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Bacillus firmus* for the biological control of *Meloidogyne hapla* and *Xiphinema americanum

Lisa M. Valencia

**Thesis submitted to the
Davis College of Agriculture, Natural Resources and Design
at West Virginia University
in partial fulfillment of the requirements for the degree of**

**Master of Science
in
Applied and Environmental Microbiology**

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Division of Plant and Soil Sciences

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2016**

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nematode, biological control, bionematicide, mode of action, chemotaxis**

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ABSTRACT

Bacillus firmus* for the biological control of *Meloidogyne hapla* and *Xiphinema americanum

Lisa M. Valencia

Root-knot nematode (RKN) and dagger nematode (DN) are serious pests of agricultural crops, including peach trees of West Virginia. RKN is a sedentary endoparasite that feeds and reproduces within the roots of host plants, causing gall formation, loss of vigor, and plant death. DN is an ectoparasite that has the potential to vector devastating plant viruses. Many control options recommended for annual crops are not appropriate for orchards and the use of chemical nematicides is limited. The endospore-forming bacterium, *Bacillus firmus* (BF), is marketed as a bionematicide, though its mode of action and efficacy in controlling certain nematodes indigenous to West Virginia are unclear. The purpose of this study was to determine efficacy and mode of action of BF as a bionematicide against the northern RKN, *Meloidogyne hapla* and DN, *Xiphinema americanum*. Direct toxicity was determined by exposing RKN and DN to various concentrations of BF over a 72-hour period. Exposure to a 10^7 CFU/ml concentration of BF caused a 15 % decrease in living RKN and an 11 % decrease in living DN by 72 hours. No effect was observed with lower concentrations. *In-vitro* attraction assays were performed to determine if the presence of BF affects nematode migration and infection rates. Filter paper discs were treated with BF and sterile soil extract (SSE) and were placed at either end of a slide covered with Pluronic gel. Approximately 150 RKN or 30-50 DN were placed in the center of each slide and the number of nematodes that left the center and migrated to each side was counted at 1, 2, 4, and 24 hours post inoculation. A similar attraction assay was performed with tomato seedlings instead of filter paper. Filter paper assays showed that 93 % of motile RKN were observed on the SSE portion of the slides compared to 7 % on the BF side by hour 24. Attraction assays using tomato seedlings showed 71 % of motile RKN were observed on the portion of slides with SSE-treated roots, compared to 29 % on the side with BF-treated roots. DN results were contrary to RKN results. Filter paper assays showed 59 % of motile DN on the BF portion of slides compared to 41 % on the SSE side, while tomato seedling assays showed no significant difference between treatments at hour 24. Infection assays using seedlings on glass slides showed an average of three RKN successfully penetrated BF-treated roots compared to 20 in SSE roots. Attraction assays in sand, comparing RKN infection of BF and SSE-treated tomato seedlings, showed no significant difference between treatments. Nematode mortality observed after exposure to BF suggests that BF produces secondary metabolites that are directly toxic to RKN and DN, though these metabolites have limited potency. Behavior of RKN in the presence of BF suggests the involvement of a chemorepellent, while the behavior of DN suggests the involvement of a chemoattractant. The results of this study indicate that the mode of action of BF is linked to the production of chemorepellent compounds, though these chemotactic factors are species specific. BF is a promising biocontrol option for the management of RKN but may not demonstrate the same measure of control against DN.

Dedication

To the many amazing people that helped make this happen and kept me sane through the process. A very special thanks to NAKS, mom, and my Baby Bugg.

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CHAPTER 1

LITERATURE REVIEW

Introduction

Plant-parasitic nematodes are serious pests of agriculture that continue to threaten the security of the commercial fruit industry in West Virginia and other Mid-Atlantic states (Kotcon, 1990; Walters and Barker, 1994; Koenning et al., 1999). Many of the nematode species that parasitize plants, most notably species of *Meloidogyne*, cause major crop losses throughout the world. The damage generated by the parasitic activity of these nematodes can be further amplified by the ability of certain species, including *Xiphinema* spp., to act as virus vectors. As the available chemical options for nematode management decline, growers diagnosed with nematode problems have limited options, many of which are unacceptable for established orchard sites. These situations call for effective bionematicide alternatives. Several studies have demonstrated that the bacterium *Bacillus firmus* is an effective option for biological control of certain nematode species. However, it is uncertain if this organism maintains similar efficacy against problematic nematodes of West Virginia fruit trees such as *Meloidogyne hapla* and *Xiphinema americanum*. Furthermore, the specific nematicidal mode of action employed by *B. firmus* remains disputed.

Plant-parasitic nematodes

Plant-parasitic nematodes are economically important agricultural pests, causing billions of dollars in crop damage annually (Handoo, 1998). As these microscopic roundworms feed on the roots of plants, they impair the health and vigor of crops, which can result in an overall decline in crop yields as well as host plant death. A 1994 survey of various crops grown in 35 states of the USA indicated that at least 25% of crop losses can be attributed to the presence of plant-parasitic nematodes (Koenning et al., 1999). This plant damage may be a direct result of nematode infection within roots and ingestion of cell contents, or may be associated with indirect consequences of nematode parasitic activity. Nematode-inflicted wounds on root tissue leave plants more susceptible to secondary infection by fungal and bacterial pathogens, and may also facilitate transmission of nematode-vectored plant viruses (Powell et al., 1971; Brown et al., 1995). Two of the most economically important nematode genera, *Meloidogyne* spp. and *Xiphinema* spp., are abundant in peach and apple orchards of West Virginia (Kotcon, 1990).

Meloidogyne spp.

Root-knot nematodes of the genus *Meloidogyne* are regarded as highly destructive pests to agricultural crops worldwide (Barbosa et al., 2004; Gugino et al., 2006; Hussain et al., 2011; Koenning et al., 1999; Wesemael et al., 2011). These sedentary endoparasites live most of their lives within the roots of their host plants, feeding on cytoplasmic material of root cells. Female root-knot nematodes use both sexual and asexual reproduction, laying upwards of 1000 eggs while embedded within the host plant roots (Chitwood and Perry, 2009). After completing embryogenesis and one molt, the larvae emerge from their eggs as second stage juveniles (J2), the infectious stage of the species, and either re-infect the same root or migrate to new infection sites. CO₂ and other volatile compounds associated with plant roots act as chemoattractants to the J2s, allowing them to orient themselves accordingly to find host plant roots (Robinson, 1995; Wang et al., 2009). Once an acceptable host root has been located, the nematode secretes degrading enzymes and uses its stylet to puncture the root tissue near the zone of elongation, and migrates inside (Wyss et al., 1992). The nematode quickly establishes a feeding site where it remains sedentary through the remainder of its life, if female, or until it molts into a mature adult, if male. Salivary secretions produced by the nematode promote the formation of several multinucleated giant cells that act as nutrient pools for the feeding nematode (Bird, 1962; Jones, 1981; Mor (Mordechai) and Oka, 2006). Root galls form around the area of infection, which ultimately inhibits the flow of water and nutrient within host plant roots.

The life cycle of root-knot nematodes is temperature dependent and is completed in approximately 600 degree days with a base temperature of 8.13°C (Bird and Wallace, 1965; Vrain et al., 1978; Starr and Mai, 1976; Lahtinen et al., 1988). Within host plant roots, J2s undergo three additional molts to develop into a mature, reproductive adult. Adult males are vermiform and mobile. Adult females are pyriform and sedentary, maintaining only the musculature in the neck region to enable feeding (Eisenback and Hunt, 2009). Each reproductive female deposits several hundred eggs on the surface of host plant roots, held together within a gelatinous matrix. The ability of these nematodes to produce large numbers of progeny in short periods of time can quickly overwhelm susceptible agricultural crops. If root-knot nematode infestations are left untreated, plants can exhibit signs of severe nutrient deficiencies such as stunting, yield loss, and death. If numbers are maintained at population levels below the damage

threshold, root-knot nematodes are considered less of an immediate threat to West Virginia peach and apple orchards, however, once they become established in these deep-rooted perennial crops, control is difficult and options are limited (Nyczepir, 1991).

Xiphinema spp.

Dagger nematodes are migratory ectoparasites belonging to the Longidoridae family. The female worms produce eggs by parthenogenesis and observation of males is rare. In contrast with many nematode species, *Xiphinema* spp. maintains a relatively low reproductive rate and a long lifespan. Their life cycle may take several months to over a year to complete and they have been reported to live 2-5 years in their natural soil environments, laying only a few eggs monthly (Flegg, 1968; Cohn and Mordechai, 1969; Brown and Coiro, 1983; Brown, 1986). These nematodes live their entire lives within the soil and feed along the growing roots of plants with a long, needle-like stylet (Cohn, 1970; Weischer and Wyss, 1976). The stylet punctures through plant cell walls, enabling the nematode to ingest the cytoplasmic material of cells. It is this method of ectoparasitism, along with their long life span, wide host range, and persistence in fallow soil that contribute to the efficiency of *Xiphinema* spp. as a vector of nepoviruses.

Dagger nematodes are notorious virus vectors that are known to transmit viruses within the genus *Nepovirus* (nematode transmitted polyhedral viruses) throughout a wide range of host plants (Brown et al., 1993; Taylor and Raski, 1964). One such virus, tomato ringspot virus (TomRSV), is the causal agent of peach stem pitting (PSP), a devastating affliction of peach trees, and apple union necrosis and decline (AUND) in apple trees (Smith et al., 1973; Parish and Converse, 1981). PSP-diseased trees possess highly disorganized xylem tissue which can enable the breakage of whole trees in just a few years (Barrat et al., 1968). AUND produces similar symptoms within apple trees, though typically less severe than those observed in peach and generally confined to the graft union (Stouffer et al., 1977). TomRSV infections cannot be cured and diseased trees must be removed and destroyed to prevent viral spread, a practice that often leads to great losses within a single orchard. Dagger nematodes responsible for transmission of TomRSV are a persistent presence in Mid-Atlantic orchards (Georgi, 1988; Jaffee et al., 1987). Kotcon (1990) determined that at least 74% of peach orchards surveyed in West Virginia harbor *Xiphinema* spp., and it can be expected that apple orchards maintain similar *Xiphinema* spp. populations.

As dagger nematodes feed on infected plants, viral particles may adhere to the stylet and esophageal lumen of the feeding nematode, establishing the nematode as a potential virus vector (Taylor and Robertson, 1970; Raski et al, 1973). Viral particles will remain attached until the nematode molts or the stylet is used to puncture root tissue of a new host plant. During feeding, dagger nematodes secrete digestive enzymes into the cytoplasm of root cells, a process that can cause attached viral particles to dislodge and be transmitted directly into the new host cell (Taylor and Robertson, 1970). The virus is then free to replicate and eventually translocate throughout the plant. These viral particles can be acquired by subsequent nematode feeding, allowing the cycle of transmission to continue. The potential for dagger nematodes to act as virus vectors complicates the issue of nematode damage thresholds. Plants parasitized by virus-free dagger nematodes show few symptoms at low to moderate nematode population densities. However, nematodes harboring viral particles can fuel the development of serious crop damage. A single dagger nematode can transfer TomRSV to a susceptible host plant, and only a few are needed to infect multiple new host plants in a matter of weeks (Mountain et al., 1992). Plants are rarely infected with TomRSV mechanically, leaving dagger nematodes as the primary mode of viral transmission. Elimination of the nematode vector is crucial for controlling the frequency of disease.

Nematode management

Traditional means of nematode management, such as crop rotation, are not always appropriate or cost effective for orchard sites. Resistant rootstocks are recommended, however, the use of rootstocks, as well as rootstock selection is based heavily on individual orchard situations (Esmenjaud et al., 1994; Fernández et al, 1994; McKenry et al., 2001). Not all cultivars are compatible with resistant rootstocks and different rootstocks offer varied resistance to nematode species, as well as other plant pathogens (Nyczepir, 1991). With the escalated concern for human and environmental safety, the ability to manage nematode populations with classic chemical nematicides is waning. Few options are available to peach and apple growers, and recommendations often involve removal of whole trees and fumigation of fallow land. Except for the insecticide spirotetramat, there are presently no chemical nematicides registered for application on bearing fruit trees in the state of West Virginia. There remains a clear need for novel biological control options that offer reliable control of nematodes within orchard settings,

while remaining environmentally benign. As it stands today, a limited number of commercialized bionematicides reporting efficacy in nematode control are available for use in various agricultural systems. Notable biological agents used as active ingredients within these products include *Pasteuria* spp., *Purpureocillium lilacinum*, and *Bacillus firmus* (Kariuki et al., 2006; Singh et al.; 2013; Keren-Zur et al., 2000). In recent years, the most successful bionematicide, based on consumer reports, has been the Bayer CropScience product, VOTiVO, a seed treatment utilizing the bacterium *B. firmus* (Wilson and Jackson, 2013).

Bacillus firmus

Bacillus firmus is a member of the genus *Bacillus*, a diverse group of gram-positive, endospore-forming bacteria. Along with the anaerobic group of Clostridia, these aerobic or facultative anaerobic organisms make up a large part of the Firmicutes phylum. Species within the genus *Bacillus* are ubiquitous in nature. A large majority of *Bacillus* spp. are soil-dwelling saprophytes, though a few species are known to have medical, agricultural, and industrial significance (Turnbull, 1996). *B. anthracis* and, occasionally, *B. cereus* are pathogenic to humans and other animals, *B. thuringiensis*-produced crystals are used as a bioinsecticide in agriculture, and certain *Bacillus* species are employed in the industrial production of useful organic compounds such as vitamins and proteins (Welkos et al., 1986; Pezard et al., 1991; Bottone, 2010; Höfte and Whiteley, 1989; Tang et al., 2004; Bajaj and Manhas, 2012). *Bacillus* spp. have a diverse range of physiological characteristics that allow them to inhabit a variety of ecological niches, including those with environmental extremes of high temperature, high pH, and high salt (Epstein and Grossowicz, 1969; Nielsen et al., 1995; Ventosa et al., 1989; Turnbull, 1996). Their endospore-forming capabilities allow them to survive unsuitable conditions for hundreds, if not millions, of years (Cano and Borucki, 1995; Errington, 2003). High bacterial densities and limited nutrient availability trigger sporulation of *Bacillus* spp., with each vegetative cell producing one dormant endospore capable of surviving UV radiation, extreme desiccation, dry and wet heat, and common disinfectants (Sonenshein, 2000; Nicholson et al., 2000). When endospores sense the return of favorable conditions, they germinate back to their vegetative form.

Because of the diversity of these organisms and the unique characteristics associated with them, *Bacillus* spp. have been subject to a great deal of research in the past several decades. They possess unique qualities that make them desirable for the biological control of nuisance

organisms such as pathogenic bacteria and plant-parasitic nematodes, and their endospore survival strategy allows them to persist over time within desiccated soil, a feature that may be exploited for convenient packaging and distribution as pesticides. *Bacillus* spp. produce an array of secondary metabolites that perform a variety of biological functions, including antagonistic activities on neighboring microorganisms and the promotion of symbiotic relationships within the rhizosphere (Sansinenea and Ortiz, 2001; Chen et al., 2008; Leelasuphakul et al., 2008). *B. subtilis*, for example, is able to produce over two dozen antibiotics of medical and agricultural significance, and devotes nearly 5% of its genome to the cause (Stein, 2005). *B. thuringiensis* produces crystals during sporulation that have insecticidal properties, a trait that has been applied to the genetic modification of agricultural crops (Schnepf et al., 1998). Wei et al. (2003) revealed that the toxicity of these proteins extends to the phylum Nematoda. The relatively new discovery of a nematocidal strain of *B. firmus* (I-1582) has prompted numerous investigations into the use of this organism to suppress agriculturally destructive nematode populations (Peleg and Feldman, 1996).

The nematocidal strain of *B. firmus* (I-1582) was first isolated from cultivated soils of Israel, quickly becoming the active biological agent of a novel bionematicide, BioNem-WP. Under laboratory conditions, Keren-Zur et al. (2000) demonstrated that applications of the *B. firmus*-based product to *Meloidogyne javanica*-infested soil caused a rapid decline in juvenile viability within a period of 3 to 5 days, and further maintained low root-knot nematode densities of less than 1 juvenile per gram of soil throughout the remainder of the experiment's 20-day period. Field trials incorporating *B. firmus* into root-knot infested sandy soils subsequently planted with tomato seedlings demonstrated that the product reduced the galling index of roots from 4-5 (an index representing severe galling) in the untreated controls, down to an index of 1-2 (an index representing mild galling), for 50 and 85 days after planting. This novel research provided strong evidence that the use of *Bacillus firmus*-based nematicides for agricultural purposes was worth further investigation.

Giannakou et al. (2003) used greenhouses infested with *Meloidogyne* spp. to compare the efficacy of BioNem-WP in controlling root-knot infection of cucumber plants to standard fumigant and nonfumigant nematicides and the well-known biocontrol agent *Pasteuria penetrans*. The study demonstrated that, though *B. firmus* was less effective as a nematicide than 1,3-dichloropropene, dazomet, and oxamyl, nematode populations were significantly lowered

with band applications of *B. firmus* compared with the untreated control, and even further reduced with broadcast applications. The study also showed that *B. firmus* was more effective at reducing root-knot nematode populations than *P. penetrans* and could suppress nematode population densities throughout the growing season, an aspect that can drastically reduce nematicide application costs for growers. Through a series of *in-vitro* and greenhouse trials, Terefe et al. (2009) presented compelling evidence that BioNem-WP was successful in controlling root-knot populations. Applications of BioNem-WP in concentrations varying from 0.5 to 2.5% (Bionem concentrations produced by adding 0.5, 1, 1.5, 2, and 2.5 grams of Bionem-WP powder formulation to 99.5, 99, 98.5, 98, and 97.5 mL of sterile water respectively) on *Meloidogyne incognita* egg masses *in-vitro*, inhibited egg hatching 98-100% by 3 days after treatment, while hatching in the control continued up to day 24. Applications of 2.5-3.0% BioNem-WP to root knot juveniles *in-vitro*, resulted in 100% inhibition of motility. BioNem-WP applied to tomato seedlings planted in sterilized soil reduced gall formation up to 91% and increased plant height and biomass. Mendoza and Sikora (2009) considered the efficacy of *B. firmus* alone and in conjunction with *Fusarium oxysporum* as a means of controlling *Radopholus similis* in banana production. They determined that *B. firmus* significantly reduced *R. similis* penetration into banana roots, while the combination of *B. firmus* and *F. oxysporum* produced a synergistic effect to lower nematode populations as well as increase shoot fresh weight greater than that of the untreated control.

Mode of action

Though products utilizing *Bacillus firmus* as an active ingredient have been found effective at reducing nematode densities in the aforementioned studies, conflicting information exists about the biology, ecology, and mode of action of *B. firmus* when used as a nematicide (Wilson and Jackson, 2013). Bionematicidal agents typically offer control of nematode populations by expressing direct toxicity, initiating plant host defense, or interfering with nematode-host interactions. From early studies of the nematicidal strain of *B. firmus*, it was proposed that the organism's mode of action involves the production of toxic secondary metabolites. The BioNem-WP patent application filed April 16, 1996 stated that "...proteolytic and collagenolytic activities play an important role in control of nematodes, either by direct effect on the cuticle of nematode, or indirectly by increasing the release of ammonia which is

known to be toxic to nematodes due to protein breakdown...” (Patent US6406690, Peleg and Feldman, 1996). The study illustrated that higher levels of proteolytic and collagenolytic activity were expressed by *B. firmus* compared to other bacterial species examined. It is well established that many species within the genus *Bacillus* are able to produce various metabolites and extracellular enzymes that have a range of antimicrobial properties. Certain strains of *B. firmus* have been shown to generate antibiotics that are lethal to other bacteria such as *Escherichia coli* and species of *Staphylococcus* (Adamu et al., 2009). This antibiotic production may explain the organism’s ability to suppress nematode densities. Mendoza et al. (2008) demonstrated the production of nematicidal bioactive metabolites by *B. firmus* in *in-vitro* tests on mobile stages of *R. similis*, *M. incognita*, and *Ditylenchus dipsaci* nematodes. Three-day-old cultures of *B. firmus* grown in trypticase soy broth (TSB) were centrifuged to separate bacterial cells from TSB and the supernatant was passed through 0.45 and 0.22- μm Millipore filters to remove any remaining cells. Observable paralysis and mortality were seen after root-knot, stem, and burrowing nematodes were incubated in 15-100% concentrations of bacteria-free culture filtrates. Culture filtrates at 100% concentration led to 25, 33, and 11% mortality of *R. similis*, *M. incognita*, and *D. dipsaci*, respectively. Suspensions consisting of 100% concentrations of bacteria-free culture filtrates applied to *M. incognita* egg masses resulted in significantly reduced hatching compared with the untreated control. Nematode mortality and hatching inhibition in the absence of bacterial cells demonstrates that *B. firmus* production of bioactive compounds is directly toxic to nematodes.

Bacillus firmus is currently marketed by Bayer CropScience and sold as a nematicide seed treatment (VOTiVO). The proposed mode of action associated with the *B. firmus* in these products involves bacterial colonization of young roots that create a “living barrier” to promote healthy root growth and reduce nematode damage. It is reported that *B. firmus* competes with nematodes for space along the root system and interferes with nematode host finding ability by consuming plant root exudates. Chemotaxis is an important factor in nematode host finding that involves the interaction of chemotactic signals with cephalic amphids of the nematode. Chemotactic factors accumulate at receptor sites within the amphids and lead to the orientation of the nematode either towards the source of a chemoattractant or away from the source of a chemorepellent (Zuckerman and Jansson, 1984). As nematodes encounter attractive root exudates within their environment, they are drawn towards the suitable host plant’s roots.

Altering these chemical signals can prevent nematodes from finding food or even repel nematodes from their food source.

The relationship between rhizobacteria and plant roots is known to have a variety of positive effects on root development, such as boosting host plant defenses, promoting plant growth, and altering plant root exudates, all of which are a potential hindrance to nematode parasitic behavior (Sikora et al, 2007). Diaz et al. (2009) demonstrated that inoculating roots of *Eucalyptus globulus* with naturally associated rhizobacteria can stimulate plant rooting, fibrosity and, biomass. The study showed that inoculating soil with various strains of rhizobacteria successfully increased *E. globulus* plant growth compared to untreated controls. A strain of *B. firmus* specifically isolated from the study, increased rooting by 43.8%. Thus, the application of *B. firmus* (I-1582) to plants may prove to be a beneficial approach to promoting plant growth as well. Dadwal and Jamaluddin (2010) demonstrated the ability to suppress the occurrence of post emergence root rot of four forest species. A 150-cc aliquot of an 11-day old *B. firmus* culture (diluted 10 times with sterilized water) was applied to soils inoculated with a pathogenic *Fusarium oxysporum* spore suspension (10^6 spores/mL) and planted with *Acacia nilotica*, *Albizia procera*, *Albizia lebbek*, and *Dalbergia sissoo* seedlings. After a 30-day period, seedlings treated with *B. firmus* showed no evidence of root rot while 13-28% of the untreated seedlings showed signs of infection. The study demonstrates that *B. firmus*'s association with plant roots can have an antagonistic effect on plant pathogenic organisms, an effect that may extend to plant-parasitic nematodes.

Rationale

Control of plant-parasitic nematodes in agricultural systems has proven to be an increasingly daunting task, further complicated by government restrictions on available chemical nematicides as well as the unreliability of available biological controls. Many growers, especially those involved in organic crop and tree fruit production, are left with little insight into how to manage problematic nematode infestations. Presently, nematicide research aims largely at exploiting the nematicidal properties of soil-dwelling bacteria and fungi to produce environmentally friendly bionematicide options. Bionematicide products utilizing *B. firmus* as the active ingredient have appealed to growers in recent years, yet information regarding the bacterium's ability to control different nematode species is limited. Research has focused, to a

large extent, on the effects of *B. firmus* applications on root-knot nematodes, namely *M. incognita* and *M. javanica*. Several studies have demonstrated the superior ability of *B. firmus* to suppress nematode motility, egg hatching, root penetration, and galling on a variety of host plants, though the organism's specific nematicidal mode of action remains disputed (Giannakou et al., 2003; Mendoza et al., 2008; Castillo et al., 2013; Crow, 2014). Understanding the direct consequences of *B. firmus* applications on nematode infested soils will confirm the value of this novel bionematicide for nematode control.

With the ability to transmit viruses such as TomRSV, dagger nematodes pose a huge threat to the commercial fruit industry, specifically peach production, in West Virginia, while root-knot nematodes remain a major pest of agricultural crops worldwide. Nematologists are limited in advice available to growers with serious nematode problems, leaving orchards vulnerable to further nematode damage. Current gaps in research on the use of *B. firmus* as a bionematicide limit the amount of information available for growers to make informed decisions regarding *B. firmus* applications. The nematicidal potential of *B. firmus* has only been researched and reported for a relatively small number of nematode species. By examining the effects of *B. firmus* on dagger and root-knot nematode species indigenous to West Virginia, this study aims at providing information on the efficacy of *B. firmus* as a bionematicide for fruit bearing trees, such as peaches and apples, in the Mid-Atlantic region of the United States.

Past studies reporting the ability of *B. firmus* to control root-knot nematode populations have considered the interaction between the bacterium and tropical root-knot nematode species such as *Meloidogyne incognita* and *M. javanica*, therefore, little is known about the nematicidal effects of *B. firmus* on the temperate root-knot nematode species, *M. hapla*. It is also unclear whether *B. firmus* limits root-knot nematode infection through the production of toxic chemicals that are lethal to the nematodes, or through interactions with host plant roots that alter root-knot nematode parasitic behavior. In this study, various direct toxicity and attraction assays were performed to determine how the presence of *B. firmus* impacts the mortality and behavior of *M. hapla*. Likewise, little is known about the use of *B. firmus* as an effective bionematicide for the control of *Xiphinema americanum*, or whether the mode of action employed by *B. firmus* to control *X. americanum* is similar to that with *Meloidogyne*.

The project documented in this thesis consisted of two studies, both of which involved determining the effect of *B. firmus* on species of plant-parasitic nematodes indigenous to West

Virginia orchards. The first study (Chapter 2) had two objectives: 1) to determine if *B. firmus* is an effective bionematicide for the control of *Meloidogyne hapla*; and 2) to verify the mode of action employed by *B. firmus* to control *M. hapla* J2s. The second study (Chapter 3) had similar objectives, but aimed to determine the interaction of *B. firmus* with *Xiphinema americanum*. In both studies, direct toxicity and attraction assays were performed to determine how the presence of *B. firmus* affects the mortality and behavior of each nematode species.

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CHAPTER 2

EFFICACY AND MODE OF ACTION OF *BACILLUS FIRMUS* AS A BIONEMATICIDE FOR THE NORTHERN ROOT-KNOT NEMATODE, *MELOIDOGYNE HAPLA*¹

Introduction

Root-knot nematodes of the genus *Meloidogyne* are sedentary endoparasites that infect a variety of host plants, many of which are important agricultural crops. The infective larval stage (J2) of these nematodes is attracted to various volatile and soluble root exudates released into the soil (Linford, 1939; Perry, 2005). The attractiveness of these chemicals prompts migration towards the host plant and penetration of the roots near the zone of elongation. Once inside the roots, female root-knot nematodes remain sedentary, molting into a mature, reproductive adult and feeding on five-seven multinucleated giant cells that develop in response to nematode salivary secretions (Favery et al., 2016). Each mature female may produce more than 1000 eggs within their lifetime.

Root-knot nematode infection induces gall formation in host tissues and disrupts the flow of nutrients, causing mild to severe damage and potentially death to host plants. It is estimated that plant-parasitic nematodes are responsible for billions of dollars in crop damage annually (Handoo, 1998). Management of root-knot nematode populations often involves the combination of several pest management practices, though some options can be inappropriate and ineffective for situations such as fruit tree production. In these cases, chemical nematicides remain the best defense. In recent years, however, many chemical nematicides have been banned or are actively being phased out due to harmful environmental effects and human and animal toxicity. With the exception of the insecticide spirotetramat (Movento), there are presently no chemical nematicides registered for use on bearing fruit trees in the United States. Thus, environmentally benign options such as biological controls are needed.

Various soil dwelling bacteria have demonstrated success in controlling root-knot nematode populations. These antagonistic organisms employ different methods, such as direct toxicity and disrupting host finding ability, to limit the severity of nematode infection within plants. *Bacillus firmus*, a gram-positive endospore-forming bacterium is the active ingredient in a nematicide currently marketed by Bayer CropScience. This organism has been shown to be effective at reducing gall formation and egg hatch, and increasing mortality of root-knot nematodes. Current

literature available on the efficacy of *B. firmus* is limited to the effects seen on tropical root-knot nematode species, such as *M. javanica* and *M. incognita*, therefore, little is known about the bionematicidal effects of *B. firmus* on temperate *Meloidogyne* species such as *M. hapla*. In addition, the bacterium's nematicidal mode of action remains uncertain. Understanding *B. firmus*'s antagonistic relationship towards *M. hapla* can help establish more effective treatment plans for growers facing root-knot nematode infestations in temperate climates, such as those located in the state of West Virginia.

Peach and apple production is a multi-million dollar business in West Virginia and other mid-Atlantic states (USDA, 2015), though growers facing *M. hapla* infestations have few options short of chemical intervention. The goal of this study was to determine if *B. firmus* has potential for controlling infection by the northern root-knot nematode *M. hapla*, and to elucidate a nematicidal mode of action for the bacterium.

Materials and methods

Bacillus firmus

A research grade formulation containing a minimum of 1.0×10^{11} CFU/gram of *Bacillus firmus* (I-1582) provided by Bayer CropScience was used in this study. A *Bacillus* isolate from this formulation (named BF1) was selected and the identity was confirmed using a range of morphological and biochemical characteristics analogous to *B. firmus* (Appendix 1). Later, rRNA gene analysis of the BF1 isolate resulted in 100% identity with *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus anthracis* and 93% identity with *Bacillus firmus* (Appendix 1). For the following experiments, the isolated BF1 culture is referred to as *B. firmus*. It was grown in 100 mL of TSB at 28°C for 24 hours to produce a concentration of approximately 10^7 CFU/mL. The day-old culture was centrifuged at 5000 rpm for 20 minutes to separate bacterial cells and TSB. The supernatant was removed and the *B. firmus* cells were re-suspended in 100 mL of sterile soil extract (SSE).

Sterile soil extract (SSE)

Sterile soil extract (SSE) was used as the control for all experiments in this study. SSE was used in lieu of sterile distilled water and TSB based on preliminary results that exhibited a larger nematode mortality rate for both cell-free sterile distilled water and cell-free TSB when

compared to cell-free SSE. SSE was prepared by combining 1000 g of a Tilsit silt loam soil obtained from the West Virginia University Organic Farm in 1 L of tap water. The soil and water were mixed thoroughly then allowed to settle for 24 hours. The water was decanted and autoclaved twice for 90 minutes each time.

Meloidogyne hapla

Root-knot nematodes used for this experiment were extracted from a pure culture (#16) of *M. hapla* isolated from strawberry from Monongalia County, WV. *M. hapla* was maintained on tomato roots as part of the nematode culture collection at West Virginia University. Perineal patterns were used to verify the *M. hapla* culture. Tomato roots were washed free of debris and eggs were extracted by submerging roots in 1% NaClO and shaking vigorously for 4 minutes. The resulting suspension was washed through stacked sieves with 250 and 25 μ m pore mesh (Hussey and Barker, 1973). Nematode eggs were placed in Baermann funnels and 1-5 day old J2s were used for the following experiments.

Tomato seedlings

Rutgers tomato seeds were surface disinfested by washing in a 70% ethanol solution for 2 minutes, then a 3% NaOCl solution for 10 minutes and immediately rinsing with sterile distilled water three times. Seeds were transferred to 0.75% trypticase soy agar (TSA) plates and incubated at 28°C for 5 days or until roots were approximately 2-4 cm. Seedlings that showed signs of microbial contamination were discarded.

Experiment 1. *Bacillus firmus* direct toxicity assay

To assess whether *B. firmus* was directly toxic to nematodes, we tested the hypothesis that *M. hapla* survival differed among various concentrations of *B. firmus* in solution. A 10⁷ CFU/mL concentration of *B. firmus* in SSE was serially diluted to produce seven *B. firmus* concentrations from approximately 10¹ to 10⁷ CFU/mL. Bacterial concentrations were enumerated using serial dilution and spread plating. Two mL of each bacterial concentration were added to 35-mm plastic dishes and uniformly spread throughout the plates. Plates with cell-free SSE were used as a control. Approximately 10 *M. hapla* J2s were added to each dish and the plates were incubated in darkness at room temperature (23 \pm 2°C). Nematode mortality was

determined at 24, 48, and 72 hours. Nonmotile nematodes were probed with a fine needle and those that did not respond were considered dead. Four replications were used for each *B. firmus* concentration and the entire experiment was conducted three times.

Experiment 2. Filter paper attraction assay

To determine if the movement of root-knot nematodes is affected by the presence of *B. firmus*, we tested the hypothesis that the number of *M. hapla* J2s that accumulate near *B. firmus* will differ from that accumulating near the SSE control. Sterile filter paper disks (12.7 mm dia) were soaked in either a 10^7 CFU/mL concentration of *B. firmus* in SSE or cell-free SSE. Approximately 2 mL of a 23% Pluronic gel medium was pipetted onto 75 X 25-mm glass microscope slides, covering the entire surface. The slides were partitioned into three labeled sections: *B. firmus* (32.5 X 25 mm), center (10 X 25 mm), and SSE (32.5 X 25 mm). Treated filter paper disks were immediately placed towards the edges of either the *B. firmus* or SSE sections of the slides, approximately 40 mm apart. No filter paper disks were placed in the centers. The slides were placed in petri dishes with a dampened paper towel and incubated at 28°C for 1 hour. After incubation, approximately 150 *M. hapla* J2s were added to the center of each slide (Figure 1). The slides with nematodes were again incubated in darkness at room temperature (23±2°C). The number of nematodes in each section of the slide was counted at 1, 2, 4 and 24 hours post inoculation. The experiment had eight replicates.

Experiment 3. Tomato seedling attraction assay

To determine if the migration of *M. hapla* towards host plant roots is altered by the presence of *B. firmus*, we tested the hypothesis that a smaller number of *M. hapla* J2s will accumulate near *B. firmus*-treated roots compared with SSE-treated roots. The roots of surface disinfested tomato seedlings were submerged in either a 10^7 CFU/mL concentration of *B. firmus* in SSE or cell-free SSE. Approximately 2 mL of a 23% Pluronic gel medium was pipetted onto 75 X 25-mm glass microscope slides, covering the entire surface. The slides were partitioned into three labeled sections: *B. firmus* (32.5 X 25 mm), center (10 X 25 mm), and SSE (32.5 X 25 mm). One tomato seedling from each treatment was immediately placed towards the edge of the appropriate section on the slides approximately 50 mm apart. No tomato seedlings were placed in the centers. The slides were placed in petri dishes with a dampened paper towel and incubated

at 28°C for 1 hour. After incubation, approximately 150 *M. hapla* J2s were added to the center of each slide and the slides were stored in darkness at room temperature (23±2°C). The nematodes in each section of the slide were counted at 1, 2, 4 and 24 hours. The experiment had five replicates.

Experiment 4. *In-vitro* infection assay

To determine if root penetration by *M. hapla* was influenced by the presence of *B. firmus*, we tested the hypothesis that fewer *M. hapla* J2s will successfully penetrate *B. firmus*-treated roots compared with SSE-treated roots. Tomato seedling roots were submerged in either a 10⁷ CFU/mL concentration of *B. firmus* in SSE or cell-free SSE. Approximately 2 mL of a 23% Pluronic gel medium was pipetted onto 75 X 25-mm glass microscope slides, covering the entire surface. One seedling of either the *B. firmus* treatment or the SSE treatment was added to each slide. Slides were placed in petri dishes with a dampened paper towel and incubated at 28°C for 1 hour. After incubation, approximately 150 *M. hapla* J2s were added to each slide close to the root tips. The slides were incubated in darkness at room temperature (23±2°C). At 48 hours post inoculation, each tomato seedling was removed from the gel medium and the roots were stained by bleaching in a 1.5% NaOCl solution for 4 minutes, rinsing with distilled water, and boiling with acid fuchsin stain for 30 seconds (Byrd et al., 1983). J2s that had successfully penetrated the roots were counted. Ten replications were used for each treatment.

Experiment 5. In-sand attraction assay (5-day)

To determine if root-knot infection in tomato roots in sand is disrupted by the presence of *B. firmus*, we tested the hypothesis that fewer *M. hapla* J2s will successfully penetrate the roots of *B. firmus*-treated roots compared with SSE-treated roots. Tomato seedling roots were submerged in either a 10⁷ CFU/mL concentration of *B. firmus* in SSE or cell-free SSE. Two tomato seedlings, one of each treatment, were planted on either side of a beaker (approximately 35 mm apart) containing 50 g of sterile sand with a 10% moisture content (approximately field capacity) (Figure 2). The tomato seedlings were left on a laboratory bench top under lights for 1 day before being inoculated with approximately 500 *M. hapla* J2s in the center of each beaker. The plants were left on the bench for an additional 5 days receiving 16 hours of light over a 24-hour period. The seedlings were then removed from the sand, gently washed free of debris and

the roots were stained. The number of nematodes that successfully penetrated the roots was determined. Ten replicates were used for each treatment.

Experiment 6. In-sand reproduction assay (40-day)

To determine if *B. firmus* can decrease the severity of *M. hapla* infection in tomato roots, we tested the hypothesis that *B. firmus*-treated roots will contain fewer *M. hapla* females and eggs per gram of root compared with SSE-treated root. Tomato seedling roots were submerged in either a 10^7 CFU/mL concentration of *B. firmus* in SSE or cell-free SSE. Two seedlings, one from each treatment, were planted on either side of a beaker (approximately 35 mm apart) containing 50 g of sterile sand with a 10% moisture content (approximately field capacity). The seedlings were left on a laboratory bench top under lights for 1 day before being inoculated with approximately 500 *M. hapla* J2s in the center of each beaker. After 5 days, the seedlings were separated and transplanted into individual beakers containing 500 g of sterile sand. The plants were left on the bench top at room temperature ($23\pm 2^\circ\text{C}$) receiving 16 hours of light over a 24-hour period and fertilizer every 3 days. After 40 days the plants were removed from their beakers, the roots were washed free of debris, and *M. hapla* eggs were extracted and counted. Roots were stained with acid fuchsin as described in the infection assay above and the number of visible females was determined. Shoot weight, dry shoot weight, and root weight were also recorded. The experiment was conducted once with 20 replicates per treatment.

Data Analysis

All data were analyzed using JMP and SAS software (JMP®, Version Pro 11, SAS Institute Inc., Cary, NC, Copyright ©2013; SAS®, Version 9.3, SAS Institute Inc., Cary, NC, Copyright ©2002-2010). The significance criterion (alpha) for all tests was 0.05 unless otherwise indicated.

Results

Experiment 1. *B. firmus* direct toxicity assay

As the concentration of *B. firmus* increased from cell-free to approximately 10^7 CFU/mL, plates became increasingly cloudy and visibility was impacted by the bacterial composition. Nematodes exposed to higher concentrations of *B. firmus*, specifically the two highest

concentrations of approximately 10^7 and 10^6 CFU/ mL were typically less active and required probing to verify living status more often than J2s in cell-free SSE. Due to the significant negatively skewed distribution, data were analyzed using a Kruskal-Wallis test by ranks and treatment means were compared with the control using the Steel ranks test. Differences among treatments were not statistically significant at 24 hours ($P = 0.29$) but by 48 hours there was a significant difference in *M. hapla* survival in dishes with 10^7 CFU/mL compared to the control ($P = 0.02$). Exposure to 10^7 CFU/mL of *B. firmus* yielded a 15% decrease in the proportion of living nematodes by 72 hours (Figure 2.3) compared to the control ($P < 0.01$).

Experiments 2 and 3. Attraction assays

Nematode movement from the center of each slide was observed within the first hour, though 50% or more of the nematodes remained in the center throughout the length of the experiment. Most nematode movement occurred in the direction of the SSE control for both filter paper and tomato seedling attraction assays. Tracks made by nematodes moving along the surface of the Pluronic gel medium were visible but were rarely seen in the *B. firmus* section of slides. Data were analyzed using a repeated measures ANOVA with irregular spacing (due to unequally spaced time points), using spatial Gaussian covariance structure. Time was considered the covariate. The attraction assays using filter paper showed a significant treatment effect over time ($P < 0.01$, Figure 2.4). After 24 hours, 16% of motile J2s were on the *B. firmus* portion of the slides compared to 93% of motile J2s at the SSE control. Attraction assays using tomato seedlings were similar with 29% of motile J2s on the *B. firmus* portion of the slides compared to 71% at the SSE control ($P = 0.01$, Figure 2.5).

Experiment 4. *In-vitro* infection assay

By 48 hours after nematodes were introduced to tomato roots on Pluronic gel, stained J2s within roots were clearly visible using a stereoscopic microscope. Data were analyzed using the nonparametric Kruskal-Wallis test by ranks because the distribution was not normal. Root infection by J2s was significantly greater in the control treatment group ($P = 0.01$) than roots treated with a *B. firmus* concentration of 10^7 CFU/mL (Figure 2.6). On average, 20 J2s successfully penetrated SSE-treated roots compared with three J2s in *B. firmus*-treated roots.

Experiment 5. In-sand attraction assay 5-day

Root-knot nematode infection was apparent in tomato roots of both treatment groups at 5 days post inoculation. Data were analyzed using a matched pairs t-test analysis. Roots treated with a 10^7 CFU/mL concentration of *B. firmus* had fewer J2s than the control ($P = 0.09$, Figure 2.7). On average, 79 J2s successfully penetrated SSE-treated roots compared with 58 J2s in *B. firmus*-treated roots.

Experiment 6. In-pot reproduction assay 40-day

At the end of the 40-day trial, tomato plants in both treatment groups showed typical root galling. Data (females per plant and eggs per female) were analyzed using a matched pairs t-test analysis. A slightly larger number of female root-knot nematodes were associated with plants from the SSE control group ($P = 0.05$, figure 2.8). An average of 54 females were found within SSE roots versus an average of 43 females found within *B. firmus* roots. The number of eggs produced per *M. hapla* female did not differ significantly between the *B. firmus* treatment and the control ($P = 0.22$, Figures 2.9). Each *M. hapla* female produced an average of 880 eggs within *B. firmus*-treated roots and 704 eggs within SSE-treated roots.

Discussion

These results indicate that *B. firmus* has the potential to affect infective *M. hapla* nematodes in a biological control context. Exposing *M. hapla* J2s to a *B. firmus* concentration of 10^7 CFU/mL resulted in a 15% decrease in living nematodes after 72 hours. Observed nematode mortality may be explained by the production of lethal secondary metabolites by *B. firmus*. Species within the genus *Bacillus* are known to produce secondary metabolites that have an array of functions, including those with antimicrobial properties (Niu et al., 2006; Zhang et al., 2012). Yilmaz et al. (2006) showed that 29 tested *Bacillus* spp. strains were inhibitory to the growth of both gram-negative and gram-positive bacteria. *B. thuringiensis* has been a successful bioinsecticide for over half a century, and produces toxic crystal proteins that form pores within the digestive tract of insects. Wei et al. (2003) revealed that the toxicity of these proteins extends to the phylum Nematoda. The patent submitted for the use of *B. firmus* as a bionematicide asserted that "...proteolytic and collagenolytic activities play an important role in control of

nematodes...” (Patent US6406690, Peleg and Feldman, 1996). Terefe et al. (2009) reported that *M. incognita* exposed to 2.5 and 3% concentrations of the *B. firmus* product, BioNem WP, resulted in 100% nematode paralysis after 24 hours, and Mendoza et al. (2008) showed that 100% cultural filtrates of *B. firmus* caused 33% mortality of *M. incognita* after 24 hours of exposure. Both studies reported higher nematode mortality after exposure to *B. firmus* culture filtrates than the 15% nematode mortality observed in the present study. Furthermore, the lethal properties of *B. firmus* were seen only when the bacterial concentration reached 10^7 CFU/mL, an indication that the potency of any secondary metabolites produced is not very high. These compound would likely be diluted within the rhizosphere and have little effect on nematodes populations in an orchard environment.

Though the statistically significant nematode mortality produced by 10^7 *B. firmus* CFU/ml may be due to the production of toxic secondary metabolites, another explanation is that high bacterial densities lead to a decrease in oxygen over time. As the *B. firmus* concentrations were increased from 0- 10^7 CFU/ml, the turbidity of SSE increased, leaving plates with the highest *B. firmus* concentration lined in a thick, cloudy film of bacteria. Such high bacterial concentrations likely results in high oxygen consumption which can cause hypoxic or anoxic conditions. Nematodes are obligate aerobes that rely solely on diffusion of oxygen from the ambient environment, therefore, this lowered oxygen availability can be detrimental. Föll et al. (1999) showed that more than 80% of *Caenorhabditis elegans*, a free-living nematode, will die after 72 hours in anoxic conditions and Qiu and Bedding (2000) demonstrated that the ability of infective *Steinernema carpocapsae* juveniles to survive in decreased oxygen conditions is low when their available energy supply is limited. In the present study, utilizing an additional treatment of cell-free culture filtrates may have confirmed the presence of toxic secondary metabolites. Also, whether nematode mortality in the present study was caused by the production of toxic chemicals or the limited oxygen availability may have been more conclusive if a steady flow of oxygen was provided to plates through the duration of the experiment.

In-vitro attraction assays comparing *M. hapla* movement on Pluronic gel in the presence of 10^7 *B. firmus* CFU/ml and cell-free SSE on filter paper showed a significant difference between treatments over time. *M. hapla* J2s were consistently more inclined to migrate away from *B. firmus* towards cell-free SSE. By 24 hours, a large proportion of nematodes could be seen accumulating on the SSE portion of slides. Similar results were seen when *B. firmus* and

cell-free SSE were applied to roots of tomato seedlings, though the observed avoidance of *B. firmus* was not as strong. A suggested nematicidal mode of action for rhizobacteria such as *B. firmus* states that bacterial colonization of roots induces systemic resistance and interferes with a plant-parasitic nematode's ability to find a suitable host (Kloepper et al., 2004). In the present study, the presence of *B. firmus* affected the movement of *M. hapla* not only when applied to tomato roots, but when applied to a non-food source (paper discs) as well. While an influence of *B. firmus* on root exudates cannot be discounted, this observation suggests the production of a chemorepellent by *B. firmus*.

Chemotaxis is a central feature of nematode food-finding behavior (Linford, 1939; Wieser, 1955; Bird, 1959; Troemel et al., 1997; Wang et al., 2009; Reynolds et al., 2011). Linford (1939) demonstrated that the ability of *Meloidogyne* spp. J2s to locate suitable hosts is not a random event, but is influenced by the presence of various plant roots, which results in the accumulation of nematodes near the zone of elongation. Zhao et al. (2000) further demonstrated that exudates from different regions of plant roots can have a positive or negative influence on nematodes. While root border cell exudates from various host plants were attractive to *M. incognita*, root tip exudates were repellent. Despite numerous studies on the subject of nematode reactions to various chemotactic factors, the specific plant-based chemical signals involved in nematode chemotaxis remain largely unexplained. It is likely that the phenomenon is governed by complex biochemical mixtures produced by plants and their associated microbiota. Thus, overwhelming the system with large quantities of an introduced organism, such as *B. firmus*, could affect these biochemical signals and disrupt nematode host finding. Vos et al. (2011) demonstrated this potential with the introduction of mycorrhizal fungi to banana plants. The fungi successfully induced nematode resistance in the host when compared to control plants. In the present study, the avoidance of *M. hapla* J2s towards *B. firmus* regardless of the absence of an available food source is an interaction occurring in other bacteria/nematode relationships. Höckelmann et al. (2004) demonstrated that cyanobacteria produce various volatile organic compounds that function to drive nematodes to and away from their associated biofilms and Neidig et al. (2011), showed that *Pseudomonas fluorescens* produces secondary metabolites that act to repel *C. elegans*.

To further support the evidence that *B. firmus* repels root-knot nematodes and interferes with nematode host-finding, the present study showed that the presence of *B. firmus* significantly

decreased *M. hapla* infection of tomato roots on Pluronic gel compared to roots treated with cell-free SSE. However, when *B. firmus* and cell-free SSE treatments were applied to tomato seedlings for the 5-day and 40-day in-pot attraction assays in sand, no significant treatment effect was observed, and tomato roots of both treatments showed varying levels of root-knot nematode infection. The inconsistencies between *in-vitro* and in-pot assays may be a reflection of the efficiency at which chemicals diffuse through media of various textures. Though Pluronic gel is a useful medium in the study of nematodes, as it mimics the three-dimensional structure of soil, the gel texture may alter the diffusion of volatile organic compounds produced by *B. firmus* compared with sand. Volatile organic compounds diffuse through the porous channels within the soil matrix (Reynolds et al., 2011) and diffusion gradients of the chemorepellent may be more complex in three-dimensional films of moisture around sand grains than in the uniform two-dimensional gel medium. The coarse texture of sandy soils allows for fast diffusion rates of organic compounds, while the same chemicals may persist on Pluronic gel longer, offering an extended period of nematode repulsion.

Bayer CropScience states that their *B. firmus* product, VOTiVO, “creates a living barrier that grows with the roots to extend protection through multiple generations of nematodes.” Durham (2013) used a rifampicin-resistant *B. firmus* mutant strain and demonstrated the organism’s ability to colonize the rhizosphere, rhizoplane, and endorhiza of corn, soybean, and cotton. Those data, along with the negative association between *B. firmus* and *M. hapla* observed *in-vitro* in the present study, indicate the plausibility of the “living barrier” hypothesis, though additional *in-vitro* experiments are needed to determine the specific interactions.

The present study presents evidence of the biocontrol potential of *B. firmus*, however, the results of these *in-vitro* and in-pot experiments give little insight into the interactions between *B. firmus* and root-knot nematodes within an orchard setting. Future studies utilizing non-sterilized soil collected from peach orchards would help determine if the presence of native microorganisms has an effect on the ability of *B. firmus* to repel nematodes and suppress infection as seen in the present study. Attraction assays on peach seedlings as well as orchard studies spanning several growing seasons would give a more meaningful assessment on the efficacy of utilizing *B. firmus* as a bionematicide in West Virginia peach orchards. Having a better understanding of the complex relationships between nematodes and antagonistic organisms, such as the interactions between *M. hapla* and *B. firmus*, will ultimately support the

development of more effective biological control options and more efficient pest management plans for growers.

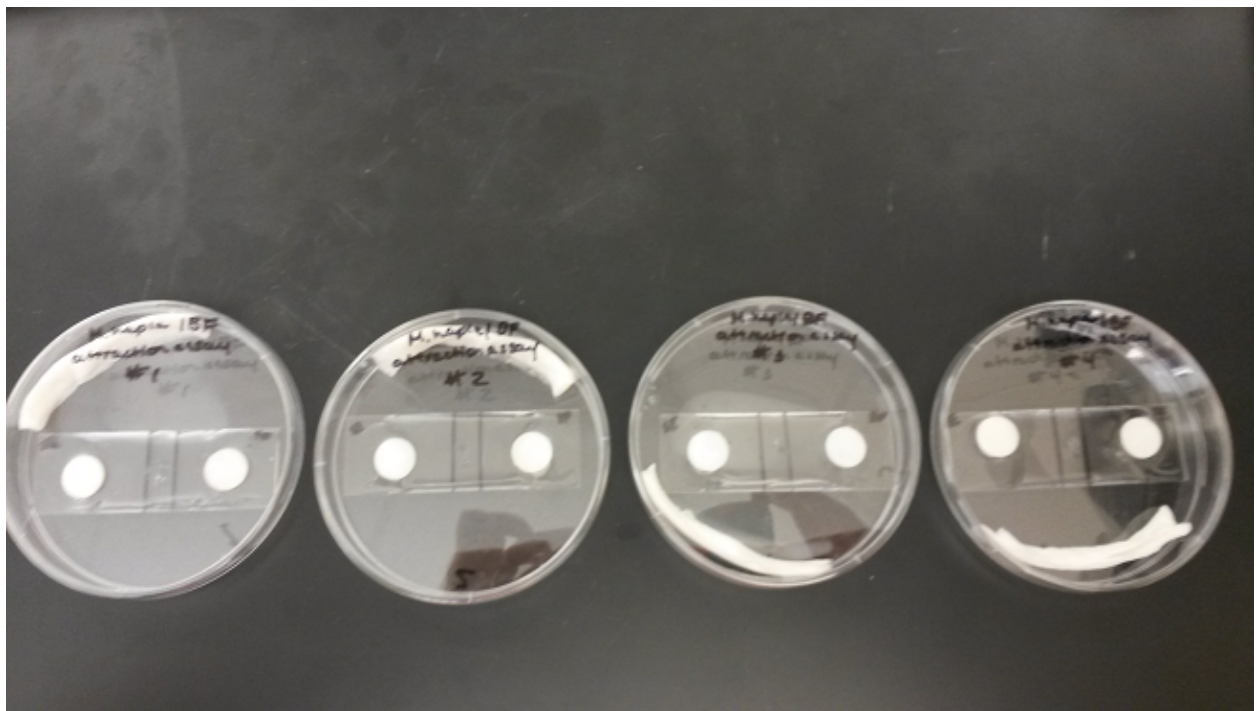


Fig. 2.1. *In-vitro* attraction assay set-up: *B. firmus*- and SSE-treated filter paper on opposite ends of slides covered in Pluronic gel.



Fig. 2.2. In-pot attraction assay set-up: Beakers containing both *B. firmus*- and SSE-treated tomato seedlings on opposite sides.

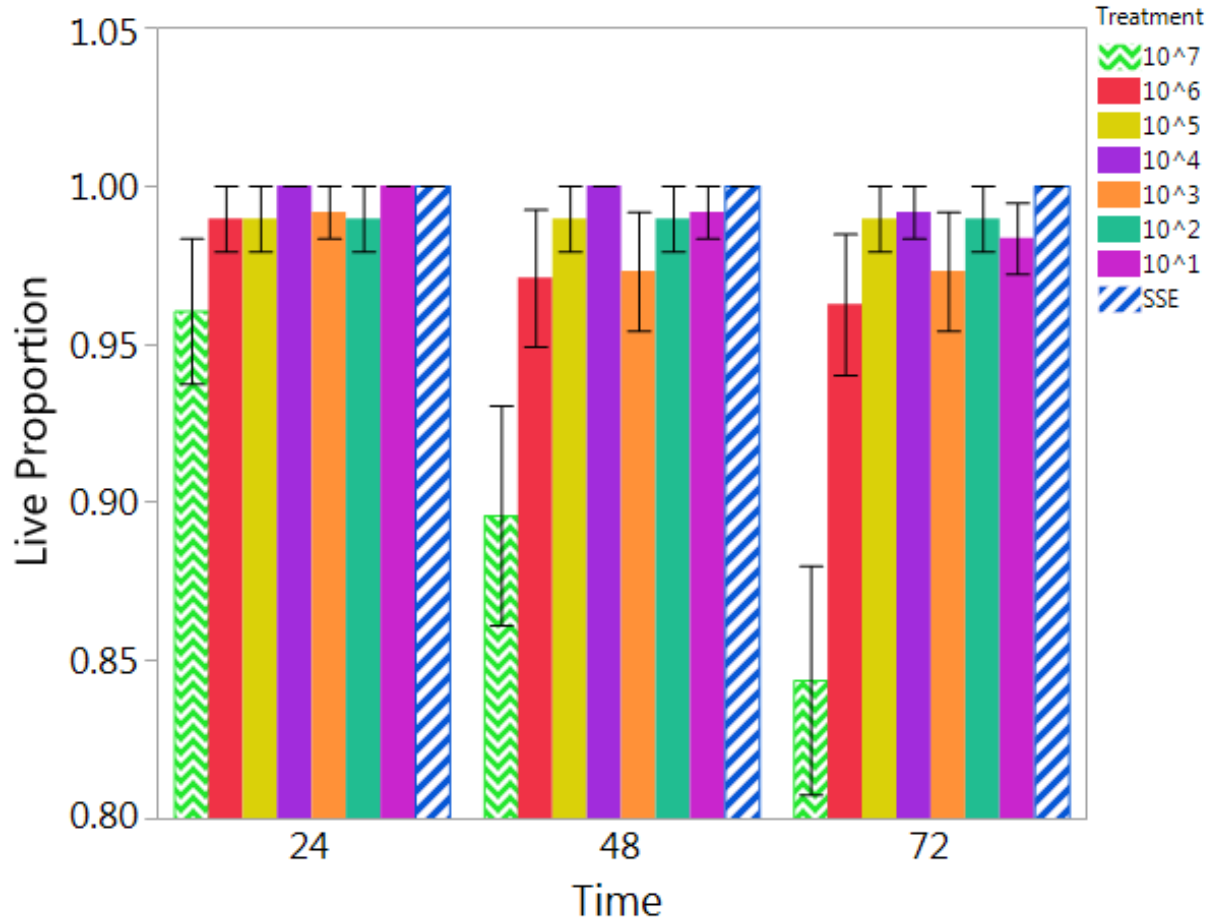


Fig. 2.3. Direct toxicity assay: Proportion of living *M. hapla* nematodes after 24, 48, and 72 hours of exposure to different concentrations of *B. firmus*. Error bars represent standard error of the mean.

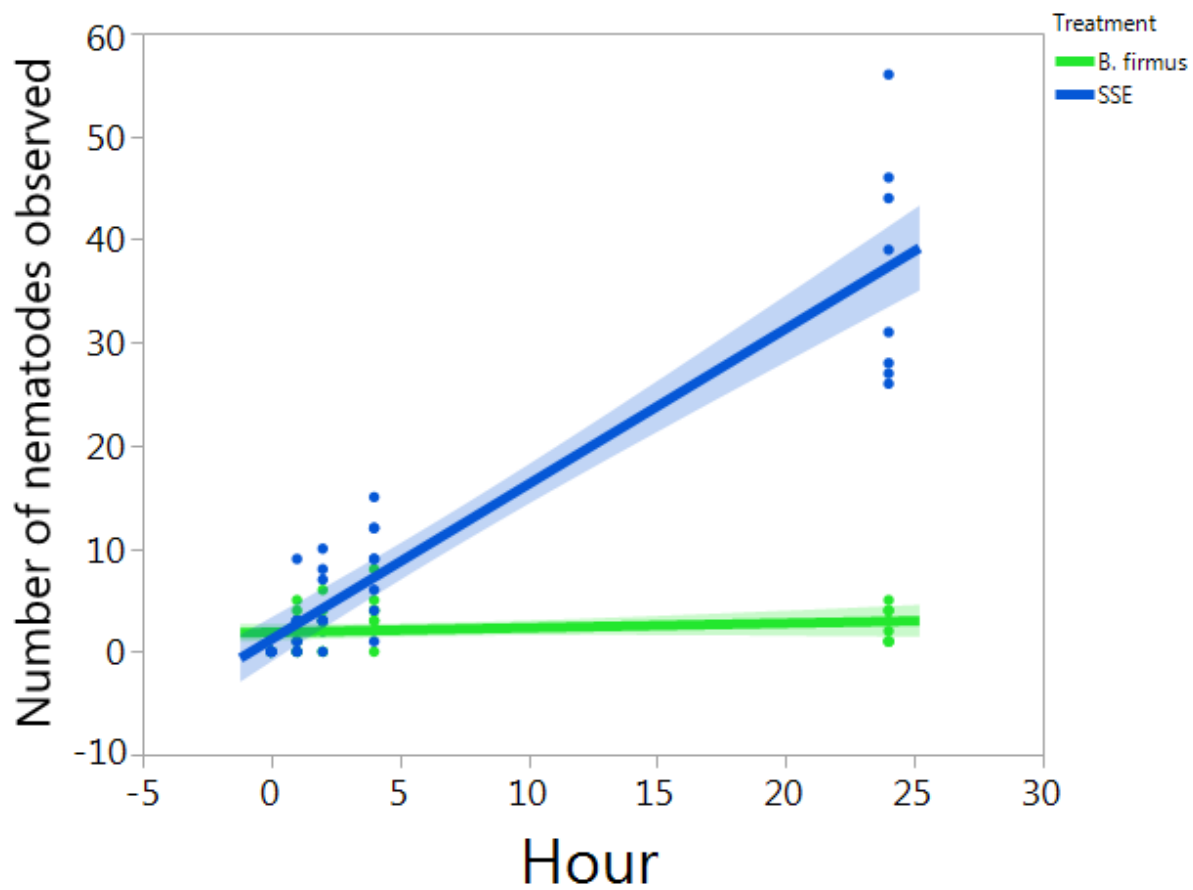


Fig. 2.4. Filter paper attraction assay: Number of *M. hapla* nematodes observed on the *B. firmus* portion of the slide vs. the SSE portion of the slide over time. The shaded regions represent confidence intervals of the slope.

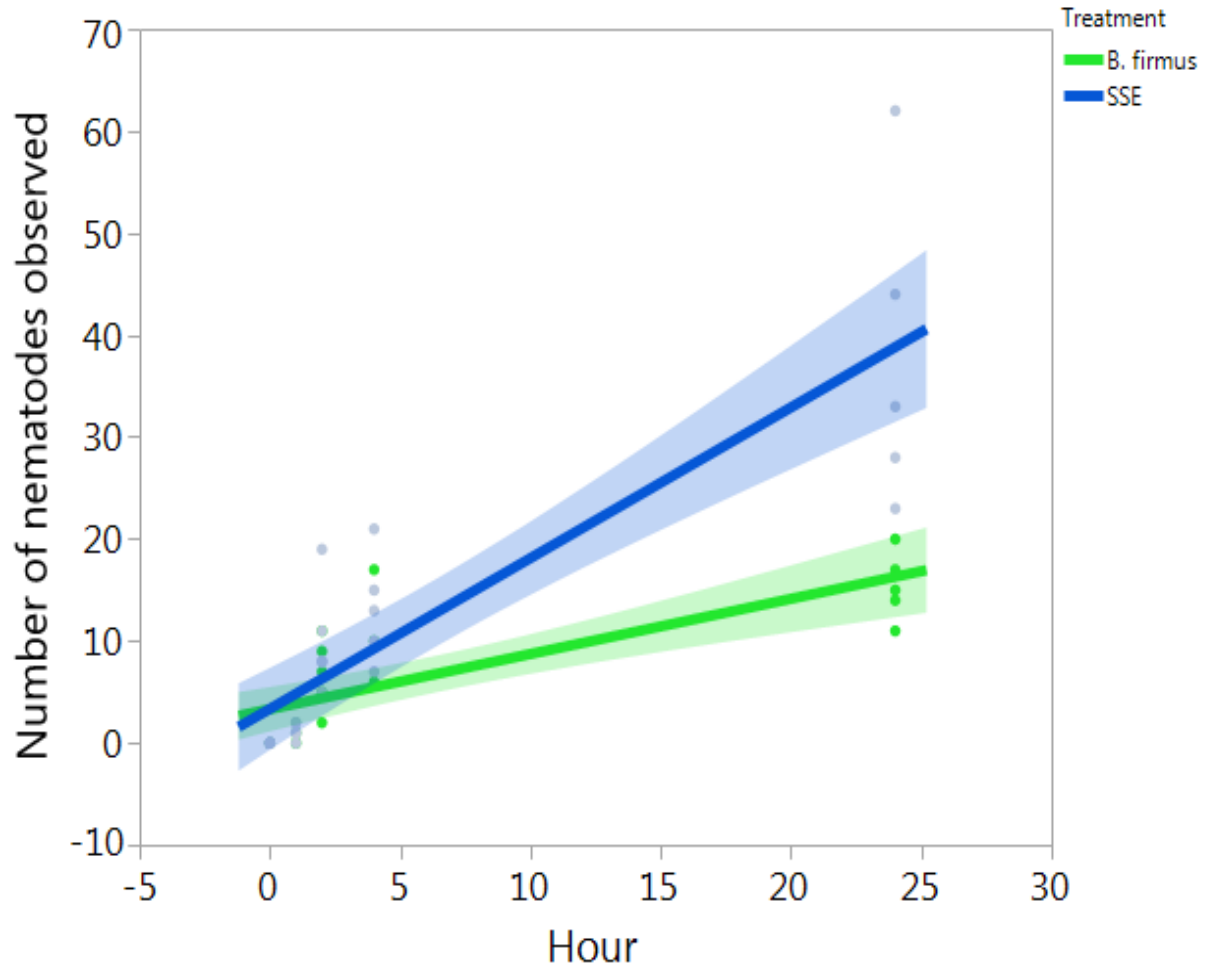


Fig. 2.5. Tomato seedling attraction assay: Number of *M. hapla* nematodes observed on the *B. firmus* portion of the slide vs. the SSE portion of the slide over time. The shaded regions represent confidence intervals of the slope.

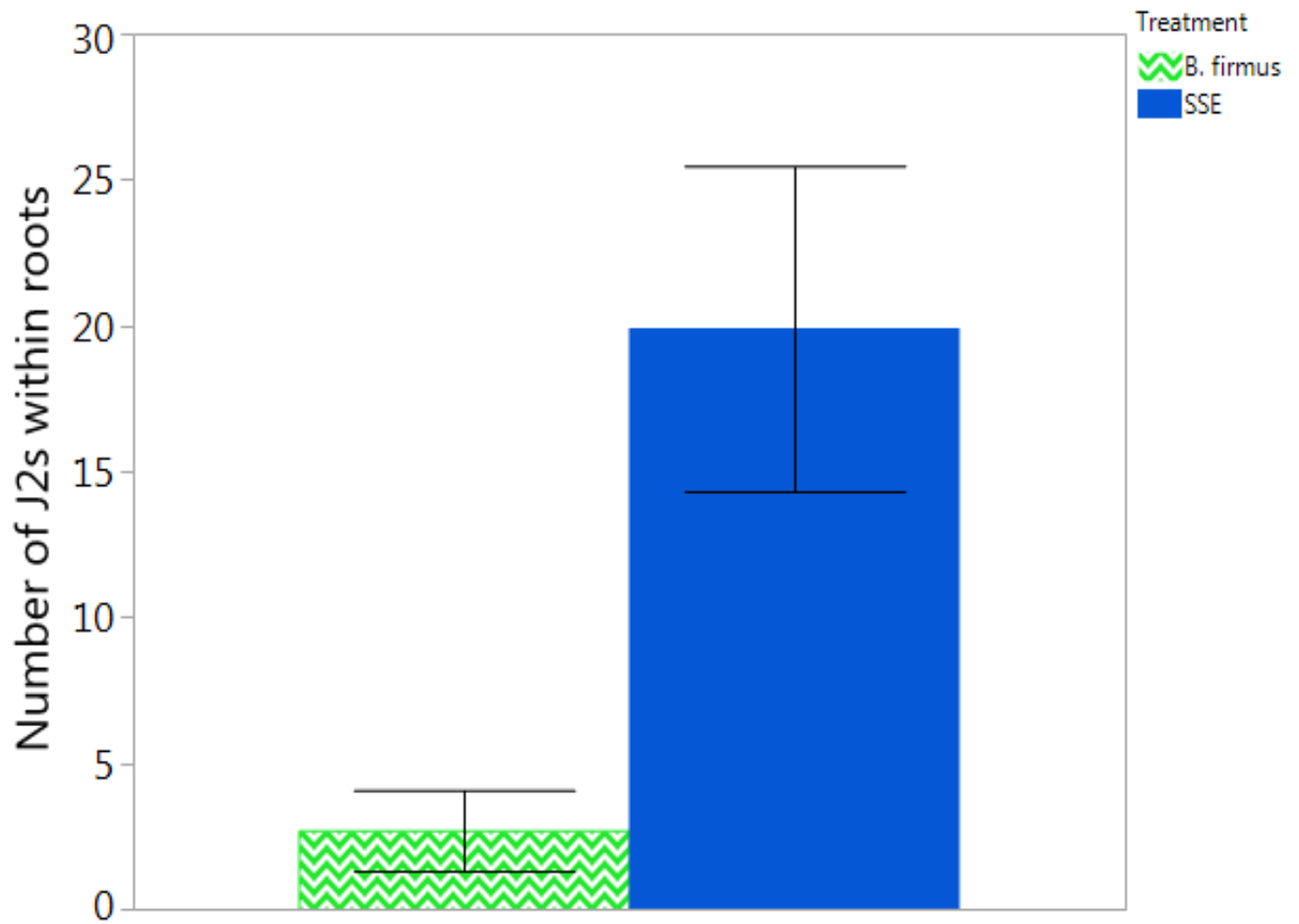


Fig. 2.6. *In-vitro* infection assay: The average number of *M. hapla* nematodes that successfully penetrated roots of *B. firmus*-treated tomato seedlings vs. SSE-treated tomato seedlings. Error bars represent standard error of the means.

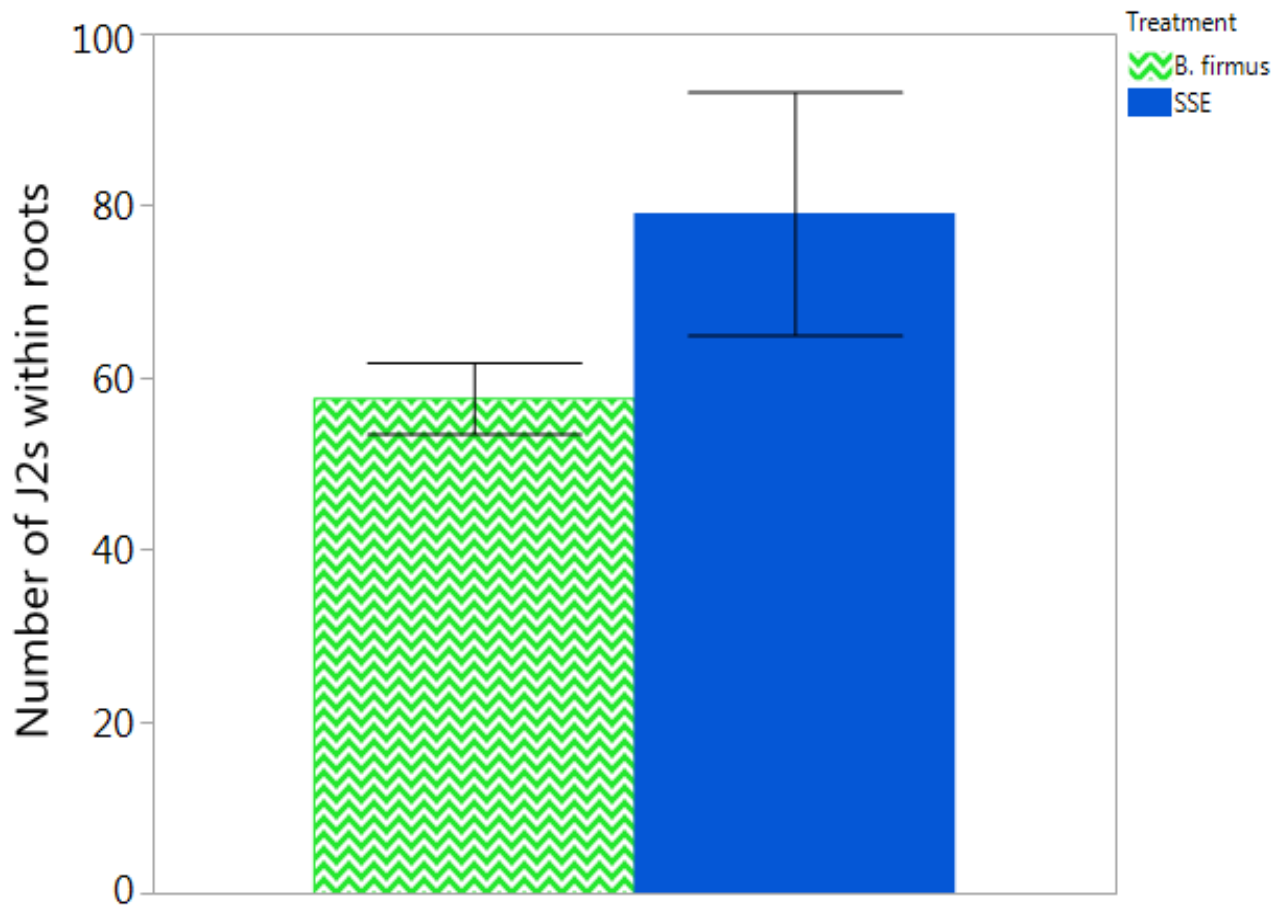


Fig. 2.7. In-sand attraction assay (5-day): The average number of *M. hapla* nematodes that successfully penetrated roots of *B. firmus*-treated tomato seedlings vs. SSE-treated tomato seedlings. Error bars represent standard error of the means.

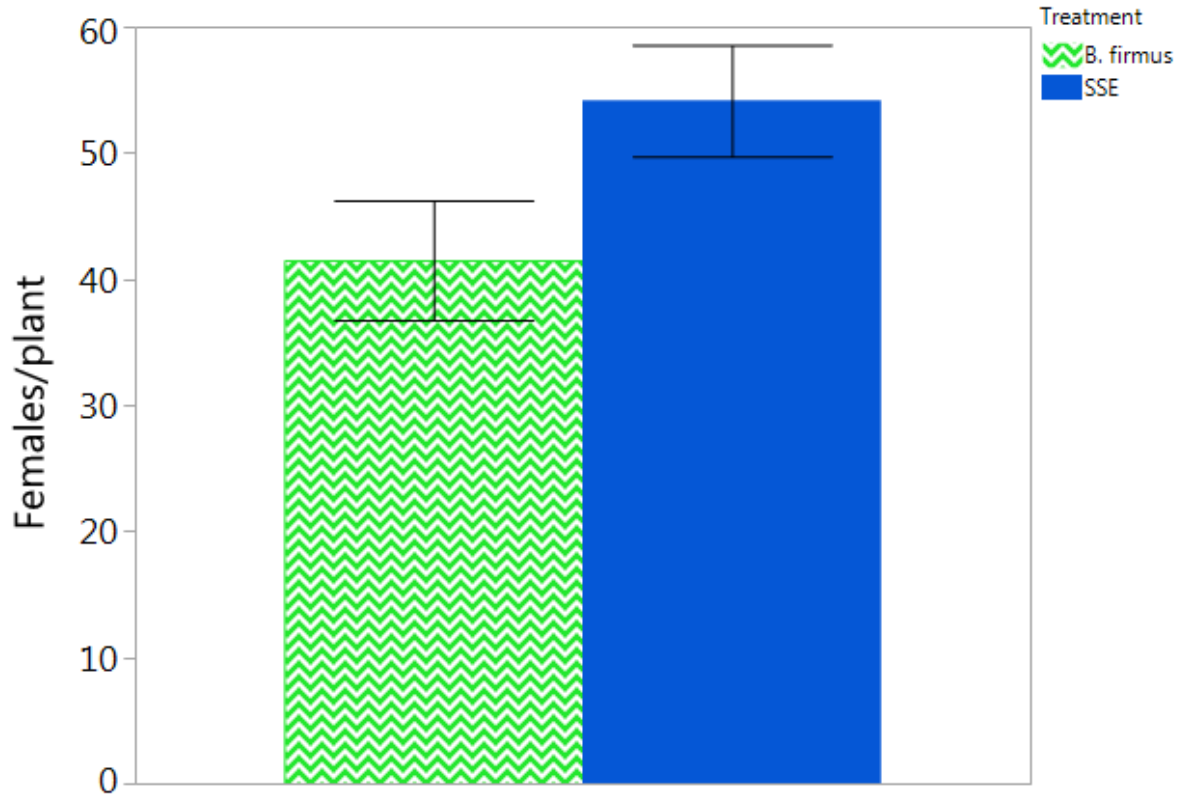


Fig. 2.8. In-sand reproduction assay (40-day): The average number of reproductive *M. hapla* females within the roots of *B. firmus*-treated tomato seedlings vs. SSE-treated tomato seedlings. Error bars represent standard error of the means.

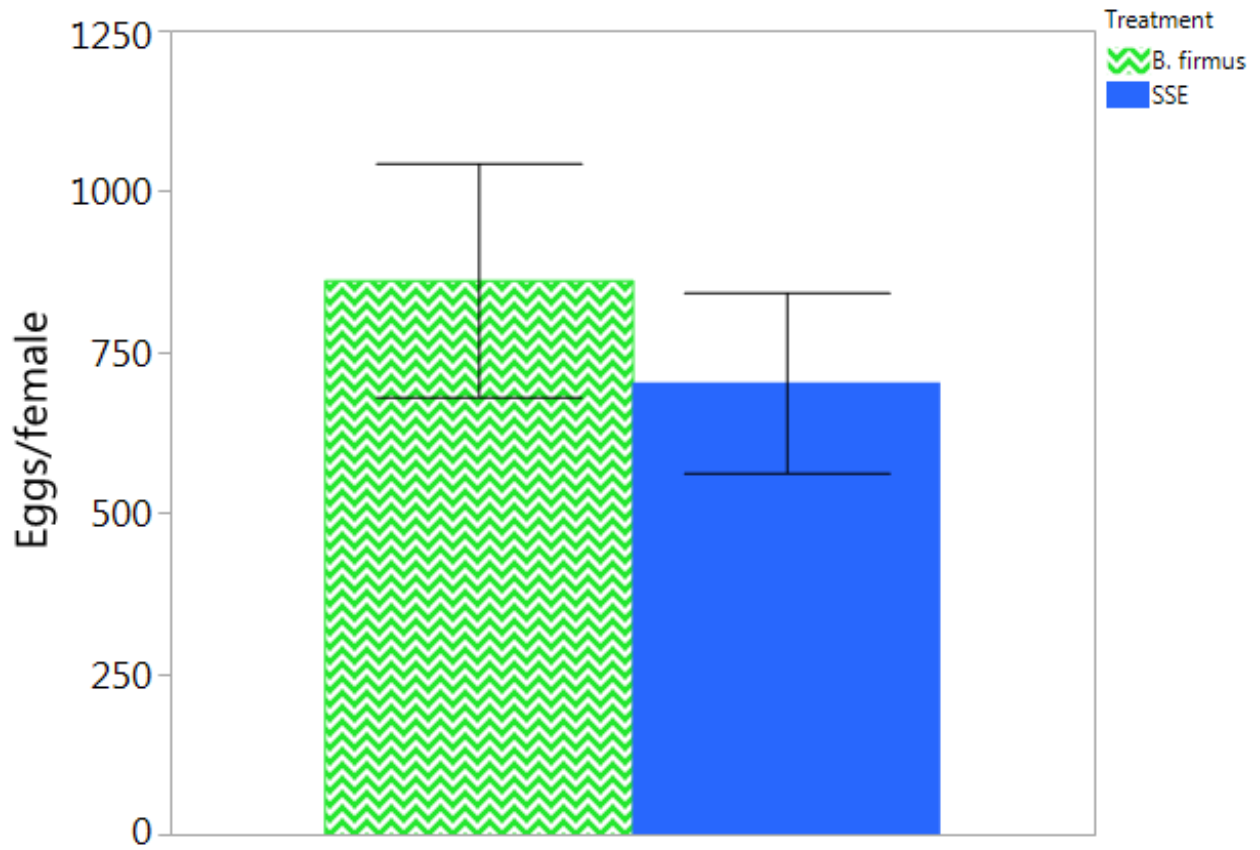


Fig. 2.9. In-sand reproduction assay (40-day): The average number of eggs per reproductive *M. hapla* female within the roots of *B. firmus*-treated tomato seedlings vs. SSE-treated tomato seedlings. Error bars represent standard error of the means.

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CHAPTER 3

EFFICACY OF *BACILLUS FIRMUS* AS A BIONEMATICIDE AGAINST *XIPHINEMA AMERICANUM*²

Introduction

Dagger nematodes of the genus *Xiphinema* are economically important plant-parasitic nematodes that cause damage to a wide range of agricultural crops worldwide. These nematodes are ectoparasites that migrate through soil, feeding on young root tips and root hairs of susceptible plants with a long, needle-like stylet. High dagger nematode populations can lead to significant yield losses and a general decline in plant health, though direct damage related to *Xiphinema* spp. infestation is generally not regarded as an immediate concern for growers. The economic importance of dagger nematodes is derived from their ability to transmit different nepoviruses (nematode transmitted polyhedral viruses), including tomato ringspot virus (TomRSV), tobacco ringspot virus (TRSV), and grapevine fanleaf virus (GFLV), to plants (Forer & Stouffer, 1982; McGuire, 1964; Raski & Hewitt, 1960).

As dagger nematodes feed on the roots of virus-infected plants, viral particles may adhere to the stylet and esophageal lumen of the nematode, initiating the potential for viral transmission to a new host plant (Taylor & Robertson, 1970). During feeding, the dagger nematode uses its stylet to puncture the plant cell wall and secrete salivary enzymes prior to ingesting the cell's cytoplasmic contents. At this time, any retained viral particles may dislodge and infect the new host. Viral particles attached to the nematode stylet are shed with each molt but may persist within adult worms for several years (Taylor & Raski, 1964; Demangeat et al., 2005). Without intervention, the longevity of viral retention and lengthy lifespan of dagger nematodes (2-5 years) allow for long-term spread of devastating plant diseases.

Management practices for nepoviruses include the use of resistant rootstocks and control of the nematode vector, though reducing *Xiphinema* spp. population sizes can prove difficult due to a wide host range and persistence in fallow soil. Combining several traditional management practices, such as fumigation and the use of cover crops, is recommended, however, these options are not suited for all agricultural situations, and government restrictions continue to limit the use of chemical nematicides. Aside from spirotetramat (Movento), which is primarily an insecticide, there are presently no registered chemical nematicides available for application on

bearing fruit trees in the US. Pre-plant nematicides and fumigants are available but these will not provide control over the life of the orchard. In recent years, focus in nematode management has shifted to the use of ubiquitous, soil-dwelling organisms as a means of controlling plant-parasitic nematode populations. While a large portion of bionematicide research has focused on endoparasites such as *Meloidogyne* spp., a variety of organisms including *Pasteuria penetrans*, *Cunninghamella elegans*, *Variovorax paradoxus*, *Pseudomonas pseudoalcaligenes*, *Bacillus mycooides*, *Bacillus sphaericus*, *Bacillus thuringiensis*, *Curtobacterium flaccumfaciens*, *Pseudomonas putida*, *Pseudomonas alcaligenes*, and *Pseudomonas viridiflava* have been reported to decrease dagger nematode population densities and damage (Ciancio, 1995; Galper et al., 1991; Aballay et al., 2012). *Bacillus firmus*, a gram-positive, endospore-forming bacterium, is the active ingredient in a nematicide currently marketed by Bayer CropScience. This organism has shown success in controlling populations of several nematode species, namely *Meloidogyne* spp., by direct toxicity and disruption of host-finding ability. Although dagger nematodes are listed as key pests controlled by this *B. firmus* product, its efficacy as a bionematicide against species of dagger nematode indigenous to West Virginia peach and apple orchards remains unclear.

Peach and apple production is a valuable industry in West Virginia and other Mid-Atlantic states, with a combined revenue exceeding 18 million dollars annually in West Virginia alone (USDA, 2015). These trees are highly susceptible to dagger nematodes and the spread of TomRSV, a virus that leads to development of peach stem pitting (PSP) and apple union necrosis diseases (AUND) (Georgi, 1988; Jaffee et al., 1987). The purpose of this study was to determine if the bacterium *B. firmus* is an effective biological control organism against the West Virginia-native dagger nematode, *Xiphinema americanum*.

Materials and methods

Bacillus firmus

A research grade formulation containing a minimum of 1.0×10^{11} CFU/gram of *Bacillus firmus* (I-1582) provided by Bayer CropScience was used in this study. A *Bacillus* isolate from this formulation (named BF1) was selected and the identity was confirmed using a range of morphological and biochemical characteristics analogous to *B. firmus* (Appendix 1). Later, rRNA gene analysis of the BF1 isolate resulted in 100% identity with *Bacillus cereus*, *Bacillus*

thuringiensis, and *Bacillus anthracis* and 93% identity with *Bacillus firmus* (Appendix 1). For the following experiments, the isolated BF1 culture is referred to as *B. firmus*. It was grown in 100 mL of TSB at 28°C for 24 hours to produce a concentration of approximately 10⁷ CFU/mL. The day-old culture was centrifuged at 5000 rpm for 20 minutes to separate bacterial cells and TSB. The supernatant was removed and the *B. firmus* cells were re-suspended in 100 mL of SSE.

Sterile soil extract (SSE)

Sterile soil extract (SSE) was used as the control for all experiments in this study. SSE was used in lieu of sterile distilled water and TSB based on preliminary results that exhibited a larger nematode mortality rate for both cell-free sterile distilled water and cell-free TSB when compared to cell-free SSE. SSE was prepared by combining 1000 g of a Tilsit silt loam soil obtained from the West Virginia University Organic Farm in 1 L of tap water. The soil and water were mixed thoroughly then allowed to settle for 24 hours. The water was decanted and autoclaved twice for 90 minutes each time.

Xiphinema americanum

Dagger nematodes were collected from an orchard in Hampshire County, West Virginia with a Lehigh-Berks Complex soil (channery fine sandy loam, Loamy-skeletal, siliceous, semiactive, mesic Typic Dystrudepts) and extracted using a sieving and sucrose-centrifugation method. One hundred cm³ of soil was placed in a pitcher, mixed with tap water, and immediately poured through a 600-µm-pore mesh sieve to remove large debris. The water was then washed through a 38-µm-pore mesh sieve and anything remaining in the sieve was collected in a tube and centrifuged at 1000 rpm for 3 minutes. The supernatant was discarded and the nematodes were re-suspended in a 60% sucrose solution. The tubes were centrifuged at 1000 rpm for an additional 3 minutes. After centrifugation, the sucrose/nematode suspension was washed through a 25-µm-pore mesh sieve to collect nematodes. Those resembling dagger nematodes of various life stages were picked from the samples and used for the following experiments. Measurements of stylet and tail lengths and anal body diameters were taken for species verification (Lamberti and Bleve-Zacheo, 1979).

Tomato seedlings

Rutgers tomato seeds were surface disinfested by washing in a 70% ethanol solution for 2 minutes, then a 3% NaOCl solution for 10 minutes and immediately rinsing with sterile distilled water three times. Seeds were transferred to 0.75% TSA plates and incubated at 28°C for 5 days or until roots were approximately 2-4 cm. Seedlings that showed signs of microbial contamination were discarded.

Experiment 1. *Bacillus firmus* direct toxicity assay

To determine if various concentrations of *B. firmus* are directly toxic to *X. americanum*, we tested the hypothesis that survival of *X. americanum* will be affected by the concentration of *B. firmus*. A 10⁷ CFU/mL concentration of *B. firmus* in SSE was serially diluted to produce seven *B. firmus* concentrations from approximately 10¹-10⁷ CFU/mL. Bacterial concentrations were enumerated using serial dilution and spread plating. Two mL of each bacterial concentration were added to 35-mm plastic dishes and uniformly spread throughout the plates. Plates with cell-free SSE were used as a control. Approximately 10 *X. americanum* nematodes of various life stages were added to each dish and the plates were incubated in darkness at room temperature (23±2°C). Nematode mortality was determined at 24, 48, and 72 hours. Nonmotile nematodes were probed with a fine needle and those that were unresponsive after probing were considered dead. Four replications were used for each *B. firmus* concentration and the entire experiment was conducted three times. Data were analyzed using ANOVA and treatment means were compared with the control using a nonparametric Kruskal-Wallis test.

Experiment 2. Filter paper attraction assay

To determine if movement of dagger nematodes is affected by the presence of *B. firmus*, we tested the hypothesis that a smaller portion of *X. americanum* will accumulate near *B. firmus*-treated filter paper compared with SSE-treated filter paper. Sterile filter paper disks (12.7 mm dia) were soaked in either a 10⁷ CFU/mL concentration of *B. firmus* in SSE or cell-free SSE. Approximately 2 mL of a 23% Pluronic gel medium were pipetted onto 75 X 25-mm glass microscope slides, covering the entire surface. The slides were partitioned into three labeled sections: *B. firmus* (32.5 X 25 mm), center (10 X 25 mm), and SSE (32.5 X 25 mm). Treated filter paper disks were immediately placed towards the edges of either the *B. firmus* or SSE sections of the slides, approximately 40 mm apart. No filter paper disks were placed in the

centers. The slides were placed in petri dishes with a dampened paper towel and incubated at 28°C for 1 hour. After incubation, 10-50 *X. americanum* nematodes of various life stages were added to the center of each slide (Figure 3.1). The slides with nematodes were again incubated in darkness at room temperature (23±2°C). The number of nematodes in each section of the slide was counted at 1, 2, 4, and 24 hours post inoculation. The experiment had eight replicates.

Experiment 3. Tomato seedling attraction assay

To determine if the migration of *X. americanum* towards host plant roots is altered by the presence of *B. firmus*, we tested the hypothesis that the number of *X. americanum* that move towards *B. firmus*-treated tomato roots differs from the number of *X. americanum* that move towards SSE-treated tomato roots. The roots of surface disinfested tomato seedlings were submerged in either a 10⁷ CFU/mL concentration of *B. firmus* in SSE or cell-free SSE. Approximately 2 mL of a 23% Pluronic gel medium were pipetted onto 75 X 25-mm glass microscope slides, covering the entire surface. The slides were partitioned into three labeled sections: *B. firmus* (32.5 X 25 mm), center (10 X 25 mm), and SSE (32.5 X 25 mm). One tomato seedling from each treatment was immediately placed towards the edge of the appropriate section on the slides approximately 50 mm apart. No tomato seedlings were placed in the centers. The slides were placed in petri dishes with a dampened paper towel and incubated at 28°C for 1 hour. After incubation, 10-30 *X. americanum* nematodes of various life stages were added to the center of each slide and the slides were stored in darkness at room temperature (23±2°C). The number of nematodes in each section of the slide was counted at 1, 2, 4, 24 and 48 hours. The experiment had five replicates.

Data Analysis

All data were analyzed using JMP and SAS software (JMP®, Version Pro 11, SAS Institute Inc., Cary, NC, Copyright ©2013; SAS®, Version 9.3, SAS Institute Inc., Cary, NC, Copyright ©2002-2010). The significance criterion (alpha) for all tests was 0.05 unless otherwise indicated.

Results

Experiment 1. *Bacillus firmus* direct toxicity assay

As the concentration of *B. firmus* increased from cell-free SSE to approximately 10^7 CFU/mL, dishes became increasingly cloudy and visibility was impacted by the turbidity of the solution. Nematodes exposed to higher concentrations of *B. firmus*, specifically the two highest concentrations of approximately 10^7 and 10^6 CFU/ mL were typically less active and required probing to verify living status more often than nematodes in cell-free SSE. Juveniles appeared to be more affected than adults, however, data were not collected for this observation. Due to a significant negatively-skewed distribution, data were analyzed using a Kruskal-Wallis test by ranks and treatment means were compared to the control using the Steel ranks test. Differences among treatments were not statistically significant at 24 or 48 hours (Figure 3.2). By 72 hours, there was a significant increase in the number of nonmotile nematodes in the 10^7 CFU/mL dishes compared to the control ($P = 0.03$), and the exposure to *B. firmus* resulted in an 11% decrease in the proportion of living nematodes.

Experiment 2. Filter paper attraction assay

Nematode movement from the center of each slide was observable within the first hour, though approximately 50% of the nematodes did not move from the centers throughout the entire length of the experiment. Data were analyzed using a repeated measures ANOVA with irregular spacing (due to unequally spaced time points), using spatial Gaussian covariance structure. Time was considered the covariate. Attraction assays using filter paper showed a significant treatment effect over time. At hours 1, 4, and 24 there were significantly more nematodes on the *B. firmus* end of the slides ($P = 0.02$, $P = 0.01$, $P = 0.01$) respectively. By hour 24, 59% of motile nematodes were observed on the *B. firmus* side compared with 41% on SSE (Figure 3.3).

Experiment 3. Tomato seedling attraction assay

Data were analyzed using a repeated measures ANOVA with irregular spacing (due to unequally spaced time points), using spatial Gaussian covariance structure. Time was considered the covariate. No significant differences in the number of dagger nematodes at each treatment were seen throughout the length of this experiment, with the exception of hour 2 ($P = 0.04$), when 70% of motile nematodes were observed on the *B. firmus* end of slides compared to 30% of motile nematodes on SSE (Figure 3.4).

Discussion

The results of this study indicate that *B. firmus* may not be a suitable candidate for the biological control of the dagger nematode *Xiphinema americanum*. Exposure to a *B. firmus* concentration of 10^7 CFU/mL, the highest concentration used in this study, showed a significant decrease in the number of living nematodes by 72 hours, suggesting the production of lethal secondary metabolites. Species of *Bacillus* are well-known for their production of secondary metabolites that have an array of properties and functions, including those with nematicidal activities. Castaneda-Alarez et al. (2015) demonstrated that cultural filtrates of *B. thuringiensis*, *B. megaterium*, and *B. amyloliquefaciens* are capable of killing 54-100% of *Xiphinema index* after 72 hours of exposure. Metabolite and enzyme analysis showed that these *Bacillus* species produce collagenases, proteases and lipases, which may play a role in their nematicidal activity. Ramezani Moghaddam et al. (2014) showed that culture filtrates of *B. cereus* and *B. pumilus* reduced egg hatch and resulted in 72-99% juvenile mortality of *Meloidogyne javanica* after 48 hours of exposure. The patent submitted for the use of *B. firmus* as a bionematicide asserted that "...proteolytic and collagenolytic activities play an important role in control of nematodes..." (Patent US6406690) (Peleg & Feldman, 1996). Mendoza et al. (2009) showed that 100% concentrations of cell-free *B. firmus* culture filtrates caused 33% mortality of *Meloidogyne incognita* after 24 hours.

The 11% mortality of *X. americanum* seen in the present study may be explained by the production of secondary metabolites, though this degree of nematode death was only observed after exposure to the highest concentration of *B. firmus* (10^7 CFU/mL). Durham (2013) showed that a rifampicin-resistant *B. firmus* mutant could maintain population densities of 10^5 , 10^7 , and 10^6 CFU/g of root in the rhizosphere of corn, cotton, and soybean, respectively, 6 weeks after planting, while population densities of the rhizoplane were measured at 10^6 CFU/g of root for each plant host. Those data suggest that *B. firmus* may be unable to maintain population densities that reflect the highest concentration used in the present study. Furthermore, since the lethal properties of *B. firmus* were observed only when the bacterial concentration reached 10^7 CFU/mL, the potency of any secondary metabolites produced may be limited. These compounds would likely be diluted within the rhizosphere and have little effect on nematode populations in realistic orchard settings. However, if the metabolites suppress nematode movement, the nematode may be induced to remain near the source of the metabolite long enough for toxic effects to occur.

An additional explanation for the significant nematode mortality observed in the present study is that high concentrations of bacteria can lead to a decrease in available oxygen over time. In the direct toxicity experiment, the increase of *B. firmus* concentrations from 0 in SSE to 10^7 CFU/mL was reflected in the turbidity of the solution within each plate. Thick films of bacteria were clearly visible within plates containing high bacterial concentrations. Metabolic activity of high concentrations of bacteria can deplete oxygen levels and create hypoxic and anoxic conditions. As aerobic organisms that rely solely on the diffusion of oxygen from the ambient environment, nematodes can be negatively impacted by oxygen depletion. Kung et al. (1990) demonstrated that mortality rates of *Steinernema carpocapsae* and *S. glaseri* increase over time as oxygen levels are decreased from 20-1%, while Föll et al. (1999) showed that more than 80% of *Caenorhabditis elegans* die after 72 hours in anoxic conditions. Future studies could utilize an additional treatment of cell-free culture filtrates to confirm the presence of toxic secondary metabolites, as well as supply a steady flow of oxygen to plates to ensure oxygen depletion does not occur.

In-vitro attraction assays comparing *X. americanum* movement on Pluronic gel in the presence of *B. firmus* and cell-free SSE on filter paper showed a significant treatment effect over time. Interestingly, nematodes were observed more often on the *B. firmus* end of slides compared to the SSE control, a contrast to attraction assays observing *Meloidogyne hapla* movement in the presence of *B. firmus* as discussed in Chapter 2. Chemotaxis is a key component of nematode behavior, where various chemical gradients signal the presence of food, potential mates, and environmental stressors, prompting nematode movement (Linford, 1939; Wieser, 1955; Bird, 1959; Simon & Sternberg, 2002; Saeki et al., 2001). Bayer CropScience reports that their *B. firmus* product, VOTiVO, "...creates a living barrier that grows with the roots to extend protection through multiple generations of nematodes..." by interfering with the nematode's ability to find host plant roots. The behavior of *X. americanum* towards *B. firmus* on filter paper observed in the present study, however, indicates the involvement of a chemoattractant produced by *B. firmus*. Because nematodes remained motile and free to migrate within the Pluronic gel throughout the length of the experiment, nematode accumulation at *B. firmus* is more likely a result of the continual lure of attractive compound(s) produced by *B. firmus* rather than the presence of chemicals affecting nematode motility. In this case, the addition of *B. firmus* to roots may lead to an accumulation of dagger nematodes near the source of the attractant, though it

remains unclear whether this interaction would result in a higher or lower degree of nematode parasitism. Aballay et al. (2012) showed that treatment of grapevines with various rhizobacteria including *B. mycooides*, *B. sphaericus*, and *B. thuringiensis* significantly reduced feeding damage by *X. index* compared with the untreated control.

Attraction assays utilizing *B. firmus*- and SSE-treated tomato seedlings showed no significant treatment effect, with the exception of hour two, when more nematodes were observed on the *B. firmus* portion of slides. Again, these data conflict with the behavior of *M. hapla* towards *B. firmus*-treated tomato seedlings observed in the tomato seedling attraction assay discussed in Chapter 2. The lack of significant treatment effect may be explained by small nematode sample sizes and the use of an unsuitable host plant. In the present study, 10-30 *X. americanum* nematodes of various life stages were placed on each slide. Throughout the length of the experiment, a relatively large portion of these nematodes remained motionless in the centers. Larger sample sizes may have compensated for this lack of nematode movement and generated more significant results. Tomato seedlings were chosen for use in attraction assays based on their ease and speed of germination, however, the parasitic association of *Xiphinema* spp. with tomato is not as strong as with other crops. Offering a more suitable host, such as corn, may have initiated a more notable nematode response (Batchelor, 1993).

The present study presents evidence of the limited potential of *B. firmus* as a bionematicide for the control of *X. americanum*. *In-vitro* experiments demonstrated that, although *B. firmus* may produce lethal secondary metabolites, these metabolites have low nematicidal activity. Additionally, the production of a chemoattractant may result in the accumulation of nematodes at host plant roots.

The observed difference in behavior of *X. americanum* seen in the present study and *M. hapla* as discussed in Chapter 2 in the presence of *B. firmus* is an interesting outcome that suggests that the effect of any chemotactic factor produced by *B. firmus* is species-specific. This contrasting behavior between nematode species is an indication that maximum nematode control may only be achieved by treating infested soils with a combination of antagonistic organisms. Mendoza & Sikora (2009) showed this trend by combining applications of a nonpathogenic strain of *Fusarium oxysporum* (considered to induce resistance within banana plants), and *B. firmus* to soils inoculated with the endoparasitic nematode, *Radopholus similis*. The combined

treatments reduced *R. similis* densities between 77 and 86% compared to a reduction between 33 and 43% seen with treatments of *F. oxysporum* alone.

While this study gave insight into the interaction of *X. americanum* and *B. firmus* in a highly controlled setting, little is known about how these interactions translate to the complex orchard environment. Additional attraction assays incorporating more suitable host plants, preferably peach and apple, and increasing dagger nematode sample sizes would allow for a more accurate interpretation of this *B. firmus*/dagger nematode relationship. The present study was useful in facilitating a better understanding of the nematicidal mode of action of *B. firmus* but, ultimately, long-term nematicide trials within orchards, utilizing applications of *B. firmus* throughout several growing seasons should be the future direction of *B. firmus* efficacy trials. Monitoring *X. americanum* populations, damage, and viral transmission over time would give the most meaningful assessment of *B. firmus* as a bionematicide against *X. americanum*.

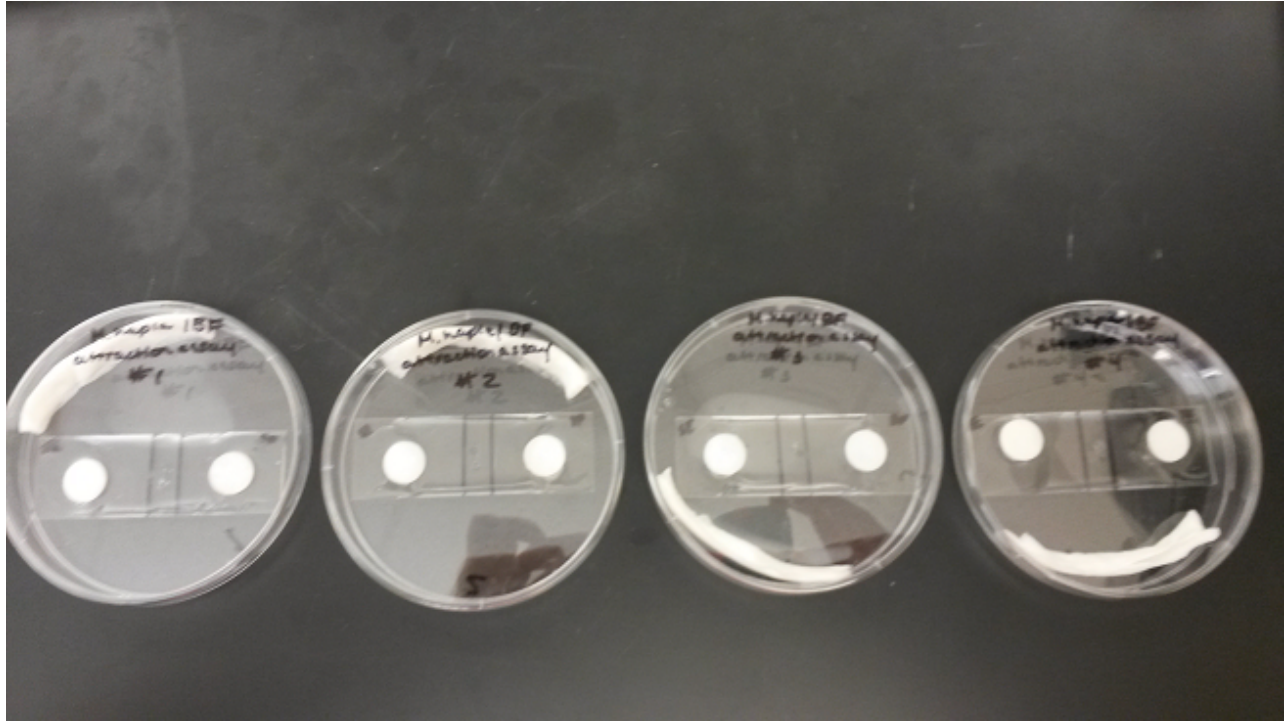


Fig. 3.1. *In-vitro* attraction assay set-up: *B. firmus* and SSE treated filter paper on opposite ends of slides covered in Pluronic gel.

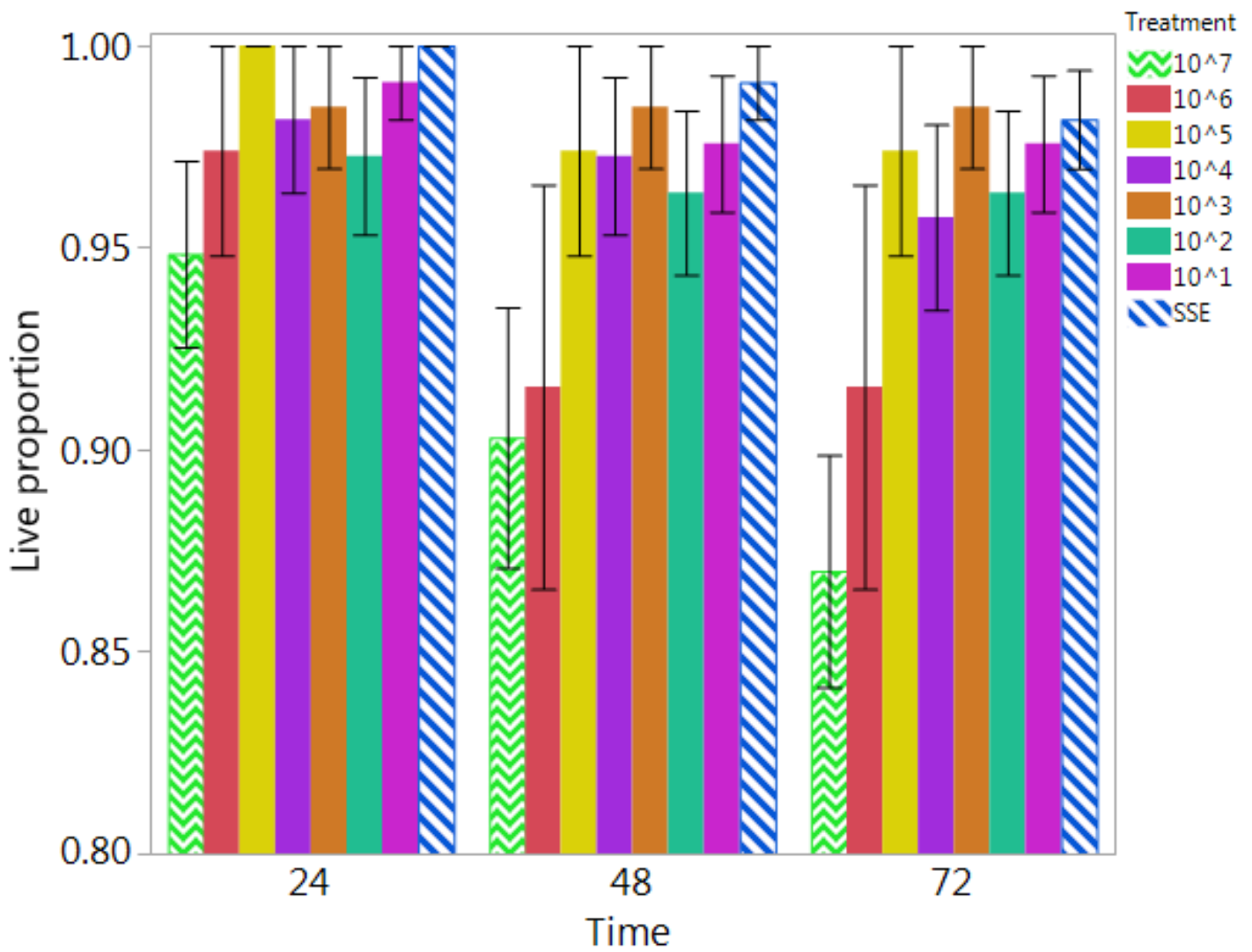


Fig. 3.2. Direct toxicity assay: Proportion of living *X. americanum* nematodes after 24, 48, and 72 hours of exposure to different concentrations of *B. firmus*. Error bars represent standard error of the mean.

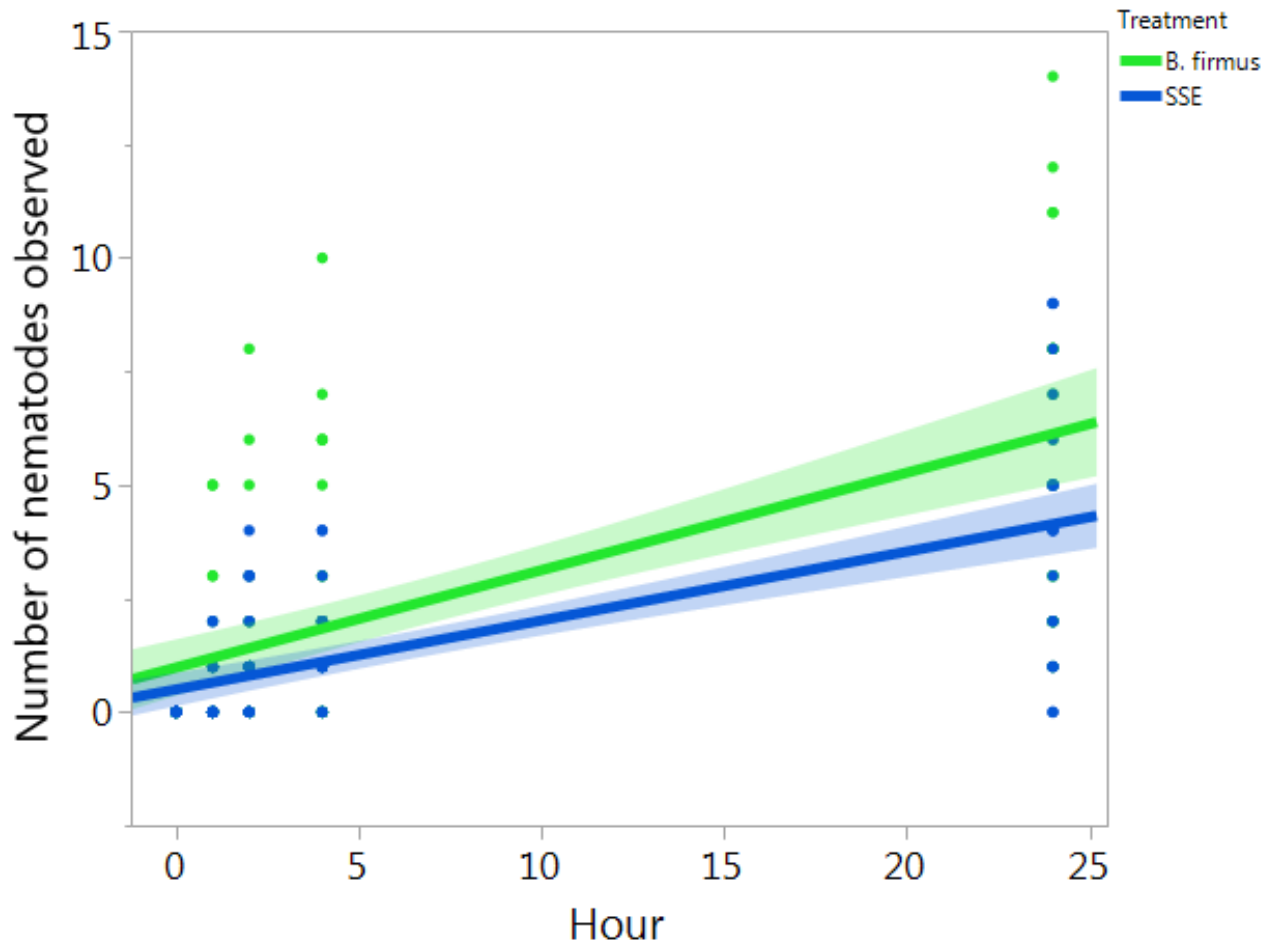


Fig. 3.3. Filter paper attraction assay: Number of *X. americanum* nematodes observed on the *B. firmus* portion of the slide vs. the SSE portion of the slide over time. The shaded regions represent confidence intervals of the slope.

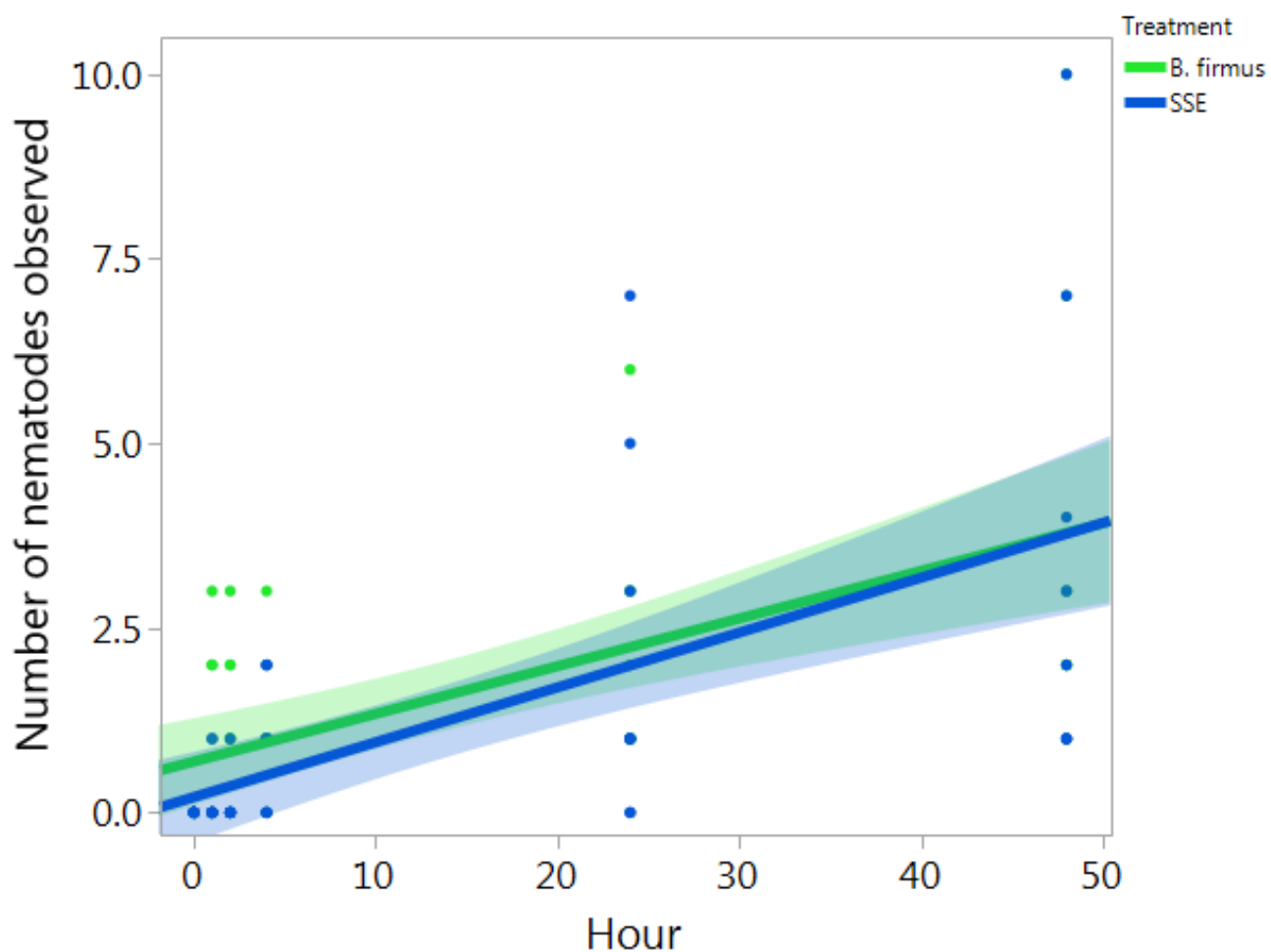


Fig. 3.4. Tomato seedling attraction assay: Number of *X. americanum* nematodes observed on the *B. firmus* portion of the slide vs. the SSE portion of the slide over time. The shaded regions represent confidence intervals of the slope.

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Appendix 1

Bacillus firmus isolation 1

A research grade formulation containing a minimum of 1.0×10^{11} CFU/gram of *Bacillus firmus* (I-1582) provided by Bayer CropScience was used to obtain the *B. firmus* isolate that was used in the studies described in this thesis. One gram of the product was placed into a flask containing 100 mL of TSB and incubated at 28°C for 24 hours. The culture was then used to streak TSA plates for isolation. Plates were incubated at 28°C for 24 hours. Following incubation, one main morphological type of colony (Morphotype 1) could be seen covering approximately 80-90% of each plate and a secondary morphological type (Morphotype 2) covered approximately 10-20% of each plate. Morphotype 1 colonies had a glossy cream-yellow coloring with entire-slightly irregular margins. Colony sizes averaged 3-5 mm after 24 hours of growth. Morphotype 2 colonies were transparent-cream colored and circular with entire margins. Colony sizes averages 1-2 mm after 24 hours of growth. Several colonies of both morphological types were chosen for staining and biochemical testing.

Gram stains showed gram-positive bacillus-shaped cells, 3-5 μm X 1.5-2 μm for both morphotypes. Spore stains showed one slightly offset endospore per vegetative cell. Both morphotypes displayed similar results for biochemical tests. Positive results included catalase production, reduction of nitrate to nitrite, starch hydrolysis, casein and gelatin decomposition, and production of acid in glucose and mannitol. Negative tests included oxidase, production of acid in lactose, urease, indole, citrate, and Voges-Proskauer. Based on morphology and biochemical testing, a Morphotype 1 isolate (BF1) was chosen to be used for the experiments described in this thesis.

rRNA gene analysis 1

Following the studies described in this thesis, 16S rRNA gene sequencing was performed on the BF1 isolate to verify the presence of *Bacillus firmus*. DNA was extracted from cells and universal 16S rRNA bacterial primers Bakt_341F 5'-CCTACGGGNGGCWGCAG-3' and Bakt-805R 5'-GACTACHVGGGTATCTAATCC-3' were used to amplify a ~450bp portion of the gene. PCR products were visualized on a 1% agarose gel under UV light to confirm the presence of a ~450bp band. Sanger sequences were generated by Eurofins MWG Operon LLC in Louisville, KY. Blasting Sanger sequences in the National Center for Biotechnology Information

(NCBI) database resulted in 100% identity with *Bacillus cereus*, *B. thuringiensis*, and *B. anthracis* and 93% identity with *B. firmus*. These results lead to re-isolation from the original *Bacillus firmus* product provided by Bayer CropScience and additional rRNA gene sequencing.

Bacillus firmus isolation 2

The research grade formulation of *Bacillus firmus* (I-1582) provided by Bayer CropScience as previously mentioned in “*Bacillus firmus* isolation 1” was used to re-isolate *Bacillus firmus* for species verification. One gram of the product was placed into a flask containing 100 mL of TSB and incubated at 28°C for 24 hours. The culture was then used to streak TSA plates for isolation. Plates were incubated at 28°C for 24 hours. Again, following incubation, one main morphological type of colony (Morphotype 1) could be seen covering approximately 80-90% of each plate and a secondary morphological type (Morphotype 2) covered approximately 10-20% of each plate. Morphotype 1 colonies had a glossy cream-yellow coloring with entire-slightly irregular margins. Colony sizes averaged 3-5 mm after 24 hours of growth. Morphotype 2 colonies were transparent-cream colored and circular with entire margins. Colony sizes averages 1-2 mm after 24 hours of growth. Six colonies of Morphotype 1 (BF 2-7) and three colonies of Morphotype 2 (BF 8-10) were chosen for staining and biochemical testing.

Gram stains showed gram-positive bacillus-shaped cells, 3-5 µm X 1.5-2 µm for both morphotypes. Spore stains showed one slightly offset endospore per vegetative cell. Both morphotypes displayed similar results for biochemical tests. Positive results included catalase production, reduction of nitrate to nitrite, starch hydrolysis, casein and gelatin decomposition, and production of acid in glucose. Negative tests included oxidase, production of acid in lactose, urease, indole, citrate, and Voges-Proskauer. Isolates BF3, 9, and 10 tested positive for production of acid in mannitol.

rRNA gene analysis 2

16S rRNA gene sequencing was performed on the BF1-10 isolates to verify the presence of *B. firmus*. DNA was extracted from cells and universal 16S rRNA bacterial primers Bakt_341F 5'-CCTACGGGNGGCWGCAG-3' and Bakt805R 5'-GACTACHVGGGTATCTAATCC-3' were used to amplify a ~450bp portion of the gene. PCR products were visualized on a 1% agarose gel under UV light to confirm the presence of a

~450bp band. Sanger sequences were generated by Eurofins MWG Operon LLC in Louisville, KY. Blasting BF1-2 sequences in the NCBI database resulted in 100% identity with *Bacillus cereus*, *B. thuringiensis*, and *B. anthracis*; BF4-7 resulted in 99% identity with *B. cereus*, *B. thuringiensis*, and *B. anthracis*; BF3 resulted in 100% identity with *Lysinibacillus*; BF9 resulted in 100% identity with *Bacillus firmus*; and BF10 was unidentifiable.