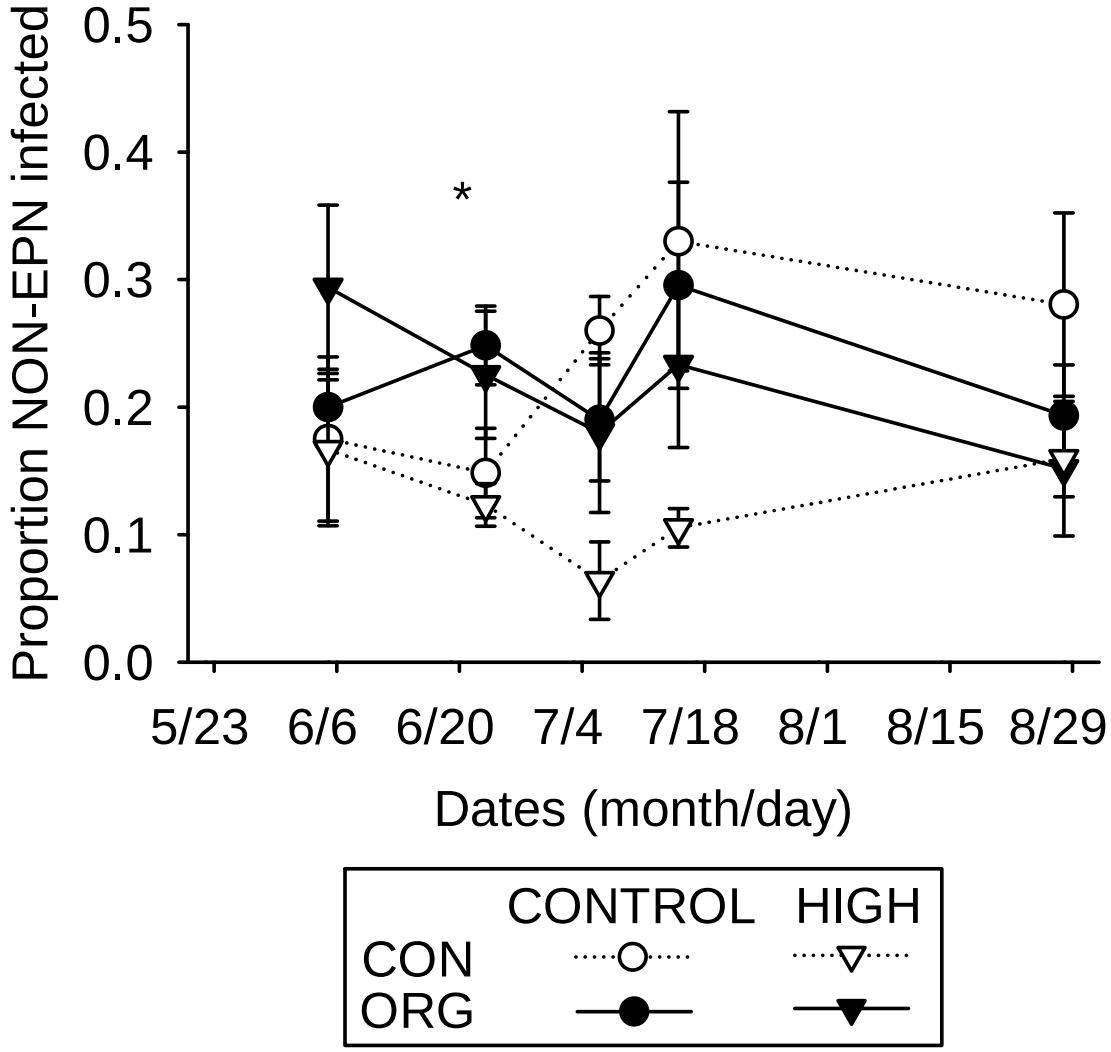

Figure 4. From the 2006 field experiment, proportion of sentinel *Galleria mellonella* hosts infected by resident entomopathogens (NON-EPNs) in potato plots receiving entomopathogenic nematodes applied at each of two rates (CONTROL and HIGH), across two fertilizer treatments, synthetic fertilizer (CON) and animal manure (ORG). Treatments: CON with CONTROL (○) and HIGH (▽) rates of EPNs applied, ORG with CONTROL (●) and HIGH (▼) rates of EPNs applied. Date of EPN application indicated by *. Means are ± 1 S. E.

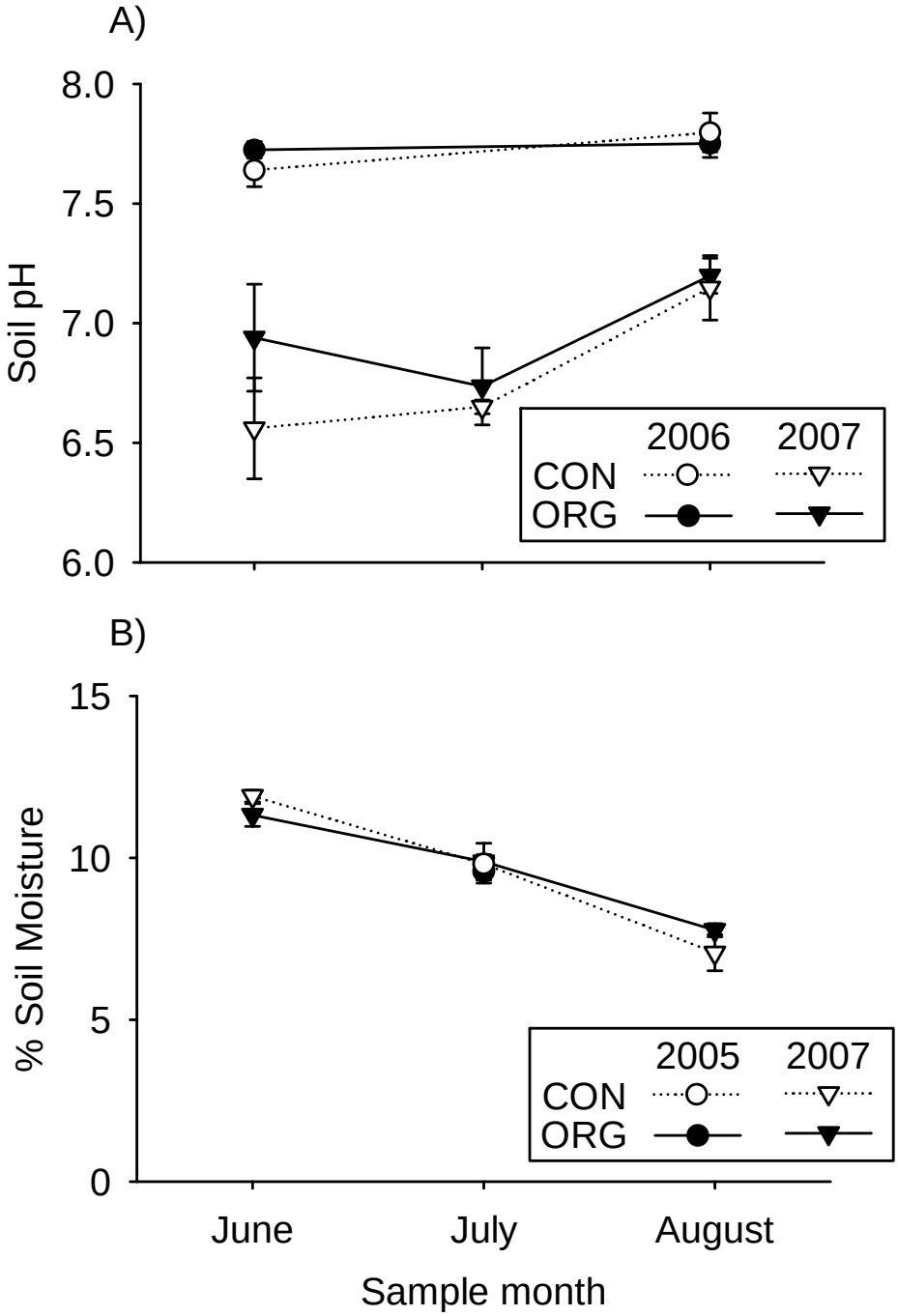

Figure 5. Measurements of a) soil pH and b) % soil moisture for two fertilizer treatments, synthetic fertilizer (CON) and animal manure (ORG) in June, July, and August. Soil pH measurements were taken in 2006 and 2007. Fertilizer treatment: CON (○) and ORG (●) for 2006, CON (▽) and ORG (▼) for 2007. Soil moisture measurements were taken in 2005 and 2007. Fertilizer treatment: CON (○) and ORG (●) for 2005, CON (▽) and ORG (▼) for 2007. Means are $\pm$ 1 S. E.

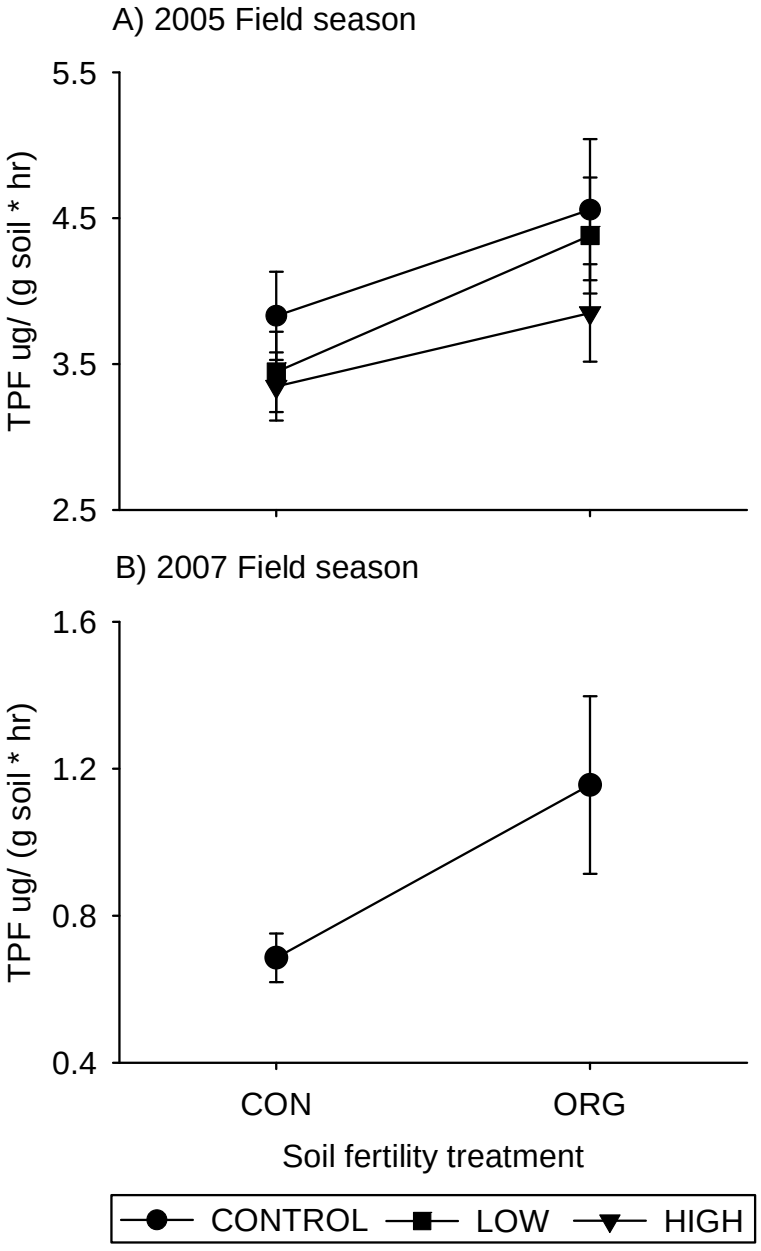

Figure 6. Soil microbial activity measurement for two fertilizer treatments, synthetic fertilizer (CON) and animal manure (CON), in a) 2005, entomopathogenic nematodes applied at each of three EPN rates, CONTROL (●), LOW (■), and HIGH (▼) and b) 2007, no entomopathogenic nematodes applied, CONTROL (●). Means are ± 1 S. E.

CHAPTER FOUR

Scared sick? Cascading effects of predator-pathogen complementarity

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Keywords: complementarity, biodiversity, functional diversity, ecosystem function, entomopathogen, predator, disease ecology

ABSTRACT

Herbivore suppression often strengthens with increasing predator biodiversity, but less is known about the role of pathogens in these relationships. Colorado potato beetle eggs and small larvae occur in plant foliage where they are attacked by a guild of generalist predators, whereas later-stage larvae burrow into the soil to pupate and are attacked by a guild of entomopathogens. Thus, predators and pathogens form spatiotemporally and ecologically distinct guilds. We manipulated predator-pathogen species richness and found that potato beetle densities decreased, and plant biomass increased, with greater enemy biodiversity. However, herbivore suppression strengthened only for predator-pathogen pairs, and not for pairings within the same natural enemy guild, suggesting that predators and pathogens form distinct functional groups. In the laboratory, exposure to the threat of predation weakened beetles' immune response. Thus, predators and pathogens exerted complementary impacts, perhaps due to a tradeoff for the herbivore in defenses against these two enemy guilds.

INTRODUCTION

Across a range of taxa and trophic levels, the dominant trend is for resource use to grow increasingly complete with greater consumer biodiversity (Cardinale *et al.* 2006). While the pattern of these relationships is well-established, its mechanistic underpinnings have proven more difficult to document (Duffy *et al.* 2007). Sometimes, species identity ('sampling') effects appear to dominate, with more diverse communities processing resources most efficiently purely because they are more likely, by chance alone, to include particularly voracious species (Huston

1997; Loreau 1998). Sampling effects result from the attributes of particular consumer species, and thus only confound true diversity effects (Loreau & Hector 2001). In contrast a second mechanism, complementarity, can occur only when multiple species are present. Complementarity occurs when consumer species differ in the subset of resources utilized, such that community-wide resource use is most complete when many species co-occur (Tilman *et al.* 2001). Unfortunately, complementary resource use can be difficult to demonstrate convincingly, with evidence often indirect and based on observing resource utilization by biodiverse communities that exceeds what can be explained by a sampling effect alone (Norberg 2000; Fridley 2001; Loreau & Hector 2001; Duffy *et al.* 2003; Cardinale *et al.* 2006). Sometimes, species can be clustered into unique "functional groups" that differ in how they utilize resources, with differences within a group relatively indistinct (Reich *et al.* 2004; Schmitz 2007). In these cases it may be functional group diversity, rather than species richness itself, that leads to improved resource exploitation at higher diversity levels (Petchey & Gaston 2006).

Herbivores are attacked by two broad classes of natural enemy, predators and pathogens (Hawkins *et al.* 1997). For predators in terrestrial systems, various studies have reported stronger (Cardinale *et al.* 2003; Wilby *et al.* 2005; Snyder *et al.* 2006), weaker (Finke & Denno 2004, 2005), or unchanged (Rodriguez & Hawkins 2000, Schmitz & Sokol-Hessner 2002, Aukema & Raffa 2004, Straub & Snyder 2006) herbivore suppression with greater predator biodiversity. Weakened herbivore suppression in diverse communities is generally attributed to intraguild predation (e.g., Finke & Denno 2004). Positive effects of predator diversity are generally thought to result from complementary feeding relationships among predators although, as at other trophic levels, evidence for complementarity is often indirect (but see Wilby *et al.* 2005; Straub & Snyder 2008). Within biodiversity studies, pathogens have generally been studied as responders

to diversity at other trophic levels (Keesing *et al.* 2006). For example, greater biodiversity among hosts can either increase or decrease community-wide infection levels, depending on the relationship between biodiversity and the abundance of highly susceptible hosts (e.g., Schmidt & Ostfeld 2001; LoGuidice *et al.* 2003; Dobson 2004; Rudolf & Antonovics 2005). We know of no studies that manipulate predator and pathogen diversity simultaneously. However, there is good reason to think that predators and pathogens might exert complementary impacts on herbivores, because herbivores deploy largely non-overlapping defenses against these two enemy classes. In response to predators, for example, herbivores often engage in avoidance behaviors (Lima & Dill 1990), or develop physical (Bernard 2004; Relyea 2003; Rinke *et al.* 2007) or chemical (Dyer 1995; Daly 1995) defenses. In contrast, the immune system repels pathogen attack (Wodarz 2007). Thus, predators and pathogens may form functionally distinct enemy classes, in that herbivores must deploy a different suite of defenses in response to each.

In a field experiment, we experimentally manipulated diversity across a community of predators and pathogens and measured the effects on herbivorous Colorado potato beetles and their host plants. We found that the strongest herbivore suppression was exerted by biodiverse natural enemy communities, an effect that cascaded to also benefit plants. However, herbivore suppression improved only when predators were paired with pathogens, and not when diversity was increased within either the predator or pathogen guilds. This suggests that predators and pathogens form distinct functional groups, with functional group diversity, rather than species richness *per se*, strengthening herbivore suppression. Because of the natural tradeoff in defenses deployed by herbivores in response to predators versus pathogens, we predict that the complementarity that we observed is widespread in nature.

METHODS

Natural history

The Colorado potato beetle (*Leptinotarsa decemlineata*) is a widespread herbivore of solanaceous plants, in both natural and agricultural systems, including potato (*Solanum tuberosum*). Potato beetle eggs, larvae and adults occur aboveground within plant foliage, while the last instar larva burrows into the soil to pupate, a lifecycle lasting roughly one month (Hare 1990). As potato beetles move between above- and belowground habitats they transition between two guilds of natural enemies, and our experiments included representatives of the major natural enemy types in both habitats. Aboveground, generalist predators dominate and include active searchers in the foliage and on the soil surface, represented in our experiments by the lady beetle *Hippodamia convergens* and the ground beetle *Pterostichus melanarius*, respectively, and sit-and-wait predators such as the damsel bug, *Nabis* spp. (Chang & Snyder 2004; Koss & 2005). Belowground, pathogens dominate, including entomopathogenic nematodes in the genera *Steinernema* and *Heterorhabditis* (we used *S. carpocapsae* and *H. marelatus*), and fungal pathogens (we used *Beauveria bassiana*) (R. A. Ramirez, personal observation). The nematodes enter its host through a natural opening and release a symbiotic bacterium that reproduces rapidly and kills the host; the nematode then reproduces and multiplies rapidly by feeding on the bacteria (Griffin *et al.* 2005). *Beauveria bassiana* can be ingested but infection usually occurs when a spore attaches to the host cuticle and germinates, producing toxins as the hyphae penetrate into the host (Boucias & Pendland 1991). As with the predators, these pathogen species span a diverse range of approaches to resource acquisition: *S. carpocapsae* is a sit-and-wait ambusher

whereas *H. marelatus* actively searches for hosts in the soil (Campbell & Gaugler 1997; Lewis 2002), while *B. bassiana* is dependent on passive transmission as burrowing larvae contact its spores. Thus, our natural enemy species pool included six species in total, three predators and three pathogens, with diverse hunting styles both within and among the predator and pathogen guilds.

Experiments

The field experiment was conducted at Washington State University's Research and Education Center in Othello, WA. Experimental units were cages constructed with plastic tubs (68 L; Sterilite Corp., Townsend, MA, USA) as the base, buried flush with the ground and filled with soil. These tubs were covered aboveground with PVC frames (0.61 m x 0.46 m x 0.30 m) enclosed with a mesh (voile) fabric screen, with a zipper on one side to allow access. To each cage we added two 2-week-old potato plants. We next added five potato beetle egg clutches (10 eggs per clutch) and 40 larvae (20 first-second and 20 third-fourth instar), collected from an adjacent potato field, to leaves of both plants in all experimental cages, allowed 24 hours for these beetles to acclimate to the cages, and then released predators and pathogens.

Natural enemy communities were assembled from our pool of six predator and pathogen species according to three diversity treatments: One Species (two replicates of each enemy in monoculture), Two Species (one replicate of each of the 15 unique species pairs) and Five Species (one replicate of each of the six unique draws of five species). The experiment also included six replicates of no-enemy controls, where neither predators nor pathogens were released. Natural enemy diversity was manipulated within a substitutive design. For predators,

density was 10, 5 and 2 individuals per species in One, Two and Five species treatments, respectively, within the range of typical field densities for these predators (Prasad and Snyder 2006; Snyder *et al.* 2006). Similarly, the nematodes were released at a rate of 25,000 nematodes per m^{-2}, and *B. bassiana* at a rate of 10^9 spores m^{-2}, in monocultures, with one half or one fifth these amounts released in Two and Five species treatments respectively. Pathogen densities were based on the results of field surveys of production potato fields, wherein rates of infection of sentinel potato beetle hosts by resident entomopathogens was c. 30% (R. Ramirez, unpublished data) – in preliminary experiments, the release rates that we used approximately reproduced this level of infection. By combining a substitutive design with the inclusion of all possible species compositions, and multiple species compositions, at each diversity level, we minimized the potential for sampling effects (Hooper *et al.* 2005).

Aboveground predators were field collected within 2 days of the initiation of the experiment using a D-vac suction sampler (Rincon Vitova, Ventura, California, USA), by hand, or using pitfall traps. Entomopathogenic nematodes were reared in the laboratory and *B. bassiana* was purchased (Mycotrol-O®, Emerald BioAgriculture, Lansing, Michigan, USA). All of the entomopathogens were applied to the soil using a spray bottle to evenly coat the area, and the cage was watered one hour prior to and after application to assist pathogens in moving into the soil. In addition, all cages were watered twice a week to maintain the high soil moisture typical of a production potato field.

After 28 days all potato beetles had reached adulthood or were in the penultimate pupal stage, and so the experiment was terminated. Each cage was hand-searched for 15 min to collect predators and adult beetles. Next, all aboveground plant material was harvested, after which surviving potato beetle pupae were destructively sampled by sifting all soil in each cage-base

through a screen (0.6-cm mesh; Forestry Suppliers Inc., Jackson, MS, USA). Harvested plant material was returned to the laboratory, dried for 7 days at 70 ºC, and weighed.

As a complementary laboratory experiment, we examined whether larvae that face the threat of predation are less-able to mount an effective immune response later in development. We adopted a commonly used insect immune response assay wherein a piece of nylon monofilament is surgically implanted into the insect; the insects' immune system responds to this foreign object by encapsulating it, with the degree of encapsulation providing a measure of immune capability (e.g., Rantala *et al.* 2000; Cotter *et al.* 2004; Kapari *et al.* 2006). This encapsulation response is also used to retard attack by entomopathogenic nematodes and fungi (Hajek & St. Leger 1994; Thurston *et al.* 1994). We transferred, singly, fourth instar potato beetle larvae into Petri dishes containing one potato leaf and a moistened dental wick, and also either a single *H. convergens* or *Nabis* spp. predator, or no predator (Control), with 6 replications per treatment. We used last-instar larvae because this stage is too large to be killed by these predators, eliminating the need to physically manipulate the predators to prevent predation (e.g., Schmitz *et al.* 1997; Nelson *et al.* 2004). After 48 hours, we implanted a nylon monofilament (2 mm long x 0.20 mm diameter, first rubbed with sandpaper) into each larva by using a syringe to create a tiny entrance hole on the dorsal side, between the pronotum and prothorax, and inserting the monofilament into the insect's hemocoel (see Kapari *et al.* [2006] for details of methodology). The larvae were kept at 18^{o} C for one hour, after which the nylon monofilament was retrieved through dissection, placed directly onto a slide, and photographed using an inverted light microscope. Portions of the filament that are encapsulated turn grey, and the proportion grey on each filament was measured using ImageJ 1.38x software (http://rsbweb.nih.gov/ij/index.html).

Analyses

For all analyses we took a conservative approach, averaging across the two replicate monocultures of each species, and also the six no-predator controls, to yield a single value for each unique species composition. All analyses were conducted using SYSTAT 11.0 software (SPSS, Chicago, IL, USA). The relationship between natural enemy biodiversity and final potato beetle density, and plant biomass, was assessed using linear regression. As a natural result of our design the Two Species treatment in the field experiment included pairs of two types: two species within the same natural enemy guild (either two predators or two pathogens), or a predator species paired with a pathogen species. We compared one- and two-guild pairs to the One Species (including, of course, a member of just one natural enemy guild) and Five Species (all including multiple representatives of both natural enemy guilds) treatments in ANOVA, followed by Tukey's posthoc test. For each of the two levels of guild diversity among natural enemy species pairs, we next compared observed beetle survivorship to that which would be expected based on the performance of constituent species in monoculture (i.e., we searched for over- or under-yielding). Our metric was D_T, a measure of non-transgressive overyielding (Loreau 1998). D_T is the proportional deviation of the ecosystem function of interest (herbivore suppression in this case) of polycultures from expected, based on the performance of constituent species when in monoculture, and is calculated using the formula (Petchey 2003):

$$D_T = \frac{\text{Observed}_{poly} - \text{Expected}_{poly}}{\text{Expected}_{poly}}$$

Within our design, D_T=0 indicates that natural enemy pairs performed as expected, and did not exhibit emergent biodiversity effects (Wardle *et al.* 1997; Hector 1998; Loreau 1998; Weis *et al.* 2007). Thus, we considered 95% confidence intervals that did not overlap with zero to be evidence for emergent positive or negative effects of natural enemy biodiversity.

Within our community of natural enemies interference among members of the natural enemy community is possible between predator species (Snyder & Ives 2001; Snyder *et al.* 2006), or if pathogens infect predators (Georgis *et al.* 1991; James & Lighthart 1994). We examined for interference among natural enemies by comparing predator recovery rates across all diversity levels, and between predator-only and predator-pathogen pairings within the Two Species treatement, using ANOVA.

For the laboratory experiment, we compared melanization rates across treatments using one-way ANOVA, followed by Tukey's posthoc test.

RESULTS

Beetle survival to the end of the experiment decreased with increasing natural enemy species richness (R^2 = 0.45, P = 0.0001; Fig.1a). This strengthening of herbivore suppression indirectly benefitted plants, with plant biomass increasing with greater natural enemy biodiversity (R^2 = 0.14, P = 0.049; Fig.1b). However, among natural enemy species pairs there was a great deal of variability in final potato beetle densities (Fig. 1a), and we next separated these data into pairings within (either predator-predator or pathogen-pathogen species pairs) versus between (predators + pathogens) natural enemy guilds, and compared the mean response of each to beetle densities in

communities including one and five natural enemy species (Fig. 2). These groupings significantly differed in the degree of beetle suppression that they exerted ($F_{3,29} = 9.69$, $P = 0.0001$). For pairings within a single natural enemy guild there was no improvement in beetle suppression as diversity increased from one to two natural enemy species ($P = 0.99$; Tukey's posthoc test). In contrast, beetle densities in predator-pathogen pairs were significantly lower than in natural enemy monocultures ($P = 0.02$), an effect that was not further significantly strengthened when multiple predator and pathogen species were included ($P = 0.31$, comparison of predator-pathogen pairs to Five Species).

Consistent with these findings, there was no evidence for overyielding by species pairs within a single natural enemy guild, with a mean response near zero (Fig. 3). In contrast, two-species communities that paired a predator and pathogen species consistently exerted stronger beetle suppression than would be predicted by the performance of constituent species in monoculture, as for the predator-pathogen pairs 95% confidence intervals did not overlap with zero (Fig. 3).

The proportion of predators recovered at the end of the study did not differ across diversity treatments ($F_{2,18} = 0.235$, $P = 0.793$; Fig. 3a), or between predator-predator versus predator-pathogen species pairs in the Two Species treatment ($F_{1,19} = 0.25$, $P = 0.623$; Fig. 3b).

For the laboratory immune-response experiment, the presence of predators significantly altered encapsulation rates ($F_{2,15} = 3.83$, $P = 0.045$; Fig. 4). The presence of both *H. convergens* and *Nabis* predators reduced later melanization of implanted filaments compared to that seen in the no-predator control ($P < 0.05$, Tukey's posthoc test, for both comparisons; Fig. 4), indicating that this cellular immune response was suppressed following exposure to these predators.

DISCUSSION

We found clear evidence that herbivore suppression strengthened with greater natural enemy diversity (Fig. 1A), an effect that cascaded to also benefit plants (Fig. 1B). Our experiments included two spatiotemporally and ecologically distinct guilds of natural enemies, predators and pathogens, with obvious niche differences between members of these two guilds. However, species within these guilds also differed in ecologically important ways, hunting styles for example, that could have led to niche differentiation at a finer scale. Thus, the improved herbivore suppression that we observed might have resulted from greater guild diversity, greater species diversity *per se*, or both, at higher levels of natural enemy species richness. Our design included all possible species pairs from our pool of six natural enemy species, and there was marked variation in response within this treatment (Fig. 2). Pairs could bring together either two members of the same natural enemy guild (either two predator species, or two pathogen species), or pair a predator with a pathogen species. For intraguild pairings there was no strengthening of herbivore suppression when moving from one to two enemy species, while, in contrast, herbivore suppression was dramatically stronger when predators and pathogens were paired (Fig. 2). Indeed, suppression was not significantly further strengthened when multiple predator and pathogen species were included within five-species communities (Fig. 2). Thus it was greater guild diversity, rather than greater biodiversity *per se*, that strengthened herbivore suppression. Apparently, predators and pathogens form distinct functional groups that exert complementary impacts on herbivores. The mechanism underlying this effect of guild diversity must be quite general, because predator-pathogen complementarity occurred across a broad range of species compositions (Fig. 1A).

Calculations of our overyielding metric, D_T, further strengthen the conclusion that predators and pathogens form complementary functional groups. For our experiment, the null expectation in the absence of emergent biodiversity effects is $D_T = 0$, meaning that the degree of herbivore suppression exhibited by diverse polycultures equals the average response across monocultures of the constituent species. Indeed, for single-guild species pairs D_T values averaged near zero (Fig. 3). However, for mixed-guild species pairs D_T values were strongly negative (Fig. 3), indicating that suppression exceeded what would be predicted based on monoculture performance. Thus, we again found evidence that predators and pathogens formed complementary functional groups, with herbivore suppression improving with greater biodiversity only because guild and species diversity were confounded.

For predators, interference among consumer species can lead to a negative relationship between biodiversity and herbivore suppression (Finke & Denno 2004; Casula *et al.* 2006). In this community intraguild predation was possible, with predators feeding on one another (Snyder & Ives 2001; Snyder *et al.* 2006), as was interguild predation, with pathogens attacking the predators (Georgis *et al.* 1991; James & Lightheart 1994). However, consistent with stronger herbivore suppression by more species-rich communities, in our field experiment we found no evidence for interference among natural enemies: predator survivorship was unchanged with greater natural enemy diversity (Fig. 4A), and did not differ between predator-predator and predator-pathogen species pairs (Fig. 4B). The latter result is consistent with a study reporting relatively weak impacts of soil-dwelling pathogens on aboveground predators, despite strong impacts on sympatric herbivores with belowground stages (Georgis *et al.* 1991). Thus, we found no evidence that negative interactions among enemy species grew with greater biodiversity.

Theory predicts that predator species that partition their attacks among different components of the prey community are particularly likely to complement one another (Wilby & Thomas 2002; Ives *et al.* 2005; Casula *et al.* 2006); resource-niche partitioning of this type is also thought to underlie biodiversity-resource consumption relationships for species at other trophic levels (Hooper *et al.* 2005). Empirical evidence from predator communities, while sparse, is beginning to accumulate support for this prediction. For example, natural enemies that partition their attacks among different life stages of the rice leaf-folder (*Marasmia patnalis*, a moth) exert complementary mortality, leading to a positive relationship between enemy diversity and herbivore suppression (Wilby *et al.* 2005). Similarly, for aphid predators on *Brassica oleracae* plants, the combination at high diversity levels of predators that differ in where they hunt in plant foliage strengthens aphid control (Straub & Snyder 2008). Further, predators that use different microhabitats are less likely to encounter and interfere with one another than are predators that use the same microhabitat (Schmitz 2007). Our predator-pathogen system combined attributes of all of these examples of natural enemy complementarity, with predators and pathogens differing in both the potato beetle stages attacked and in their physical location within the environment. Thus, niche separation on both axes may have contributed to the predator-pathogen complementarity that we observed.

Our laboratory experiment provided preliminary evidence for niche partitioning of another type, based not on partitioning by prey stage or spatial location, but rather on the differing defenses that the herbivore must deploy in response to predator versus pathogen attack. Potato beetle larvae that had previously been exposed to predators exhibited a weakened immune response when challenged with a foreign body (a surgically implanted piece of nylon thread; Fig. 4). This assay quantifies the cellular immune response that potato beetles use to fight off attack

by entomopathogenic nematodes and fungi (Hajek & St. Leger 1994; Thurston *et al.* 1994). To repel predators, in contrast, potato beetles exude, from a series of dorsal glands, an autogenous dipeptide that is toxic to predators (Daloze *et al.* 1986). For the Colorado potato beetle replenishing the defensive toxin can take c. 1 week (Daloze *et al.* 1986), suggesting that there is a substantial physiological cost associated with production of this chemical (as is the case for toxins produced by other beetle species [Grill & Moore 1998; Labeyrie *et al.* 2003]). Thus, repeated deployment of the chemical defense against predators likely reduces resources available to mount an effective immune response (e.g., Rigby & Jokela 2000). This physiological tradeoff also could explain how predator-pathogen complementarity spans the spatiotemporal separation between these two natural enemy guilds.

It now is clear that biodiversity mediates the impact of pathogens on communities. For example, in both mammalian and plant communities, the addition of relatively resistant species can slow disease transmission (Keesing *et al.* 2006). Also, by inducing apparent competition, pathogens can speed species invasions and alter regional biodiversity (Torchin & Mitchell 2004). Thus, pathogens both respond to and impact biodiversity at other trophic levels. The work reported here demonstrates that pathogens themselves also constitute an important functional component of natural enemy biodiversity. Broadly, the defenses that prey/hosts use to repel predator attack – shifting habitat use (e.g., Schmitz *et al.* 1997), deployment of toxic chemicals (Dyer 1995), physical defenses (Rinke *et al.* 2007), etc. – are often different than and non-overlapping with the immune responses (Wodarz 2006) deployed in response to attack by pathogens. Thus, the predator-pathogen complementarity that we found is likely to be common in nature. Combined with, for example, complementary dynamical impacts of these two natural

enemy guilds (Dwyer *et al.* 2004), predator-pathogen complementarity may play a key role in strengthening resource (prey/host) exploitation by bio-diverse natural enemy communities.

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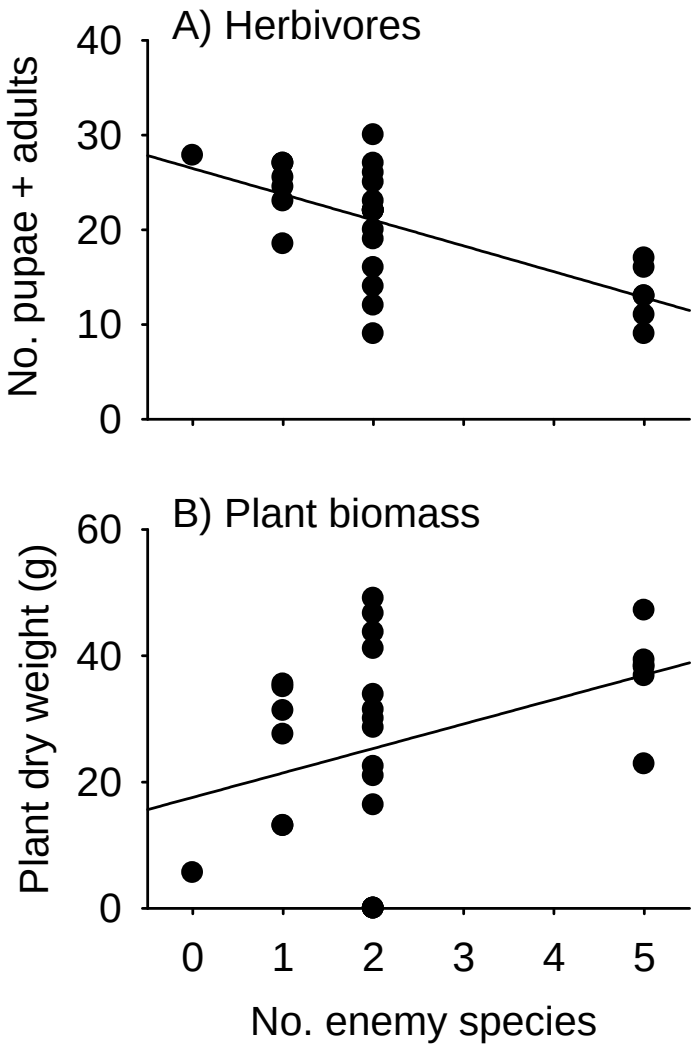

Figure 1. At the end of the field experiment, (a) potato beetle survivorship and (b) plant biomass, versus natural enemy species richness.

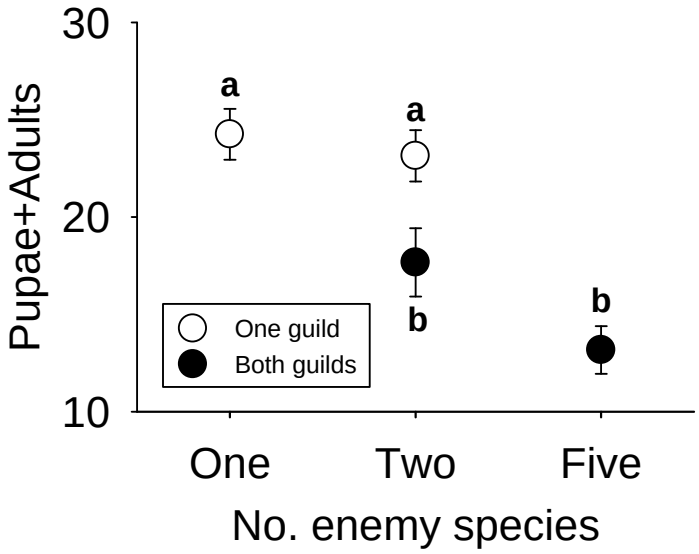

Figure 2. Potato beetle survivorship by species/guild diversity groupings. Data are means $\pm$ 1 S.E.; letters indicate significant differences among groups ($P < 0.05$, Tukey's posthoc test).

Figure 3. Overyielding (D_T) values for species pairs including one versus both natural enemy guilds. The dashed line indicates zero; data are means ± 95% confidence intervals.

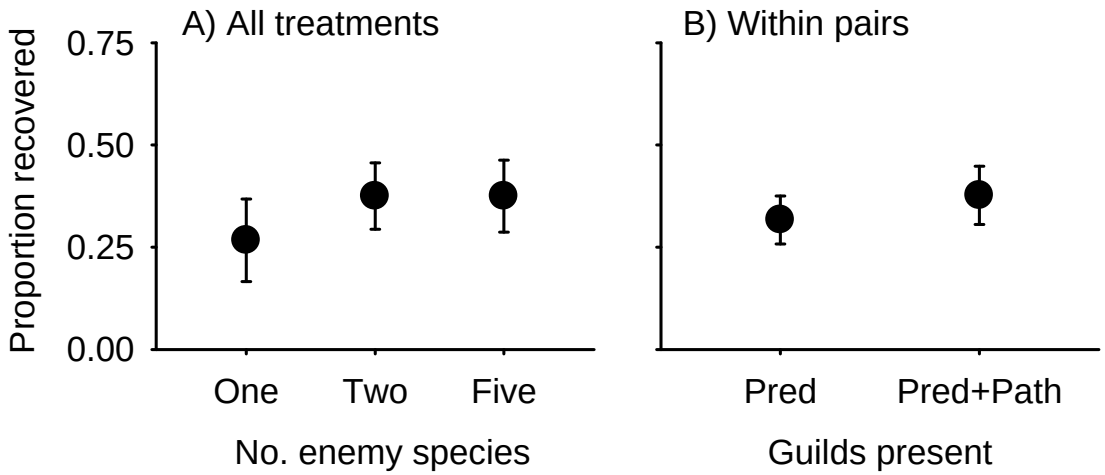

Figure 4. Proportion of predators recovered (a) across levels of natural enemy species richness, and (b) in the presence (Pred+Path) versus absence (Pred) of pathogens. Data are means $\pm$ 1 S.E.

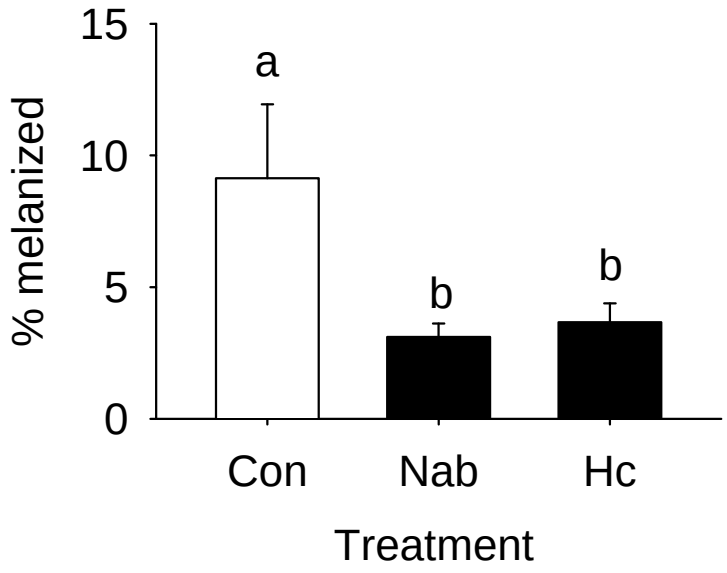

Figure 5. Proportion melanization of a surgically-implanted filament in potato beetle larvae earlier exposed to predators. Predator treatments: Con, no-predator control; Nab, *Nabis* spp. predator; Hc, *Hippodamia convergens* predator. Data are means $\pm$ 1 S.E.

CONCLUSIONS

Growers are implementing multiple strategies to improve crop health and control different pests in the soil. However, it is unclear how crop and pest management impact biological control by entomopathogens.

In the first study, sentinel insect prey were used to measure entomopathogen activity in commercial fields that did or did not utilize mustard green manures. Rates of entomopathogen infection on average were lower in fields where mustard bio-fumigants were applied, compared to those not receiving mustard bio-fumigants. In laboratory bioassays, extracts from two mustard cultivars differing in glucosinolate levels were tested against the abilities of a diverse group of EPN species to infect insect hosts. EPN infection rates were lower in laboratory arenas receiving mustard extracts than the water control, and lower still when EPNs were exposed to extracts from plants with high versus low glucosinolate levels. These results suggest that the use of mustard bio-fumigants for the control of plant parasitic nematodes has the potential to interfere with the biocontrol of insect pests using entomopathogens.

In the second study, the effects on *Heterorhabditis marelatus* and *Steinernema carpocapsae* EPNs of a mixture of chicken and cow manure were compared to those of the synthetic dry formulated complete fertilizer. EPN infection rates were lower, and EPN persistence shorter, in plots receiving animal manure than in those receiving synthetic fertilizer. There was no evidence that resident entomopathogens, soil pH, or soil moisture contributed to the negative effect of animal manure on EPNs. However, microbial activity was greater in manure than synthetic fertilizer plots. These results suggest that biotic resistance from increased microbial activity and not competition by resident entomopathogens, or altered moisture levels or soil chemistry, reduced EPN effectiveness in plots treated with animal manure. Consequently,

there may be a conflict between using animal manures to manage soil fertility, and EPNs as insect biocontrol agents.

Conservation of a diverse community of natural enemies has been achieved by reducing broad-spectrum pesticide use. In the third study, we manipulated predator-entomopathogen species richness to test a) if Colorado potato beetle control strengthens with increasing natural enemy diversity and b) if aboveground predators interact with belowground entomopathogens. Potato beetle densities decreased, and plant biomass increased, with greater enemy biodiversity. However, potato beetle control strengthened only for pairings of predators with entomopathogens, and not for pairings within the same natural enemy guild. Thus, predators and entomopathogens exerted complementary impacts, perhaps due to a tradeoff for the potato beetle in defenses against these two natural enemy guilds.

The research presented here examined the effects on insect control by entomopathogens of mustard bio-fumigation, animal manure application, and the conservation of aboveground predatory insects. Overall, these studies suggest that there are complex interactions between these management practices and biological control using entomopathogens. Conservation of natural enemies, including entomopathogens, is pivotal to the overall success of biological control, whereas mustard biofumigation and the use of animal manures as fertilizers both reduce the success of entomopathogens as biocontrol agents.