Characterizing A Soybean Cyst Nematode Mycobiome From Waseca Long-term Soy-corn Rotation Experiment in Search of Fungal Biological Control Agents and Bio-nematicides

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DEEPAK RAJENDRAN

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Prof. Senyu Chen and Dr. Kathryn E. Bushley

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This dissertation is dedicated to
William Ernest Henley

Out of the night that covers me,
Black as the pit from pole to pole,
I thank whatever gods may be
For my unconquerable soul.

In the fell clutch of circumstance
I have not winced nor cried aloud.
Under the bludgeonings of chance
My head is bloody, but unbowed.

Beyond this place of wrath and tears
Looms but the Horror of the shade,
And yet the menace of the years
Finds and shall find me unafraid.

It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate,
I am the captain of my soul.

Abstract

The Soybean Cyst Nematode (SCN, *Heterodera glycines*) is the most economically consequential pathogen of the soybean plants worldwide. Integrated management of this pathogen currently relies on using genetic resistance and crop rotation with a non-host such as corn. Additional help from chemical and biological agents could lead to better management of this pathogen by supplementing the biological limitations of genetic resistance and economical limitations of using a non-host crop. Identifying natural antagonists of SCN is a good first step towards finding potential biocontrol agents. In this study, I have cultured and identified fungi from 6000 SCN cysts obtained from a long-term soy-corn rotation experiment with no nematicide applied, over a period of 3 years. ITS barcode sequences from all the isolates (about 5000 from 6000 cysts) obtained from these cysts, as well as their colony morphologies were used to identify the fungi against NCBI database. ITS sequences were then used to cluster them into 326 OTU groups based on at least 99% sequence similarity. OTU cluster representatives were evaluated *in vitro* for their ability to produce anti-nemic metabolites and to directly parasitize SCN eggs. Ten high anti-nemic metabolite producers ('A' through 'J') and ten high parasitic fungi ('K' through 'T') were then evaluated *in vivo* using a growth chamber-based cone-tainer assay. Top three performers in the cone-tainer assay, isolates 'D', 'E' and 'T' were subsequently evaluated in greenhouse potted plant assays. Isolates 'E' and 'T' were as good as commercially available biocontrol agent Melocon WG, even at 40-400 folds lower concentration.

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Chapter 0: **INTRODUCTION**

The soybean cyst nematode (*Heterodera glycines*, SCN) was discovered as a significant pest of soybean in 1954 in North Carolina in the USA, and subsequently spread across a few counties in Arkansas, Missouri, Kentucky, and Tennessee, as well as few other pockets in North Carolina (Winstead and Skotland 1955). In 2014, bolstered by the increase in planting of soybean, the SCN has become a significant plant health issue all over the agrarian eastern continental United States as well as Hawaii and Puerto Rico, and southern Canada (Tylka and Marett 2014). In the USA, SCN accounts for up to 30% of all soybean yield lost to disease, amounting to a little over 2.7 million metric tons a year (Allen et al. 2017). It is a major problem of this important protein and oilseed crop of the United States, the largest producer of soybean with an annual output of 117.3 million metric tons in 2016 (White and Honig 2017).

The life cycle of SCN includes several life stages that can be targeted by natural antagonists. However, the survival structures or cysts, which consist of the female bodies containing hundreds of eggs protected by a melanized layer of cuticle, are an important target stage for biocontrol. The eggs in cysts can survive up to 10 years or longer in soil and can produce viable and infective second-stage juvenile nematodes (J2) when conditions are favorable. Thus, reducing the egg numbers in cysts in soil will ensure significant reduction in nematode inoculum.

The idea of using biological agents to control nematodes is as old as nematology itself, as Nathan Cobb, the father of nematology first suggested using

predatory nematodes to control plant-parasitic nematodes (Cobb 1917). Biological control or biocontrol is scientifically attractive goal, albeit often not as commercially successful in most cases compared to chemical control methods. It has become increasingly attractive since methyl bromide has been proven harmful to the environment, livestock, and humans and subsequently banned from use. (Duniway 2002). Although there are several chemical alternatives to methyl bromide available in the market today, their mechanisms are either poorly understood or not extremely specific to the target nematodes. Furthermore, biocontrol also offers the option to isolate bioactive metabolites from microorganisms and develop them into bionematicides, in addition to using the organism by itself. It is also a good idea to develop biocontrol agents and bionematicides to work synergistically with currently employed practices such as genetic resistance, crop rotation, and other chemical nematicides as well, for a well-rounded, integrated nematode management.

Several microorganisms have been tested as potential antagonists against SCN, including both fungi and bacteria, with *Pasteuria* spp. being the most important bacterial agent. However, only a few bacterial and fungal biocontrol agents have been commercialized thus far, mostly in the genera *Bacillus* or *Pasteuria* for bacteria, and *Paecilomyces* and *Pochonia* for fungi (Li et al. 2015; Chen and Dickson 2012). Bionematicides of bacterial origin, especially Abemectin, have seen some commercial success, including for use on *Heterodera avenae* (Zhang et al. 2017). However, eukaryotic fungi and other fungal-like organisms that can sporulate, have been increasingly reported as potential biocontrol agents, especially for root-knot nematodes, but have had fewer commercial successes than biocontrol agents for SCN

(Tranier et al. 2014; K. M. Musil 2016). Filamentous fungi could overwinter, produce spores and remain in the soil for longer periods of time than their non-sporulating counterparts (Jung et al. 2014); and thus, may reduce reapplication rates and associated costs. Given that the major soybean producing states (Iowa, Illinois, Minnesota, the Dakotas, and Ohio) (USDA 2016) have harsh winters with fallow fields and conditions unfavorable for any growth, filamentous and sporulating fungi could be the most viable option for biocontrol, yet few have been commercially developed. Furthermore, desiccated spores enable easy storage and transport of the organism without losing viability for long periods compared to live vegetative cells. Desiccated fungal spores could also be easily formulated to be applied as soil drenches like in the case of Melocon WG or coated on seeds as in the case of Poncho/Votivo (BAYER 2012, 2015).

There are several reviews that discuss biological control of several plant-parasitic nematodes using fungi. This review aims to specifically summarize studies that have tried to understand the fungal communities associated with one nematode, SCN, in the US. A detailed review of biocontrol of all plant-parasitic nematodes by different kinds of fungal parasites such as nematode-trapping fungi, obligate endoparasites of SCN juveniles, and female and egg parasites, on different stages of nematode development, is presented in a book chapter by Chen and Dickson (2012). Therefore, this review will focus mainly on members of culturable fungal communities from SCN that can be studied *in vitro* as well as *in vivo* for their biocontrol properties. For a good biological control agent, in addition to being able to colonize nematodes *in vitro,* the ability to tolerate abiotic and biotic stresses to survive

in soil or colonize roots is an important consideration. Fungistasis, or competition between fungi, as well as host preferences (Mauchline et al. 2004) and abiotic stresses such as varying conditions of temperature, humidity, pH and moisture in the soil environment must be evaluated before a biocontrol agent is tested in the field. Several reviews have discussed the role of nematode trapping fungi that utilize various trapping devices such as nets, rings, and knobs, in control of nematodes (Mankau 1980; Kerry 1988; Siddiqui and Mahmood 1996; Chen and Dickson 2012). This review will not discuss trapping fungi as they often are influenced by other soil organisms and competition, and are often fastidious to culture (Persmark et al. 1996; Jaffee et al. 1998; Li et al. 2011). This article aims to summarize some of the common fungal taxa reported in the literature, associated with SCN, as well as anti-nemic metabolites from fungi that have been studied for their potential against SCN and other cyst nematodes. This review also discusses various soil amendments that have been shown to reduce SCN levels in the soil, which may have indirect effects on fungal communities in the soil affecting SCN.

0.1 Common culturable fungi (Mycobiome) associated with SCN cysts and eggs

The diversity of fungi and fungal-like organisms is vast, and soil is a very complex habitat that hosts all kinds of fungi with varying modes of nutrition. Obligate parasites of SCN are highly virulent and effective nematode parasites, but they have limitations as viable biocontrol options as they cannot be cultured easily on artificial media and other fungi usually outcompete them in soil. On the other end of the

continuum, true saprophytes can be easily cultured on artificial media, but generally may not colonize the SCN as pathogens. Thus, facultative saprophytes that are both effective parasites of nematodes, but can also be cultured on artificial media have promise as potential biocontrol agents.

Several studies have investigated the fungi associated with the SCN from several locations of the United States(Supplementary Figure 0-1) indicates the major fungal genera isolated from SCN cysts (culturable mycobiome studies from the US) on a fungal tree generated by the Joint Genome Institute (JGI) fungal program and the MycoCosm initiative (Grigoriev et al. 2011, 2014). Figure 0-2 indicates major soybean producing states in the USA (Economic Research Service USDA 2017) and locations from where SCN cyst mycobiomes have been isolated, analyzed, and summarized in this report. Most often, studies that report potential biocontrol fungi seek natural parasites and hence culture fungi from cysts or females or eggs or J2 of SCN. *Heterodera glycines* cyst, female and egg parasites are relatively easy to locate and isolate from the environment as they are the sedentary overwintering structures in the soil. It would be practical and beneficial to target the immobile and resistant life stages, which are survival structures that allow the hundreds of eggs in each cyst to survive dormant for years in soil.

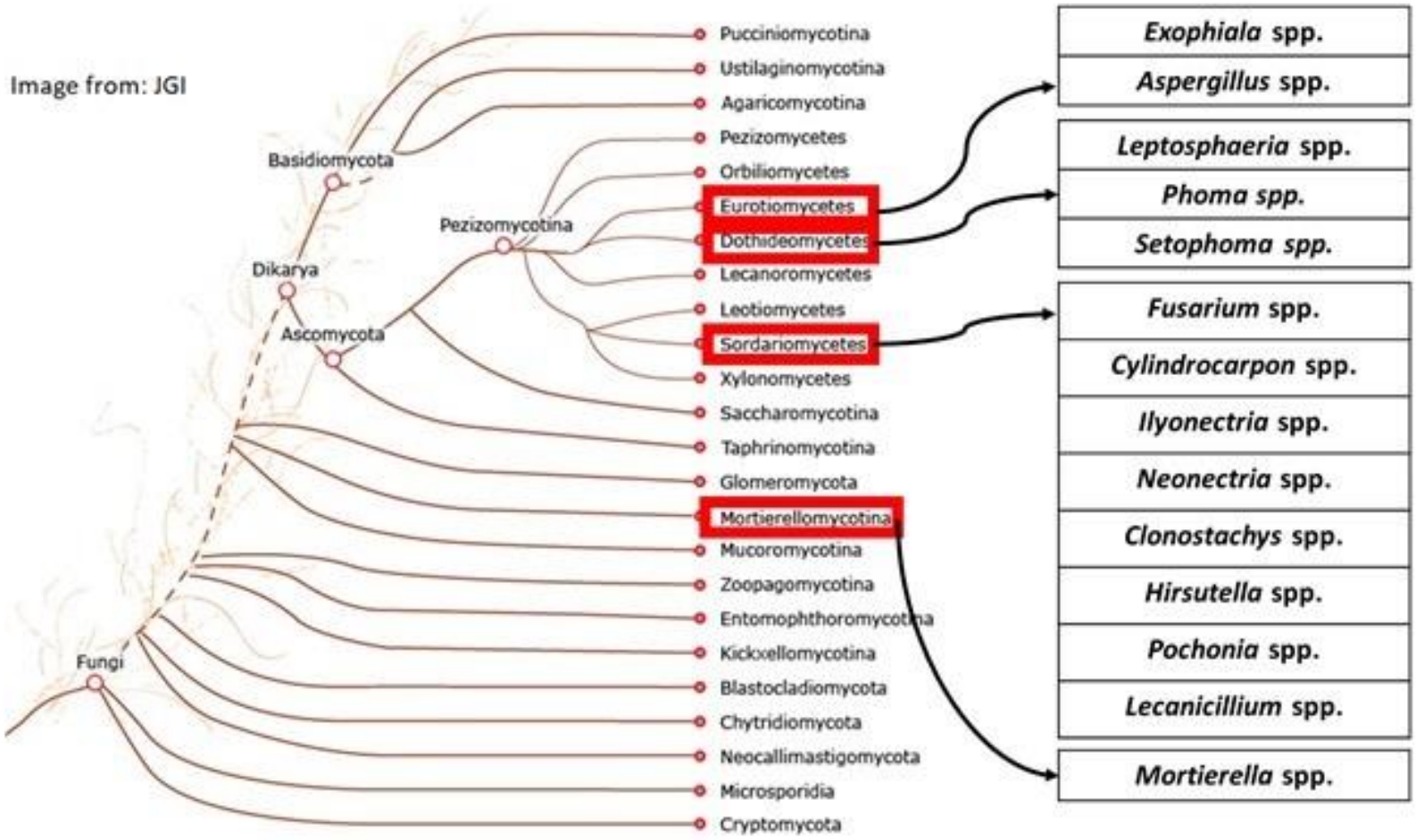


Figure 0-1. Common fungi isolated from SCN cysts (US SCN mycobiome studies) and their relationship within the Kingdom Fungi.

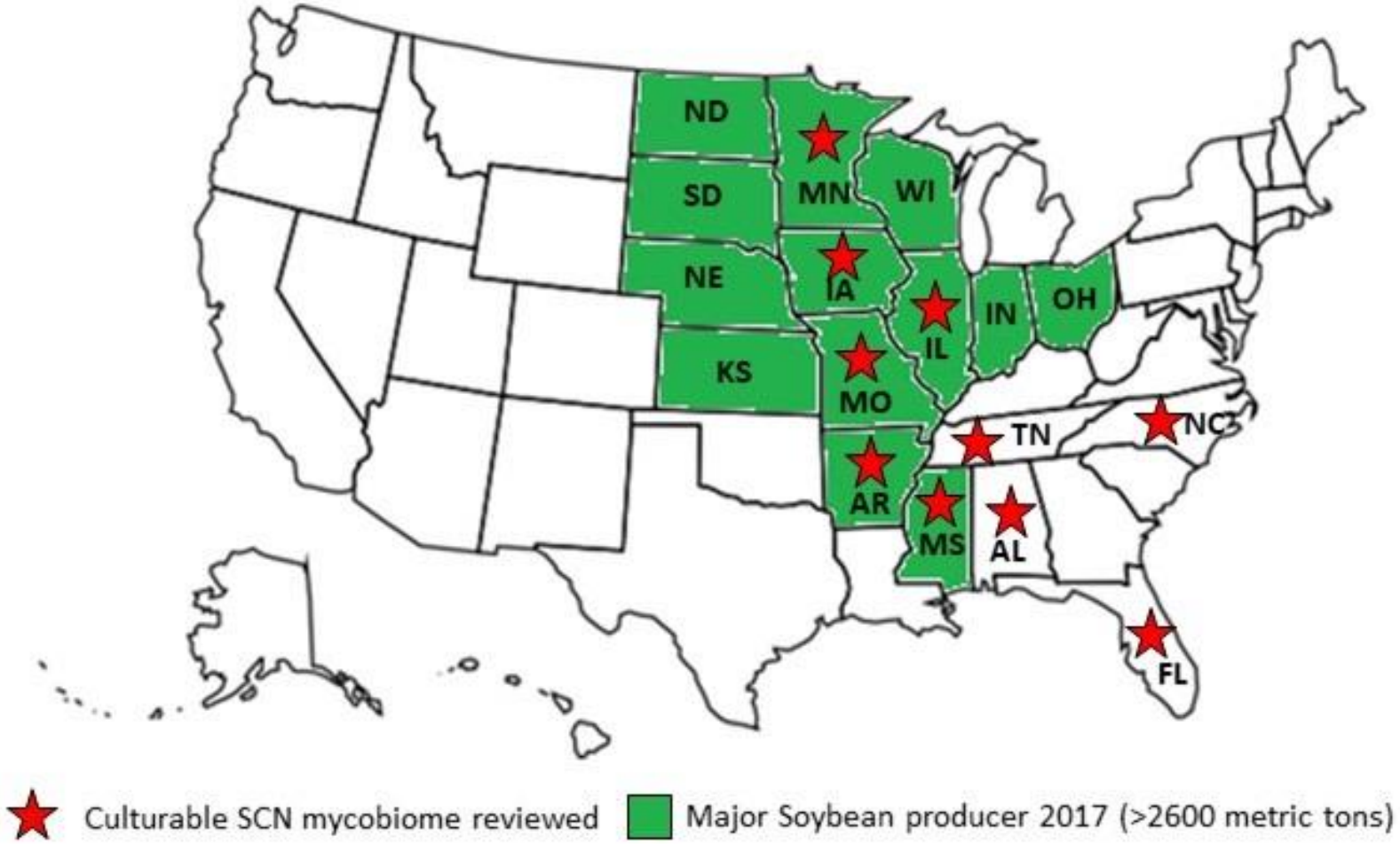


Figure 0-2. Top soybean producing states (colored green) and locations where SCN mycobiome was analyzed (red stars).

0.1.1 Family Nectriaceae: *Fusarium*, *Cylindrocarpon*, and other genera

Assessments of fungal genera associated with SCN cysts and eggs as potential biocontrol agents have been reported for more than three decades. These studies have reported *Fusarium* spp., to be highly associated with SCN cysts, the most common species being *F. oxysporum* and *F. solani.* However, using molecular techniques for species identification, we know now that these groups form large species complexes that may harbor cryptic species and are highly diverse (O'Donnell et al. 2008, 2009; Muraosa et al. 2014).

Several studies have examined the culturable mycobiome or the collection of culturable fungi from SCN in the US. A study published from Alabama by Gintis et al. (1983) looking at fungal diversity associated with several stages of cysts, ranging from the cream colored females attached to the root tissue to the brown cysts encasing eggs that are dispersed into soil, provided insights into the importance of the genus *Fusarium*. *Fusarium oxysporum* and *F. solani* are omnipresent soil fungi that help degrade residual plant materials. Fungi in these large species complexes have highly diverse lifestyles. There are several members in this group of fungi that are specific pathogens of plants, animals and even humans (Rajendran and Ramakrishnan, 2015; Zhang et al., 2006; Zhang et al., 2007) while there are those that are saprophytes in the soil and could become pathogenic when proper plant hosts are present, and some endophytes that could increase fitness of plants against pathogens such as nematodes (Banihashemi and Dezeeuw 1973; Waweru et al. 2014). Some may also colonize SCN cysts or produce metabolites that hinder the lifecycle of the nematode. In this study

(Gintis et al. 1983), *Fusarium* spp. were isolated at a very high frequency from all stages of SCN cysts and the authors also discuss that several other studies prior to this publication found similar results. For example, in a similar study conducted on cream-colored females that were still attached to the soybean roots, from four different locations in North Carolina, 31% of all fungi isolated were *Fusarium* spp. (Gintis et al. 1982). Similarly, *Fusarium* spp., especially *F. oxysporum* and *F. solani* have been reported as the most prevalent fungi associated with SCN cysts in a study in two soybean fields in Illinois (Carris et al. 1989)**.** Over a period of three years, this study observed a high frequency of *Fusarium* and closely related fungi within Nectriaceae from both locations. In fact, both studies reported that anywhere between 30 to 60% of all fungi isolated from the cysts belonged to *Fusarium* or other allied genera such as *Cylindrocarpon* or *Neocosmospora*. An older and broader study done across the southern states of Arkansas, Florida, Mississippi, and Missouri (Morgan-Jones et al. 1981), in which 250 cysts from one field from each state were studied, also reports to have observed more *Fusarium* than any other genera. Nearly 38% of all cyst fungi from Arkansas, 61.5% from Florida, 50% from Missouri and 52% from Mississippi locations were either *F. oxysporum* or *F. solani*. Chen et al. (1994) also reported a high frequency of this genus from soybean fields in Florida and almost 37% of all fungi isolated from SCN cysts in Tennessee fields were also from the genus *Fusarium*. While examining the effects of various management practices on the SCN mycobiome, Bernard et al. (1996) also discovered that *Fusarium* spp. were the most frequently isolated from SCN females from both the field and from roots grown in greenhouse. Chen and Chen (2002) conducted a large state-wide study in Minnesota involving 4,500 SCN cysts, 4,500 females and 45,000 eggs across 45 fields from 26

counties. Their reports also indicate similar trends. It was also noted by Gintis et al. (1983) that continuous cropping of soybean or soybean monoculture increased the frequency of *F. oxysporum* and *F. solani.* In our recent study done in Minnesota, on a long-term soy-corn rotation system, *Fusarium* was the most frequently isolated genus, with about 30% of all fungi isolated from 6,000 mature SCN cysts (Haarith et al. 2019). Although further *in vitro* testing is needed to determine their virulence and ability to parasitize and kill SCN eggs within cysts, one cannot overlook their possible potential important role in biocontrol of SCN, given their abundance and taxonomic complexity.

Beyond the genus *Fusarium*, other fungi from the family Nectriaceae appear to also be common inhabitants of SCN cysts. *Cylindrocarpon destructans* and *C. olidum* are two other fungi that have been reported in these studies (Morgan-Jones et al. 1981; Gintis et al. 1982, 1983; Carris et al. 1989; Chen et al. 1994) to be commonly isolated from SCN. There have also been reports of *Cylindrocarpon* spp., found associated with potato cyst nematodes (PCN) in the tropics (Rodriguez-Kabana and Morgan-Jones 1988)**.** Many Nectriaceae fungi are morphologically similar. Hence, many of the earlier studies based only on morphology may not have resolved the different Nectriaceae genera. Sterile fungi that do not produce any spores are difficult to identify morphologically. Hence, there is a need for molecular studies to carefully dissect and better characterize the taxonomy of this group, in addition to morphological characterization. In our culturable SCN cyst mycobiome (Haarith, Hu, D. Kim, David N Showalter, Chen, and Bushley 2019), of the top 14 genera that made up about 80% of all fungi isolated, Nectriaceae other than *Fusarium* spp. such as *Ilyonectria* spp., *Cylindrocarpon* spp., and *Neonectria* spp. contributed to about 10%

of all fungi isolated. Therefore, the net contribution of this fungal family to the mycobiome was about 40%.

0.1.2 Family Clavicipitaceae: *Pochonia*

All the previously discussed regional studies on SCN cyst fungal diversity that reported high frequency of *Fusarium* spp. also found several members of closely related families within the order Hypocreales, including Cordycipitaceae and Clavicipitaceae. The most common fungus from Clavicipitaceae isolated from cysts is *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium, Metacordyceps chlamydosporia, Cordyceps chlamydosporia, Diheterospora chlamydosporia*). Many other studies have shown *P. chlamydosporia* to have potential to antagonize SCN. Interestingly, it was Gintis et al. (1983) who first reported the relationship of *P. chlamydosporia* with SCN cysts although its association with the genus *Heterodera* was previously reported by Tribe (1977), who hypothesized it was a potential nematode pathogen. The pioneering work of Kerry et al. (1982) showed that *P. chlamydosporia* was able to control the cereal cyst nematode. Morgan-Jones et al. (1983) showed that this fungus directly inhibited the hatching of *Meloidogyne arenaria* (root-knot nematode) by colonization and hyphal penetration. In a study conducted on sugar beet cyst nematode, *Heterodera schachtii*, Crump (1991) and Crump and Irving (1992) observed 75% biocontrol while Muller (1982) and Siddiqui and Mahmood (1995) reported a loss of reproductive capacity of *H. schachtii* due to *P. chlamydosporia.* The loss of female fecundity over a period will ensure a significant reduction in the number of cysts and eggs that could cause disease either later in the season or in the following year. *Pochonia chlamydosporia* was also

frequently isolated (5%) from eggs in a study examining fungal parasitism of SCN in different cropping-tillage regimes (Bernard et al. 1996). Although no successful field trials of *P. chlamydosporia* have been reported in the United States against SCN, at the time of authoring this manuscript, Tobin et al. (2008) from the UK reported success in controlling PCN, *Globodera pallida* and *G. rostochiensis*, in the field using *P. chlamydosporia.* In our SCN cyst mycobiome study, *Pochonia* spp. contributed to about 4% of all fungi isolated (Haarith, Hu, D. Kim, David N Showalter, Chen, and Bushley 2019). A recent review by Manzanilla-Lopez et al. (2013) discusses both toxicity and parasitism exhibited by this fungus as well as the many efforts that are underway to test the durability of this fungus in real agricultural environments and the practical issues of implementing *P. chlamydosporia* as a biocontrol agent.

0.1.3 Family Cordycipitaceae: *Lecanicillium*

Previously known as *Verticillium lecanii*, *Lecanicillium lecanii* has also shown promise as a potential biocontrol agent. It is noteworthy that classification based on morphology previously placed the two fungi, *P. chlamydosporia* and *L. lecanii*, in the genus *Verticillium*, while recent molecular analyses clearly showed that *Verticillium* is polyphyletic, and places these fungi in different families, Clavicipitaceae and Cordicypitaceae, respectively (Sung et al. 2007). This further emphasizes the need for molecular studies to identify natural fungal parasites of SCN. Recent rDNA evaluations have separated the entomopathogenic strains of *V. lecanii* into a separate genus (*Lecanicillium)* and resolved it into several species (Koike et al. 2011)**,** each with differing abilities to colonize SCN. Owing to its ability to colonize insects, *Lecanicillium* spp., are not new to the biocontrol literature. A strain of *L. lecanii*

isolated from SCN was studied for its ability to colonize various nematode structures. Microscopic evaluations revealed that within 16 h of incubation, the fungus colonized SCN females, cysts, eggs, and the gelatinous matrix (Meyer and Wergin 1998). Several species of *Lecanicillium* have been used to control aphids and other insects as well as plant pathogenic fungi (Faria and Wraight 2007; Kim et al. 2010). Protoplast fusion using nitrate non-utilizing (Nit) mutants of commercially available *L. muscarium, L. longisporium* and other *L. muscarium* strains capable of controlling insects and other phytopathogens were used to attempt to create a super-agent that could control insects, SCN, as well as some phytopathogenic fungi (Koike et al. 2011; Shinya, et al. 2008b, 2008a). Their findings indicated that many of these isolates had high toxicity effects on nematode eggs and culture filtrates from those strains produced visible developmental damage in SCN eggs. They also report the loss of female fecundity and direct parasitism of SCN eggs by *Lecanicillium* spp. As these fungi possess more than one mode of action, such organisms are good candidates for biocontrol.

0.1.4 Family Ophiocordycipitaceae: *Hirsutella* and *Purpureocillium*

Hirsutella spp. are one of the most discussed group of fungi with respect to biological control of SCN, with new species being recently discovered in Minnesota (Chen et al. 2000; Chen and Liu 2007; Liu and Chen 2005). *Hirsutella rhossiliensis* and *H. minnesotensis* are two fungi that have been found to possess promising biocontrol abilities in a greenhouse study (Chen and Liu 2005). Greenhouse studies of their abilities to control SCN numbers were done alongside development of specific PCR based quantification methods for detecting this fungus in soil (Zhang et al.,

2006; Zhang et al., 2008). Unlike *Fusarium* or other Nectriaceae discussed earlier, *Hirsutella* spp. are not egg parasites, but are obligate parasites of J2 and other vermiform motile stages. In a study evaluating suppression of SCN in long-term soybean monoculture, two different fields were compared with each other and with a soybean-corn rotation plot. *Hirsutella rhossiliensis* was observed to have parasitized most of the J2s in this field (Chen 2007a). However, as discussed earlier and in other reports, obligate parasites such as *Hirsutella* pose challenges for development of biocontrol strategies (Chen 2004; Jaffee and Zehr 1985) and commercial development as they are slow growing and have limited saprophytic abilities.

Many reviews to date have discussed the presence of *Purpureocillium lilacinum*, named based on its ability to produce purple spores, as a key component in nematode suppressive soils (Sun and Liu 2000). It is a commonly occurring natural soil fungus, which was initially identified using morphology as *Paecilomyces lilacinus*, and was later given its own genus (*Purpureocillium*) within the family Ophiocordycipitaceae, based on molecular analyses showing that *Paecilomyces* is a polyphyletic genus (one genus represented in several phylogenetic clades), and its medical importance as an opportunistic animal and human pathogen (Luangsa-Ard et al. 2011; de Sequeira et al. 2017; Demitsu et al. 2017; Trinh and Angarone 2017; Sung et al. 2007). However, many agronomically important strains of this fungus are not necessarily animal or human pathogens. In fact, *P. lilacinum* YES-2 has been studied for biological control of plant nematodes, while strain QLP12 has been used against fungal pathogens (Lan et al. 2017; Aminuzzaman et al. 2013). Unlike *Hirsutella* spp. it has been found primarily associated with nematode eggs (Mwaheb et al. 2017).

0.1.5 Family Herpotrichiellaceae: *Exophiala*

The genus *Exophiala* can be traced back to 1980s and beyond, as a commonly isolated natural antagonist to SCN (Wrather and Anand 1984**).** In all the studies that have been discussed in this review focused on identifying fungal communities associated with SCN cysts, *Exophiala* spp., have been consistently reported. However, it has been isolated with far lower frequency than that of *Fusarium* spp., *Cylindrocarpon* spp., *M. chlamydosporia,* or *Lecanicillium* spp. It would be a valuable insight to evaluate the role of this genus in SCN infested soils. Some of the common species identified with SCN were *E. equina, E. salmonis*, and *E. pisciphila,* but *E. oligosperma* has been found on free-living marine nematodes (Bhadury et al. 2009). *Exophiala pisciphila* was shown moderately pathogenic to SCN eggs (Chen et al. 1996). *Exophiala* spp. contributed to about 5% of the culturable SCN cyst mycobiome in our recent study (Haarith, Hu, D. Kim, David N Showalter, Chen, and Bushley 2019). Further studies are needed to determine their role in natural control of nematodes and potential of any species and isolates as commercial biocontrol agents.

A summary of all the common fungi in SCN mycobiomes are listed in Table 0-1. It is evident that there are several common genera of fungi that have been isolated across all these diverse mycobiome studies done in the US. It is possible that both the root exudates of soybean plants as well as the niche microcosm of SCN as an organism could both influence the selection of only a few groups of fungi to be associated with SCN (Haarith et al. 2019).

Table 0-1. Common fungi isolated from SCN mycobiome studies in the USA.

Fungal Class	Fungal Family	Fungal Genus	Isolated from				References
			Females	Cysts	Eggs	Juveniles	
Sordariomycetes	Nectriaceae	*Fusarium*	Yes	Yes	Yes		(Morgan-Jones et al. 1981; Gintis et al. 1982, 1983; Carris et al. 1989; Chen et al. 1994; Bernard et al. 1996; Chen and Chen 2002b; Haarith et al. 2019)
		Cylindrocarpon		Yes	Yes		(Carris et al. 1989; Chen and Chen 2002b; Haarith et al. 2019)
		Neocosmospora	Yes	Yes			(Morgan-Jones et al. 1981; Gintis et al. 1982, 1983; Carris et al. 1989; Chen et al. 1994; Bernard et al. 1996)
		Ilyonectria		Yes			(Carris et al. 1989)
		Neonectria		Yes			(Haarith et al. 2019)
	Bionectriaceae	*Clonostachys*		Yes			(Haarith et al. 2019)
	Clavicipitaceae	*Pochonia*	Yes	Yes			(Gintis et al. 1983; Carris et al. 1989; Bernard et al. 1996; Haarith et al. 2019)
	Cordycypitaceae	*Lecanicillium*	Yes	Yes			(Gintis et al. 1982, 1983; Carris et al. 1989; Chen et al. 1994; Haarith et al. 2019)
	Ophiocordycipitaceae	*Hirsutella*				Yes	(Chen, Liu, et al. 2000; Chen and Liu 2005, 2007; Liu and Chen 2005)
		Purpureocillium	Yes	Yes			(Gintis et al. 1983; Carris et al. 1989; Bernard et al. 1996; Haarith et al. 2019)
Eurotiomycetes	Herpotrichiallaceae	*Exophiala*	Yes	Yes	Yes		(Morgan-Jones et al. 1981; Gintis et al. 1982, 1983; Carris et al. 1989; Chen et al. 1994; Chen and Chen 2002b; Haarith et al. 2019)

Dothidiomycetes	Leptosphaeraceae	*Leptosphaeria*		Yes			(Chen and Chen 2002b; Haarith et al. 2019)
	Didymellaceae	*Phoma*	Yes	Yes			(Morgan-Jones et al. 1981; Gintis et al. 1982, 1983; Carris et al. 1989; Chen et al. 1994; Bernard et al. 1996; Haarith et al. 2019)
	Phaeosphaeriaceae	*Setophoma*		Yes			(Haarith et al. 2019)
Mortierellomycetes	Mortierellaceae	*Mortierella*		Yes			(Haarith et al. 2019)

0.2 Fungal metabolites in fungal-SCN interactions

I have discussed fungi that can colonize eggs and other stages of nematodes and feed off several nematode stages. As lysotrophic organisms, fungi rely on the production of extracellular metabolites and enzymes to externally break down substrates into simpler organic molecules before assimilating them (Richards and Talbot 2018). Some fungi also make specialized secondary metabolites for fitness advantages in specific ecological niches (Fox and Howlett 2008). Acids, alkaloids, terpenes, enzymes, and antibiotics are some of the commonly produced fungal metabolites that have been cited to be involved in fungal interactions with nematodes (Huang et al. 2015; Dihingia et al. 2017). Most of what is known about fungal-nematode interactions at the molecular level come from studies of the model organism *Caenorhabditis elegans*, not the SCN (Li et al. 2015).

In several studies, fungi have been isolated from SCN cysts and eggs, identified, and tested for the effects of their culture filtrates on nematode morphology and physiology. The ability of fungi to produce metabolites toxic to SCN or other nematodes in submerged liquid culture or on solid substrates such as rice medium in the laboratory might not mirror the natural environment in soil. However, it is useful to identify compounds that have high toxicity and specificity towards SCN and other nematodes which are also (a) safe to other soil inhabitants, animals, and humans (b) cheap and easy to synthesize, and (c) biodegradable. These could potentially be useful biopesticides in agriculture or as pharmaceuticals.

Strains from the genus *Fusarium* are currently being explored for their potential to produce secondary metabolites and toxins. In a recent study, culture filtrates of *Fusarium oxysporum* strain 162 were analyzed for nematicidal activity (Bogner et al. 2016). The fungus was cultured on rice media and then extracted with ethanol. Several fractions were obtained using other organic solvents and chromatography techniques. Six new compounds were reported for the first time from *Fusarium.* Four compounds were found to be significantly detrimental to SCN and RKN and one, 4-hydroxybenzoic acid, was found to be almost as effective in killing nematodes as the nematicide carbofuran (Bogner et al. 2016). The same fungus was first isolated in Kenya as a tomato endophyte and several groups from China and Europe have since investigated its potential as a nematode biocontrol agent. In fact, this particular strain of *F. oxysporum* was subjected to submerged fermentation and the culture broth was tested on a diverse array of nematodes belonging to different ecological niches and was found to have negative effects on almost all of them except for fungivores (Hallmann and Sikora 1996). This study used a gliotoxin-inducing fermentation medium, specifically designed to induce mycotoxins, and found 20 of their 34 culture filtrates to be effective against nematodes.

Similarly, a study of a strain of *F. equiseti* isolated from SCN was grown in potato dextrose agar and the entire petri-dish of media and fungus were homogenized into potato dextrose broth, subjecting the strain to both solid-substrate and submerged fermentation. Culture filtrates inhibited SCN hatch significantly when compared to water and uninoculated potato dextrose broth media (Nitao et al. 1999). The study used four other media (yeast-lactose, cornmeal, soybean and V8 juice) as hatch controls and deduced that all microbial growth media inhibited hatch when compared

to water, even without fungi growing in them (Nitao et al. 1999). In another study, when SCN J2s were exposed to culture filtrates, *Paecilomyces lilacinus* and *Stagonospora* spp. were found most effective, while *F. oxysporum, E. pisciphila*, *Gliocladium catenulatum* and *Pyrenochaeta* spp. culture filtrates did not produce detectable effects. Furthermore, the study also tested two kinds of media, Czapek-Dox broth and malt extract broth, the latter being more effective in inducing metabolite production (Chen et al. 2000). Comparing the two media, Czapek-Dox broth has 30 g/L sucrose as a carbon source while malt extract broth has only 17 g/L of carbon in the form of malt extract. Carbon starvation is often reported as a condition necessary to initiate secondary metabolism (Stappler et al. 2016).

Analysis of a known SCN infested field in China, resulted in a mycobiome of 253 isolates belonging to 17 different genera and 23 species. Only 9.1% of all these isolates produced culture filtrates that significantly inhibited SCN hatch (Meyer et al. 2004). Filtrates from all *Cylindrocarpon* strains and 25% of all *Fusarium* strains exhibited significant decrease in hatch while 6% of *Fusarium* strains and about 8% of *Pochonia* strains stimulated hatch. Interestingly, the study also measured pH of the culture filtrates and found no significant differences between those that inhibited and stimulated SCN egg hatch. It is noteworthy that the composition of mycobiome in this Chinese soybean field was not very different from those isolated in the USA as discussed previously.

Several studies have investigated the components in culture filtrates with activity against SCN. Linoleic acid and several other chlorinated aliphatic compounds from *Lachnum papyraceum* were reported to have nematicidal and even anti-microbial properties, therefore supporting the idea that soil microbial communities are

interdependent and strongly interacting (Anke et al. 1995). One compound may have multiple effects on several soil inhabitants. This makes *in vivo* testing compulsory to screen for good biocontrol agents. Apart from beauvericin and enniatins, there are many new toxins being isolated from *Fusarium* spp., termed emerging fusaritoxins, such as fusaproliferin and moniliformins (Jestoi 2008). It will be informative to test some of these toxins against SCN, as a majority of the SCN mycobiome appears to be composed of *Fusarium* spp. Some *Fusarium* metabolites have been tested against nematodes other than the SCN and found to be effective bio-nematicides. At 100 µg/mL concentration *in vitro,* bikaverin and fusaric acid were able to kill about 50% of all of pinewood nematodes treated over a 2 day period (Kwon et al. 2007). Flavipin, isolated from the culture broth of a *Chaetomium globosum* strain, were also shown to produce anti-nemic compounds in culture. Culture broth was screened for ability to curb egg-hatch and mobility of both SCN and RKN and subsequently flavipin was purified and identified using nuclear magnetic resonance (NMR) spectroscopy to be the active ingredient. This pilot study revealed that the compound had good results *in vitro* while greenhouse assays showed no difference from the control, indicating that bioavailability of this compound in soil environment needs to be investigated (Nitao et al. 2002).

The J2 is the most exposed life stage of the SCN to soil microbes, as they move through soil towards soybean roots to establish a feeding site. Until they molt for the first time and subsequently hatch out of eggs, J2s are protected by the chitinous and glycoprotein rich eggs, which are in turn encased within the melanized SCN female body or cyst (Hirshmann 1981). Nematode cuticle is very similar to animal skin as it is primarily made up of collagen and other glycoproteins, while the

shell of eggs is rich in chitin (Cox et al. 1981; Niblack et al. 2006). Therefore, an egg-parasitic fungus needs to produce chitinases, collagenases, and carboxypeptidases while J2 parasites would primarily have to produce collagenases, other carboxypeptidases and Carbohydrate-Active enZymes (CAZymes) (Larriba et al. 2014; Page et al. 2014). Some predatory fungi also produce small molecule acids that are detrimental to nematodes (Anke et al. 1995; Stadler et al. 1993). Similar tactics are used by egg and J2 parasites as well.

Secreted fungal enzymes with the ability to breakdown cuticle or the outer layer of eggs or antagonize plant-parasitic nematodes also have a role in control of nematodes, including SCN. The first serine protease from a nematode parasitic fungus was identified and reported from *Pochonia rubescens* (Lopez-Llorca 1990). Since then, several extracellular serine proteases from nematode parasitic fungi have been purified and in some cases, the genes involved in their synthesis have been cloned (Yang et al. 2007; Tunlid et al. 2017). Serine proteases can help degrade nematode cuticle, thereby giving fungi access to the nematode body to derive nutrition (Tunlid and Jansson 1991; Zhang et al. 2008; Tunlid et al. 2017). *Hirsutella rhossiliensis* is well known as an obligate SCN J2 parasite (Liu and Chen 2000, 2001). A thermostable serine protease from *H. rhossiliensis* was recently isolated and purified, and was also proven to have nematicidal activity against SCN (Wang et al. 2007). Similarly, upon analysis of the genome of *Purpureocillium lilacinum*, a close relative of *Hirsutella* spp., several CAZymes, 13 polyketide synthase clusters, 10 non-ribosomal peptide synthase clusters, and several other genes or clusters involved in production of secondary metabolites were found, including one responsible for producing the antibiotic leucinostatin (Wang et al. 2016). The proteinase VCP1 from

Pochonia chlamydosporia is shown to be highly host specific as its activity on *Meloidogyne incognita* eggs was significantly better than on those of *Globodera rostochiensis* (Segers et al. 1996). Whole genome sequencing and comparative genomics could also greatly accelerate discoveries of new nematicidal compounds (Prasad et al. 2015).

The first chitinase shown to have activity against nematodes, Chi43, was also purified from the genus *Pochonia* (Tikhonov et al. 2002). As discussed earlier, chitinases help fungi weaken the nematode eggshell but are mainly useful to fungi in terms of hyphal growth. The endochitinase was purified from *V. chlamydosporium* (now *Pochonia chlamydosporia*) and *V. suchalasporium* grown in submerged fermentation in a semi-liquid, colloidal chitin medium for about 20 days. Microscopic evaluations of the effect of this enzyme of PCN, a close relative of SCN, revealed peeling of eggshells post exposure (Tikhonov et al. 2002).

A summary of this metabolite discussion is presented in Table 0-2. Although there are several small molecules, organic acids and enzymes isolated and proven to be antagonistic to SCN in vitro, they must be evaluated in vivo before they could become potential bionematicides. Greenhouse studies on bioavailability and specificity, followed by *in vitro* studies of human and animal toxicity of these compounds are important next steps, and are yet to be reported.

Table 0-2. Specific compounds characterized from common fungi discussed in this chapter.

Fungal origin	**Fungal compound**	**Class of compound**	**References**
Chaetomium sp.	Flavipin	Aryl hydrocarbon	(Nitao et al. 2002)
Fusarium spp.	4-hydroxy benzoic acid	Organic acid	(Bogner et al. 2016)
	Bikaverin	Organic heterocyclic hydrocarbon	(Kwon et al. 2007)

	Fusaric acid	Organic acid	(Kwon et al. 2007)
Lachnum sp.	Linoleic acid	Organic acid	(Anke et al. 1995)
	Serine proteases	Protease enzyme	(Lopez-Llorca 1990; Segers et al. 1996; Wang et al. 2007)
Pochonia spp.	Chitinase	Chitinase enzyme	(Tikhonov et al. 2002)
Purpureocillium sp.	Leucinostatin	CAZ-yme	(Wang et al. 2016)

0.3 Indirect fungal biocontrol through soil amendments

Crop rotation with non-host crops such as corn, and reduced tillage, have been reported as viable methods for SCN control (Melakeberhan et al. 2015). The ability of non-host crops to reduce the nematode population densities stems from the inability of SCN to invade or establish feeding sites in non-hosts and the subsequent starvation of the nematode to death, but also could be due to a change in the soil microbiome through changes in plant root exudates and plant residues. Addition of such exudates and botanical compounds to the soil, therefore, is also a way to promote biological control of SCN or any other nematode (Riga et al. 2001). There are many routes by which organic amendments to soil can potentially be detrimental to SCN, apart from crop rotation (Chitwood 2002; Oka 2010). One of the ways cover crops and non-host rotation could reduce nematode population densities is by favoring nematode antagonistic fungi in the soil environment (Oka 2010). In a recent metabarcoding study observing the mycobiome of a long-term soy-corn rotation experiment, Hu et al. (2018) noted that SCN egg densities (eggs/100 cm^3 of soil) were reduced in fields where soybean was interspersed with 5 years of corn. This was correlated with a change in species richness and rhizosphere beta diversity, possibly explained by the effects of root exudates and crop residues primarily.

Crop rotation with corn will not be favorable economically if corn prices decline considerably while there is no change or a decline in the global demands and prices of soybean. Instead, pre-planting with cover crops such as sunn hemp (*Crotalaria juncea*) have been studied and found effective in lowering nematode inoculum levels, apart from symbiotic relationships with nitrogen fixing bacteria (Wang et al. 2002). In a greenhouse study, sunn hemp was one of the most effective rotation crops in lowering SCN population density (Warnke et al. 2006). *Crotalaria striata* was especially found to be effective in controlling SCN (Valle et al. 1995). Several other cover crops have also been suggested and tested. In recent studies of African indigenous vegetables such as African nightshades (*Solanum villosum* and *S. scabrum*) and African spinach (*Amaranthus dubius* and *A. cruentus*), both field surveys and field trials, the nightshades (non-tuberous Solanaceae plants) significantly reduced the PCN population when used as a cover crop before growing potatoes (Chitambo et al. 2019). In the same study, in addition to the absence of any cysts on the roots of the nightshade, egg viability of cysts isolated from the rhizosphere of nightshade were shown to have reduced viability. In another study exploring cover crops, Brassicaceae such as brown mustard (*Brassica juncea*) and winter canola and winter rapeseed (*B. napus*) and field rye (*Secale cereal*) reduced several important bacterial and fungal soybean diseases including foliar diseases such as septoria leaf blight and root diseases such as sudden death syndrome, indicating the indirect manipulation of the microbial community in that soil (Wen et al. 2017). The study also reported significant reduction of SCN eggs post field rye and rapeseed cover cropping.

Although changing crops and using cover crops changes the phytobiome, there is evidence that monoculture may be more effective at controlling SCN numbers by aiding accumulation of natural fungal antagonists in the soil. When 10% of suppressive soil from three long-term soybean monoculture (5 or more years) fields in China were mixed individually with 90% sterile soils, all three mixtures still retained the ability to suppress SCN compared to controls (Sun and Liu 2000). The same study observed an abundance of *P. lilacinum* in all three suppressive soils. Similarly, the accumulation of *H. rhossiliensis* in these soils was attributed to soil suppression of SCN, from evaluating long-term monoculture soil in potted-plant experiments in a greenhouse in Minnesota (Chen 2007b). An increase in rhizosphere *Purpureocillium lilacinum* and *Pochonia chlamydosporia*, along with some Pseudomonad bacteria, was observed and negatively correlated with SCN numbers in soil from several long-term soybean monoculture plots (Hamid et al. 2017a).

Certain polymers such as chitosan have been shown to affect fungi and plants alike (Lopez-Moya et al. 2019). The nematode egg parasite *Pochonia chlamydosporia* was seen to have increased protease production and appressorial differentiation in the presence of chitosan, thereby being more efficient in parasitizing nematode eggs (Escudero et al. 2016). Similarly, chitin from crustacean shells, a polymer from which chitosan is produced by deacetylation, also increased certain fungal groups such as *Lecanicillium lecanii* and *Geotrichum candidum* when added to soil between 0.5% and 4% (w/w) (Rodríguez-Kábana et al. 1984). The same study also noted an overall increase in the amount of fungi and actinobacteria post chitin amendment. Manure and domestic wastes have also been studied for their ability to control SCN in the field (Bao et al., 2013; Reynolds et al., 1999; Yeates et al., 2006). Digested swine

manure with enriched concentrations of ammonia and volatile fatty acids were tested and found to heavily inhibit SCN egg hatch, as well as be lethal to hatchlings or juveniles, reducing their ability to infect soybean seedlings (Xiao et al. 2008). Similarly, a combination of cow manure and chemical fertilizer or simply the addition of fertilizer alone altered the nematode community structure in an organic continuous soybean monoculture field, reducing the population of parasitic nematodes (Li et al. 2018). A simple explanation for these observations might be the change in the carbon: nitrogen ratio in the soil environment, which further influences the soil microbial community structure. Therefore, one cannot discount the possibility of these amendments directly and positively supporting fungal communities in the soil that are also natural antagonists to SCN, in addition to any possible directly toxic effects on SCN itself.

Although such studies are extremely difficult to accomplish, there have been some studies that have tracked the decrease of nematodes correlated with addition of organic matter to the soil as well as an increase in fungal groups such as *Trichoderma* spp., *Penicillium* spp., and *Aspergillus* spp., which have all been implicated in nematode biocontrol (Hoitink and Boehm 1999; Hoitink et al. 1997) and have been isolated from SCN cysts as detailed in the previous sections. In another study, *P. chlamydosporia* was observed to thrive in fields with increased input of organic matter (Viaene and Abawi 2000). Studies aimed at characterizing how soil amendments alter the soil microbiome coupled with modern *in vitro* culture and molecular analyses of affected nematophagous fungi should enable detailed studies to dissect the major factors contributing to these indirect effects.

Soil is a very complex environment with many interacting partners; these interactions are hence multi-faceted and complex. A good biocontrol agent against SCN should not only be able to parasitize and/or produce toxins *in vitro,* but must also do the same in soil, while overcoming the effects of other microbes present in the soil. Several fungal genera have been routinely isolated from the SCN and have shown potential antagonism. In the absence of effective chemical control methods, screening and selecting good biocontrol agents that can thrive in several soils and effect biocontrol will complement the cultural practices already in place. It is noteworthy that a majority of culturable fungi for the SCN belong to the order Hypocreales and the family Nectriaceae, and their presence in all SCN soybean fields studied indicates possible soybean-SCN-fungal tri-partite interactions and co-evolution between these organisms. It is also evident that these fungi use both parasitism and toxicity as mechanisms to attack SCN. Apart from fungal organisms directly employed for biocontrol, specific compounds produced by these organisms could become future biopesticides. In this multifaceted scheme of interactions, a more thorough understanding of the effects of organic amendments to soil on microbial communities will aid in effective management of these potential biocontrol organisms. Known fungal taxa associated with the SCN are a starting point for future investigations to build on existing knowledge of biocontrol agents and potential bionematicides to develop more effective strategies for control of the SCN.

University of Minnesota has several research and outreach stations across the state of Minnesota. The southern research and outreach station (SROC) is situated in Waseca and is home to a 30-year old soy-corn rotation experiment, with both nematicide treatment and non-nematicide treatment, in a complete randomized block

design. In this dissertation, I have studied SCN cysts from the non-nematicide treated plots, for fungi that are associated with them. I wanted to isolate and identify all the culturable fungi and compare such a collection of fungi (SCN cyst mycobiome) to previous such studies. I further wanted to screen this mycobiome using both *in vitro* and *in vivo* assays to identify fungi that could potentially be developed into commercial biocontrol agents against SCN.

Chapter 1: **CULTURABLE SCN CYST MYCOBIOME**

The idea of biocontrol of nematodes using microbes has generated several studies focused on identification of candidate biocontrol organisms (Chen and Liu, 2005; Kim et al., 2010; Aminuzzaman et al., 2013; Lan et al., 2017). However, only a few microbes have been developed into commercial nematicides in the U.S. market to tackle the SCN (e.g. Poncho Votivo^{TM}, Bayer), and only a handful are derived from fungi (Chen and Dickson, 2012; Anderson, 2014). An increase in market demands for organic food production adds to the importance of developing sustainable and nontoxic solutions such as biological control using fungi for management of the SCN.

With advances in sequencing technologies and biocomputing, it has been possible to identify both culturable and nonculturable fungi within agricultural ecosystems. High-throughput metabarcoding analyses have enabled characterization of the community composition of different agricultural soil environments (Taberlet and Coissac 2012). Hu et al. (2018b), recently published a detailed account of the community structure of fungi within SCN cysts in the same agricultural fields discussed in this study, using metabarcoding of the fungal ITS barcode region. However, metabarcoding studies are subject to multiple biases that can make accurate estimation of the abundance of specific taxa problematic (Amend et al. 2010). A culture-based approach offers an alternative assessment of fungal biodiversity and the relative abundance of specific taxa within agroecosystems and provides living cultures that can be evaluated both *in vitro* and *in vivo* for their biological control potential. Several culture based studies have shown clear

associations between the SCN and several genera of fungi in the cysts or soil (Kerry, 1988; Carris et al., 1989; Chen et al., 1994; Chen and Chen, 2002; Aminuzzaman et al., 2013), which may include parasites, commensals, or opportunistic saprobes of SCN cysts. In fact, some of these fungal genera have shown promising biological control properties against nematodes in single isolate *in vitro* and greenhouse bioassays (Cayrol et al. 1989; Nitao et al. 1999; Shinya, Watanabe, et al. 2008; Shinya, Aiuchi, et al. 2008a). However, to date, there has not been significant broad-scale commercial success of biologicals against nematodes.

Soil is a complex environment and the interactions between potential biocontrol agents and the SCN, as well as their interactions with other microbes living in soil, may be influenced by multiple biotic and abiotic factors. Therefore, candidate biological control organisms tested in axenic or monoxenic conditions, such as *in vitro* or *in vivo* assays, may not successfully translate into biocontrol under field conditions. For a good biological control agent to be effective in the field, it must be able to survive under varying biotic, soil, and climatic conditions. Competition with other soil microbes and the impact of environmental factors is often overlooked while screening for biocontrol agents in controlled environments. Therefore, investigating the natural mycobiome of the SCN cyst over crop rotation in an agricultural system, over multiple years, is an important step towards understanding these complex interactions, and in identification of taxa that can survive and persist amongst other members of the natural mycobiome of the SCN cyst. It is also possible that some of the members isolated from this mycobiome may be tested for their biocontrol potential.

Long-term crop rotation (LTR) plots are extremely useful for studying the effects of plant hosts and various biotic and abiotic aspects of a crop ecosystem, while

minimizing the impacts of the confounding time component of the environment. Hence, this study utilized a long-term soybean-corn rotation system located in southern Minnesota that has been in annual rotation, 5 years of corn-soybean rotation following 5 years monoculture of the alternate crop, or continuous monoculture for over 30 years, to characterize the culturable SCN cyst mycobiome. Previous studies of nematode communities at this site showed that populations of the SCN have steadily declined with increasing years of corn monoculture but increased in plots with increasing years of soybean monoculture (Grabau and Chen 2016; Hu et al. 2018). The main objectives of this study were to: (i) characterize the composition of the culturable mycobiome of SCN cysts across a 3 year period from 2014 to 2016; and (ii) to determine the impacts of crop rotation on the frequency of fungal taxa in SCN cysts, including certain fungi previously described as potential biocontrol agents, across different crop rotation regimes.

1.1 Materials and methods

1.1.1 Experimental system – Long-term crop rotation (LTR) plots

The experimental system consisted of long-term soybean-corn rotation plots on Nicollet clay loam, located at the Southern Research and Outreach Center in Waseca, MN (44°04'N, 93°33'W). The individual plots were 4.572 x 7.62 m in size and planted in 6 rows across the width of the plot. Each crop rotation treatment consisted of four replicate plots that were arranged in a randomized complete block design to minimize factors such as localized soil microenvironments. A detailed report on the experimental system has been previously published (Grabau and Chen 2016a). Although split plots were previously maintained in each replicate plot, one with nematicide and the other without nematicide, only the non-nematicidal treatments were sampled for this study to find

natural fungal antagonists of the SCN. The crop rotations consisted of continuous monoculture, annual rotations and 5-year rotations (Supplemental Figure 1-1). Continuous monoculture of corn (DKC50-82 RIB) and SCN susceptible soybeans (Pioneer P91Y90), denoted as Cc and Ss respectively, have been planted with Roundup ready varieties since the 1990s (when Roundup ready varieties were available); but have been in continuous rotation and monoculture of these crops since 1983. Annual rotations of corn and soybeans are denoted Ca and Sa, respectively. Similarly, each year of the 5-years of cropping soybean following 5 years of corn are denoted S1 through S5 for soybean years 1-5 while each year of corn following 5 years of soybean cropping are denoted C1 to C5 for corn years 1-5 (Supplemental Figure 1-1). The plots were tilled conventionally using a field chisel both in the fall after harvest and in the spring before planting. Corn plots were fertilized with 224.4 kg/ha of nitrogen during planting. As varieties of both corn and soybeans used in the study were Round-up ready, glyphosate (Roundup), herbicide was used to manage post-emergence weeds. Endigo, a mixture of the insecticides thiamethoxam and lambda-cyhalothrinm, was used at 0.245 kg/ha for aphid control.

1.1.2 Sample collection

Because of the very low abundance of SCN cysts in the longer-term corn rotations, only plots from soybean rotations (Sa, S1, S5, and Ss) as well as annual rotation of corn (Ca) were considered for this study. Cysts were collected during mid-season (July) between 47-64 d post planting, and post-harvest during the fall season (October) each year, over a 3 year period from 2014 to 2016 from the four replicate plots of each crop rotation treatment (Supplemental Figure 1-2). Ten soil cores 6 inches deep were sampled from the 2^{nd} and 4^{th} rows of each of the 6-row plots and pooled within each

replicate plot. SCN cysts were extracted by a modified hand-decanting method (Chen et al. 2001). About 4 kg of soil from each plot was soaked separately in water and aggregates were broken up using a stirrer fitted to an electric drill. For the first year of soybean after 5 years of corn (S1), the cyst density was very low, and more soil was needed to be sampled to extract 50 cysts from those plots. The resultant soil suspension was further diluted with a strong jet of water and left undisturbed for 30 s for aggregates and heavy particles to settle, allowing cysts to float to the top. The top layer was then decanted into a nested sieve system of pore sizes 850 µm and 250 µm. This was repeated 5 times. The thick slurry post-decantation was then re-diluted with a water jet and decanted into the sieve system two more times to maximize cyst recovery. Debris collected on the top 850 µm sieve was discarded while the cyst-rich fraction collected on the 250 µm sieve was subjected to dual flotation-centrifugation using a 63% (w/v) sucrose solution (Jenkins, 1964). Cysts from the top layer were further separated from lighter debris manually under a stereoscope.

1.1.3 Fungal isolation from cysts

Fifty SCN cysts were randomly selected from each of four replicates per treatment during both mid and fall (post-harvest) seasons over all 3 years. Thus, there were a total of six sampling time points in the study. Cyst surfaces were sterilized by treating them with 0.5% NaOCl solution for 3 min, followed by rinsing in distilled water. Individual cysts, numbered from 1 through 50, for each plot and replicate, were placed on separate water agar (WA) plates and incubated at 25 °C for 2 weeks. Mycelial plugs of 2 mm in diameter from all WA plates with fungal mycelia growing out of the cyst were individually transferred onto full strength Potato Dextrose Agar (PDA) with 100 ppm streptomycin and 50 ppm chlortetracycline (APDA) and incubated at 25°C for 2 weeks.

Each isolate was given a unique code using year and season code, plot number, and cyst number.

1.1.4 Fungal morphotyping and genus identification

During each sampling time point, pure cultures of fungal isolates obtained from all the cysts from that time point were grown for 2 weeks and grouped into morphogroups, based on similar morphology and pigmentation on APDA. A representative isolate (morphotype) was randomly selected for every five isolates in a morphogroup for molecular identification. Morphotypes were grown on APDA layered with cellophane at 25°C for 2 weeks and fungal mycelia of each representative morphotype were collected and lyophilized overnight. DNA extraction and subsequent amplification of the internal transcribed spacer (ITS) fungal barcode region were carried out using REDExtract-N-Amp^{TM} tissue PCR kit from Sigma-Aldrich using the ITS1F and ITS4 primers (White et al. 1990) according to the manufacturer's protocol. PCR products of about 600 bp were visualized on 1% agarose gel and were sequenced using Sanger sequencing. Sequences were trimmed at both ends to a minimum length of 350 bp, based on signal quality using an in-house python program, dhtrim.py (Byun and Haarith 2018), and genera were identified by searching against the NCBI nucleotide database (nt) using BLAST (Madden, 2013).

1.1.5 Community analysis

All fungal isolates were identified using the best BLAST hit of the sequenced ITS fungal barcode of the representative morphotype(s) of each group. When multiple morphotypes were sequenced from a single morphogroup, all mapped to an identical taxon, confirming that the morphological classifications were consistent with molecular

identification. Thus, members of a morphogroup were assigned to genus based on the identity of their representative morphotype(s). We used the criterion of $\geq$ 97% identity of the BLAST hit to classify morphogroups to the genus level. A few morphogroups whose BLAST hits were <97% identity or whose morphotypes could not be confidently assigned to the genus level were classified as unknown and identified based on their best BLAST hit in the Genbank nt database. A percent composition at the genus level was calculated for each year of sampling, each sampling season, and for every crop sequence.

Alpha diversity of the culturable SCN cyst mycobiome was analyzed using the Shannon-Weaver method (Smith and Wilson 1996). The Shannon Index (H) was estimated using the formula $H = -\sum_{i=1}^{s} p_i \ln(p_i)$ and the evenness (E) was estimated using the formula $E = H/\ln(N)$, where p is the count of the i^{th} fungal genera of s genera in each category (Year, Sampling season, Crop sequences, Replicate Block) and N is the total number of fungal genera detected in the respective category. Both H and E values were compared across sampling time points and crop sequences using ANOVA and Tukey's HSD test.

Since the combined data of percentage relative abundances of all 62 genera and the unknown taxa across 3 years could not be fit to a linear model (Shapiro and Wilk 1965), changes in beta-diversity of fungal communities sampled across crop sequences and season were tested using PERMANOVA (Kelly et al. 2015) and visualized using non-parametric multidimensional scaling (NMDS) based on their Bray-Curtis distances (Kruskal 1964). Because of variation observed between years, PERMANOVA analyses were performed separately for each year of the experiment. All analyses were performed in the R (R Core Team 2013) statistical software using the 'vegan' package (Okansen et al. 2018) and figures were made using the 'ggplot2' package (Wickham 2009a). The

PERMANOVA, implemented using the 'Adonis' function in 'vegan' package, was applied using the non-linear ordination model to study the effects of crop sequences, sampling season (Mid and Fall), and replicate blocks both singly and as interactions separately within each year (Bray-Curtis Distance Matrix ~ Block + Sampling season + Crop sequences + Sampling season*Crop sequences). Since several crop sequences were not adequately sampled in the first year of the study 2014 (Ca and Sa) and one in 2015 (S1) (Supplemental Figure 1-2), these treatments were dropped from analyses in 2014 and 2015, respectively. Significant differences between the variations of samples around the centroids were calculated using the beta-dispersion 'betadisper' function in 'vegan' and a post-hoc Tukey-HSD test was applied to the vectors to assess pairwise differences in beta-diversity. Many studies have shown the risk of losing biological meaning of data by applying log transformations, especially for count data (O'Hara and Kotze 2010; Stroup 2015). Thus, we did not transform our count data except for estimating the relative abundances as a percentage.

The relative abundance of each of the 14 most common genera, individually over the six sampling time points, was normally distributed. Thus, changes in relative abundance of each of these genera was analyzed separately using linear regression analysis followed by an ANOVA and Tukey-HSD tests using the same model as used for PERMANOVA: (Formula: Percentage Composition (relative abundance) ~ Block + Sampling season + Crop sequences + Sampling season*Crop sequences). Tests for correlation between relative abundance of fungal genera with SCN egg density per 100 cc soil in these plots was tested with the Spearman Rank test implemented in the base R package using the cor.test() function (R Core Team 2013).

1.1.6 *Fusarium* spp. diversity analyses using EF1α locus

The elongation factor 1-α (EF1α) locus was amplified from one random isolate from each of the ITS-based clusters identified to the genus *Fusarium*. They were aligned with reference sequences from eight different species complexes obtained from the Fusarium-ID database (O'Donnell et al. 2012) using the Clustal Omega built-in program within BioEdit freeware (Hall 2011). Except for the *F. oxysporum* species complex, all other species complexes were represented by more than one species from the database. The alignment file was then processed using the RAxML program version XSDSE 8.2 (Stamatakis 2014) through the CIPRES online gateway (Miller et al. 2010) to obtain the cladogram file with bootstrap values. The output from CIPRES was then visualized using the online iTOL (interactive Tree Of Life) tool (Letunic and Bork 2006).

1.2 Results

1.2.1 The culturable SCN cyst mycobiome is dominated by *Fusarium* and other Nectriaceae

Of a total of 6,000 SCN cysts that were examined over the 3-year period for fungal growth on WA, most cysts contained fungi (>5,000 isolates in total) that grew out on WA and were subsequently grown in pure culture. Only a single fungus was obtained from a cyst. A total of 72, 300, and 329 morphotypes were isolated during Mid-season sampling time points, and 124, 310 and 500 morphotypes were isolated during the Fall sampling time points of 2014, 2015, and 2016, respectively (Supplemental Figure 1-3). Based on 'blastn' searches of the ITS sequences of representative morphotype(s) from each morphogroup to the NCBI nucleotide database (nt), the fungi isolated were assigned

to 62 known genera, while representatives of 30 morphogroups could not be accurately assigned to the genus level (Supplemental Figures 1-4 and 1-5).

The SCN cyst culturable mycobiome composition was visualized by sampling seasons and crop sequences in each year (Figure 1-1). The 14 most abundant genera constituted about 79% of all fungi cultured from cysts across the three years. These 14 most abundant fungal genera belonged primarily to the class Sordariomycetes (*Clonostachys, Cylindrocarpon, Fusarium, Ilyonectria, Mariannaea, Neonectria*, and *Pochonia*) and Dothideomycetes (*Alternaria, Phoma, Paraphoma, Setophoma* and *Leptosphaeria*), with one Eurotiomycete (*Exophiala*), and one Mortierellomycotina (*Mortierella*). Among the Sordariomycetes, the bulk of the mycobiome was made up of *Fusarium* and closely related genera such as *Ilyonectria, Cylindrocarpon, Mariannaea* and *Neonectria,* which like *Fusarium,* all belong to the single family Nectriaceae within the order Hypocreales (Sung et al., 2007, 2008) (Figure 1-2, Supplemental Table 1-1). Additionally, *Clonostachys* and *Pochonia,* are closely related to families Bionectriaceae and Clavicipitaceae in order Hypocreales in Sordariomycetes. The Dothideomycete genera *Alternaria, Phoma, Paraphoma, Setophoma* and *Leptosphaeria* are also all close relatives within the single order Pleosporales. Apart from unknown morphogroups not mapping with high confidence to genus, *Fusarium* (Avg 28.46%) was always the most abundant taxon, followed by *Ilyonectria* (Avg 11.22%), *Clonostachys* (Avg 4.05%), *Cylindrocarpon* (Avg 5.5%), *Exophiala* (Avg 6.92%), and *Leptosphaeria* (Avg 5.25%) (Figure 1-2).

Similarly, although the rank order of the relative abundance of the other 14 dominant taxa varied across sampling seasons, this same group of 14 taxa were consistently the most common taxa isolated at each sampling point (Supplemental Table

1-1). Morphogroups identified to genus that individually comprised less than 1% of the mycobiome community combined, made up a total of 8.69% of the total mycobiome community and formed a long tail of rare taxa typical of most fungal communities (Shade et al., 2014) (Supplemental Figure 1-4). Several genera that include species previously considered as putative biological control agents against plant pathogenic nematodes, including *Arthrobotrys, Trichoderma, Purpureocillium, Hirsutella* and *Dactylella* (Chen and Dickson 2012; Stirling 2015), were among those genera that each contributed less than 1% of total fungi isolated over the study period (Supplemental Figure 1-4).

Although the SCN cyst mycobiome was dominated by ascomycetes, several basidiomycetes such as *Bjerkendera, Ceratobasidium, Coprinellus,* and *Trametes* were

among these rare taxa (Supplemental Figure 1-4).

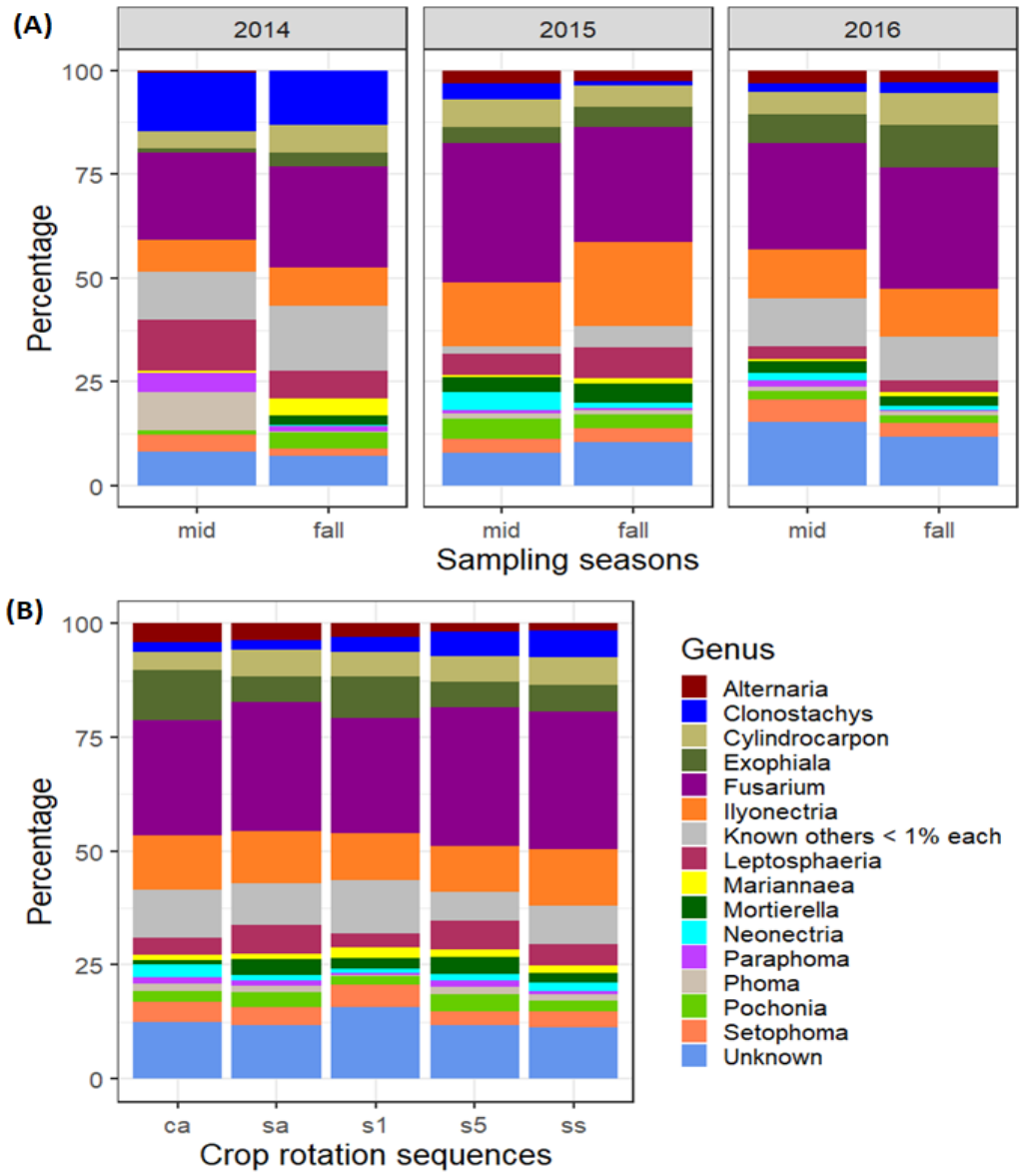


Figure 1-1. Mean relative abundances of fungi in Waseca culturable SCN cyst mycobiome by (A) Sampling Seasons or the six time points of sampling (B) various crop sequences by year (rotation "ca" was not sampled in 2014).

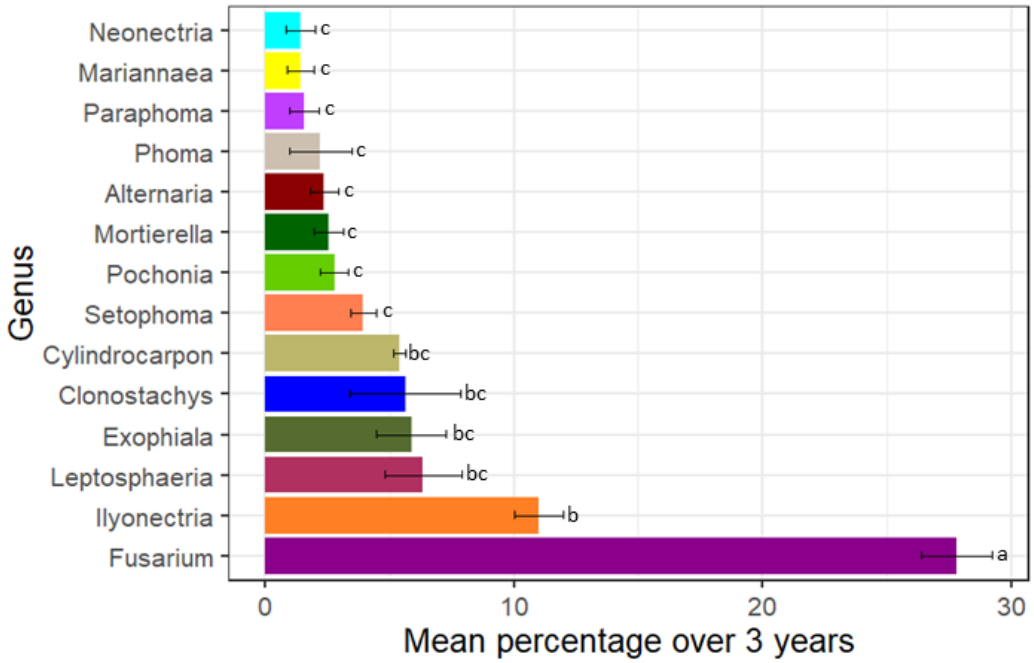


Figure 1-2. Relative abundances of the 14 most frequently isolated fungal genera of the culturable SCN cyst mycobiome, expressed as a percentage of the total fungal isolates collected over the complete three-year period (2014-2016). Letters indicate grouping based on Tukey-HSD on means.

Approximately 10% of isolates that were from morphogroups that could not be confidently identified to the genus level with a BLAST hit of $\geq$ 97% identity. These were assigned to the category of unknown taxa, a majority of which individually comprised less than 1% of total isolates (Supplemental Figure 1-5). Interestingly, among morphotypes that could not be identified to genus, several could only be classified to the kingdom level, potentially representing novel groups of fungal taxa. However, one morphogroup that could not be identified to genus was classified as 'related to Hyalorbilia' in family Orbiliaceae, which contains most of the nematode trapping fungi (Supplemental Figure 1-5). When summed together, those morphotypes not identified to genus contributed roughly 6% of the total 3-year mycobiome.

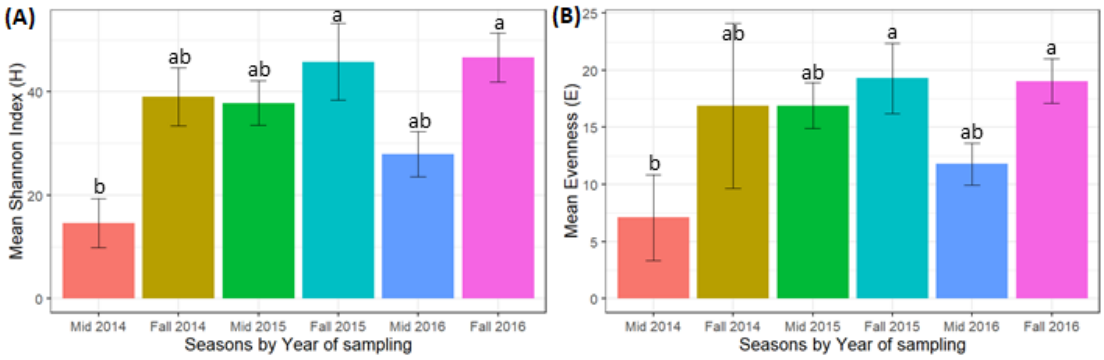


Figure 1-3. Alpha diversity: (A) Shannon index (H) and, (B) Evenness (E) of the culturable SCN cyst mycobiome for each of the six sampling seasons across three years. Letters indicate grouping based on Tukey-HSD on means.

1.2.2 Alpha-diversity across crop sequence and season

The complete mycobiome data at the genus level was used to calculate alpha-diversity. Shannon Index (H) and evenness (E) were calculated for each genus for all sampling points and analyzed by year, sampling season, crop sequence, and plot replicate. The 2014 mid-season sampling had lower H and E values compared to the other five sampling seasons (Figure 1-3).

Both H and E values showed an increasing trend from the first year of soybean (S1) to the fifth year of continuous soybean (S5) after 5 years of corn cropping (Figure 1-4). The S5 plots had the highest H and E values of all crop sequences, while annual corn rotation (Ca) had the lowest values and both annual soybean rotation (Sa) and continuous long-term monoculture of susceptible soybean (Ss) had intermediate values (Figure 1-4). No significant differences in either of these diversity measures were found between the seasons within any given year, nor among plot replicates. However, when fungal diversity was measured as the total number of different genera detected, diversity was higher in the fall seasons than in the mid-seasons (Supplemental Table 1-2).

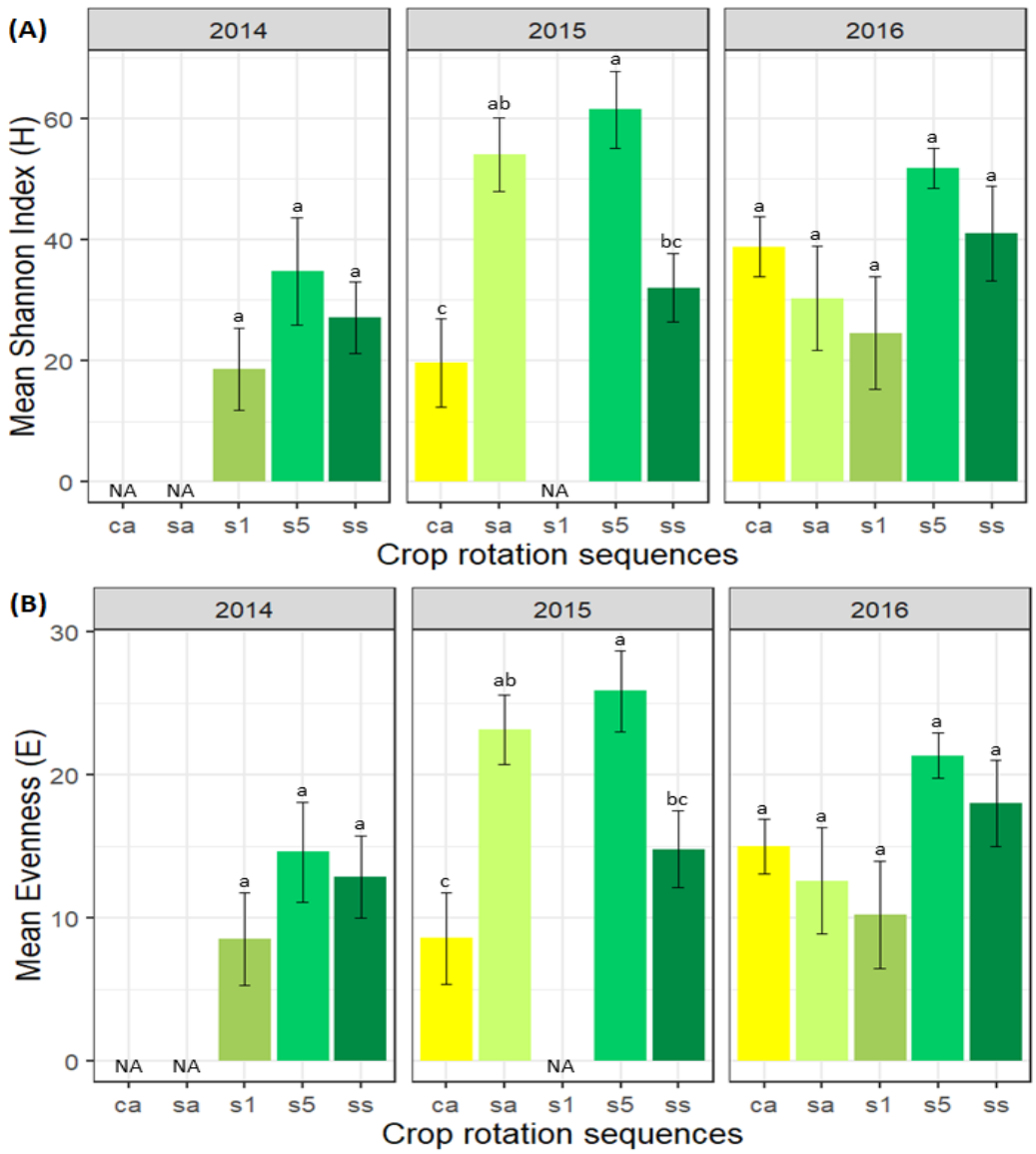


Figure 1-4. Alpha diversity: (A) Shannon index (H) and (B) Evenness (E) of the culturable SCN cyst mycobiome by crop sequences for each year. Letters indicate grouping based on Tukey-HSD on means.

1.2.3 Beta-diversity across crop sequence and season

When communities were analyzed with PERMANOVA for each year, the effect of crop sequences on fungal communities was significant in all 3 years (Table 1-1). Significant seasonal differences in communities between mid and fall seasons, as well as

significant interactions between crop sequences and sampling seasons, were observed only in 2016 (Table 1-1).

Differences in the dispersion or variation between samples within a factor were visualized using nonmetric multidimensional scaling (NMDS) plots (Figures 1-5, 1-6). Across sampling years, 2014 showed much higher dispersion compared to other years (Figure 1-5A). The midseason fungal communities, especially midseason of 2014, were generally more highly dispersed compared to those from the fall season (Figure 1-5B). However, in the PERMANOVA analysis, only beta-dispersion between the mid and fall seasons in 2016 was significantly different (Table 1-4).

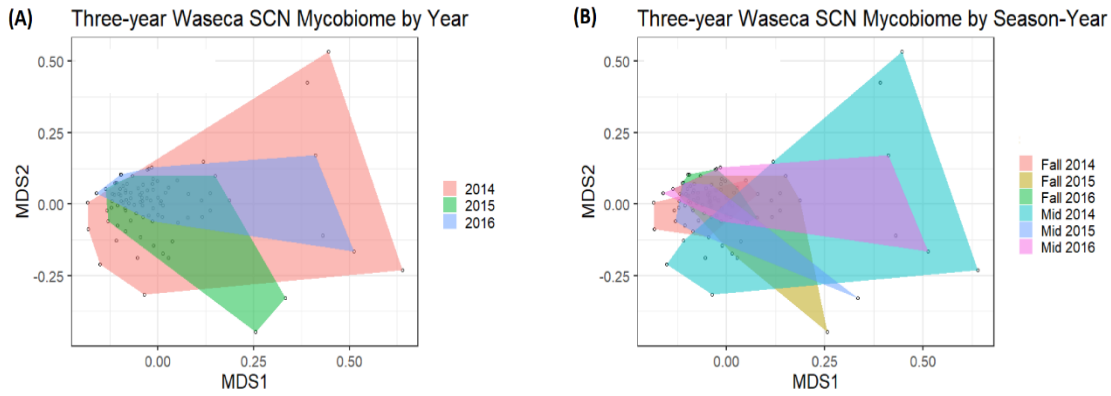


Figure 1-5. NMDS of fungal communities of the Waseca culturable SCN cyst mycobiome by (A) Year of sampling and (B) Sampling seasons over the complete three-year period from 2014 to 2016.

When analyzed across crop sequences, both 2014 and 2016 showed higher dispersion in early years of soybean monoculture (S1) in 2014 and in both S1 and Sa in 2016 than in later years of soybean monoculture (Ss and S5) (Figure 1-6). There was significantly more dispersion in early years of soybean monoculture S1 compared to long-term monoculture (Ss) in 2014, while in 2016, there was significantly more dispersion

between soybean in annual rotation with corn (Sa) compared to the five-year monoculture (S5). In contrast, in 2015 the long-term monoculture (Ss) appeared more dispersed than other soybean treatments (Figure 1-6), but only the Ca treatment showed significantly more dispersion than the Sa treatment (Table 1-2).

*Table 1-1. PERMANOVA of SCN cyst mycobiome data analyzed separately in each of three years (2014-2016). Bray-Curtis Distance Matrix ~ Block + Sampling season + Crop sequences + Sampling season*Crop sequences.*

PERMANOVA for 2014

	Df	Sum of Sq	Mean Sum of Sq	F.model	R sq	Pr(>F)
Blocks	1	0.3266	0.32655	1.89536	0.08395	0.026*
Crop rotation sequences	2	0.5785	0.28924	1.67878	0.14871	0.015*
Seasons	1	0.2329	0.23288	1.35169	0.05987	0.182
Crop rotation sequences*Seasons	2	0.3400	0.16998	0.98658	0.06079	0.480
Residuals	14	2.4121	0.17229		0.62008	
Totals	20	3.8899			1	

PERMANOVA for 2015

	Df	Sum of Sq	Mean Sum of Sq	F.model	R sq	Pr(>F)
Blocks	1	0.0917	0.09166	0.64043	0.02248	0.774
Crop rotation sequences	3	0.4482	0.14942	1.04401	0.10992	0.402
Seasons	1	0.0862	0.08623	0.60253	0.02115	0.769
Crop rotation sequences*Seasons	3	0.4464	0.14879	1.03967	0.10946	0.440
Residuals	21	3.0054	0.14312		0.73700	
Totals	29	4.0780			1	

PERMANOVA for 2016

	Df	Sum of Sq	Mean Sum of Sq	F.model	R sq	Pr(>F)
Blocks	1	0.1395	0.13948	1.1326	0.02528	0.298
Crop rotation sequences	4	0.8878	0.22196	1.8024	0.16093	0.004**
Seasons	1	0.1933	0.19326	1.5694	0.03503	0.098
Crop rotation sequences*Seasons	4	0.8484	0.21209	1.7223	0.15377	0.007**
Residuals	28	3.4481	0.12315		0.62499	
Totals	38	5.5170			1	

Significance indicated as p ≤ 0.01 '*', 0.01 '**', and 0 001 '***'

Table 1-2. Tukey HSD test on Beta-dispersion of SCN cyst mycobiome data analyzed using PERMANOVA individually in each of three years (2014-2016).

Beta-Dispersion

	2014	**2015**	**2016**
Mid - Fall	0.232	0.567	**0.035***
s5-s1	0.060	NA	0.21
sa-s1	NA	NA	0.974
ss-s1	**0.021***	NA	0.854
sa-s5	NA	0.996	**0.049***
ss-s5	0.866	0.105	0.736
ss-sa	0.585	0.066	0.473
s1-ca	NA	NA	0.61
s5-ca	NA	0.595	0.936
sa-ca	NA	**0.038***	0.24
ss-ca	NA	0.968	0.991

Significance indicated as p ≤ 0.01 '*', 0.01 '**', and 0001 '***'

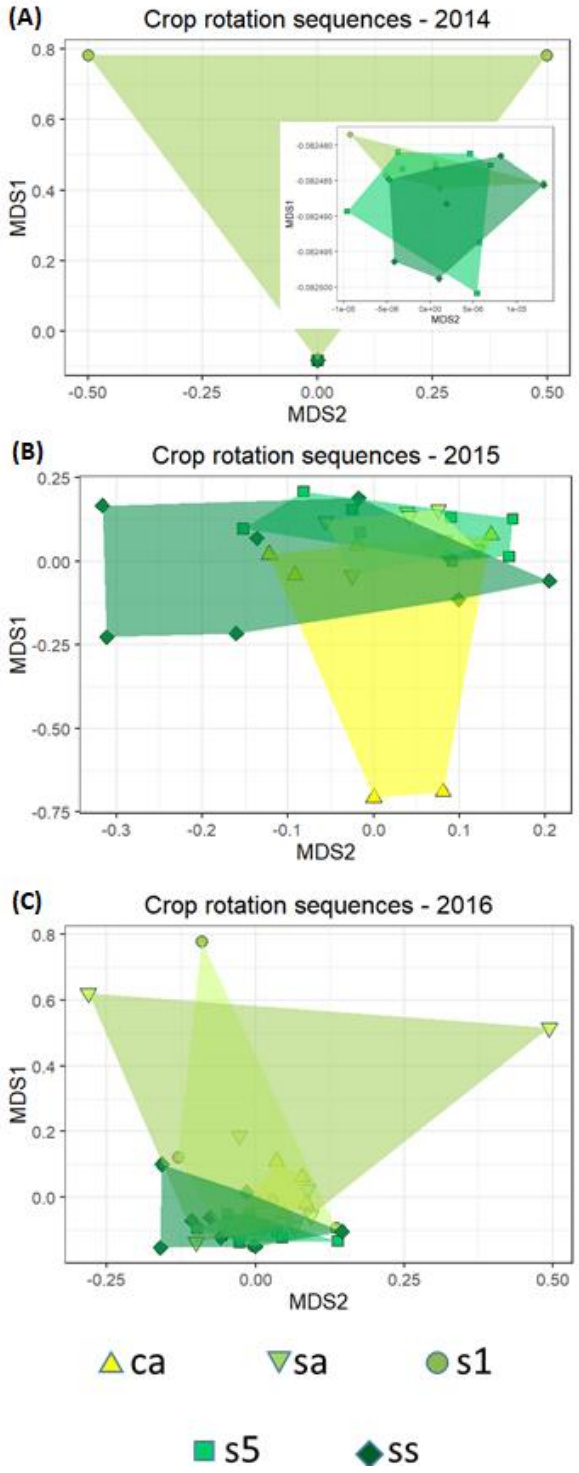


Figure 1-6. NMDS of fungal communities' analyses of the Waseca culturable SCN cyst mycobiome by various crop rotation sequences from which they were sampled, for each of three years (A) 2014 (B) 2015 (C) 2016. High dispersion of different samples from the S1 crop rotation treatment in 2014 reduced separation between other treatments to a single point (bottom point of S1 triangle). The inset shows the relationships between the other treatments (S5 and Ss) at a smaller scale.

1.2.4 *Fusarium* spp. diversity: EF1α

A genetic relationship cladogram was constructed (Figure 1-7) based on the EF1α sequences with reference sequences from the FUSARIUM-ID database. The majority of the isolates were either in the *Fusarium solani* species complex (FSSC) or the *F. oxysporum* species complex (FOSC). Of the 36 total cluster representatives, 18 clustered with the *F. solani* reference NRRL 45880 strain and 10 clustered with *F. oxysporum* reference NRRL 34936. Four additional clusters with *F. nisikadoi* reference NRRL 25179 and *F. commune* NRRL 28387 in the *Fusarium nisikadoi* species complex were observed. A total of three representatives clusters in the *Fusarium tricinctum* species complex (FTSC) along with *F. avenaceum* JN811693.1 and *F. acuminatum* JN811692.1 were found. Only one isolate clustered in the *Fusarium sambucinum* species complex. No isolates were found clustering in *F. dimerum*, *F. fujikuroi* or *F. equiseti* species complexes.

1.3 Discussion

Several previous studies conducted in various parts of the US have investigated the fungi associated with the SCN cyst (Winstead and Skotland, 1955; Morgan-Jones et al., 1981; Gintis et al., 1983; Carris et al., 1989; Chen and Chen, 2002;). However, this is the first study to systematically address the diversity of fungi in the culturable SCN cyst mycobiome across multiple crop sequences in a long-term crop rotation experiment using both culturing and an ITS fungal barcoding approach. The LTR system in Waseca, southern Minnesota was set up to study various aspects associated with the soybean-corn crop rotation system, the most common crop rotation system in the USA and many other soybean production areas globally. In our study, 14 fungal genera comprised the majority

of fungi isolated from SCN cysts, each individually representing greater than 1% and collectively greater than 79% of all fungi isolated in this study (Figure 1-2).

When analyzing the relative abundance of each of these 14 genera individually for each year across crop rotation treatments using a linear model, the genus *Fusarium* was consistently the most commonly isolated genera from SCN cysts across all sampling time points and crop sequences, followed by *Ilyonectria*, *Leptosphaeria*, *Clonostachys, Exophiala,* and *Cylindrocarpon* (Figures 1-1, 1-2). In previous studies conducted in various other soybean growing states in the United States, *Fusarium* and closely related genera in Nectriaceae contributed anywhere between 30-60% of fungi cultured from SCN cysts (Morgan-Jones et al. 1981; Carris et al. 1989; Chen et al. 1994). Some previous morphological studies were unable to classify some fungi to genus and species due to the presence of sterile somatomorphs that could not be identified due to lack of spore production (Gintis et al. 1983). In our study, *Fusarium* contributed 28.46% of all fungi cultured across 3 years.

However, when *Fusarium* was combined with other closely related taxa within Nectriaceae, such as *Ilyonectria, Mariannaea*, *Cylindrocarpon,* and *Neonectria*, together these comprised an average of 48.21% of the total mycobiome, consistent with these previous studies. *Cylindrocarpon sensu lato* has been described previously as a potential nematode egg parasitic genus (Rodriguez-Kabana and Morgan-Jones 1988; Dackman 1990; Chen and Chen 2002). Although we recognize that *Cylindrocarpon* is now considered paraphyletic, with different clades falling within or sister to *Neonectria* and *Ilyonectria* (Chaverri et al. 2011a), all known *Cylindrocarpon* isolates belong within the Nectriaceae.

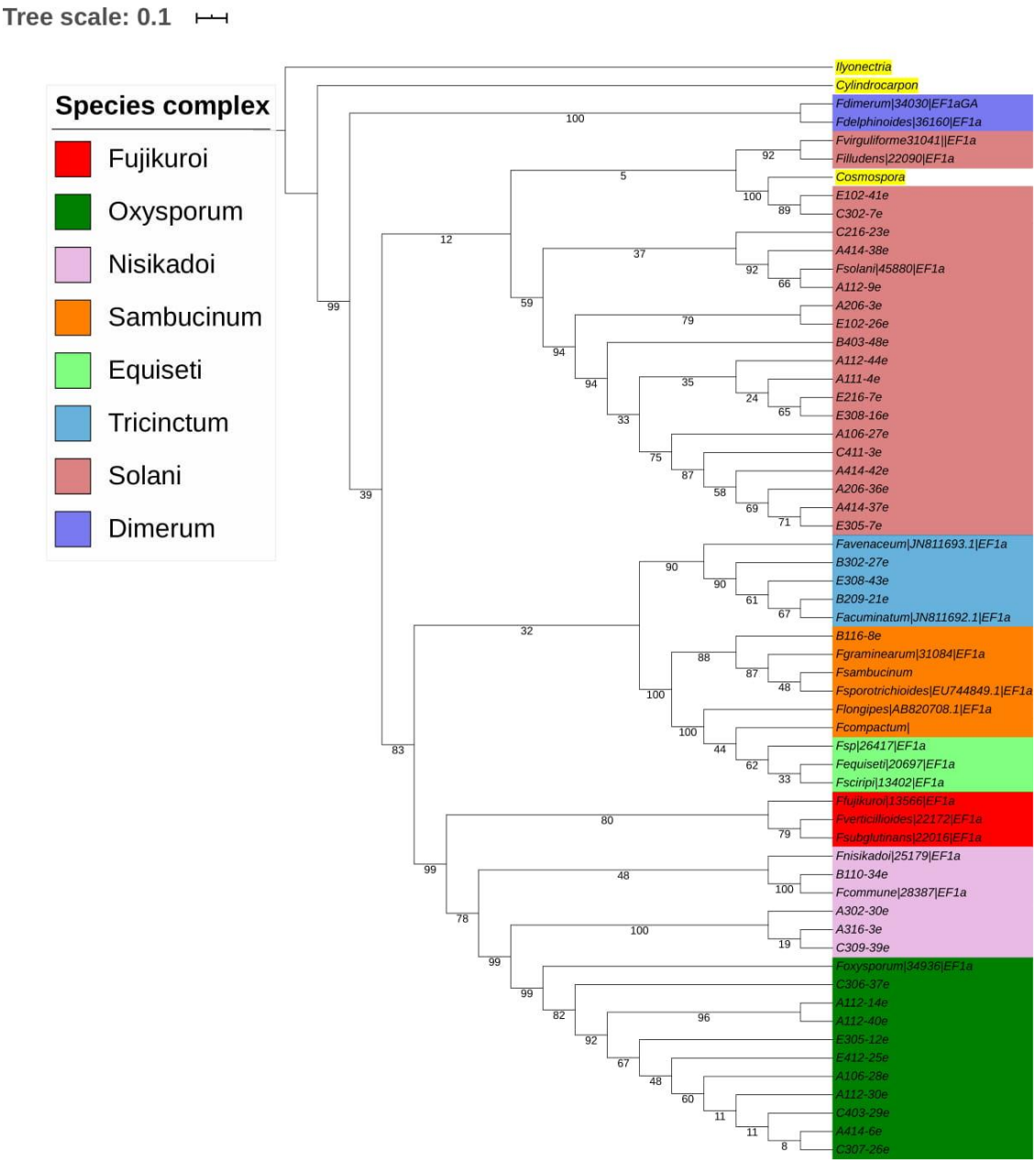


Figure 1-7. Cladogram showing relationships of different Fusarium species cluster representatives, clustering with different reference sequences belonging to different species complexes. Bootstrap values are shown on each branch.

Table 1-3. Tukey HSD analyses on Crop sequences for the ANOVA performed on the most frequent 14 genera, analyzed individually for each year individually. Note that 'ca' and 'sa' in 2014 and 's1' in 2015 were excluded because of limited sampling of these crop sequences.

Genus	2014			2015						2016									
	s1-s5	s1-ss	s5-ss	ca-sa	ca-s5	ca-ss	sa-s5	sa-ss	s5-ss	ca-sa	ca-s1	ca-s5	ca-ss	sa-s1	sa-s5	sa-ss	s1-s5	s1-ss	s5-ss
Alternaria	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-
Clonostachys	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	*	-	-
Cylindrocarpon	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Exophiala	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ilyonectria	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Leptosphaeria	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mariannaea	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mortierella	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neonectria	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*
Paraphoma	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phoma	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pochonia	-	-	-	-	-	-	-	-	-	*	-	-	-	*	*	-	-	-	-
Setophoma	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Significance indicated as p ≤ 0.01 '*', 0.01 '**', and 0 001 '***'

Even though there was a significant reduction in relative abundance when rotated with corn, *Fusarium* was consistently the most abundant genera in samples from all crop rotation treatments and sampling time points. Further investigation using multi-locus sequence analysis (MLSA) is required to determine species level classification for taxa within the highly diverse genus *Fusarium* (O'Donnell et al. 2012).

Although the rank of relative abundance of the 14 most abundant taxa varied across the six sampling points (Figure 1-1, Supplemental Table 1-1), *Fusarium* was always the most abundant and the other 13 taxa were always ranked among the most common 14 genera at all sampling points (Supplemental Table 1-1). The structure of the fungal mycobiome isolated from cysts that is dominated by a few abundant taxa resembles, in some respects, that of microbial communities from other specialized environments such as human skin, where a few bacterial taxa dominate the community in high relative abundance, with variation among less abundant taxa (Hulcr et al. 2012). Previous studies have noted that the fungi able to colonize the specialized environment of nematode cysts are likely a highly filtered and restricted set compared to those found in bulk soils, which may result in a similar mycoflora inhabiting cysts from different geographic and soil environments (Morgan-Jones et al. 1981).

Fungi colonizing SCN cysts include both opportunistic saprotrophs feeding on cyst contents as well as true parasites of eggs and SCN larvae that require specialized enzymes such as proteases, chitinases, and collagenases to penetrate and digest the SCN cyst wall, nematode egg shells and cuticle (Mutua et al., 2012; Lai et al., 2014). Previous studies of microbial communities inhabiting other highly specialized environments, such as human skin, have also show greater phylogenetic relatedness than would be expected by chance among the dominant members of the community (Hulcr et al. 2012). This

pattern may result from the ability to colonize such a specialized environment having evolved as a shared trait among closely related taxa (Costello et al. 2009; Hulcr et al. 2012). We observed that fungal communities isolated from cysts in our long-term rotations experiments were consistently dominated by the same set of 14 abundant genera, of which 5 of the 14 belong to the family Nectriaceae (*Cylindrocarpon, Fusarium, Ilyonectria, Mariannaea, Neonectria*) and an additional two genera belong to closely related families in Bionectriaceae (*Clonostachys*) and Clavicipitaceae (*Pochonia*), all within the single Sordariomycete order Hypocreales. Similarly, five of the 14 taxa were closely related genera within the single Dothideomycete order Pleosporales (*Alternaria, Phoma, Paraphoma, Setophoma* and *Leptosphaeria)*. Thus, our data suggest at least two independent origins in fungi of the ability to colonize the specialized microenvironment of the SCN cyst in Sordariomycetes and Dothideomycetes, leading to higher than expected phylogenetic relatedness of taxa that share this trait.

Long-term monoculture of crops has been suggested to contribute to the development of suppressive soils through an increase in the abundance of microbes antagonistic to host-specific plant pathogens (Weller et al. 2002; Mazzola 2004; Schlatter et al. 2017), including fungal antagonists of plant parasitic nematodes (Kerry et al. 1982; Chen et al. 2001; Chen 2007; da Silva et al. 2018). In contrast, crop rotation with a non-host crop species may also help to control the SCN pathogen by reducing its abundance under a nonhost rotation crop (Chen et al. 2001; Grabau and Chen 2016a, 2016b; Cheng et al. 2018). However, how crop rotation and continuous monoculture in SCN infested fields affects entire fungal communities, which include both saprobic and parasitic taxa, in bulk soil (Song et al. 2016) and rhizosphere soil (Hamid et al. 2017b) especially in the

microenvironment of the SCN cyst (Hu et al. 2017, 2018), has not been well characterized.

Some nematode trapping and endoparasitic fungi of the juvenile stage 2 (J2), such as *Hirsutella minnesotensis*, have host-density dependent population dynamics, in which populations of the nematode parasite and parasitism rates increase in response to increasing host abundance (Jaffee et al. 1992 1993). However, many egg-parasitic fungi also possess saprotrophic ability (Rodriguez-Kabana and Morgan-Jones 1988) or may colonize the rhizosphere or plant roots as endophytes (Zavala-Gonzalez et al. 2015). Thus, they may not depend as fully on the nematode host for nutrition and could demonstrate a weaker correlation with the density of their hosts, as has been observed for nematode trapping fungi that possess greater saprotrophic ability (Persmark et al. 1996). Chen and Chen (2002a), however, showed that the frequency of several fungi colonizing cysts, and the egg-parasitic index of cysts, were positively correlated with egg-density across multiple fields in southern Minnesota, suggesting some degree of correlation between egg-parasites and density of SCN eggs. Similarly, Bourne and Kerry (1998) observed that the egg parasite *Verticillium* (Syn: *Pochonia*) *chlamydosporia*, followed a density-dependent relationship with abundance of several root-knot nematode species, but found that a minimum nematode density was required to increase growth of *P. chlamydosporia* in the rhizosphere. This study, as well as previous research in these research plots, has shown increased density of SCN eggs in longer term soybean monoculture (Grabau and Chen 2016a, 2016b; Hu et al. 2018b) (Supplemental Table 1-6). However, only two genera of egg-colonizing fungi, *Ilyonectria* and *Neonectria* within Nectriaceae, were found to be positively correlated with SCN egg density at the single sampling time point of midseason of 2016 (Supplemental Table 1-3). Egg-densities in

these fields were very low (<3000 eggs/100 cc soil) during the study period (2014-2016), and differences in egg-densities between crop sequences were small (Hu et al. 2018b). It is possible that low egg-densities during the study period may have impacted our ability to detect significant differences in relative abundance of fungal egg parasites resulting from density-dependence effects. Previous research at this site has suggested that the relative abundance of the more obligate endoparasite of J2 worms, *Hirsutella rhossiliensis*, varied significantly with crop sequences and may contribute more to control of populations of the SCN in these fields (Chen et al. 2001). Most *Hirsutella* species are not easily culturable and were detected in very low abundance in our study (<1%) (Supplemental Figure 1-4).

The fungi isolated from cysts include both opportunistic saprobes which feed on the cyst contents as well as true egg-parasites. Among the major 14 contributors to the SCN cyst mycobiome in our study, several genera have been previously identified as containing species capable of directly parasitizing SCN eggs or producing compounds that may kill nematode larvae within eggs (*Fusarium, Leptosphaeria, Exophiala, Clonostachys, Cylindrocarpon, Setophoma, Pochonia, Mortierella, Phoma, Paraphoma*) (Chen et al. 1996; Chen and Chen 2003; Meyer et al. 2004; Manzanilla-Lopez et al. 2013). Two genera, *Clonostachys* (Zou et al. 2010) and *Pochonia, P. chlamydosporia* (Kerry et al. 1993; Kerry and Crump 1998; Mauchline et al. 2004; Manzanilla-Lopez et al. 2013) in particular, have been studied for their potential biocontrol of nematodes. When analyzing the relative abundances of each of the most abundant 14 genera individually for each year, using a linear model, several of these egg-parasitic taxa (*Fusarium, Leptosphaeria, Clonostachys,* and *Pochonia*), as well as two additional genera (*Alternaria* and *Neonectria),* differed significantly by crop sequence (Table 1-5).

However, several other putative egg-parasites (*Cylindrocarpon and Exophiala)* were not significantly affected by crop sequence (Table 1-5). Among the two candidate biocontrol SCN egg parasites, *Pochonia* showed a trend of increasing abundance with increasing years of soybean monoculture in 2015 and significant differences between the S1 and S5 crop sequences in 2016, while *Clonostachys* showed a trend of increasing abundance with increasing years of soybean monoculture in 2014 and significant differences between the S1 and S5 crop sequences in 2016 (Table 1-6, Supplemental Figure 1-7). However, these patterns were not consistent across all years and neither species were significantly positively correlated with SCN egg density (Supplemental Table 1-3). Overall, our results do not support a strong effect of crop rotation on relative abundance of fungal egg-parasites and are similar to results of a study of a 7-year tillage-cropping experiment that showed little effect on rates of fungi parasitizing SCN eggs (Bernard et al. 1996).

Seasonal variation was also observed for some taxa in our study. While much of the variation observed in 2014 may have resulted from limited sampling in midseason of 2014, *Clonostachys* (Bionectriaceae) differed significantly in relative abundance in midseason of 2015, and *Exophiala* (Chaetothyriomycetes) showed significantly different relative abundance in fall of 2016 (Table 1-5). In a previous metabarcode analysis of fungal cyst communities from these same rotation fields (Hu et al. 2018b), a greater abundance of taxa within Nectriaceae was observed at midseason. Several factors could contribute to this trend. As SCN egg-density was also higher at midseason (Hu et al. 2018b), these results could reflect an increase due to higher abundance of the nematode host. However, our study did not support a strong relationship between fungal relative abundance and SCN egg-density.

Alternatively, these results may suggest that the soybean plant has a role in promoting growth of cyst colonizing fungi that associate with the plant root or rhizosphere. Many host plants recruit specific microbial taxa to the rhizosphere or root environment through production of root exudates and other signals (Berendsen et al. 2012; Lahrmann et al. 2013; Philippot et al. 2013). At midseason, soybean plants are actively growing and secreting root exudates, which may promote diversity and proliferation of root and rhizosphere associated fungi (Broeckling et al. 2008). Carris et al. (1989) suggested that root endophytes may colonize cyst nematode females and cysts while still on the root or in the nearby rhizosphere soil. *Fusarium* are common root endophytes and pathogens (Naseri and Mousavi 2015; Ranzi et al. 2017), while several other members of Nectriaceae are potential pathogens of soybean roots (Naito et al. 1993). Additionally, other groups of egg-parasitic taxa (e.g. *P. chlamydosporia*) (Bourne and Kerry 1998; Zavala-Gonzalez et al. 2015) as well as some nematode trapping fungi (Bordallo et al. 2002), have also been shown to colonize plant roots endophytically or to proliferate in the rhizosphere. Other authors have suggested that members of *Fusarium* may be early opportunistic colonizers of SCN cysts that can feed saprotrophically on the cyst contents and endocuticle (Morgan-Jones et al. 1981). Colonization of young females and cysts by fungi inhabiting the root and rhizosphere environment is one possible explanation for the dominance of *Fusarium* and other Nectriaceae fungi in our cyst mycobiome.

Analyses of alpha-diversity using the Shannon index showed that both richness (H) and evenness (E) in diversity were the lowest in 2014, especially at midseason, while diversity at both time points in 2015 and 2016 were slightly higher but not significantly different from each other. We also observed substantial differences in variation among

the most abundant 14 taxa in 2014, and it is likely that a difference in sampling effort during the first sampling point in midseason 2014 contributed to the lower diversity observed in 2014. Fewer cysts and hence fewer fungi were obtained and analyzed from midseason of 2014 (Supplemental Figure 1-3).

However, fungal diversity when measured as number of different genera detected was generally higher in the fall season than in the midseason (Supplemental Table 1-2). Variation in both abiotic factors, such as temperature, as well as biotic factors such as plant root exudates and changes in soil nutrients over the course of the growing season, are likely factors influencing this increase in diversity. In addition to seasonal variation and growth and development of the plant, the increase in diversity of fungi within cysts in the fall (Supplemental Table 1-2) could also be due to an increase in numbers of cysts produced on the soybean roots over the growing season. It is currently unknown whether cysts are colonized primarily by root inhabiting fungi or by soil fungi. As discussed, as the soybean plants mature over the growing season, secretion of root exudates may promote increased diversity of fungi in the root and rhizosphere environments (Broeckling et al. 2008), thus increasing the diversity of fungi available to colonize cysts. However, once cysts are dislodged from roots, they may also persist in the soil. If fungi from soil are the primary source of inoculum for cysts, it is also possible that the increase in the diversity of fungi colonizing cysts observed later in the season is simply due to increased likelihood of colonization by fungal colonists with increasing age of the cysts. There is also evidence that once a fungus colonizes a cyst, other fungal species may be excluded by priority effects through competition (Chen and Chen 2003), supported in our study by isolation of only a single fungal strain from cysts. Priority effects and exclusion

of other fungi through competition could also increase species diversity over the growing season and contribute to the slightly increased diversity observed in the fall season.

Both indices of Shannon diversity were also higher in longer years of soybean monoculture (S5 and Ss) compared to early years of soybean monoculture (S1) and annual rotation with corn (Ca), although these differences were only significant in 2015 (Figure 1-4). Carris et al. (1989) reported a higher diversity and a more stable fungal community in a field suppressive towards SCN compared to a field with higher levels of egg-density. Our results showed the highest levels of diversity after 5 years of soybean monoculture (S5) and intermediate levels of diversity in the long-term soybean monoculture (Figure 1-4).

Differences in sampling during 2014 could have contributed to the greater dispersion and variation observed in fungal community composition in 2014 visualized by NMDS (Figure 1-4). Across the six sampling time points, the midseason appeared more variable than the fall season in 2014 and 2016, but not 2015, on the NMDS plot (Figure 1-5). The physiological stage of the soybean plant and increased production of root exudates discussed above may have contributed to the increased dispersion observed at midseason.

Another important factor that could have influenced the fungal community composition or β-diversity is crop sequence. Because of the differences observed in community composition across years in the NMDS plots (Figure 1-5), differences across crop sequences and season were analyzed separately for each year using PERMANOVA. Crop sequences significantly impacted community composition or β-diversity in all three years, while sampling season significantly influenced community composition and

interacted with crop sequences only in 2016 (Table 1-3). In both 2014 and 2016, the fungal communities in the first year of soybean monoculture (S1) or annual rotation (Sa) were the most variable, while later years of soybean (S5 and Ss) were less variable (Figure 1-4), with significant differences in beta-dispersion between S1 and Ss in 2014 and Sa and S5 in 2016 (Figure 1-6, Table 1-5). Greater diversity in S1 and Sa crop sequences is most likely explained by crop rotation, which may create a more diverse community by supporting both fungi that proliferate under corn as well as those that proliferate under soybean compared to monoculture of each crop (Broeckling et al. 2008; Zhou et al. 2017).

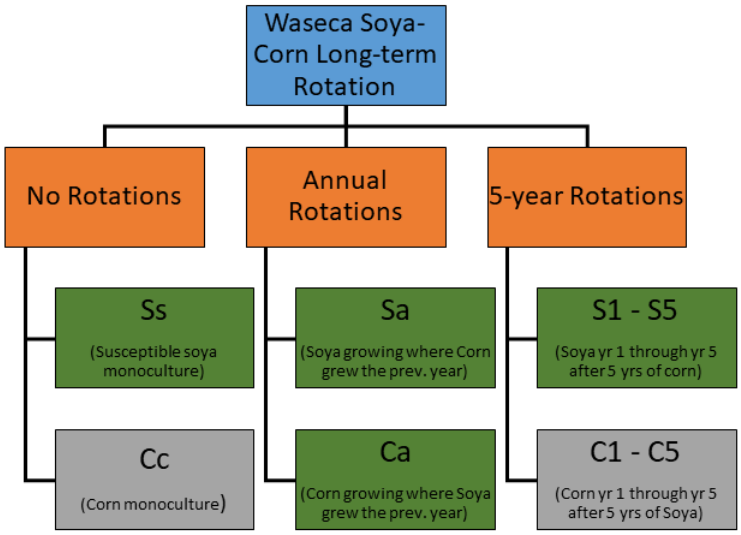
Previous studies suggest that EF1α is a better locus to identify *Fusarium* isolates to species level, especially from soil, than the ITS region, which is commonly considered as the barcode for fungal identification (Zhang et al. 2006). However, multi-locus sequence alignment techniques are the most reliable and preferred for *Fusarium* spp. identification (O'Donnell et al. 2012). Using reference sequences from the Fusarium ID database to identify isolates to the species complex level found that most isolates belonged to the 'oxysporum' or 'solani' clade. It is noteworthy that none of the isolates aligned with *F. virguliforme* given that many studies seem to suggest SCN-SDS interactions (McLean and Lawrence 1993; Gao et al. 2006; Marburger et al. 2014).

1.4 Conclusions

The majority of the culturable mycobiome associated with SCN cysts from a long-term corn-soy crop rotation experiment were consistently comprised of 14 highly abundant genera. *Fusarium* was the most abundant taxon recovered from SCN cysts during all six sampling seasons between 2014 and 2016. Although there was variation in

the relative abundance of these 14 taxa at different sampling points and across crop rotations, we did not observe strong effects of crop sequences or SCN egg density on the relative abundance of these genera, and the same 14 genera were consistently the most frequently isolated in all samples. These findings support the hypothesis that only a limited number of fungal taxa are able to colonize and grow within the specialized environment of the SCN cyst. We found that a majority of the 14 highly abundant taxa represented closely related genera within the family Nectriaceae or families closely related to Nectriaceae within the single order Hypocreales (Sordariomycetes) or within the single order Pleosporales (Dothideomycetes). These findings suggest the evolution of the ability to colonize the SCN cyst in at least two orders of fungi, leading to higher than expected phylogenetic relatedness due to a shared adaptation among these closely related taxa. Previous studies of fungal communities in SCN cysts using morphological methods show that *Fusarium* and closely related fungi in Nectriaceae comprise anywhere from 30-60% of the SCN cyst mycobiome and agrees well with the frequency detected in our results (48.21 %). This study furthers our understanding of the fungi colonizing the SCN cysts, as well as some of the biotic factors influencing these communities, but both *in vitro* and *in vivo* testing of representative members from all these taxa is crucial to determine their potential for biological control and ecological roles in this specialized environmental niche.

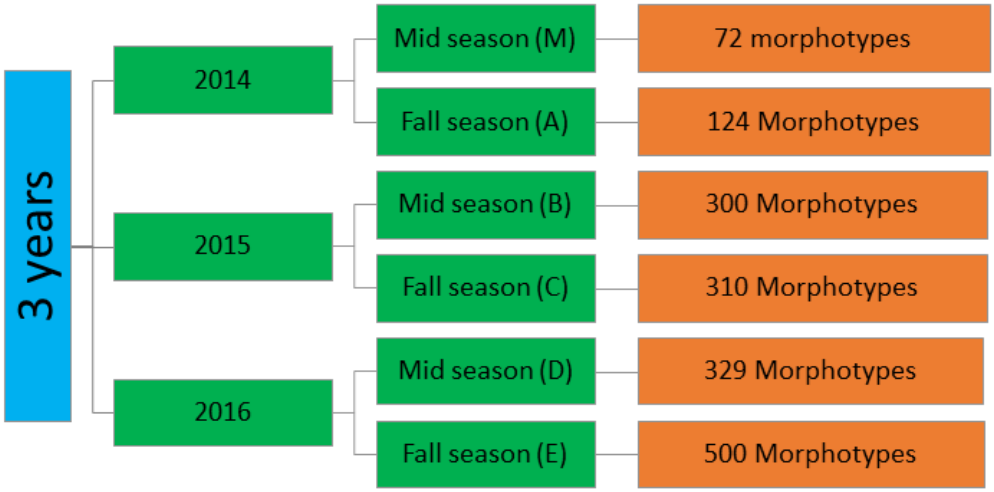
1.5 Supplementary Figures



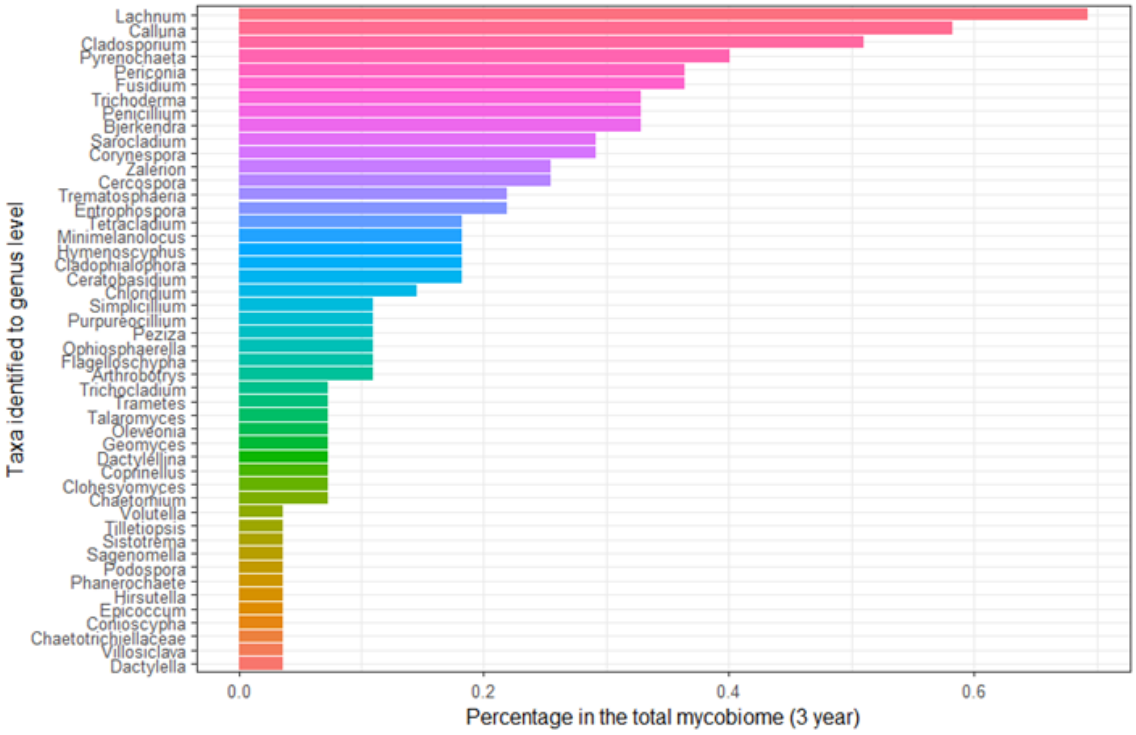
Supplemental Figure 1-1. Crop sequences in Waseca Soya-Corn Long-term Rotation system. Green colored rotations associated with soya bean were considered for this study.

Sampling time point	2014 Mid	2014 Fall	2015 Mid	2015 Fall	2016 Mid	2016 Fall
Sampling code	M	A	B	C	D	E
	Plot numbers from which Fungi were obtained					
	No Sa Fungi	105	No S1 Fungi	104	101	101
	106	106	No Ca Fungi	105	102	102
	111	111	106	106	105	105
	112	112	110	110	106	106
	No Ca Fungi	No Ca Fungi	116	116	116	116
	205	205	209	209	204	204
	206	206	210	210	209	209
	209	209	211	211	211	211
	216	216	No S1 Fungi	214	215	215
	No Ca Fungi	No Ca Fungi	216	216	216	216
	302	302	302	302	302	302
	303	303	303	303	303	303
	314	314	306	306	305	305
Crop Rotation Key	316	316	307	307	307	307
Ss	No Ca Fungi	No Ca Fungi	No S1 Fungi	309	308	308
S1	No Ss Fungi	406	403	403	403	403
S5	No Sa Fungi	407	No S1 Fungi	405	406	406
Sa	No S1 Fungi	409	406	406	407	407
Ca	No S5 Fungi	414	407	407	412	412
	No Ca Fungi	No Ca Fungi	411	411	413	413

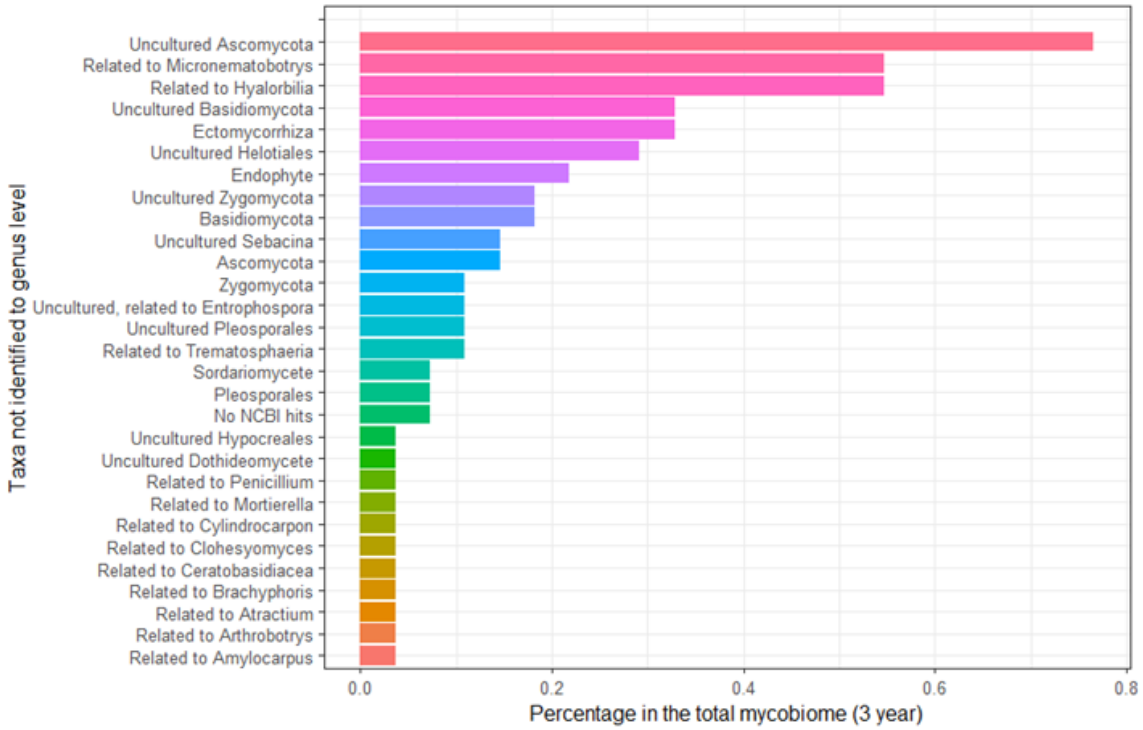
Supplementary Figure 1-2. Cyst sampling map showing plots sampled, their rotations and codes for each sampling time point. Only 3 replicates were sampled during "M" or midseason of 2014. No "Ca" rotation plots were sampled in 2014, during both seasons. No cysts were found in "S1" rotation plots during "B" or 2015 midseason, after 5 years of Corn grown on that plot.



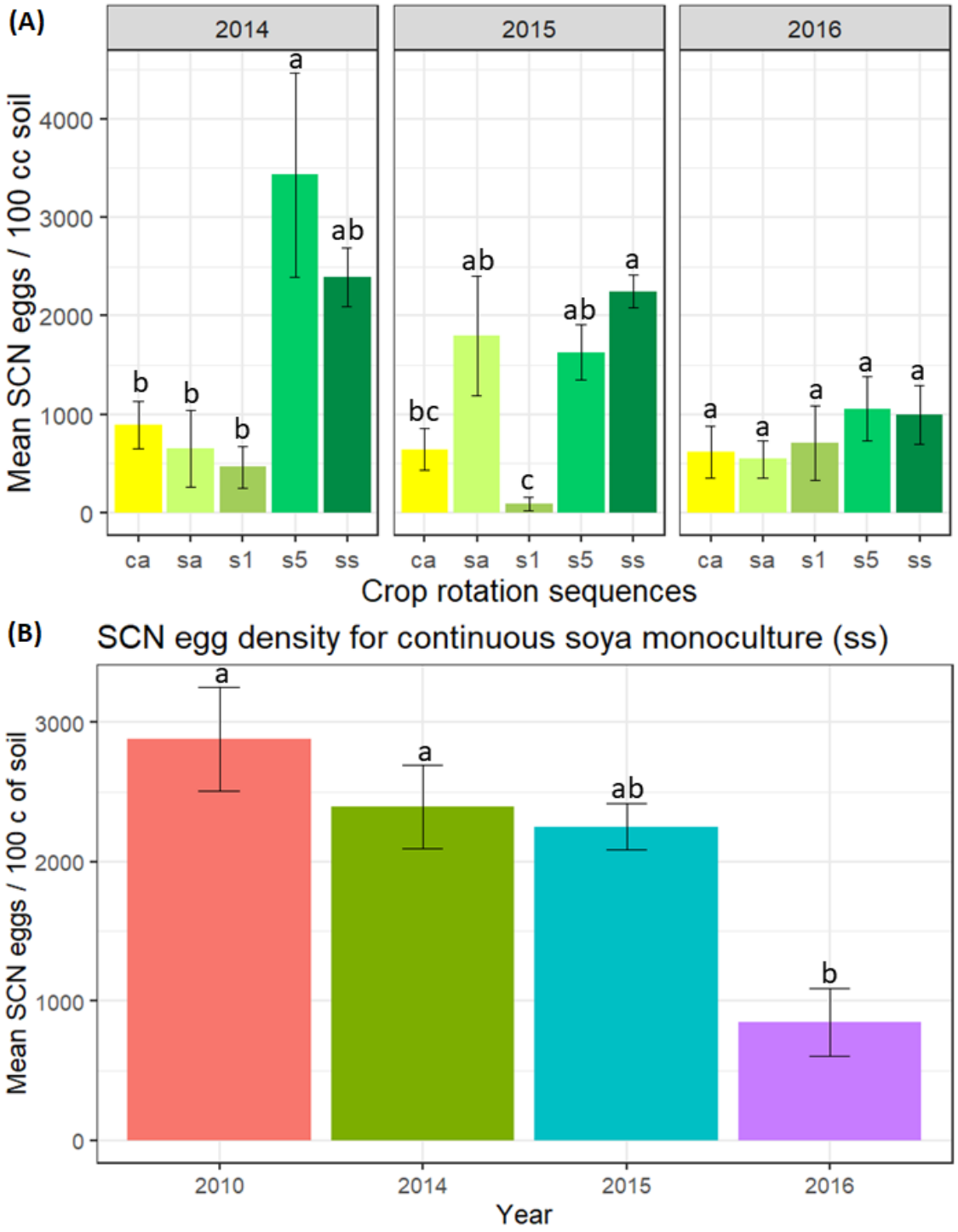
Supplementary Figure 1-3. Fungal morphotypes of the culturable SCN cyst mycobiome by sampling seasons, over the 3-year period (2014-2016).



Supplementary Figure 1-4. Average relative abundance of morphotypes identified to genera with BLAST % identity >97% that each contributed less than 1% to the total 3-year mycobiome dataset.



Supplementary Figure 1-5. Average relative abundance of morphotypes that could not be identified to genus with a BLAST % identity > 97%. Identifiers are the top BLAST hit in Genbank nt database. Each morphogroup contributed less than 1% to the total 3-year mycobiome dataset.



Supplementary Figure 1-6. Mean and standard error of SCN egg-density/100 cc soil A) across crop sequence within each year of the study (2014-2016) and B) across a longer time spans (1999-2004 and 2011-2016) of the long-term crop rotation.

1.6 Supplementary Tables

Supplementary Table 1-1. Top 14 taxa that were identified to genus level, ranked based on relative abundance in percentage within each sampling time point across the six sampling time points over three years.

	2014				2015				2016			
	Mid		Fall		Mid		Fall		Mid		Fall	
Genus	%	Rank	%	Rank	%	Rank	%	Rank	%	Rank	%	Rank
Fusarium	21.05	1	24.29	1	33.41	1	27.48	1	25.49	1	29.14	1
Clonostachys	14.04	2	12.96	2	4.00	7	0.96	12	1.98	10	2.58	8
Leptosphaeria	12.28	3	6.68	5	4.94	4	7.35	3	3.08	7	2.88	7
Phoma	9.09	4	0.21	13	1.19	12	0.90	13	0.85	13	1.03	13
Ilyonectria	7.60	5	9.11	3	15.29	2	20.45	2	11.87	2	11.53	2
Paraphoma	4.55	6	1.50	11	0.72	14	0.54	14	1.71	12	0.29	14
Cylindrocarpon	4.09	7	6.68	4	6.59	3	5.11	4	5.27	4	7.74	4
Setophoma	4.09	8	1.82	10	3.29	10	3.35	8	5.27	5	3.49	5
Exophiala	1.17	9	3.44	8	4.00	8	4.95	5	7.03	3	10.17	3
Pochonia	1.17	10	3.85	7	4.94	5	3.35	7	2.20	9	1.82	10

Mariannaea	0.65	11	4.28	6	0.72	13	1.26	10	0.64	14	1.03	11
Alternaria	0.58	12	0.20	14	3.06	11	2.72	9	3.30	6	2.88	6
Mortierella	0.00	13	2.14	9	3.58	9	4.67	6	2.78	8	2.06	9
Neonectria	0.00	14	0.21	12	4.30	6	1.26	11	1.71	11	1.03	12
Nectriaceae	33.40		44.58		60.31		55.55		44.99		50.47	

Supplementary Table 1-2. Analysis of total number of genera identified, and total number of fungal isolates obtained from SCN cysts across our three-year study period using ANOVA and post-hoc Tukey HSD tests. This may be considered an alternative measure of alpha diversity.

	Number of Genera		**Number of Isolates**		
Factor(s)	**Significance**	**P-value**	**Significance**	**P-value**	**Method**
Year	***	4.73E-11	***	6.90E-09	ANOVA
2014-2015	***	0	***	9	Tukey HSD
2014-2016	***	0	***	0	Tukey HSD
2015-2016	**	0.033	*	0.048	Tukey HSD
Sampling Season	***	2.12E-08	***	3.06E-09	ANOVA
Mid-Fall	***	0	***	0	Tukey HSD
Crop rotation sequences	***	2.62E-06	***	4.27E-09	ANOVA
s1-ca	-	0.999999	-	0.999983	Tukey HSD
s5-ca	***	3.79E-05	***	1E-07	Tukey HSD
sa-ca	**	0.019144	**	0.008311	Tukey HSD
ss-ca	-	0.062898	**	0.011089	Tukey HSD
s5-s1	***	3.11E-05	***	1E-07	Tukey HSD
sa-s1	**	0.016596	**	0.011089	Tukey HSD
ss-s1	-	0.055605	**	0.014693	Tukey HSD
sa-s5	*	0.403034	*	0.035576	Tukey HSD
ss-s5	-	0.188532	*	0.027532	Tukey HSD
ss-sa	-	0.99212	-	0.999983	Tukey HSD
Year x Sampling season	*	0.012751	-	0.222274	ANOVA
Year x Crop sequences	***	5.73E-07	***	0.000869	ANOVA
Sampling season x Crop sequences	***	0.001868	***	6.21E-05	ANOVA
Year x Sampling season x Crop sequences	-	0.445702	-	0.803307	ANOVA

Supplementary Table 1-3. Spearman correlation - Fungal abundance in the mycobiome vs SCN egg densities for Soya S1, S5, and Ss rotations.

2014 Mid					**2014 Fall**				
	Taxa	**Rho value**	**P. value**	**P. value adj.**		**Taxa**	**Rho value**	**P. value**	**P. value adj.**
	Alternaria	0.044	0.893	0.929		Alternaria	NA	NA	NA
	Clonostachys	0.117	0.717	0.929		Clonostachys	0.342	0.277	0.929
	Cylindrocarpon	-0.092	0.777	0.929		Cylindrocarpon	-0.124	0.701	0.929
	Exophiala	-0.075	0.816	0.929		Exophiala	0.415	0.180	0.929
	Fusarium	0.404	0.193	0.929		Fusarium	-0.342	0.277	0.929
	Ilyonectria	-0.136	0.672	0.929		Ilyonectria	0.146	0.650	0.929
	Leptosphaeria	0.165	0.609	0.929		Leptosphaeria	0.287	0.366	0.929
	Mariannaea	-0.219	0.495	0.929		Mariannaea	-0.270	0.396	0.929
	Mortierella	NA	NA	NA		Mortierella	0.116	0.719	0.929
	Neonectria	NA	NA	NA		Neonectria	NA	NA	NA
	Paraphoma	0.463	0.130	0.929		Paraphoma	-0.451	0.141	0.929
	Phoma	-0.096	0.767	0.929		Phoma	NA	NA	NA
	Pochonia	0.145	0.652	0.929		Pochonia	-0.066	0.837	0.929
	Setophoma	0.146	0.651	0.929		Setophoma	-0.281	0.377	0.929
2015 Mid					**2015 Fall**				
	Taxa	**Rho value**	**P. value**	**P. value adj.**		**Taxa**	**Rho value**	**P. value**	**P. value adj.**
	Alternaria	-0.655	0.078	0.929		Alternaria	-0.152	0.719	0.929
	Clonostachys	0.317	0.444	0.929		Clonostachys	-0.187	0.657	0.929
	Cylindrocarpon	-0.313	0.450	0.929		Cylindrocarpon	0.076	0.858	0.929
	Exophiala	-0.240	0.568	0.929		Exophiala	0.293	0.482	0.929

	Fusarium	0.452	0.267	0.929		Fusarium	-0.238	0.582	0.929
	Ilyonectria	-0.071	0.882	0.929		Ilyonectria	-0.238	0.582	0.929
	Leptosphaeria	0.431	0.286	0.929		Leptosphaeria	-0.599	0.117	0.929
	Mariannaea	-0.592	0.122	0.929		Mariannaea	0.293	0.482	0.929
	Mortierella	0.026	0.952	0.965		Mortierella	0.096	0.821	0.929
	Neonectria	0.133	0.754	0.929		Neonectria	-0.382	0.351	0.929
	Paraphoma	-0.247	0.555	0.929		Paraphoma	NA	NA	NA
	Phoma	-0.062	0.883	0.929		Phoma	-0.577	0.134	0.929
	Pochonia	-0.204	0.629	0.929		Pochonia	-0.108	0.799	0.929
	Setophoma	0.300	0.470	0.929		Setophoma	0.000	1.000	1.000
2016 Mid					**2016 Fall**				
	Taxa	**Rho value**	**P. value**	**P. value adj.**		**Taxa**	**Rho value**	**P. value**	**P.v alue adj.**
	Alternaria	0.044	0.893	0.929		Alternaria	-0.388	0.213	0.929
	Clonostachys	0.245	0.443	0.929		Clonostachys	0.300	0.344	0.929
	Cylindrocarpon	0.540	0.070	0.929		Cylindrocarpon	-0.106	0.743	0.929
	Exophiala	0.192	0.549	0.929		Exophiala	-0.081	0.803	0.929
	Fusarium	0.095	0.770	0.929		Fusarium	0.158	0.625	0.929
	Ilyonectria	**0.672**	**0.017**	**0.929**		Ilyonectria	0.053	0.871	0.929
	Leptosphaeria	0.403	0.194	0.929		Leptosphaeria	-0.179	0.578	0.929
	Mariannaea	NA	NA	NA		Mariannaea	-0.150	0.642	0.929
	Mortierella	0.155	0.630	0.929		Mortierella	0.250	0.434	0.929
	Neonectria	**0.609**	**0.035**	**0.929**		Neonectria	-0.112	0.728	0.929
	Paraphoma	0.488	0.108	0.929		Paraphoma	NA	NA	NA
	Phoma	0.306	0.333	0.929		Phoma	-0.330	0.294	0.929
	Pochonia	-0.354	0.259	0.929		Pochonia	0.108	0.738	0.929

	Setophoma	0.401	0.196	0.929		Setophoma	-0.025	0.939	0.965

Chapter 2: **IN-VITRO SCREENING OF THE SCN CYST MYCOBIOME FOR BIOCONTROL AGENTS**

The SCN reproduces at an extremely rapid rate. Within one growing season, even if only 3% of the available egg inoculum reproduces, the population of SCN may multiply 1000 times (SCN Coalition 2016). The eggs within cysts produced during each growing season can accumulate and remain viable in soil for nearly a decade (Chen 2011; Koenning 2004), making eradication of the SCN from fields nearly impossible. These eggs and cysts are the main sources of inoculum for the disease. However, their populations can be managed to mitigate their economic impacts. Cultural practices such as rotating with a non-host crop like corn, and genetic resistance have been the most important control methods for SCN in the field (Chen and Dickson 2012). Unfortunately, the most commonly deployed genetic resistance is losing effectiveness and new sources of genetic resistance are still being assessed for their potential to generate agronomically-acceptable soybean varieties (Niblack et al. 2008). In this current scenario, there is a need for developing biological control as an important component of integrated nematode management.

Fungi are promising candidates for biological control as many of them can overwinter as spores in the soil, enabling their persistence in agroecosystems. As prolific producers of secondary metabolites with bioactivity against nematodes, they also represent an unexplored resource for novel bionematicides (Mousa and Raizada 2013;

Hallmann and Sikora 1996). Natural antagonists of the SCN such as nematophagous fungi are promising candidates for biological control and are increasingly popular in organic production systems (Haarith et al. 2019). Nematophagous fungi have several modes of antagonizing nematodes, which may target different stage of the nematode life cycle. Nematode trapping fungi produce special hyphal structures to trap vermiform nematodes in the soil, while others such as *Hirsutella rhosilliensis* and *H. minnesotensis* are endoparasites that also target the vermiform stage of the life cycle. Others directly parasitize SCN larvae/ developing juveniles in eggs within the cysts (Chen and Dickson 2012; Hu et al. 2018). All these nematophagous fungi have been shown to use direct parasitism, secretion of nematicidal metabolites, or both mechanisms to kill their prey (Chen et al. 1994; Chen et al. 2000). Testing for these two mechanisms *in vitro* is a first step in screening for biocontrol potential in fungi.

The egg-parasites of the SCN, which destroy the source of new infective vermiform juveniles, are particularly attractive as potential biological control agents. An important aspect for a potential biocontrol fungus is the ability to grow well in artificial culture using limited nutrients to produce copious spores. The production of secreted metabolites with bioactivity against nematodes is another important mechanism. These metabolites may help the fungi weaken or paralyze their nematode prey and could also enable biocontrol fungi to be more competitive and counteract “fungistasis” or the inhibition by other microorganisms in the complex soil environment (Kerry 1988). Furthermore, these metabolites could also be developed into environmentally safe nematicides, even if the producing fungus is potentially pathogenic to plants. One the few commercially available products from fungi, for example, is a bionematicide (DiTera®) derived from the potential plant pathogen *Myrothecium verrucaria* (Warrior et al. 1999;

Valent BioSciences 2010). Other desirable traits of candidate biological control agents needed to overcome bottlenecks to commercialization are important considerations (Fravel 1999; Whipps and Lumsden 2009). The ability to sporulate well to enable easy formulation and application methods, viability in efficacy, and retention of activity during transport and storage are the most significant ones to be considered in screening for biological control agents.

We recently characterized the SCN cyst mycobiome from SCN cysts isolated from a long-term soybean-corn rotation experiment in Waseca, Minnesota, USA (Haarith et al. 2019; Hu et al. 2018). In this study, we have screened this SCN cyst mycobiome for fungal isolates with the *in vitro* ability to (i) effectively parasitize SCN eggs, (ii) inhibit SCN eggs and juveniles with secreted metabolites, and (iii) grow quickly and make copious amounts of spores on artificial media. We have also developed a cost-effective and reliable fluorescence-based visualization of fungal-egg parasitism using Calcofluor White M2R, a chitin-binding dye, and Propidium iodide, a live-cell exclusion DNA binding dye to visualize invasion of SCN eggs by the fungal hyphae and cell death in the nematode embryo respectively.

2.1 Materials and Methods

2.1.1 Fungal isolates

All fungal strains were obtained from a long-term soy-corn rotation experiment at the University of Minnesota Southern Research and Outreach Station as detailed in chapter 1 (Haarith et al. 2019). Morphologically similar fungi were grouped together into morphogroups and divided into subgroups for each five members of a morphogroup. One

random isolate from each subgroup was selected as a morphotype for sequencing of the ITS1 region for molecular identification. These morphotypes were identified to fungal genus based on BLAST hits of more than 97% similarity to their respective ITS fungal barcode gene to the NCBI database. ITS sequences of all morphotypes were then clustered, using the USEARCH (Edgar 2010) run in QIIME (Caporaso et al. 2010), into clusters based on sequence similarity of 99% or more (Strom et al. 2019) and these were compared back to morphogroups to assess accuracy of the morphogroup classifications. Each cluster was also assigned a cluster representative strain by the USEARCH algorithm. All isolates were stored as mycelial plugs in 20% glycerol at -80°C until needed for subsequent assays. A random representative of each cluster, or two to three representatives for clusters with more than five representatives, were tested from each USEARCH cluster, for their ability to grow on ¼ PDA from the glycerol stock after the freeze-thaw. Clusters with only one representative were excluded from *in vitro* assays if the representative morphotype did not grow from the glycerol stock.

2.1.2 Axenic SCN cysts

SCN eggs were obtained from the nematology research laboratory at Southern Research and Outreach Center in Waseca and were rinsed three times with autoclaved distilled water. The eggs were then decontaminated by incubating them at 25°C for 8 h in an aqueous solution of 100 ppm each of streptomycin and chlor-tetracycline with 50 µg/ mL Fungin (Invitrogen) to remove any bacterial and fungal contaminants. The eggs were subsequently rinsed using autoclaved distilled water and hatched in an aseptic environment in a 4 mM $ZnCl_2$ solution using mesh support and sterile coffee filters as described in a previous report (Chen and Liu 2005). Fresh second-stage juvenile (J2) nematode hatchlings were filtered through sterile coffee filters into autoclaved distilled

water. Hatchlings were then inoculated on week-old susceptible soybean seedlings grown in 6-inch bleached cone-tainers filled with autoclaved sand:soil mixture (80:20; v/v) by applying 5 mL of nematode suspension (at least 1000/mL) in a three-inch deep hole created parallel to the roots. The system was maintained in a clean growth chamber with a HEPA filter to filter out fungal spores under daily 14 h light and 27°C and 10 h dark and 22°C, for 28 days post-inoculation. The soybean plants were watered using autoclaved tap water and no fertilizers were used. The soil and roots were then washed to recover SCN cysts using modified hand floatation and sucrose centrifugation as described previously (Chen et al. 2001). These cysts were surface sterilized using a 0.5% NaOCl bleach solution for 3 min and decontaminated with antibiotics, as discussed above. Cysts were assessed prior to each experiment to certify that no fungal or bacterial growth was observed when incubated on PDA and in PDB for 7 days at 25°C.

2.1.3 Parasitism assay

Ten cysts were placed in a circle of 2.5 cm diameter on water agar. A 1-cm square plug of a representative cluster isolate was placed in the center of this circle and incubated at 25°C until the fungal colony edge reached the cysts. After the colony contacted the cysts, the plates were incubated for two weeks at 25°C. Then, five random cysts were examined under a light microscope and an egg parasitic index (EPI) was assessed for each cyst based on the percentage of eggs colonized in each cyst (Chen et al. 1996; Chen and Chen 2002a) on a scale of 0-10 (0-100% of eggs colonized). An average EPI was calculated for each representative fungal isolate (Figure 2-1A). Additional glycerol stocks were made for the top 10 highly parasitic fungal isolates and stored at -80 C. The parasitism assay was repeated for these 10 isolates from the newly prepared glycerol stocks after one year in storage.

2.1.4 Widefield fluorescence microscopy

The remaining five parasitized cysts from each water agar plate were collected by flooding the surface with autoclaved distilled water. The eggs within were released from their cysts using tissue pestles (Carolina®) in 1.5 mL Eppendorf tubes, and simultaneously labelled with the fluorescent dyes propidium iodide (Invitrogen™) and Calcofluor White M2R (Sigma-Aldrich®). Several modifications were made to the manufacturers' protocols: for propidium iodide staining, RNase and antifade reagent treatments were not performed; for Calcofluor White M2R staining, samples were rinsed several times with 2X SSC medium following labeling. Solutions were eluted following centrifugation at 5,000 RPM for 1 minute after each step.

Widefield fluorescence microscopy of labelled samples was conducted at the University of Minnesota University Imaging Center (St. Paul, Minnesota). Images were obtained using a C11440-42U30 digital CMOS camera (Hamamatsu®) on an NIS-Elements Viewer (Nikon®) through Z-stack of foci of interest using DAPI (excitation: 325 ~ 375 nm, emission: 435 ~ 485 nm) and DsRed (excitation: 590 ~ 650 nm, emission: 590 ~ 650 nm) filters for Calcofluor White M2R and propidium iodide, respectively. A DIC N2 LWD T-C Eclipse microscope condenser prism (Nikon®) was used. Exposures were adjusted manually on a case-by-case basis to ensure visual clarity of microscopic features.

Greyscale Tagged Image File Format (.tif/.tiff) files obtained through microscopy were analyzed using the Extended Depth of Field plugin algorithm on the NIS-Elements Viewer to obtain a focused image. This focused image was processed using Photoshop CS5 (Adobe®) for image processing. The Unsharp Mask filter was applied (amount:

150%, radius: 3 pixels, threshold: 0 levels) to sharpen microscopic features. The levels tool was also manually adjusted for individual images to increase image contrast. Color corrections were made for Calcofluor White M2R and propidium iodide images.

2.1.5 Hatch-inhibition and J2 mortality assays

2.1.5.1 Fermentation and recovery of metabolites

All cluster representatives were incubated on Spezieller Nahrstoffarmer Agar (SNA) at 25 °C with 12 h light-dark cycles for 15 days. The plates were flooded with 4 mL of sterile, ice cold 0.2% Tween-20 solution, and the solution was gently pipetted on the fungal colony 4-5 times. The tween solution was then examined under a light microscope using a haemocytometer for the presence of fungal spores. Fungal isolates that did not produce any spores (95 isolates) were eliminated from this assay. For each isolate, 10^6 spores were separately inoculated into 50 mL of ¼ strength Czapek-Dox Broth (CDB) and incubated at 25 °C for 15 days at 120 rpm. The flasks were then cooled overnight to 4 °C and the contents were centrifuged at 4000 rpm for 15 min to separate the fungal hyphal biomass. The hyphal biomass was frozen at -80 °C while the supernatant was recovered and sterilized by passing through sterile SFCA syringe filters of 0.45 um and 0.20 µm. Sterile supernatants were frozen at -20 °C until used for the assay (Figure 2-1B).

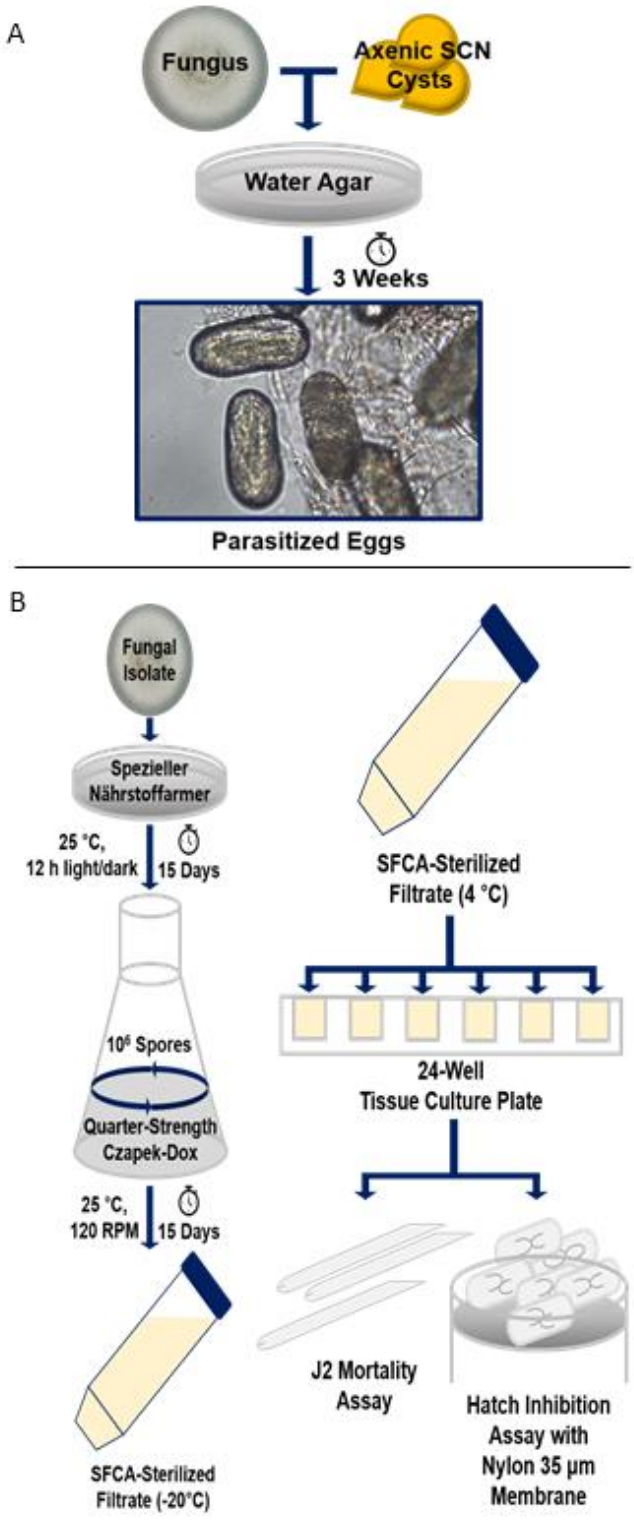


Figure 2-1. (A) Fungal direct parasitism assay on water agar, (B) fungal culture filtrate assays to test inhibition of SCN egg hatch and J2 mortality.

2.1.5.2 Hatch inhibition assay

Frozen supernatants were transferred to 4 °C to thaw slowly for 24 h before the assay. Nylon membranes of 35 µm pore size were supported (by pressing the membrane between the mouth and the hollowed lid) on homemade modified 1.5 mL centrifuge tube tops to form a J2 permeable filter that fit over a standard 24-well tissue culture plate well. These filters were autoclaved and used as supports for egg hatch. Above each well, 500 SCN eggs were suspended on a sterile filter over 2 mL of sterile supernatant and incubated at 27 °C for 48h. The eggs were thus kept in contact with the supernatants without being inundated in them. The filters were then removed along with unhatched eggs and the number of J2s hatched was counted in each well (Figure 2-1B). The experiment was done in triplicate for each supernatant with ¼ CDB and autoclaved distilled water controls. An average percent inhibition was calculated for the supernatant of each cluster representative isolate, as well as the media control using the following formula

$$(1 - \frac{No.\ of\ hatchlings\ in\ test\ solution}{No.of\ hatchlings\ in \frac{1}{4} CDB}) \times 100$$

The hatch-inhibition assay was repeated for these 10 isolates from the newly prepared glycerol stocks, after one year in storage.

2.1.5.3 J2 mortality assay

Sterile supernatants of the ten fungi showing the most effective egg hatch inhibition were also studied for their ability to reduce J2 viability. In a 24-well plate setup, a suspension of 100 µL containing 50 freshly hatched SCN J2s was added in each well with 1 mL of supernatant and incubated at 25 °C for 24 h and 48 h. J2 mortality was estimated under an inverted microscope, using 1 M NaOH stimulation as described by

Chen and Dickson (2000) (Figure 2-1B). Percentage mortality was calculated for all culture filtrates, media and water controls. The experiment was conducted in triplicate and repeated twice.

2.1.6 Statistical analyses

The EPI and hatch-inhibition data were analyzed each year (for each experiment run) using the formula "EPI/hatch-inhibition%~isolates" and isolates were grouped based on the Least Significant Difference or LSD method using 'agricolae' package in R (R Core Team 2013) and Bonferroni correction for *p*-values. For the J2 mortality assay, data from all six replicates were combined and analyzed using the formula "J2 mortality%~isolates" and grouped based on the LSD method.

2.2 Results

2.2.1 Mycobiome represented by 326 OTU clusters

In a previous study, we examined 6,000 SCN cysts for fungal growth after surface sterilization. Fungi isolated from these cysts were grouped into morphogroups based on colony similarities on PDA. From each morphogroup, a random isolate for every five isolates in the morphogroup (morphotype) was selected for ITS sequencing and identification. ITS region amplification and subsequent Sanger sequencing was successful for 1,540 morphotypes (~94% success rate). The morphotypes were assigned to genus based on the first twenty BLAST hits (Haarith et al. 2019) and the mycobiome was found to be dominated by 14 genera (Haarith et al. 2019). For this study, the sequences of the 1,540 morphotypes were clustered into 326 OTU clusters based on 99% or higher sequence similarity amongst their ITS regions using USEARCH. For the most part, all the

representative morphotypes from a single morphogroup clustered together, providing support for their correct grouping based on morphology alone. However, most of the genera identified were represented by more than one cluster, suggesting the clustering based on ITS was potentially able to distinguish groups below the genus level. Out of 326 clusters, 173 clusters had five or fewer members and a single morphotype representative, while the remaining 153 clusters had more than five members and at least two morphotype representatives (Supplemental Table 2-1). The most abundant genus, *Fusarium*, comprised a majority (~30%) of the isolates in the total mycobiome (Haarith et al. 2019) and was represented by 467 morphotypes that clustered into 28 clusters. The next most abundant genera, *Ilyonectria*, included 119 morphotypes in 12 clusters; 106 morphotypes of *Cylindrocarpon* in 12 clusters; 64 morphotypes of *Exophiala* in 11 clusters; and 43 morphotypes of *Clonostachys* in four clusters (Supplemental Table 2-1).

2.2.2 *In vitro* screening approach identifies potential biocontrol fungi

A total of 237 morphotype isolates representing 174 clusters successfully grew from the glycerol stocks. In the 174 clusters, several morphotypes from the same cluster successfully grew for the genera *Fusarium*, *Ilyonectria, Cylindrocarpon, Leptosphaeria,* and *Mortierella*. Those isolates that grew, were subjected to at least two different *in vitro* biocontrol analyses as detailed below (Figure 2-2).

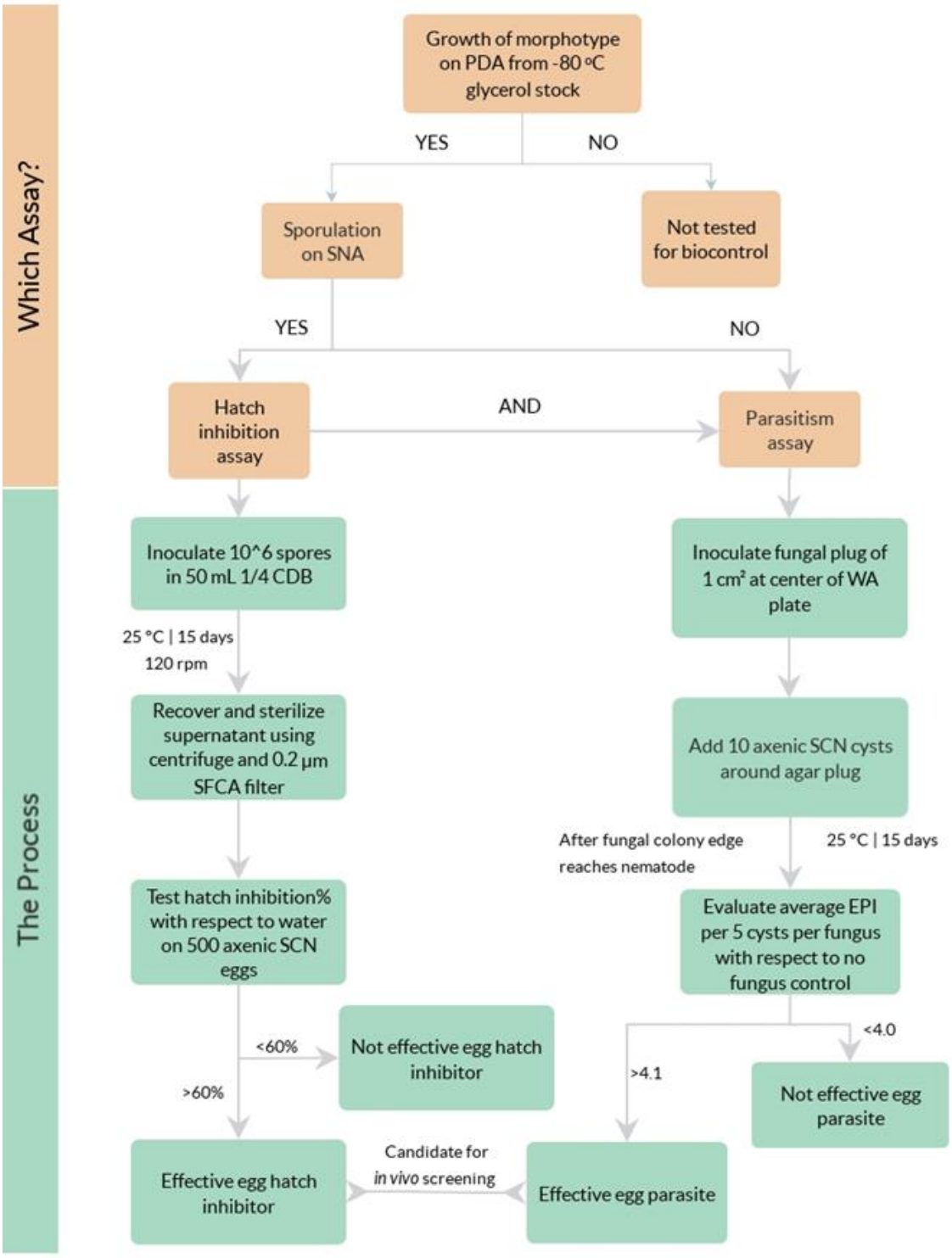


Figure 2-2. Workflow for In vitro screening process to identify isolates with high parasitism, egg-hatch inhibition, or J2 toxicity. Only top 10 hatch inhibiting supernatants were also tested for J2 mortality (not indicated in this flowchart)

2.2.2.1 Egg parasitism assay – few effective parasites

The Egg Parasitic Index (EPI) or the percentage of eggs colonized by the fungus in a cyst at two weeks from initial contact was expressed on a scale of 0 to 10 (0-100% colonization). The EPI of the 237 morphotypes belonging to 174 clusters were evaluated using light microscopy and the frequency distribution of EPI was calculated (Figure 2-3A). About 83.54% (198) of all isolates tested had an EPI of 4 or less and were deemed to have low EPI; 10.12% (24) had a moderate EPI between 4.01 and 6; and 4.22% (10) had a high EPI between 6.01 and 8. Only five morphotypes from five different clusters (3 *Fusarium*, 1 *Mortierella* and 1 *Alternaria*), which make up for about 2% of all isolates tested, had very high EPI between 8.01 and 10, meaning 80.1 – 100% of all eggs in the cysts were colonized by those fungi within 2 weeks from initial contact. Three out of five of the isolates with very high EPI belonged to three different *Fusarium* clusters. However, the mean EPI of all *Fusarium* isolates did not vary significantly from the mean EPI of other genera. Additionally, both very high and low EPI isolates were often found within the same cluster. The EPI of the most parasitic fungal isolates varied slightly between the two experiments conducted. Some of the 10 highly egg parasitic fungal isolates showed a slightly reduced EPI when tested from a second glycerol stock, compared to the initial screen, but all still had an EPI above 4.5 (Figure 2-3B). Light micrographs of the representative cysts from each of the 10 highly egg parasitic fungi showed that the inoculated fungi had established in both the egg matrix and the eggs themselves (Supplemental Figure 2-1). The control eggs incubated on water agar without a fungal plug were observed to be devoid of any colonization.

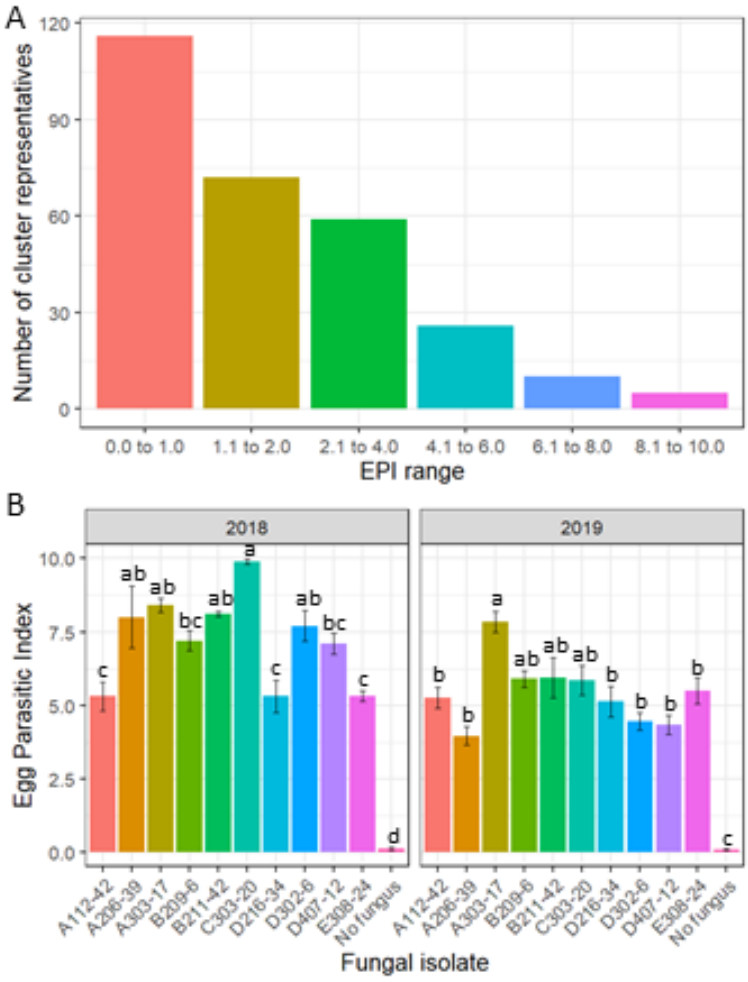


Figure 2-3. (A) Frequency distribution of EPI (egg parasitic index) of all fungi tested, (B) EPI measurement for the 10 most highly parasitic fungal isolates estimated in 2018 after reviving cultures from an initial -80 °C glycerol stock and in 2019 after reviving from a secondary -80 C glycerol stock. Significant groups assigned based on LSD test within each year's data and error bars indicate standard error.

2.2.2.2 Microscopic visualization of egg parasitism

In order to visualize fungi parasitizing SCN eggs and confirm that these are true egg-parasites and not opportunistic saprobes of the cyst matrix or walls, we developed a fluorescent microscopy protocol to simultaneously stain fungal hyphae with calcofluor white (blue) and apoptotic nuclei in the nematode eggs using propidium iodide fluorescence (red). Compared to the controls, which showed a diffuse signal for both dyes, the majority of eggs inoculated with fungi showed visible evidence of fungal

hyphae penetrating eggs and brighter or more punctate signal for apoptotic cells within the nematode egg (Figure 2-4). However, different fungal isolates showed different patterns of colonization. In some cases (isolates A303-17, C303-20 and D216-34), a dense network of fungal mycelia could be seen to have growth throughout the egg and replaced the J2s within the eggs. In contrast, a majority of eggs from cysts inoculated with isolates B211-42 were empty at the end of the two-week incubation period, when visualized using fluorescent microscopy. On the other hand, dead apoptotic cells of J2s inside eggs were observed in a majority of isolates, with particularly strong signals inside eggs that were inoculated with isolates A206-39, B209-6, D302-6, D407-12 and E308-24.

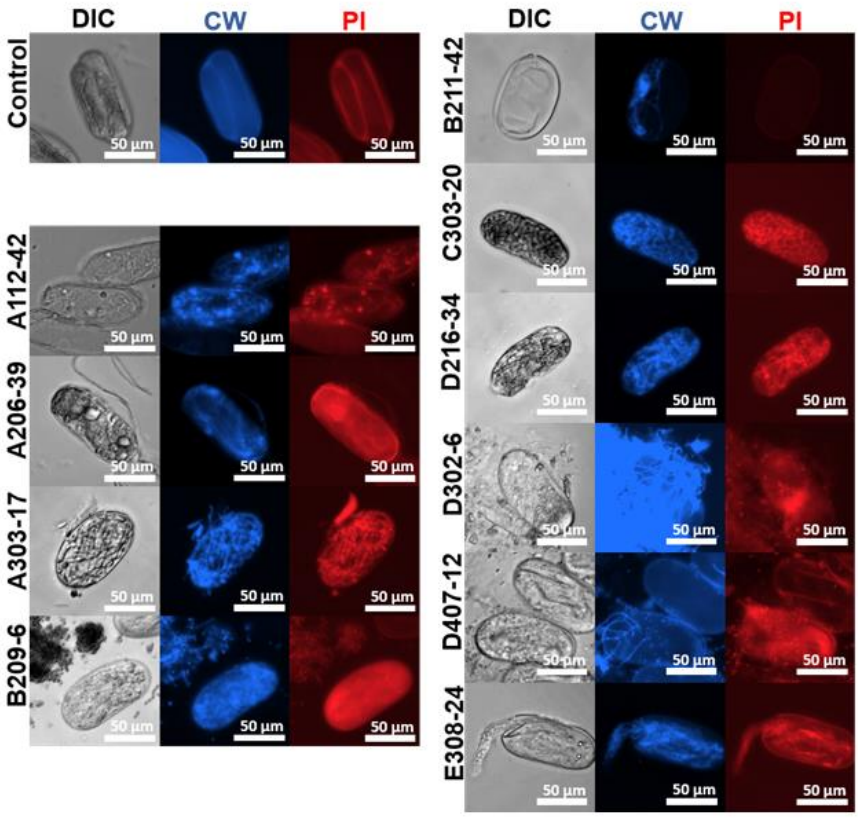


Figure 2-4. Visualization of egg parasitism using differential interference contrast (DIC) and fluorescent microscopy. Calcofluor white (CW) binds chitin and traces the fungal hyphae. Propidium iodide (PI) fluoresces red when bound to active apoptotic nuclei.

2.2.2.3 Egg hatch-inhibition – many hatch-inhibiting fungi

Only 142 isolates out of the 237 morphotypes for which the EPI was evaluated p roduced spores and were subsequently assayed for hatch-inhibition potential. Filter sterili zed 15-day-old culture filtrates from these isolates as well as a mock inoculated broth con trol were evaluated for their ability to inhibit SCN egg hatch. About 35% of all isolates te sted were able to inhibit egg hatch between 60.1-80% more than the ¼ CDB control. Seve nteen percent of isolates had 80.1-100% more hatch-inhibition compared to ¼ CDB contr ol (Figure 2-5A).

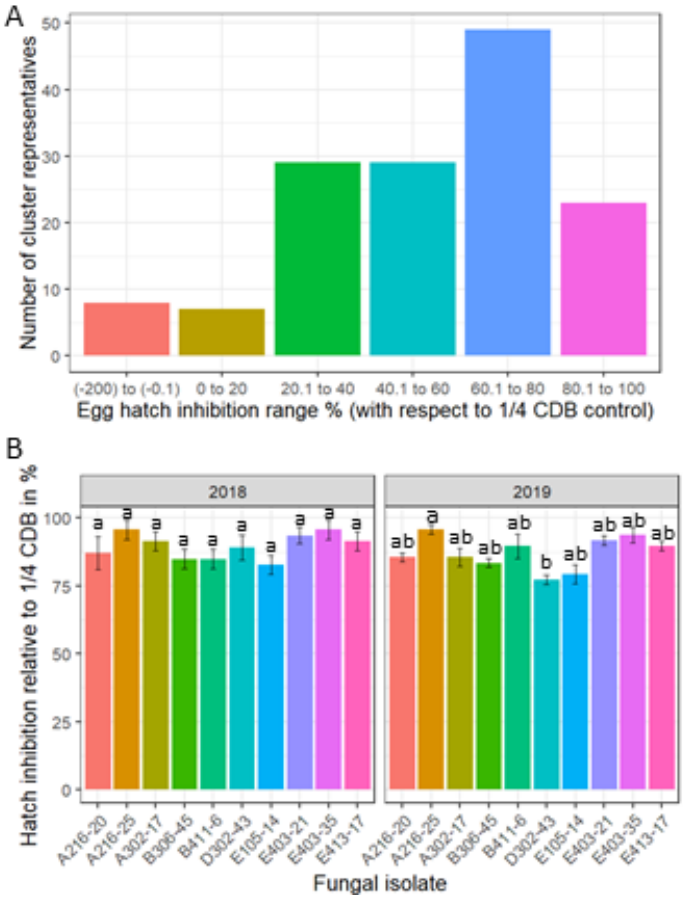


Figure 2-5. (A) Frequency distribution of hatch-inhibition of all isolates screened, (B) Hatch inhibition for the 10 highly inhibitory fungal isolates estimated in 2018 after reviving from an initial -80 °C glycerol stock and in 2019 after reviving from a secondary -80 C glycerol stock. Significant groups assigned based on LSD test within each year's data and error bars indicate standard error.

Culture filtrates of isolate E403-35 had the highest inhibition of 95.68%. On the other end of the spectrum, eight isolates stimulated better hatch compared to media contro l (E303-41, -213%; D302-9, -68%; B302-19, -34%; E305-10, -32%, E406-34, -10%; A10 5-47 and A112-40, -8%; and D407-12, -4%). Mock inoculated media alone inhibited egg hatch at about 35% compared to water controls. Therefore, only E303-41 and D302-9 filtr ates could be considered as hatch-stimulants. The inhibitory potentials of the culture filtra tes of the top 10 isolates were significantly higher than mock-inoculated ¼ CDB control. But, they are not significantly differ from each other and are all relatively well above 75% hatch inhibition. The ability of these isolates to produce nematicidal metabolites was not a ffected by glycerol cryopreservation (Figure 2-5B).

2.2.2.4 Hatch-inhibitors also kill J2s

The 10 isolates with high potential for inhibiting SCN egg-hatch also exhibited significant toxicity towards freshly hatched J2s (Figure 2-6). All culture filtrates had a final pH between 5.8 and 6.3 (data not shown). Culture filtrates from isolate A302-17 was the most effective at 24 h incubation while there were no significant differences between the mortality rates of ¼ strength Czapek-Dox broth and sterile deionized water. At 48 h incubation, isolates A216-25, D302-43, E105-14, E403-35 and E413-17 had killed almost all the J2s. The ¼ CDB medium control also showed higher mortality at 48 h compared to sterile deionized water. It is noteworthy that mortality in the culture filtrates of B306-45 and B411-6 was not different between 24 h and 48 h time-points.

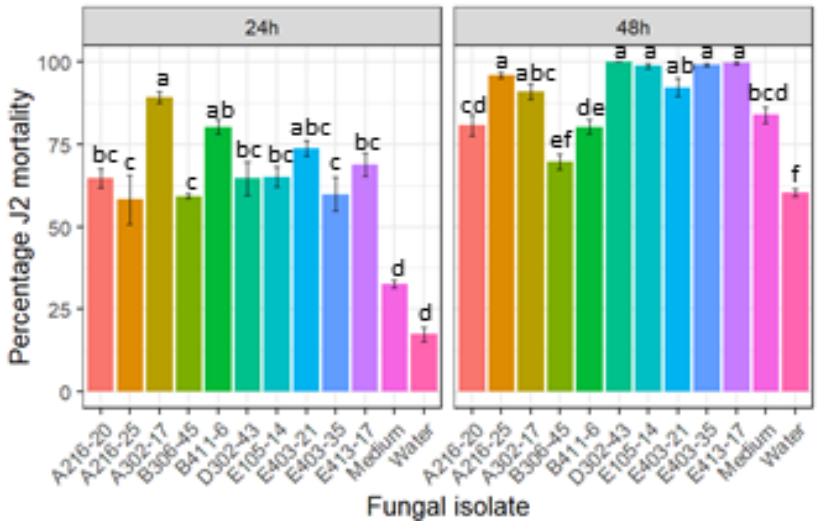


Figure 2-6. Juvenile (J2) mortality estimates for the top 10 hatch-inhibiting culture supernatants at 24 h and 48 h time points. Mortality in 1/4 CDB medium and water as references. Significant groups assigned based on LSD test and error bars indicate standard error.

2.3 Discussion

Fungi are promising candidates for biological control of the SCN, yet only a few studies have systematically screened the fungi found within SCN cysts for direct parasitism of eggs (Chen et al. 1996; Chen et al. 2000; Chen and Chen 2002b, 2003; Hu et al. 2018), inhibition of egg hatch and direct toxicity to J2s (Meyer et al. 1990; Nitao et al. 1999; Mukhtar and Pervaz 2003). In this study, we have used all these three techniques to identify potential biocontrol candidates. The fungal isolates screened in this study were isolated from a large number of cysts (6,000), therefore hopefully capturing most of the diversity of fungi inhabiting SCN cysts in this field. Here, we developed and implemented an efficient and high-throughput screening pipeline to assess the biocontrol potential within this SCN mycobiome. Isolates were first grouped based on colony morphology into morphogroups, then we sequenced the ITS region of a random representative morphotypes for every five members of a morphogroup, and these

sequences were further clustered into 326 groups based on 99% ITS sequence similarity (OTU clustering). Clustering corroborated groupings based on morphology and only a few isolates were sorted into two different clusters representing the same genera. Many genera were represented by several clusters (Supplemental Table 2-1). *Fusarium* and *Ilyonectria*, the most abundant genera in this mycobiome (Haarith et al. 2019), are both difficult to resolve into species using only the ITS region (Chaverri et al. 2011b; O'Donnell et al. 2012). Therefore, clustering based on 99% ITS sequence similarity was used here primarily as a tool to narrow down our screening process and these clusters may or may not represent clusters at the species level. A total of 326 clusters were identified and we were able to screen representatives from over half of them (174 clusters). Unfortunately, we were not able to revive representatives from some clusters on ¼ PDA after cryopreservation in glycerol at -80 °C and these were excluded from further screening. Not all fungi react the same way to a particular preservation technique (Morris et al. 1988; Ilyas and Soeka 2019). In fact, in a recent viability study done on preserving *Beauveria bassiana* strains, cryopreservation with glycerol consistently underperformed and reduced the fitness of the fungus (Oliveira et al. 2011). Due to the sheer number of isolates we had to handle, we only relied on cryopreservation, a technique that is quite frequently used (Nakasone et al. 2004).

SCN cysts and eggs are the sedentary stages of the nematode and serve as the source of new inoculum and can remain viable in soil for nearly a decade (Chen 2011). Therefore, the ability to parasitize and/or kill SCN eggs is a beneficial trait for a biological control agent. Many fungi that can colonize cysts need not be good parasites of SCN eggs, and might simply be commensals or saprobic (Kerry 1988; Carris et al. 1989; Chen et al. 2000). Many studies have reported parasitic indices for fungi based on the EPI

observed in cysts recovered from the field from which these fungi were isolated (Chen and Chen 2002b, 2003; Hu et al. 2018). These approaches may not indicate the exact age of the cysts recovered, nor the age of the relationship between the cyst and the associated fungus. It is impossible to deduce the age of the cysts and the contact time between the infected cysts and the fungal mycelia. However, different stages of cyst and eggs have been recovered and co-incubated with mycelia and studied previously, and young females were found the most susceptible (Chen et al. 1994). Therefore, a good parasite might have a low EPI because it had less time of contact compared to a poor parasite that has had more time of contact with the cyst before they were collected from the field. Additionally, obligate fungi that cannot be cultured may be responsible for parasitism of eggs in the field, rather than the culturable fungi that were isolated from the cyst. Thus, in this study, we used an *in vitro* egg parasitism assay using axenic SCN cysts to directly evaluate fungal parasitism of SCN cysts and eggs. Previous studies showed that eggs at embryonic stages are more susceptible to fungal parasitism than the eggs in juvenile stages of development (Chen and Chen 2003). In this study, all cysts were collected at 30 days post infection of soybean plants and were roughly of the same age. All fungi also had the same amount of contact time (2 weeks) with the cyst and eggs before their EPI was deduced. We found that 16.5% of all fungi tested had an EPI of 4 or higher, meaning that they could parasitize 40% or more of the eggs in the cysts within two weeks of mycelial contact. As discussed, the ability to maintain biocontrol activity during storage is an important consideration for biocontrol organisms. Therefore, we also evaluated the EPI of these 10 isolates with the highest EPI, after preserving them in glycerol at -80 °C for one year. Although the EPI was reduced for many isolates, it remained well above 4.5, indicating these isolates retained their parasitic ability.

When tested from the initial glycerol stock, all of the 10 most effective parasitic isolates had very high EPI. However, isolates A206-39, D302-6, C303-20 and D407-12 showed a decrease of about 3 index points (30% reduction in parasitism) when grown and tested from a secondary glycerol stock. Isolates A112-42, D216-34 and E308-24 retained their EPI above 5 in both years. We subcultured these fungi in order to preserve them in glycerol at -80 °C for a secondary stock. Subculturing followed by storage in glycerol stock, revival on PDA and subsequent testing on WA could have made fungi lose some of their ability to infect nematodes and to become more saprobic. It has been shown previously that parasitic fungi can lose virulence when subcultured on artificial media. A nematode parasitic fungus *Drechmeria coniospora* was observed to lose virulence increasingly with number of sub-cultures or transfers from one petri dish of medium to another (Zuckerman et al. 1989). It is also possible that the different batches of HG type 0 egg populations that were tested to have contributed to this variation. However, in this study, the fungi revived from a secondary glycerol stock still retained an EPI of 4.5 or more, well above our initial cut-off of 4 index points, and could still be considered as effective SCN egg parasites. This study, like many other previous studies done on a variety of fungi, reveals the need to examine different preservation methods that could retain the parasitism of the cultures over long periods of time (Zuckerman et al. 1989; Humber 1997; Sanchez-Pena and Thorvilson 1995; Nakasone et al. 2004). Sporulation was another important criterion for our biocontrol screening. For commercial success of a biocontrol agent, spore formulations can be more easily stored and transported without losing viability (Wraight et al. 2001; Parnell et al. 2016). Many spores withstand abiotic stresses and can be desiccated easily to be used in seed coatings or as powders to be

mixed in the fertilizer tanks of planters. Spores could also be suspended in irrigation water and applied near the roots where they are needed to combat nematodes.

Although light microscopy can help differentiate parasitized eggs from normal eggs with a developing embryo or juvenile stages, it is not adequate to observe the hyphal structures within individual eggs. In fact, significant amount of skill and training is necessary to tell apart eggs colonized by hyphae, eggs with an under-developed embryo, and eggs that are apoptotic using light-microscopy. Fluorescence microscopy is hence an interesting tool to visualize the progression of parasitism as it could help differentiate fungal tissue from that of the nematode (Escudero and Lopez-Llorca 2012). Previous studies have used Calcofluor white M2R dye to visualize fungal features and their interactions with other organisms, including nematodes, due to its ability to bind chitin and β-glucans (Elad et al. 1983; Segers et al. 1994; Zavala-Gonzalez et al. 2015). This fluorescent dye binds non-specifically to chitin and cellulose and fluoresces blue. In our egg-parasitism experiments, since there were no plant materials, this dye bound to chitin in the cell wall of fungi and helped visualize hyphae. The outer layer of nematode eggs are made of vitelline and chitin is usually in the middle structural support layer (Bird and McClure 1976; Wharton 1980). Therefore, intact eggs do not bind chitin and only have a faint background calcofluor fluorescence as seen in our controls (Figure 2-4). We also used a live-cell exclusion dye, propidium iodide, which non-specifically intercalates with DNA of dead cells, to track cell death in the nematode within the parasitized eggs (Shapard et al. 2012; Ferreira et al. 2015). The combination of these two dyes helped us track fungal growth and parasitism in our assay by showing the extent of colonization by fungi emitting blue florescence and red fluorescence indicating nematode death. Thus, we have developed an affordable and rapid method to confirm egg-parasitism by fungi as a

part of this high-throughput screening of the mycobiome for biocontrol agents. With this technique, we were able to visualize some fungal isolates which colonized the egg and replaced the nematode embryo and juvenile within the egg entirely with hyphae, while others hollowed out the egg, but hyphae were not observed. Different fungal isolates also showed different levels of apoptosis in the juveniles within the egg, although almost all of them showed some level of apoptosis (Figure 2-4). This could indicate either differences in the speed of parasitism or the mechanism (biotrophic vs necrotrophic) of parasitism, or both. Further focused investigations are necessary to ascertain specific mechanisms of parasitism.

Fungi are known producers of several secondary metabolites and could employ direct parasitism and indirect influence on nematodes using these metabolites. Several studies have tried to characterize the effect of fungal metabolites and culture filtrates on nematode hatching and or nematode juvenile mobility/mortality. Not all fungi that were able to grow after cryopreservation could produce spores when incubated on a sporulation medium for two weeks. All fungi that did not sporulate by the end of 2 weeks were observed for 4 weeks until the medium dried out and only a few sporulated at the end of 4 weeks, but only sparsely. If we did not get enough spores to inoculate our ¼ CDB flasks (10^6 spores in 50 mL), we did not evaluate that isolate for its ability to produce metabolites. Many studies have used hyphae or hyphal plugs to inoculate media in search of metabolites. A study that evaluated *Verticillium lecanii* (syn *Lecanicillium lecanii*) strains used mycelial agar plugs to inoculate liquid broth to study the production of nematotoxic metabolites (Shinya et al. 2008a). This *Verticillium* study also looked at the effect of fungal metabolites on the development of fertile embryos into J2s and observed that some metabolites indeed affect nematode development. Most studies that investigate

the effect of fungal metabolites on egg hatching incubate for 1 week or more (Nitao et al. 1999; Massoud et al. 2002; Meyer et al. 2004). Therefore, our study was limited to a 48 h incubation, which would only study the effect of metabolites on hatch inhibition of mature eggs that are ready to hatch. Another study that investigated fungi in rhizosphere of plants for their ability to produce nematotoxic metabolites also inoculated fungal mycelial plugs (Qureshi et al. 2012). As the growth rate and biomass production per unit area for different fungi differ from each other, we chose to inoculate conidial spores, to control the inoculum levels and make comparisons on the metabolite production based on equal inoculum amongst the different isolates.

Thus, only those fungi that sporulated were studied further for their ability to produce metabolites as spores can be better quantified and standardized as inoculum. We used ¼ strength CDB for secondary metabolite production for several reasons. It is a defined medium and is thus easier to evaluate active metabolites from other components in the media. The ¼ strength medium more rapidly achieves carbon starvation, which can induce fungi to produce secondary metabolites (Nitsche et al. 2012). Among isolates that produced spores, the production of antagonistic metabolites was significantly more frequent in this mycobiome than direct parasitism. Fungi are known to produce secondary metabolites, some of which are thought to enable them to compete in soil and other environments with highly diverse or competitive microbial communities (Magan and Aldred 2008). The control ¼ CDB medium also inhibited SCN egg hatch more than sterile deionized water, but significantly less than most other media tested. In a previous study that tested the effect of different media on metabolite production, a basal level of media antagonism was also observed for most media (Nitao et al. 1999).

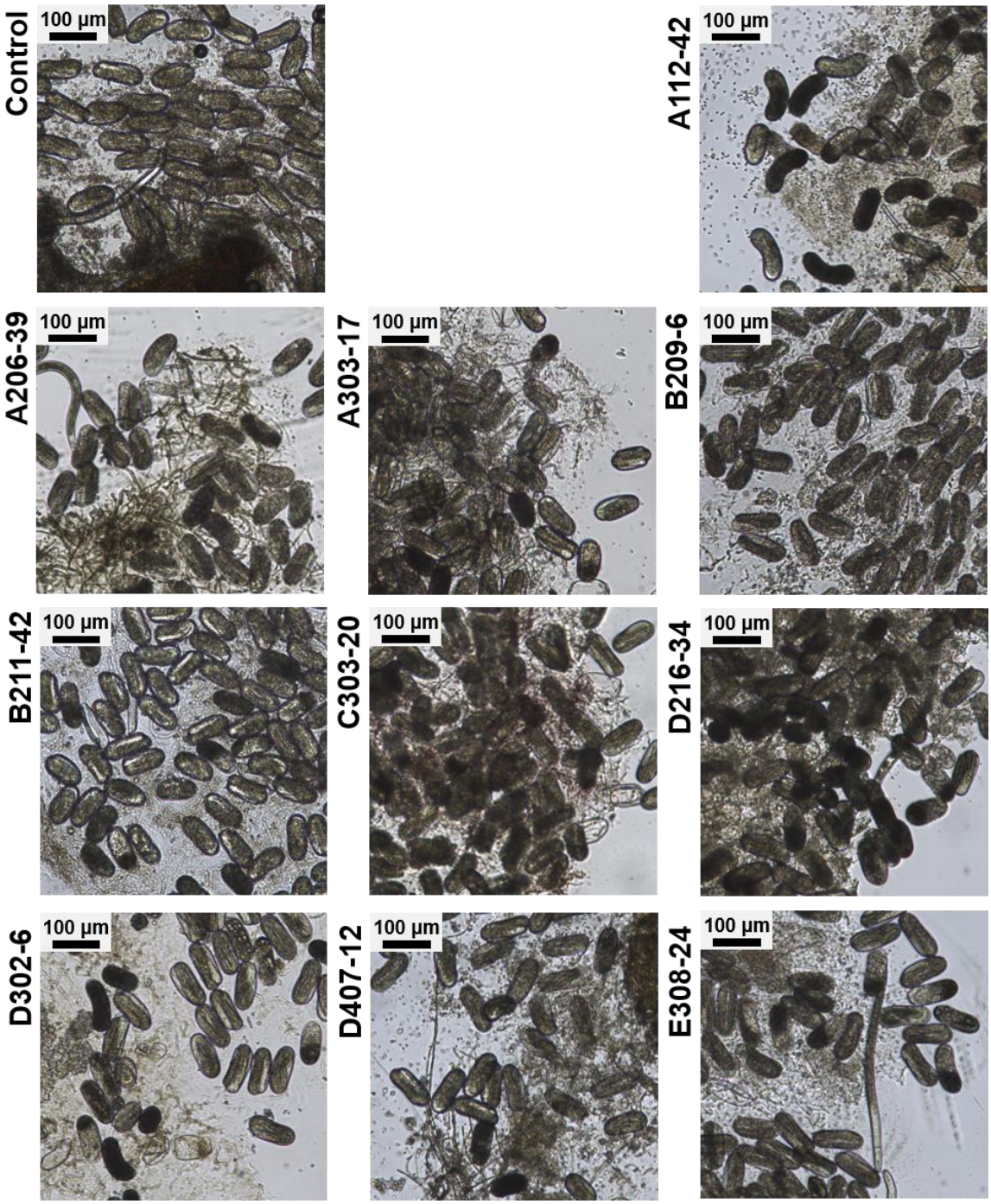
Unlike the EPI, the ability of fungi to produce anti-nemic metabolites did not diminish after sub-culturing and secondary cryopreservation. In addition to testing culture-filtrates from egg parasitic fungi on egg hatch-inhibition, we also tested the ability of the culture-filtrates from the 10 most effective hatch-inhibitors on J2 mortality. Many studies have previously investigated metabolite production as an important aspect of biocontrol. However, it is possible for metabolites to bind to sand and soil, reducing bioactivity when applied to soil. For example, flavipin was isolated from culture filtrate of a *Chaetomium globosum* strain using bioassay-guided fractionation. Although the compound had good *in vitro* effects, it did not inhibit nematodes when percolated through nematode-infected pots of plants (Nitao et al. 2002). Testing culture filtrates *in planta* on SCN infested plants is an important next step in screening for bionematicides. In fact, as discussed, there is already a commercial biocontrol product in the market, DiTera, which is comprised of the dried culture filtrate of an isolate of *Myrothecium verrucaria*. This application method may especially important if the culture filtrates have several nematotoxic compounds with synergistic or additive effect on each other (Bogner et al. 2016; Kundu et al. 2016).

2.4 Conclusions

High-throughput screening methods were used to identify candidate biocontrol agents from a large culturable mycobiome containing over 5,000 fungal isolates. Only a small percentage of fungi isolated from SCN cysts were strong parasites of SCN eggs, while a larger proportion were shown to produce compounds with bioactivity of egg-hatch inhibition or toxicity towards J2s. We identified 20 different sporulating candidate biological control agents, 10 each of high egg-parasitic and high toxic metabolite producing isolates. We tested the reproducibility of their ability to parasitize SCN eggs or

to inhibit SCN egg hatch in two separate experiments over two years after subculturing and cryopreservation. Future studies will evaluate these isolates *in planta* to determine their biocontrol potential on an SCN-susceptible soybean line.

2.5 Supplementary Figures



Supplementary Figure 2-1. Light microscopy of SCN cyst EPI assays done for the top 10 parasitic isolates.

2.6 Supplementary Tables

Supplementary Table 2-1. USEARCH OTU clustering results with NCBI and UNITE IDs for each cluster. Consensus NCBI IDs were assigned using top 20 BLAST hits of the USEARCH representative morphotypes.

Cluster	UNITE ID	NCBI BLAST ID	No. of morphotypes
1	UI sordariomycete	*Fusarium sp.*	50
2	Fusarium solani	*Fusarium sp.*	64
3	Ilyonectria macrodidyma	*Ilyonectria sp.*	29
4	UI ascomycete	*Fusarium sp.*	6
5	UI sordariomycete	*Fusarium sp.*	9
6	Exophiala equina	*Exophiala equina*	12
7	UI ascomycete	*Setophoma sp.*	10
8	UI nectriaceae	*Cylindrocarpon sp.*	31
9	UI pleosporales	*Leptosphaeria sp.*	10
10	Ilyonectria macrodidyma	*Cylindrocarpon sp.*	12
11	UI ascomycete	*Setophoma sp.*	24
12	UI sordariomycete	*Fusarium sp.*	3
13	Fusarium concentricum	*Fusarium sp.*	27
14	UI sordariomycete	*Fusarium sp.*	41
15	Fusairum sp.	*Fusarium sp.*	17
16	Hyalorbilia fusispora	*Hyalorbilia sp.*	16
17	Hyalorbilia fusispora	Hyalorbiliacea	13
18	Fusarium keratoplasticum	*Fusarium sp.*	33

19	Mortierella elongata	*Mortierella elongata*	3
20	Exophiala equina	*Exophiala spp.*	10
21	Pochonia chlamydosporia	*Pochonia chlamydosporia*	10
22	UI Hypocreales	*Mariannaea sp.*	8
23	Exophiala equina	*Exophiala equina*	15
24	UI Helotiales	*Berberis oiwakensis*	3
25	UI pleosporales	*Leptosphaeria sp.*	51
26	Corynespora cassiicola	*Corynespora cassiicola*	7
27	Clonostachys rosea	*Clonostachys sp.*	5
28	UI ascomycete	*UI ascomycete*	7
29	Mortierella elongata	*Mortierella elongata*	1
30	UI sordariomycete	*Fusarium sp.*	10
31	Ilyonectria macrodidyma	*Ilyonectria sp.*	2
32	Ilyonectria macrodidyma	*Ilyonectria sp.*	38
33	Hyalorbilia fusispora	*Hyalorbilia sp.*	1
34	Neonectria sp.	*Neonectria sp.*	6
35	Mortierella elongata	*Mortierella elongata*	4
36	UI ascomycete	*Periconia sp.*	5
37	UI ascomycete	*Fusarium sp.*	5
38	Lachnum sp.	*Lachnum sp.*	2
39	UI Pezizales	*Micronematobotrys sp.*	1
40	UI Agaricomycete	*UI Agaricomycete*	8
41	Exophiala equina	*Exophiala sp.*	9

42	UI nectriaceae	*Cylindrocarpon sp.*	4
43	Mortierella sarnyensis	*UI Zygomycete*	5
44	Micronematobotrys verrucosus	*Micronematobotrys sp.*	3
45	Ilyonectria macrodidyma	*Ilyonectria sp.*	4
46	UI pleosporales	*Calluna vulgaris*	7
47	Nectria ramulariae	*Nectria sp.*	5
48	Hyalorbilia fusispora	*Hyalorbilia sp.*	11
49	UI ascomycete	*Alternaria sp.*	1
50	Sarocladium bactrocephalum	*Sarocladium strictum*	4
51	Gibberella tricinta	*Fusarium sp.*	1
52	UI nectriaceae	*Cylindrocarpon sp.*	8
53	UI Hypocreales	*Mariannaea sp.*	7
54	Fusarium keratoplasticum	*Fusarium sp.*	1
55	Clonostachys rosea	*Clonostachys sp.*	12
56	UI pleosporales	*Mortierella elongata*	4
57	Exophiala bonariae	*Fusarium sp.*	5
58	Ilyonectria macrodidyma	*Ilyonectria sp.*	1
59	Lindgomyces apiculatus	*Trematospaheria sp.*	3
60	UI pleosporales	*Pyrenochaeta sp.*	5
61	Exophiala sp.	*Exophiala sp.*	2
62	Penicillium catenatum	*Penicillium sp.*	3
63	UI ascomycete	*Setophoma sp.*	19

64	Cladosporium delicatulum	*Cladosporium sp.*	2
65	UI ascomycete	*Zalerion sp.*	3
66	Penicillium vinaceum	*Penicillium sp.*	4
67	UI nectriaceae	*Cylindrocarpon sp.*	9
68	Talaromyces stollii	*Talaromyces spp.*	4
69	Mortierella elongata	*Mortierella elongata*	23
70	UI ascomycete	*UI ascomycete*	2
71	Plenodomus biglobosus	*Entrophospora sp.*	3
72	Pochonia chlamydosporia	*Pochonia chlamydosporia*	20
73	Exophiala sp.	*Exophiala sp.*	4
74	UI Helotiales	*Berberis oiwakensis*	2
75	Ilyonectria macrodidyma	*Ilyonectria sp.*	5
76	Exophiala opportunistica	*Exophiala opportunistica*	3
77	UI sordariomycete	*UI Sordariomycete*	2
78	Penicillium astrolabium	*Penicillium sp.*	2
79	Clonostachys rosea	*Clonostachys sp.*	19
80	UI sebacinales	*Sebacina sp.*	3
81	UI ascomycete	*Fusarium sp.*	76
82	Cladosporium delicatulum	*Cladosporium sp.*	2
83	UI Helotiales	*UI Helotiales*	1
84	UI pleosporales	*Leptosphaeria sp.*	2
85	Bjerkandera adusta	*Bjerkandera adusta*	2
86	Ophiosphaerella sp.	*Ophiosphaerella*	1

87	Purpureocillium lilacinum	*Purpureocillium lilacinum*	3
88	Fusarium solani	*Fusarium sp.*	2
89	UI nectriaceae	*Fusidium sp.*	3
90	UI Hypocreales	*Mariannaea sp.*	2
91	UI pleosporales	*UI pleosporales*	2
92	UI ascomycete	*Setophoma sp.*	6
93	Hyalorbilia fusispora	*Hyalorbilia juliae*	7
94	Neonectria sp.	*Neonectria sp.*	1
95	Alternaria alternata	*Alternaria alternata*	2
96	UI pleosporales	*Calluna vulgaris*	4
97	UI Fungus	*UI Fungus*	2
98	Trichocladium opacum	*Trichocladium opacum*	3
99	UI ascomycete	*Chloridium sp.*	2
100	UI pleosporales	*UI pleosporales*	1
101	UI Helotiales	*UI Helotiales*	3
102	Gibberella zeae	*Fusarium graminearum*	1
103	UI sordariomycete	*UI ascomycete*	2
104	UI nectriaceae	*Atractium crassum*	2
105	Lachnum sp.	*Lachnum sp.*	1
106	Simplicillium lamelicola	*Simplicillium lamelicola*	1
107	UI nectriaceae	*Fusarium sp.*	2
108	UI orbiliaceae	*Dactylellina sp.*	2
109	Exophiala equina	*Exophiala sp.*	5

110	UI orbiliaceae	*UI ascomycete*	2
111	Gibberella tricinta	*Fusarium sp.*	9
112	flagelloschypha minutissima	*flagelloschypha minutissima*	2
113	Hyalorbilia fusispora	*Hyalorbilia juliae*	1
114	Hyalorbilia fusispora	*Hyalorbilia juliae*	13
115	Pochonia chlamydosporia	*Pochonia chlamydosporia*	5
116	UI sordariomycete	*Fusarium equiseti*	4
117	Mortierella sp.	*Mortierella sp.*	2
118	Trichoderma koningiopsis	*Trichoderma sp.*	1
119	Trichoderma evansii	*Trichoderma hamatum*	1
120	UI Helotiales	*Tetracladium sp.*	3
121	UI Helotiales	*Berberis oiwakensis*	3
122	UI Hypocreales	*Cylindrocarpon sp.*	3
123	UI Lasiosphaeriaceae	*Podospora sp.*	1
124	Exophiala sp.	*Exophiala sp.*	1
125	Dinemasporium spiniicis	*Junewangia lamma*	1
126	Neonectria sp.	*Neonectria sp.*	2
127	UI ascomycete	*Alternaria sp.*	1
128	Tilletiopsis washingtonensis	*Tilletiopsis sp.*	1
129	UI Pyronemataceae	*UI Pyronemataceae*	1
130	Fusarium keratoplasticum	*Fusarium sp.*	1
131	Massariosphaeria sp.	*Clohesyomyces*	2
132	UI orbiliaceae	*Arthrobotrys sp.*	1

133	Cylindrocarpon sp.	*Nectria sp.*	1
134	UI ascomycete	*UI ascomycete*	2
135	UI Hypocreales	*UI Hypocreales*	1
136	UI Dothidiomycete	*Helicosporium sp.*	1
137	Cladosporium halotolerans	*Cladosporium sp.*	2
138	UI Sordariomycetes	*UI Diaporthales*	1
139	Sagenomella oligospora	*Sagenomella oligospora*	1
140	Glarea sp.	*Hymenoscyphus sp.*	1
141	Simplicillium lamellicola	*Simplicillium lamelicola*	1
142	UI Tremellodendropsidales	*UI Agaricales*	1
143	UI Cladosporiaceae	*Cladosporium sp.*	2
144	Penicillium citreonigrum	*Penicillium sp.*	1
145	UI ascomycete	*Tetracladium sp.*	1
146	UI ascomycete	*UI Helotiales*	3
147	UI sordariomycete	*Neonectria sp.*	2
148	UI Auriculariales	*Exidiopsis sp.*	1
149	Pseudogymnoascus roseus	*Geomyces sp.*	1
150	Mortierella horticola	*UI Fungus*	1
151	UI ascomycete	*Geomyces sp.*	1
152	Pyrenochaetopsis leptospora	*Leptosphaeria sp.*	1
153	Conlarium sp.	*Conlarium sp.*	1
154	UI Phaeosphaeriaceae	*Stagonospora sp.*	1
155	Hyalorbilia fusispora	*Hyalorbilia juliae*	1

156	UI Auriculariales	*Exidiopsis sp.*	1
157	No blast hit	*No blast hit*	1
158	UI Nectriaceae	*Cylindrocarpon sp.*	1
159	Cosmospora arxii	*Cosmospora sp.*	1
160	UI ascomycete	*Wongia sp.*	1
161	UI Hypocreales	*Neonectria sp.*	2
162	UI Helotiales	*Tetracladium sp.*	2
163	Penicillium corylophilum	*Penicillium sp.*	1
164	Hyalorbilia fusispora	*Hyalorbilia juliae*	2
165	Ceophora sp.	*UI Fungus*	2
166	Lindgomyces apiculatus	*Clohesyomyces sp.*	2
167	Hyalorbilia fusispora	*Dactylella sp.*	1
168	UI ascomycete	*Chloridium sp.*	1
169	UI Fungus	*UI Fungus*	1
170	UI Nectriaceae	*Fusarium sp.*	1
171	UI sebacinaceae	*Sebacina sp.*	1
172	UI sordariomycete	*UI ascomycete*	1
173	UI Helotiales	*UI Helotiales*	1
174	UI Fungus	*UI Fungus*	1
175	UI ascomycete	*Chloridium sp.*	1
176	Ceratobasidium sp.	*Ceratobasidium sp.*	1
177	Epicoccum nigrum	*Epicoccum nigrum*	1
178	Fusarium pseudensiorme	*Fusarium sp.*	1

179	Cladophialophora chaetospira	*Cladophialophora chaetospira*	1
180	UI Fungus	*UI ascomycete*	1
181	UI Ceratobasidiaceae	*Ceratobasidium sp.*	1
182	Chaetomium globosum	*Chaetomium globosum*	1
183	UI pleosporales	*Calluna vulgaris*	1
184	Clonostachys rosea	*Clonostachys sp.*	6
185	Leproplaca xantholyta	*UI ascomycete*	1
186	UI Helotiales	*UI Helotiales*	1
187	Simplicillium lamelicola	*Simplicillium lamelicola*	2
188	UI Agaricomycete	*Trametes sp.*	1
189	UI ascomycete	*Myrmecridium sp.*	1
190	Nectria ramulariae	*Neonectria sp.*	1
191	UI sordariomycete	*UI ascomycete*	1
192	Mortierella elongata	*Mortierella sp.*	2
193	UI Magnaporthaceae	*Junewangia sphaerospore*	1
194	Diaporthe caulivora	*Diaporthe caulivora*	1
195	UI pleosporales	*UI pleosporales*	1
196	Murispora hawksworthii	*Leptosphaeria sp.*	1
197	UI Fungus	*Mortierella sp.*	1
198	Dendryohion nanum	*Dendryphion nanum*	1
199	Chaetosphaeria sp.	*Chaetosphaeria sp.*	1
200	UI Fungus	*UI Fungus*	1

201	Conioscypha minutispora	*Conioscypha sp.*	1
202	Exophiala sp.	*Exophiala sp.*	1
203	Lachnum sp.	*Lachnum sp.*	2
204	Penicillium aethiopicum	*Penicillium sp.*	1
205	Sistotrema sp.	*Sistotrema sp.*	1
206	UI ascomycete	*Myrmecridium sp.*	1
207	UI Ceratobasidiaceae	*Ceratobasidium sp.*	1
208	Lachnum sp.	*Lachnum sp.*	2
209	Lindgomyces apiculatus	*fungal endophyte*	1
210	Cladophialophora chaetospira	*Cladophialophora chaetospira*	2
211	UI sordariomycete	*UI ascomycete*	2
212	UI Fungus	*UI ascomycete*	2
213	Purpureocillium lilacinum	*Purpureocillium lilacinum*	1
214	UI Nectriaceae	*Volutella ciliata*	1
215	UI Helotiales	*UI Helotiales*	1
216	UI pleosporales	*UI pleosporales*	1
217	Phanerochaete sp.	*Phanerochaete sp.*	1
218	Brachyphoris oviparasitica	*Brachyphoris sp.*	1
219	UI Sebacinaceae	*Sebacina sp.*	1
220	Dendryohion nanum	*Dendryphion nanum*	1
221	Cistella albidolutea	*Cistella sp.*	1
222	UI Fungus	*UI Fungus*	1

223	Acremonium persicinum	*Acremonium persicinum*	1
224	UI Sordariomycete	*UI Diaporthales*	1
225	UI Herpotrichiellaceae	*Chaetothyriales sp.*	1
226	Nectria laetidisca	*Nectria laetidisca*	1
227	Purpureocillium lilacinum	*Purpureocillium lilacinum*	1
228	Simplicillium lamelicola	*Simplicillium lamelicola*	1
229	UI Nectriaceae	*Cylindrocarpon sp.*	1
230	Microidium phyllanthi	*UI Fungus*	1
231	Talaromyces sayulitensis	*fungal endophyte*	1
232	Fusarium concentricum	*Fusarium sp.*	1
233	UI sordariomycete	*Cylindrocarpon sp.*	1
234	UI Agaricomycete	*UI Agaricomycete*	3
235	UI Halosphaeriaceae	*UI Fungus*	1
236	UI sebacinales	*Sebacina sp.*	2
237	Trichoderma petersenii	*Trichoderma sp.*	2
238	Talaromyces stollii	*Talaromyces spp.*	1
239	UI Auriculariales	*Exidiopsis sp.*	1
240	flagelloscypha minutissima	*flagelloschypha minutissima*	1
241	Dinemasporium spiniicis	*UI Fungus*	2
242	UI Helotiales	*UI Helotiales*	1
243	UI pleosporales	*Calluna vulgaris*	1
244	UI sordariomycete	*Fusarium sp.*	1
245	UI pleosporales	*Periconia sp.*	1

246	UI Tremellodendropsidales	*UI Agaricales*	1
247	UI pleosporales	*Leptosphaeria sp.*	1
248	UI pleosporales	*Leptosphaeria sp.*	1
249	Micronematobotrys verrucosus	*Micronematobotrys verrucosus*	3
250	Trichoderma longibrachiatum	*Trichoderma longibrachiatum*	1
251	Parasola lilatincta	*Prasola sp.*	1
252	UI Mortierellomycota	*UI Mortierellomycota*	1
253	Ilyonectria macrodidyma	*Ilyonectria sp.*	1
254	UI Tremellodendropsidales	*UI Agaricales*	1
255	UI Orbiliaceae	*Arthrobotrys sp.*	1
256	UI Orbiliaceae	*Monacrosporium sichuanense*	1
257	UI Orbiliaceae	*Arthrobotrys yunnanensis*	1
258	flagelloscypha minutissima	*flagelloschypha minutissima*	1
259	UI Orbiliaceae	*Arthrobotrys yunnanensis*	1
260	Mortierella sp.	*Mortierella sp.*	1
261	Mortierella sp.	*Mortierella sp.*	1
262	UI Pleosporaceae	*UI ascomycete*	1
263	Lindgomyces apiculatus	*Trematosphaeria hydrela*	2
264	Massariosphaeria sp.	*Clohesyomyces sp.*	1
265	Lindgomyces apiculatus	*Trematosphaeria hydrela*	3

266	UI Nectriaceae	*Cylindrocarpon sp.*	1
267	Ilyonectria macrodidyma	*Neonectria sp.*	1
268	Ilyonectria macrodidyma	*Ilyonectria sp.*	1
269	Trichoderma evansii	*Trichoderma hamatum*	1
270	Ilyonectria macrodidyma	*Ilyonectria sp.*	1
271	UI Nectriaceae	*Cylindrocarpon sp.*	22
272	UI Auriculariales	*Oliveonia sp.*	1
273	Lindgomyces lemonweirensis	*fungal endophyte*	1
274	UI Pezizales	*UI Fungus*	9
275	Peziza buxea	*Peziza sp.*	2
276	UI Melanommataceae	*UI pleosporales*	1
277	Dinemasporium spiniicis	*UI Fungus*	1
278	Phlebia tremellosa	*Phlebia tremellosa*	1
279	Hirsutella rhossiliensis	*Hirsutella sp.*	1
280	Ilyonectria macrodidyma	*Ilyonectria sp.*	45
281	UI Nectriaceae	*Fusarium sp.*	1
282	Didymella americana	*Phoma sp.*	2
283	UI Sordariomycete	*Fusarium sp.*	67
284	UI Sordariomycete	*Atractium crassum*	3
285	UI Pleomassariaceae	*UI Dothidiomycete*	1
286	UI ascomycete	*Setophoma sp.*	35
287	Ilyonectria macrodidyma	*Ilyonectria sp.*	5

288	UI Helotiales	*Berberis oiwakensis*	7
289	Cistella albidolutea	*Cistella sp.*	1
290	Lachnum sp.	*Lachnum sp.*	1
291	Ilyonectria macrodidyma	*Ilyonectria sp.*	16
292	Neonectria sp.	*Neonectria sp.*	2
293	Stagonosporopsis lupini	*Staganosporopsis sp.*	1
294	Diaporthe caulivora	*Diaporthe caulivora*	1
295	UI Pleosporales	*Paraphoma radicina*	9
296	Lachnum sp.	*Lachnum sp.*	4
297	UI Pyronemataceae	*UI Pyronemataceae*	1
298	Hyalorbilia fusispora	*Hyalorbilia juliae*	2
299	UI Fungus	*UI Fungus*	1
300	Penicillium decumbens	*Penicillium sp.*	1
301	Massariosphaeria	*Trametosphaeria*	1
302	Coprinellus curtus	*Coprinellus curtus*	1
303	Peziza buxea	*Peziza sp.*	1
304	UI Sordariales	*UI Fungus*	1
305	Ophiosphaerella sp.	*Ophiosphaerella sp.*	2
306	Sarocladium bactrocephalum	*Sarocladium sp.*	1
307	Cladosporium delicatulum	*Cladosporium sp.*	3
308	UI Hypocreales	*Mariannaea sp.*	5
309	Lindgomyces apiculatus	*Trematosphaeria hydrela*	1
310	UI Dothideomycetes	*UI pleosporales*	1

311	UI Nectriaceae	*Fusidium sp.*	5
312	Trichoderma virens	*Trichoderma virens*	1
313	UI Ceratobasidiaceae	*Periconia sp.*	1
314	UI Auriculariales	*Exidiopsis sp.*	1
315	Exophiala salmonis	*Exophiala sp.*	1
316	Cladorrhinum oecundissimum	*Ceophora sp.*	1
317	Podospora sp.	*Podospora sp.*	1
318	Ophiosphaerella sp.	*Ophiosphaerella narmari*	1
319	UI Agaricomycete	*Trametes sp.*	1
320	Podospora sp.	*Podospora sp.*	1
321	Purpureocillium lilacinum	*Purpureocillium lilacinum*	2
322	Ilyonectria robusta	*Ilyonectria sp.*	1
323	Cladophialophora chaetospira	*Cladophialophora sp.*	1
324	Fusarium sp.	*Fusarium sp.*	26
325	Lachnum sp.	*Lachnum sp.*	2
326	UI Nectriaceae	*Cylindrocarpon sp.*	14

Chapter 3: **IN VIVO SCREENINGS OF CANDIDATE FUNGAL BIOCONTROL ORGANISMS AGAINST SCN**

The soybean cyst nematode (SCN; *Heterodera glycines*) is the most consequential plant pathogen of the global soybean industry (Tylka and Marett 2014). Soybean comprises 57 % of all internationally traded crops, with the United States contributing 39 million tons of the 172 million tons of soybeans traded internationally in 2015 (Thoenes 2016; USDA-FAS 2018). The obligate plant pathogenic SCN causes an estimated 30 % of all soybean disease yield loss in North America, resulting in a combined estimate of USD 1.5 billion yield loss in the U.S. and Canada in 2014 alone (Allen et al. 2017). The SCN continues to pose a major problem to soybean agriculture due to the prolonged viability of SCN eggs, hardiness of the cyst structure in which the eggs are encased and protected, abundant reproduction during a single growing season, and genetic diversity among populations that allows the nematodes to overcome plant resistance (Niblack 2005; Niblack et al. 2008).

Currently available tools for the integrated management of SCN are limited. Chemical control of nematodes had involved the application of potent nonspecific fumigants such as methyl bromide (Duniway 2002) and 1,3-dichloropropene, especially on soybean fields (Barnekow et al. 1995). However, the environmental and human health concerns surrounding widespread fumigant application have led to their ban (Ristaino and Thomas 1997). Highly specific and/or low toxicity nematicides remain under several stages of development and commercial application. The employment of resistant soybean

cultivars needs reimagination due to SCN populations overcoming resistance associated with breeding line PI (Plant Introduction) 88788, a source of resistance present in more than 90 % of all currently used soybean cultivars (Niblack et al. 2008; Kandoth et al. 2011). Alternating different sources of resistance might be a better approach than using only PI 88788 varieties, to prolong durability of plant resistance (Chen 2020). Crop rotation with nonhost crops, particularly corn, and using soybean varieties that are resistant to SCN remain the only widespread methods of managing SCN populations (Porter et al. 2001). Biological control (biocontrol) of SCN offers an environmentally favorable addition to the arsenal of integrated pest management strategies against SCN. Potential new biocontrol agents could work in synergy with chemical and genetic resources to improve SCN management.

While microbes have promising properties for biocontrol of economically consequential plant parasitic nematodes, there has been limited success in development of effective products. Currently, commercially available microbial biocontrol agents include the seed treatments Poncho/VOTiVO (*Bacillus firmus* strain I-1582; Bayer CropScience, Inc.), CLARIVA® pn (*Pasteuria nishizawae*, Syngenta, Inc.), MeloCon® WG (*Paecilomyces lilacinus* strain 251, Certis USA, L.L.C.), and a fungal-free fermentation product of *Myrothecium verrucaria*, DiTera® (Valent BioSciences). Numerous greenhouse and microplot studies have demonstrated either the ineffectiveness of such biocontrol seed treatments at managing plant parasitic nematode populations *in vivo*, or suggest the necessity of integrated pest management strategies to reduce nematode populations below damaging levels (Crow 2013; K. Musil 2016; Beeman and Tylka 2018; Xiang et al. 2017; Ebadi et al. 2018). Once introduced to soil, promising biocontrol candidates face numerous abiotic and biotic factors that limit their efficacy. Abiotic

factors, such as nutrient content, soil pH, temperature, moisture and soil type affect microbial survival (Burpee 1990; Amir and Alabouvette 1993). The fungistatic effect of soil microbial communities that may outcompete biocontrol agents for nutrients and/or produce volatile compounds and other inhibitory chemicals contribute additional hurdles to real-life application of fungal biocontrol agents (Lockwood 1977; de Boer et al. 2003; Garbeva et al. 2011). Therefore, there is a need to discover new biocontrol agents and identify the most robust and effective isolates for integrated SCN management.

We recently characterized the culturable SCN cyst mycobiome from cysts from long-term soy-corn rotation plots in Waseca, Minnesota (Haarith, Hu, D. gyu Kim, et al. 2019). Certain genera, including *Fusarium*, *Ilyonectria*, *Cylindrocarpon*, *Exophiala*, *Purpureocillium*, *Trichoderma* and *Pochonia*, were isolated frequently, in concurrence with previous studies investigating the culturable SCN cyst mycobiome (Morgan-Jones et al. 1981; Gintis et al. 1983; Carris et al. 1989; Chen et al. 1994; Bernard et al. 1996; Chen and Chen 2002a; Chen 2007a). While these genera represent the most common fungi isolated in association with SCN cysts, a plethora of less-well known and less-abundant fungal genera also were isolated in our study (Haarith, Hu, D. gyu Kim, et al. 2019). In a subsequent study, we also screened this mycobiome to identify members with *in vitro* biocontrol potential against SCN (Haarith et al. 2020). Numerous bottlenecks in the screening process limit the types of microbes that can be commercialized (Fravel 1999; Whipps and Lumsden 2009). The ability to abundantly and efficiently reproduce (sporulate) in artificial conditions allows easy formulation and application of a biocontrol product. Other considerations include the number of mechanisms for biocontrol such as direct parasitism and the production of secondary metabolites, as well as long-term viability of stored cultures. We applied these principles in selecting 20 candidate fungal

biocontrol isolates, 10 demonstrating high direct parasitism towards SCN eggs and 10 producing hatch-inhibitory and/or nematotoxic secondary metabolites (Haarith et al. 2020). In the same study, we saw little correlation between these two properties.

In this study, we have evaluated the translatability of *in vitro* nematode-antagonistic properties of the 20 candidate fungal biocontrol isolates in growth chamber and greenhouse conditions. The objectives of this study were to 1) evaluate the reproduction of HG type 7 SCN on soybean plants challenged against each of the 20 candidate fungi in growth chamber cone-tainer assays, and 2) further evaluate the biocontrol potential and effects on plant growth and health of top cone-tainer performers, individually and in combination, in a greenhouse potted-plant assay against HG type 7 SCN, alongside commercially available biocontrol products against SCN.

3.1 Materials and Methods

3.1.1 Soybean, Nematode and Fungal Inoculum Preparation

SCN susceptible ‘Sturdy’ soybean seeds were sterilized using chlorine gas. A petri dish containing a single layer of soybeans was placed in a desiccator. Chlorine gas was produced by adding 5 mL of glacial hydrochloric acid to 50 mL of 10 % household bleach solution in a separate beaker placed along with the seeds inside the desiccator. The desiccator was sealed immediately with petroleum jelly and left undisturbed for about 12 h. The soybeans were aired out in a laminar airflow chamber to remove any residual chlorine gas and stored sealed at 4°C until use.

Eggs of SCN population SY-97, HG Type 7, were obtained from the University of Minnesota Southern Research and Outreach Station, Waseca, and decontaminated

using a cocktail of antibiotics (Haarith et al. 2020). Eggs were added to 50 mL of autoclaved distilled water in a 500 mL Erlenmeyer flask containing 50 ppm streptomycin, 100 ppm chlortetracycline and 250 μM Fungin™ (InvivoGen©). Eggs in antibiotic solution were incubated for 8 h at 25°C. Antibiotics-treated eggs were rinsed thoroughly with autoclaved distilled water prior to usage, within one week of preparation.

Twenty fungal biocontrol candidates denoted ‘A’ through ‘T’ (Haarith et al., 2020) were inoculated on quarter-strength potato dextrose agar (PDA) medium from -80°C glycerol stocks. Following seven days incubation at 20°C, fungal colonies were subcultured and inoculated on Spezieller Nährstroffarmer agar (SNA) medium plates to induce sporulation. Sporulation cultures were incubated at 24°C at a 12 h light/dark cycle for 14 days. Sporulating cultures were subsequently flooded with chilled 2 mL autoclaved 0.2 % aqueous Tween 20. Spores were harvested by repeatedly pipetting the 0.2 % Tween 20 onto the colony surface. Spore concentrations were determined using a hemocytometer. Spore suspensions were stored at 4°C until use, within seven days of harvesting.

3.1.2 Soil Preparation

SCN-free Nicollet clay loam soil was obtained from long-term soybean-corn rotation plots at the Southern Research and Outreach Center in Waseca, Minnesota. Fine sand was mixed in with the black soil in a 3:2 ratio and autoclaved for 2 h, dried for 24 h followed by another cycle of 2 h autoclaving and drying. This soil mixture was used for both the cone-tainer and pot assays.

3.1.3 Growth Chamber: Cone-tainer Assays

Six-inch deep plastic cone-tainers were used in the growth chamber study as growth vessels. Cone-tainers and glass marble stoppers used in the cone-tainers were soaked in 50 % household bleach solution overnight, followed by rinsing with distilled water, drying and wiping with 70 % ethanol immediately before use. Cone-tainer racks and troughs were also sterilized with 10 % household bleach solution and 70 % ethanol. Growth chamber spaces were thoroughly vacuumed, then disinfected with 50 % household bleach solution and 70 % ethanol. They were also heated to 55 °C for 8 h and cooled to 27 °C before the plants were setup. High-efficiency particulate air (HEPA) units were installed in all growth chambers to prevent cross-contamination.

Cone-tainer assay trials were prepared within growth chamber spaces and organized in a randomized complete block design. Glass marbles were first placed at the bottom of each cone-tainer. Soil was then thoroughly compacted to 1-inch depth to create a plug. Soil was added to 1-inch depth and compacted to plug the bottom of the cone-tainer and additional loose soil added to 4.5-inch depth. Antibiotics-treated SCN eggs were directly applied to the soil at 2.5-inch and 4.5-inch depths, each application equivalent to half the total nematode egg count of each inoculation level (3,000 eggs total per cone-tainer for “low” inoculation; 10,000 eggs total per cone-tainer for “high” inoculation). Two ‘Sturdy’ soybean seeds were then added to each cone-tainer and covered with soil. Growth conditions were set to 27°C during 14 h of light, and 22°C during 10 h of darkness. Plants were watered at 8- to 10-h intervals twice a day with about 5 mL autoclaved tap water.

Seven days following soybean seedlings' emergence from the soil surface, the cone-tainers were thinned to one plant each. Fungal spores of the respective isolate were either inoculated at a low or high level (1 x 10^5 spores/plant for "low" inoculation; 1 x 10^6 spores/plant for "high" inoculation). Low nematode inoculation was paired with low fungal inoculation (3,000 SCN eggs + 1 x 10^5 spores) and high nematode inoculation was paired with high fungal inoculation (10,000 SCN eggs + 1 x 10^6 spores). All treatments were evaluated with five cone-tainers per treatment. Due to resource constraints, 6 random isolates ('F', 'G', 'I', 'N', 'S' and 'T') were evaluated in one growth chamber trial, while the remaining 14 isolates were evaluated in a subsequent trial, staggered by 30 days, both with appropriate controls. The first 6 isolates will henceforth be called as Block A cone-tainers while the remaining will be called Block B cone-tainers.

Sixty days following fungal spore inoculation, a combination of sucrose flotation and sieving methods were used to wash the soil and infected soybean roots to obtain cysts (S. Chen et al. 2001). Cysts collected on a 250 μm mesh were centrifuged at 3,500 RPM for 10 minutes in 63 % sucrose solution to separate debris out. The floating cysts were decanted, and total number of cysts retrieved per growth vessel were manually counted under a stereoscope. Once cyst enumeration was complete, eggs were released from the cysts, collected on a 25 μm mesh, and adjusted to 50 mL egg suspensions. Total egg count was calculated from the average of three counts of 500 μL egg suspension aliquots on an inverted light microscope.

We also developed a numerical disease index of 1 through 5 to evaluate plant health based on phenotype at the end of 60 dpi and assessed plant health for the cone-tainers (Table 3-1). A score of 1 meant a completely healthy, green soybean plant while a score of 5 meant complete necrosis of the plant.

Table 3-1. Plant disease index – Plant health scoring rubric for cone-tainer experiments.

Plant disease index	Description
1	Healthy plant, no signs of stress
2	Leaves with minor chlorosis
3	Withering of chlorotic leaves with minor necrosis
4	Withering of leaves with completely necrotic leaves
5	Complete necrosis of plant – no standing stem with leaves

3.1.4 Greenhouse: Pot Assays

Six-inch diameter clay pots were used in the greenhouse study. They were rinsed with tap water and autoclaved prior to use. All pot assay trials were prepared and conducted within a greenhouse space. Antibiotics-treated SCN eggs were incorporated into soil at a concentration of 30,000 eggs per liter of soil by mixing and kneading soil and egg suspension in large autoclave bags. One liter of soil was added to each pot and seeded with 5 chlorine-sterilized 'Sturdy' soybeans. Pots were watered at 8- to 10-h intervals twice a day with 50 mL greenhouse tap water. Growth conditions were set to 27 °C during 14 h of light, and 22 °C during 10 h of no light. Artificial supplemental lighting was used in the greenhouse whenever necessary – 4 PM to 8 AM. One week following soybean seedlings' emergence from the soil surface, the pots were thinned down to 3 plants each. In this experiment, there was only one level of nematode inoculum (3,000 eggs/ 100 cm^3 of soil) but two levels of fungal inoculum (low and high). All treatment conditions were set up with five pots per treatment. The treatments included low (1×10^5 spores/ plant) and high (1×10^6 spores/ plant) levels of the three fungal isolates, an equal mixture of the three isolates at the high level (3.33×10^5 spores each/ plant),

recommended levels of commercially available SCN control products MeloCon® WG (4.1×10^7 spores/plant or 1.23×10^8 spores/15 cm diameter pot), DiTera (1.68 g/m^2 or 30 mg/ 15 cm diameter pot), Poncho/VOTiVO (0.13 mg active ingredients/ seed) and a 'no fungus' control. We also tested isolate 'T' as a formulation in non-fat milk as the bulking agent for application (1×10^6 spores/gram of non-fat dry milk) and lyophilized, at the high level, compared to an appropriate non-fat milk control. The pots were randomly located in the greenhouse space and were moved within the greenhouse space every two weeks at random, to minimize greenhouse microcosm effects.

Sixty days post fungal inoculation, shoots were harvested by cutting at the base of the crown and the roots were carefully separated from the soil. The plant roots were massaged and gently shaken to remove any nematodes or sandy soil attached to them. The soil from each pot was thoroughly homogenized manually, and a sample of 100 cm^3 soil was taken from each pot for estimating nematode reproduction. From this sample, cysts were floated out and crushed to release eggs as previously described for the cone-tainer assay. Only eggs from 100 cm^3 of soil were enumerated for the pot assays. The plants were dried at 35°C for 72 h and their root and shoot dry biomass were determined.

3.1.5 Data analyses and visualization

Means and standard errors for all variables assessed in the study (cysts, eggs, eggs/root biomass, and plant height, disease index, total biomass) were analyzed using R statistical software (R Core Team 2013). Analyses of variances were computed using the formula variable~fungal isolate using the aov() function and the least significant differences amongst the means were analyzed using the LSD test() function in

the“agricolae” library package (de Mendiburu 2020). All analyses were visualized using the “ggplot2” library package (Wickham 2009b).

3.2 Results

3.2.1 Growth Chamber Cone-tainer Assays

3.2.1.1 Block A cone-tainers

At high inoculation, where each cone received 10,000 SCN eggs and 1 x 10^6 fungal spores, only isolates ‘F’ and ‘N’ had significantly lower numbers of cysts than the ‘no fungus’ control. Isolates ‘G’ had higher numbers of cysts overall. Other isolates (I, S, T) were comparable to X, the ‘no fungus’ control. (Figure 3-1A). At low inoculation, where 3,000 SCN eggs and 1 x 10^5 fungal spores were inoculated in each cone for each of the treatment, all isolates had significantly lower cyst numbers compared to the ‘no fungus’ control. However, the cyst numbers did not significantly differ across the fungal treatments (Figure 3-1A).

When the cysts were crushed and the eggs were enumerated, the high inoculation, ‘no fungus’ control treatment (X) had an unusually low egg density (eggs/ 100 cm^3 soil), even lower than ‘G’ and ‘N’ fungal-inoculated treatments (Figure 3-1B). Only cone-tainers inoculated with isolate ‘T’ had comparably low levels of eggs to the ‘no fungus’ control. Egg numbers for isolates ‘F’ and ‘I’ also had lower, but not significantly different, egg-numbers compared to the ‘no-fungus’ control. In the low inoculation experiment, only isolate ‘S’ had significantly lower egg numbers than the ’no fungus’ control and all other isolates (Figure 3-1B). Isolates ‘I’ and ‘T’ had lower egg numbers, although these were not significantly different from the “no-fungus” control, while

isolates ‘G’ and ‘N’ had the highest amounts of eggs (Figure 3-1B). No cysts or eggs were found in cone-tainers that were only inoculated with fungi.

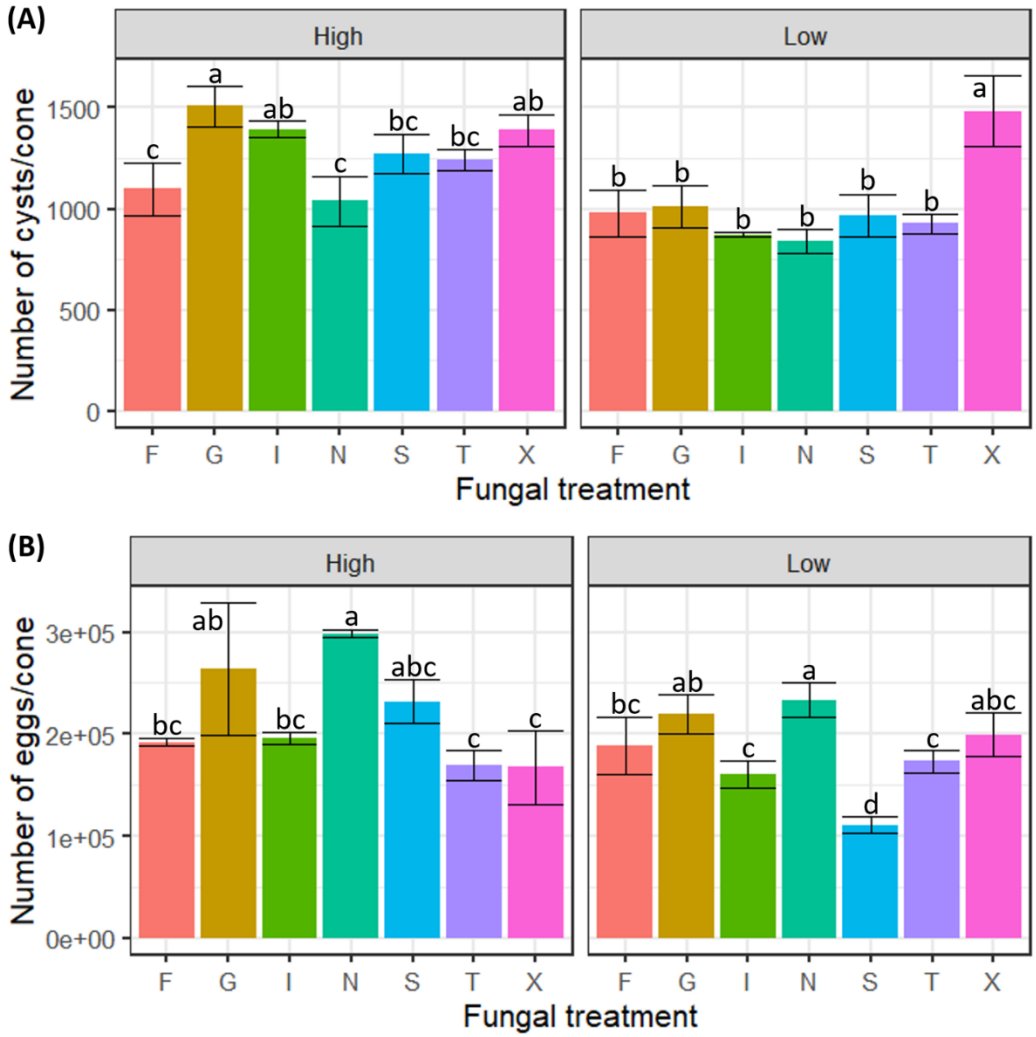


Figure 3-1. (A) Mean cyst numbers for block A cone-tainers inoculated with fungi, with or without SCN. (B) Mean egg density (eggs/100 cm3 cone-tainer) for block A cone-tainers inoculated with fungi, with or without SCN. Error bars are standard error with LSD groups as lowercase alphabets. High: 10,000 SCN eggs + 1 x 10^6 spores. Low: 3,000 SCN eggs + 1 x 10^5 spores.

In terms of plant height, in nematode plus fungal inoculation at high levels, isolate ‘T’ produced significantly taller plants than any other isolate (Figure 3-2A). The height of plants inoculated with the other fungal isolates were not significantly different from those of the ‘no fungus’ control plants (Figure 3-2A). At lower inoculation, isolates ‘G’ and ‘N’

produced significantly taller plants than the rest of the isolates, and all the other isolates were not significantly different from the 'no fungus' control (Figure 3-2A). When only fungi and no nematodes were inoculated, the plants were generally taller than when nematodes were also inoculated; 16-21 cm range vs 10-18 cm range (Figure 3-2B).

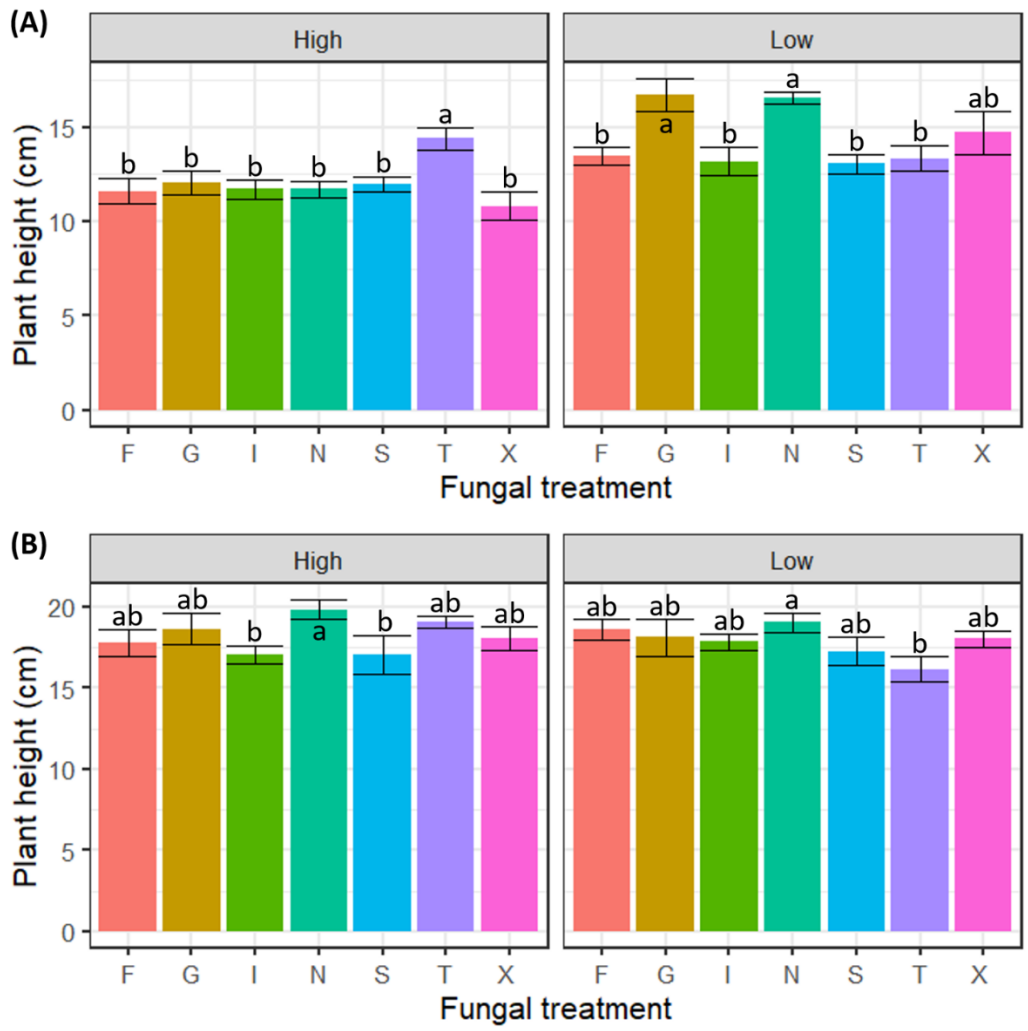


Figure 3-2. Mean plant height in cm for block A cone-tainers (A) inoculated with fungi and SCN (B) inoculated with only fungi. Error bars are standard error with LSD groups as lowercase alphabets. High: 10,000 SCN eggs + 1 x 10^6 spores. Low: 3,000 SCN eggs + 1 x 10^5

Plant heights of all isolates were not statistically different from to the 'no fungus' control in both low and high inoculations of fungi alone. However, isolate 'N' produced taller plants than 'I' and 'S' at high inoculation, and taller than 'T', at low inoculation.

Isolate ‘T’ showed a notable increase in height between the high and low inoculations (Figure 3-2B).

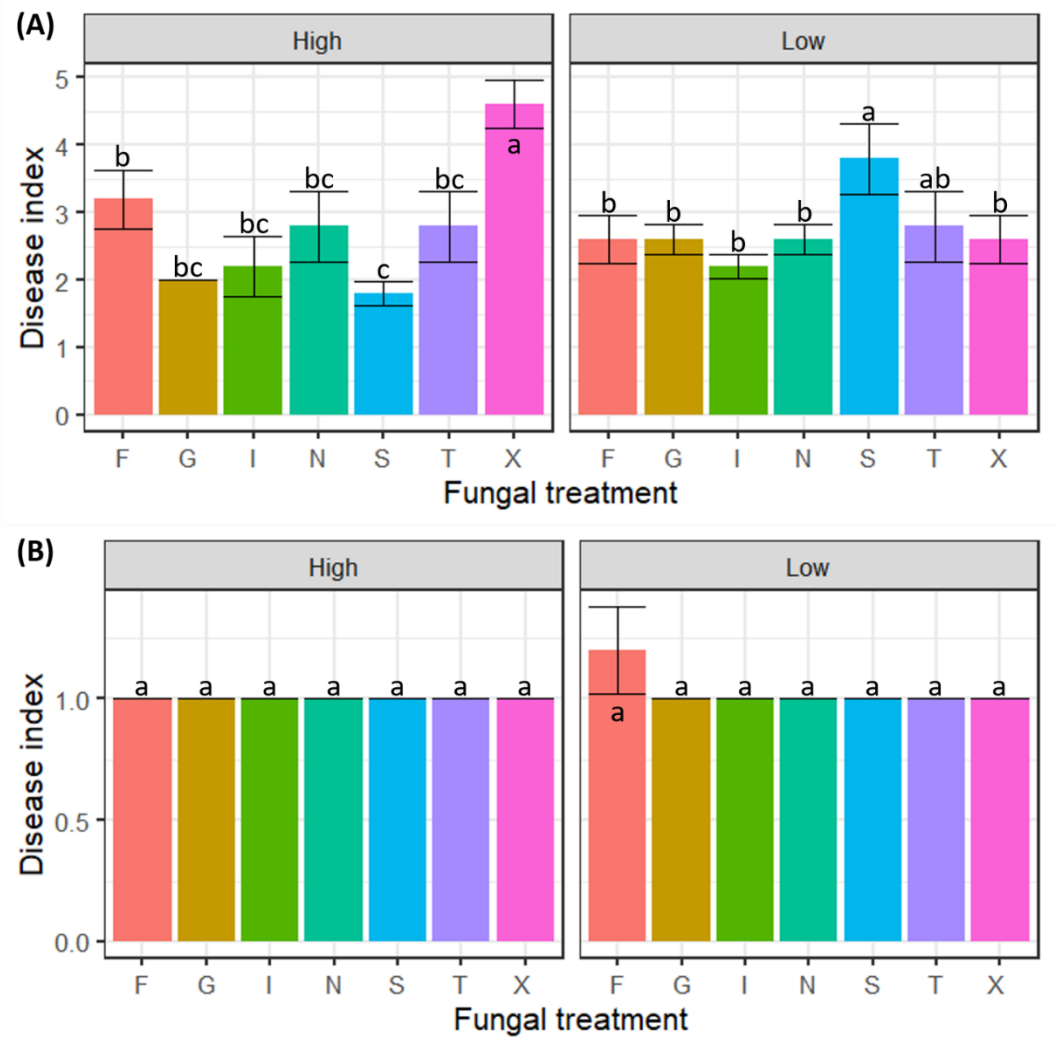


Figure 3-3. Mean disease index for block A cone-tainers (A) inoculated with fungi and SCN (B) inoculated with only fungi. Error bars are standard error with LSD groups as lowercase alphabets. High: 10,000 SCN eggs + 1 x 10^6 spores. Low: 3,000 SCN eggs + 1 x 10^5 spores.

With respect to plant health, it is noteworthy that all the ‘no fungus’ control plants (‘X’) inoculated with nematodes died at 40 dpi in the high inoculation experiment and their disease indices were more than 4.5 (Figure 3-3A). All isolates at the high inoculation level had live plants at the end of the experiment and had significantly lower

disease indices compared to the ‘no fungus’ control. Isolate ‘S’ had the lowest disease index among the isolates in the high inoculation experiments, but only significantly lower than ‘F’ (Figure 3-3A). No difference in disease indices was detected among other isolates. However, in the low inoculation experiments with both nematodes and fungi, isolate ‘S’ had a significantly higher disease index than the ‘no fungus’ control. The disease indices of all isolates except ‘S’ did not differ significantly from the ‘no fungus’ control or each other (Figure 3-3A). When nematodes were inoculated on to the plants, both at high and low levels, the plants were under some disease stress irrespective of the fungal treatment. Aside from isolate ‘S’, most treatments scored between 2 and 3.5 on the disease index, in contrast to about 4.5 in the no-fungus control. In contrast, when only fungi were inoculated on the plants, all plants were healthy (disease index ~1) and comparable to the ‘no fungus’ control at both low and high inoculation levels (Figure 3-3B).

3.2.1.2 Block B cone-tainers

At high inoculation levels of both fungi and nematodes, isolate ‘P’ produced significantly higher numbers of cysts compared to the ‘no fungus’ control (Figure 3-4A). Isolate ‘O’ also had slightly higher mean cyst numbers but was not significantly different from the control. Isolates ‘A’, ‘D’, and ‘R’ had significantly lower cyst numbers compared to the ‘no fungus’ control (Figure 3-4A). Isolates ‘B’ and ‘E’ also had lower, but not statistically different, cyst numbers compared to the control. At low co-inoculation levels of fungi and nematodes, similar to the high inoculation experiments, isolates ‘M’, ‘O’ and ‘P’ had significantly higher numbers of cysts compared to the ‘no fungus’ control and isolates ‘A’, ‘D’, ‘C’ and ‘Q’ (Figure 3-4A). Isolates ‘A’ and ‘C’ had significantly lower number of cysts compared to isolates ‘H’, ‘J’, ‘K’. No significant

differences were detected amongst other treatments, including the ‘no fungus’ control (Figure 3-4A).

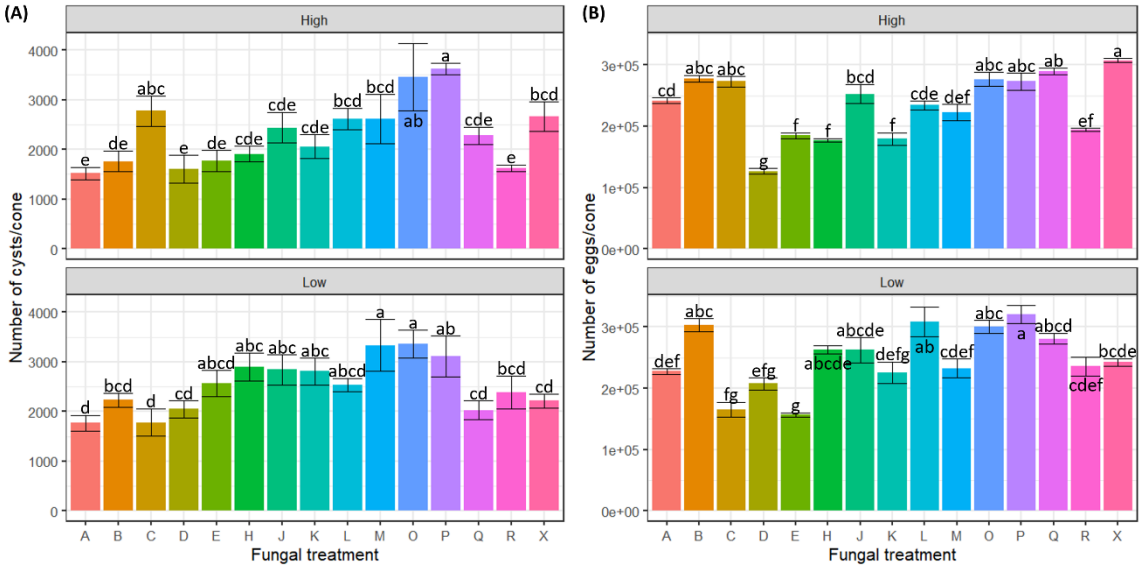


Figure 3-4. (A) Mean cyst numbers for block B cone-tainers inoculated with fungi, with or without SCN. (B) Mean egg density (eggs/100 cm^3 cone-tainer) for block A cone-tainers inoculated with fungi, with or without SCN. Error bars are standard error with LSD groups as lowercase alphabets. High: 10,000 SCN eggs + 10^6 spores. Low: 3,000 SCN eggs + 10^5 spores.

Despite only having a moderate number of cysts (Figure 3-4A), the ‘no fungus’ control had the greatest number of eggs in the high level of inoculation experiments (Figure 3-4B). A majority of isolates (‘A’, ‘D’, ‘E’, ‘H’, ‘J’, ‘K’, ‘L’, ‘M’, and ‘R’) all produced significantly lower egg densities compared to the ‘no fungus’ control. Isolate ‘D’ in particular had significantly lower egg-densities than all other isolates and reduced the egg counts by about 58 %. Isolates ‘E’, ‘H’, and ‘K’ reduced egg population density by about 40 % and isolate ‘R’ by about 33 %, compared to the ‘no fungus’ control (Figure 3-4B). At the low inoculation levels of fungi and nematodes, only isolates ‘C’ and ‘E’ had significantly lower egg densities compared to the ‘no fungus’ control. Isolate ‘D’ had the next lowest egg-density but was not significantly lower than the control. Amongst the

other isolates, isolate ‘P’ had significantly higher egg density compared to the control (Figure 3-4B), while isolates ‘B’, ‘L’, and ‘O’ also had higher mean egg densities but were not significantly different from the control. No cyst or eggs were recovered from cone-tainers with only fungal inoculations.

There were no significant differences in heights of most plants inoculated with different fungal isolates at high inoculation levels when nematodes were co-inoculated (Figure 3-5A), Only isolate ‘A’ produced significantly shorter plants compared to the ‘no fungus’ control (Figure 3-5A). At low inoculation level, none of the isolates showed significant differences in height compared to the ‘no fungus’ control (Figure 3-5A).

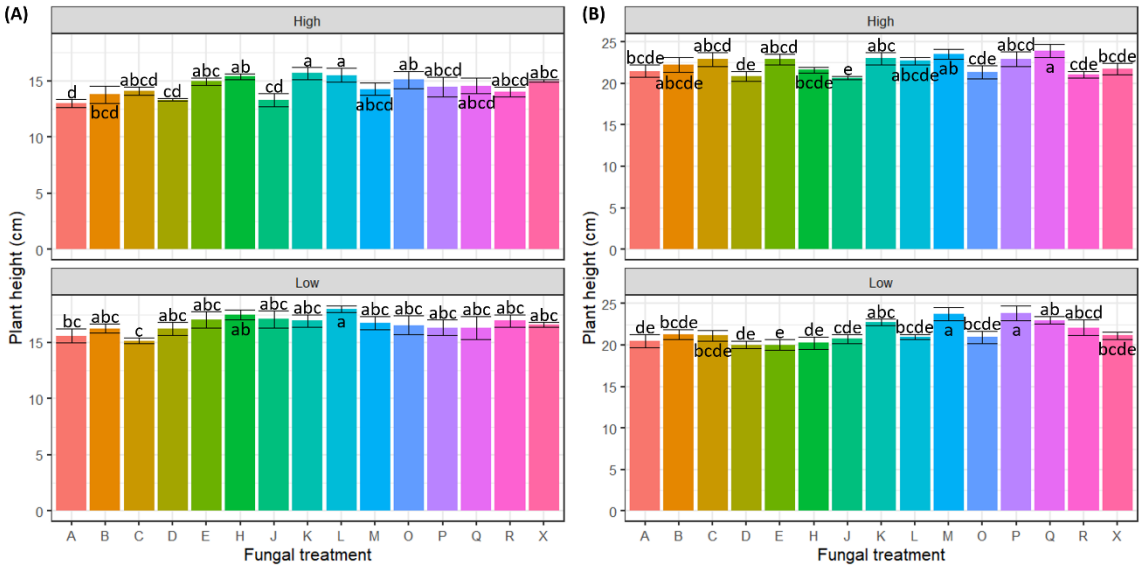


Figure 3-5. Mean plant height in cm for block B cone-tainers (A) inoculated with fungi and SCN (B) inoculated with only fungi. Error bars are standard error with LSD groups as lowercase alphabets. High: 10,000 SCN eggs + 1 x 10^6 spores. Low: 3,000 SCN eggs + 1 x 10^5 spores.

When only fungi were inoculated on plants, only the isolate ‘Q’ produced significantly taller plants at the high inoculation level, and ‘M’ and ‘P’ produced significantly taller plants at the low inoculation level compared to the “no fungus’ control. Although there are slight differences detected among other isolates, there were no

significant differences in plant heights amongst these isolates or the ‘no fungus’ control plants (Figure 3-5B). However, it is evident that nematode inoculation produced shorter plants in general. SCN inoculated plants were 15-18 cm tall, while those with only fungi ranged between 20-24 cm.

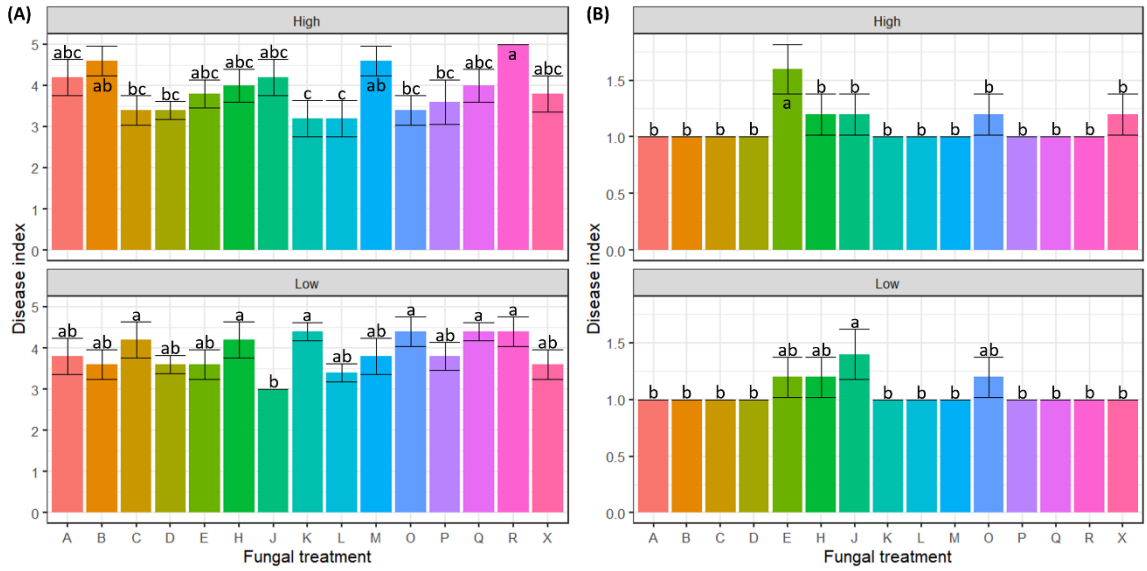


Figure 3-6. Mean disease index for block B cone-tainers (A) inoculated with fungi and SCN (B) inoculated with only fungi. Error bars are standard error with LSD groups as lowercase alphabets. High: 10,000 SCN eggs + 1 x 10^6 spores. Low: 3,000 SCN eggs + 1 x 10^5

The plant disease indices for the plants inoculated with both nematodes and fungi were higher than those with just the fungal inoculation. At high inoculation, isolates ‘C’, ‘D’, ‘K’, ‘L’, ‘O’, and ‘P’ had slightly, but not significantly, lower disease indices (<3.5) than the ‘no fungus’ control (Figure 3-6A). In contrast, plants with nematodes and isolate ‘R’ were all dead by the end of the experiment and scored 5 on the disease index. At low co-inoculation level, isolate ‘J’ scored a slightly lower disease index compared to other isolates and the ‘no fungus’ control. Isolates ‘B’, ‘D’, ‘E’, ‘J’, ‘L’, ‘M’ and ‘P’ were all below 4 index points, but were not significantly different from the ‘no-fungus’ control.

When no nematodes were co-inoculated, at both high and low levels, plants were healthy and scored disease indices between 1 and 1.5 (Figure 3-6B).

Considering both cone-tainer experiments, only isolates 'D', 'E' and 'T' showed consistent and substantial reduction of nematode reproduction (eggs) at both levels of inoculation and had lower disease indices. Hence, these three isolates were chosen for further testing in a greenhouse pot assay.

3.2.2 Greenhouse Pot Assays

It is important to remember that for the greenhouse pot assays, the nematode inoculation levels were set at a constant of 3,000 SCN eggs/ 100 cm^3 of soil across all the different fungal treatments. The low and high levels for these assays only refer to the levels of fungal inoculation of 1 x 10^5 spores/ plant or 1 x 10^6 spores/ plant respectively.

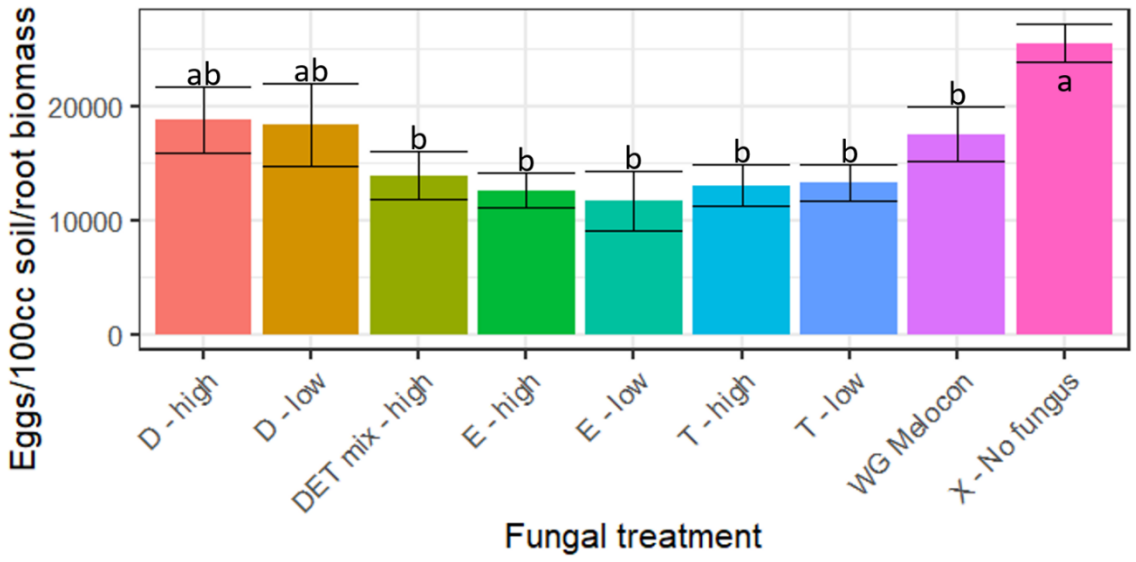


Figure 3-7. Mean egg density/ g dry root biomass of pots treated with different isolates at both high (1 x 10^6) and low (1 x 10^5) levels of fungal spore inoculation per plant and SCN eggs at 3,000/100 cm^3 of soil. 'X' is the no fungus control and WG MeloCon® WG is the commercial fungal biocontrol applied at 1.23 x 10^8 spores per pot recommended rate. Error bars are standard error with LSD groups as lowercase alphabets.

3.2.2.1 First experiment

In the first experiment, isolate 'E' and 'T', the DET mixture (equal mix of isolates 'D', 'E' and 'T' applied at 1 x 10^6 spores/ plant – high level), and the commercial control Melocon® WG (applied at 4.1 x 10^7 spores/ plant – recommended level) significantly reduced nematode reproduction (SCN egg density/ g root biomass) compared to the 'no fungus' control (Figure 3-7). Isolate 'D' also reduced egg-density, but not significantly, compared to the control. Isolate 'D' and Melocon® WG produced egg concentrations 33 % lower than the 'no fungus' control, while isolates 'E', 'T' and the DET mixture reduced SCN numbers per unit root biomass by 50 % compared to the 'no fungus' control.

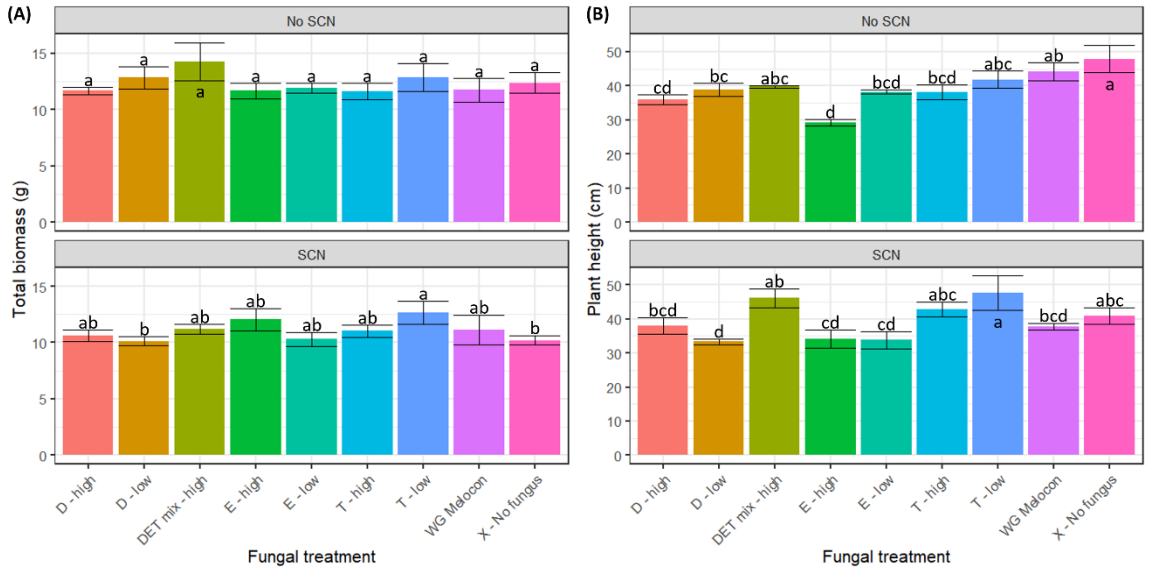


Figure 3-8. ***Exp. 1:*** *(A) Mean total plant dry biomass in g (B) Mean plant height in cm, with and without SCN for different fungal treatments. 'X' is the no fungus control and MeloCon® WG is the commercial fungal biocontrol applied at 1.23 x 10^8 spores per pot recommended rate. Error bars are standard error with LSD groups as lowercase alphabets.*

When nematodes and fungi were co-inoculated, the total biomass across the treatments did not vary much (Figure 3-8A). Treatment 'T - low' had the highest biomass

and was statistically greater than the ‘no-fungus’ control and isolate ‘D’ at the low level. However, ‘T - low’ total biomass did not differ significantly from the other treatments. When only fungi were inoculated on the plants, there were no significant differences in total biomass across the treatments, including the ‘no fungus’ control, although the DET mixture and isolate ‘T’ were slightly higher (Figure 3-8A).

Treatment ‘T - low’ also produced significantly taller plants than the rest of the fungal treatments, with the exception of the DET mixture and ‘T-high’ treatments (Figure 3-8B). The DET mixture also produced plants that were significantly taller than isolate ‘D-low’ and isolate ‘E’ applied at both levels. However, although the DET mixture and ‘T’ treatments produced plants that were slightly taller than most treatments, they were not significantly taller than the ‘no fungus’ control. It is important to note that isolate ‘E’ at both levels of inoculation, as well as treatment ‘D - low’ produced the shortest plants in the experiment. When only fungi and no nematodes were inoculated, ‘E - high’ produced the shortest plants while ‘no fungus’ control plants were the tallest (Figure 3-8B). All other treatments did not differ significantly amongst each other. The ‘no fungus’ control was etiolated, unlike other treatments in the greenhouse conditions, for both no nematode and nematode conditions.

3.2.2.2 Second experiment

In the second experiment, MeloCon® WG, applied at 4.1 x 10^7 spores/plant (recommended rate) and treatment ‘E - low’ applied at a much lower rate (1 x 10^5 spores/plant) both performed slightly better and showed lower nematode numbers than the ‘no fungus’ control and other treatments. However, there were no statistically significant differences amongst any of the treatments, including the ‘no fungus’ control. The commercial bacterial+chemical product, Poncho/VOTiVO, had the highest numbers

of SCN reproduction (Figure 3-9). In general, SCN reproduction was at least 3-fold higher during the second experiment compared to the first one (Figures 3-7 and 3-9).

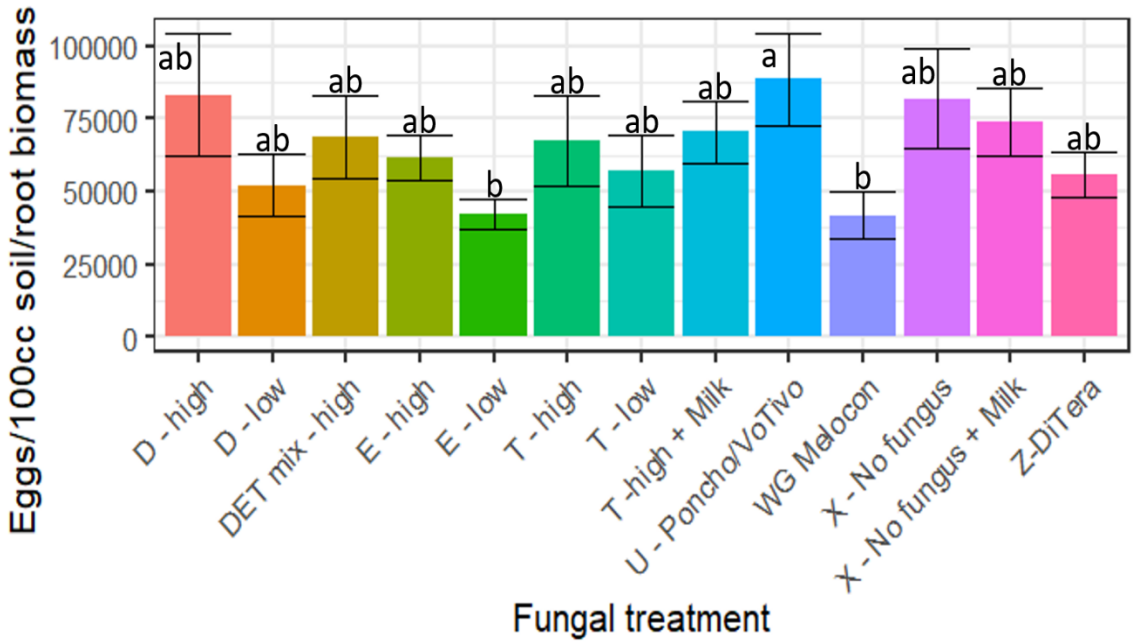


Figure 3-9. ***Exp. 2:*** *Mean egg density/ g dry root biomass of pots treated with different isolates at both high (1 x 10^6) and low (1 x 10^5) levels of fungal spore inoculation per plant and SCN eggs at 3,000/100 cm^3 of soil. 'X' is the no fungus control and WG MeloCon® WG is the commercial fungal biocontrol applied at 1.23 x 10^8 spores per pot recommended rate. Error bars are standard error with LSD groups as lowercase alphabets.*

The total biomass across treatments did not vary significantly across the treatments when fungi and nematodes were co-inoculated (Figure 3-10A). In the absence of nematodes, with only fungi inoculated, only Melocon® WG was significantly higher than the 'no fungus' control (Figure 3-10A). However, the DET mixture, 'E - high' and 'T - low' were also slightly higher than the other treatments (excluding 'T - high + Milk' and 'X – no fungus + Milk') and were not significantly different from the 'no fungus' control. When isolate 'T' was bulked up and lyophilized with non-fat dry milk, it had slightly, but not significantly, higher biomass compared to 'T - high', both with and

without nematodes. Similarly, the 'X – no fungus + Milk' had a significantly higher biomass compared to the 'no fungus' control treatment (Figure 3-10A).

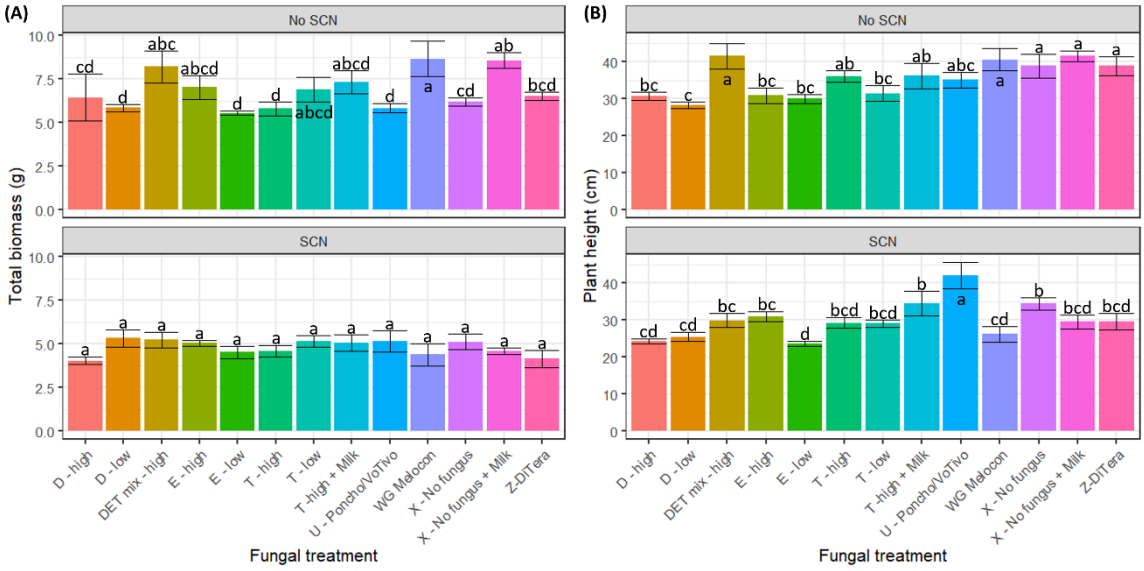


Figure 3-10. ***Exp. 2:*** *(A) Mean total plant dry biomass in g (B) Mean plant height in cm, with and without SCN for different fungal treatments. 'X' is the no fungus control and MeloCon® WG is the commercial fungal biocontrol applied at 1.23 x 10^8 spores per pot recommended rate. Error bars are standard error with LSD groups as lowercase alphabets.*

As in the first experiment, plants with no fungus and Poncho/VOTiVO were significantly taller compared to other fungal treatments and the 'no-fungus' control when both nematodes and fungi were co-inoculated (Figure 3-10B). In contrast, all the other treatments including Melocon® WG produced shorter plants. When there were no nematodes added, isolates 'D' and 'E' at both levels and isolate 'T' at the low level produced significantly shorter plants compared to MeloCon®, DiTera, and the 'no-fungus' control (Figure 3-10B).

3.2.3 Pod development

In the first experiment, when plants were inoculated with both nematodes and fungi, treatment 'E - high' produced significantly more pods per pot compared to 'E-low',

'T-high', and the 'no-fungus' control (Figure 3-11A), which produced the least number of pods. All the other treatments had an intermediate level of pods per pot, in between those of 'E - high' and 'no fungus' control and were not significantly different amongst themselves. When only fungi were inoculated, there were no significant differences across the fungal treatments, including the 'no fungus' control (Figure 3-11A), although the DET mixture and isolate 'T' produced slightly more pods than other treatments.

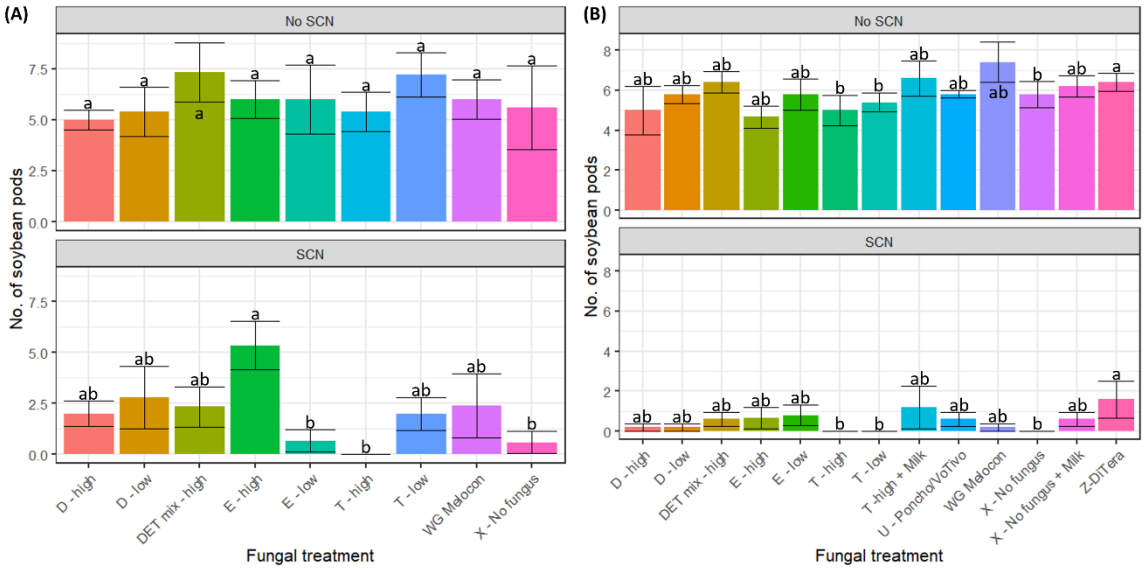


Figure 3-11. Mean pod production per pot for (A) first greenhouse experiment (B) second greenhouse experiment, with and without SCN, for different fungal treatments. 'X' is the no fungus control and WG MeloCon® is the commercial fungal biocontrol applied at 1.23 x 10^8 spores per pot recommended rate. Error bars are standard error with LSD groups as lowercase alphabets.

The pattern for pod development was similar in the second experiment. In the presence of both fungal and nematode inoculum, 'T - high', 'T - low', and the 'no fungus' control developed the least number of pods per pot, significantly lower than DiTera®, which had the highest numbers of pods (Figure 3-11B). All the other isolates had intermediate numbers of pods and not statistically different from to each other or the 'no fungus' control. When only fungi were inoculated, treatment 'D - low', the DET mixture,

‘E - low’, ‘T - high + Milk’ and Melocon® WG had slighter greater, but not significantly, more pods than the ‘no fungus’ control. In both experiments, numbers of pods were considerably reduced across treatments in the presence of nematodes compared to the fungal only inoculations.

3.3 Discussion

In this study, we have evaluated the *in vivo* biocontrol potential of 20 candidate fungal agents that were identified through *in vitro* screening of a large SCN cyst mycobiome (Haarith et al. 2020). We designed the cone-tainer assays as an initial *in planta* screen to identify the three isolates that most reduced nematode reproduction under both low and high nematode and fungal inoculum, while not being detrimental to plant growth or health. For the cone-tainer assay, the ability of fungal isolates to reduce nematode numbers was given more weight than their effects on plants. This principle is similar to the Female Index (FI) used to assess genetic resistance in soybean varieties (Riggs et al. 1981). The cone-tainer is not an optimal environment for the plants to grow. Hence, we developed and used a disease scale (Table 1), instead of measuring plant biomass and pod development, for the cone-tainer assays. For this initial screen, we tested six isolates first, due to limitations of growth chamber space, and tested the remaining 14 isolates in a separate experiment. Both experiments maintained identical light and temperature regimes in the growth chambers and each experiment had its own ‘no fungus’ control. All these experiments were conducted in growth chambers of the same make and model, and the same running conditions. Nonetheless, because of the potential for slight variation between experiments, these two experiments were evaluated separately in comparison to their respective control.

We did not observe any of the fungi to be pathogenic on the plants when inoculated without the nematodes. It is noteworthy that the difference between low and high fungal inoculation was ten-fold, while the difference between low and high nematode inoculation was only about three-fold. According to the University of Minnesota extension guidelines (University of Minnesota 2018), farmers are advised not to grow even resistant soybean varieties when there are more than 10,000 eggs/100 cm^3 of soil. Therefore, the high-level nematode inoculation at 10,000 eggs/100 cm^3 soil was included to screen for isolates that can show some effect even at that high level. In general, if there are 3,000 SCN eggs/100 cm^3 of soil, it is recommended to use a resistant variety. For these experiments, we used 'Sturdy', a susceptible soybean variety, to give the nematodes the upper hand in this interaction. Thus, the conditions for this initial screen were stressful conditions for the plant and intended to identify only those fungi with a strong effect on reducing nematode populations. From this initial screen, we chose the three isolates that most significantly reduced the egg-densities and performed well at both the low and high inoculation levels for further greenhouse testing.

Previous research suggests that fungal isolates may either reduce the number of nematode infections on plants or decrease the fecundity of nematodes (Muller 1982; Crump 1991; Crump and Irving 1992; Zaki A Siddiqui and Mahmood 1995) by reducing the number of eggs contained in each cyst. Therefore, analyzing cyst numbers alone will not fully capture rates of reproduction of the nematode. Furthermore, irrespective of the number of cysts, the number of eggs in soil is the most important measure as they are the actual source or inoculum and new nematode infections. We evaluated the numbers of both the cysts and eggs for the cone-tainer assays to measure these different aspects of nematode reproduction. In the block A cone-tainers, although isolates 'S' and 'T' had

lower egg counts than the 'no-fungus' control at the low level, only isolate 'T' consistently had low egg numbers at both low and high inoculation levels (Figure 3-1B). In block B cones, although isolates 'C', 'D' and 'E' had lower egg population densities than other isolates at the low inoculation level, isolate 'C' did not perform well at the high inoculation level (Figure 3-4B). Thus, only those isolates that most reduced egg population densities at both the low and high levels ('D', 'E', and 'T') were selected for further testing in the greenhouse pot assay.

For the greenhouse pot assays, we measured the egg density in a 100 cm^3 sample of soil normalized with root biomass (eggs/100 cm^3 soil/ g dry root biomass) and did not enumerate all the cysts from the pot, due to time and labor constraints and the fact that egg density is the most meaningful measure of nematode reproduction. For these pot assays, we also tested an equal mixture of all three isolates (DET mixture) inoculated at the high level (total 1 x 10^6 spores/plant and 3.33 x 10^5 spores of each isolate) to investigate any possible synergistic effects.

The first set of pots were set-up in mid-November 2019 and harvested at the end of January 2020. The second experiment was set up towards the end of December 2019 and harvested in the first week of March 2020. Although greenhouses were heated and maintained the same light: dark regime, slight temperature variations and differences in the amount of artificial supplementary light usage could explain the differences in results between the two experiments. The nematode proliferation for the same initial inoculum was three folds higher across the second experiment, especially the 'no fungus' control (Figures 1, 3). In fact, Barker et al., (1975) reported a several fold increase in SCN reproduction under metal halide and incandescent light sources than under natural light. Similarly, Hill and Schmitt (1989) showed that slight variations in temperature could

impact nematode reproduction under greenhouse conditions. Although greenhouse temperatures were set at 27 °C during 14 h of light, and 22 °C during 10 h of no light for both experiments, it is possible that soil temperatures in the two experiments could have been slightly different and affected SCN activities including hatching, penetration, growth and reproduction. The plant biomass across all the treatments decreased in the second experiment compared to the first (Figures 3-8A and 3-10A) by nearly half, which could be due in part to the increased nematode proliferation. Similarly, the plant height range for first experiment was between 35 cm and 50 cm for nematode and no nematode conditions respectively (Figure 3-8B), while, for the second experiment, the height was lower and ranged between 22 cm and 45 cm (Figure 3-10B). We included several additional commercially available biocontrol products as controls in the second experiment. Isolate 'T' was also applied as a formulation of lyophilized spores with non-fat dry milk as a bulking agent. For these reasons, these two experiments have also been evaluated separately.

With respect to nematode control, in the first experiment, isolates 'E' and 'T', and the DET mixture performed as well as the commercial biocontrol agent MeloCon® WG applied at the recommended concentration of 6 pounds of granules (1×10^{10} spores/ g) per acre. For a 6-inch pot, considering 6 inches of topsoil, each pot was inoculated with a total of 1.23×10^{8} spores MeloCon® WG. Therefore, for three plants per pot, we inoculated 4.1×10^{7} spores of MeloCon® WG per plant compared to 1×10^{6} (high) or 1×10^{5} (low) spores of our isolates per plant. The DET mixture, isolates 'E' and 'T', and MeloCon® WG all showed significant reductions in egg counts compared to the 'no-fungus' control. Isolate 'D' also showed slightly lower egg-densities but did not differ significantly from the 'no-fungus' control. Although not statistically significant, the DET

mixture and isolates ‘E’ and ‘T’ also showed slightly lower SCN reproduction than MeloCon® WG.

Biological agents interact with both the plant and the nematodes. Therefore, it is crucial to evaluate their effects on plants, as well as on nematode reproduction. As a 6-inch pot is a more realistic and less restrictive environment for root growth than cone-tainers and the greenhouse space is a more natural environment for plant growth than growth chambers. Therefore, for pot experiments, we evaluated additional plant health and growth parameters. Furthermore, we have expressed nematode reproduction in terms of egg density per gram root dry biomass (Stetina and Young 2006; Giné et al. 2013), as potential effects of biological agents may include increasing plant root growth, thereby increasing root surface available for nematode reproduction.

In the second experiment, where nematode densities were much higher, our isolates at the low inoculation level, and the commercial agents MeloCon® WG and DiTera®, resulted in slightly lower nematode population density than the ‘no fungus’ control. In particular, isolate ‘E’ and MeloCon® WG showed the greatest reduction in nematode reproduction, with isolate ‘E’ performing as well as MeloCon® WG at a much lower application rate. In contrast, Poncho/VOTiVO had the highest nematode reproduction of all treatments, even higher numbers than the ‘no fungus’ control. As discussed earlier, we hypothesize that the differences in light and heat conditions in the greenhouse in this second experiment may have created more favorable conditions for nematode reproduction in the second experiment, and the rate of nematode hatching and reproduction might have outpaced the capacity of 1×10^6 or 1×10^5 initial fungal spores applied as inoculum. The higher application rate (40-400 times that of our fungal

inoculum) of MeloCon® WG spores could explain its better performance despite higher nematode reproduction rates.

It is necessary to consider the biology of the SCN when interpreting statistical results of biocontrol experiments, especially with respect to nematode reproduction. Unlike a simple unit like total biomass or plant height, nematode reproduction is normalized for both volume and root biomass because its reproduction is dependent on initial population densities. At low population density, reproduction can be exponential. In the first pot experiment, although a mean of 16,000 eggs/ 100 cm^3 / g root biomass is not significantly different from 11,000 eggs/ 100 cm^3 / g root biomass, an extra inoculum of about 4,000 eggs/ 100 cm^3 / g root biomass could still be biologically significant for plants, especially above a threshold of 10,000 egg/100 cm^3, where SCN becomes very damaging to plant health. It is interesting that low level inoculations of both isolates 'D' and 'E' performed better than their higher-level inoculations. This could mean that the dose-dependent effect for these isolates need not be collinear.

Plants from 'no fungus' control and Poncho/VOTiVO inoculations were etiolated and hence, taller. However, their biomass was lower than the other treatments (Figures 3-8 and 3-10). In contrast, isolate 'E' and the DET mixture inoculations produced shorter plants with higher biomass, comparable to MeloCon® WG applied at 40-400 folds higher (Figures 3-8 and 3-10). Additionally, the numbers of nematodes in these three treatments were lower compared to other treatments (Figures 3-7 and 3-9). These three treatments also had higher soybean pod production (Figure 3-11). These results indicate that isolate 'E', the DET mixture and MeloCon® WG can manage plant stress due to nematodes better than the other treatments. It is also highly likely that the isolates complement each other in the DET mixture. For example, both isolate 'T' and the DET mixture slightly

increased plant height and biomass compared to either isolate ‘D’ or ‘E’ alone and performed comparably in terms of nematode control to isolate ‘E’ alone. Given the strong performance of our isolates at equivalent or much lower application rates compared to commercially available products, these isolates show promise as new biocontrol agents for the SCN.

In conclusion, we have tested 20 fungal isolates with high *in vitro* biocontrol ability for their *in vivo* efficacy using a growth chamber cone-tainer assay, as well as a greenhouse pot assay. In the growth chamber assays, we identified three isolates that produced robust results under both high (1×10^6 fungal spores + 10,000 eggs/ 100 cm^3) and low (1×10^5 fungal spores + 3,000 eggs/ 100 cm^3) inoculation levels. These three isolates were further evaluated to be as effective as several commercially available biocontrol products, even at 40 to 400-fold lower spore concentrations in greenhouse assays. We also observed positive effects of a mixture of our isolates on all four parameters tested (nematode control, plant height, plant biomass, plant reproduction). Future studies will further explore the potential of mixtures in different ratios and assess these promising biocontrol fungi in field trials.

Chapter 4: **BIOPROSPECTING BIOMOLECULES FOR BIOCONTROL**

There are different modes by which a biocontrol fungus could affect nematodes and the two main modes are direct parasitism and producing production of anti-nemic metabolites (Haarith et al., 2020). As fungi produce a variety of metabolites, biocontrol fungi could also be prospected for metabolites that can help control the pathogens. With respect to biocontrol, their metabolites could also be evaluated as a bionematicide or as a supplement to existing chemical control.

There are currently few effective natural products available in the commercial market to control nematodes. There are only a few commercial products of fungal origin that are available to control nematodes (Chapter 0). Different strains of *Aspergillus* spp., *Pochonia* spp., *Purpureocillium* spp., and *Trichoderma* spp., have been routinely tested against nematodes, mostly root knot nematodes, but with limited success against cyst nematodes, especially SCN (Abd-Elgawad and Askary 2018). At present, MeloCon® WG is the only formulation of fungal spores available as a living biocontrol agent (BAYER 2015) that is available in the US markets to control nematodes. DiTera®, a killed mixture of fermentation filtrate and hyphae derived from a *Myrothecium verrucaria* strain (Valent BioSciences 2010) is one of the only commercial nematode control products derived from fungi. The identity and quantification of the active molecules have not been determined and hence the fermentation-dependent production and efficacy may vary from batch to batch. Identifying the active molecule(s) provides information that can allow for chemical synthesis, structure activity relationship studies, and structure

optimization. If the compounds cannot be synthesized efficiently, the identification of the active molecules also allows for more precise quantification in extracts produced during fermentation for normalization of the active products. Identification of an active compound will also help in understanding the mode of action of bionematicide and better evaluate the specificity and toxicity.

When seeking to identify anti-nemic compounds, bioactivity-guided fractionation using iterative bioassays and chromatographic separations is a useful approach for isolating the specific molecule responsible for the activity from raw fermentation products (Nitao et al. 2002). In Chapter 2, I tested fungal filtrates for hatch inhibition and identified isolate 'D' as one of the top ten fungi from the SCN cyst mycobiome showing strong (>90% inhibition compared to distilled water control). In this chapter, I have attempted to fractionate the SCN egg hatch-inhibiting supernatant of isolate 'D' with the goal of identifying the active metabolite(s).

4.1 Materials and methods

4.1.1 Fungal inoculum preparation

One 1 cm^2 fungal plug of isolate 'D' was inoculated on each of the 50 SNA plates and incubated at 25°C with 12 h day-night cycle for 15 days. Spores were harvested by adding 3 mL of sterile 0.2% aqueous solution of Tween20 to each plate and pipetting it vigorously throughout the surface. The spore suspension was centrifuged at 23,000 g for 20 min at 4°C and resuspended in autoclaved distilled water.

4.1.2 Metabolite production

Prepared fungal spores were inoculated at 10^6 spores in 50 mL of ¼ strength Czapek-Dox Broth (CDB) in a 125 mL Erlenmeyer flask and incubated in a shaker incubator set at 125 rpm and 25°C for 15 days. The flasks were then chilled overnight to 4°C. Mycelia and mycelial balls were separated out using sterile filter paper and the supernatant was sterilized through a 0.2 µm SFCA filter with the help of vacuum. The sterilized supernatant was frozen until use. A total of four 125 mL Erlenmeyer flasks were combined to yield approximately 200 mL of supernatant.

4.1.3 Step 1: Crude separation

200 mL of supernatant was added on top of a 5 cm diameter buchner funnel packed with 10 cm of reversed phase C18 matrix under vacuum. The supernatant was initially fractionated using a step gradient of solvent (200 mL, 75% water to 100% methanol, 25% steps) A total of 5 fractions including the initial flow through were obtained. The solvent was evaporated from each fraction using a rotary vacuum evaporator and taken up in DMSO (200 ug/ uL) to generate crude extracts. Aqueous solutions were made by dissolving the crude extracts in autoclaved distilled water to a final concentration of 1% (v/v) to perform hatch inhibition assays to test for activity.

4.1.4 HPLC separation

The active fractions of crude extracts were further separated using reversed phase HPLC (Grace Vision HTC18 classic 5 µm column of length 250 mm and internal diameter of 10 mm). The mobile phases were water and acetonitrile at a flowrate of 3 mL/ min, for a total time of 20 min. The gradient used varied slightly for the different active fractions (Table 4-1). The chromatograms were visualized at 220 nm and 280 nm UV

wavelengths and an automated fraction collector was used to collect fractions (Figures 4-1, 4-2). The fractions were then dried down using a rotary vacuum evaporator and taken up in DMSO (200 ug/ uL). These will henceforth be referred to as fractions and were used as 1% aqueous solution for the hatch inhibition assay evaluation.

Table 4-1. Elution gradients for the different crude fractions

Crude fraction 1			Crude fraction 2		
Time (min)	**A% (Water)**	**B%(ACN)**	**Time (min)**	**A% (Water)**	**B%(ACN)**
3	95	5	3	95	5
14	60	40	14	70	30
15	0	100	15	0	100
18	0	100	18	0	100
20	95	5	20	95	5

4.1.5 Egg-hatch inhibition assay

The egg-hatch inhibition assay, as described in chapter 2, was used to guide fractionation. For this assay, 500 SCN eggs suspended in 100 µL of autoclaved distilled water were placed on a 30 µm nylon mesh filter. The filter was then placed inside one of the wells in a 24-well tissue culture plate. 2 mL of the supernatant or test solution (1% aqueous solutions of fractions in DMSO) was added such that they came into contact with the eggs but did not submerge them. Test solutions and appropriate controls, including autoclaved distilled water or 1% DMSO mixture were also included. The plates were incubated at 27 °C for 72 h and healthy J2 hatchlings that passed through the nylon membrane were counted after removing the membrane inserts with unhatched eggs. All tests were done in three replicate wells. Hatch inhibition relative to control was calculated using the formula:

$$(1 - \frac{No.\ of\ hatchlings\ in\ test\ solution}{No.of\ hatchlings\ in\ DMSO\ control}) \times 100$$

For the HPLC fractions test, the inhibition was expressed per µg of solids as different amounts were obtained from evaporating the different fractions. Hence different concentrations were used in the assay. Whenever possible, 200 ug/ well of the fractions were tested. When only lower amounts were available, they were split amongst the three wells. All activity was normalized to the amount of fraction tested per well.

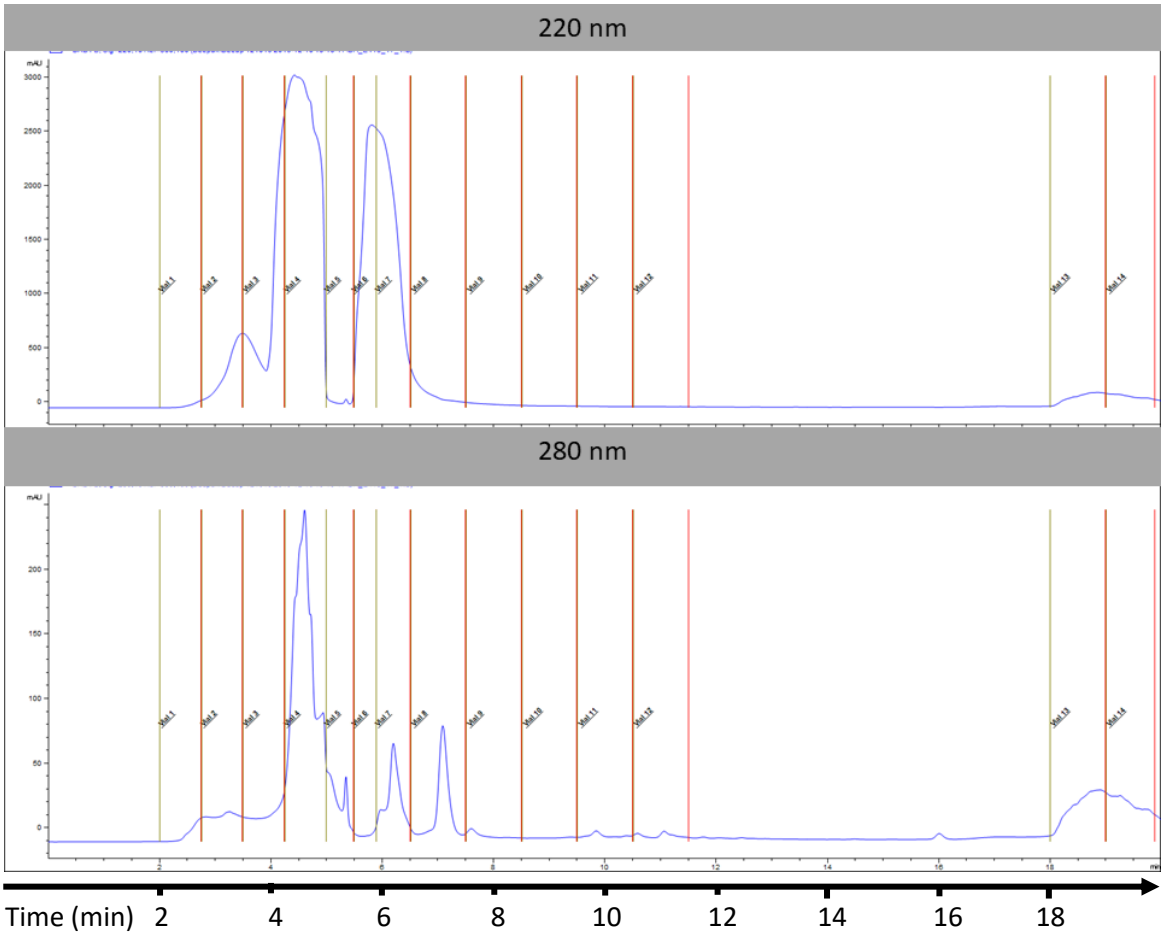


Figure 4-1. HPLC chromatogram of the first C18 crude fraction. Region between two vertical bars denote a fraction.

4.2 Results

4.2.1 Crude separation

A total of 1.4 g of solids were obtained from 200 mL of supernatant, distributed across the different crude fractions and a majority of that was distributed between the first two fractions, especially the second (75% water; 25%methanol) (Table 4-2). Only the first two crude extracts showed any hatch inhibition against SCN eggs compared to the control (Table 4-2). The flow through had about 10.5% hatch inhibition compared to the DMSO control, while the second crude extract showed about 38% hatch inhibition compared to the DMSO control (Table 4-2). Therefore, only the first two crude extracts were subsequently separated using HPLC.

Table 4-2. SCN egg hatch inhibition activity of crude extracts.

Fraction	Mobile phase	compound mass (g)	Average percent inhibition (with respect to 1%DMSO)	amount added to each well (ug)
1	100% water 0% methanol	0.6372	10	6372
2	25% methanol	0.502	38	10040
3	50% methanol	0.0108	-24	216
4	75% methanol	0.0165	-72	330
5	100% methanol	0.2358	-31	2358

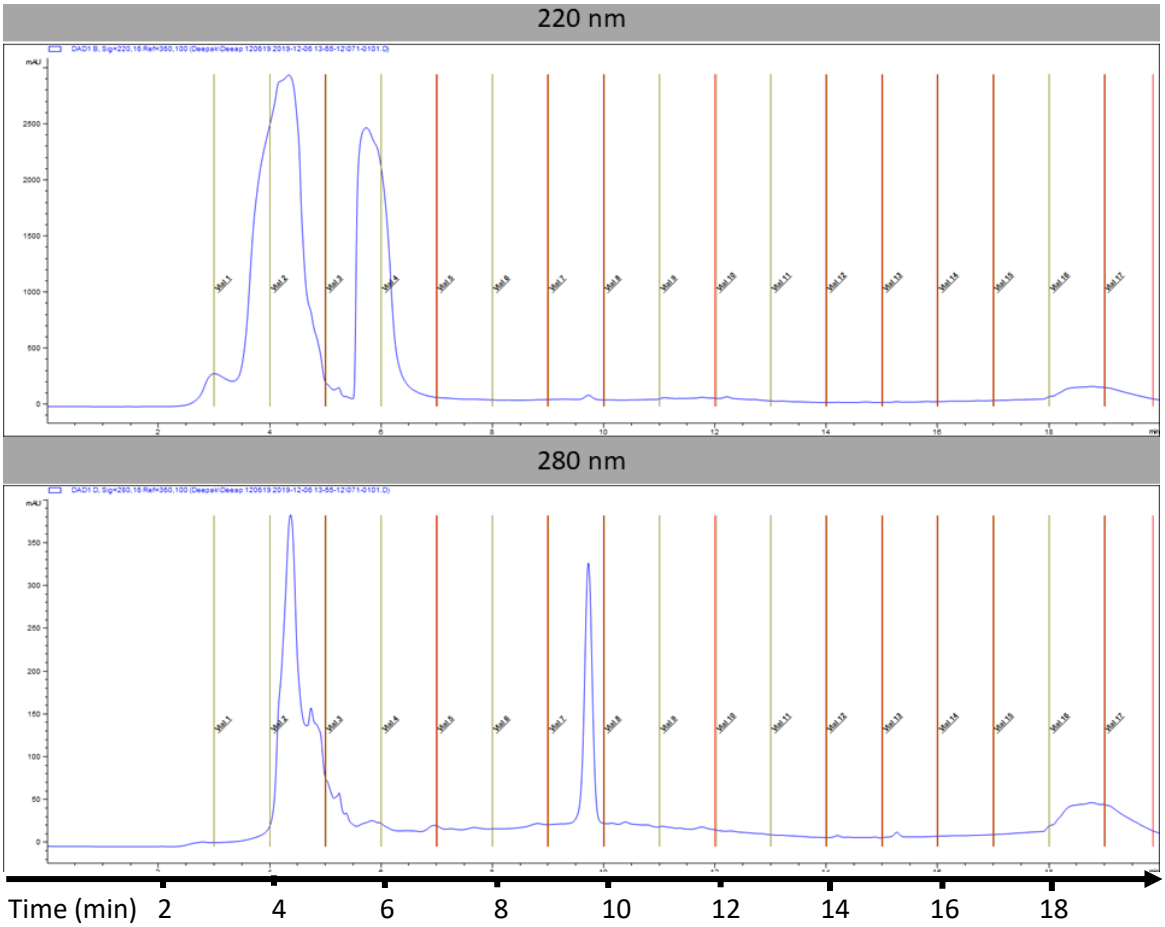


Figure 4-2. HPLC separation of the second C18 fraction. Region between two vertical bars denote a fraction.

4.2.2 HPLC separation

The first crude fraction was separated into 14 different fractions while the second crude fraction was separated into 17 different fractions (Figures 4-1, 4-2). The different fractions and the weight of solids from each fraction after solvent removal are summarized in Table 4-3.

When test solutions were normalized by concentration, only HPLC fractions 13 and 14 from the first crude extract had hatch inhibition activity (Figure 4-3). Similarly, of the 17 fractions of the second crude extract, only the 15th fraction showed hatch inhibition activity when normalized (Figure 4-4).

Table 4-3. Solid compound weights of different HPLC fractions of the two crude C18 fractions.

FIRST C18 CRUDE FRACTION		SECOND C18 CRUDE FRACTION	
FRACTION	**compound weight (ug)**	**FRACTION**	**compound weight (ug)**
1	530	1	700
2	2260	2	25740
3	3160	3	760
4	27660	4	390
5	230	5	80
6	280	6	100
7	1210	7	90
8	40	8	100
9	80	9	80
10	50	10	30
11	1300	11	60
12	1000	12	10
13	900	13	110
14	200	14	70
WASTE	580	15	20
CRUDE	545	16	20
		17	280
		WASTE	1040
		CRUDE	7310

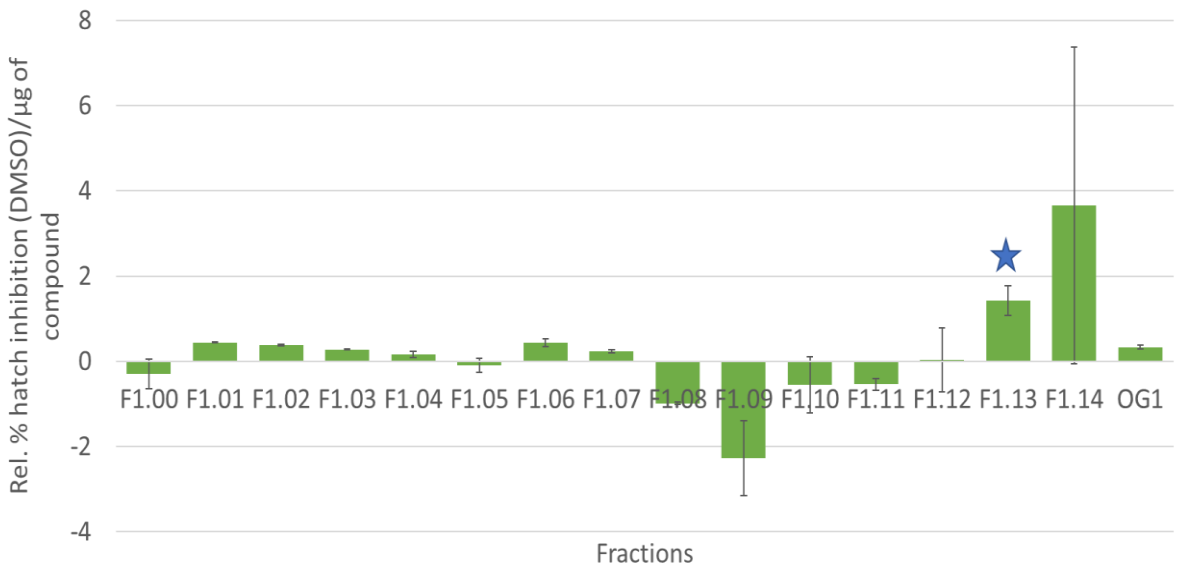


Figure 4-3. Hatch inhibition relative to 1% DMSO for the HPLC fractions of the first crude C18 crude fraction. OG1 is the original crude fraction.

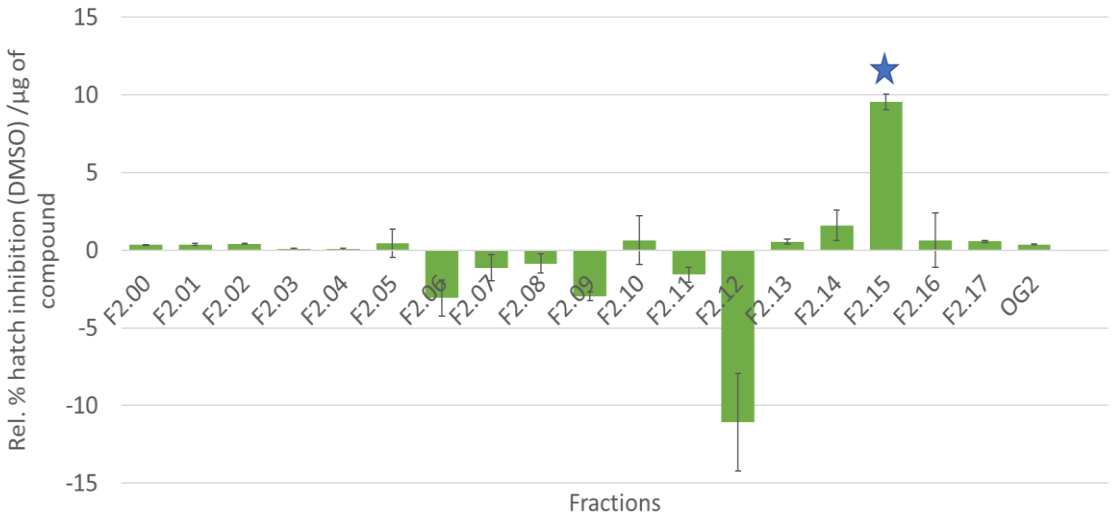


Figure 4-4. Hatch inhibition relative to 1% DMSO for the HPLC fractions of the second crude C18 crude fraction. OG2 is the original crude fraction.

4.3 Discussion

My initial egg hatch inhibition assays discussed in Chapter 2 used a 15-day old culture of ¼ strength CDB inoculated with 10^6 spores/50 mL media. To scale-up production, I did not attempt to increase the volume of fermentation, but instead used multiple flasks of the same volume (50 mL). Scaling-up fermentation processes require careful engineering. A change in the shape, size, or material of the reactor can affect the gas exchange, heat exchange and other micro-environment dynamics of the bioreaction (Ales Prokop 1983). Therefore, for the purposes of this dissertation, I cultured more flasks of the same scale, instead of scaling it to a larger volume. Filtrates from all the individual flasks were pooled at harvesting and sterilized together using the vacuum filtration system. The SCN egg hatch inhibition activity was tested for the supernatant before it was frozen and separated further. Bioactivity was tested at every separation step. Bioactivity guided fractionation is an effective tool to identify active compounds from fermentation. We had already established the hatch inhibition assay as an effective *in*

vitro screening technique (Haarith et al., 2020). Therefore, I have used this technique to guide metabolite fractionation in this chapter.

Since most of the activity fractionated into the first two fractions that had either 100% or 75% water, it is evident that the activity is from a fairly polar compound in the fermentation broth. However, during the HPLC separation, the active fractions eluted at a later time point with a higher concentration of acetonitrile, a less polar solvent. As our fractions are still not very pure, the active fraction could have a mixture of small polar molecules, non-polar and amphipathic molecules. This suggests that our active compound could be a small sugar or peptide or any other small amphipathic molecule such as a glycosylated compound. In fact, some nematode trapping fungi have been shown to produce small glucosides that mimic nematode pheromones to attract nematode prey (Hsueh et al. 2013). The fractions obtained by HPLC (Figures 4-1 and 4-2) are not yet pure and require additional chromatography and further analyses to identify the active compound (s). Since these fractions were isolated in very small yields (< 1mg), this would also require re-fermentation and scaling up of cultures to obtain enough material for multiple HPLC steps. However, the bioassays have proven to be sensitive enough to detect activity at all stages of purification, and this should be technically feasible. If these fractions can be purified as individual compounds, NMR spectroscopy, mass spectrometry and other analytical techniques will be used to determine their structures. The identification of the active compounds will be essential for considering mechanisms of action, provide leads for identifying additional active analogs from other active biocontrol fungi, and allow for the study of structure activity relationships.

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