MULTIFACETED BIOCONTROL METHODS AGAINST THE COLUMBIA ROOT KNOT

NEMATODE, *MELOIDOGYNE CHITWOODI*, AND THE COLORADO POTATO BEETLE,

LEPTINOTARSA DECEMLINEATA, PESTS OF POTATOES IN WASHINGTON STATE

By

DONNA RENEE HENDERSON

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To the faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of DONNA RENEE

HENDERSON find it satisfactory and recommend that it be accepted.

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Chair

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Multifaceted biocontrol methods against the Columbia Root Knot nematode, *Meloidogyne chitwoodi*, and the Colorado Potato Beetle, *Leptinotarsa decemlineata*, pests of potatoes in Washington State

ABSTRACT

by Donna Henderson, Ph. D

Washington State University

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Chair: Ekaterini Riga

Mustard seed meal from *Brassica carinata* has shown both nematicidal as well as insect oviposition deterring properties against the root knot nematode *Meloidogyne chitwoodi*, and the Colorado Potato Beetle (CPB), *Leptinotarsa decemlineata*, respectively. In addition, entomopathogenic nematodes (EPN) have shown biocontrol potential against both CPB and *M. chitwoodi*. The objectives of this research were to 1) determine whether entomopathogenic nematodes can reduce *M. chitwoodi* populations, 2) determine whether entomopathogenic nematodes can infect CPB larvae, 3) determine whether mustard seed meal can decrease *M. chitwoodi* populations, 4) determine whether the two methods (mustard seed meal and EPN) can be used simultaneously against both the CPB and *M. chitwoodi,* and 5) determine if the addition of mustard seed meal amendment deters CPB oviposition. Field and greenhouse trials were conducted in WSU Prosser, WA and WSU Pullman, WA in 2006 and 2007. Mustard seed meal

alone, EPN, and combination of mustard seed meal and EPN significantly reduced the percent culled potato tubers, percent infection, and infection index due to *M. chitwoodi* in field trials. In the greenhouse trials, mustard seed meal, *S. feltiae* and *S. riobrave*, and treatment combinations of mustard seed meal with species of EPN reduced *M .chitwoodi* infection significantly. Both species of EPN were able to infect CPB larvae in the field and greenhouse, regardless of mustard seed meal amendment. The oviposition rates of CPB in the field were reduced significantly on mustard seed meal amended plants compared to the untreated control. In the greenhouse there were no effects of mustard seed meal amendment on CPB plant choice. EPN and mustard seed meal have shown promising potential as biological control methods to control both the above ground herbivorous insect pest, CPB, and the below ground nematode pest of potato tubers, *M. chitwoodi*. This multifaceted approach will provide potato growers with new options for sustainable control of both the root knot nematode and the insect pest.

LIST OF FIGURES

LIST OF TABLES

DEDICATION

To my husband and sons, Amukela, Ayize, and Musa Gwebu. And to my mother Deanna, who was the first scientist and inspiration in my life.

TABLE OF CONTENTS

General Introduction

The Colorado potato beetle, *Decemlineata leptinotarsa,* and the Columbia root knot nematode, *Meloidogyne chitwoodi,* are common and important pests of potato in Washington State. This dissertation addresses the biocontrol potential of *Brassica carinata* seed meal and entomopathogenic nematodes as treatments against both pests, and the interactions observed in using the two biocontrol methods together. Chapter one contains a literature review addressing the biology, chemical control, and current biocontrol research for each pest. Chapter two is a publication article formatted for submission to the peer-reviewed journal Biological Control, and is entitled "Biocontrol potential of *Brassica carinata* seed meal and entomopathogenic Nematodes against the Columbia Root Knot Nematode, *Meloidogyne chitwoodi*, and the Colorado potato Beetle, *Leptinotarsa decemlineata*, pests of potatoes in Washington State". Chapter three is a publication article formatted under the ESA style guide for submission to the peer-reviewed journal Economic Entomology and is entitled "The effect of *Brassica carinata* mustard seed meal amendment on oviposition and plant choice of Colorado Potato Beetle (Coleoptera: Chrysomelidae). For all chapters with multiple authors all writing, experimental work, and conception of ideas were done by the first author, Donna Henderson. Ekaterini Riga, supervisor, and William E. Snyder, co-supervisor, contributed as advisors for experimental design and analysis.

CHAPTER ONE

Literature Review

Meloidogyne chitwoodi

Biology

Meloidogyne chitwoodi (Golden, O'Bannon, Santo, and Finley), commonly called the Columbia root knot nematode is a plant parasitic nematode of potato, *Solanum tuberosum*, L. This endoparasite damages the tuber, causing warting on the tuber skin and necrotic spots within the tuber (Strand et al., 1986). These symptoms decrease the marketability of the tubers as the potato processing industry has a tolerance of less than ten percent infection (culls) caused by the root knot nematode (Santo et al., 2003). Potato is among the most important agricultural crops grown in the Pacific Northwest (PNW) and is a good host for *M. chitwoodi* (Karssen and Moen, 2006). Processors have stringent restrictions for *M. chitwoodi* tuber infection, requiring farmers to abide to a low economic threshold of one nematode per 250 cc soil (Cram et al., 2007). This threshold is due to the high reproductive potential of *M. chitwoodi*, which makes this nematode expensive and hard to control.

The high reproductive potential of *M. chitwoodi* in the Pacific Northwest is in part due to its ability to survive low temperatures, a characteristic of other cool climate root-knot nematodes (Santo and O'Bannon, 1981). Not only can the nematode survive the winter well, but it can also continue reproduction at lower temperatures than can other species of root knot. The base temperature at which *M. chitwoodi* development can occur is 5 °C (Santo and O'Bannon, 1981).

In the Pacific Northwest farmers typically plant potatoes in April, when soil temperatures begin to warm up to 5 °C, allowing the nematode to resume its lifecycle and infect potato plants early in the season compared to other cool climate nematodes (*M. hapla, M. nassi*) (Santo and O'Bannon, 1981). *Meloidogyne chitwoodi* begins its development very early in the potato growing season therefore it is possible to have 5-6 reproductive cycles before harvest (Santo and O'Bannon, 1981). Not only can *M. chitwoodi* reproduce well in cooler weather, but it also has developed survival strategies to avoid chemical fumigants. *Meloidogyne chitwoodi* can migrate down one meter in the soil (Pinkerton et al., 1987), too deep to be reached by fumigants, and then later migrates upwards quickly to the rhizosphere where the population of *M. chitwoodi* normally resides.

Meloidogyne chitwoodi has a lifecycle strategy that enables re-infection of the plant host. The infection of the host begins when the J2 infective stage of the nematode penetrates the roots. Once inside the host, the J2 juvenile establishes feeding sites in the plant roots or in the tuber. *Meloidogyne chitwoodi* may penetrate the growing root tips and migrate to the zone of elongation or may enter through the lenticel openings of potato tubers to the vascular ring (Karssen and Moens, 2006). Once a feeding site has been established the female remains sedentary, creating a multinucleate giant cell for sustained feeding. Giant cells result from nematode salivary secretions of J2 during feeding (Karssen and Moens, 2006). The J2 feed on the stele region, and several cells in the pericycle begin to enlarge (hypertrophy) and become multinucleated and coenocytic (nuclear division without cell wall formation). Two to twelve giant cells are produced per nematode head (Karssen and Moens, 2006). After giant cells form the J2 undergoes 2nd and 3rd molts within 2-3 weeks. The 2nd molt becomes a J3 stage, and the 3rd molt becomes the J4 stage. During the 3rd and 4th molts, no feeding occurs. At the 4th molt

stage the sex of the nematode can be distinguished. The J4 stage males mate with the female or exit the root, but males are rarely detected in natural populations and most females reproduce through parthenogenesis (Karssen and Moens, 2006). The J4 stage female remains sedentary in the root and feed off the giant cells, and 7 days later a gelatinous egg sac is produced outside her body. After egg production the female ceases feeding. Eggs hatch in about 13 days at temperatures around 24 ºC (Santo et al., 1994), and the lifecycle is repeated.

Chemical Control

Columbia root knot nematode infestations are most commonly controlled through the use of fumigants and nonfumigants such as Telone II (1,3 dichloropropene) and/or Vapam (sodium methyldithiocarb) applied in the fall followed by Mocap (ethoprophos), applied in the spring prior to planting potatoes to reduce populations of plant parasitic nematode. The cost for Telone II is approximately $252/ acre, versus $70/ acre for Vapam, and $250/ acre for Mocap (John Wilson, personal communication). The total cost for potato production is $1500/acre (John Wilson, personal communication), with nematode control comprising roughly one-third of the cost of potato production. Chemical fumigants or nonfumigants are also toxic to humans and environments where applied and can also harm the beneficial organisms which contribute to soil health (Haydock et al., 2006). Additionally, the frequent use of metam sodium has led to its bio-degradation in the soil (Karpouzas et al., 2002) which along with the harmful impacts of fumigant use, has lead to discussions that these chemicals may be phased out in the future (Noling, 2002).

Entomopathogenic Nematodes

Biology

Entomopathogenic nematodes (EPN) are parasites of insect larvae in the soil (and, less commonly in some adult insects) which enter the larvae through natural openings of the anus, mouth or spiracles (Grewal et al., 1997a; Poinar, 1979). EPN are obligate parasites, with only one stage, the third juvenile, capable of residing outside of the insect in the soil prior to infection. EPN share a highly specialized symbiotic relationship with the bacterium in their gut (Boemare, 2002). Specific genera and species of bacteria and nematodes occur together. The bacterium, *Xenorhabdus* spp. is found in Steinernematids and resides in the anterior part of the intestine. *Photorhabdus* spp. is found in Heterorhabditids, and resides in the intestinal tract (Boemare, 2002). Once the entomopathogenic juveniles have entered the hemoceol of the insect larvae they will release the bacteria through the anus (Steinernematids) (Poinar, 1996) or through the mouth (Heterorhabditids) (Ciche and Ensign, 2003). The bacteria then begin to multiply and release chemicals (indole/stilbene derivatives; xenocoumacins; xenorhabdins; ammonia) to rapidly kill the host and prevent colonization of the insect cadaver by other organisms (Akhurst and Boemare, 1990). The EPN itself will also release toxins inside the host (Burman, 1982), and will then feed on the bacteria and the host tissue and complete 1-3 reproductive cycles within the host until resources are depleted. The EPN then exits as a third stage infective juvenile. In order to complete reproduction the amphimictic Steinernematids require a male and female juvenile to enter the host larvae, while the Heterorhabditids are hermaphroditic. The host range of EPN is quite broad in laboratory EPN (Poinar, 1986). However, ecological position and foraging behaviors narrow the host range significantly in the field (Campbell and Gaugler, 1993).

Suppression of Plant Parasitic Nematodes

Allelopathy is the suppression of the root knot nematode through the nearby release of toxins from the entomopathogenic nematodes or their bacterial symbionts. Allelopathic suppression of root-knot nematodes using entomopathogenic nematodes has been successful in some cases. Root knot nematodes and entomopathogenic nematodes occupy the same space in the soil, but otherwise do not share similar life cycles. The suppressive interaction observed between the root knot nematode and EPN has been hypothesized to occur in three ways (Grewal et al., 1999): 1) suppression of root knot nematodes through physical presence and competition for space (Bird and Bird, 1986), 2) allelochemicals released by the EPN or its bacterial symbiont which repel or suppress plant parasitic nematodes (Hu et al., 1995), or 3) application of EPN results in increased nematode antagonists in the soil (Ishibashi and Kondo, 1987; Ishibashi and Choi, 1991). In the laboratory, the EPN has the ability to infect an insect host which otherwise is not a host in the field due to proximity. However, in the field these same EPN will target insects at different zones in the soil depending on EPN searching behavior (Campbell and Gaugler, 1993). There are three types of searching behaviors: ambushers, cruisers, and ambushers/cruisers. The ambushers reside in the top soil, awaiting mobile insect prey, while the cruisers migrate further down around the rhizosphere, and the ambushers/cruisers employ both searching methods (Campbell and Gaugler, 1993). These differences in searching behaviors must be considered when targeting a plant parasitic nematode population that resides around the rhizosphere.

Root knot nematodes reside around the rhizosphere during the growing season, proximal to the cruisers or cruiser/ambushers such as *S. glaseri, S. riobrave, S. feltiae,* and *H. bacteriophora*. Several of these nematodes have been tested on other root knot nematodes (*M.*

hapla, *M. incognita*) with relative success (Perez and Lewis, 2004; Perez and Lewis, 2001; Lewis et al., 2001). In particular, *S. feltiae* has been shown to negatively affect *M. incognita* root penetration and egg hatch. Filtrates from the symbiotic bacterium *Xenorhabdus* repel *M. incognita,* or directly killed them upon exposure (Grewal et al., 1999). Evidence suggests that the suppression of plant parasitic nematodes by entomopathogenic nematodes can be explained by the role of the bacterial symbionts as a toxic repellant to the plant parasitic nematode. Among these chemicals, the stilbene derivatives and ammonia produced by the bacteria are selectively nematicidal (Hu, et al., 1995). In addition, bacterial filtrate toxicity tests indicate a stronger toxicity towards the juvenile stage than the egg stage (Grewal et al, 1999). Grewal and co-authors (1999) found that the release of live entomopathogenic nematodes did not suppress *M. incognita* root penetration. Therefore, the suppression of plant parasitic nematodes with live entomopathogenic nematodes may be challenging (Grewal, et al, 1999). This research favors allelopathy as the hypothesis for plant parasitic nematode suppression. There is research to support live entomopathogenic nematodes as the cause of plant parasitic nematode suppression (Grewal, et al., 1997b), but it is also hypothesized that the release of allelochemicals from cadavers of inundatively applied entomopathogenic nematodes may be the cause for the decline in plant parasitic nematodes. For example, in studies of the symbiotic bacterium and the EPN, bacterial symbionts caused 98-100% mortality to *M. incognita* at 15% concentration *Xenorhabdus* spp. (Grewal et al., 1999). The suppressive effect of the bacteria was shown to be due to the ammonium hydroxide produced by the bacterial symbiont; bacterial ammonium hydroxide fractions had the same suppressive effect as the bacteria alone (Grewal et al, 1999). However, there are also three antibiotics which have been isolated from *Xenorhabdus* and *Photorhabdus* bacteria: indole/stilbene derivatives (Paul, et al., 1981; Richardson, et al., 1988),

xenocoumacins (Paul et al., 1981) and xenorhabdus (McInerney et al., 1991 a, b). These antibiotics are important for maintaining the nematode/bacteria relationship so that the bacteria can remain the only microbe able to colonize the nematode or the insect cadaver (Boemare, 2002). It is possible that the cadavers of infected insects may slowly release allelochemicals into the rhizosphere during the infection period (Jagdale et al., 2002), and this time period of release may be governed by the biology of the specific entomopathogenic nematode. For example, *S. feltiae* requires nine days to complete reproduction after insect infection in comparison to *Heterorhabditis bacteriophora,* which averaged a seven day time period before juvenile emergence (Unlu and Ozer, 2003). The discrepancy in reproductive time periods between species or genera of entomopathogenic nematodes may result in differing time periods of allelochemical release into the rhizosphere. The extended periods of allelochemical release from some species or genera of entomopathogenic nematodes but not others may offer an explanation for the varied results of previous studies entomopathogenic nematode species (*S. feltiae, S. riobrave, S. glaseri, H. bacteriophora*) for their ability to suppress plant parasitic nematodes (Perez and Lewis, 2004; Perez and Lewis, 2001; Lewis et al., 2001).

Infection of Insects

Both Heterorhabtids and Steinernematids have wide host ranges across several families of insects under laboratory conditions (Kaya and Gaugler, 1993; Poinar, 1986). However, these nematodes are soil-borne, and target insects which reside or have a lifecycle stage in the soil habitat. Therefore, the host range of the EPN narrows considerably in the field (Akhurst, 1990; Bathon, 1996; Georgis et al., 1991; Peters, 1996). There are numerous species of

entomopathogenic nematodes that have been found worldwide. However, only a few have been selected for mass production for insect pests. These include the widely used *S. carpocapsae* which was among the first entomopathogenic nematodes investigated for biological control of the Colorado potato beetle (CPB) (Stewart et al, 1998; Veremchuk, 1974) and numerous other insects. Other commercially produced entomopathogenic nematodes include *S. glaseri, S. feltiae, S. riobrave, S. scapterisci, H. bacteriophora, H. megidis,* and *H. marelatus.* Several genera and species of EPN are effective against the CPB, such as *H. marelatus* (Armer et al., 2004), although this nematode had limited reproduction in the beetle in the field (Armer et al., 2004). Other successful nematodes against CPB include *S. feltiae* and *S. glaseri* (Toba et al., 1983; Wright et al., 1987) and the most common commercially produced EPN (Armer et al., 2004; Berry and Reed, 1997; Wright et al., 1987; Nickle and Cantelo, 1992; Nickle et al., 1994).

Green Manures and Seed Meal

Biofumigation Mechanism

Brassica spp. such as *B. carinata, B. napus* (canola, rapeseed), *B. alba* (white mustard), *B. hirta* (yellow mustard), and *Sorghum* spp. such as *Sorghum vulgare var. sudanenseare* (Sudangrass) are till-under crops that can be incorporated into the soil to release active nematicidal compounds (Brown and Morra, 1997; Riga et al., 2003). *Brassica* plants contain inert levels of the glucosinolate compounds and the enzyme myrosinase in their plant tissue (Brown and Morra, 1997). Breaking the plant tissue via tillage in the presence of water brings the two compounds together to complete a hydrolysis reaction, where the enzyme myrosinase acts to hydrolyze the available glucosinolates (Figure 1) (Bones and Rossiter, 1996). Seed meals work through the same mechanism. However, the source product is not the foliar plant tissue but the

defatted (oil extraction) seed waste product of the plant. For both green manures and seed meals the resulting products are isothiocyanates, nitriles, epithionitriles, and thiocyanates (Brown and Morra, 1997) which have been found to have fungicidal, herbicidal and nematicidal properties (Riga and Collins, 2005; Riga et al., 2003). Some glucosinolate products are systemic in the plant, translocated through the plant from the amended soil. In particular, the thiocyanate ion SCN^- produced by hydrolysis of 4-OH benzyl glucosinolate in *Sinapis alba* is one of the most important systemically translocated products. Indole glucosinolates in *B. napus* tissues will also produce SCN^- (Dr. Matt Morra, University of Idaho; personal communication).

Nematode Biofumigation

Green manures and seed meals have become more commonplace as alternative methods of nematode control (Ekaterini Riga, personal communication). There have been many successful studies of nematode biofumigation, with most success coming from cultivars with certain types of glucosinolate byproducts. Hydrolysis products of glucosinolates were tested on *M. javanica*, and it was found that of the many glucosinolate products, 2-phenylethyl, benzyl, 4-methylthiobutyl, and prop-2-enyl isothiocyanate showed the highest nematicidal activity, with an LD50 at concentrations of 11, 15, 21, and 34 M (Lazzeri et al, 2004). In another study, 2-propenyl (sinigrin) from cultivar *B. juncea* significantly reduced *M. javanica* survival at a concentration of 2.82 μmol ml^{-1} (Zasada and Ferris, 2004). Other Brassica green manures include a rapeseed cultivar (Jupiter) that was found to reduce *M. chitwoodi* nematode populations significantly (Mojtahedi et al., 1991). Non-brassicaceous green manures that have been successful are cultivars of Sudangrass. Specifically, *S. vulgare var. sudanenseare* (Sudangrass)

cultivars Trudan 8 and Sordan 79 were effective at reducing field populations of *M. chitwoodi* (Mojtahedi et al., 1993).

Figure 1. The myrosinase-glucosinolate reaction resulting in isothiocyanates, nitriles, and thiocyanate products.
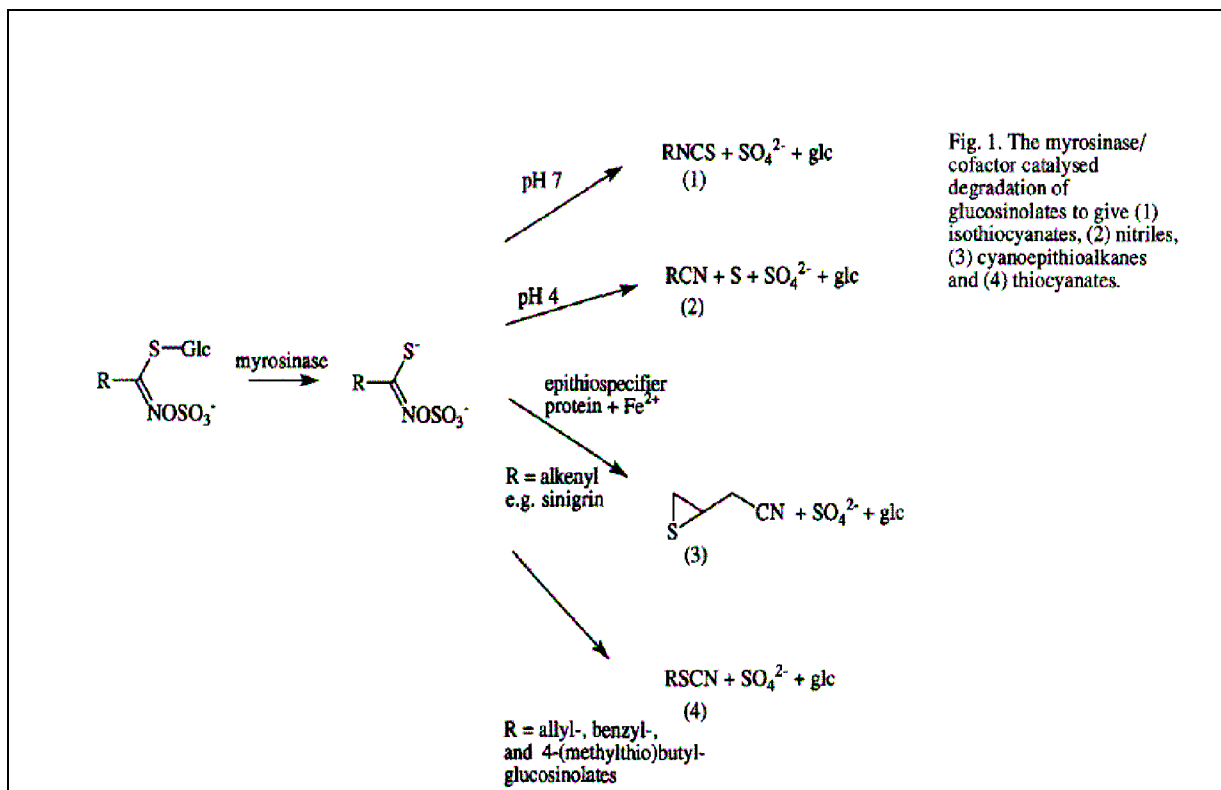
Fig. 1. The myrosinase/cofactor catalysed degradation of glucosinolates to give (1) isothiocyanates, (2) nitriles, (3) cyanoepithioalkanes and (4) thiocyanates.

Figure adapted from Bones and Rossiter, 1996.

Control options for the Columbia root knot nematode are limited by economic viability. The majority of commercial potato growers rely on chemical nematode control despite the high cost because of its consistency and effectiveness (John Wilson, personal communication). The growers are often not interested in biocontrol or green manure methods in comparison to the familiarity and ease of chemical control (John Wilson, personal communication). However, in the case of organic growers, alternative control options are of interest because of the limited availability of control methods that meet the regulations required of organic growers (Ekaterini Riga, personal communication). Nonetheless, some conventional growers may be interested in combining biocontrol methods with synthetic nematicides or with green manures or seed meals. As of yet, neither entomopathogenic nematodes, green manures, nor seed meals have been tested as biocontrol options specifically against *M. chitwoodi*.

Entomopathogenic nematodes range in price depending on the type. For example, at a rate of 7.5 billion/ ha, *S. carpocapsae* (Millenium®) can cost $100/ ha, *S. feltiae* (Nemasys®) can cost up to $500/ ha and *S. riobrave* (BioVector®) can cost up to $412/ ha (Greg Wahl, BeckerUnderwood, 2007). Considering that the cost of applying EPN is comparable to root knot nematode synthetic nematicide costs, more research must be done to validate the use of biocontrol options such as entomopathogenic nematodes, green manure crops, and seed meals against *M. chitwoodi* to ensure that the control option can be effective as well as economical.

Decemlineata leptinotarsa

Biology

Colorado Potato Beetle (CPB), *Decemlineata leptinotarsa* (Say) (Coleoptera: Chrysomelidae) is an economically important insect pest of potato crops throughout the United States and Canada (Pedigo, 2002). In addition, CPB was recently introduced as a pest of potato to Europe (Pedigo, 2002). CPB adults measure 9.5 mm long and 6.4 mm wide, with ten black stripes lengthwise of the body on yellow or orange elytra (Pedigo, 2002). Eggs are laid in egg clusters on the underside of potato leaves. There are four stages of larvae developing from eggs into 1st to 4th instars (Pedigo, 2002). Larvae are red or orange with rows of black spots along the sides of the body. Adult CPB over-winter in the soil, females usually mate prior to over-wintering, and will emerge in the spring ready to lay the first set of eggs (Pedigo, 2002). CPB emergence coincides with potato plant sprouting (Pedigo, 2002), usually in June in Washington State, USA. Adults feed, mate and lay eggs, and the females oviposit up to 300 eggs over a 5 week period (Pedigo, 2002). The eggs hatch in 4-9 days, and larvae feed and molt for 2-3 weeks. The number of days to complete a developmental stage is dependent on the temperature (Ferro et al., 1985), with each stage dependent on the accumulation of growing degree days (Figure 2) (Bourgeois, unpublished).

Chemical Control

CPB infestation has traditionally been controlled using rotations of several different classes of chemicals such as neo-nicotinoid insecticides (imidacloprid, thiamethoxam and clothianidin), pyrethroids, carbamates among several others throughout the season (Alyokhin et

14

al., 2007) contributing to the overuse of chemicals in potato crops. New chemical pesticides are manufactured for CPB control due to the ability of CPB to evolve resistance to almost every chemical introduced into the market (Alyokhin et al., 2007; Forgash, 1985).

Targeting Both Pests Simultaneously

Both the CPB and *M. chitwoodi* are poikilothermic organisms: thus, their metabolism is regulated by environmental temperature. Growth and development of both organisms is dependent on the accumulation of successive hours of heat units, resulting in completion of a growth stage in their respective lifecycles. *Meloidogyne chitwoodi* resides around the potato rhizosphere, except during times of high stress such as cold, heat, or fumigation where it can migrate downwards in the soil. *Meloidogyne chitwoodi* has been actively recovered below 1 m in the soil, indicative of its ability to survive fumigation and migrate up to the root zone following fumigation (Pinkerton et al., 1987). Ideally, biological control of this organism must target the partially exposed sedentary stage of the female nematode; otherwise, the infective juvenile may easily escape into the roots or soil to avoid being targeted. Thus, we hypothesized that targeting *M. chitwoodi* during its sedentary egg laying stage may provide the best opportunity for controlling this pest.

The CPB also has a life-stage that is proximal to the potato plant rhizosphere. The 4th instar larva must drop down from the potato foliage into the soil, and burrow up to 5 cm to complete pupation (Pedigo, 2002). We hypothesized that, ideally, biological control should target of the burrowing 4th instar CPB as it is immobile, vulnerable in the soil without a hard outer insect cuticle, and has exposed entry routes to pathogens via its mouth, spiracle, and anus.

15

Using this information, we believe that the application of biological control agents can be most effective against both organisms if applied to coincide with those specific lifestages, simultaneously. In order to accomplish this, knowledge of the phenology of each organism can be used to predict when the accumulation of heat units will allow the organism to reach each lifestage, and the timing of the biological control can be applied when the two lifestages are most vulnerable (i.e., sedentary female egg laying [Table 1) and CPB 4th instar larvae, [Table 2)). The 4th instar larval stage of the Colorado potato beetle is susceptible to entomopathogenic nematodes, and this stage often coincides with the vulnerable stages of *M. chitwoodi* (i.e. egg laying and juvenile hatch). The lifestages which are most vulnerable to biological control using entomopathogenic nematodes may be targeted using the combined growing degree day phenology models for *M. chitwoodi* and the Colorado potato beetle (Figure 2) and current weather data.

Temperature data for development of growing degree days (Figure 2) were taken from WSU-PAWS (AgWeatherNet) online weather archives for Prosser, WA from April 2006 to September 2006 (http//:www.weather.wsu.edu). Air temperatures (ºC) were used in degree-day calculations. Phenology models of *Meloidogyne chitwoodi* (Table 1, Pinkerton et al., 1991) and the Colorado potato beetle (Table 2, Bourgeois, G. unpublished) were used to construct the combined phenological growing degree day model for Prosser, WA.

There are several biological control agents and products that can be amended in the soil to potentially target both plant parasitic nematodes and the 4th instar larval stage of the Colorado potato beetle. This review discussed the biocontrol products which have shown potential against either pest, but have not been investigated for simultaneous control of both pests. The potential for biocontrol using several methods to control both pests have been discussed. The phenology

models of both pests can also potentially be used to target these specific vulnerable life stages at the same time for more effective control.

Table 1. Phenology model of *Meloidogyne chitwoodi* (adapted from Pinkerton et al, 1991).

Host: Russet Burbank Potatoes	DD (°C)
Tuber initiation:	450-500
First generation females produce egg masses:	600-800
Second generation hatch:	950-1100
Juveniles in tubers (1st generation)	988-1166
Third generation hatch:	1500-1600
Total DD for first generation time:	1000
Total DD for subsequent generation time:	500-600

*Degree Days (DD)
**Base temperature of 5 °C

Figure 2. Predictive timing of life cycle stages based upon growing degree day accumulation and phenology models of *Meloidogyne chitwoodi* and the Colorado potato beetle.
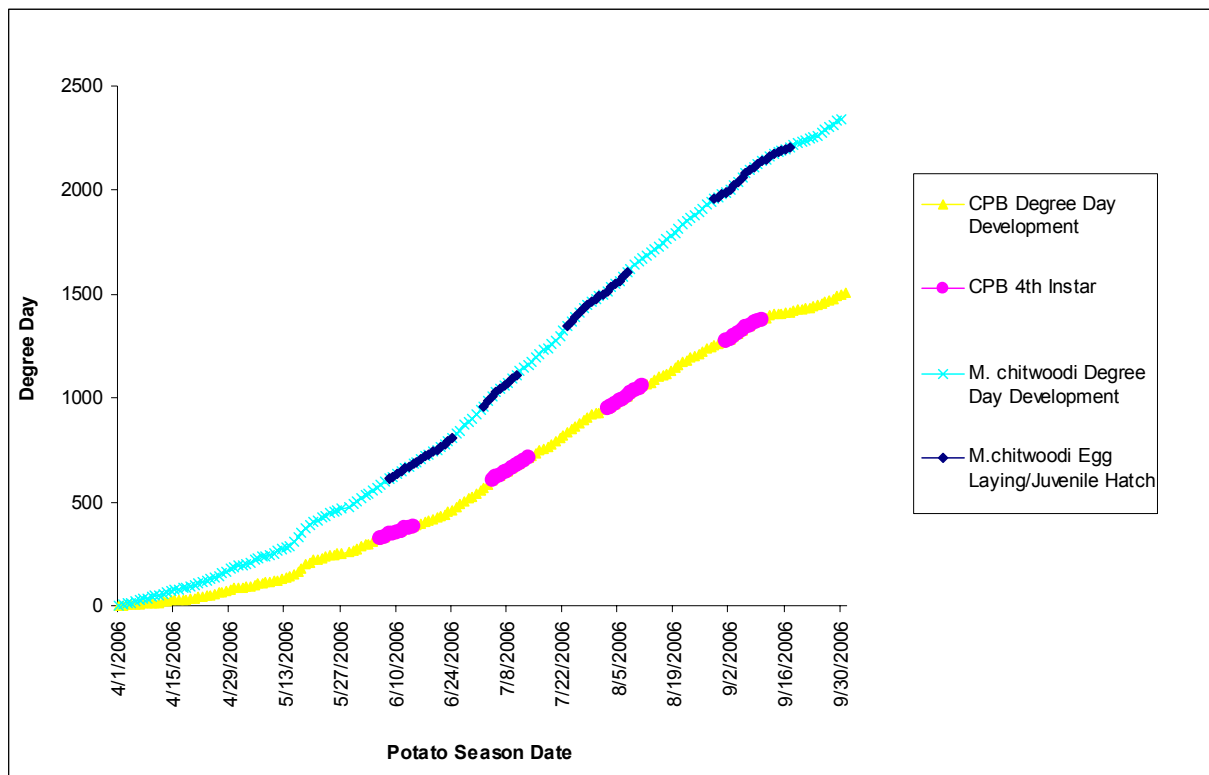
Table 2. Phenology model of the Colorado potato beetle (Bourgeois, G. unpublished).

Life Cycle Stage	Degree Days *Between* Each Stage	Accumulated Degree Days
Sowing Date to Adult Spring Emergence	110	110
Emergence to Egg Laying	48	158
Egg laying to L1/L2 larvae	90	248
L1/L2 to L3/L4	72	320
Total Life Cycle (Egg-Adult)**	–	272

*Degree Days (DD)
**Excludes DD for Adult Spring Emergence
***Base Temperature 10 ºC

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CHAPTER TWO

Biocontrol potential of *Brassica carinata* mustard seed meal and entomopathogenic nematodes against the Columbia Root Knot Nematode, *Meloidogyne chitwoodi*, and the Colorado Potato Beetle, *Leptinotarsa decemlineata*, pests of potatoes in Washington State

D. Henderson [a] E. Riga [a1], W.E. Snyder [b]

[a] Department of Plant Pathology, Washington State University, Pullman and Washington State University, IAREC, 24106 N Bunn Rd., Prosser, WA 99350

[b] Department of Entomology, Washington State University, Pullman, WA 99164

Mustard seed meal and entomopathogenic nematodes have shown biocontrol potential against the root knot nematode, *Meloidogyne chitwoodi*, and the Colorado Potato Beetle (CPB), *Leptinotarsa decemlineata,* on potato. Field and greenhouse trials were conducted in 2006 and 2007. Seed meal of *Brassica carinata* was applied in a field at the Washington State University (WSU) Research and Extension Center in Prosser, WA, at 2.5 ton/ ha, 15 days pre-plant of potatoes. Two applications of the entomopathogenic nematodes (EPN) *Steinernema feltiae* or *S. riobrave* were applied at planting and again at mid-season, at a rate of 7.5 billion IJ/ ha and 5 billion IJ/ ha, respectively. EPN infection of 4th instar CPB larvae was evaluated at mid-season. Greenhouse trials were conducted in WSU Pullman, WA to replicate field experiments using tomatoes as the host plants. Roots of tomato seedlings were stained and number of *M. chitwoodi*

[1] Corresponding author: Ekaterini Riga
Tel: (509) 786-9256 Fax: (509)786-9370
E-mail: riga@wsu.edu

females in roots was recorded after two months. CPB larvae infection was tested in pots during the second EPN application (5 billion IJ/ ha). The percent of CPB larvae infected by *S. feltiae* or *S. riobrave* were recorded. *Steinernema feltiae* and mustard seed meal amendment significantly reduced the number of *M. chitwoodi* females in tomato roots. Mustard seed meal alone, EPN, and combination of mustard seed meal and EPN significantly reduced the percent culled potato tubers, percent infection, and infection index in field trials. However, combination of mustard and EPN reduced the efficacy of both control methods below that expected based on their individual impacts. In the greenhouse mustard seed meal, *S. feltiae* and *S. riobrave*, and combination of mustard seed meal and these species, significantly reduced *M. chitwoodi* infection on tomato roots. Both species of EPN caused significant infection of CPB larvae in the field and greenhouse, regardless of mustard amendment.

Keywords: Entomopathogenic nematodes; Brassica mustard seed meal; *Leptinotarsa decemlineata*; *Meloidogyne chitwoodi*; Biological control

1. Introduction

Among the most important pests of potato, (*Solanum tuberosum* L.) in Washington State, are a herbivorous insect, the Colorado potato beetle (CPB) *Leptinotarsa decemlineata* (Say), (Coleoptera: Chrysomelidae) and the Columbia root knot nematode, *Meloidogyne chitwoodi* (Golden, O'Bannon, Santo, and Finley). Above ground, CPB can completely defoliate potato plants in 1-2 generations (Hare, et al, 1980). The most extensive damage caused by the larval stages of the beetle (Hare, 1980). Below ground, potato tubers are attacked by the plant parasitic nematode *M. chitwoodi*, causing warting symptoms on the tuber skin (Cram et al., 2007).

The ability of *M. chitwoodi* to survive freezing temperatures and begin reproduction in cooler temperatures (5 °C) groups this nematode with other cool climate root-knot nematodes like *M. hapla* (Santo and O'Bannon, 1981). In the Pacific Northwest farmers typically plant potatoes in April, when temperatures begin to warm up to 5 °C, allowing the nematode to resume its lifecycle and infect potato plants early in the season compared to other cool climate nematodes. *Meloidogyne chitwoodi* begins development very early in the potato growing season, making it possible to have 5-6 reproductive cycles before harvest (Santo and O'Bannon, 1981). *Meloidogyne chitwoodi* can cause warting symptoms on the tuber skin, and necrotic spots within the tuber (Strand et al., 1986). These symptoms decrease the marketability of the tubers to potato processors, who have a tolerance of less than ten percent infection (culls) by *M. chitwoodi* (Santo et al., 2003). Potato farmers fumigate the soil with Telone II (1,3 dichloropropene) and/or Vapam (sodium methyldithiocarb) in the fall and may also apply Mocap (ethoprophos) in the spring prior to planting potatoes as strategies to reduce populations of the plant parasitic nematode (Cram et al., 2007). The use of both insecticides and fumigants may deplete above ground and below ground diversity (Haydock et al., 2006), as well as increase the risks of toxic runoff into groundwater (Karpouzas et al., 2002).

The lifecycles of CPB and *M. chitwoodi* coincide in the same soil habitat around the potato rhizosphere during the growing season (Pedigo, 2002; Santo et al., 1994). This should make both pests ideal targets for using one or two biological control methods to simultaneously target them. In the lifecycle of CPB, eggs develop through 1-4 instars, and upon reaching the 4th instar larval stage the larvae drop into the soil to pupate and emerge as adults a few days later (Pedigo, 2002). This burrowing stage leaves CPB pupae vulnerable to soil dwelling entomopathogenic nematodes (EPN) (Cantelo and Nickle, 1992; Nickle et al., 1994; Toba et al,

1983; Armer et al., 2004). EPN are nematodes that are pathogenic to CPB (Cantelo and Nickle, 1992) larvae, but have also been shown to reduce plant parasitic nematode populations (Grewal et al, 1997; Jagdale, et al, 2002; Perez and Lewis, 2004; Perez and Lewis, 2001; Lewis et al., 2001) although the mechanism is still unknown. The suppressive interaction observed between root knot nematodes and EPN has been hypothesized to occur in three ways (Grewal et al., 1999): 1) suppression of root knot nematodes through physical presence and competition for space (Bird and Bird, 1986), 2) repulsion or suppression of plant parasitic nematodes as EPN or their bacterial symbionts release allelochemicals that act to repel or suppress plant parasitic nematodes (Grewal et al., 1999; Hu, et al., 1995), or 3) inundatively released EPN on plant parasitic nematodes may increase nematode antagonists in the soil (Ishibashi and Kondo, 1987; Ishibashi and Choi, 1991).

Another biocontrol method against root knot nematode is the use of biologically derived products with nematicidal properties. In particular mustard (*Brassica* spp.) plants are used as biofumigants because they contain glucosinolate compounds which are hydrolyzed into isothiocyanates after soil incorporation and irrigation (Morra and Kirkegaard, 2002). These isothiocyanate compounds have pesticidal and nematicidal properties that are dispersed throughout the soil upon tillage and irrigation (Bones and Rossiter, 1996; Riga and Collins, 2005; Riga et al., 2003). Mustard seed meal is a waste product generated from the mustard seed oil extraction process, and can also be used for biofumigation. There is growing interest from Washington state potato farmers regarding use of plant material for biofumigation (Ekaterini Riga, personal communication). Of the variety of glucosinolate compounds tested against *M. incognita*, the glucosinolate sinigrin (2-propenyl) has shown to have the most nematicidal activity against the root knot nematode (Lazzeri et al., 2004). *Brassica carinata* contains high

proportions of the glucosinolate sinigrin (2-propenyl) (Leoni et al., 2004), and the mustard seed meal has been commercially produced as "BioFence" (Triumph Italia S.p.A. Livorno, Italy). The *B. carinata* mustard seed meal has a N composition greater than 6% and a low residual oil level (2.6%) (Lazzeri et al., 2004). The chemical composition of the mustard seed meal was found to contain 163.4 μmol g-1 of glucosinolates, 98% of type 2-propenyl glucosinolate (sinigrin) and a sufficient level of myrosinase enzyme to catalyze glucosinolate hydrolysis (Leoni, et al., 2004). Although mustards and other crops have been used to target root knot nematodes, it has not been investigated whether mustard seed meal *(Brassica carinata)* can decrease *M. chitwoodi* populations or whether mustard seed meal can be used simultaneously with EPN.

A 2 x 3 multi-factorial experiment was used to examine all combinations of mustard seed meal (+,-), and EPN species (none, *S. feltiae, S. riobrave*) against *M. chitwoodi* percent tuber infection (external symptoms) and tuber infection index (internal symptoms). The following hypotheses were tested: 1) mustard seed meal of *B. carinata* can decrease *M. chitwoodi* populations, 2) *S. feltiae* or *S. riobrave* can infect 4th instar CPB larvae and cause mortality while controlling *M. chitwoodi,* and 3) mustard seed meal amendment has an effect on EPN infectivity of CPB and EPN suppression of *M. chitwoodi*.

2. Materials and Methods

2.1 Field Experiments

Field plots in Washington State University (WSU) Irrigated Agriculture Research and Extension Center (IAREC) in Prosser, WA in 2006 and 2007 were set up with 5 replications in a randomized complete block design to control for the uneven distribution of *M. chitwoodi* in the field. Plots with soil type sandy loam were 2.4 x 6 m with 0.30 m inter-row spacing and 3 rows

per plot. The middle row was used for sampling because it was buffered by two border rows. Mustard seed meal of *B. carinata* (supplied by Dale Gies: High Performance Seed Company, Moses Lake, WA) was applied at 2.5 ton/ha or 4.42 kg per plot on May 30, 2006 and May 1st, 2007 followed by ~5 cm of irrigation. The mustard seed meal is a commercial product "Biofence" (Triumph Italia S.p.A. Livorno, Italy) produced from *B. carinata* sel. ISCI 7 seeds produced through a proprietary partial defatting method that limits glucosinolate and myrosinase degradation (Lazzeri et al., 2002).

Russet Burbank potatoes were planted 15 days post-mustard seed meal application on June 15, 2006 and May 15, 2007. On the same day as potato planting, entomopathogenic nematodes *S. feltiae* strain 75 (Nemasys®) or *S. riobrave* strain 355 (BioVector®) (Supplied by Becker Underwood, UK) were applied at 7.5 billion IJ/ha with 2.3 L of water per plot using a backpack sprayer. EPN were sprayed after 5pm to avoid UV and heat damage to the EPN (Smits, 1996). The 2 x 3 factorial randomized complete block design examined the interaction between mustard: not applied (-), versus applied (+), and EPN species: none, *S. feltiae* or *S. riobrave*. Treatments were mustard seed meal, *S. feltiae, S. riobrave*, mustard seed meal + *S. feltiae,* mustard seed meal + *S. riobrave*, synthetic nematicide Mocap® (Rhone-Poulenc, Inc.) as a control, and an untreated control. There were 5 replications in the field for each year 2006-2007.

2.1a EPN Monitoring

The presence of EPN after the first treatment application and prior to the second application was evaluated for the time it takes for the excess population of EPN to subside to pre-treatment or residual levels. An insect-baiting technique was used to detect the presence of EPN in the soil (Woodring and Kaya, 1988). The presence of EPN in the field was assessed

using *Galleria melonella* L. (Lepidoptera: Pyralidae) wax worms (Supplied by Rainbow Mealworms) on August 4th, 2006. One 118 ml perforated bucket (perforated to allow for water drainage and ventilation) was filled with soil (from each plot), and ten wax worms were placed in each bucket. The buckets were then capped and left in the field for 48 hours to bait the EPN out of the soil. Wax worms were collected August 7th, 2006 and placed into Petri dishes in the laboratory (25 ºC) inside darkened plastic containers to maintain humidity. The wax worms were observed for discoloration, and dissected for EPN infectivity 4-5 days later.

2.1b Field Soil Sampling for Plant Parasitic Nematodes

Prior to the first treatment applications, soil samples were taken with a field core probe on May 30th, 2006 and May 1st, 2007 to quantify the presence of *M. chitwoodi* in the field. Three samples to the depth of 30.5 cm were taken from each middle row plot and combined and put into cold storage (4 ºC). Within 1-2 weeks, total nematodes were extracted from 250 cc of the homogenized field soil by centrifugal-flotation technique (Byrd et al., 1966) using a series of 35 μm, 400 μm, and 500 μm pore-seives, and were analyzed for number and identity of plant parasitic and free-living nematodes.

2.1c Colorado Potato Beetle Field Infection

CPB populations were sufficiently high to conduct bioassays at potato mid-season on August 8, 2006 and July 6, 2007. To determine EPN infection of CPB a bioassay was conducted according to the technique by Amer and co-authors (2004), with slight modifications. Two 118

ml perforated buckets (perforated on the bottom and top to allow for water drainage and ventilation) were filled with soil (from each plot). EPN were then sprayed in the field at 5 billion IJ/ ha using a backpack sprayer (2.3 L water per plot). After spraying the soil, including the soil filled buckets, ten CPB 4th instar larvae were placed in each bucket. The buckets were then capped and left in the field for 48 hours for infection to take place. Buckets were taken from the field and CPB larvae were put inside individual Petri dishes. The Petri dishes were maintained in the laboratory inside dark plastic containers (to maintain sufficient humidity levels) at 25 ºC to observe for mortality and EPN infection. After 4-5 days, the larvae were dissected using a Leica MZ95 dissecting microscope (Leica Microsystems GmbH, Wetzlar, Germany) and recorded as infected if EPN were visibly present.

2.1d Potato Tuber Infection Ratings

Potato plots were harvested on October 30, 2006 and October 15, 2007. Middle rows of each plot were dug with a potato harvester and bagged into burlap sacks and put into cold storage (4 ºC) until processed (within 2-4 weeks). Potato tubers from the middle row (2.4 x 6 m) of experimental plots were weighed, counted, and sorted using a Lectro Tek® Singulator (Lectro Tek, Inc, Wenatchee, WA, USA) for culls, #1 and #2 tubers. Twenty potato tubers were randomly chosen from each plot and assessed for percent total *M. chitwoodi* infection and infection index of tubers. To assess percent infection, the numbers of tubers with visible root knot symptoms on the outer tuber skin were counted as infected. To assess infection index, 20 potato tubers were peeled and inspected under a magnifying lens with light for presence of females in the potato cortex. The number of females were counted and assigned an infection

rating using the infection index scale of 0 = 0 females, 1 = 1-3, 2 = 4-5, 3 = 6-9, 4 = 10-50, 5 = 100+, 6 = 200+ (Bridge and Page, 1980).

2.2 Greenhouse Experiments

Greenhouse trials were conducted at WSU Pullman, WA in 2006 and 2007, in a completely randomized design with ten replications and repeated three times at different dates. Pots (25 cm^2) filled with 500 g of fumigated 2:1 sand:soil mixture was used in each experiment. The greenhouse was kept at a 16:8 (light:dark) photoperiod and at average temperature of 27 ºC. *Meloidogyne chitwoodi* eggs were inoculated into each pot at 2 eggs/g soil at the same time as the application treatments. Mustard seed meal was applied by mixing 500 g of soil with 2.5 ton/ ha or 2.61 g per pot of *B. carrinata* 'Biofence' seed meal. The mustard seed meal was thoroughly mixed with the soil in a plastic bag prior to putting soil into the pots. *Steinernema feltiae* and *S. riobrave* were applied at 7.5 billion ij/ ha or 7652.28 IJ/ pot by creating a small hole in the soil and pipetting in the EPN. Treatment combinations of mustard seed meal and EPN were prepared by first mixing the mustard seed meal with the soil, potting the soil and then applying *M. chitwoodi* and the respective EPN species, *S. feltiae* or *S. riobrave*. Ten days after treatment, five-week-old tomato seedlings (*Lycopersicum esculentum* var. Rutgers Select) were transplanted into the pots containing treated soil. CPB larval infection was tested in pots during the second EPN application, at a rate of 5 billion IJ/ ha or 5101.53 IJ/ pot, by placing 5 larvae in cloth mesh bags and burying them 5 cm in the potted soil prior to EPN application. EPN were applied in the same manner as the first application using a pipette to inoculate them into a small indentation in the soil. CPB larvae were observed for infection and mortality and dissected for EPN reproduction using the Leica MZ95 dissecting microscope (Leica Microsystems GmbH,

Wetzlar, Germany). The percent of CPB larvae infected by *S. feltiae* or *S. riobrave* were then recorded. After two months, the experiment was terminated and the roots of the tomato seedlings were stained with acid-fuchsin (Byrd et al., 1983) and the number of *M. chitwoodi* females per gram of dry roots was recorded.

3. Results

3.1 Field Experiments

3.1a Potato Tuber *Meloidogyne chitwoodi* Infection

A 2 x 3 multi-factorial experiment was conducted to examine the interaction between mustard seed meal: not applied (-), versus applied (+), and EPN species: none, *S. feltiae* or *S. riobrave,* and were analyzed using factorial analyses of variance (Proc GLM, SAS Institute Inc, 2003). Percent potato tuber infection, infection index data, and yield data were analyzed as a randomized complete block design with a 2 x 3 factorial interaction and further examined for effects within each factor level (slice option; slice EPN/mustard). There was a significant interaction between 2006 and 2007 for percent tuber infection ($F_{1, 49} = 5.71$; $P = 0.0208$), and tuber infection index ($F_{1,49} = 6.75$; $P = 0.0123$); therefore, each field season will be discussed separately.

In the 2006 field experiment, there was a strong but only marginally significant mustard x EPN interaction for potato tuber infection index, due to the antagonistic effect of combining mustard seed meal treatment with *S. feltiae* ($F_{2, 25} = 3.11$; $P = 0.0621$) (Figure 3). Without the addition of mustard seed meal, the EPN treatments significantly reduced infection ($F_{2, 25} = 5.62$; $P = .0097$) of potato tubers by *M. chitwoodi*, due to the ability of *S. feltiae* to reduce infection.

Steinernema riobrave treatment alone was not able to reduce tuber infection due to *M. chitwoodi*. Without the addition of EPN treatments, mustard treatment alone reduced infection ($F_{1, 25}$ = 5.91; P = 0.0226). Only the combination of EPN species *S. riobrave* with the mustard seed meal treatment significantly reduced tuber infection caused by *M. chitwoodi* ($F_{1,25}$ =4.48; P = 0.0445).

There was a significant mustard x EPN interaction ($F_{2,25}$ = 5.67; *P* = 0.0093) (Figure 4) for potato tuber percent infection. Mustard seed meal reduced percent tuber infection by *M. chitwoodi* ($F_{1, 25}$ = 12.54; P = 0.0016), an effect independent of EPN application. The combination of mustard seed meal and EPN species treatments reduced the efficacy of *S. feltiae* ($F_{1, 25}$ = 1.8; P = 0.1915), and improved the efficacy of *S. riobrave* ($F_{1, 25}$ = 3.07; P = 0.0921).

In the 2007 field experiment, there was a significant mustard x EPN interaction ($F_{1,20}$ = 4.90; P = 0.0185) for tuber infection index due to the reduction in infection when mustard was applied (Figure 5). The addition of EPN to mustard reduced the efficacy of mustard. Without the addition of EPN, mustard alone significantly reduced *M. chitwoodi* infection ($F_{1,20}$ = 31.70; P < 0.0001). The addition of *S. feltiae* to mustard seed meal reduced the efficacy of mustard ($F_{1,20}$ = 2.82; P = 0.1089) more so than mustard with *S. riobrave* ($F_{1,20}$ = 3.71; P = 0.0684). The factor EPN without mustard was significant at reducing *M. chitwoodi* tuber infection ($F_{2,20}$ = 9.15; P = 0.0015), with similar reduction in *M. chitwoodi* tuber infection for both species, *S. feltiae* and *S. riobrave* treatments (Table 3).

There was not a significant mustard seed meal x EPN interaction for *M. chitwoodi* percent tuber infection ($F_{2,20}$ = 0.69; P = 0.5144) (Figure 6) in the field experiment of 2007. Neither species of EPN treatments was able to reduce the percent tuber infection ($F_{2, 20}$ = 2.0; P = 0.1616) alone. However, the addition of mustard seed meal to both EPN species significantly reduced *M. chitwoodi* infection compared to EPN alone (mustard + *S. riobrave*; $F_{1, 20}$ = 5.07; P =

0.0358, mustard + *S. feltiae*; $F_{1, 20}$ = 13.8; P = 0.0014). The treatment of mustard seed meal alone was significant ($F_{1, 20}$ = 13.38; P = 0.0016) at reducing *M. chitwoodi* tuber infection.

Figure 3. Infection index of *Meloidogyne chitwoodi* after treatments of EPN species (none, *Steinernema. feltiae, S. riobrave)* and *Brassica carinata* seed meal (+/-) from the 2006 field experiment.
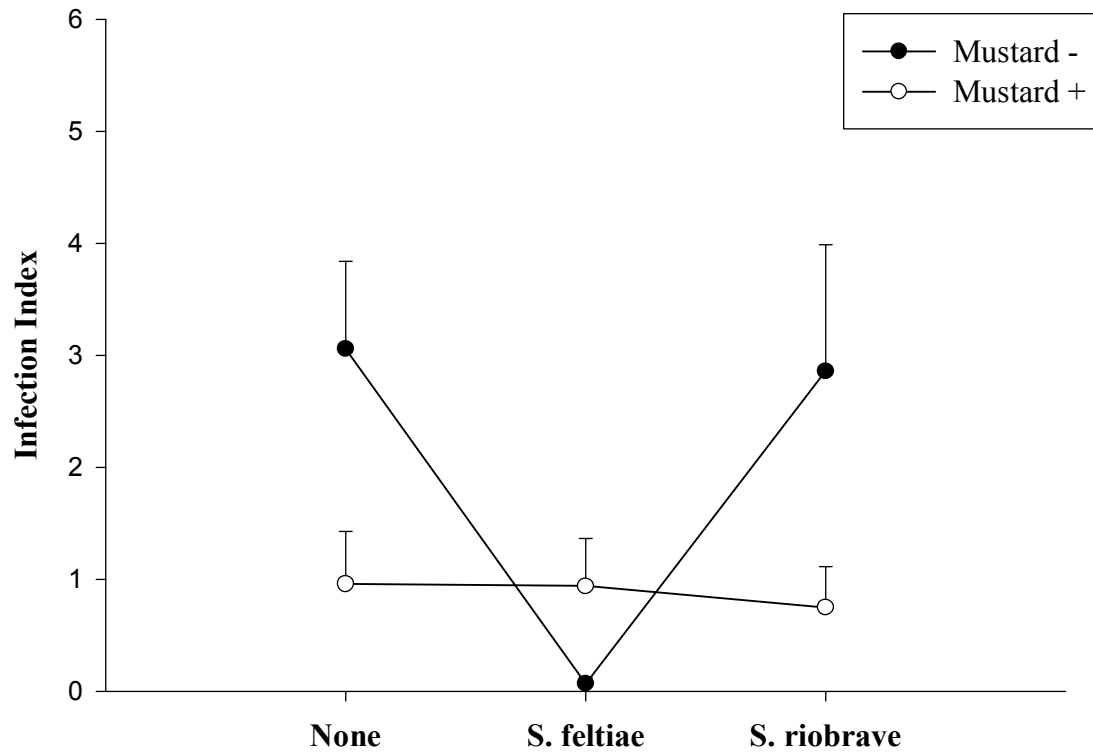
Infection index is the actual number of observed females based on a scale of 0 = 0 females, 1 = 1-3, 2 = 4-5, 3 = 6-9, 4 = 10-50, 5 = 100+, 6 = 200+ (Bridge and Page, 1980).
*Data are means +/- SE

Figure 4. Percent potato tubers infected by *Meloidogyne chitwoodi* after treatments with EPN species (none, *Steinernema feltiae, S. riobrave)* and *Brassica carinata* seed meal (mustard +/-) from the 2006 field experiment.

*Data are means +/- SE

Figure 5. Infection index of *Meloidogyne chitwoodi* after treatments of EPN species (none, *Steinernema. feltiae, S. riobrave)* and *Brassica carinata* seed meal (+/-) from the 2007 field experiment.
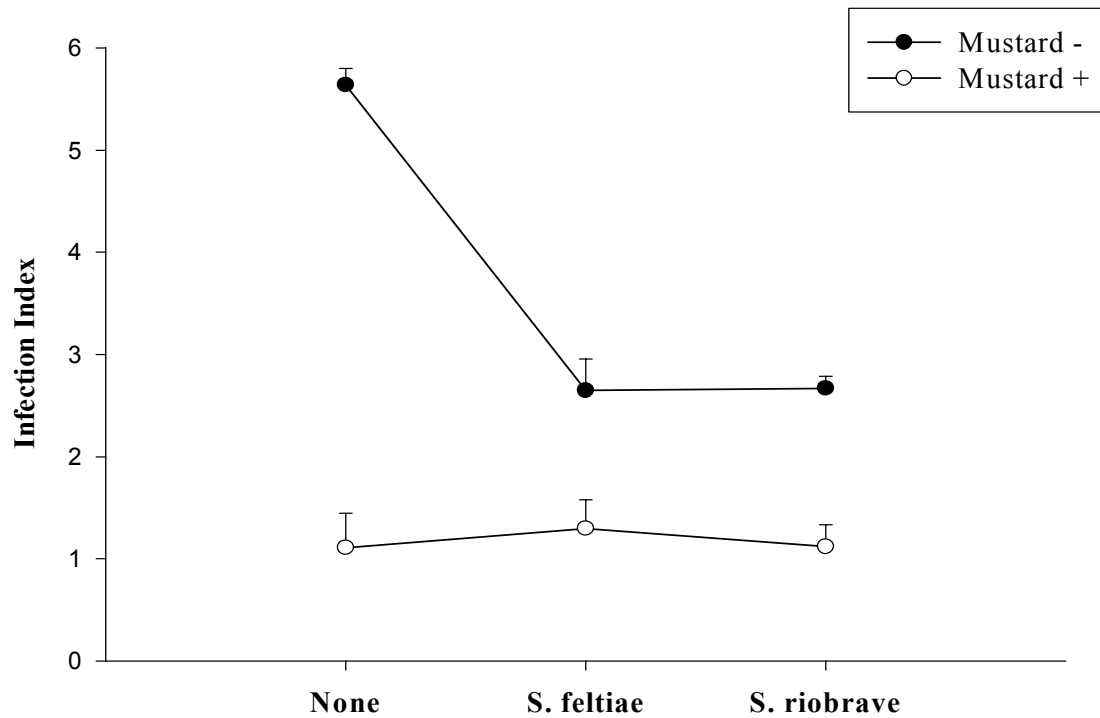
Infection index is the actual number of observed females based on a scale of 0 = 0 females, 1 = 1-3, 2 = 4-5, 3 = 6-9, 4 = 10-50, 5 = 100+, 6 = 200+ (Bridge and Page, 1980).
*Data are means +/- SE

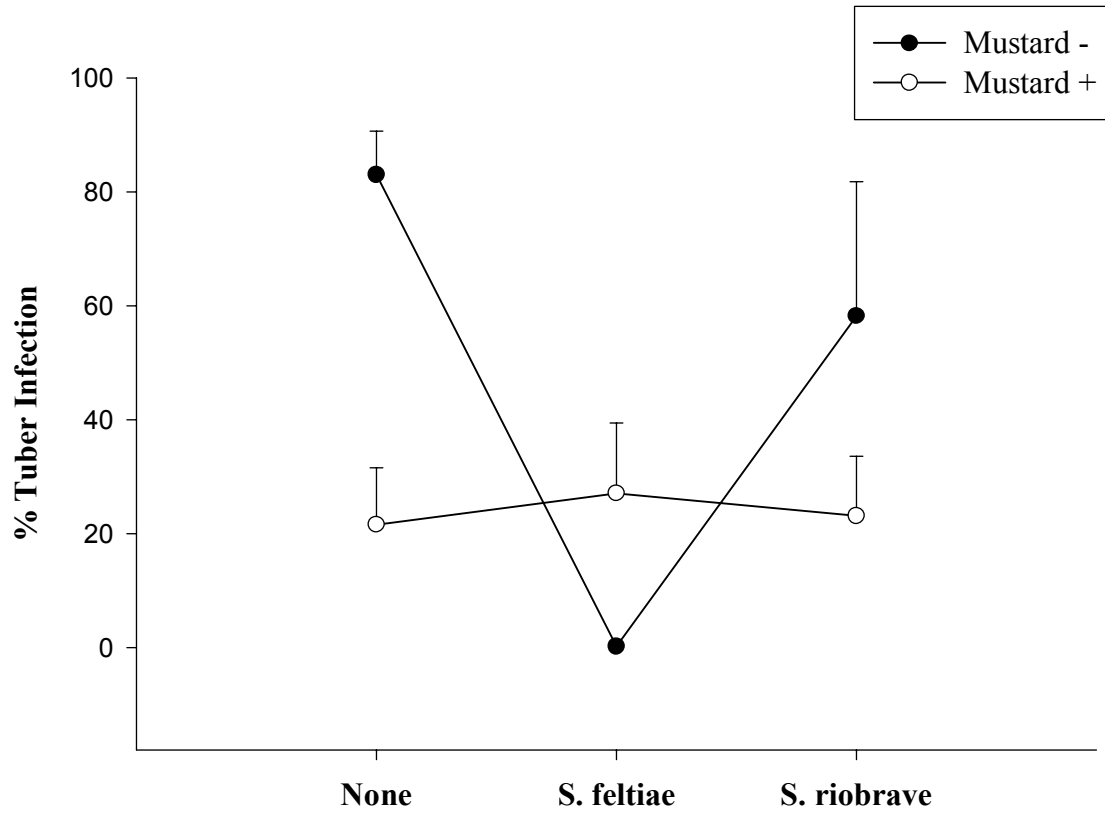**Figure 6. Percent potato tubers infected by *Meloidogyne chitwoodi* after treatments with EPN species (none, *Steinernema feltiae, S. riobrave)* and *Brassica carinata* seed meal (mustard +/-) from the 2007 field experiment.**
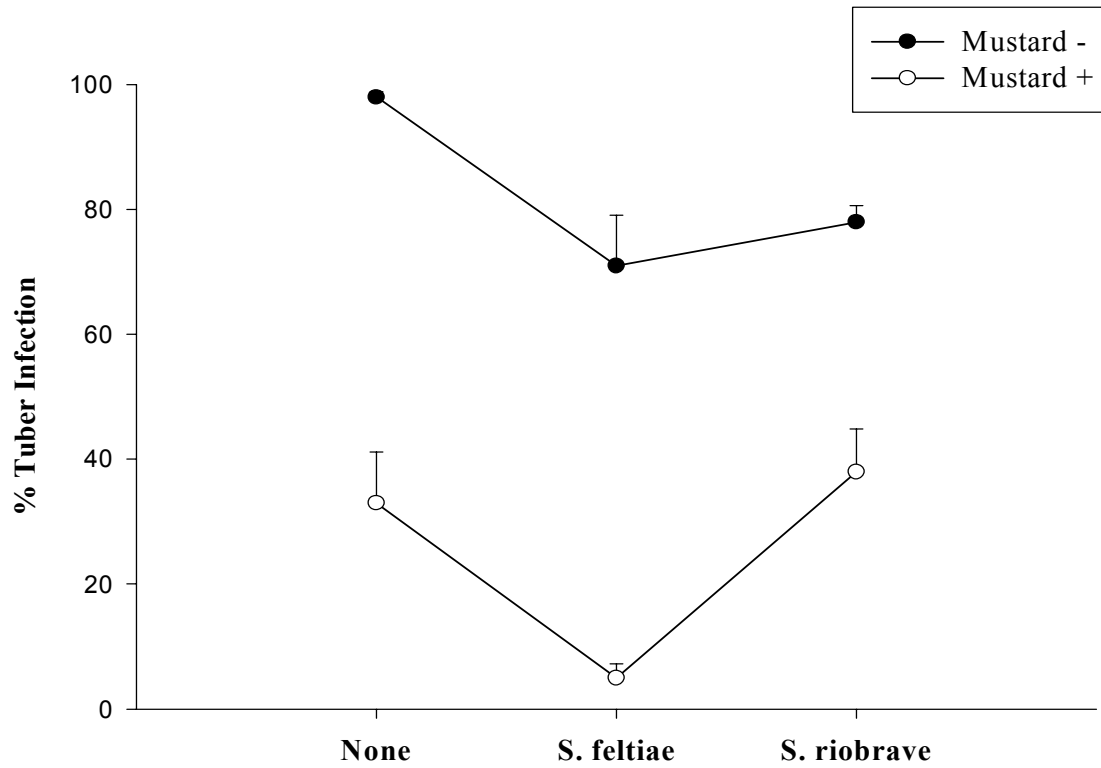
*Data are means +/- SE

Table 3. Percent tuber infection and tuber infection index ratings for potato tuber evaluation in field experiments 2006-2007.

Treatment	% Tuber Infection		Infection Index	
	2006	2007	2006	2007
Steinernema feltiae	0.2	71	.07	2.65
S. riobrave	58.2	78	2.86	2.67
Brassica carinata	21.55	33	0.96	1.11
S. feltiae x B. carinata	27.1	5	0.94	1.30
S. riobrave x B. carinata	23.1	38	0.75	1.12
Control	83	98	3.06	5.64
Mocap	0	15	0	0.23

3.1b Potato Tuber Yield

Grade #1 and #2 Tubers

Grade #1 tubers are the highest quality of shape, size and appearance that are accepted in the potato market. Grade #2 tubers are the next lower grade with slight shape or appearance blemishes, but considered acceptable for the potato market (United States standards for grades of potatoes). All data were taken from the middle row of each 2.4 x 6 m plot. In 2006, there was a strong mustard seed meal and EPN interaction for the number of #1 tubers ($F_{2,20}$ = 3.75; P = 0.0413). This interaction was due to the increased number of #1 tubers for *S. feltiae* treatments and increased weight of #1 tubers ($F_{2,20}$ = 3.78P = .0406), but reduction in weight when *S. feltiae* and mustard seed meal treatments were combined (Table 4). For the field experiments of 2007, there was no significant interaction between mustard seed meal and EPN for the number or total weight of #1 or #2 tubers (Table 5). Although there was not a significant effect of *B. carinata* and *S. feltiae,* and *S. riobrave* treatments on yield in both years, both *Brassica carinata* and *S. feltiae* were observed to increase the total weight of #1 tubers in comparison to the control. Although the effect was not significant, both EPN treatments *S. feltiae* and *S. riobrave* resulted in the most #2 tubers in 2006 (Table 4) and 2007 (Table 5).

Table 4. The effect of mustard seed meal (*Brassica carinata*), *Steinernema feltiae* and *S. riobrave* (EPN), and combination of mustard seed meal and EPN on the number and total weight of grade #1, #2 and culled potato tubers for the field experiment of 2006.

Treatment	#1	#1 Wt	#2	#2 Wt	#Cull	Cull Wt	Total Wt (g)
Steinernema feltiae	113	16610	5	843	4	877	18330
S. riobrave	85	12134	6	1118	4	838	14090
Brassica carinata	111	17701	3	784	3	891	19376
S. feltiae x B. carinata	101	13702	2	438	4	820	14960
S. riobrave x B. carinata	106	15354	2	569	4	905	16828
Control	83	10640	2	604	4	590	11834
Mocap	146	25960	57	5161	4	258	31379

*All weight (Wt) in gram (g) units

Table 5. The effect of mustard seed meal (*Brassica carinata*), *Steinernema feltiae* and *S. riobrave* (EPN), and combination of mustard seed meal and EPN on the number and total weight of grade #1, #2 and culled potato tubers for the field experiment of 2007.

| Treatment	#1	#1 Wt	#2	#2 Wt	#Cull	Cull Wt	Total Wt (g)
Steinernema feltiae	185	25940	13	2239	2	484	28663
S. riobrave	172	23680	12	2090	3	594	26364
Brassica carinata	202	29291	9	1842	1	160	31293
S. feltiae x B. carinata	174	22001	8	1488	1	267	23756
S. riobrave x B. carinata	175	24459	5	1128	1	174	25761
Control	182	25846	8	1462	2	324	27632
Mocap	235	35842	57	2031	2	160	38033

*All weight (Wt) in gram (g) units

Culled Tubers

Culled tubers are classified based upon parameters of disease, i.e. soft rot and malformed shape (United States standards for grades of potatoes). In 2006, there was an antagonistic yet insignificant interaction between mustard seed meal and EPN treatments for percent culled tubers ($F_{2,20} = 2.77$; $P = 0.0867$), *S. feltiae* treatments reduced percent culls, but the combination of *S. feltiae* with mustard seed meal increased the percent culls ($F_{1,20} = 4.83$; $P = 0.0194$). Similarly in 2007, there was a strong but non-significant interaction between mustard treatment and EPN for percent culled tubers ($F_{2,20} = 1.03$; $P = 0.3736$). Mustard seed meal treatment ($F_{1,20} = 12.02$; $P = 0.0024$) significantly reduced the percent culled tubers.

3.1c Colorado Potato Beetle Field Infection

Colorado potato beetle infection by EPN species, *S. feltiae, S. riobrave*, or untreated control applications were analyzed using Proc GLM (SAS Institute Inc, 2003), followed by Fishers LSD. There were no interactions between the years 2006 and 2007; therefore, data were combined for further analysis. *Steinernema feltiae* infected CPB larvae at a rate of 95%, *S. riobrave* infected CPB larvae at a rate of 93%, and the combination of mustard seed meal amendment with *S. feltiae* infected CPB larvae at a rate of 94%. Mustard seed meal with *S. riobrave* infected CPB at 90%. All treatments were significantly different than the untreated control and Mocap ($F_{10,49} = 126.34$; $P < 0.0001$) (Figure 7).

Figure 7. Percent of Colorado potato beetle larvae infected with EPN species, *Steinernema feltiae,* and *S. riobrave*, and combination of *Brassica carinata* seed meal x EPN species in 2006 and 2007.
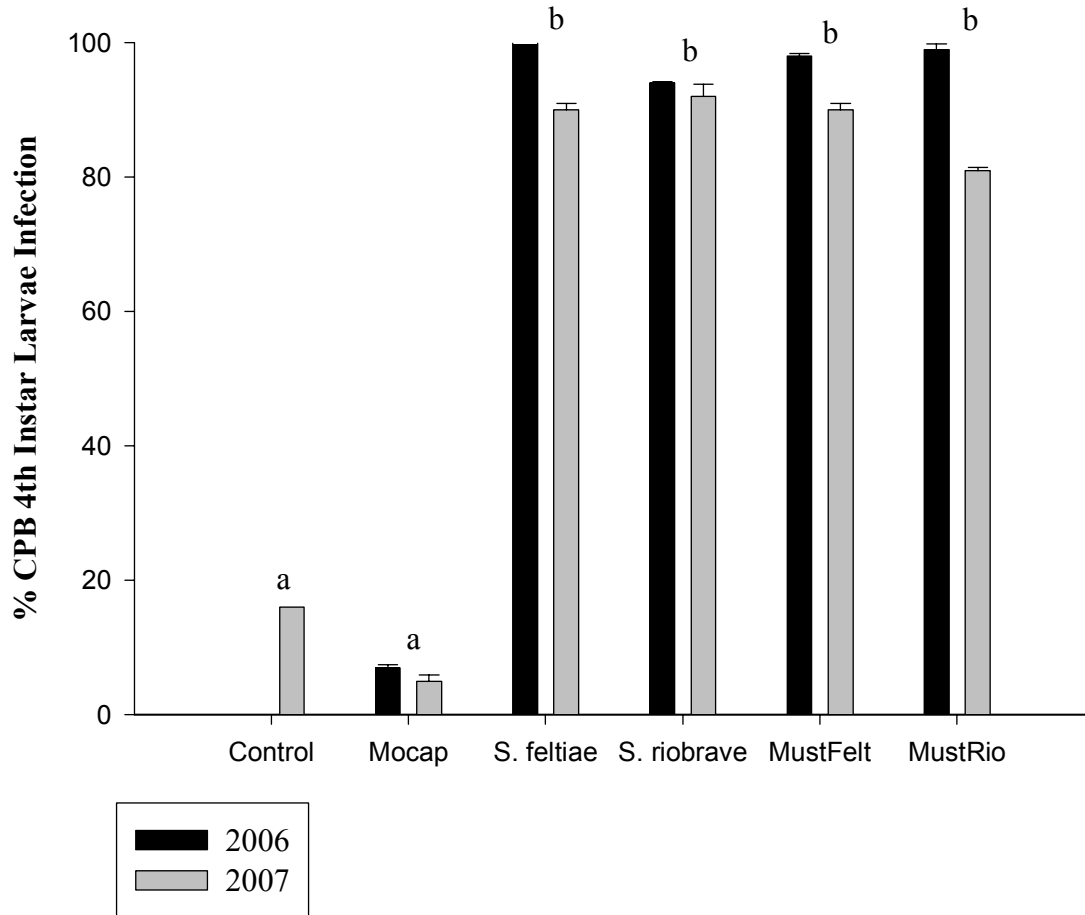
*Different letters indicate significant differences between means.
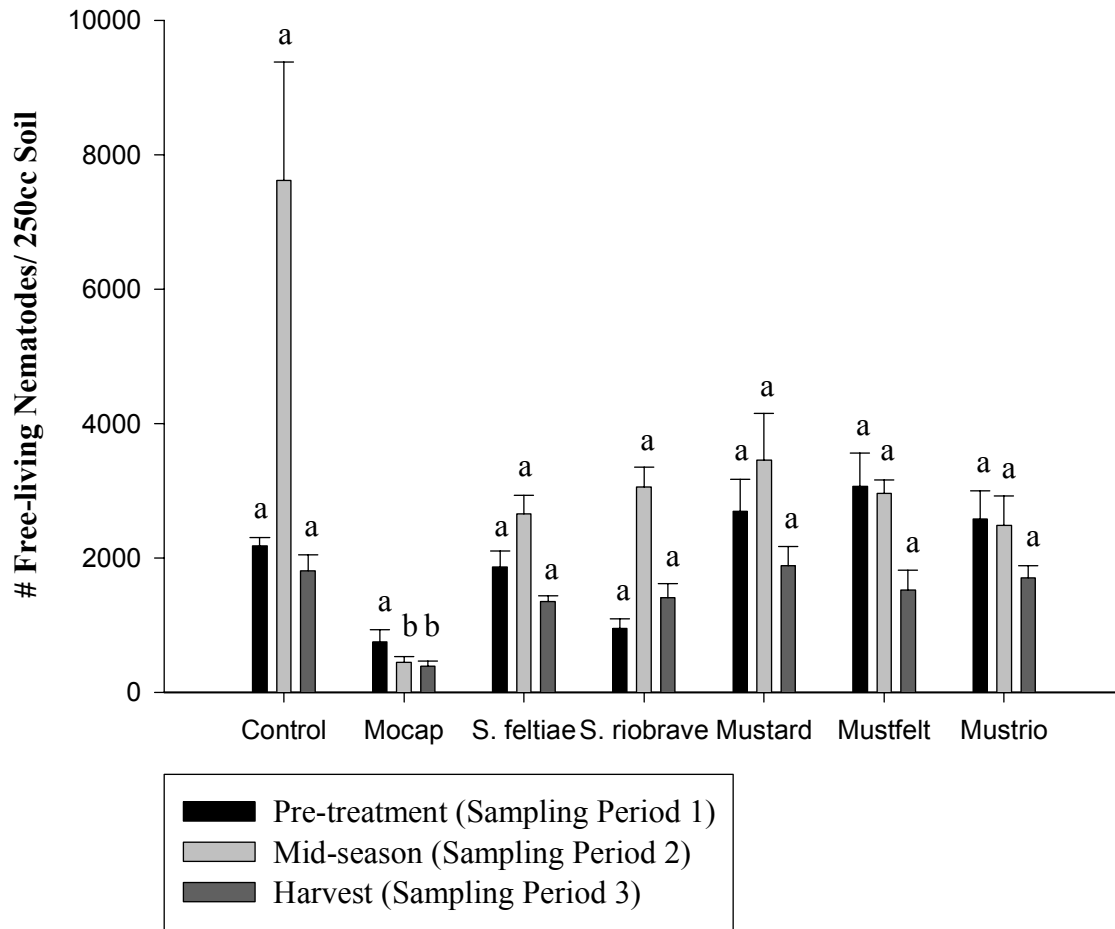*Data are means +/- SE

3.1d Nematode Population Changes in the Soil

Populations of *M. chitwoodi* and free living nematodes in the soil were analyzed using SAS 9.1, Proc GLM, repeated measures or Fishers LSD (for effects within one sampling date) (SAS Institute Inc, 2003). If the variances in populations were unstable, data transformation log(x+1) or log (x) was performed (Noe, 1985). Populations varied across all field plots and treatments and the variances were not homogeneous, therefore a log(x+1) transformation of data was performed to stabilize the variance before analysis. For each specific sampling time (pre-treatment, mid-season, harvest) *M. chitwoodi* populations were analyzed (Proc GLM, Fishers LSD) for significant differences.

Meloidogyne chitwoodi populations from pre-treatment (pre-plant) samples were not significantly different between replications or treatments for 2006 or 2007. In the 2006 field season, there was no significant difference between treatments or replications after the second EPN application treatments were applied (mid-season), or between *M. chitwoodi* nematode populations at harvest (post-treatment). Populations varied across all field plots and treatments and the variances were not homogeneous in 2006; common among natural helminthic systems (Noe, 1985), therefore, a log(x) transformation of data was performed to stabilize the variance before analysis. There were interactions between years 2006 and 2007, therefore, the data were analyzed separately. Proc GLM and Fishers LSD (SAS Institute Inc, 2003) were used in the analysis. In 2006, there were violations of assumptions of normality (Shapiro-Wilks) and the data were log transformed before analysis. There were no significant differences in the initial free-living populations, indicating uniform populations between all replications and treatments prior to the experiment. In the second sampling time period, there was a significant reduction in free-living nematodes in the treatment mocap compared to all other treatments ($F_{6, 24} = 3.87$; P =

0.0076) (Figure 8). In the third sampling date, there was also a significant reduction in free-living nematodes in the treatment mocap ($F_{6, 24}$ = 3.66; P = 0.0101) (Figure 8). In the field experiment of 2007, there was no significant difference between initial free-living populations amongst treatments. In the second sampling date (mid-season), there was a significant reduction in free-living nematodes between treatments control, mocap, *S. feltiae*, and *S. riobrave* ($F_{6, 24}$ = 4.46; P = 0.0036) (Figure 9). There were no significant differences between treatments in the third sampling date (harvest).

Figure 8. Effect of treatments on the free-living nematode populations at three different sampling dates during the 2006 growing season.

*Different letters indicate significant differences between means.
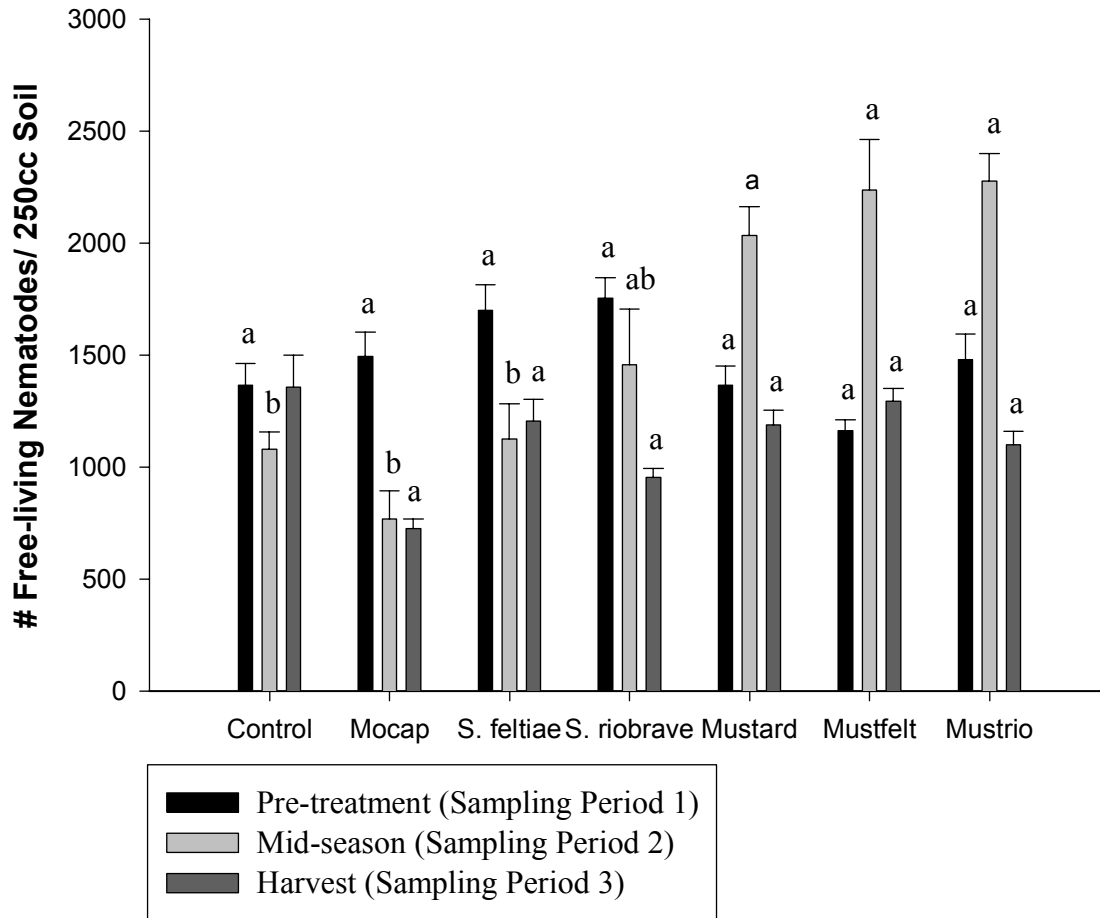*Data are means +/- SE

Figure 9. Effect of treatments on the free-living nematode populations at three different sampling dates during the 2007 growing season.

*Different letters indicate significant differences between means.
*Data are means +/- SE

3.2 Greenhouse Experiments

The multi-factorial experiment investigated the interaction between mustard seed meal: not applied (-), versus applied (+), and EPN species: none, *S. feltiae* or *S. riobrave*, and were analyzed using factorial analyses of variance (Proc GLM, SAS Institute Inc, 2003). *Meloidogyne chitwoodi* infection of tomato roots data was analyzed as a completely randomized design with a 2 x 3 factorial interaction and further examined for effects within each factor level (slice option; slice EPN/mustard seed meal). The CPB bioassays for EPN infection were analyzed using Proc GLM, followed by Fishers LSD (SAS Institute Inc, 2003). There was no interaction between the three greenhouse trials in the *M. chitwoodi* evaluation or CPB infection bioassays; therefore, the respective data were combined for further analysis. There was a significant mustard seed meal x EPN interaction ($F_{2,172}$ = 59.07; P < 0.0001) (Figure 10). The interaction was due to the ability of the treatment *S. feltiae* to reduce *M. chitwoodi* infection on tomato roots, yet infection increased when mustard seed meal was combined with *S. feltiae*. Both the treatments *S. feltiae* and *S. riobrave* lowered the infection rate of *M. chitwoodi* on tomato roots ($F_{2,172}$ = 40.03; P < 0.0001) (Table 6). When mustard seed meal was combined with either EPN species, mustard seed meal slightly reduced the efficacy of *S. feltiae* ($F_{1,172}$ = 11.65; P < 0.0008) but improved the efficacy of *S. riobrave* ($F_{1,172}$ = 18.95; P < 0.0001).

In the CPB infection bioassays, there was a significant difference between treatments ($F_{6,143}$ = 37.05; P < 0.0001). There was no difference between EPN species. *Steinernema feltiae* infected CPB larvae at 73%, *S. riobrave* infected CPB at 70%. In the treatment combinations of EPN species with mustard seed meal *S. feltiae* x mustard seed meal had a 49.3% infection rate, and *S. riobrave* x mustard seed meal had a 52.6% infection rate (Figure 11).

Table 6. Effect of EPN species (none, *Steinernema feltiae, S. riobrave*) and *Brassica carinata* (seed meal +/-) on the number of *Meloidogyne chitwoodi* females per gram dry tomato root in a greenhouse experiment.

Treatment	# *M. chitwoodi* females/ g dry root
S. feltiae	48.17
S. riobrave	87.04
B. carinata	54.61
S. feltiae x B. carinata	99.98
S. riobrave x B. carinata	20.96
Control	236.13

Figure 10. Interaction between treatment combinations of EPN species (none, *Steinernema feltiae, S. riobrave*) and *Brassica carinata* (seed meal +/-) on the number of *Meloidogyne chitwoodi* females per gram dry tomato root in a greenhouse experiment.
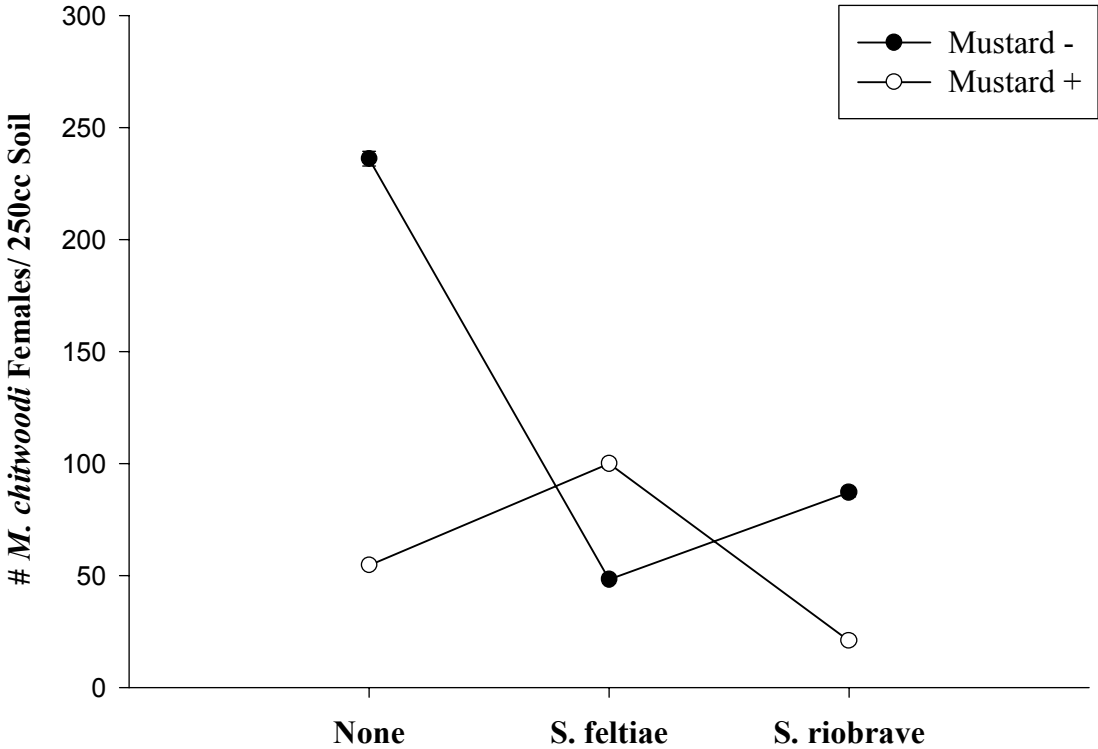
*Data are means +/- SE

Figure 11. Colorado potato beetle infection rate by two EPN species (*Steinernema feltiae, S. riobrave*) alone and in combination with *B. carinata* seed meal soil amendment in the greenhouse.
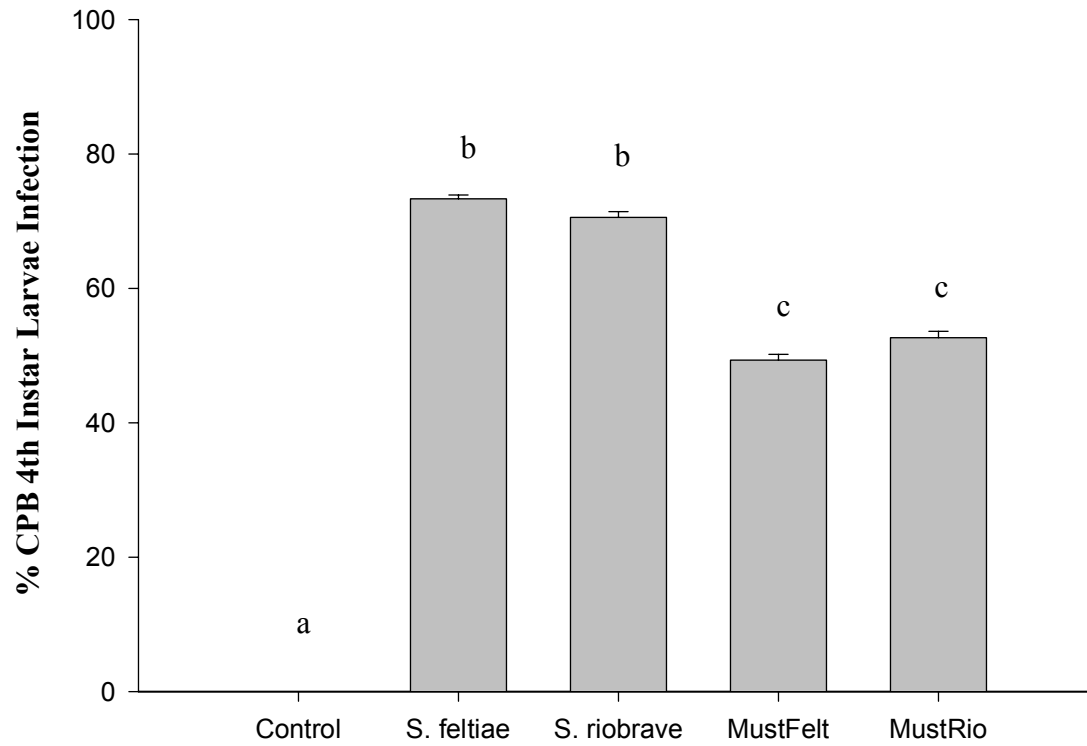
*Different letters indicate significant differences between means.
*Data are means +/- SE

4. Discussion and Conclusions

Entomopathogenic nematodes (EPN) and mustard seed meal soil amendment are both biological control methods that have previously been investigated for suppression of several *Meloidogyne* spp (Grewal et al., 1997; Jagdale et al., 2002; Perez and Lewis, 2004; Perez and Lewis, 2001; Lewis et al., 2001). In many cases, a certain species of EPN or cultivar of mustard seed meal would suppress *Meloidogyne* spp better than another species or cultivar. Therefore, the current study chose EPN species and *Brassica* cultivars previously found to be most effective against *Meloidogyne* spp. and the Colorado potato beetle. The potato plant is attacked by the CPB aboveground, and *M. chitwoodi* belowground; however, both pests have lifecycles which coincide within the same soil habitat. It was hypothesized that one or two biological control methods applied in the soil could inhibit both pests. The use of EPN was chosen because of the ability of EPN to infect CPB insect larvae and suppress *M. chitwoodi* in the same soil habitat. *Brassica carinata* seed meal was also chosen as a biological control product because of the high amount of the chemical glucosinolate sinigrin which has a chemical by-product (2-propenyl) effective against various species of *Meloidogyne* spp. (Lazzeri et al., 2004). However, it was unknown if the two biological control methods would be able to work simultaneously, as the isothiocyanate products of mustard seed meal are known to be nematicidal against plant parasitic nematodes but could also be nematicidal against EPN. It was unknown if the morphology or life habit of EPN would enable it to avoid the nematicidal effects of mustard seed meal.

In the present study, two species of EPN (*Steinernema feltiae, S. riobrave*), in combination with and without mustard seed meal (*B. carinata*) were investigated for suppression of *M. chitwoodi* and infection of the CPB. Of the two species of EPN tested, it was found that *S. feltiae* or mustard seed meal was more effective at reducing *M. chitwoodi* internal and external

tuber symptoms in the 2006 field studies. However, in 2007 results of the treatments differed from 2006. Both species of EPN were able to reduce the internal symptoms, infection index, but did not significantly reduce the external symptoms, percent infection. Mustard seed meal treatment alone significantly reduced the internal and external tuber symptoms. However, the addition of EPN to mustard seed meal appears to have reduced the efficacy of the mustard seed meal treatments in 2006 but not in 2007. The differences in external tuber ratings, percent infection, between the two years of the study could be attributed to the significantly higher nematode population in the 2007 field soil relative to 2006. The yield results correspond to the *M. chitwoodi* tuber infection results. In 2006, *S. feltiae* treatments resulted in increased number of #1 tubers, while mustard seed meal treatments increased the total weight of #1 tubers. In 2007, mustard seed meal treatments increased the total #1 and total weight of tubers. Mustard seed meal treatments in both years resulted in the largest total weight of #1 tubers, followed by an increased total weight of #1 tubers by the treatment *S. feltiae*. In both years, treatments of EPN species *S. feltiae* and *S. riobrave* produced the most #2 tubers.

In the greenhouse, the EPN or mustard seed meal treatments alone reduced infection to a greater extent than combinations of the two treatments. The results of the greenhouse trials are consistent with the field trial of 2006. The combination of treatments mustard seed meal and EPN show that *S. riobrave* alone was not able to reduce infection as well as *S. feltiae*, but the combination of treatments *S. riobrave* with mustard seed meal enhanced the ability of *S. riobrave* to reduce infection. However, mustard seed meal caused an antagonistic effect in the ability of *S. feltiae* to reduce tuber infection by *M. chitwoodi*. Nonetheless, all EPN, mustard seed meal and combination of mustard seed meal and EPN treatments significantly reduced *M. chitwoodi* infection compared to the untreated control.

In the CPB bioassays, the ability of EPN to infect CPB was tested, including the ability of the EPN to infect CPB larvae in the presence of mustard seed meal. In the field study, there was no difference in the ability of either EPN species to infect CPB, alone or in combination with mustard seed meal. However, in the greenhouse there was a stronger inhibitory action of mustard seed meal treatment on EPN infectivity. This effect may be due to the shorter period between the application times of mustard seed meal and EPN treatments in the greenhouse than in the field. CPB infection bioassays were conducted approximately 2 months after mustard seed meal soil amendment in the field experiments, and 1 month after mustard seed meal amendment in the greenhouse experiments. In other studies of mustards used as biofumigants, it was found that most isothiocyanates will be released into the soil within 4 days of incorporation (Morra and Kirkegaard, 2002). This indicates that timing should have an effect on the amount of volatiles released from the mustard treatments throughout the duration of the experiments. In the present study, mustard seed meal amendment was applied before planting, and for the CPB bioassay, EPN was applied several months after the mustard seed meal treatment. This time delay could account for the ability of the EPN to infect the CPB without interference from the mustard seed meal. The greenhouse trials indicate a lower infection rate than in the field, and this may be indicative of the method. In the field, EPN was sprayed onto soil within buckets and larvae were placed on top of treated soil. In the greenhouse, larvae were placed inside cloth mesh bags and placed into soil prior to EPN application, adding a barrier in comparison to the bucket method used in the field study. The mesh bags were not wet, which may have impeded the ability of the EPN to pass through and infect the CPB inside. However, taking this barrier into account, there was still sufficient larval infection in the greenhouse trials to indicate the ability of EPN to kill a single generation of CPB larvae in field conditions.

Results from this study showed a greater ability of *S. feltiae* to reduce *M. chitwoodi* infection in the field in comparison to *S. riobrave*. This difference may be due to the differing biology of the two nematode species. The length of time each species requires in order to complete reproduction in the insect host is different. *Steinernema feltiae* requires nine days to complete reproduction after insect infection in comparison to *Heterorhabditis bacteriophora*, which averaged a seven day time period before juvenile emergence (Unlu and Ozer, 2003). These differences may have an effect on the timing of the suppressive ability of the EPN. It is possible that the cadavers of infected insects or the EPN themselves release allelochemicals at varying periods of time into the rhizosphere during the infection period, and this period of release may be governed by the biology of the specific entomopathogenic nematode. The differences in infection and reproductive time periods between species or genera of entomopathogenic nematodes may result in varying time periods of allelochemical release into the rhizosphere (Unlu and Ozer, 2003). For example, in terms of EPN infection of wax worms in the laboratory, *S. riobrave* required only 4-5 days to infect the wax worm larvae, compared to 8-10 days before larvae were infected by *S. feltiae,* with corresponding delays in completion of reproduction in the wax worm (D. Henderson, unpublished data). The differing periods of allelochemical release from *S. feltiae* compared to *S. riobrave* may explain the results of the present study. The discrepancy in effectiveness between species of EPN is common, and this explanation may be applicable to the varied results comparing different entomopathogenic nematodes of previous studies investigating plant parasitic nematodes suppression (Grewal et al., 1997; Jagdale et al., 2002; Perez and Lewis, 2004; Perez and Lewis, 2001; Lewis et al., 2001). In summary, *S. feltiae* was more effective at reducing *M. chitwoodi* infection of potato, but the difference in *M. chitwoodi* suppression in the field between the two field trials indicates that

further research is needed to optimize the efficacy of these nematode species. *Brassica carinata* seed meal was consistent in both field trials, but suppression of *M. chitwoodi* was not adequate for the needs of potato producers, which have an extremely low tolerance for presence of *M. chitwoodi* in the field (1 juvenile/ 250 cc soil) (Cram et al., 2007). Further research is needed to better understand the interaction of EPN and mustard and improve their efficacy. For example, if the timing was staggered to allow the mustard seed meal to work independently of EPN, this might enhance the ability of the mustard seed meal to reduce *M. chitwoodi*, but allow a mid-season application of EPN to target a generation of CPB. Furthermore, it would be interesting to combine a fall green manure application with spring applied mustard seed meal amendment or EPN application to find out if levels of *M. chitwoodi* would be suppressed to acceptably low levels in the field.

In the analysis of free-living and *M. chitwoodi* nematode populations in the soil, there were no significant differences between treatments for control of *M. chitwoodi* in this soil analysis. However, the free-living nematode populations were adversely affected by the chemical fumigant Mocap, yet the differences were not significantly different than the control or *S. feltiae* applications after the first treatment applications. It is only after the second EPN application that the treatment Mocap, untreated control, *S. feltiae*, and *S. riobrave* lowered populations of free-living nematodes significantly compared to all other treatments. The populations of free-living nematodes leveled out at the end of the season, and were not significantly different from each other. Additionally, these soil observations show that the effect of *S. feltiae* on free-living nematodes was most pronounced after the second EPN application to the same levels as Mocap, while *S. riobrave,* mustard seed meal, or combinations of mustard and EPN did not affect the free-living nematodes. Although Mocap and *S. feltiae* reduced the free-

living nematodes, the nematodes were able to repopulate the soil by the end of season, to levels that were comparable between treatments. These results are promising for both biocontrol and chemical control; if neither method has a long-lasting impact on the populations of free-living nematodes then this is a positive outcome for future studies on soil health. Soil health is often analyzed by the composition or trophic diversity of free-living and plant parasitic nematodes that reside in the soil (Neher, 2001). The trophic diversity of nematodes positively correlates with the nitrogen cycling and decomposition processes critical to soil health (Neher, 2001). This study indicates that biocontrol and chemical control can be used without adversely affecting the soil health long term.

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Acknowledgments

CHAPTER THREE

The Effect of *Brassica carinata* Mustard Seed Meal Amendment on Oviposition and Plant

Choice of Colorado Potato Beetle (Coleoptera: Chrysomelidae)

D. Henderson [a], E. Riga [a2], W.E. Snyder [b]

[a] Department of Plant Pathology, Washington State University, Pullman, WA and Washington

State University, IAREC, 24106 N Bunn Rd., Prosser, WA 99350

[b] Department of Entomology, Washington State University, Pullman, WA 99164

The potential effects of mustard seed meal on the biology of the Colorado potato beetle,

Decemlineata leptinotarsa (Say), have not yet been investigated. In 2006 and 2007, oviposition

rates of Colorado potato beetle confined to feed on mustard seed meal amended or non-amended

potato plants were investigated in the field. The experiment was repeated in the greenhouse, and

the beetles were given a plant host choice and free range to consume and oviposit on randomly

arranged non-amended or amended potato plants. The host plant choice was not significantly

different between plants grown in mustard seed meal amended and non-amended soil in the

greenhouse. Oviposition rates of Colorado potato beetle on potato plants grown on mustard seed

meal amended soil in the field experiments were reduced significantly compared to the non-

amended soil treatment for field experiments of 2006 and 2007. In the greenhouse experiments,

[2] Corresponding author. Ekaterini Riga
Tel: (509) 786-9256 Fax: (509)786-9370
E-mail address: riga@wsu.edu

there was not a significant reduction in oviposition rates (number of egg clusters). However, there was a significant reduction in the size of the egg clusters oviposited by the beetles on mustard seed meal amended potato plants compared to non-amended plants.

1. Introduction

Colorado potato beetle, *Decemlineata leptinotarsa* (Say), (Coleoptera: Chrysomelidae) is an economically important insect pest of potato crops throughout the world (Pedigo, 2002). If Colorado potato beetle is left unchecked it is able to completely defoliate an entire crop by mid-season and may reduce yield by two-thirds (Hare, 1980). Colorado potato beetle infestation has traditionally been controlled using rotations of several different classes of chemicals such as neo-nicotinoid insecticides (imidacloprid, thiamethoxam and clothianidin), pyrethroids, and carbamates among several others throughout the season contributing to the overuse of chemicals in potato crops. There are new chemical pesticides manufactured for Colorado potato beetle control due to the ability of the Colorado potato beetle to evolve resistance to several chemicals introduced into the market (Alyokhin et al., 2007; Forgash, 1985).

Adult Colorado potato beetles over-winter in the soil and female Colorado potato beetles usually mate prior to over-wintering, and emerge in the spring ready to lay the first set of eggs (Pedigo, 2002). Adults feed, mate and lay eggs, with the females able to oviposit up to 300 eggs over a 5 week period (Pedigo, 2002). Eggs are laid in clusters on the underside of potato leaves, hatch in 4-9 days, and larvae feed and molt for 2-3 weeks (Pedigo, 2002). Numerous studies have investigated control methods for the Colorado potato beetle, but few have been adopted as effective control strategies. The Colorado potato beetle is difficult to manage as it is continually evolving resistance to new chemical insecticides such as imidacloprid, thiamethoxam and

clothianidin (Alyokhin et al., 2007). Other methods of alternative control have been investigated in attempts to combat this insect such as the use *Bacillus thuringiensis* Berliner subsp. *Tenebrionis*. However, the Colorado potato beetle has also begun to evolve resistance to this biopesticide, as well (Whalon et al., 1993).

The Colorado potato beetle is a herbivorous insect of plants within the *Solanaceae* family, which have naturally high levels of foliar glycoalkaloids (Carman et al., 1986). The beetle is able to detoxify the glycoalkaloids it consumes, but the glycoalkaloids do have detrimental effects on the biology of this organism. For example, the glycoalkaloids solanine and chaconine can negatively affect biological organisms as acetlycholinesterase inhibitors (Roddick, 1989) and are capable of causing cell membrane lysis (Roddick et al., 1988). In particular, the glykoalkaloids solanine and chacocine have been found to reduce growth rates of Colorado potato beetle (Hare, 1987). Although the Colorado potato beetle is able to avoid toxicity caused by consuming natural glycoalkaloids levels, high levels can actually stress the Colorado potato beetle enough to impact some biological processes (Hare, 1987). Glycoalkaloids levels can be manipulated through increasing the N fertilizer, which results in higher levels of foliar potato plant glycoalkaloids solanine and chacocine than in plants grown in lower N fertilized plots (Armer et al., 2004). Mustard seed meal soil amendment, although used primarily as a biofumigant, also adds more organic matter to the soil, including higher N levels than non-amended soil. The range of biological processes impacted by consuming higher levels of glycoalkaloids in the Colorado potato beetle is uncertain. Mustard and mustard plant products can be used as a biofumigation method for treating plant parasitic nematode infestations instead of standard chemical treatments (Brown and Morra, 1997). Mustard soil amendments can either be in the form of green manures in which case, the mustard plants are grown and then

incorporated into the soil. In addition, mustard soil amendments can be found in the form of commercially prepared soil amendments, such as mustard seed meal (Leoni et al., 2004). Mustard seed meal is the waste byproduct of the mustard seed oil industry. Oil is extracted from the seed through a process called defatting (pressing), and the seed remnants are discarded. However, the seed still contains the original biofumigant properties and can be used as a soil amendment for treating plant parasitic nematodes (Leoni et al.,2004).

Although mustard plants are used as green manures and soil amendments, the volatiles characteristic of these plants may either attract or repel insect herbivory (Reddy and Guerrero, 2004; Mewis et al., 2002). Once the mustard plant tissue is broken, volatiles are released that can be inhibitory to insect herbivory, or attract some insects to the plant. Phytophagous insects have olfactory and visual cues which are used to locate plants for oviposition and feeding. Insects which feed on glucosinolate containing plants use the hydrolysis products for host-plant finding and oviposition (Mewis et al., 2002). However, other insects may react to these same stimulants as deterrents and avoid these plants for feeding and laying eggs (Reddy and Guerrero, 2004). Mustard plants contains inert levels of glucosinolates and myrosinase that are released upon tissue degradation (such as plant tissue maceration) to catalyze the production of volatile isothiocyanates which have pesticidal properties. If mustard is used as a soil amendment its volatiles are released into the soil upon tillage and irrigation (Bones and Rossiter, 1996). This mustard seed meal is applied to the soil as an amendment and is more commonly used for biofumigation of nematodes. However, the mustard seed meal may also have the added benefit of repelling insects from oviposition or choosing plant hosts that have been grown on mustard seed meal amended soil.

This study examines the potential effects of mustard seed meal in the oviposition and plant choice behavior of Colorado potato beetles. The objectives of this study were to: 1) determine if potato plants grown in *Brassica carinata* mustard seed meal amended soil deterred Colorado potato beetle from oviposition, and 2) determine if the *B. carinata* mustard seed meal amendment will affect Colorado potato beetle plant host choice in the greenhouse.

2. Materials and Methods

2.1 Colorado Potato Beetle Oviposition Field Experiments

Field plots in Washington State University, IAREC, Prosser, WA in 2006 and 2007 were set up in a randomized complete block design with 5 replications. Plots were 2.4 x 6 m with 0.30 m inter-row spacing and three rows per plot, the middle row being buffered by two border rows. Soil was loamy sand consisting of 84.20% sand, 10.60% silt, and 5.20% clay and had a pH 6.7. Fertilizer was applied (402.5 kg actual Nitrogen/ ha; 113.25 kg actual Phosphorus/ ha; 85 kg actual Potassium/ ha; 45.35 kg actual Sulfur/ ha; 2.25 kg actual Boron/ ha) to both the mustard seed meal amended and non-amended soil prior to potato planting.

Seed meal of *Brassica carinata* was applied at 4.4 kg per plot or 2.5 ton/ ha on May 30, 2006 and May 1, 2007 followed by ~5 cm of irrigation. The *B. carinata* seed meal is a commercial product "Biofence" (Triumph Italia S.p.A. Livorno, Italy) produced from *B. carinata* sel. ISCI 7 seeds through a proprietary partial defatting method which limits glucosinolate and myrosinase degradation (Lazzeri et al., 2002). The *B. carinata* seed meal has a N composition greater than 6% and a residual oil level of 2.6% (Lazzeri et al., 2004). The chemical composition of the mustard seed meal was previously characterized, containing 163.4 µmol g-1 of

glucosinolates, 98% of type 2-propenyl glucosinolate (sinigrin) and a adequate level of myrosinase enzyme to catalyze glucosinolate hydrolysis (Leoni et al., 2004).

Russet Burbank potatoes were planted fifteen days post-application on June 15, 2006 and May 15, 2007. The Colorado potato beetle adults were observed feeding on potato plants on July 20, 2006 and July 6, 2007. Mesh sleeve cages of dimensions 17 x 38 cm were sewn to leave a sack-like opening at one end. This design allowed easy fitting of the mesh sleeve cages over individual potato stems. Sleeve cages were fitted over the stems of three randomly chosen potato plants in each experimental plot. Three male and 4 female beetles were collected from each plot and inserted into the mesh sleeve cages and the open end was tied close using a string to prevent the escape of the beetles. Beetles were observed mating and closely observed for the first set of egg oviposition by the females. If the beetles consumed too much leaf area the cages were repositioned on a different stem of the same plant. The number of egg clutches $\geq$ 5 eggs/ cluster was recorded for each of the three plants in each plot after the first oviposition event was observed. The experiment was terminated after observation of oviposition. There were five replications in the field for each treatment. Data were analyzed using Proc GLM, followed by Fishers LSD, (SAS Institute Inc, 2003).

2.2 Colorado Potato Beetle Host Choice and Oviposition

Potato plants were transplanted from the field into 3.6 L pots using field soil from the respective amended or non-amended plots on June 15, 2006 and arranged into a completely randomized design with 12 each of non-amended and mustard amended plants, and repeated in three different cages. The potato plants were taken from mustard seed meal amended soil and

non-amended soil and transplanted into pots using the original field soil. The potted potato plants were randomly arranged inside four plastic crates (0.9 x 1.2 m). The four crates were aligned in tandem to form a continuous platform for beetles to traverse between crates. The crevices between crates and pots were filled with potting media to allow for a level walking surface surrounding all of the plants. This arrangement allowed the easy movement by the beetles between individual plants. The crates were closed off on all edges with cardboard to keep beetles from wandering off the experimental site. The experimental setup was kept inside large mesh cages in the greenhouse at a temperature of 26 ºC ± 2 ºC. Plants were watered every other day until the experiment was terminated. Beetles were released at a density of 3 beetles per plant at randomly chosen points between potato plants. Beetles were allowed to randomly move between plants until the first oviposition events were observed. The experiment was terminated after the first oviposition event at approximately 24 hours. The number of egg clutches ≥ 5 eggs per cluster was recorded for each of the plants after the first oviposition event was observed. Additionally, the number of individual eggs was counted for each egg clutch on every plant, and organized into four size categories, 5- 10, 11- 20, 21- 50, and 51- 200. The number of beetles on each plant was recorded at the time of oviposition to determine the host plant choice. The data were analyzed using Proc npar1way, Kruskall Wallis test (SAS Institute Inc, 2003).

3. Results

There was no interaction between years in the field trials or between the three greenhouse trials; therefore, the respective data were combined for final analysis. Oviposition rates of Colorado potato beetle on plants grown on mustard seed meal-amended soil in the field experiments were reduced significantly ($F_{10, 49} = 5.59$; $P < 0.0001$) compared to the control or

non-amended soil treatment for 2006 and 2007 field experiments (Figure 12). On average, there were approximately 6.27 egg clusters laid on the plants grown in non-amended soil, while plants grown in mustard seed meal amended plants consistently had fewer ~3.14 egg clusters oviposited on the plants.

In the greenhouse experiments, there was no significant difference between oviposition rates or the number of egg clusters. However, there was a significant difference in the number of eggs laid by the beetles on mustard seed meal-amended plants (Figure 13). Significantly more egg clusters of ranges 5-10 eggs (Chi-Square = 8.848, df =1; P = 0.0032), and 51-200 eggs (Chi-Square = 5.5630, df =1; P = 0.0183) were laid on non-amended plants. The host plant choice was not significantly different between plants grown in mustard seed meal-amended and non-amended soil (t_{70} = -1.58; P = 0.1189). In the greenhouse experiment, there was no difference in height of the transplanted potato plants (t_{70} = -0.92; P = 0.3628).

Figure 12. Oviposition rates of Colorado Potato Beetle on mustard seed meal amended and non-amended (control) potato plants in 2006 and 2007 field experiments.
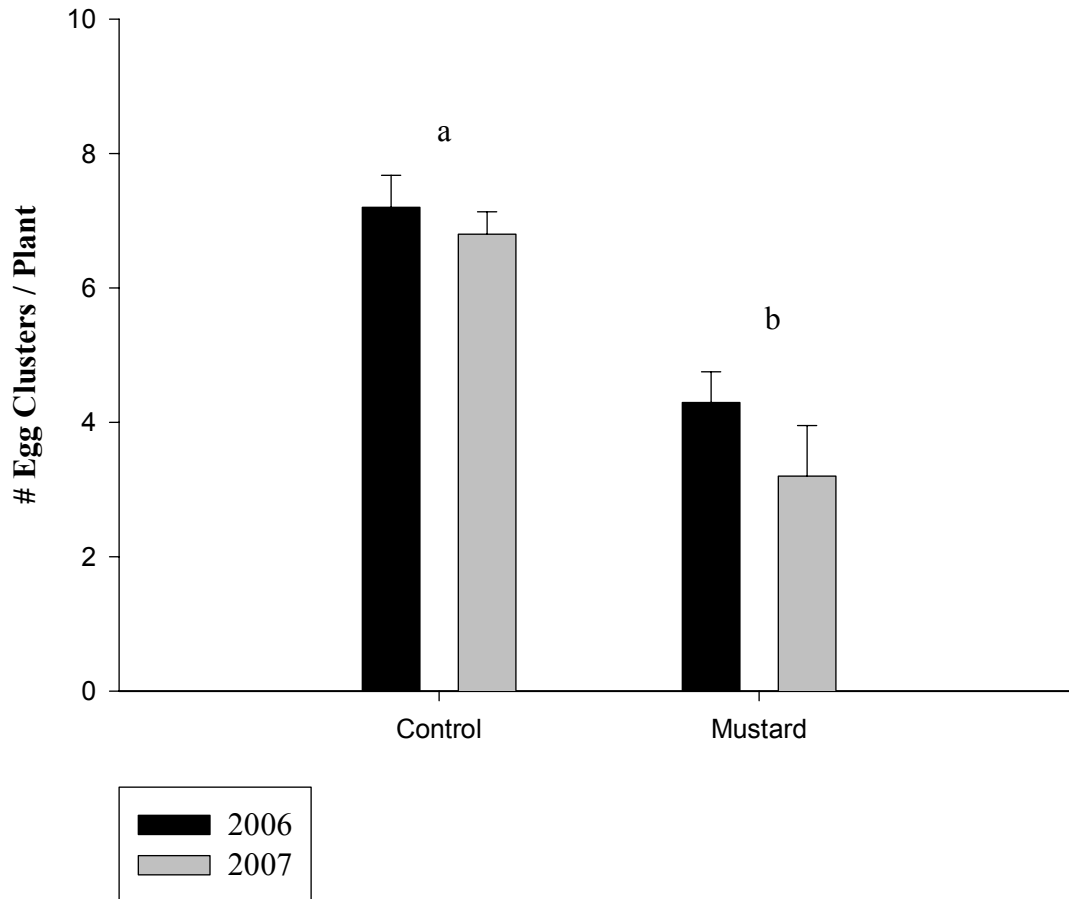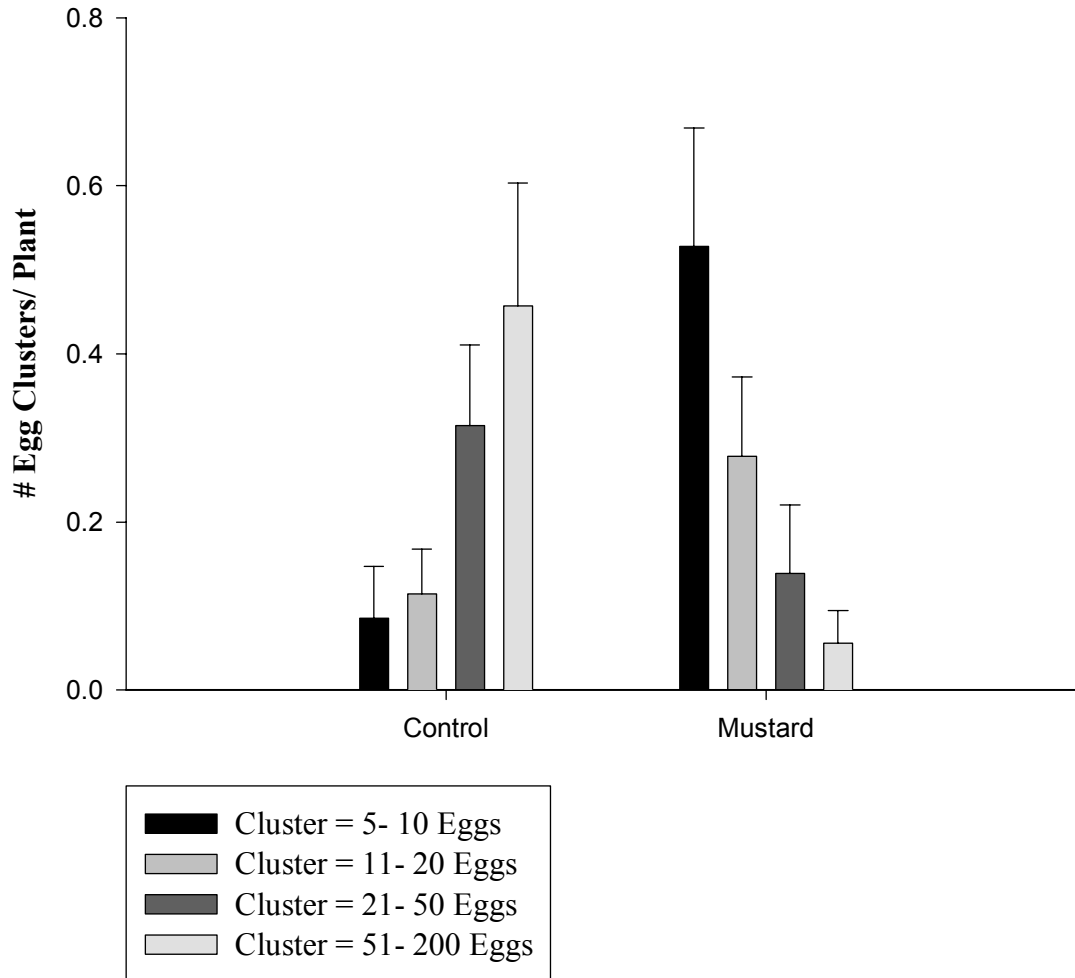

*Different letters indicate significant differences between means
**Egg cluster = $\geq$ 5 eggs/ cluster
***Data are means +/- SE

Figure 13. Oviposition rates of Colorado Potato Beetle on mustard seed meal amended and non-amended potato plants across four cluster size ranges in the greenhouse experiments.

*Data are the mean +/- SE

4. Discussion and Conclusions

In the 2006 and 2007 field experiments there was a significant reduction in the number of Colorado potato beetle egg clusters laid on potato plants grown in mustard seed meal amended soil compared to potato plants grown in non-amended soil. This was a 50 percent reduction in the oviposition rate of beetles on mustard seed meal amended plants than the non-amended control plants in the field. In the greenhouse experiments there were a significant reduction in the number of egg clusters and total eggs laid on the mustard seed meal-amended transplanted potato plants compared to the non-amended plants.

In the field, it is likely that the increase in N levels in the mustard seed meal-amended soil yielded plants with higher levels of plant glycoalkaloids than in non-amended soil based upon previous studies of increasing N levels (Armer et al., 2004). The beetles in the field experiments were confined to cages and unable to make a choice of plant foliage consumption. It is hypothesized that the Colorado potato beetles caged in mustard seed meal amended cages were exposed to higher levels of glycoalkaloids than the non-amended plants. This increase in consumption of plant glycoalkaloids may have stressed the Colorado potato beetles (Armer et al., 2004; Hare, 1987). Imbalances in plant minerals has been found to negatively affect Colorado potato beetle oviposition rates and reduce larval development times (Alyoykin et al., 2005). An increase in glycoalkoloids may have placed enough stress on female beetles to require more energy be channeled toward detoxification than on egg production and oviposition. The greenhouse trials show a reduction in oviposition rates, in both the number of egg clusters and the amount of eggs laid on mustard seed meal amended plants. The beetles may have preferred to lay larger clutches of eggs on non-amended plants to ensure the offspring can feed on plants

containing lower levels of glycoalkoloids, consistent with other studies showing the deleterious affects of high glycoalkoloid consumption (Hare, 1987). At the time of oviposition, there were no significant differences observed in the number of beetles consuming plant foliage from mustard seed meal-amended or non-amended plants. It is unknown whether the beetles were selective in choosing to feed on the non-amended or mustard seed meal amended plants prior to oviposition. The mustard seed meal amended soil might still have retained some mustard seed meal activity, releasing volatile isothiocyanates which are naturally pesticidal (Brown and Morra, 1997). However, because there were no significant differences in the beetle plant host choice, it is unlikely that the mustard seed meal amendment was emitting volatiles which were a deterrent to the Colorado potato beetle plant choice.

In the field the beetles were confined to feed on a mustard seed meal amended or non-amended plant while in the greenhouse there was freedom of choice between plants. The amount of energy the beetle might use to detoxify the higher levels of glycoalkoloids in the mustard seed meal-amended plants might have detracted from the energy required to produce and lay eggs. This is reflected in the greenhouse results, where beetles chose to lay larger number of total eggs on non-amended plants, perhaps so larvae feed on lower levels of glycoalkoloids and enhance their chances of survival.

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