

**IMPROVED AND SUSTAINABLE MANAGEMENT OF  
ECONOMICALLY IMPORTANT PLANT-PARASITIC  
NEMATODES OF FIELD CROPS AND FUNGAL DISEASES OF  
SOYBEAN AND HEMP IN TENNESSEE**

**A Dissertation Presented for the  
Doctor of Philosophy  
Degree  
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## DEDICATION

I dedicate my work to God who always keeps and gives me favor in life, my late brother Wilson O. Akinrinlola who taught me how to read and set me up for a successful academic career, my brother Ilesanmi O. Akinrinlola who supported my academic pursuits, and my beloved wife *Ayanfe* Oluwayemisi Akinrinlola for her unreserved supports for me during my Ph.D. program.

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## ABSTRACT

Nematodes and diseases can limit field crops and hemp production in Tennessee. Therefore, seven research objectives were undertaken to improve the management of the crops: First, the prevalence and density of plant-parasitic nematode populations were evaluated across many field crop acreages in Tennessee through a survey from 2018 through 2020. Second, the virulence phenotypes of soybean cyst nematode (SCN), (*Heterodera glycines* [HG]), populations were assessed in Tennessee fields using the HG types test. Third, the impact of the virulent SCN HG type 1.2.5.7 on soybean nitrogen fixation activity and growth was evaluated on different soybean genotypes in the greenhouse. Fourth, the impact of different densities of *Macrophomina phaseolina* [Mp] microsclerotia on charcoal rot disease severity and soybean damage were assessed. Fifth, the impact of soybean varieties on Mp density in soil and plant tissue was evaluated using greenhouse tests. Sixth, the causative pathogen of greenhouse hemp powdery mildew in Tennessee was identified using PCR and DNA sequencing. Finally, multiple fungicides were screened for the control of hemp powdery mildew and leaf spots in the greenhouse and field, respectively.

Fourteen nematode taxa occurred across samples. The most prevalent nematodes across samples occurred at 83% (spiral, *Helicotylenchus* spp.), 29% (lance, *Hoplolaimus*), 25% (SCN, *Heterodera glycines*), 23% (lesion, *Pratylenchus*), 20% (reniform, *Rotylenchulus*), 19% (stunt, *Tylenchorhynchus*), and 13% (root-knot, *Meloidogyne*). Reniform, RKN, SCN, and lesion nematodes occurred at high densities in samples. All tested SCN populations were virulent (HG-types 1.2.5.7, 2.5.7, and 7), and HG-type 1.2.5.7 had varying effects on soybean NF and growth among susceptible cultivars, but no effect on the resistant cultivar. *Macrophomina phaseolina* density of about 327 CFU/gram of soil caused charcoal rot disease on a susceptible soybean. Moderately resistant soybean cultivar suppressed Mp densities in soil. *Golovinomyces ambrosiae* was identified as the pathogen causing hemp powdery mildew. All tested fungicides

suppressed powdery mildew, with Bonide sulfur, Cinnerate, Exile, MilStop, Regalia, and Sil-Matrix being the most effective fungicides.

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## **CHAPTER I. REVIEW OF LITERATURE**

## ***Part 1: Plant-parasitic nematodes of field crops and biological control agents of nematodes***

### ***Major plant-parasitic nematodes***

Over 4,100 species of plant-parasitic nematodes have been described, and annual crop loss caused by nematodes is estimated at \$US80 billion worldwide (Jones et al., 2013). The most frequently observed or economically important plant-feeding nematodes include root-knot nematodes (*Meloidogyne incognita*, *M. hapla*, and *M. javanica*), soybean cyst nematode (*Heterodera glycines*), lesion nematode (*Pratylenchus* spp.), dagger nematode (*Xiphinema americanum*), reniform nematode (*Rotylenchulus* spp.), spiral nematode (*Helicotylenchus pseudorobustus*), and stunt nematode (*Tylenchorhynchus claytoni*) (Bernard, 1980; Jones et al., 2013). Plant-parasitic nematodes are classified into four main categories based on their association and methods of feeding on hosts. They are classified as migratory ectoparasites, migratory endoparasites, semi-endoparasites, or sedentary endoparasites (Lambert & Bekal, 2002).

### ***Migratory ectoparasites.***

These nematodes live outside of their host plants and use their long or short stylet to feed on the epidermal cells (external surface) of the plant roots (Lambert & Bekal, 2002). Migratory ectoparasites exhibit the most common type of feeding style among all plant-parasitic nematodes. Some ectoparasites (*Ditylenchus*, the stem nematode) are foliar parasites that feed mainly on epidermal cells of young leaves, stems, and flower primordia (Manzanilla-Lopez et al., 2004). Most ectoparasites are root feeders, possessing a short or a long feeding stylet. The short stylet ectoparasites feed on outer epidermal and cortical cells and root hairs. Examples include *Tylenchorhynchus* (stunt), *Trichodorus* (stubby-root), *Paratrichodorus* (stubby-root), and some *Helicotylenchus* (spiral) (Manzanilla-Lopez et al., 2004). Some examples of long stylet ectoparasites include *Belonolaimus* (sting), *Criconemella* (ring), *Longidorus* (needle), and *Xiphinema* (dagger). These nematodes can insert their long stylet deep into root tissues via young growing tip (Manzanilla-Lopez et al., 2004).

### ***Migratory endo-parasites.***

Some examples of migratory endoparasites include *Pratylenchus* (lesion), *Hoplolaimus* (lance), some *Helicotylenchus* (spiral), some *Rotylenchus* (reniform), some *Ditylenchus* (stem nematode), *Aphelenchoides* (leaf nematode), *Bursaphelenchus* (pinewood nematode), and *Scutellonema* (yam nematode) (Manzanilla-Lopez et al., 2004). These nematodes enter host roots, migrating through and feeding upon host cortical cells. This migration destroys cells and facilitates the entry of other plant pathogens, but the nematodes do not form permanent feeding cells in their hosts (Lambert & Bekal, 2002).

### ***Semi-endoparasites***

*Rotylenchulus* spp., (reniform nematode) and *Tylenchulus* (citrus nematode) are examples of semi-endoparasites (Manzanilla-Lopez et al., 2004). They embed the anterior part of their body in the root, with the posterior portion projected outside swollen. At this sedentary stage, the nematode forms a permanent feeding site for a long-lasting nutrient supply (Lambert & Bekal, 2002).

### ***Sedentary endoparasites.***

Examples of sedentary nematodes include *Heterodera glycines* (soybean cyst nematode) and *M. incognita*; *M. hapla*; and *M. javanica* (the root-knot nematodes). These nematodes will completely enter and become embedded in the host tissue, and move toward the developing vascular cells to form a permanent feeding site for a continuous nutrient supply (Acedo et al., 1984; Lambert & Bekal, 2002).

### ***Typical features and life cycle***

All plant-feeding nematodes possess a hollow protrusible mouthpart referred to as a stylet which they use to probe and feed on plant tissues. They typically affect crop growth directly by feeding on roots or indirectly by altering symbiotic nitrogen fixation activity in legumes (Elhady et al., 2020; Ko et al., 1984). The typical life cycle of plant-

parasitic nematodes begins with eggs, followed by four juvenile stages (J1 to J4), and ends at adult. Depending on the nematode species and conditions, plant-parasitic nematodes may require from two to six weeks to complete a life cycle. At the end of a life cycle, adult females can produce about 50 to 1,000 eggs per female, depending on the nematode species and environmental influences (Lambert & Bekal, 2002; Mitkowski, 2003; Wesemael et al., 2014).

### ***Management***

The amount of crop damage caused by nematodes depends on the host species, the nematode species and their population density, and environmental factors (Mitkowski and Abawi, 2003). The understanding of these factors plays an important part in the effective management of nematodes in fields. Different strategies are combined to develop integrated nematode management programs. Examples of integrated nematode management strategies include crop rotation, cultivar selection, soil management, chemical control, and monitoring of nematode population density in fields (Sikora et al., 2021).

### ***Soybean cyst nematode***

The use of resistant varieties and crop rotations with non-hosts, combined with the use of reduced or no-till cropping, are the most effective approaches to control SCN. Typically, populations of SCN have limitations in their ability to feed and reproduce on soybean cultivars. Populations are categorized into different pathotypes or HG types with varying virulence phenotypes based on their ability to feed and reproduce on different resistant cultivars (Mitchum et al., 2007; Niblack et al., 2002). Once the virulence phenotypes of SCN populations in a field are identified, a soybean cultivar with resistance to those SCN populations should be selected and used with other strategies to reduce nematode impact. The most common SCN-resistant cultivars derived from PI 88788 have become ineffective against most SCN populations across the United States (Gardner et al., 2017; McCarville et al., 2017). Whenever possible, these cultivars should be avoided when selecting resistance cultivars. In addition to utilizing a resistant cultivar,

non-host crops including alfalfa, barley, corn, oat, potato, sorghum, sugar beet, sunflower, and wheat can be planted in the next one or two seasons to reduce SCN density in fields. It is also recommended that different resistance types should be incorporated into crop rotations. It is essential to avoid the selection and increase of new virulent SCN populations on the resistant cultivar being grown (Young, 1982). In Tennessee, the thresholds for soybean damage (adapted from Mehl et al., 2018) range from low (200 eggs/100cm<sup>3</sup>) to medium (>2000<5000 eggs/100cm<sup>3</sup>) to high damage (5000+ eggs/100cm<sup>3</sup>) (Mehl et al., 2018). Additionally, other supportive tactics including nematicidal seed treatment, fertility, and weed management may be integrated to reduce nematode population, plant stress, and potential breeding place for the nematodes.

### ***Root-knot nematode***

Consisting of about 100 known species, root-knot nematodes (RKN) are an economically important pathogen of field crops. They are sedentary obligate endoparasites that enter host tissue, migrate to the cortical tissue, form a giant cell feeding site, and remain in the host feeding on nutrients from the site (Mitkowski & Abawi, 2003; Moens et al., 2009). A distinguishing feature of RKN is the ability to form galls on host roots (Kyndt et al., 2013). Suggested rotation crops for RKN in field crops include grain sorghum, peanut, and rice. Rice should not be included in a rotation if a field is infested with *Meloidogyne graminicola*, which is an important parasite of rice (Soares et al., 2022). These crops can effectively lower populations below economic levels, to less than 60 nematodes/100cm<sup>3</sup> for soybean (Kirkpatrick et al., 2014) or about 100 nematodes/100cm<sup>3</sup> for cotton (Mueller, 2012). Corn is usually a good host to RKN, being able to support large reproduction of the nematode with little to no crop loss. So corn should not be grown in infested fields if the field will be rotated to soybean or cotton, to avoid damage. This is because the population density of RKN may be extremely high after corn even if no damage was observed on corn. Fields with low to moderate population densities can be grown with resistant cultivars and supported with other protective practices. Some examples of RKN-resistant cotton cultivars with moderate resistance to RKN include PHY 367 WRF, ST 5458B2RF, ST 4288B2RF, and

DP 174 RF (Mueller, 2012). Soybean cultivars vary in their host status to different species of RKN nematodes, and cultivars with the highest resistance to most RKN species should be selected to reduce the damage by most species. There are high-yielding soybean cultivars in the maturity groups II to IV with resistance to RKN (Kruger et al., 2008). Caution should be exercised to select cultivars with resistance to other nematodes including soybean cyst nematode that may be present in the field in addition to RKN. Seed treatments are common practices used to reduce the effects of nematodes on crop yields. Abamectin, Fluopyram, and *Bacillus firmus* are commonly used seed treatments against nematodes (Eisenback, 2018). Also, additional protective practices include the use of finely chopped hay residues (alfalfa, oat, lespedeza, flax), solarization, and suppressive crops such as marigold and rapeseed have been effectively used to manage RKN in vegetable fields (Bernard, 2018).

### ***Lesion nematode***

Lesion nematode is a polyphagous migratory endoparasite capable of attacking an extensive host range of more than 400 plant species (Davis & MacGuidwin, 2000). Soybean, corn, peanut, potato, rice, wheat, and vegetables are among the most common economically important hosts of lesion nematodes. Due to the large host range, non-host crops are very limited. Some of the crops or cover crops that have been identified as poor hosts or have moderate resistance to lesion nematode include African and French marigolds, pearl millet (cv. HGM 100), Sudax, and Saia oats (Warner and Bird, 2020). These crops can reduce lesion nematode below economic thresholds when included in rotation or used as cover crops where applicable. The lowest threshold for lesion nematodes is estimated to be 20, 50, 200 nematodes/100 cm<sup>3</sup> of soil for soybean (Mehl et al., 2018), cotton, and corn, respectively (Dickerson et al., 2000). Also, cover crops such as ryegrass or canola used as green manures are recommended to inhibit the increase of low-density populations in Nebraska (<https://cropwatch.unl.edu/potato/lesion>). Resistance is limited for lesion nematodes among most host crops, but some soybean cultivars with moderate resistance to the nematodes have been identified (Chowdhury et al., 2022). Also, there are suggestions that soil microbial antagonists and weed management can

contribute to reducing lesion nematode population density in fields (Brown & Smart, 1985; Davis & MacGuidwin, 2000; Kandel et al., 2013). Soil fumigation is the most common effective practice used on fields infested with high lesion density to lower population levels to below damage thresholds. Metham-sodium and Telone II (1,3-dichloropropene) are recommended effective soil fumigants for nematodes (Riedel & Rowe, 1985; <https://cropwatch.unl.edu/potato/lesion>). Non-fumigants are effective for lowering lesion nematode density in fields. Aldicarb (Temik) is an example of a nonfumigant nematicides still available that may offer lasting systemic protection against nematodes (Davis & MacGuidwin, 2000).

### ***Reniform nematodes.***

Reniform nematodes are semi-endoparasites of more than 300 host plant species, with the most economically important hosts including cotton, soybean, sweet potato, and vegetables (Ayala & Ramírez, 1964). The nematode can cause damage when soil density reaches about 210 nematodes/100 cm<sup>3</sup> soil at planting or 1,050 nematodes/ 100 cm<sup>3</sup> at harvest for cotton (Newman 1998; Bernard, 2018), while soybean damage can occur at 500 nematodes/100cm<sup>3</sup> (Kirkpatrick et al., 2014). Crop rotation with corn, grain sorghum, and peanut can lower population densities to below damage thresholds. Rotation to corn can reduce reniform nematode by up to 31%. Also, other non-host crops including winter grain crops such as wheat, rye, oats, and barley have been recommended (Kelly, 2016). Growing resistant varieties is one of the most effective practices to reduce nematode damage. While no cotton variety with resistance to reniform is currently available in Tennessee (Kelly, 2016), varieties with resistance to the nematode have been shown to effectively reduce population density in a field elsewhere (Grabau, 2022). Also, multiple cultivars of soybean with resistance to reniform nematodes are available and can be used to prevent damage and suppress nematode density increase in fields (Robbins et al., 1999). Nearly 350 species of weeds have been found to support reniform nematode reproduction (Inserra et al., 1989; Robinson et al., 1997). Proper weed management can contribute to lowering the nematode density in the fields. Other control options include nematicides and seed treatments (e.g. Abamectin, Thiodicarb, or *Bacillus firmus*). Foliar

applications of Vydate, a systemic nematicide, combined with seed treatments can effectively suppress nematode populations in the field and prevent crop damage (Mueller et al., 2012).

### ***Biological control agents of plant-parasitic nematodes***

#### ***Bacteria***

##### ***Pasteuria penetrans***

Many reports have indicated the biological control activity of *P. penetrans* against RKN (Mankau, 1975; Brown and Smart, 1985; Sayre, 1980). Brown and Smart (1985) reported that about 20 spores/RKN juvenile inhibited tomato root infection by RKN. At seven days after inoculation, the spores prevent RKN gall formation by over 500%, and at 21 days, root penetration was inhibited by over 1,000%. The bacterial spores were more effective against *M. javanica* than against *M. incognita* and *M. arenaria*.

##### ***Bacillus spp.***

Numerous strains of *Bacillus* species are known for controlling plant-parasitic nematodes. They are attractive biological bacteria due to their heat and desiccation-tolerant endospores. This structure gives *Bacillus*-based products the ability to survive under extreme conditions and a long shelf-life. *Bacillus thuringiensis*, *B. subtilis*, *B. firmus*, and *B. amyloliquifaciens* are the most common species used in biological control. A strain of *B. thuringiensis* was reported to suppress *M. impala* on lettuce (Chen et al., 2000). Likewise, *B. subtilis* strain GBO3 and *B. cereus* strain C4 reduced the number of *M. incognita* root galls on pepper (Kokalis-Burelle et al., 2002). A formulation containing *B. subtilis* strain GB03, *B. amylolifaciens* strain GB99, and chitin reduced RKN gall numbers on tomatoes. A strain of *B. firmus* significantly paralyzed or kill juveniles of *Radopholus similis*, *M. incognita*, and *Ditylenchus dipsaci* after incubation in the bacterial filtrate (Mendoza et al., 2008).

### ***Burkholderia capacia***

*Burkholderia capacia* has been indicated for the control of plant-parasitic nematodes through a non-enzymatic antagonism. Culture filtrates of the bacterium were found to inhibit RKN egg hatching and second-stage juvenile (J2) mobility. Likewise, two strains of *B. capacia* were reported to reduce the number of *M. incognita* eggs on bell pepper by up to 69% (Meyer et al., 2001). Also, a commercial formulation of the bacterium was reported to reduce the population of *Aphelenchoides fragariae* on hosta foliage by up to 85% (Jagdale & Grewal, 2002). An additional report suggested that *B. capacia* exhibited efficacy against RKN but was ineffective against SCN (Noel, 1990).

### ***Pseudomonas spp.***

Several species of *Pseudomonas* have been indicated for suppressing populations of many plant-parasitic nematodes. Siddiqui and Shaukat (2003) reported the suppressive activity of *P. fluorescens* strain CHA0 and *P. aeruginosa* strain IE-6S against *M. javanica* on tomato. Hackenberg et al. (2000) reported the efficacy of *P. chloroaphis* strain Sm3 against lesion nematode, *Pratylenchus penetrans* on strawberry. Also, isolates of *P. aeruginosa* protected sesame against the nematode (Kumar et al., 2009). Nagachandrabose (2020) reported that liquid bio-formulations containing *P. fluorescens*, *Purpureocillium lilacinum* and *Trichoderma viride* suppressed egg reproduction by potato cyst nematodes, *Globodera rostochiensis* and *Globodera pallida*.

### ***Fungi***

#### ***Paecilomyces spp.***

*Paecilomyces lilacinus* strain 251 is a registered biological control agent for nematodes in many countries including the United States. According to Martéz et al. (1996), *P. lilacinus* can suppress *M. incognita* J2 in tomato fields by 40 to 70%. Also, Goswami et al., (2008) found that the fungus suppressed tomato root galls (*M. incognita*) by 39%. However, in two experiments, a commercial formulation containing *P. lilacinus* did not reduce densities of *M. marylandi* on a golf course (Starr et al., 2007), and strain

251 did not reduce galling in tomatoes when used on tomatoes grown in a soil heavily infested with *M. incognita* in the greenhouse (Kaşkavalci et al., 2009). This suggests that the nematode suppressive activity of the fungus may vary depending on the nematode species and variations in environments.

### ***Pochonia chlamydosporia***

*Pochonia chlamydosporia* is commonly associated with nematode-suppressive soils. It is known as a parasite of root-knot and cyst nematode eggs, it can colonize the roots of several plant species, and can induce plant defense mechanisms (Ghahremani et al., 2019). The fungus was shown to reduce tomato root galling by *M. javanica* (Sorribas et al., 2003). *Pochonia chlamydosporia* was found to significantly reduce nematode density in a *Globodera pallida*-infested field (Tobin et al., 2008). Ghahremani et al. (2019) reported that two *P. chlamydosporia* isolates, M10.43.21 and M10.55.6, induce systemic resistance against *M. incognita* in tomatoes. The M10.43.21 isolate decreased infection (43%), reproduction (59%), and female fertility (27.6%), while the isolate M10.55.6 only consistently decreased nematode reproduction (35–47.5%).

### ***Trichoderma spp.***

*Trichoderma* spp. is known for antagonizing and suppressing nematode populations using several mechanisms including parasitism, antibiosis, lytic enzyme production (Zhang et al., 2014), or competing for space and nutrients and modifying the rhizosphere in favor of host plants. Additionally, some can induce hormone-mediated plant-defense mechanisms against nematodes (Poveda et al., 2020). Goswami et al., (2008) reported that *T. harzianum* reduced *M. incognita* root galls on tomatoes by 47%. Oyekanmi et al. (2007) reported that a *T. pseudokoningii* isolate suppressed *M. incognita* egg masses on soybean in pot experiments. Another species, *T. longbranchiatum*, significantly decreased the number of cysts and females of *H. avenae*, and inhibited juvenile development in soils and roots in greenhouse trials (Zhang et al., 2014).

## ***Nematodes***

### ***Predatory diplogastrid nematodes***

Predatory diplogastrid nematodes are nematode predators that are known as nematode control agents due to their high reproduction rates, short life cycles, and being culturable. Bilgrami et al. (2008) showed that the diplogasterid nematode species, *Mononchoides gaugleri*, suppressed populations of *Ditylenchus* spp., *Aphelenchoides* spp., *Tylenchorhynchus* spp., and *Tylenchus* spp. by up to 45%, compared to uninoculated turfgrass microplots.

### ***Steinernema and Heterorhabditis***

Smitely et al (1992) reported that a mixture of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* decreased the population of *Tylenchorhynchus* spp. by 59% at 5 weeks after application in irrigated turfgrass plots. In another report, *S. riobrave* suppressed populations of *Meloidogyne* spp., *Belonolaimus longicaudatus*, and *Crictonemella* spp. by 84 to 100% at 4 and 8 weeks after application into turfgrass golf courses (Grewal et al., 1997). Also, soil application of *S. carpocapsae* and *H. bacteriophora* individually reduced root galls, eggs, and egg mass formation by *Meloidogyne javanica* on cucumber. A list of biocontrol organisms of plant-parasitic nematodes is summarized in Table 1.1 below.

**Table 1.1. List of biological control agents for plant-parasitic nematodes**

| Potential biocontrol organism | Nematode spp/ stage                                                      | Test condition                                                                                                                                         | References                     |                                                |
|-------------------------------|--------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------|------------------------------------------------|
| Bacteria                      | <i>Pasteuria penetrans</i>                                               | <i>Meloidogyne</i> spp galls on tomato                                                                                                                 | Microplot                      | Chen and Dickson, 1998                         |
|                               | <i>Pasteuria penetrans</i>                                               | <i>Meloidogyne</i> spp. galls on peanut                                                                                                                | Field                          | Kariuti and Dickson, 2007                      |
|                               | <i>Burkholderia capacia</i>                                              | <i>Meloidogyne</i> spp galls on pepper, and <i>Aphelenchoides fragariae</i> on hosta foliage                                                           | Glasshouse                     | Meyer et al., 2001<br>Jagdale and Grewal, 2002 |
|                               | <i>P. aeruginosa</i>                                                     | <i>Heterodera cajani</i> on sesame                                                                                                                     | Field                          | Kumar et al., 2009                             |
|                               | <i>P. chloroaphis</i>                                                    | <i>Pratylenchus penetrans</i> , on strawberry                                                                                                          | Glasshouse                     | Hackenberg et al., 2000                        |
|                               | <i>Streptomyces costaricanus</i> and <i>Bacillus thuriengiensis</i>      | <i>Meloidogyne</i> spp. galls on lettuce                                                                                                               | Microplots                     | Chen et al., 2000                              |
|                               | <i>Bacillus subtilis</i> and <i>B. Cereus</i>                            | <i>Meloidogyne</i> spp. galls on pepper                                                                                                                | Field                          | Kokalis-Burelle et al., 2002                   |
|                               | <i>B. subtilis</i> and <i>B. amyloliquifaciens</i>                       | <i>Meloidogyne</i> spp. galls on tomato                                                                                                                | Field                          | Koka and Dickson, 2003                         |
|                               | <i>B. firmus</i> and plant extracts                                      | <i>Meloidogyne</i> spp. galls on tomato                                                                                                                | Glasshouse, and tomato nursery | Giannakou et al., 2004                         |
| Fungi                         | <i>Paecilomyces lilacinus</i>                                            | <i>Meloidogyne</i> sp J2 on tomato. But did not suppress <i>Rotylenchus reniformis</i> and <i>H. cotylenhus</i>                                        | Field                          | Goswanni et al., 2007                          |
|                               | <i>Pochonia chylamydosporia</i>                                          | <i>Globodera pallida</i>                                                                                                                               | Field                          | Tobin et al., 2008                             |
|                               | <i>Pochonia chylamydosporia</i>                                          | <i>Meloidogyne</i> spp. galls on tomato                                                                                                                | Field                          | Wei et al., 2009                               |
|                               | <i>Trichoderma harzianum</i>                                             | <i>Meloidogyne incognita</i> on eggplant but did not reduce galling.                                                                                   | Microplots                     | Rao et al., 1998                               |
|                               | <i>Trichoderma harzianum</i>                                             | <i>Meloidogyne incognita</i> galls on tomato                                                                                                           | Field                          | Goswanni et al., 2008                          |
|                               | <i>T. pseudokoningii</i>                                                 | Failed to inhibit galls formation <i>Meloidogyne incognita</i> on soybean, but reduced egg mass in a pot experiment                                    | Field and greenhouse           | Oyekanmi et al., 2007                          |
| Nematodes                     | <i>Mononchoides gaugleri</i>                                             | Suppressed populations of several nematodes including <i>Ditylenchus</i> , <i>Aphelenchoides</i> , <i>Tylenchorhynchus</i> , and <i>Tylenchus</i> spp. | Micro plot                     | Bilgrami et al., 2008                          |
|                               | <i>Steirnerinema carpocapsae</i> and <i>Heterorhbditis bacteriophora</i> | Suppressed <i>Tylenchorhynchus</i> sp. in irrigated plots                                                                                              | Turfgrass plots                | Smitley et al., 1992                           |
|                               | <i>Steirnerinema riobrave</i>                                            | Suppressed <i>Melodoigyne</i> , <i>Belonolaimus longidaudatus</i> , and <i>Criconemella</i> spp populations                                            | Turfgrass plots                | Grewal et al., 1997                            |

## ***Part 2: Charcoal rot of soybean by *Macrophomina phaseolina****

*Macrophomina phaseolina* (Tassi) Goid is the causative organism of charcoal rot of soybean (*Glycine max*), an important disease that causes soybean yield loss and reduces seed quality worldwide (Mengistu et al., 2007; Mengistu et al., 2011; Kaur et al., 2012). With an annual yield loss that can be up to 27 million tons in the U.S, charcoal rot is the second most important soybean pathogen in the U.S behind soybean cyst nematode and is responsible for the highest soybean loss in the Southern U.S States (Allen et al., 2017; Wrather and Koenning, 2009). The fungus causing the disease, *M. phaseolina* (Tassi) Goid, can attack over 500 species of plants and causes disease in many other economical crops, including corn (*Zea mays* L.), sorghum (*Sorghum moench*), cotton (*Gossypium* L.), and tobacco (*Nicotiana tabacum*) (Su et al., 2001).

### ***Prevalence in agricultural soils***

*M. phaseolina* (Tassi) Goid occurs in all agricultural soils in plant residues in the form of microsclerotia during winter (Smith et al., 2014). The fungal microsclerotia can persist and remain viable in the soil for more than 10 years (Baird et al., 2003). Usually, the density of microsclerotia in soil and other factors determine the severity of the next disease incidence. Gray et al (1991) observed that soil microsclerotial density was positively related to charcoal rot incidence in a sorghum field, and Mengistu et al. (2009) investigated the roles of soil management practices against charcoal rot severity and soybean yields and found that practices that reduced microsclerotial density in fields can be used to suppress charcoal rot severity and maintain soybean yields.

### ***Infection process***

During warm weather, when soil becomes warm and ambient temperature increases above 30°C, microsclerotia near the roots of soybean plants will germinate in response to root exudates and penetrate the roots (Gupta et al., 2012). The fungus infiltrates roots by producing specialized structures known as germ tubes on its primary hyphae. The germ tubes possess a needle-like end known as an appressorium which is used for invading the epidermal cell. Infection occurs by either breaking through the walls using mechanical pressure and enzymatic activity that breaks the cell wall (Ammon

et al., 1974) or by entering via natural openings or wounds on the root surfaces (Ammon et al., 1975). Once inside the root, the hyphae will occupy intercellular and intracellular spaces within the root, pressing against the nearby cells (Bressano et al., 2010; Short, 1978). This invasion often results in the death of multiple cells and disruption of nutrient and water movement in the vascular tissue, thus resulting in the killing of the infected roots, and eventually the plant. Usually, the infection begins earlier at the vegetative stage but becomes vividly evident on infected plants beginning from the flowering or pod setting stage when the plant begins to die (Meyer, 1974). The death of plant tissue induces the fungus to form microsclerotia. The microsclerotia and remaining fungal hyphae colonize the dead plant tissue until the tissue completely decays. After decay, mycelium and microsclerotia are liberated into the soil and become the inoculum source for the next infection cycle in the next season (Mengistu et al., 2009).

### ***Symptoms***

Seedborne infections may reduce seedling emergence. Infected seedlings develop lesions on hypocotyls below the cotyledons. Lesions first appear reddish-brown and then become silver-gray before turning dark-brown or black (Meyer, 1974). For older plants, the infection may result in reduced plant vigor, leaf yellowing, wilting, and premature death of the plants, with the leaves still attached to the petioles (Smith et al., 2014). The most common signs associated with the disease include gray streaks on the lower stem and taproots, small black specks (microsclerotia) in the epidermis, and lower stems and taproots of infected plants, giving them a charcoal (blackish) appearance. Often, a reddish-brown discoloration may occur in the pith and vascular tissues of the lower stem and root (Smith et al., 2014).

### ***Factors affecting charcoal rot severity***

*Macrophomina* microsclerotia can survive in the soil for 2-15 years depending on environmental conditions (Baird et al., 2003). The germination of the microsclerotia in soybean fields is promoted by high soil temperature (28-35°C), high C: N ratios of amendments, low moisture, low soil compaction, and high oxygen concentrations

(Manici et al., 1995; Mengistu et al., 2009; Meyer, 1974). Also, microsclerotia size and density, and history of the fields can promote *Macrophomina* infections. Generally, large size microsclerotia produce more germ tubes and cause more infections (Kaşkavalci et al., 2009; Short, 1978). This suggests that the presence of a high density of large microsclerotia in fields will likely increase fungal germination and infection rate. Also, fields that have been grown consistently with soybean or other *Macrophomina* hosts will probably support severe charcoal rot infections. Furthermore, some farm practices can promote *Macrophomina* infection. The microsclerotia survive in infected plant residues, hence the presence of residues of highly susceptible soybean plants in fields can promote infection (Short et al., 1980). Also practicing soil tillage may expose microsclerotia to soybean seeds or emerging seedling roots, increase soil aeration, nutrient support for microsclerotia germination, and promote more infection (Mengistu et al., 2009).

### ***Management***

Strategies to control charcoal rot include the use of resistant cultivars, seed treatments, and cultural practices such as tillage, rotation, irrigation, and seeding rate management (Smith et al., 2014). Generally, management efforts often focus on lowering the pathogen inoculum propagules in the field (Gupta et al., 2012; Mengistu et al., 2009). This is because the pathogen cannot be eliminated once in a field, as it can survive under various environmental conditions. Also, there is no complete resistance against the pathogen (Papavizas, 1977; Smith and Carvil, 1997).

### ***Crop rotation and resistance cultivars***

Rotation to non-hosts for 2-3 years can reduce microsclerotia density in highly infested fields. Also, rotation to corn and grain sorghum for 1 year can keep microsclerotia density below a damaging threshold. Rotation to cotton, sorghum, or corn has been reported to reduce charcoal rot inoculum in fields grown with soybean (Francl et al., 1988). Also, in a cowpea field, charcoal rot inoculum density was significantly reduced when the field was planted with fonio (*Digitaria exilis*) or millet (*Panicum miliaceum*) (Ndiaye, 2007). Limited soybean cultivars with moderate susceptibility to

*Macrophomina* have been reported (Smith & Carvil, 1997), and additional moderately resistant genotypes were identified (Mengistu et al., 2007) that can be used to reduce the impact of charcoal on soybean yield (Smith & Carvil, 1997).

### ***Other strategies***

No-till can protect soil moisture, keep soil oxygen level optimum, reduce soil temperature, and prevent the spread of microsclerotia across the field, thereby reducing the chances of microsclerotia germination and infection (Mengistu et al., 2009). Likewise, planting soybean at reduced seeding rates can help to reduce stress by conserving soil moisture, and early planting of early maturity group soybean can help soybean plants escape the stress window of high temperature during the flowering stage, reducing the impact of charcoal rot (Wrather et al., 2007). Also, certified fungicide or biocontrol-treated seeds can help protect germinating soybean seeds and young seedlings from infection and reduce potential damage (Gupta et al., 2012; Mishra & Dantre, 2017). Drought stress and nutrient (such as nitrogen and phosphorus) deficiency can make plants more vulnerable to infection by soilborne pathogens including *Macrophomina*. Adequate water and nutrient supply to plants can keep plants healthy and reduce charcoal rot impact (Gupta et al., 2012).

## ***Part 3. Status and major diseases of hemp in Tennessee***

### ***Brief history***

Hemp is among the newest cash crops in Tennessee, and has described has been described as the new “gold rush” by Tennessee farmers since its reintroduction (Kelman, 2019). Hemp was a major cash crop for Tennessee and the United States up to the mid-1930s. In 1850, Tennessee farmers produced over 500 tons of hemp. However, in 1970, *Cannabis* production, including hemp and marijuana, was banned in the U.S (Walker 2018). Hemp and marijuana belong to the same plant species (*Cannabis sativa*), although hemp differs from marijuana. The psychoactive compound tetrahydrocannabinol (THC) is very low ( $\leq 0.3\%$ ) in hemp and is considered too low to be intoxicating, compared to that of marijuana ( $>0.3\%$  THC, usually much higher), which is intoxicating (Cherney and

Small, 2016). Recently, hemp was reintroduced as a field and glasshouse crop in Tennessee and several other states while marijuana remains illegal.

### ***Use and demand for hemp products***

Hemp is mainly cultivated for fiber, grain, seed oil, and essential oil. Hemp fiber is useful in making textiles, building materials, mulch, paper, and biofuels. The grain (seed) can be used as food, feed products, or as a source of oil that can be put into several uses. Certain oils from hemp can be used for food and in making personal care and industrial products (paints, solvents, and lubricants). Also, cannabidiol (CBD), which is a non-psychoactive compound found in hemp, is reported to offer broad wellness benefits (Cherney and Small, 2016). Consequently, there is a nationwide demand for hemp, which creates an opportunity for Tennessee growers to profit from growing hemp. Thus, hemp production acreage has surged in the state.

### ***Tennessee hemp production acreage***

Tennessee hemp acreage has steadily increased after the re-introduction of hemp as an agricultural crop by the 2014 Agricultural Act (aka. 2015 Farm Bill) (Tennessee Department of Agriculture, 2021). Hemp acreage has increased from approximately 650 acres/year in 2015 to 4,800 acres/year in 2020 (Self, 2021). Production acreage increased by 100% between 2018 and 2019. In 2018, hemp production costs were approximately \$2,301/acre in Tennessee (Tennessee Department of Agriculture, 2021). At this rate, it means hemp growers spent over \$11 million on hemp acreage in Tennessee in 2020. A large portion of the acreage is under high tunnel and greenhouse or indoor systems. The cultivated indoor space was up to 8 million sq. ft. (184 acres, 74 hectares), with growers spreading across all regions of the state (Tennessee Department of Agriculture, 2021).

### ***Major diseases of hemp in Tennessee***

Many diseases can affect all hemp production systems. The most frequently reported diseases of hemp in Tennessee are bud rot, leaf spots, southern blight, and

powdery mildew (Hansen et al., 2020a). Fusarium foliar and flower blights, fusarium wilt, and bacterial leaf spots are also common in some other states (Thiessen et al., 2020).

### ***Powdery mildew***

Powdery mildew is the most common disease in the greenhouse. Powdery mildew pathogens are supported by warm temperatures and humid conditions that are often present in the greenhouse. This makes the greenhouse environments favorable to powdery mildew development and spread. Powdery mildew spreads by asexual conidial spores that are carried by air currents from infected sources to healthy leaves within the greenhouse. After 2 to 3 days on the healthy leaves, the conidial spores germinate and initiate secondary infections (Punja et al., 2019).

### ***Symptoms***

Early signs and symptoms appear as white powdery growths, made up of fungal hyphae and conidia, on upper leaf surfaces and stems. As the hyphae penetrate the leaf tissues, they absorb nutrients and water, impair photosynthesis, and subsequently initiate secondary symptoms such as leaf distortion, necrosis, and leaf drop. In severe cases, powdery mildew can result in reduced plant health, poor yields, or plant death (Britt 2020; Gauthier, 2020).

### ***Host range***

Powdery mildew can affect a wide range of hosts and is caused by many fungal species in genera including *Erysiphe*, *Leveillula*, *Golovinomyces*, *Podosphaera*, and *Sphaerotheca*. Several species of *Golovinomyces* are known to cause powdery mildew on diverse crops in the United States and elsewhere. *Golovinomyces spadiceus* was reported to cause powdery mildew on Green and Gold in Tennessee (Trigiano et al., 2018); okra in Georgia (Moparathi et al., 2018); *Helianthus* in Washington State (Moparathi et al., 2018); and industrial hemp in Kentucky and New York (Szarka et al., 2019; Cala et al., 2019). *G. ambrosiae* was reported on whorled sunflower in

Tennessee (Trigiano et al., 2016), and *G. cichoracearum* sensu lato was found on medical cannabis in Canada (Pépin et al., 2018).

### ***Management***

Effective management of powdery mildew can be achieved by integrating cultural and chemical management tactics. These tactics include the use of less susceptible or resistant cultivars, removal of inoculum sources, and chemical fungicides (Pfeuffer et al., 2017). Although there is no known hemp cultivar with complete resistance to powdery mildew, studies from Tennessee (Hansen et al., 2020b) and New York (Stack et al., 2021; Cala et al., 2019) have shown that many CBD hemp cultivars exhibit low to moderate susceptibility to powdery mildew. On greenhouse hemp, maintaining low relative humidity, removing infected leaves, and applying fungicides can suppress powdery mildew (Britt, 2020). Many commercial greenhouse growers are attracted to organic or bio-pesticides because of their minimum re-entry intervals, low human toxicity, and their perceived or real reduced environmental impact (Gradish et al., 2010; Tjosvold & Koike, 2001). Many organic fungicides are registered for hemp in the United States (Environmental Protection Agency, 2021) and many of them have been approved for use on hemp in Tennessee. Some options include Double Nickel, Carb-O-Nator, Defguard, Exile, Guarda, Lifeguard, Oxidate, Ramgo, Regalia, Sil-Matrix, Stargus, and ZeroTol (Tennessee Department of Agriculture, 2022).

### ***Southern blight***

Southern blight of hemp is caused by the fungus *Athelia rolfsii* (anamorph *Sclerotium rolfsii*) (Pane et al., 2007). This fungus is known to cause major problems in many economic crops including vegetables, fruits, and field crops (Meadows et al., 2019). Occurring worldwide, it causes damage to tomato, pepper, bean, carrot, watermelon, apple, tobacco, cotton, potato, soybean, and many other plant species. It usually infects lower stems but may infect any part of a susceptible plant under favorable conditions. It survives on dead organic matter as mycelium in the absence of susceptible hosts but also survives as sclerotia in soil. During warm (81-95°F), humid conditions,

sclerotia germinate and hyphal growth occurs and infects susceptible plants. Depending on the type of plants or plant parts infected, the disease symptoms may vary, but typically infected plants wilt and eventually die (Mullen, 2001).

### ***Symptoms***

Sudden plant wilt and chlorosis, leaf necrosis, and plant death are immediate symptoms. Later, stem decay and sclerotia formation occur near the soil line. A hyphal mat may spread along the soil surface near the base of the decayed stalk (McPartland et al., 2000).

### ***Management***

Resistant cultivars can reduce infection and lower crop damage. ‘Dwarf’, ‘medium’, and ‘tall’ were reported as cultivars with some level of resistance to the southern blight pathogen (McPartland et al., 2000). Soil solarization can kill the pathogen. Adequate weeding will eliminate alternative hosts, and fallow periods can eliminate germinated sclerotia of the fungus from the soil. Biocontrol with *Trichoderma*, applying mycorrhiza fungi or urea to the soil, and drenching of soil with formalin can protect hemp against the disease (McPartland et al., 2000).

### ***Fusarium wilt***

Fusarium wilt is caused by two subspecies of *Fusarium oxysporum* including *Fusarium oxysporum* f. sp. *vasinfectum* and *Fusarium oxysporum* f. sp. *cannabis* (McPartland & Hillig, 2004). Fusarium wilt can reduce hemp fiber quality and yield, and seed yields, with crop loss reaching up to 90% if unchecked (McPartland & Hillig, 2004). The disease can be very devastating to hemp to the extent that historically, in the 1980s, the fusarium wilt fungi were adopted by the U.S government in an attempt to destroy illegal cannabis cultivation (McPartland and Hillig, 2004). The fungus overwinters in the form of chlamydospores in soil or mycelium on debris (McPartland, 1996). In spring during warm weather, chlamydospores germinate and produce hyphae to infect young roots of seedlings or enter through wounds of older plant parts. The hyphae will spread

within the root and produce microconidia which block water passage in the xylem, interrupting water flow, and killing the plant (McPartland, 1996). A review of hemp (cannabis) diseases caused by diverse *Fusarium* species has been recently published, and it expands on five *Fusarium* species complexes that can cause disease in hemp (Gwinn et al., 2022).

### ***Symptoms***

Initial symptoms include small, dark, irregular spots on lower leaves. Later, the leaf suddenly turns yellow and begins wilting, leaf tips curling upward as infections become severe. Wilted leaves dry and appear yellow-tan in color and remain on plants without falling off. Wilted stems also appear yellow-tan and stem xylem tissue shows reddish-brown discoloration when split-opened (McPartland et al., 2000).

### ***Management***

Crop rotation to non-host crops (wheat has been suggested) can reduce disease pressure, and fusarium wilt-resistant cultivars ('Fibramulta 151') can reduce disease and prevent potential crop damage. Proper nutrient management, including avoiding excess nitrogen and maintaining optimal calcium and potash support plants against wilt disease. Soil pH should be maintained near neutral. Supplying soil with organic materials and manure can support natural soil *Fusarium*-antagonist microbes (McPartland and Hillig, 2004). Biocontrol fungi such as *Trichoderma*, *Bulkoldera*, and *Streptomyces* are reported to be effective against *Fusarium* wilt when used combined with *F. oxysporium* biocontrol product. Chemical control options are limited to treating seeds with fungicides and applying soil fumigants (McPartland et al., 2000).

### ***Hemp leaf spots***

Leaf spot diseases are among the most common diseases of hemp. Hemp leaf spots are caused by bacterial and fungal species. Among frequently reported hemp leaf spots include anthracnose leaf spot (D. Szarka et al., 2020), bacterial leaf spot (Thiessen et al., 2020), *Cercospora* leaf spot (Marin et al., 2020a), *Curvularia* leaf spot (Marin et al., 2020b), *Corynespora* leaf spot, hemp (*Bipolaris*) leaf spot (Desiree Szarka et al., 2020),

Septoria leaf spot (Rahnama et al., 2021), and Helminthosporium leaf spot (Thiessen et al., 2020). *Curvularia* and *Bipolaris* were associated with leaf spots in Tennessee (Akinrinlola & Hansen, 2021). Leaf spot diseases can be very detrimental to hemp plants. Leaf spot incidence can range from 50 to 100%, covering parts or whole plant foliar parts, and severity and damage can be up to 100% (Akinrinlola & Hansen, 2021; Szarka et al., 2020).

### ***Symptoms***

Leaf spot symptoms can slightly vary among organisms and hemp cultivars. A typical symptom starts as small round lesions having a tan center and brown to dark or purple margins. Yellow halos and packs of conidiophores may be visible to a naked eye or through a hand lens at the center. As the disease becomes severe and lesions coalesce, large lesions with more irregular shapes and dark/brown centers known as leaf blights may result. Leaves often turn yellow and fall off prematurely (Akinrinlola & Hansen, 2021; Szarka et al., 2020). In most leaf spots, symptoms often start from lower/older leaves and inner canopy and spread throughout the plant canopy (Marin et al., 2020a; Rahnama et al., 2021).

### ***Bipolaris leaf spot***

*Bipolaris* is an important fungus with a wide host range and causes diseases in many other valuable crops including rice, corn, wheat, barley, and sorghum. Rice brown spot is caused by a *Bipolaris* species and was responsible for the famine that resulted in human starvation in India in 1943 (Manamgoda et al., 2014). Although little is known about the fungus in hemp, typically it is known to survive as thick-walled conidia or mycelium during harsh conditions or in the absence of its host. During favorable warm humid conditions, it develops and infects young plant roots, crowns, or other below-ground parts. As infections spread to above-ground parts, conidia develop and spread with the aid of wind or rain splash to cause secondary infections (Creswell and Harris, 2014).

## ***Management***

Presently the best control option for hemp leaf spots is to avoid susceptible cultivars. Resistant or moderately resistant cultivars can reduce disease severity and prevent yield loss to leaf spots. Cultivars with a significant level of resistance were identified in Tennessee (Hansen et al., 2020b). Likewise, fungicides with varying levels of efficacy against leaf spots have been identified (Akinrinlola & Hansen, 2021). Some examples of fungicides with known efficacy that have been recommended to control leaf spots on hemp include Exile, Stargus, and Regalia (Akinrinlola & Hansen, 2021). It is important that growers read, understand, and follow fungicide labels before applying them to crops to ensure fungicides are applied safely and legally.

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**CHAPTER II. NEMATODE POPULATION DYNAMICS AND  
*HETERODERA GLYCINES* HG TYPES IN TENNESSEE, 2018-2020**

## Abstract

Field crops occupy a large portion of Tennessee farm acreage, but plant-parasitic nematodes (PPNs) can reduce their productivity. Multiple PPNs are found across Tennessee fields but there is no current information on their prevalence and density in field crops. Additionally, information on soybean cyst nematode [SCN] (*Heterodera glycines*) virulence potentials (HG types) on resistant soybean can support SCN management, but the information is currently lacking for Tennessee SCN. This study was conducted from 2018 through 2020 to (1) determine the population density of PPN associated with field crops in Tennessee, and (2) determine virulence phenotypes of SCN in the state by the HG types. Soil samples (378) were collected from 25 counties, and populations of nematode juveniles or eggs present in 100 cm<sup>3</sup> of each sample were extracted by the decanting method. HG type testing was conducted for SCN found in samples that had at least 65,000 eggs/100 cm<sup>3</sup> of soil. Seven SCN resistant indicator lines and ‘Williams 82’ (susceptible) soybean seedlings were infested with 2,000 eggs of SCN at planting, and SCN females that formed on the roots of the soybean were collected and used to estimate the female index on each resistant line. HG type number was assigned to SCN populations based on the female index on each resistant line. Fourteen nematode species were found at different frequencies and densities across the samples. Spiral (83%), lance (29%), SCN (25%), stunt (19%), lesion (23%), reniform (20%), and root-knot nematodes (13%) were found most frequently. Lance, reniform, root-knot, SCN, and spiral nematode populations occurred in relatively high frequencies or densities that may require close monitoring or immediate control measures. Also, all the screened SCN populations were identified as the virulent HG types 1.2.5.7 (67%), 2.5.7 (22%), and 7 (11%). None of the screened SCN populations were the non-virulent HG type 0. This information will support nematode management and field crop production in Tennessee.

## Introduction

Field crops occupy a large portion of Tennessee farm acreage. Large acres are regularly cultivated for soybean, cotton, corn, and wheat in the state. Approximately 1.7 million acres of soybean, 280,000 acres of cotton, 870,000 acres of corn, and 300,000 acres of wheat were cultivated in Tennessee in 2020 (U.S Department of Agriculture\_ National Agricultural Statistics Service, 2022). Field crop production can be limited by biotic and abiotic constraints. Nematodes are among the most important crop limiting biotic factors (Bernard, 1980). Among the most predominant and economically important of these species are root-knot nematodes (*Meloidogyne incognita*, and *M. hapla*) soybean cyst nematode [SCN] (*Heterodera glycines*), lesion nematode (*Pratylenchus scribneri*), reniform nematode (*Rotylenchulus reniformis*), dagger nematode (*Xiphinema americanum*), spiral nematode (*Helicotylenchus pseudorobustus*), and stunt nematode (*Tylenchorhynchus claytoni*) (Bernard, 1980).

All plant-feeding nematodes possess a hollow protrusible mouthpart referred to as a stylet which they use to probe and feed on plant tissues. They typically affect crop growth directly by feeding on roots or indirectly by altering symbiotic nitrogen fixation activity in legumes (Elhady et al., 2020; Ko et al., 1984). They can live as migratory ectoparasites, migratory endoparasites, semi-endoparasites, or sedentary endoparasites (Lambert & Bekal, 2002). Migratory ectoparasites (e.g. dagger nematode, *X. americanum*; stunt nematode, *Tylenchorhynchus claytoni*) stay outside of their host plants and use their stylets to feed on the cells of the plant roots (Lambert & Bekal, 2002). Whereas migratory endoparasites (e.g. lesion nematode, *P. scribneri*) enter the host roots and migrate through the cortex, feeding on cell cytoplasm and causing tissue damage. Migratory endoparasites do not form permanent feeding cells (Lambert & Bekal, 2002). Semi-endoparasitic nematodes (e.g., reniform nematode; *Rotylenchulus reniformis*) partially penetrate their host so that the anterior portion is embedded while the posterior portion is exposed. The nematode then forms a permanent feeding site for a long-lasting nutrient supply (Lambert & Bekal, 2002). Sedentary endoparasites (e.g. SCN, *H. glycines* and root-knot nematodes, *M. incognita*, *M. hapla*, and *M. javanica*) completely enter and become embedded in the host tissue, and move toward the

developing vascular cells to form a permanent feeding site for a continuous nutrient supply (Acedo et al., 1984; Lambert & Bekal, 2002). The life cycle of plant-parasitic nematodes begins with eggs, followed by four juvenile stages (J1 to J4), and adult. Depending on the nematode species and conditions, a nematode may take two to six weeks to complete a life cycle. At the end of a life cycle, adult females can produce about 50 to 1,000 eggs per female, depending on the nematode species and environmental influences (Lambert & Bekal, 2002b; Mitkowski, 2003; Wesemael et al., 2014).

The amount of crop damage caused by nematodes depends on the host species, prevailing environmental conditions, the nematode species, and the population density of the nematodes (Mitkowski and Abawi, 2003). Nematodes are managed mainly by growing resistant or tolerant cultivars, rotating to non-hosts, and using nematicides to suppress nematode population densities (Sikora et al., 2021). Generally, information on nematode population density is used as a guide or threshold for making management decisions. These thresholds are based on the number of nematode infective juveniles (J2) or eggs found in a representative soil sample, usually a 100 or 500 cm<sup>3</sup> soil sample (Barker & Olthof, 1976). There is no current information on the density of the economically important nematodes associated with field crops in Tennessee. This information is essential to provide nematode management guidance for Tennessee growers and guide future research efforts.

The soybean cyst nematode was estimated to cause more than 123 million bushel yield loss in annual soybean production between 2010 and 2014 in the United States and Canada (Allen et al., 2017). With soybean at \$16/bushel, SCN costs producers over \$2 billion loss annually. The nematode has been mainly controlled by growing resistant/tolerant soybean cultivars, rotating to non-host crops, and nematicides. However, SCN populations with the ability to feed and reproduce on the most common resistance source used in commercial cultivars are evolving and eliciting yield loss on resistance cultivars, because of the prolonged reliance on a sole resistance source (Niblack et al., 2008; Yan & Baidoo, 2018). This circumstance has spurred researchers to investigate and categorize SCN populations based on their capability to feed and reproduce on resistant indicator lines. This categorization is done with the HG Type test

on SCN populations to place them into different biotypes or HG (*Heterodera glycines*) types (Niblack et al., 2002; Tylka, 2016). The HG type test is conducted by culturing an SCN population on the roots of the seven resistant indicator lines (numbered 1 to 7) possessing the known sources of SCN resistance used in commercial cultivars, and a standard susceptible cultivar. The reproduction of the SCN population is compared between each indicator line and the susceptible cultivar. When the density of an SCN population on an indicator line is greater than 10% of the SCN population on the susceptible cultivar, the population is considered capable of feeding on the indicator line, and hence the SCN population HG Type will carry the number of that indicator line. For example, an ‘HG type 1’ SCN population can feed and reproduce on indicator line 1. This means the population density on indicator 1 is greater than 10% of its density on the susceptible cultivar. Similarly, an “HG type 1.2” population can successfully feed and reproduce on indicator lines 1 and 2. Knowing the HG type of an SCN population can help to make informed management decisions, specifically about what sources of resistance should be sought to manage specific SCN populations. However, there is currently no available data on the HG types of the SCN populations associated with field crops in Tennessee.

The objectives of this study were therefore to (1) determine the population density of major plant-parasitic nematodes associated with field crops in Tennessee, and (2) determine the HG types or virulence phenotypes of SCN in the state.

## **Materials and methods**

### ***Soil sample sources***

A total of 378 soil samples were collected across 25 counties in Tennessee from 2018 through 2020. The samples were either submitted by county agents, crop consultants, growers, or collected by the members of the field crop pathology team from row crop fields. Fields were sampled predominantly in the fall after the harvest of soybean, corn, or cotton. Each sample comprised of approximately 20 soil cores collected per 20 acres of land, from 6 to 8 inches deep in the soil, using a 2.5-cm diameter soil probe.

### ***Determination of nematode density in samples***

Each soil sample was mixed by passing it through a 0.6-cm-mesh metal screen. The screen was cleaned between samples. A 100-cm<sup>3</sup> subsample was taken from each sample for nematode extraction. The nematode extraction was conducted by the Arkansas Nematode Diagnostic Laboratory at the University of Arkansas using the decanting method (Van Bezooijen, 2006). The numbers of nematode juveniles (J2) and SCN eggs found in each subsample were recorded.

### ***SCN HG types***

#### ***Extraction of SCN females from samples.***

Soil samples having a moderate to high SCN egg density (>2,000/100cm<sup>3</sup>) screened were selected for HG-type testing. The remaining sample was either used alone or mixed with some additional soil to grow a susceptible soybean cultivar, 'Hutchinson', for 30 to 60 days to increase the SCN population in the soil to have enough nematodes for the HG type test. After 30 or 60 days, SCN females were collected from the roots and rhizosphere soil of Hutchinson using a sugar flotation-centrifugation method (Jenkins, 1964). First, the root system was soaked in water for at least 15 min, after which most of the remaining soil attached to the roots was gently removed into the water by hand. The roots were then placed onto an 850 µm-pore sieve nested over a 250 µm-pore sieve. Roots were sprayed with a strong water stream to dislodge SCN females from the roots into the 250µm-pore sieve. The soil in the bucket was thoroughly mixed, stirred by hand, and then carefully decanted through the 850µm-pore sieve. All the materials collected in the 250µm sieve were then rinsed with water and decanted into a clean beaker. Further separation and extraction of the SCN females were done by using the sucrose centrifugation method, using a dense sugar solution containing 605g table sugar dissolved in 500 ml water (Jenkins, 1983).

### ***Extraction of eggs from SCN females.***

After extraction, the SCN females were crushed using a rubber stopper attached to a drill press in a small 250µm pore sieve over a 75µm pore sieve nested on a 25µm pore sieve. After rinsing with a strong pressure water stream, the eggs were poured into a clean beaker and diluted to 20 to 40mL egg solution. The density of eggs present in 1mL of the egg solution was determined on a counting disk under a microscope, and the total egg number was extrapolated. SCN populations with sufficient eggs (> 65,000 eggs) were used to perform the HG type test. Populations with a lower egg yield were inoculated onto the roots of susceptible soybean again to reproduce for 30 to 60 days to increase the population to the required density and provide an active population for the HG type test.

### ***Determination of the HG type.***

Nine SCN populations that had a sufficient population density were used to perform the HG type test following the method described in previous studies (Acharya et al., 2016; Niblack et al., 2002, 2003, 2009; Zheng & Chen, 2011) with some modification. Soybean indicator lines used for the HG type testing included PI 548402 ('Peking', indicator 1), PI 88788 (2), PI 90763 (3), PI 437654 (4), PI 209332 (5), PI 89772 (6), PI 548316 ('Cloud', 7), and a susceptible check ('Williams 82'), which were obtained from the USDA soybean germplasm collection at the University of Illinois, Urbana-Champaign. Seeds were germinated on moist germination paper in a growth chamber at 28°C for three days. One seedling of each line was planted into a PVC tube (2.5 cm wide, 23 cm long) filled with a mixture of pasteurized Mason Sand and field soil in a 3:1 ratio, respectively. The tubes were arranged in a completely randomized design with five replicates in a rectangular 5-gallon bucket (D:W:H: 40:30:20cm). Spaces between the tubes were filled with soil to keep the tubes in place. At planting, each soybean seedling was infested with approximately 2,000 SCN eggs contained in 1 mL of water. The bucket was maintained in a growth chamber at 28°C and 16-hours of daylight for 30 days. The plants were watered to optimum moisture level each day and fertilized weekly with Miracle-Gro Plant Food (The Scotts Company, Marysville, OH) at the rate

of 14g per 3.8 L. After 30 days, the tubes were removed from the bucket and soaked in water for 15 min, then plants were carefully. Female SCN were collected from the roots as previously described. All females collected from each indicator line and Williams 82 were counted under a microscope, and the data were used to calculate the female index (FI) for each line, as follows:  $FI = (\text{average number of females found on indicator line} / \text{average number of females found on susceptible line}) \times 100$ . An SCN population with an FI equal to or greater than 10% on any indicator line was assigned the number of the indicator line as the HG type. Each population was evaluated twice except for P26 and P28 populations, which died out after the first evaluation.

### ***Data analysis***

Descriptive statistics were obtained for the survey data using JMP Pro 14 (SAS Institute, Cary, NC), and the analysis of variance (ANOVA) was performed for the HG type data to compare the number of SCN females collected from the roots of each soybean line using SAS 9.4 (SAS Institute, Cary, NC).

## **Results**

### ***Sample sources***

A total number of 378 soil samples were collected from 2018 through 2020 from 25 Tennessee counties (Table 2.1). The highest number of samples per county was 118, 44, and 31 samples from Crockett, Gibson, and Henry counties, respectively. Carroll, Lawrence, and Obion counties had only 1 sample each. Information about the crop that was grown in the fields during the sampling year was provided for 372 samples (Figure 2.1). The number of fields for each crop type was 150 for soybean, 54 for cotton, 47 for corn, 10 for wheat, and the remaining fields were either grown with cover crops, weeds, unknown crops, or under fallow (Figure 2.1). The “unknown crops” were samples that had no information about the crops that were grown in the fields during the sample year. Information about the names of soybean cultivars grown in fields was provided for 70 samples. The top three soybean cultivars in the list were Asgrow 47×6 from 22 fields, Asgrow 43×8 from 11 fields, and Asgrow 43×7 from 7 fields (Figure 2.2).

### ***Nematode diversity and distribution***

***Across samples.*** All of the 378 samples, except one, had at least one type of plant-parasitic nematode detected in them (Table 2.1). Fourteen plant-parasitic nematode genera (dagger, lance, lesion, needle, pin, reniform, ring, root-knot, sheath (*Hemicycliophora*), SCN, spiral, sting (*Belonolaimus* spp.), stubby-root, and stunt nematodes) were found at different frequencies and densities in the sampled county samples (Table 2.1).

***Across counties.*** No nematode was detected in one county (Lawrence). Five counties (Carroll, Obion, Overton, Tipton, and Warren) had a low nematode diversity (2 to 3 nematodes). Eleven counties (Benton, Chester, Coffee, Giles, Hardin, Henderson, Henry, Madison, Marion, Marshall, and Shelby) had a moderate nematode diversity with 4 to 7 nematode genera detected. The remaining eight counties (Cannon, Crockett, Dyer, Gibson, Haywood, Lauderdale, McNairy, and Weakley) were high in nematode diversity, with 8 to 11 genera detected (Table 2.1).

### ***Nematode frequency and density***

***Density across samples.*** The frequency and average population densities of the nematodes per 100 cm<sup>3</sup> of soil are presented in Table 2.2. Spiral nematode (occurring at 83%), lance nematode (29%), and SCN (25%) were the most frequently found nematodes across samples (Table 2.2). The average population density per sample for the nematodes was 88 J2s for spiral nematodes, 22 J2s for lance nematodes, and 22 J2s or 779 eggs for SCN (Table 2.2). Likewise, root-knot (occurring at 25%), lesion (23%), and reniform (20%) nematodes, which are economically important, were found at an average density of 78, 20, and 1,764 J2s per sample, respectively. The maximum density found for each nematode was 562 J2s for root-knot, 100 J2s or 4,567 eggs for SCN, 115 J2s for lesion, and 16,669 J2s for reniform per sample (Table 2.2). Dagger; pin; sting (7%), stubby-root (3%), and sheath; needle; and ring (1%) nematodes were the least found nematodes across the samples. The nematodes were found with an average density of 16 J2s (pin),

13 J2s (dagger and sheath), 8 J2s (needle and ring), 7J2s (sting), and 2 J2s (stubby-root) per sample across positive samples (Table 2.2).

***Density across counties.*** Across the 25 counties, spiral nematode was found in 24 counties (96%) with an average of 90 J2s. SCN was found in 18 counties (72%) with an average of 741 eggs (or 25 J2s). Lesion nematode was found in 19 counties (76%) with an average of 22 J2s. Reniform and root-knot nematodes were found in 2 and 13 (52%) counties (8% and 52%), with an average of 913 and 89 J2s, respectively, per sample (Table 2.2).

### ***Population density across fields***

The density of each nematode across each field type varied (Figure 2.3). Dagger nematode was found in cotton, corn, soybean, and wheat fields at an average juvenile density of 8 (Cotton and Wheat), 13 (Soybean), and 15 (Corn) J2s per sample. Lance nematode (*Hoplolaimus* sp) was found across fields (corn, cotton, soybean, wheat, fallow, and cover crops) at an average density that ranged between 8 J2s (cover crops) and 22 J2s (cotton and corn) per sample. Lesion nematode was found across all fields at an average population density that ranged between 8 J2s (cover crops) and 29 J2s (cotton) per sample. Needle nematode (*Longidorus*) was found in weed-covered and unknown fields at an average population density of 8 J2s per sample. Pin nematode (*Paratylenchus*) was only found in soybean and unknown fields at an average population density of 11 and 31 J2s per sample, respectively. Sheath nematode was found in corn and soybean fields at an average population density of 8 J2 and 15 J2s per sample, respectively. Soybean cyst nematode was found across fields grown with soybean, corn, cover crops, unknown, cotton, wheat, and fallow. SCN eggs were detected only in soybean, cover crops, unknown, cotton, and fallow fields. No SCN egg was found in cotton and wheat fields. Average SCN-eggs density per sample ranged from 58 (Weed-cover) to 701 (Soybean) across fields. Likewise, SCN J2s were detected only in soybean, corn, cover crops, unknown, wheat, and fallow fields. No SCN J2 was found in cotton and weed-covered feeds. Average SCN J2s per sample density ranged from 10 (Wheat)

to 38 (Fallow) across fields. Spiral nematode was found in all field types, with an average density that ranged from 34 J2s (Wheat) and 188 J2s (Fallow) per sample. Sting nematode was found in corn, cotton, soybean, wheat, and cover crops fields at an average population density that ranged between 1 (cover crops) and 15 J2s (Cotton) per sample. Stubby-root nematode was found in corn, cotton, soybean, and unknown fields at an average population density ranging between 8 (unknown) and 27 J2s (soybean) per sample. Stunt nematode was found in all fields except in fallow fields. The average density ranged between 8 J2s (Weed cover and Wheat) and 30 J2s (Corn) per sample. Reniform nematode was found in cotton, soybean, unknown, and wheat fields at an average population density ranging between 38 (Wheat) and 2,782 J2s (Soybean) per sample. Reniform density in cotton fields averaged 1,267 J2s per sample. Ring nematode was found in soybean and unknown fields at an average population density of 8 J2s per sample. Root-knot nematode was found in all fields except in fallow fields at an average population density that ranged between 8 J2s (Wheat) and 145 J2s (Soybean) per sample.

#### ***SCN density across soybean fields.***

Soybean fields were grown with eleven soybean cultivars and each either had only SCN eggs, J2s, or both (Figure 2.4). Of those fields, SCN eggs per sample ranged from 58 (PI40T26X) to 1,490 eggs (Asgrow 43×7), while J2 densities ranged from 2 (CL3900) to 23 (Asgrow 43×7) per sample (Figure 2.4). No SCN was found in fields grown with Asgrow 38x8, Asgrow 46x7, Becks 366, and Warren 4911. No SCN eggs were found in fields grown with CL 3900 LL, CL 4850 LL, and Asgrow 47x6 soybean cultivars (Figure 2.4).

#### **SCN HG types**

##### ***SCN reproduction on soybean lines, female index, and HG types.***

The population (cysts/plant) of each test population on the soybean lines is presented in Table 2.3. Reproduction on the susceptible soybean Williams 82 ranged from 69 cysts to 434 cysts per plant. Six populations had an FI  $\geq 10\%$  on Indicator line 1 (PI548402). The FI of the populations ranged from 10 to 28%. Eight populations had a FI

$\geq 10\%$  on indicator lines 2 (PI88788) and 5 (PI209332). The FI of the populations ranged from 27 to 106% on PI88788, while it ranged from 22 to 62% on PI209332. All nine populations had a FI  $> 10\%$  on indicator line 7 (PI548316). The FI ranged from 13 to 706% on the line. No population had a FI  $\geq 10\%$  on indicator lines 3 (PI90763), 4 (PI437654), and 6 (PI89772). Female indices on the lines ranged from 0 to 8% (Table 2.3). The tested populations were identified as three HG types: 1.2.5.7, 2.5.7, and 7. Six populations were identified as HG type 1.2.5.7, two were HG type 2.5.7, and one was HG type 7. No population was HG type 0 or with a FI  $< 10\%$  on all indicators (Table 2.3).

## **Discussion**

The number of samples from each county varied widely. The top three highest samples came from three West Tennessee counties (Gibson, Crockett, and Henry), which are among the leading producers of field crops in Tennessee with 248,145, 125,830, and 95,541 field crop acreages in 2020, respectively (Bowling and Smith, 2021). Their large field crop acreage likely accounts for their high number of samples compared to other counties, because of more farms to sample. Although Obion, Carroll, and Lawrence counties had the lowest number of samples, they are high field crop-producing counties with 196,856, 97,353, and 59,974 acreages in field crops, respectively (Bowling and Smith, 2021). The numbers of samples received from these counties were small because the number of growers that participated in the nematode survey from the county was low compared to that of other high field crop-producing counties. Future sampling efforts should be done to allow a similar number of samples to be collected from counties with similar field crop acreage. All (14) plant-parasitic nematode genera that were screened for were found in this survey, similar to the survey conducted in Tennessee by Bernard (1980) where the same genera were previously found. From our sample data, the diversity of nematodes per county was associated with the number of samples processed per county. Hence, the nematode diversity in some counties with a low number of samples (e.g. Carroll, Obion, Overton, and Warren) may be underestimated in this data set.

Results suggest that spiral, lance, and SCN are the most abundant nematodes

among those screened for across sample areas (Table 2.1). Spiral nematodes were associated with 83% of the samples, lance with 29% of the samples, and SCN with 25% of the samples (Table 2.2). Spiral nematodes are ectoparasites that feed on the epidermis and cortical cells of the host plants. Some species may shift to a semi-endoparasite feeding mode, inserting their anterior body part into host roots. They are known to have multiple hosts including fruit crops, vegetables, agronomic crops, ornamental plants, forages, turfgrasses, weeds, and plants in natural habitats (Crow, 2013). Our results suggested an increased prevalence of spiral nematodes within Tennessee compared to the previous survey (Bernard, 1980). Bernard (1980) associated 67% of samples and all tested counties with spiral nematodes, while 83% of samples and 96% of counties tested from 2018 through 2020 had spiral nematodes.

Spiral nematodes are also widespread elsewhere in the United States. In Iowa, spiral nematodes were associated with 77% of cornfield samples (Tylka et al., 2011), and in Ohio, 94% of tested fields had spiral nematodes (Wilson & Walker, 1961). However, spiral nematodes often occur as non-pests or minor parasites, even at a very high density on most of their hosts (Bernard & Montgomery-Dee, 1993; Bernard, 2018). Populations of spiral nematodes can be greater than 2,000 nematodes per 100cm<sup>3</sup> on sunflower and rapeseed without causing damage (Bernard & Montgomery-Dee, 1993; Bernard, 2018). Other hosts like soybean, cotton, corn, bermudagrass, seashore paspalum, and creeping bentgrass can incur a loss due to spiral nematode loads (Crow, 2013). A density as high as 500 nematodes/100 cm<sup>3</sup> soil was reported to cause damage to corn in Iowa (Tylka et al., 2011). Our findings indicate that spiral nematodes are not currently a major concern to field crop growers in Tennessee as the average density found was 88 nematodes/100 cm<sup>3</sup> and only a few samples (0.02%) had over 500 spiral nematodes/100cm<sup>3</sup>. A study to evaluate the effect of the highest density on corn and soybean production as well as continued monitoring of spiral nematode populations across Tennessee are recommended.

Lance nematodes (*Hoplolaimus* spp.) are migratory ectoparasites and endoparasites. They feed on the outside of the root but eventually enter and embed themselves either partially or completely within the root of their host (Supramana et al.,

2001). Lance nematodes have a wide host range, but some of their most economical hosts are cotton, soybean, and turfgrass (Crow & Brammer, 1969; Crow, 2005; Holguin et al., 2015, 2016). Lance nematode species have been associated with field crops, forage, pasture, woody ornamentals, turf, and vegetables in Tennessee (Bernard, 1980) and our study supports that lance nematode can be harbored by cotton, soybean, corn, wheat, weeds in fallow fields, and cover crops. Weeds and cover crops may serve as alternative hosts to maintain this nematode. Our study also suggests an increase in lance nematode prevalence compared to previous findings. Bernard (1980) found that 19% of samples and 27% of counties had lance nematodes, compared to 29% of samples and 68% of counties in this study. Densities of 60 lance nematodes/100 cm<sup>3</sup> can cause stunted root growth in soybean (Yan et al., 2016) and 75 nematodes/100 cm<sup>3</sup> can damage cotton, and cause up to 25% in yield loss (Koenning et al., 2004). The average density found in the present study was 22 nematodes/100 cm<sup>3</sup> soil, with only ~ 7% of lance populations greater than 60 nematodes/100 cm<sup>3</sup> of soil and 100 nematodes/100 cm<sup>3</sup> being the maximum density found. This suggests that only a few populations of lance nematodes that were found in this study have the potential to cause damage on Tennessee field crops including soybean, cotton, and corn.

Soybean cyst nematode is the most important soybean pathogen in the United States, as it is responsible for over \$1.5 billion in yield loss annually (Allen et al., 2017), and is widespread across all soybean growing countries and regions (Schmitt et al., 1992). Our results showed that SCN was associated with different field types at varying frequencies and densities. Variability in nematode densities across different areas or counties may be due to variability in soil properties, host plant, soybean cultivars, SCN aggressiveness, and sampling time (Bernard, 1980; Nigh, 1980; Salahi Ardakani et al., 2014). The five highest population densities (2,381, 2,369, 1,353, 1,076, and 950 eggs/100 cm<sup>3</sup>) were recovered from top-soybean producing counties (Weakley, Chester, Henry, Hardin, and Crockett counties, respectively). This may likely be due to the planting of soybean cultivars that are favorable to SCN population increase. Results showed that soybean cultivars Asgrow 29x8, Asgrow 43x7, and Asgrow 43x8 were associated with higher SCN densities compared to other cultivars. Likewise, sample

numbers may have contributed to the frequency of high-density populations observed as counties with high sample numbers were among those with the greatest population densities. Only 25% of the samples tested had SCN populations in them, and the majority of the populations were very low (average density was 22 J2s and 799 eggs/100 cm<sup>3</sup> of soil). Also, the maximum densities detected were 100 J2s and 4,569 eggs per 100 cm<sup>3</sup> of soil. SCN can cause high damage when densities are greater than 5,000 eggs/100 cm<sup>3</sup> of soil, moderate damage between 2,000 to 5,000 eggs/100 cm<sup>3</sup> of soil, low damage between 200 and 2000 eggs/100 cm<sup>3</sup> of soil, and trace between 1 and 200 eggs/100 cm<sup>3</sup> of soil (Mehl, 2018). None of the densities found in this study were greater than 5,000 eggs/100 cm<sup>3</sup> of soil, and of the samples that had SCN, only 11% had more than 2000 eggs/100 cm<sup>3</sup> of soil. This implied that SCN population densities are not yield-limiting in the majority of the fields sampled. These lower densities are most likely due to the regular crop rotation to corn or cotton, non-host crops for SCN, which is common practice in Tennessee. Hence, the use of resistant cultivars or rotation to non-hosts in fields with low to moderate SCN densities is still recommended to prevent further increase of the nematode density and to avoid potential yield loss. Furthermore, compared to other states in the U.S, our results suggested that SCN infestation in Tennessee may be lower than that of some other states. For example, the 25% SCN occurrence in Tennessee field crop acres from this study was lower than that of 70% in soybean fields in Michigan (Schumacher-lott, 2011), 68% in soybean fields in Minnesota (Chen and Grabau, 2018), and 30% in soybean fields North Dakota, (Yan & Baidoo, 2018). It appears that these states reported greater SCN infestation compared to the present study because they only sampled soybean fields. The infestation occurrence in TN is higher than that of North Carolina, 22%, and Kansas state, 15% (Todd and Powers, 2018).

Lesion (23%), reniform (20%), stunt (19%), and root-knot (13%) nematodes were the next most common nematodes found. Root lesion nematode (*Pratylenchus*) is the third most economically important nematode after SCN and root-knot nematode on soybean (Allen et al., 2017; Jones et al., 2013). It is a migratory endoparasite capable of attacking more than 400 plant species (Davis & MacGuidwin, 2000). Results showed that

lesion nematodes occurred at low incidences across fields with 23% of samples containing them, but were present in most counties, with 76% occurrence. Most of the populations were generally low ( $<20$  nematodes/100 cm<sup>3</sup>). Up to 34% of the populations were greater than 20 nematodes/100 cm<sup>3</sup> of soil in density, which is the lowest damage threshold for soybean (Mehl et al., 2018). It is unclear why lesion population densities are low in this survey. Resistance and non-host crops are very limited, but some cotton cultivars with moderate resistance to some lesion nematode species have been reported (Chowdhury et al., 2022). There are suggestions that soil microbial antagonists, certain weather conditions, and weed management can contribute to reducing lesion nematode densities in fields (Brown & Smart, 1985; Davis & MacGuidwin, 2000; Kandel et al., 2013). These factors may have contributed to the low density observed in this survey.

More than 300 plant species can host reniform nematode (*Rotylenchulus*), but cotton, soybean, sweet potato, and vegetables are major hosts (Ayala & Ramírez, 1964). Our results suggested that reniform nematode occurrence is low in Tennessee, occurring in 20% of all samples; although this is an increase in prevalence, as previous studies found it in 5% of samples (Newman 1998). Similarly, the population density in the current study averaged 1,764 nematodes/100 cm<sup>3</sup> whereas the previously reported population density was only as high as 268 nematodes/200 cm<sup>3</sup> of soil for a survey conducted in 1997 (Newman 1998). In a subsequent survey done in 2014-2015, the nematode densities were as high as 5,000/100 cm<sup>3</sup> of soil in several samples (Bernard, 2018). Economic thresholds for reniform nematode for cotton in Tennessee were 210 juveniles/100 cm<sup>3</sup> at planting or 1,050 adults + juveniles / 100 cm<sup>3</sup> at harvest (Newman 1998; Bernard, 2018). The thresholds for soybean in Arkansas ranged between 500 to 1,000 nematodes/100cm<sup>3</sup> (Kirkpatrick et al., 2014). Current results showed that reniform nematodes are in the economic thresholds for cotton and soybean in a few fields. The average densities were 1,764 (across 74 positive samples) and 913 (across two positive counties) nematodes/100 cm<sup>3</sup>, and the greatest density detected was 16,669/100 cm<sup>3</sup>. However, this density occurred less frequently, in only 20% of samples and 8% of tested counties. Specifically, the samples with reniform were mostly from cotton and soybean fields. This is most likely because of the rotation of the two crops. Rotation to corn or

grain sorghum has been recommended to reduce high reniform populations. Other control options include nematicides and seed treatments (e.g. Abamectin, Thiodicarb, or *Bacillus firmus*). Foliar applications of Vydate combined with nematicide seed treatments are also found to suppress nematode populations (Mueller et al., 2012).

Stunt nematodes (*Tylenchorhynchus* spp.) are obligate migratory ectoparasites of many plants including field crops, forage, turf, and vegetables (Bernard 1980; Niblack & Bernard, 1985). Legumes are major hosts of stunt nematode while corn, cotton, and peanut are poor hosts (Mehl et al., 2018). As low as 200 and 400 nematodes /100 cm<sup>3</sup> of *Tylenchorhynchus claytoni* can cause damage to corn and soybean, respectively (Barker & Olthof, 1976). Several genera and species have been identified, so hosts may respond differently depending on the genera or species (Barker & Olthof, 1976; Niblack & Bernard, 1985). From this survey, stunt nematode occurrence was very low, being found in only 19% of samples at an average density of 24 nematodes/ 100 cm<sup>3</sup> of soil and the greatest density detected was 92 nematodes/100 cm<sup>3</sup>. However, the nematode occurred more frequently (64%, 16 counties) across counties, showing a slight increase based on previous survey studies where seven species of *Tylenchorhynchus* were isolated from fields across 12 counties in Tennessee (Niblack & Bernard, 1985; Bernard 1980). Notably, the nematode's density and occurrence in Tennessee in our study are lower than that of other states. In a North Carolina state survey, stunt nematodes were found in 83% of samples at an average density of 343 nematodes/500 cm<sup>3</sup> of soil (Schmitt, 1988). Overall, the present results indicate that no immediate drastic management effort is required for stunt nematodes, other than continued monitoring before or after planting each season. Nevertheless, a study is needed to understand the host preference among stunt nematode species in Tennessee.

Four species of root-knot nematodes (*Meloidogyne graminis*, *M. hapla*, *M. incognita*, and *M. javanica*) were previously identified in Tennessee (Bernard 1980). In the past, root-knot nematodes have rarely been a major pest of field crops in Tennessee. Root-knot nematodes mostly cause damage in sandy soils (Mueller et al., 2012) and the majority of the soils tested in this survey were silt loam. Regardless, recently root-knot nematodes have been devastating in two soybean fields in Tennessee, cutting yield by

about 50% in the past 5 years (Heather Kelly, *Personal communication*). They can also become a major problem in vegetable crops. Root-knot nematode was found in 94% of samples collected from commercial tomato fields at high densities, over 5,000 nematodes/100 cm<sup>3</sup> (Bernard 1980; 1981; Stockdale, 1985). A previous Tennessee survey reported that only 41% of samples from diverse sources including field crops and others, had root-knot nematodes (Bernard 1980); but in the present survey, only 13% of samples had root-knot nematodes, indicating that root-knot nematodes were not predominantly associated with Tennessee field crops.

However, the root-knot nematodes densities in this study are at levels that can elicit damage to crops. The highest density found was 562 nematodes/100 cm<sup>3</sup> of soil. As low as approximately 10 root-knot nematodes/100 cm<sup>3</sup> can cause damage to soybean (Mehl, 2018), and as low as 40 nematodes/100 cm<sup>3</sup> in pre-plant soil can cause damage to cotton in sands to sandy-loam soil (Mueller et al., 2012). Nearly 86% of the densities found in the present survey were greater than 10 nematodes/100 cm<sup>3</sup>, indicating the need for immediate management strategies to prevent crop loss. Suggested rotation crops for RKN include grain sorghum, peanut, and rice. These crops can effectively lower populations below economic levels of less than 60 nematodes/100cm<sup>3</sup> for soybean (Kirkpatrick et al., 2014) or about 100 nematodes/100cm<sup>3</sup> for cotton (Mueller et al., 2012). Chopped hay residues such as alfalfa, oat, lespedeza, and flax have been used to suppress *M. incognita* in commercial tomato fields, and nematicides can be effective at protecting yield. Other recommended non-nematicidal methods for commercial field operators and home gardens include biological control, solarization, and biofumigation (Bernard, 2018). Additional studies are needed to understand the population dynamics and host preference of root-knot nematode species that are associated with field crops in Tennessee.

Results suggested that dagger (*Xiphinema* sp.), pin, sheath, stubby-root (*Trichodorus* spp. [syn *Trichodorus* spp.]), sting, needle, and ring nematodes are the least frequent nematodes, occurring only in 1 to 7% and 12 to 40% of samples and counties, respectively. Dagger nematodes are ectoparasites that can feed on the roots of multiple host plants using their long stylet (Heve et al., 2015). Dagger nematodes can damage or

kill their hosts, especially by feeding on the roots, and/or infecting susceptible hosts with viral and wilting diseases (Evans et al., 2007). This ability to transfer viruses to plants during feeding is a major concern. Dagger nematode species have been isolated from multiple crops in Tennessee including field crops, forage, pasture, trees, turf, and vegetables. A previous survey identified that dagger nematodes occurred in 32% of samples across 18 counties (Bernard, 1980). The present study suggests that dagger nematodes continue to be a non-significant threat to field crops in Tennessee.

Likewise, pin nematodes are ectoparasites that are capable of shifting to an endoparasitic feeding mode. They are known to have multiple hosts and can cause root injury, poor development, or yield loss in some hosts (Munawar et al., 2021; Wang et al., 2016). Pin nematode has been previously found in Tennessee in 51% of samples (Bernard, 1980; Niblack & Bernard, 1985). There were no data that indicated any harmful concern about the nematode. The 2% occurrence and density of 16 nematodes/100 cm<sup>3</sup> in the present study further suggest that there is no concern for the nematode in Tennessee. Sheath nematode was previously found in less than 1% of samples in Tennessee (Bernard, 1980). In the present study, it was found at a similar occurrence (1%), with an average density of 13 nematodes/100 cm<sup>3</sup>. This nematode is known to have a wide host range including crops, fruits, and trees. Some known hosts include celery, cabbage, cowpea, bean, okra, lettuce, tomato, cucurbits, grapefruit, and tobacco (Nguyen & Trinh, 2021). On a tomato host, 5,000 nematodes per plant resulted in growth reduction (Chitambar, 1993). The present result shows no implication for any concern for sheath nematode in Tennessee field crops.

Stubby-root nematodes, (*Trichodorus*, *Paratrichodorus* spp.) are ectoparasites that can feed on multiple host crops including corn, sorghum, turfgrass, cabbage, mustard, tomato, eggplant, sugarcane, peanut, and soybean. Host roots frequently become stunted or stubby-like in appearance. Also, they can transfer viruses to the infected host (Crow, 2004). A previous survey in Tennessee isolated stubby root nematodes from 6% of samples and five counties, but none were found in field crop samples (Bernard, 1980). The results of this study indicated that stubby root nematodes are not a concern to field crops.

Needle (*Longidorus* sp.) and sting (*Belonolaimus* sp.) nematodes are among the most economically important ectoparasites. The nematodes prune the lateral roots of the host seedlings, which then become stunted and necrotic. Needle nematodes have been associated with corn, turf, mint, sorghum, cottonwood, and grape (Ye & Robbins, 2004). A needle nematode species, *Longidorus breviannulatus*, corn needle nematode, is very destructive to corn in sandy soils. As low as 100 nematodes/100 cm<sup>3</sup> can kill corn seedlings. Likewise, the sting nematode, *Belonolaimus* spp., can cause total corn yield loss with 30 nematodes/100 cm<sup>3</sup> (Tylka, 2018). *Longidorus elongatus* was previously reported in Madison County, but no sting nematode has been reported in Tennessee (Bernard, 1980). In the present study, needle nematodes were found in Cannon, Gibson, and Haywood counties at an average density of 8 nematodes/100 cm<sup>3</sup>, but the populations were not associated with corn. This implied that needle nematode was locally present in some fields in the state, and continuous monitoring is needed to prevent yield loss from the nematode. Sting nematode was found in six counties at low densities ( $\leq 15/100$  cm<sup>3</sup>) and frequency (2%), indicating that the nematode does not pose any immediate threat at present. Continued monitoring of the nematode is recommended since the populations were detected in corn, soybean, and wheat fields.

Ring nematodes (*Criconebella* spp.) are not considered an important pest to Tennessee agriculture. Certain species of the nematodes are frequently associated with crops like blueberry, corn, cotton, peanut, soybean, vegetables, grapes, and peaches in other states (Hajihassani et al., 2018). The results of our study showed that ring nematodes are not prevalent in Tennessee field crops presently. We encourage growers to continue to monitor their fields to track any potential population increases.

*Heterodera glycines* biotypes or HG type designation and female index of SCN populations provide insights into the aggressiveness or the parasitic ability of the populations on SCN resistant indicators and cultivars (Niblack et al., 2002; Tylka, 2016). Our study shows that most of the tested SCN populations reproduced more than 10% of their population on the susceptible soybean on the indicator lines Peking (PI 548402), PI 88788, PI 209332, and PI 548316. Six populations (67%) reproduced successfully on PI 548402 (Peking), eight populations (89%) successfully reproduced on PI 88788 and PI

209332, and all nine populations (100%) reproduced on PI 548316 (Cloud). This indicates that growing cultivars derived from these resistance lines in the infested fields would result in increased SCN density and possible yield loss. Similar results have been reported from several states in the United States (Table 2.4). In a Missouri survey in 2005, 80% of SCN populations successfully reproduced on PI 88788 and 70% reproduced on PI 209332 and PI 548316 (Cloud) (Mitchum et al., 2007). Likewise, in a Nebraska survey between 2008 and 2014, 47% reproduced on PI 88788, 30% reproduced on Peking, and 88% of populations reproduced PI 548316 (Broderick, 2016). In South Dakota, 7%, 19%, 25%, and 75% of populations reproduced successfully on Peking, PI 209332, PI 88788, and PI 548316, respectively (Acharya et al., 2016). These values are relatively low compared to the 67% (Peking) 89% (PI 88788 and PI 209332), and 100% (PI 548316) in the present study (Table 2.4). These results indicated that SCN populations have adapted to the most commonly used resistance sources in every place they have been consistently grown, which is the PI88788 source that is now cited to be in over 90% of commercial cultivars (Cook et al., 2012; Kim et al., 2016; Mitchum, 2016; Niblack et al., 2008). Additionally, it is not surprising that all but one and all populations had female indices  $\geq 10$  on PI88788 and PI209332 and PI1548316 (HG Type 2.5.7), respectively, due to these lines all deriving their SCN resistance from the same gene (Cook et al., 2012; Kim et al., 2016; Mitchum, 2016; Niblack et al., 2008). However, it should be noted that there are differences in reproduction potentials among SCN populations with the same HG type. For example, female indices of the populations ranged from 10 to 28 on PI548402, from 23 to 106 on PI88788, from 22 to 62 on PI209332, and from 13 to 191 on PI548316 (Table 2.3). Likewise, our results also showed that other sources of resistance including PI 90763, PI 437654, and PI 89772 still maintain their capability to suppress SCN populations and prevent yield loss. None of the tested populations were able to successfully reproduce on the lines (FI<10).

In conclusion, this study identified the occurrence and density of fourteen plant-parasitic nematodes that can become a threat to field crop production in Tennessee. Among them, spiral, lance, SCN, and root-knot nematode populations demand close attention as these nematodes were either present in increasing predominance or density.

Also, the HG type testing identified that 100% of SCN populations tested had developed the capability to feed on PI 88788–derived resistance cultivars. This information will support Tennessee growers in managing plant-parasitic nematode populations and preventing yield loss.

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**Table 2. 1. Nematode population distribution across samples and counties**

| Nematode    | Occurrence in samples (N=378) <sup>a</sup> |     |                 | Occurrence in counties (N=25) |     |                 | Maximum |
|-------------|--------------------------------------------|-----|-----------------|-------------------------------|-----|-----------------|---------|
|             | Positive <sup>b</sup>                      | (%) | Average density | Positive                      | (%) | Average density |         |
| Dagger      | 25                                         | 7   | 13              | 10                            | 40  | 11              | 38      |
| Lance       | 108                                        | 29  | 22              | 17                            | 68  | 21              | 115     |
| Lesion      | 88                                         | 23  | 20              | 19                            | 76  | 22              | 115     |
| Needle      | 3                                          | 1   | 8               | 3                             | 12  | 8               | 8       |
| Pin         | 8                                          | 2   | 16              | 4                             | 16  | 15              | 46      |
| Reniform    | 74                                         | 20  | 1,764           | 2                             | 8   | 913             | 16,669  |
| Ring        | 3                                          | 1   | 8               | 3                             | 12  | 8               | 8       |
| Root-knot   | 51                                         | 13  | 78              | 13                            | 52  | 89              | 562     |
| SCN Eggs    | 82                                         | 22  | 799             | 18                            | 72  | 741             | 4,569   |
| SCN J2      | 93                                         | 25  | 22              | 20                            | 80  | 25              | 100     |
| Sheath      | 3                                          | 1   | 13              | 2                             | 8   | 12              | 15      |
| Spiral      | 314                                        | 83  | 88              | 24                            | 96  | 90              | 931     |
| Sting       | 8                                          | 2   | 7               | 6                             | 24  | 7               | 15      |
| Stubby-root | 11                                         | 3   | 2               | 5                             | 20  | 16              | 54      |
| Stunt       | 70                                         | 19  | 24              | 16                            | 64  | 22              | 92      |

<sup>a</sup> N = The total number of soil samples or counties tested for nematodes

<sup>b</sup> The number of positive samples or counties and the percentage value are presented to the right of each value

<sup>c</sup> Average density (Nematodes/100cm<sup>3</sup>) was calculated based on the sample in which nematode was detected

**Table 2. 2. Soybean cyst nematode (SCN) reproduction (female/plant) on indicator lines and Williams 82 and resultant female index and HG Type for 9 populations from Tennessee.**

| Indicator                                                        |                  | P12 <sup>c</sup> |    | P16     |    | P23     |   | P24     |    | P21     |    | P26    |   | P28     |   | P54     |     | P109    |                     |   |
|------------------------------------------------------------------|------------------|------------------|----|---------|----|---------|---|---------|----|---------|----|--------|---|---------|---|---------|-----|---------|---------------------|---|
| PI548402                                                         | [1] <sup>b</sup> | 5                | b  | 11      | b  | 55      | b | 70      | bc | 11      | c  | 3      | b | 21      | c | 34      | cde | 44      | b                   |   |
| PI88788                                                          | [2]              | 84               | ab | 34      | b  | 102     | b | 124     | b  | 46      | ab | 13     | b | 146     | b | 80      | bcd | 67      | b                   |   |
| PI90763                                                          | [3]              | 0                | b  | 1       | b  | 2       | b | 5       | c  | 2       | c  | 0      | b | 0       | c | 3       | e   | 3       | b                   |   |
| PI437654                                                         | [4]              | 0                | b  | 0       | b  | 1       | b | 5       | c  | 1       | c  | 1      | b | 0       | c | 14      | de  | 2       | b                   |   |
| PI209332                                                         | [5]              | 49               | ab | 28      | b  | 115     | b | 54      | bc | 41      | ab | 2      | b | 164     | b | 100     | abc | 74      | b                   |   |
| PI89772                                                          | [6]              | 0                | b  | 0       | b  | 0       | b | 9       | c  | 2       | c  | 0      | b | 1       | c | 2       | e   | 4       | b                   |   |
| PI548316                                                         | [7]              | 151              | a  | 62      | ab | 125     | b | 91      | bc | 40      | b  | 30     | b | 153     | b | 132     | ab  | 96      | b                   |   |
| Williams 82                                                      |                  | 79               | ab | 114     | a  | 434     | a | 247     | a  | 69      | a  | 228    | a | 294     | a | 165     | a   | 238     | a                   |   |
| P-value                                                          |                  | 0.0005           |    | <0.0001 |    | <0.0001 |   | <0.0001 |    | <0.0001 |    | 0.0004 |   | <0.0001 |   | <0.0001 |     | <0.0001 |                     |   |
| Female index <sup>d</sup> of SCN on resistant soybean indicators |                  |                  |    |         |    |         |   |         |    |         |    |        |   |         |   |         |     |         | FI≥10% <sup>e</sup> |   |
| PI548402                                                         | [1] <sup>b</sup> | 7                |    | 10      |    | 13      |   | 28      |    | 16      |    | 1      |   | 7       |   | 20      |     | 18      |                     | 6 |
| PI88788                                                          | [2]              | 106              |    | 30      |    | 23      |   | 50      |    | 67      |    | 6      |   | 50      |   | 48      |     | 28      |                     | 8 |
| PI90763                                                          | [3]              | 1                |    | 1       |    | 0       |   | 2       |    | 2       |    | 0      |   | 0       |   | 2       |     | 1       |                     | 0 |
| PI437654                                                         | [4]              | 0                |    | 0       |    | 0       |   | 2       |    | 2       |    | 0      |   | 0       |   | 8       |     | 1       |                     | 0 |
| PI209332                                                         | [5]              | 62               |    | 24      |    | 27      |   | 22      |    | 60      |    | 1      |   | 56      |   | 60      |     | 31      |                     | 8 |
| PI89772                                                          | [6]              | 0                |    | 0       |    | 0       |   | 4       |    | 3       |    | 0      |   | 0       |   | 1       |     | 2       |                     | 0 |
| PI548316                                                         | [7]              | 191              |    | 54      |    | 29      |   | 37      |    | 58      |    | 13     |   | 52      |   | 80      |     | 40      |                     | 9 |
| HG Type                                                          |                  | 2.5.7            |    | 1.2.5.7 |    | 1.2.5.7 |   | 1.2.5.7 |    | 1.2.5.7 |    | 7      |   | 2.5.7   |   | 1.2.5.7 |     | 1.2.5.7 |                     |   |

<sup>a</sup> Average number of females from five replications conducted twice except for P26 and P28 which were only evaluated once

<sup>b</sup> Number 1 to 7 in [ ] represents the HG type indicator number of each indicator line used in SCN HG type testing

<sup>c</sup> Values with the same letter within a column are not significantly different (Tukey's HSD,  $\alpha=0.05$ )

<sup>d</sup> Female Index = (number of females on resistant PI / number of females on Williams 82)  $\times$  100

<sup>e</sup> Number of SCN populations that had a Female Index (FI)  $\geq$  10% on each indicator line

**Table 2.3. Comparing SCN population reproduction on indicator lines in other states and Tennessee**

| State        | Soybean line                 | SCN population (%) | References           |
|--------------|------------------------------|--------------------|----------------------|
| Missouri     | PI 88788                     | 80                 | Mitchum et al., 2007 |
|              | PI 209332, PI 548316 (Cloud) | 70                 |                      |
| Nebraska     | PI 548402 (Peking)           | 30                 | Broderick, 2016      |
|              | PI 88788                     | 47                 |                      |
|              | PI 548316                    | 88                 |                      |
| South Dakota | PI 548402 (Peking)           | 7                  | Acharya et al., 2016 |
|              | PI 88788                     | 25                 |                      |
|              | PI 209332                    | 19                 |                      |
|              | PI 548316 (Cloud)            | 75                 |                      |
| Tennessee    | PI 548402 Peking             | 67                 | The present study    |
|              | PI 88788, PI 209332          | 89                 |                      |
|              | PI 548316 (Cloud)            | 100                |                      |

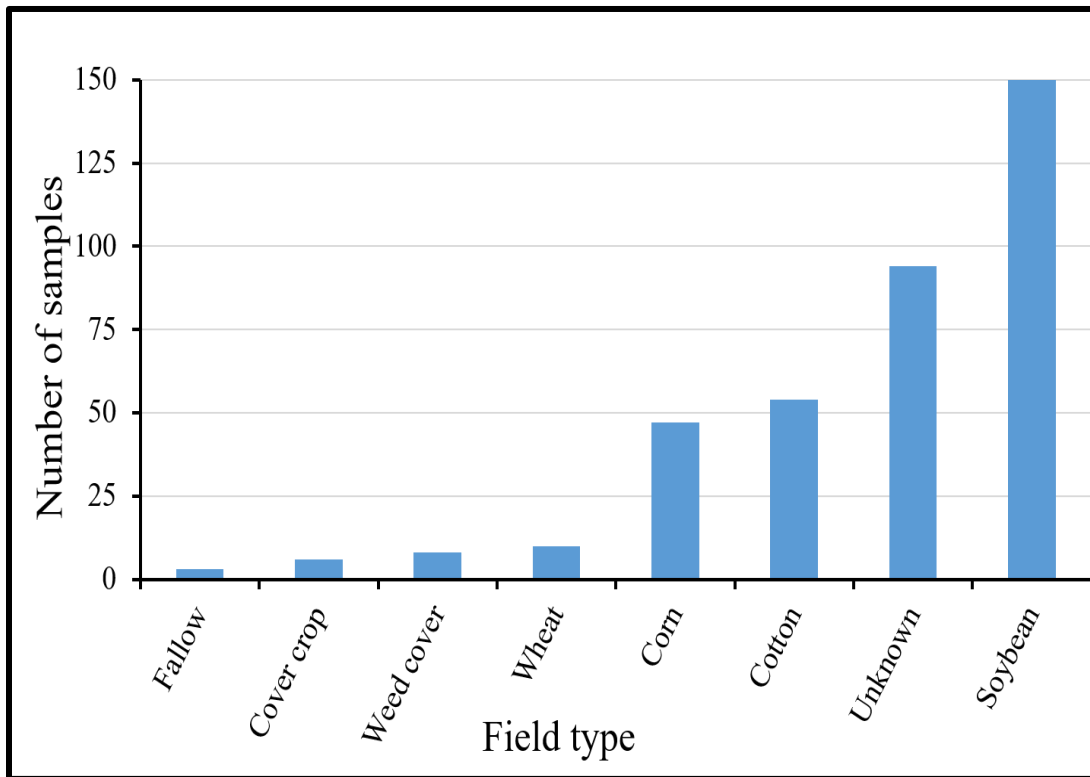


**Figure 2. 1.** Number of samples from different row crops collected from 2018 through 2020 in Tennessee for plant-parasitic nematode screening. Total number of samples = 372. Unknown = no data provided for the crops grown in the field.

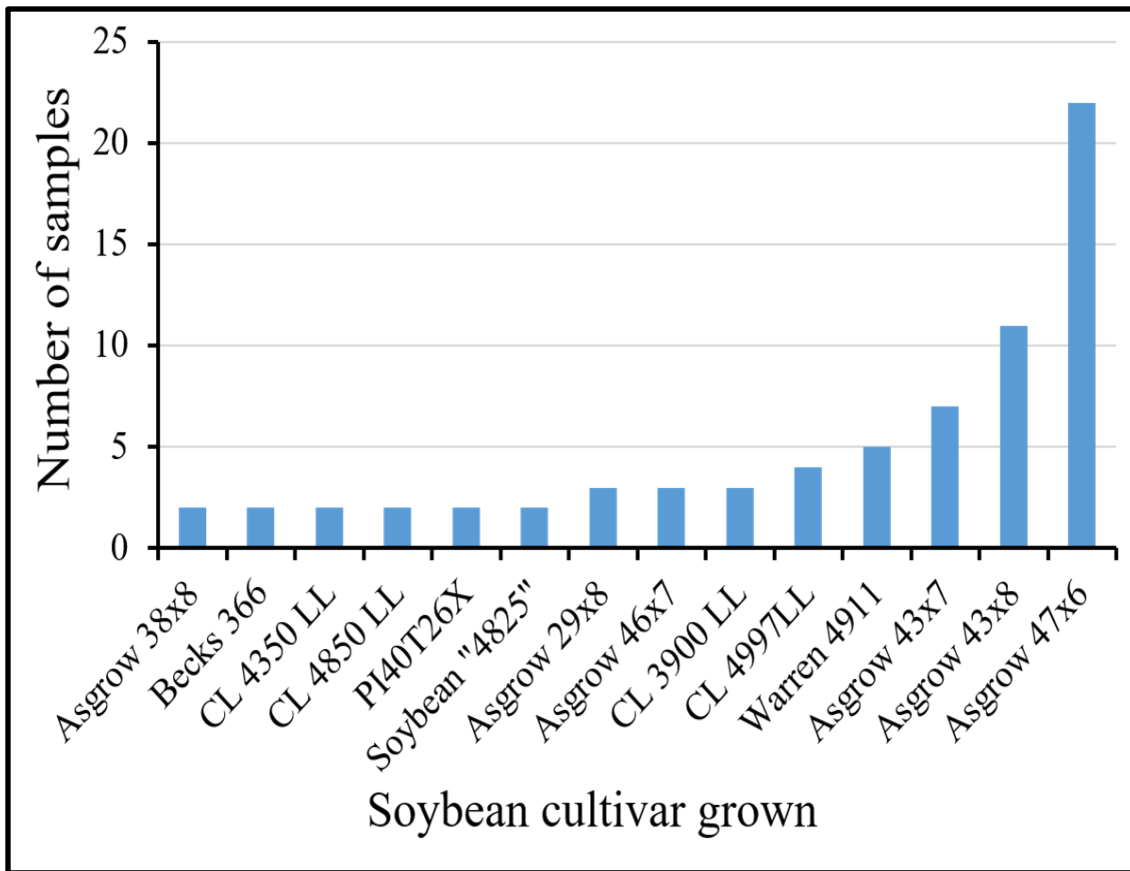


**Figure 2. 2.** Number of samples per field grown with soybean cultivar among 70 fields (samples) collected from Tennessee for nematode analysis from 2018 through 2020.

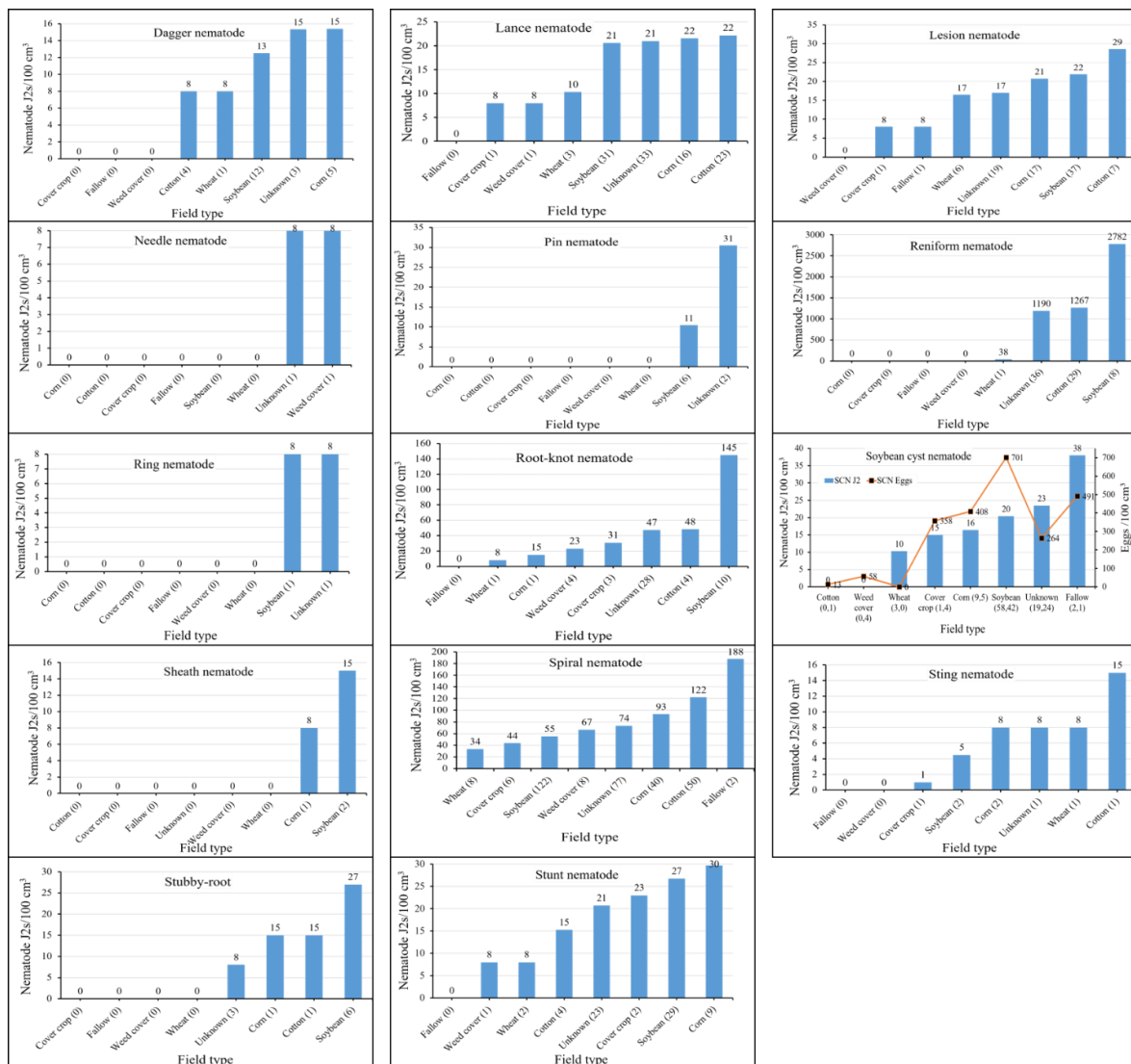


**Figure 2. 3.** Average nematode densities across 372 fields sampled for nematode analysis. The number of fields (samples) that had each nematode type is indicated in the parenthesis ( ) after each field type on the x axis.

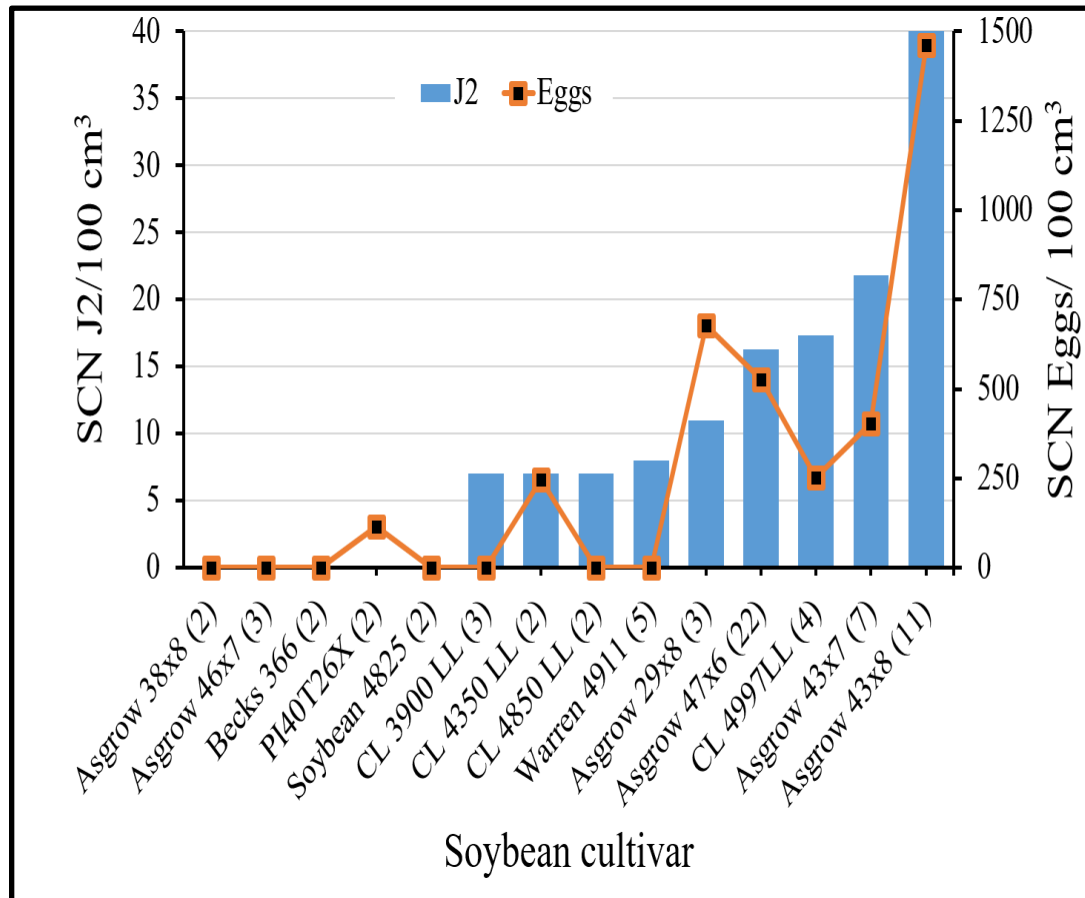


**Figure 2. 4.** Soybean cyst nematode (SCN) average densities associated with the cultivars grown among 70 fields sampled for nematode analysis. Number of fields (samples) per each cultivar is indicated in the parenthesis ( ) after each cultivar on the x ax

**CHAPTER III. IMPACT OF *HETERODERA GLYCINES* HG TYPE  
1.2.5.7 ON SOYBEAN NITROGEN FIXATION AND GROWTH  
PARAMETERS**

Data presented in this chapter are being reviewed for publication in the Journal of Crop Improvement under the title: Akinrinlola, R., Kelly, H., Sinclair, T., and Shekoofa, A. (2021). *Heterodera glycines* HG type 1.2.5.7 causes a decrease in soybean nitrogen fixation and growth variables. Rufus Akinrinlola, Heather Kelly, and Avat Shekoofa conceptualized and designed the experiments. Rufus Akinrinlola executed the experiments, collected, analysed the data, and wrote the paper manuscript. All authors commented on the initial versions of the manuscript. All authors approved the final manuscript.

### **Abstract**

Soybean cyst nematode (SCN), *Heterodera glycines*, HG type 1.2.5.7 is the most virulent and widespread SCN biotype in the southern states of the USA, but its effect on soybean (*Glycine max* [L.] Merr.) nitrogen fixation (NF) activity and growth is not well documented. The objectives of this study were to (1) measure the impact of HG type 1.2.5.7 on the NF activity during the early vegetative stage of three soybean genotypes and (2) compare the impact on soybean leaf and root development and growth. The NF activity of two susceptible genotypes ('Ellis' and 'Williams 82') and one resistant genotype (PI 88788) infested with 10,000 eggs was measured using an in-situ flow-through acetylene reduction assay. The total NF activity of infested Williams 82 was decreased by 30% but that of PI 88788 and Ellis was not decreased, compared to non-infested plants. Leaf color and leaf area were decreased in Williams 82 but not in the other two genotypes. Root surface area was decreased by SCN only in Ellis. These results showed that SCN HG type 1.2.5.7 had differential impacts on NF activity and growth of different soybean genotypes, providing novel information on the interaction of a virulent SCN biotype with NF activity of soybean genotypes with resistance or varying SCN-susceptibility.

## Introduction

Soybean cyst nematode (SCN), *Heterodera glycines*, is the most economically important soybean (*Glycine max* [L.] Merr) pathogen in North America. More than \$1.5 billion in yield losses are attributed to SCN in the USA and Canada each year (Bradley et al., 2021.; Jones et al., 2013). The nematode causes crop loss by invading soybean roots and forming a multinucleated-cell feeding site, called a syncytium, in the vascular tissues of the root. It forms the syncytium by producing secretions that dissolve vascular tissue cell walls within the infected roots. The dissolution of the cell walls and intense nuclear activity in the syncytia thus cause plant-produced photosynthates and nutrients to flow preferentially into the syncytium (Acedo et al., 1984; Sobczak & Golinowski, 2009). The nematode uses these photosynthates and nutrients for growth and reproduction (Lambert and Bekal, 2002). This process deprives the plant of nutrients and causes damage within the vascular tissue that can also affect symbiotic nitrogen fixation (NF) activity (Acedo et al., 1984; Asmus and Ferraz, 2002).

Many researchers have studied how various SCN biotypes can impair NF activity (Hussey and Barker, 1976; Ko et al., 1984; Lehman, 1971; McGinnity et al., 1980). However, the SCN biotypes most prevalent in Tennessee have not been investigated for activity against NF. Soybean cyst nematode HG type 1.2.5.7 is the most prevalent SCN biotype in Tennessee, based on unpublished data (Akinrinlola et al., unpublished) and a previous report (Young, 1990). The HG type 1.2.5.7 is a virulent SCN biotype that has adapted to and is capable of feeding and reproducing on the most frequently grown resistant soybean cultivars in the USA (Niblack et al., 2008; Sikora and Noel, 1991). The biotype is also known as race 2 under the SCN Race Classification Scheme (Niblack et al., 2002). According to a study conducted in North Carolina, race 2 SCN biotype (an equivalent of HG type 1.2.5.7) does not impact NF activity (Lehman, 1971), although no data were presented to support the claim. It is important to know if a high NF activity soybean variety can help to prevent significant damage or yield loss in fields infested with HG type 1.2.5.7. Thus, this research effort was undertaken to measure the NF activity of three soybean genotypes inoculated with an SCN HG type 1.2.5.7 population extracted from Tennessee soil.

Generally, NF activity by leguminous plants has been conventionally evaluated using an *in-vitro* acetylene reduction assay (ARA). The *in-vitro* ARA relies on the measurement of acetylene reduction in detached nodules or root segments to estimate NF activity (Hussey and Barker, 1976; Ko et al., 1984; Lehman, 1971; McGinnity et al., 1980). The *in vitro* ARA approach does not consider the intimate transport link between intact plants and the nodules. Hence, the approach does not recognize the deleterious impacts of root/nodule excision, flushing of nodules with water, incubation time, and temperature on NF activity, which can decrease NF activity of the nodules (Parsons et al., 1992). Additionally, NF activity measured on excised roots instead of an intact plant makes it impossible to perform a repetitive NF evaluation of the same plant. Unlike the *in-vitro* assay, an *in-situ* continuous-flow ARA allows the measurement of NF activity on undisturbed intact plants, overcoming the limitations associated with root excision, allowing repetitive NF evaluation of the same plant (Sinclair et al., 2007; Weisz et al., 1985). The repetitive evaluation of NF in actively growing plants makes it possible to determine how SCN affects NF activity at various times and growing intervals. Such information can provide insight into when soybean NF is vulnerable or insensitive to SCN activity.

The main objective of the study was to assess the impact of SCN HG type 1.2.5.7 on NF activity during the early development stage of three soybean genotypes at various time intervals. A secondary objective was to evaluate the possible impact of SCN infestation on root and leaf growth parameters.

## **Materials and Methods**

### ***Test SCN population***

The SCN HG Type 1.2.5.7. used for the study was extracted from a soil sample collected from a field in West Tennessee. The field had been rotated between corn (*Zea mays* L.) and SCN-resistant soybean cultivars at least for the past three years. After extraction from the soil sample, the population was increased on the roots of seven SCN-resistance indicator soybean lines in a preliminary test to confirm the biotype as HG type 1.2.5.7. using the HG-type test (Niblack et al., 2002).

### ***Soybean genotypes***

Details of the soybean genotypes (PI 88788, ‘Williams 82’ and ‘Ellis’) tested in this study are provided in Table 3.1. The genotypes were selected because they are frequently used by growers and researchers (Bernard and Cromeens, 1988; Jiao et al., 2015; Koenning, 2004; Lambert et al., 2005; Pantalone et al., 2017). Genotype PI 88788 is a maturity group III breeding line introduced from China and is important to both growers and breeders. More than 90% of SCN-resistant cultivars in the USA were developed using PI 88788 (Niblack et al., 2008), but currently, these resistant cultivars are being damaged by virulent SCN biotypes, causing major concerns to growers (Niblack et al., 2008). ‘Williams 82’ is an SCN-susceptible maturity group III cultivar and is an important breeding line because of its desirable high-yielding capability (Acharya et al., 2016; Bernard & Cromeens, 1988; Jiao et al., 2015). Similarly, ‘Ellis’ is a high-yielding, maturity group IV, SCN-susceptible soybean variety developed and released by the University of Tennessee. ‘Ellis’ is resistant to southern root-knot nematode (*Meloidogyne incognita*) and stem canker (caused by *Diaporthe phaseolorum*) and tolerant to frog-eye leaf spot (caused by *Cercospora sojina*), which further makes it desirable to growers and plant breeders (Pantalone et al., 2017).

### ***Infestation of soybean seedlings with SCN eggs***

To ensure that a fresh SCN population was used for infestation, the population was increased on the roots of susceptible soybean cultivar ‘Hutcheson’ in a greenhouse at 27°C for 30 days, after which the root systems were removed from pots, and the nematode cysts were collected. The eggs were extracted according to a standard procedure (Riggs and Schmitt, 1991) and diluted with water to make an egg suspension for the infestation of the seedlings of the soybean genotypes. Three-day-old seedlings, germinated using seed germination papers in a growth chamber at 28°C, were infested with the egg suspension. One mL of egg suspension containing approximately 10,000 eggs was directly applied to the radicle of each seedling using a multi-repeater pipette (Fisher Scientific, Portsmouth, NH). Another set of seedlings of each genotype without

egg infestation was used as a reference treatment (control).

The seeds of PI 88788 and Williams 82 were obtained from USDA Germplasm Resources Information Network (GRIN), whereas those of Ellis were supplied by Dr. Avat Shekoofa (University of Tennessee). The seeds were germinated on seed-germinating paper in a growth chamber at 27 °C for three days. One seedling was transplanted per pot containing pasteurized soil mix comprised of one-part loam and three parts Manson sand by volume. The soil texture was 90% sand, 4% silt, and 6% clay. The pot was custom-made using polyvinyl chloride (PVC) pipe (10-cm diameter and 30-cm tall) and had a toilet flange attached to the top so that a lid could be installed to seal the pot during NF measurement. The pot was sealed at the bottom using a PVC cap. The cap had a small inlet hole through which gas was introduced into the pot for continuous airflow during NF measurement. To prevent soil from clogging the inlet hole, a plastic net was placed at the bottom of the pot.

At transplanting, each pot was inoculated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum* (Verdesian, Cary, NC) inoculum diluted with water. These plants were grown in a greenhouse in a completely randomized design. The maximum and minimum temperatures in the greenhouse were 37°C and 20°C, respectively, and those of the potting soil was 30°C and 20°C, respectively. The pots were watered every other day and fertilized once a week with an N-minus fertilizer. Twenty days after transplanting, pots were sealed airtight at the top with a two-piece lid that was sealed around the plant stem and attached to the pot. The primary leaves of all plants were removed to avoid interference in sealing the pots. After sealing, measurements of NF activity were taken on nine different days between 22 days and 45 days after transplanting using the *in situ* acetylene reduction assay.

### ***Measurement of NF activity via in-situ flow-through acetylene reduction assay***

This assay allows for the estimation of NF activity by measuring the rate of reduction of acetylene gas (C<sub>2</sub>H<sub>2</sub>) to ethylene (C<sub>2</sub>H<sub>4</sub>). The protocol for the assay was described by Devi and Sinclair (2013). Before NF measurement, air with no acetylene was flowed at 1 L min<sup>-1</sup> through all pots for 30 min, through the inlet at the bottom of the

pots, and out via the outlet at the top of the pots. This was done to flush the pots of possible accumulation of CO<sub>2</sub> and replenish the oxygen levels in the pots. After this, a gas mixture comprised of acetylene and air (1: 9 ratio) was flowed into the pots through the bottom inlet at 1 L min<sup>-1</sup> for 15 min to reach a steady state of ethylene production as a result of acetylene reduction. After reaching steady-state, three 1-mL gas samples were collected in syringes from the outflow from each pot. Gas samples were also collected from the inflow gas (acetylene introduced into the root zone). After sample collection, air with no acetylene was again flowed through all pots for an hour to flush out all acetylene gas from the pots. The ethylene component of the gas samples was analyzed with a flame ionization gas chromatograph (Shimadzu Model 5710A, Shimadzu Corporation, Kyoto). The ethylene gas concentration of the inflow gas was subtracted from the outflow concentration to obtain ethylene production values for the nodules. The ethylene concentration was converted to an ethylene production rate ( $\mu\text{moles C}_2\text{H}_4 \text{ plant}^{-1} \text{ minute}^{-1}$ ) by accounting for the flow rate through the pots.

#### ***Measurement of leaf growth parameters***

Leaf color and leaf area were measured as indicators of leaf development response to the nematode infestation. Leaf color was measured six times (at least twice per week) between 22 days and 45 days after transplanting, using a Soil Plant Analysis Development (SPAD)-502 meter (Konica-Minolta, Tokyo). Typically, the SPAD meter value is positively associated with chlorophyll level (Markwell et al., 1995). The SPAD meter values of three trifoliolate leaves were recorded during each sampling and the mean value was recorded per plant. After harvest, the leaf area for all leaves per plant was measured with an LI-3100C Leaf Area Meter (Li-Cor, Lincoln, NE).

#### ***Measurement of leaf N content***

At harvest, all leaves per plant were collected and dried in an oven at 60°C for 5 days. The leaves were ground in a Krups stainless steel spice and coffee grinder (Krups, Solingen, Germany), and the Dumas dry combustion method was used to determine nitrogen concentration in 150 mg of the sample using a LECO Truespec CN analyzer

(Leco Corporation, St Joseph, MI) (Caliskan and Makineci, 2015).

### ***Measurement of root growth parameters***

After harvest, the soil core was removed from each pot and carefully washed under running tap water to remove the soil. The roots were separated from the shoot. The roots were collected on an 850-µm-pore sieve. Total root length (TRL) and total root surface area (TRSA) were measured for each plant using a modification of the method described in Villordon and Clark (2018). Clean plant roots were stained with toluidine blue, submerged in water in a water-proof tray, and scanned using a specialized Epson Perfection V850 Pro Scanner (Epson Electronics, Suwa-shi, Nagano, Japan). TRL and TRSA of the scanned root images were then analyzed using the WinRHIZO Root Image Analysis Software (Regent Instruments Inc., Ch Ste-Foy, Québec, Canada).

### ***Final densities of SCN on soybean roots***

After harvest, SCN cysts were extracted from soybean roots and soil. The cysts were crushed and the eggs were collected using standard procedures (Riggs and Schmitt, 1991).

### ***Data analysis***

Mixed model analysis of variance (SAS V94, Cary, NC) was applied to the NF activity, leaf, and root data, to compare nematode-infested plants and uninfested plants for each genotype. A repeated-measures ANOVA was used to determine the effect of infestation on overall NF activity, and a simple ANOVA was used to determine the effect of infestation on NF activity on a sampling day basis. The least squares means were separated based on Fisher's LSD test at  $P < 0.05$ . The SCN treatment was the fixed effect and replicate was the random effect. The percent decrease in NF activity of infested plants compared to non-infested ones was determined using an equation adapted from Akinrinlola et al. (2018).

$$\frac{(En - Ei)}{En} \times 100$$

where  $E_n$  and  $E_i$  represent mean values of ethylene generated each day by uninfested ( $E_n$ ) and SCN-infested plants ( $E_i$ ). A simple linear regression procedure was used to determine if the final SCN population density on each genotype was related to the NF activity of the genotype. The SCN density was the independent variable, whereas the NF activity was the dependent variable. Finally, the relationship between SCN density and root growth parameter was determined by performing a Spearman simple correlation analysis. Spearman correlation was used for the analysis because it allowed the data to be analyzed without concern for ensuring normality of data distribution.

## **Results**

### ***Impact of infestation on NF activity.***

Out of the nine observations of NF activity, the activity of SCN-infested plants of PI 88788 was low ( $P = 0.03$ ) during only two observations as compared to non-infested plants (Figure 3.1a). The low NF activity was observed on the seventh (Sept. 18) and ninth (Sept. 23) observations, which were 38 and 43 days after infestation, respectively (Figure 3.1a). However, the overall NF activity mean (across all observations) for the SCN-infested plants of PI 88788 was not lower than that of the uninfested plants ( $P = 0.09$ ). Out of nine observations, the NF activity of the infested plants of Williams 82 was low in only three observations as compared to uninfested plants. Low NF activity was observed on the first (Sept. 4), fourth (Sept. 11), and eighth (Sept. 20) observations ( $P=0.03, 0.04$ , and  $0.01$ ), which were at 22, 29, and 40 days after infestation, respectively (Figure 3.1b). The overall NF activity mean for the SCN-infested plants of Williams 82 was lower than that of uninfested plants ( $P = 0.04$ ) (Table 3.2). The NF activity of Ellis was not affected by SCN infestation ( $P = 0.9$ ) (Figure 3.1c).

### ***Impact of infestation on leaf color***

The overall mean leaf color of Williams 82 was decreased ( $P = 0.04$ ) compared to non-infested plants. For analysis based on each sampling time, in two out of the six observation dates the leaf color decreased on infested plants ( $P = 0.02, 0.001$ ) compared to uninfested (Figure 3.2). Leaf color was not impacted by SCN on PI 88788 and Ellis

(Table 3.3).

#### ***Impact of infestation on leaf area***

The mean value for the total leaf area per plant of non-infested plants was 371, 430, and 517 cm<sup>2</sup> for Ellis, Williams 82, and PI 88788, respectively. The leaf area of nematode-infested Williams 82 was decreased ( $P = 0.01$ ) by 30% compared to non-infested plants. The leaf area of the infested plants of PI 88788 ( $P = 0.7$ ) and Ellis ( $P = 0.9$ ) was not decreased by the SCN infestation (Figure 3.3).

#### ***Impact of infestation on leaf N content***

The leaf N concentration for non-infested plants was 18, 21, and 25 mg g<sup>-1</sup> for PI 88788, Williams 82, and Ellis, respectively. The leaf N concentration was not impacted by SCN infestation.

#### ***Impact of infestation on root length and surface area***

The mean value for total root length (TRL) per plant of non-infested plants was 1200, 1194, and 974 cm for PI 88788, Williams 82, and Ellis, respectively. The TRL values were not impacted by SCN infestation (data not shown). The mean value for the total root surface area (TRSA) per plant was 110, 66, and 59 cm<sup>2</sup> for PI 88788, Williams 82, and Ellis, respectively. The TRSA of infested plants of Ellis was decreased compared to non-infested ( $P = 0.03$ ) by up to 31%, but the TRSA of Williams 82 and PI 88788 was not affected by the SCN infestation (Figure 3.4).

#### ***SCN egg densities***

The density of SCN eggs on Ellis and Williams 82 was similar but higher than that on PI 88788. The density (eggs plant<sup>-1</sup>) was approximately 66,000 on Ellis, 78,000 on Williams 82, and 18,000 eggs plant<sup>-1</sup> on PI88788 (Figure 3.5). Simple regression analysis showed that SCN density on Williams 82 was associated with a 30% decrease in its NF activity ( $P = 0.02$ ). Root surface area of Ellis was negatively correlated with nematode egg density ( $r = -0.38$ ,  $P = 0.02$ ). No relationship was found between SCN

density and NF activity on PI 88788 and Ellis, and no relationship was found between SCN density and root length on any of the three genotypes.

## **Discussion**

The first objective of this study was to evaluate the impact of SCN HG type 1.2.5.7 on the NF activity at the early stages of plant development of three soybean genotypes at various time intervals. This objective tested the hypothesis that SCN will decrease NF to different levels among the three tested genotypes. The results showed that SCN did not impact NF activity on Ellis (Table 3.2), even though SCN population density on it was relatively high compared to the population on the resistant line PI 88788 (Figure 3.5). This finding is surprising and did not support the initial hypothesis that SCN will decrease NF activity in the variety Ellis. It was expected that NF activity would decrease in Ellis because it is susceptible to SCN. This result indicates that Ellis exhibits an “SCN-insensitive NF” trait, but it is unknown what impact this could have on yield level when grown in fields infested with virulent SCN biotypes.

The results also showed that the SCN population did not affect the nitrogen fixation activity of PI 88788 (Table 3.2). This study was based on the hypothesis that the test SCN population will feed and reproduce enough on PI 88788 to decrease NF activity. This hypothesis was not supported by the finding that the total NF activity of PI 88788 was not decreased by the nematode infestation (Table 3.2). The results showed that SCN population density on PI 88788 was relatively low compared to that of Williams 82 and Ellis (Figure 3.5), thus this may support the observation of a lack of decrease in NF activity on PI 88788. This could also mean that even though this SCN population is virulent on PI 88788, it was not sufficiently virulent to cause a reduction in NF, or the population just does not have an effect on the NF of PI 88788 like Ellis.

The results for Williams 82 did support the hypothesis that SCN infestation would decrease NF activity. The NF activity of Williams 82 was decreased in three out of nine individual observations (Figure 3.1b), as well as in the total NF activity (Table 3.2). Two of the three times NF activity decreased was early during the experiment, indicating that SCN can begin to decrease NF activity on a susceptible soybean as early as 22 days to 29

days after infestation. In addition, a decrease in NF activity was observed towards the end of the experiment at 40 days after infestation (Figure 3.1b). This may imply that SCN can continue to decrease the NF of susceptible soybean throughout the infection period. However, the fact that there was no impact on NF during some periods of six observations, probably disputes this or indicates that other factors may have affected the impact of SCN on NF activity during the no-impact periods. One factor that may have contributed to the variability in the impact of SCN on NF is the SCN life cycle. The three SCN impacts on NF occurred at 22, 29, and 40 days after infestation. These periods seem to coincide around the SCN life stage with maximum adult activity (Schmitt et al., 2004).

The present study is the first to show directly that SCN HG type 1.2.5.7 decreased NF activity on a susceptible cultivar, i.e., Williams 82 (Figure 3.1b). While it decreased NF on Williams 82, it did not impact NF negatively on Ellis. This indicates that not all soybean cultivars labeled as “susceptible to SCN” will exhibit a low NF activity when infested by virulent SCN biotypes. Thus, regarding NF activity, not all susceptible cultivars are sensitive to SCN HG type 1.2.5.7. What is unknown is whether susceptible cultivars with SCN-insensitive NF activity can incur or prevent yield loss to SCN infestation. If such susceptible cultivars can prevent yield loss to SCN infestation, it means an SCN-insensitive NF trait may be a rapid cultivar-screening criterion when looking for a susceptible soybean to grow in SCN-infested fields. Such cultivars can prevent high yield losses to virulent SCN biotypes. Furthermore, the result of this study indicates that labeling a variety as susceptible based on SCN reproducibility may be misleading, especially if SCN reproduction will not affect major physiological activities in soybean. Soybean susceptibility status should be based on both SCN reproduction and its effects on the physiological attributes.

The second objective of this study was to evaluate the impact of SCN infestation on soybean root and leaf growth. The results showed that leaf area was decreased by 30% on SCN-infected Williams 82 plants, but no decrease was observed for PI 88788 or Ellis (Figure 3.3). These results agree with previous studies (Asmus and Ferraz 2002; Schans and Arntzen, 1991). Asmus and Ferraz (2002) reported a decrease in soybean leaf area at

different SCN population densities. Likewise, a report by Schans and Arntzen (1991) also showed that the potato cyst nematode, *Globodera pallida*, decreased leaf area of potato plants, and different populations of *Heterodera avenae* (the cereal cyst nematode) decreased leaf area of triticale (x *Triticosecale* Wittmack) plants.

Leaf color was slightly decreased (5%) by SCN in Williams 82 in the present study. Similar to this result, a decrease in soybean leaf color by SCN has been previously reported (Asmus and Ferraz, 2002). Asmus and Ferraz (2002) attributed the decrease in leaf color by SCN to a decrease in leaf chlorophyll content. Leaf N and leaf color were affected by SCN differently in the present study; while leaf N was not decreased by SCN in any of the soybean genotypes, leaf color was decreased by SCN in Williams 82. This indicates that the impact of SCN on leaf color in this study was not associated with leaf N content; other unidentified factors may also be affecting soybean leaf color.

Root length was not affected by SCN in this study, but root surface area was decreased in Ellis (Figure 3.4). This indicates that nematode infections may have more impact on root diameter than on root length. It may also indicate that selecting and growing soybean varieties with a large root system growth potential may help to reduce potential soybean damage or yield loss caused by virulent SCN populations. In summary, this study showed that the effect of the virulent SCN biotype (HG type 1.2.5.7) on NF activity varied among soybean genotypes. The virulent SCN biotype (HG type 1.2.5.7) population had a low reproduction rate and did not affect NF activity in the PI 88788 genotype. This means that the population was not virulent enough to affect NF activity in the resistant genotype. But it is unknown whether a similar observation will occur in SCN-resistant cultivars developed from the PI 88788 since most of the PI-88788-derived resistant cultivars may have a lesser resistance potential. Reproduction of SCN was not associated with NF decrease in Ellis. This means that a soybean genotype can support a high SCN population without experiencing a loss in NF activity (e.g., in Ellis), but it is unknown whether such an attribute can prevent yield loss from SCN infection. This attribute needs to be tested to find out whether such an “SCN-insensitive NF activity” trait can be used as a criterion for rapid screening of susceptible genotypes to prevent yield loss from SCN infestation. Also, when SCN affected NF in a susceptible genotype

(e.g., Williams 82), the impact was not continuous but occurred at different stages. Looking at the periods in which the impacts were observed, it seems possible that SCN impacts the NF activity of susceptible genotypes mostly when the SCN population is high and active. This implies that it takes the presence of a high SCN population with high activity to affect NF activity in susceptible soybean. Finally, SCN affected leaf color, leaf area, and root area but did not affect leaf N and root length. A decrease in leaf color and leaf area is a frequent occurrence associated with SCN activity in soybean. This means SCN exhibits a high tendency to cause damage to soybean by impairing these major physiological traits. Any approach that can strengthen soybean leaf color and leaf area, or the growing of cultivars with high leaf and root growth potentials may help to lessen the SCN damaging effect in the field. SCN activity did not affect leaf N and root length in this study, which may mean that an unidentified factor may influence the effect of SCN on leaf N, while damage to root length by SCN is rare.

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## Appendix

**Table 3. 1. Details of test soybean genotypes**

| Genotype    | Susceptibility to SCN | Use           | Maturity group | Year of introduction / release | Origin/ Developed by    | Reference                 |
|-------------|-----------------------|---------------|----------------|--------------------------------|-------------------------|---------------------------|
| Ellis       | Susceptible           | Commercial    | IV             | 2013                           | University of Tennessee | Pantalone et al. 2017     |
| PI 88788    | Resistant             | Breeding line | III            | 1930                           | China                   | Bernard and Cremeens 1988 |
| Williams 82 | Susceptible           | Breeding line | III            | 1981                           | USDA-ARS                | Bernard and Cremeens 1988 |

**Table 3. 2. Effect of *Heterodera glycines* on total NF activity of three soybean genotypes.**

| Soybean genotype  | Total N <sub>2</sub> -fixation activity<br>( $\mu\text{moles C}_2\text{H}_4 \text{ plant}^{-1}\text{minute}^{-1}$ ) | Standard error<br>of the mean | DF | Mean<br>squares |
|-------------------|---------------------------------------------------------------------------------------------------------------------|-------------------------------|----|-----------------|
| Williams 82 only  | 8.53a*                                                                                                              | 0.45                          | 1  | 86.2            |
| Williams 82 + SCN | 6.93b                                                                                                               | 0.42                          |    |                 |
| P - value         | 0.04                                                                                                                |                               |    |                 |
|                   |                                                                                                                     |                               |    |                 |
| PI 88788 only     | 8.5a                                                                                                                | 0.54                          | 1  | 49.3            |
| PI 88788 + SCN    | 6.9a                                                                                                                | 0.38                          |    |                 |
| P - value         | 0.09                                                                                                                |                               |    |                 |
|                   |                                                                                                                     |                               |    |                 |
| Ellis only        | 6.5a                                                                                                                | 0.70                          | 1  | 0.21            |
| Ellis + SCN       | 6.6a                                                                                                                | 0.70                          |    |                 |
| P - value         | 0.94                                                                                                                |                               |    |                 |

\*Means followed by different letters in a column are different significantly (LSD test,  $\alpha = 0.05$ )

**Table 3. 3. Effect of *Heterodera glycines* on leaf color of three soybean genotypes**

| Soybean genotype  | Leaf color (SPAD values) | Standard error of the mean | DF | Mean squares |
|-------------------|--------------------------|----------------------------|----|--------------|
| Williams 82 only  | 33.2a*                   | 0.48                       | 1  | 49.6         |
| Williams 82 + SCN | 31.7b                    | 0.33                       |    |              |
| P - value         | 0.04                     |                            |    |              |
|                   |                          |                            |    |              |
| PI 88788 only     | 31.5a                    | 0.36                       | 1  | 11.3         |
| PI 88788 + SCN    | 31.0a                    | 0.29                       |    |              |
| P - value         | 0.20                     |                            |    |              |
|                   |                          |                            |    |              |
| Ellis only        | 33.5a                    | 0.43                       | 1  | 32.4         |
| Ellis + SCN       | 32.0a                    | 0.43                       |    |              |
| P - value         | 0.08                     |                            |    |              |

\*Means followed by different letters in a column are different significantly (LSD test,  $\alpha = 0.05$ )

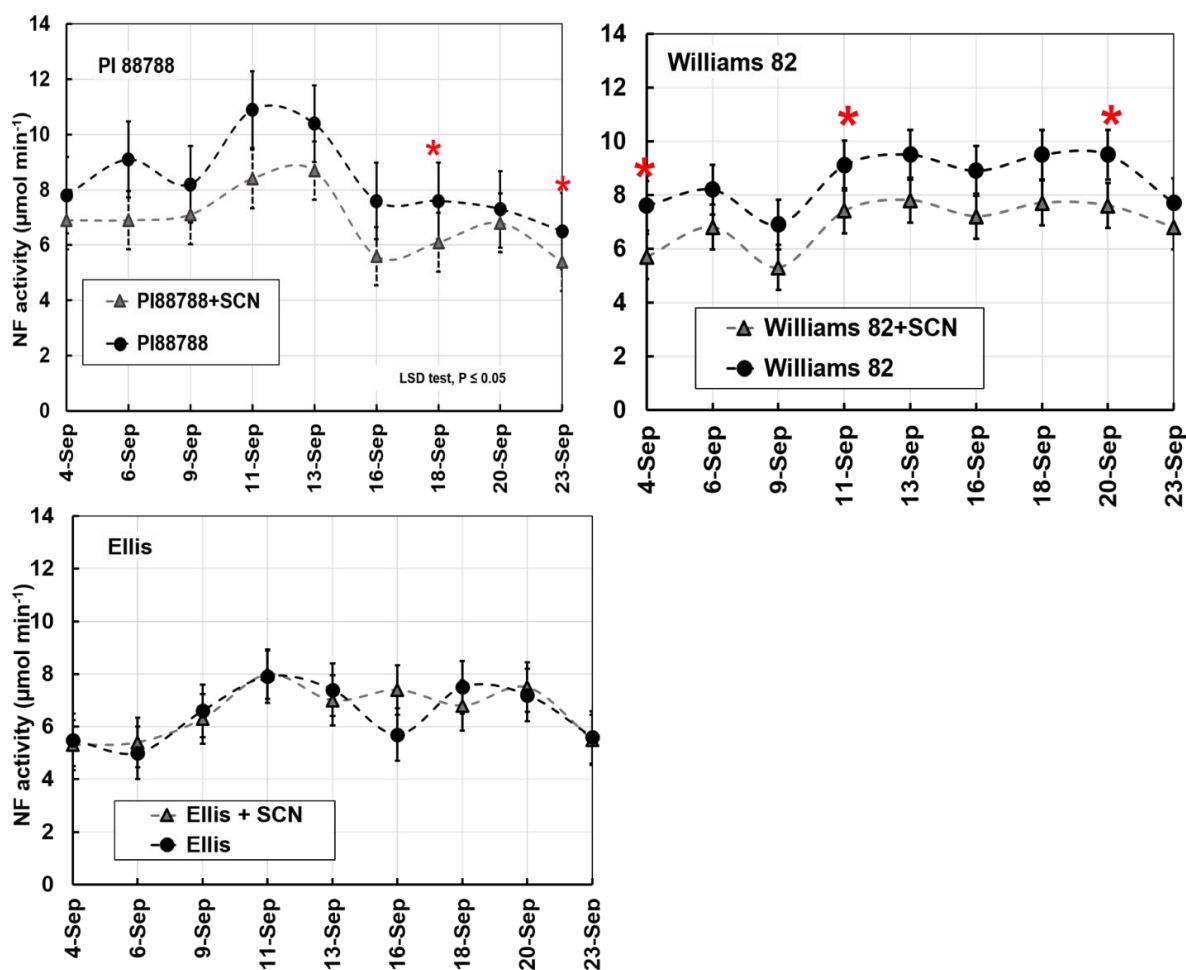


**Figure 3.1.** Impact of SCN on NF activity on soybean genotypes (a) PI88788, (b) Williams 82, and (c) Ellis. An asterisk denotes mean values statistically different at the given sampling date. (LSD test,  $\alpha = 0.05$ ).

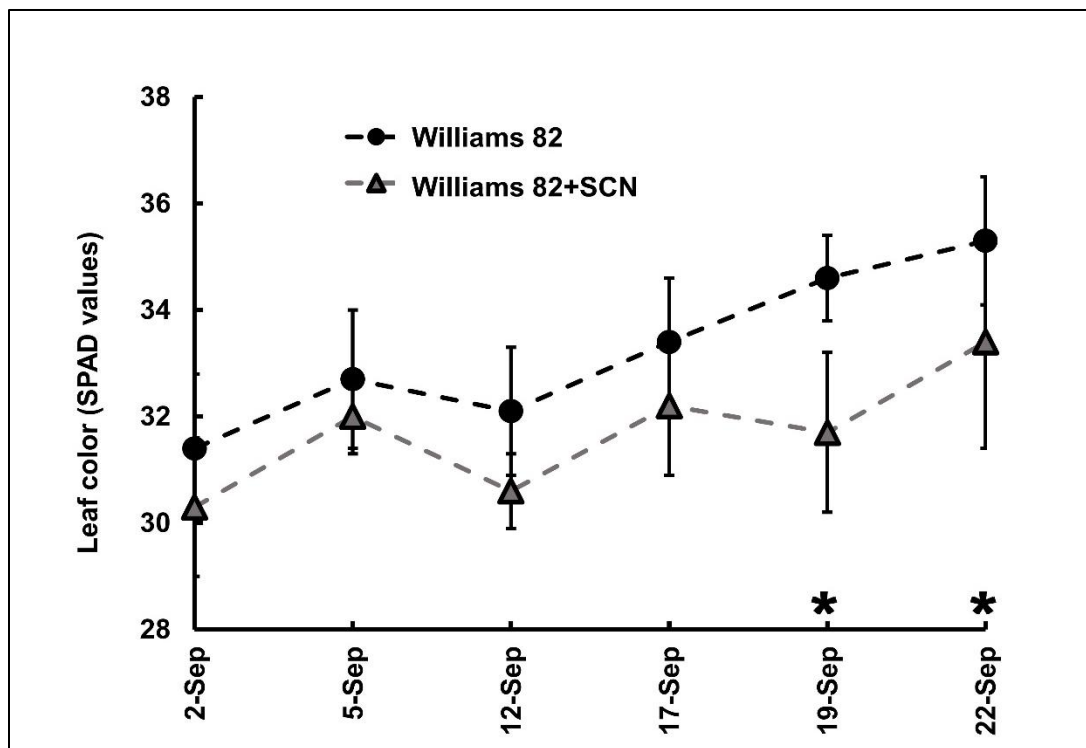


**Figure 3.2.** Effect of SCN on leaf color. An asterisk denotes values statistically different (LSD test,  $\alpha = 0.05$ ).

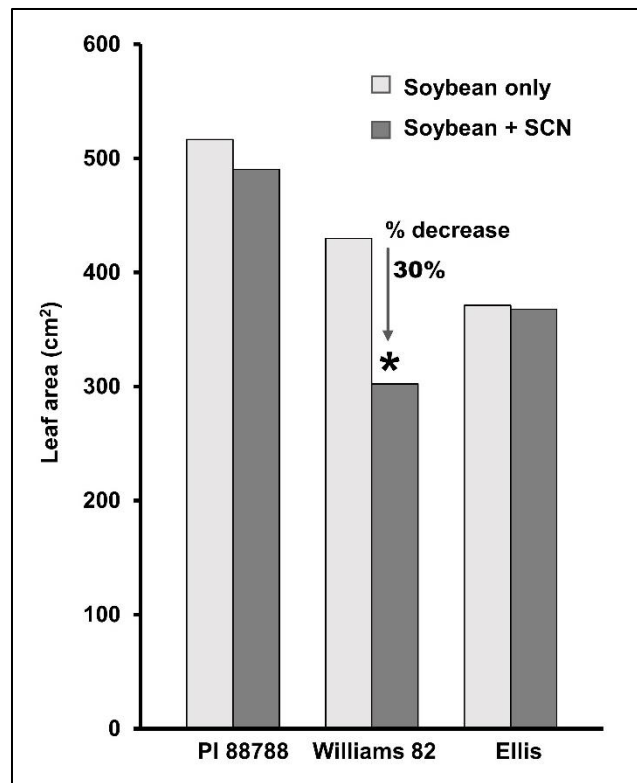


**Figure 3.3.** Impact of SCN on leaf area of soybean genotypes. An asterisk denotes values statistically different (LSD test,  $\alpha = 0.05$ )

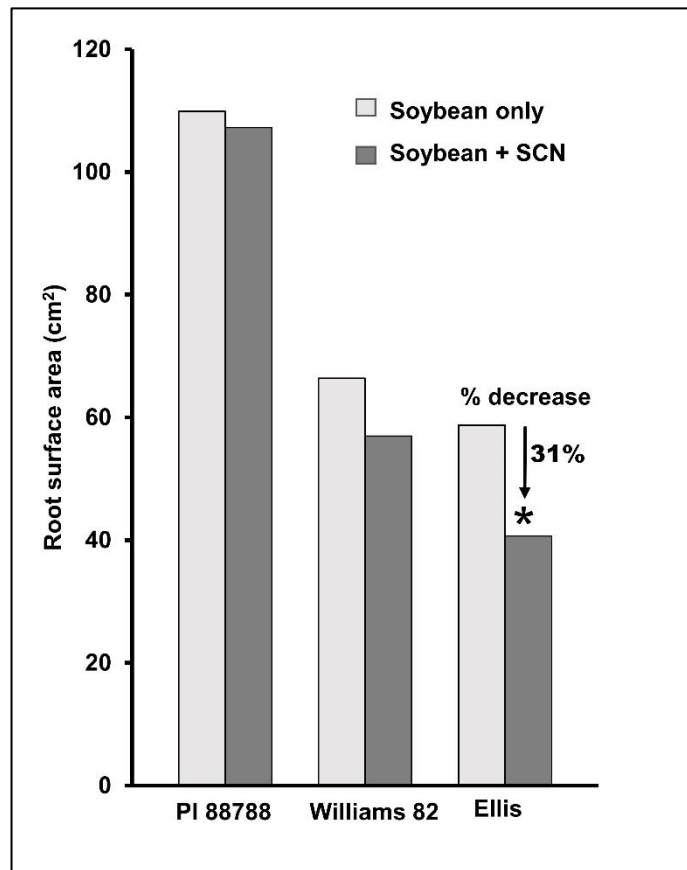


**Figure 3.4.** Impact of SCN on soybean root surface area. An asterisk denotes values statistically different (LSD test,  $P < 0.05$ ).

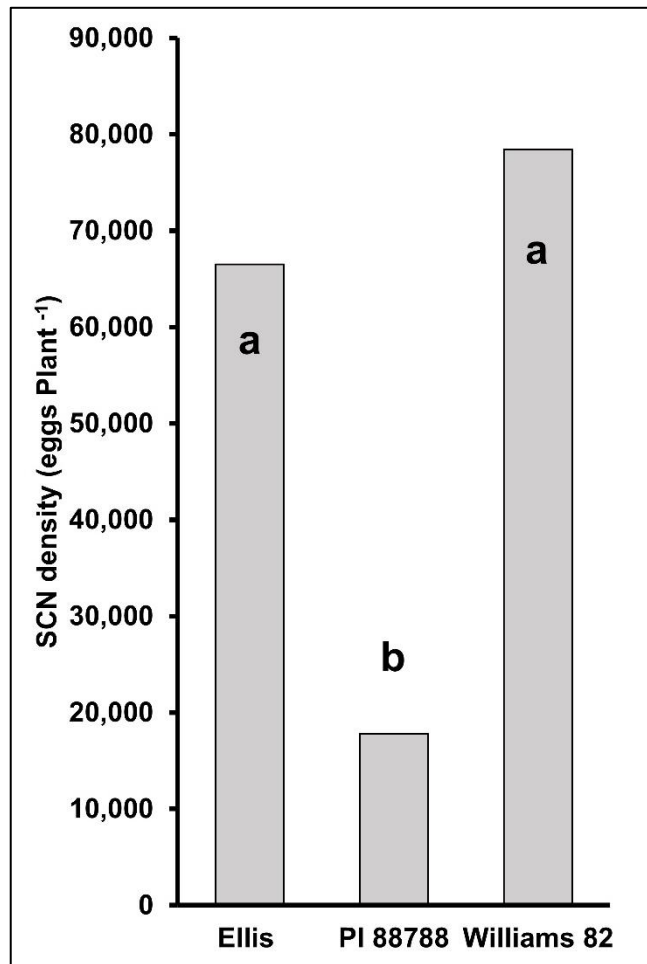


**Figure 3. 5.** Density of SCN eggs on the roots of test soybean genotypes at harvest. Bars with different letters are significantly different (LSD test,  $P < 0.05$ ).

**CHAPTER IV. *MACROPHOMINA PHASEOLINA*  
MICROSCLEROTIAL PATHOGENICITY ON SOYBEAN, AND  
SOYBEAN VARIETY INFLUENCE ON CHARCOAL ROT**

## Abstract

*Macrophomina phaseolina* (*mp*) (Tassi) Goid causes charcoal rot on soybean, reducing soybean seed quality and yields worldwide. The severity of the disease can be affected by *mp* microsclerotial density and soybean variety. There is a need to assess the influence of different microsclerotial densities on soybean. Hence, a greenhouse study was conducted to (1) identify the impact of microsclerotial inoculum density on charcoal rot disease severity on soybean, (2) determine the effect of microsclerotial inoculum densities on soybean growth and yield, and (3) evaluate the impact of two soybean varieties (susceptible LS98-0358 and moderately resistant DT97-4290) on the resulting microsclerotial density in soils and plant tissues. Three densities (142,660, 332,873, and 380,427 colony forming units (CFU)/treatment) resulted in charcoal rot symptom development on the susceptible soybean, but none of the densities developed disease on the resistant. Soybean growth and yield parameters were not significantly different among microsclerotial treatments across both varieties. Compared to LS98-0358, DT97-4290 had a significantly lower microsclerotial density at harvest from the highest inoculum density tested. Results suggest densities of *M. phaseolina* inoculum that are equivalent to 327 CFU/gram of soil can cause disease and yield loss in a greenhouse setting, but a moderately resistant soybean may aid charcoal rot suppression in fields. Field testing of the highest microsclerotial density is recommended to provide additional information.

## Introduction

*Macrophomina phaseolina* (Tassi) Goid causes charcoal rot worldwide, affecting soybean seed quality and reducing yields (Kaur et al., 2012). Charcoal rot is the second most damaging soybean disease in the United States behind soybean cyst nematode, and the number one cause of soybean yield loss in the Southern U.S States (Allen et al., 2017). Approximately 27 million tons of soybean were lost annually to charcoal rot between 1996 and 2007 in the United States (Wrather and Koenning, 2009). *M. phaseolina* is known to cause disease in many other economically important crops, including corn (*Zea mays* L.), sorghum (*Sorghum* Moench), cotton (*Gossypium* L.), and tobacco (*Nicotiana tabacum*), and attacks over 500 species of plants (Su et al., 2001).

*M. phaseolina* can adapt to diverse habitats and conditions and is found in agricultural soils where it overwinters as microsclerotia in soil and on residues (Smith et al., 2014). During low soil moisture and warm weather, the microsclerotia germinate and can infect soybean plants (Arias et al., 2013). The initial infection often remains latent until later when soil moisture becomes limiting, temperature becomes warmer (28 – 35 °C), and during reproductive growth stages, starting from R5 to R7, when plants undergo stress as they started to senescence (Short et al., 1978; Hershman, 1993). This makes the early stage of reproductive growth stages the most appropriate for detecting the early phase of charcoal rot infection (Short et al., 1978; Mengistu et al., 2009). The disease can affect soybean seedlings, mature plants, and seeds. Usually, seed-borne charcoal rot will affect seedling emergence, and infected seedlings can become discolored or die as early as a few days after seedling emergence. The disease can interrupt water movement in infected mature plants due to the fungus colonizing and clogging the vascular tissue, thereby resulting in reduced plant vigor, leaf yellowing, wilting, and ultimately plant death, where leaves stay attached to petioles of dead plants (Smith et al., 2014). Often a reddish-brown discoloration may occur in the pith and vascular tissues of the lower stem and root of infected mature plants that died from charcoal rot (Smith et al., 2014; ElAraby et al., 2003). Splitting of the stem of the dead plant often reveals many gray to black streaks or specks appearing as specks of charcoal rot on the lower stem and

taproots. These specks are the microsclerotia of the fungus that are the surviving structures.

The management of charcoal rot is limited due to the lack of completely disease-resistant soybean varieties (Papavizas, 1977; Smith and Carvil, 1997), and the fungal microsclerotia cannot be eliminated once present in a field (Papavizas, 1977). The microsclerotia can survive under diverse environmental conditions (Csöndes et al., 2012). The propagules can persist in fields with or without residue for many years and serve as an infection source for the next growing season (Short et al., 1980). Depending on environmental conditions and association with residues, microsclerotia can remain viable for 2 to 15 years (Baird et al., 2003). They can also persist in low nutrient soil at above 30 °C for up to 3 years (Short et al., 1980). The density of microsclerotial in the soil and the soybean variety grown can affect the severity of charcoal rot disease (Arias et al., 2013; Mengistu et al., 2007). There are limited reports that suggest to some degree that initial microsclerotial density can affect charcoal rot on some crops. For example, Gray et al. (1991) reported that microsclerotial densities were positively associated with charcoal rot incidence on sorghum, and Mengistu et al. (2009) showed in a field study that the initial microsclerotial density affected soybean charcoal rot severity and yields loss. Additional studies are needed to identify what microsclerotial density can elicit disease and cause yield loss on soybean, which would provide baseline information and improve charcoal rot management.

While field studies can provide reliable data on the impact of pathogens on crops in a natural environment, they are more demanding in time, labor, and cost. Greenhouse studies provide a less demanding alternative to field studies and can remove the impact of other soil pathogens and environmental factors. No greenhouse study has been reported on the impact of *M. phaseolina* microsclerotia density on soybean or the impact of different soybean varieties on microsclerotial inoculum development in the soil. Evaluating the impact of microsclerotial density on soybean in the greenhouse can provide useful information for predicting charcoal rot development and severity, which can aid management recommendations. Also, there is a need to know the dynamics of microsclerotial density as influenced by different soybean varieties, especially regarding

their potential to suppress or favor the increase of the introduced microsclerotia. Understanding the extent to which a variety can increase or decrease soil inoculum provides valuable information for charcoal rot management. Subsequently, economic or damage thresholds can be determined for susceptible and moderately resistant varieties to guide management decisions and prevent yield loss. Therefore, the objectives of this study were to: (1) identify the impact of microsclerotial inoculum density on charcoal rot disease severity of soybean, (2) determine the microsclerotial inoculum densities on soybean growth and yield, and (3) evaluate the impact of two soybean varieties (susceptible and moderately resistant) on the resulting microsclerotial density in soils and plant tissues.

## **Materials and methods**

### ***Soybean varieties and fungal isolate***

The soybean varieties and fungal isolate used for this study were provided by Dr. Alemu Mengistu, USDA Soybean Pathologist. The soybean varieties were DT97-4290, moderately resistant (Paris et al., 2006), and LS 98-0358 which is susceptible to charcoal rot (Mengistu et al., 2007). The fungal isolate (TN-4) was collected from the research Field 2 in Jackson Tennessee in 2006.

### ***Microsclerotial inoculum preparation***

The microsclerotial inoculum was produced using methods described in Mengistu et al. (2007). Briefly, millet seed (400 mL by volume) were soaked for 18 to 20 h in 4 L of a solution containing distilled water and 40 g of table sugar, 0.5 g of yeast extract, and 0.25 g of tartaric acid per liter. The solution was decanted and the remaining millet was placed in the autoclavable bag, where a tube 5 cm in diameter and 10 cm in length was inserted halfway into the autoclavable bag and a cotton plug was inserted into a tube before autoclaving at 121 °C for 30 min. Thirty 0.5 cm diameter plugs from 1-week-old cultures of *M. phaseolina*, grown on acidified potato dextrose agar (PDA), were used to inoculate the millet through the tube. The bag was incubated at 30 °C for 3 weeks, with periodic shaking to spread the inoculum within the bag. After 3 weeks, the millet was

completely colonized and darkened with microsclerotia and allowed to air dry, then stored in sealed plastic containers at 4 °C until use.

The propagules of *M. phaseolina* inoculum were estimated by the colony-forming units (CFU) methods as described in Mengistu et al. (2007). A sub-sample of infested millet was ground into residue using mortar and pestle. One gram of the residue was placed in a 2 ml microtube and disinfected with 1 mL 10% sodium hypochlorite by vortexed three times in 1-minute cycles. The disinfected residue was poured into a sterile 45 µm mesh sieve and then rinsed with sterile water into another sterile test tube. The sterile residue mixture (about 10mL) was then mixed with 50 mL rifampicin–supplemented–potato dextrose agar (PDA) at 54°C. Rifampicin stock solution (2.5 % w/v) was prepared using methanol and was applied to PDA at 0.1% (v/v). The PDA–residue mixture was evenly poured into five pre-labeled Petri plates and set inside a hood chamber to gel. The plates were incubated at 30 °C for 5 days, and microsclerotial colonies in all the plates were counted and recorded as CFU per gram infested millet. Then eight microsclerotial treatments consisting of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 grams of microsclerotial-infested millet were prepared as the microsclerotial treatments, which corresponded to 47,553, 95,107, 142,660, 190,213, 237,767, 285,320, 332,873, and 380,427 total CFUs, and two non-inoculum treatments consisting of 0 and 4.0g of sterile millet were used as controls (Table 4.1).

### ***Greenhouse experiment design***

Three-gallon plastic pots were filled with a pasteurized potting mix composed of loam soil and sand mixed in a 3:1 ratio by volume. Microsclerotial treatments were evenly placed in a circular furrow made at 6 cm depth within the soil and covered with soil until not visible. Eight seeds were then sown in the soil directly over the furrow circumference. Each treatment was replicated four times, organized in a randomized complete block design, and repeated. There was a total of 10 treatments per soybean variety and the experiments were maintained in a greenhouse where the average temperature varied from 24°C (night) to 31°C (day) with a 16-hr photoperiod. Plants were watered as needed and fertilized once a week with Miracle-Gro plant nutrients (The

Scotts Miracle-Gro Company, Ohio). Plant stands were thinned to 6 plants per pot at the V1 stage, and the tests were maintained to maturity (growth stage R7).

### ***Sampling and data collection***

Data were collected at the R7 growth stage. *Macrophomina phaseolina* microsclerotia are known to be abundant in the vascular tissues of charcoal rot infected plants at the R7 growth stage, immediately after plant start senescences (Short et al., 1978; Megistu et al., 2007). Three random samples were collected per pot by cutting the stem at the cotyledonary node. Secondary roots and the rhizosphere soils were collected from the taproot into paper bags, while the taproot of each plant was collected into a separate paper bag. The samples remained in the greenhouse for up to 20 days for drying. Following this, data related to charcoal rot severity, plant heights, pods and seeds per plant, seed size, yields, and microsclerotial densities at harvest were collected.

### ***Charcoal rot severity, and soybean growth and yield***

Samples were removed from paper bags and washed with tap water to remove the soil, and the taproot was split into two halves longitudinally. Disease severity was rated on a 1 to 5 scale, based on the microsclerotial load where 1 = no discoloration, 2 = very few microsclerotia without tissue discoloration, 3 = vascular tissue partly covered with microsclerotia and discolored, 4 = vascular tissue discolored with numerous microsclerotia, and 5 = highly discolored and covered in dark microsclerotia (Mengistu et al., 2007). Plant heights were measured from the cotyledonary node upwards, and the number of pods and seeds per plant was counted manually. Seeds were weighed and seed size (average weight of 100 Seeds) was determined for each treatment by dividing the total weight of seeds per plant by the number of seeds per plant and multiplying by 100. Also, yield (kg/ha) was determined based on 247,105 plants per ha (100,000 plants per acre) per the University of Tennessee's recommended final soybean population (Davidson, 2018).

### ***Microsclerotial densities at harvest***

Densities of microsclerotia from bulk and rhizosphere soils and stem and root tissues of the soybean varieties were estimated using the colony-forming units (CFU) method (Mengistu et al., 2007) as previously described. Soil samples were collected from bulk and rhizosphere regions and ground separately using mortar and pestle, and the microsclerotia present in 1 g of the soil was determined for each. Also, taproots collected from each pot (after rating for charcoal rot severity) were ground using the Wiley Mill Model 4 (Philadelphia, PA) milling machine (the machine was thoroughly cleansed between samples with a suction device to prevent cross-contamination) and the microsclerotia present in 0.005 g tissue was determined and multiplied by 200 to arrive at the microsclerotia per 1 g tissue.

### ***Data analysis***

Analysis of variance (ANOVA) was used to determine any difference in plant height, pod number, seed number, seed size, and yield among treatments using JMP version 15.2 (SAS Institute, Cary, NC), and means were separated using Tukey's HSD or Student's T ( $\alpha = 0.05$ ). Pearson correlations were conducted in SAS Version 9.4 (SAS Institute, Cary, NC) to show relationships between inoculum treatments and disease severity and plant growth variables.

## **Results**

### ***Impact of microsclerotial treatments on charcoal rot severity***

Analysis of variance indicated variety and microsclerotial treatments had significant effects on multiple parameters. The only significant interactions were between variety and microsclerotial treatments on the severity and *M. phaseolina* CFUs from bulk soil (Table 4.2; 4.3). While microsclerotial treatments did not affect severity on the moderately resistant variety, DT97-4290, they did affect severity across the susceptible variety, LS98-0358, where microsclerotial treatments of 1.5, 3.5, and 4.0 g of inoculum caused a significantly greater charcoal rot severity compared to controls (Table 4.3). Similarly, *M. phaseolina* CFUs from bulk soil were not different across microsclerotial

treatments on the moderately resistant variety, and only the greatest microsclerotial treatment of 4.0 g of inoculum had significantly greater *M. phaseolina* CFUs from bulk soil than any other treatment on LS98-0358 (Table 4.3).

#### ***Impact of microsclerotial treatments on soybean growth and yield parameters***

There was no significant difference in plant height, pods and seeds, seed size, and yields per plant between treatments across both varieties (Tables 4.2 and 4.4). Pods and seeds per plant varied from 16 to 26 and 36 to 58, respectively, across the susceptible variety (LS98-0358) and from 18 to 25 and 36 to 51, respectively, across the moderately resistant variety (DT97-4290) (Table 4.4). Plant height varied from 55 to 56 cm across the susceptible variety, LS98-0358, and from 80 to 92 cm across the moderately resistant variety, DT97-4290 (data not shown). Likewise, seed size and yields varied from 13.7 to 16.7 g and 2.9 to 4.9 kg/ha, respectively, across the susceptible variety, LS98-0358, and from 15.9 to 19.3 g and 4.1 to 5.6 kg/ha across the moderately resistant variety, DT97-4290 (Table 4.4). Treatments with high microsclerotial density (3.0, 3.5, and 4.0g) had numerically low yields across both varieties (Table 4.4). Further analysis of the susceptible variety showed that there was a moderate positive correlation between microsclerotial treatments and disease severity (Rho 0.4, P=0.02).

#### ***Impact of variety on charcoal rot and growth parameters***

Variety significantly influenced disease and growth parameters evaluated in this study (Table 4.5). The susceptible variety had greater severity and CFUs from the taproot than the moderately resistant variety, as well as having fewer pods, smaller seed size, height, and lower yield compared to the moderately resistant variety (Table 4.5).

### **Discussion**

Results suggested that three microsclerotial treatments of 1.5, 3.5, and 4.0 grams, representing about 142,000, 332,00, and 380,000 CFU/treatment, can elicit charcoal rot on a susceptible soybean, but a moderately resistant variety may be unaffected by the densities. A previous study showed that soil microsclerotia density was correlated with

charcoal rot severity, but moderately resistant soybean varieties harvested from charcoal rot-infested fields had little or no visually detectable charcoal rot disease (Mengistu et al., 2007). The present result provides additional insights related to the microsclerotial density that can induce charcoal rot on susceptible soybean in the greenhouse. Results suggested that none of the microsclerotial treatments were capable of reducing growth on the susceptible variety tested. This suggests that the densities tested may not cause severe enough damage to crop growth. However, results showed that high inoculum densities can cause a non-significant yield loss on susceptible soybean without a reduction in plant height. Primarily, charcoal rot disease is known for reducing water movement in susceptible cultivars and causing yield loss (Doubledee et al., 2018). Interruption of water movement can contribute to yield loss incurred by a susceptible soybean caused by a high density inoculum infection.

Additionally, our data suggest that susceptible varieties are capable of increasing soil microsclerotia density while a moderately resistant variety is capable of decreasing the density. This is especially likely to occur in fields with high microsclerotial densities. In this study, the moderately resistant variety consistently had a lower microsclerotial density compared to the susceptible variety. The microsclerotial treatment of 4.0 gram on the moderately resistant variety had a lower CFU value from taproot stem tissue, bulk, and rhizosphere soils, compared to the same treatment on the susceptible variety. This indicates that the two soybean varieties have different capacities to support *M. phaseolina* inoculum development. A crop rotation program with a moderately resistant soybean could reduce charcoal rot severity by decreasing the pathogens' microsclerotial density in the field.

In conclusion, densities of *M. phaseolina* inoculum that are equivalent to 327 CFU/gram of soil can cause disease in a greenhouse setting. Moderately resistant soybean varieties have the potential of preventing significant disease development by reducing the pathogen inoculum density. Growing a moderately resistant soybean in a charcoal rot-infested field may reduce charcoal rot inoculum propagules in the field. Investigation of the highest microsclerotial density tested in this study in a microplot field trial would provide additional information and insights into the impact of *M. phaseolina*

microsclerotial density on charcoal rot severity and subsequent soybean damage and yield loss.

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## Appendix

**Table 4. 1. Estimation of *Macrophomina phaseolina* colony forming units (CFU) for each microsclerotial treatment.**

| Treatment <sup>a</sup> | Estimated initial CFU |
|------------------------|-----------------------|
| No-inoculum            | 0                     |
| Sterile millet (4g)    | 0                     |
| T 0.5                  | 47,553                |
| T 1.0                  | 95,107                |
| T 1.5                  | 142,660               |
| T 2.0                  | 190,213               |
| T 2.5                  | 237,767               |
| T 3.0                  | 285,320               |
| T 3.5                  | 332,873               |
| T 4.0                  | 380,427               |

<sup>a</sup> Values listed after T indicate number of grams of inoculum for each treatment

**Table 4. 2. Standard least squares p-values of fixed effects of variety and microsclerotial treatments.**

| Variable                          | Variety | Microsclerotial treatments <sup>a</sup> | Variety x Microsclerotial treatments |
|-----------------------------------|---------|-----------------------------------------|--------------------------------------|
| Height (cm)                       | <0.0001 | 0.5                                     | 0.4                                  |
| Number of pods                    | 0.03    | 0.07                                    | 0.3                                  |
| Number of seeds                   | 0.4     | 0.07                                    | 0.9                                  |
| Severity <sup>b</sup>             | <0.0001 | 0.0004                                  | 0.0004                               |
| Yield (kg/ha)                     | <0.0001 | 0.08                                    | 0.1                                  |
| Rhizosphere soil CFU <sup>c</sup> | 0.5     | 0.004                                   | 0.2                                  |
| Bulk soil CFU <sup>c</sup>        | <0.0001 | <0.0001                                 | 0.0005                               |
| Taproot CFU <sup>c</sup>          | 0.002   | 0.3                                     | 0.4                                  |
| Seed size <sup>d</sup>            | <0.0001 | 0.2                                     | 0.4                                  |

<sup>a</sup> Microsclerotial treatments included 2 controls (no inoculum and 4 g of sterile millet) and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 grams of microsclerotial-infested millet

<sup>b</sup> Severity was based on rating split stems on a 1 to 5 scale, based on the microsclerotial load where 1 = no discoloration, 2 = very few microsclerotia without tissue discoloration, 3 = vascular tissue partly covered with microsclerotia and discolored, 4 = vascular tissue discolored with numerous microsclerotia, and 5 = highly discolored and covered in dark microsclerotia

<sup>c</sup> Colony-forming units (CFU)

<sup>d</sup> Seed size was estimated by dividing total seed weight by total number of seeds and multiplied by 100, to get 100 seed weight

**Table 4. 3. Variety x microsclerotial treatment interaction on the severity and bulk soil *Macrophomina phaseolina* colony-forming unit (CFU)**

| Variety                                                      | Microsclerotial treatment <sup>a</sup> | Severity <sup>b</sup> | Bulk Soil CFU <sup>c</sup> |
|--------------------------------------------------------------|----------------------------------------|-----------------------|----------------------------|
| LS98-0358 variety<br>susceptible to charcoal rot             | Sterile millet                         | 1 b                   | 1 b                        |
|                                                              | No inoculum                            | 1 b                   | 0 b                        |
|                                                              | 0.5                                    | 1 b                   | 56 b                       |
|                                                              | 1.0                                    | 2 ab                  | 41 b                       |
|                                                              | 1.5                                    | 3 a                   | 41 b                       |
|                                                              | 2.0                                    | 2 ab                  | 106 b                      |
|                                                              | 2.5                                    | 2 ab                  | 78 b                       |
|                                                              | 3.0                                    | 2 ab                  | 126 b                      |
|                                                              | 3.5                                    | 3 a                   | 76 b                       |
|                                                              | 4.0                                    | 3 a                   | 327 a                      |
| DT97-4290 variety<br>Moderately resistant to<br>charcoal rot | Sterile millet                         | 1 b                   | 0 b                        |
|                                                              | No inoculum                            | 1 b                   | 0 b                        |
|                                                              | 0.5                                    | 1 b                   | 32 b                       |
|                                                              | 1.0                                    | 1 b                   | 16 b                       |
|                                                              | 1.5                                    | 1 b                   | 18 b                       |
|                                                              | 2.0                                    | 1 b                   | 46 b                       |
|                                                              | 2.5                                    | 1 b                   | 23 b                       |
|                                                              | 3.0                                    | 1 b                   | 35 b                       |
|                                                              | 3.5                                    | 1 b                   | 46 b                       |
|                                                              | 4.0                                    | 1 b                   | 52 b                       |
| <i>P</i> - Value                                             |                                        | 0.0004                | < .0001                    |

<sup>a</sup> Microsclerotial treatments included 2 controls (no inoculum and 4 g of sterile millet) and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 grams of microsclerotial-infested millet

<sup>b</sup> Severity was based on rating split stems on a 1 to 5 scale, based on the microsclerotial load where 1 = no discoloration, 2 = very few microsclerotia without tissue discoloration, 3 = vascular tissue partly covered with microsclerotia and discolored, 4 = vascular tissue discolored with numerous microsclerotia, and 5 = highly discolored and covered in dark microsclerotia

<sup>c</sup> Colony forming units (CFU)

**Table 4. 4. Differences of microsclerotial treatments on yield parameters and *Macrophomina phaseolina* density in the rhizosphere soil**

| Variety                                                   | Microsclerotial treatment <sup>a</sup> | Number of pods/plant | Number of seeds/plant | Seed size | Yield (kg/ha) | Rhizosphere CFU <sup>b</sup> |
|-----------------------------------------------------------|----------------------------------------|----------------------|-----------------------|-----------|---------------|------------------------------|
| LS98-0358 variety<br>susceptible to charcoal rot          | sterile millet                         | 21                   | 44                    | 14.0      | 4.2           | 1 b                          |
|                                                           | 0.0                                    | 21                   | 46                    | 16.7      | 4.5           | 0 b                          |
|                                                           | 0.5                                    | 19                   | 41                    | 15.8      | 4.2           | 28 ab                        |
|                                                           | 1.0                                    | 19                   | 42                    | 16.5      | 4.5           | 29 ab                        |
|                                                           | 1.5                                    | 19                   | 41                    | 14.8      | 4.1           | 31 ab                        |
|                                                           | 2.0                                    | 25                   | 55                    | 14.3      | 5.2           | 65 ab                        |
|                                                           | 2.5                                    | 20                   | 47                    | 15.8      | 4.4           | 35 ab                        |
|                                                           | 3.0                                    | 21                   | 44                    | 13.8      | 4.2           | 61 ab                        |
|                                                           | 3.5                                    | 18                   | 39                    | 14.8      | 3.9           | 71 ab                        |
|                                                           | 4.0                                    | 19                   | 41                    | 15.3      | 4.0           | 128 a                        |
| DT97-4290 variety<br>Moderately resistant to charcoal rot | sterile millet                         | 25                   | 51                    | 17.9      | 5.4           | 0 b                          |
|                                                           | 0.0                                    | 21                   | 45                    | 17.5      | 4.7           | 0 b                          |
|                                                           | 0.5                                    | 20                   | 43                    | 18.8      | 4.8           | 40 b                         |
|                                                           | 1.0                                    | 20                   | 44                    | 19.2      | 5.1           | 29 b                         |
|                                                           | 1.5                                    | 21                   | 44                    | 18.6      | 5.0           | 40 b                         |
|                                                           | 2.0                                    | 24                   | 51                    | 18.3      | 5.6           | 67 b                         |
|                                                           | 2.5                                    | 22                   | 48                    | 15.9      | 4.6           | 34 b                         |
|                                                           | 3.0                                    | 22                   | 47                    | 17.7      | 4.9           | 58 b                         |
|                                                           | 3.5                                    | 18                   | 38                    | 18.4      | 4.4           | 83 b                         |
|                                                           | 4.0                                    | 18                   | 36                    | 19.3      | 4.1           | 49 b                         |
| <i>P</i> - Value                                          |                                        | 0.0819               | 0.0733                | 0.2059    | 0.0800        | 0.008                        |

<sup>a</sup> Microsclerotial treatments included 2 controls (no inoculum and 4 g of sterile millet) and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 grams of microsclerotial-infested millet

<sup>b</sup> Colony-forming units (CFU) of *M. phaseolina* per 1 gram of rhizosphere soil recovered after soybean maturity

**Table 4. 5. Variety effect on charcoal rot severity and taproot *Macrophomina phaseolina* colony forming units (CFU).**

| Variety       | Severity <sup>a</sup> |   | Taproot CFU <sup>b</sup> |   | Height (cm) |   | Pods/plant |   | Seed size (g) <sup>c</sup> | Yield (kg/ha) |     |   |
|---------------|-----------------------|---|--------------------------|---|-------------|---|------------|---|----------------------------|---------------|-----|---|
| DT97-4290-MR  | 1                     | b | 85                       | b | 33          | a | 21         | a | 18.1                       | a             | 4.9 | a |
| LS98-0358-SCR | 2                     | a | 571                      | a | 22          | b | 19         | b | 15.0                       | b             | 3.8 | b |

<sup>a</sup> Severity was based on rating split stems on a 1 to 5 scale, based on the microsclerotial load where 1 = no discoloration, 2 = very few microsclerotia without tissue discoloration, 3 = vascular tissue partly covered with microsclerotia and discolored, 4 = vascular tissue discolored with numerous microsclerotia, and 5 = highly discolored and covered in dark microsclerotia

<sup>b</sup> Colony-forming units (CFU)

<sup>c</sup> Seed size was estimated by dividing total seed weight by total number of seeds and multiplied by 100, to get 100 seed weight

**CHAPTER V. EFFICACY OF ORGANIC FUNGICIDES AGAINST  
HEMP POWDERY MILDEW CAUSED BY  
*GOLOVINOMYCES AMBROSIAE* IN THE GREENHOUSE IN  
TENNESSEE**

## Abstract

Hemp powdery mildew (*Golovinomyces ambrosiae*) is a common disease of hemp in greenhouses in Tennessee. Efficacy data can support the use or approval of new fungicides against the disease in Tennessee. Therefore, three greenhouse experiments were conducted to assess the efficacy of commercial and experimental fungicides against powdery mildew. ‘BaOx2’ or ‘Sweetened’ hemp cultivars, which are susceptible to powdery mildew, were inoculated with a conidial suspension 1-day before or 1 or 7-days after the first fungicide application. Two additional fungicide applications were made at 7-day intervals by thoroughly spraying the plants using a hand-held sprayer. Control plants were sprayed with water only. Weekly disease incidence and severity ratings were made three times. Disease index (DI) and the area under the disease progress curve were calculated. All treatments significantly reduced powdery mildew disease index in each trial. Compared to the control plants, disease reductions ranged from 76% to 100%. Bonide sulfur, Luna Experience, and MilStop exhibited “excellent” efficacy, reducing disease by 96% to 100%. Cinnerate, Exile, IP-1, Regalia, and Sil-Matrix exhibited “very good” efficacy, reducing disease by 86% to 95%. Defguard, IP-2, IP-V1\*, Regalia\*, and Stargus exhibited “good” efficacy, reducing DI by 76% to 85%. IP-V4 exhibited “fair” efficacy, reducing DI by 56% to 70%. These findings provide useful information to Tennessee hemp growers for the control of hemp powdery mildew.

## Introduction

Tennessee hemp acreage has steadily increased after the re-introduction of hemp as an agricultural crop by the 2014 Agricultural Act (TDA; Tennessee Department of Agriculture, 2021). Hemp acreage has increased from approximately 650 acres/year in 2015 to 4,800 acres/year in 2020 (Self, 2021). In 2018, hemp production costs approximately \$2,301/acre in Tennessee (Tennessee Department of Agriculture, 2021). At this rate, it means hemp growers spent over \$11 million on hemp acreage in Tennessee in 2020. A large portion of the acreage was under high tunnel and greenhouse or indoor systems. The cultivated indoor space was up to 8 million sq. Ft. (184 acres, 74 hectares), with growers spreading across the West, Middle, and East Tennessee regions (Tennessee Department of Agriculture, 2021).

Many diseases can affect all hemp production systems. The most frequently reported diseases of hemp in Tennessee are bud rot, leaf spots, southern blight, and powdery mildew (Hansen, 2020a). Among these, powdery mildew is the most common in the greenhouse. Powdery mildew pathogens are supported by warm temperatures and humid conditions that are often present in the greenhouse. This makes the greenhouse environments favorable to powdery mildew development and spread. Powdery mildew spread occurs by asexual conidial spores that are carried by air currents from infected sources to healthy leaves within the greenhouse. After 2 to 3 days on the healthy leaves, the conidial spores germinate and initiate secondary infections. The infection then results in early signs and symptoms that appear as white powdery growths, made up of fungal mycelium and conidia, on upper leaf surfaces and stems. As the mycelium and conidia penetrate the leaf tissues, they absorb nutrients and water, impair photosynthesis, and subsequently initiate secondary symptoms such as leaf distortion, necrosis, and leaf drop. In severe cases, powdery mildew can result in reduced plant health, poor yields, or plant death (Britt, 2020; Gauthier, 2020).

Powdery mildew can affect a wide range of hosts and is caused by many fungal genera including *Erysiphe*, *Leveillula*, *Golovinomyces*, *Podosphaera*, and *Sphaerotheca*. Several species of *Golovinomyces* are known to cause powdery mildew on diverse crops in the United States and elsewhere. *Golovinomyces spadiceus* was reported to cause

powdery mildew on green and gold in Tennessee (Trigiano et al., 2018); okra in Georgia (Moparathi et al., 2018a); helianthus in Washington State (Moparathi et al., 2018b); and industrial hemp in Kentucky (Szarka et al., 2019). *Golovinomyces ambrosiae* was reported on whorled sunflower in Tennessee (Trigiano et al 2016), while Pépin et al., (2018) reported *G. cichoracearum* sensu lato on medical cannabis in Canada. The pathogen causing powdery mildew on hemp in Tennessee is unknown. It is crucial to identify powdery mildew pathogens of hemp in Tennessee to facilitate further studies and understanding of the pathogens to provide information to support disease diagnostics and management.

Effective management of powdery mildew can be achieved by integrating cultural and chemical management tactics. These tactics include the use of less-susceptible or resistant cultivars, removal of inoculum sources, and chemical fungicides (Marine et al., 2010). Although there is no hemp cultivar with known complete resistance to powdery mildew, studies from Tennessee and New York showed that many CBD hemp cultivars exhibit low to moderate susceptibility to powdery mildew (Hansen et al., 2020b; Stack et al., 2021). On greenhouse hemp, maintaining low relative humidity, removing infected leaves, and applying fungicides can suppress powdery mildew (Britt, 2020). Many commercial greenhouse growers are attracted to organic or bio-pesticides because of their minimum re-entry intervals, low human toxicity, and their perceived or real reduced environmental impact (Gradish et al., 2010; Tjosvold & Koike, 2001). Many organic fungicides are registered for hemp in the United States (Environmental Protection Agency, 2021), and a few of these have been approved for use in Tennessee (Tennessee Department of Agriculture, 2021). Recently, four organic fungicides including Defguard, Exile, Regalia, and Stargus were tested against hemp powdery mildew and leaf spot in a field trial (Akinrinlola and Hansen, 2021). Although the fungicides showed excellent to moderate efficacy in the field, their efficacy in the greenhouse is unknown, and therefore these products need to be tested in the greenhouse. Also, as more fungicides have recently been approved for hemp in Tennessee, their efficacy needs to be independently validated against powdery mildew to inform Extension recommendations. Continued production of hemp in greenhouses in Tennessee may promote the dispersal, incidence,

and severity of powdery mildew in greenhouses across the state, thus calling for the need for more tested products to be available for use in the state. This is essential to provide diverse fungicide options for Tennessee hemp growers and to reduce the development of fungicide resistance in powdery mildew fungi. Therefore, the objectives of this study were to (1) identify the fungal species causing powdery mildew on hemp in Tennessee, and (2) determine the efficacy of fungicide products against powdery mildew in the greenhouse.

## **Materials and methods**

### ***Molecular identification and phylogenetic analysis of the pathogen***

The molecular identification of the pathogen was achieved by the amplification of three genetic regions using appropriate primer pairs: internal transcribed spacer (ITS) region using ITS-4/ITS-5 (5'-TCCTCCGCTTATTGATATGC-3'/5'-GGAAGTAAAAGTCGTAACAAGG-3'), intergenic spacer (IGS) region using IGS-12a/NSIR (5'- AGT CGT TGG ATT AGT GGC CG -3'/ 5'- GAG ACA AGC ATA TGA CTA C -3'), and the beta-tubulin (tub2) gene using TubF1/TubR1 (5'- AGG TTC ACC TCC AGA CTG G -3'/ 5'- CCA GCA CGA ACA GCA TCC AT -3') (Qiu et al., 2020). Each PCR reaction was made up of a 20-µl mixture consisting of 10 µl of 2x Phire plant PCR buffer (Thermo Fisher Scientific, Waltham, MA), 1 µl each of 10 mM forward and reverse primers, 0.4 µl of Phire Hot Start II DNA polymerase buffer (Thermo Fisher Scientific, Waltham, MA), 7.6 µl of sterile distilled water, and 5 µl PM conidia solution or SDW (negative control). The conidia used for the beta-tubulin PCR reaction were first lysed by exposure to 5% Chelex before being used for the PCR reaction (Hirata & Takamatsu, 1996). The PCR reactions were run using a Bio-Rad T100 thermal cycler (Bio-Rad, Hercules, CA) using the following programs: for the ITS and IGS regions; 5 min at 98°C, then 40 cycles of 98°C for 5 s, 52°C for 5 s, and 72°C for 20 s, and a final phase of 72°C for 1 min; and the beta-tubulin gene, 5 min at 95°C, then 30 cycles of 95°C for 1 min, 52°C for 30 s, and 72°C for 2 min, and a final phase of 72°C for 8 min; The PCR products were viewed on 1.5% agarose gel stained with Gel Red, and successfully amplified products were then sequenced by Eurofins Genomics (Louisville,

KY). Sequences were searched against the NCBI nucleotide database using the Basic Local Alignment Search Tool (BLAST), and search queries with greater than 95% coverage and identity were recorded. Sequences generated in this study were deposited in GenBank. BLAST search results were further validated through the construction of a neighbor-joining tree. ITS, IGS, and beta-tubulin nucleotide sequences of the powdery mildew isolate (TN isolate) and 12 reference isolates belonging to *G. ambrosiae* (n=5), *G. latisporus* (n=5), *G. circumfusus* (n=1), and *G. magnicellatus* (n=1) (Table 5.1) (Qiu et al., 2020) were aligned using Muscle in Mega X (Kumar et al., 2018). The aligned sequences were concatenated and used to generate a neighbor-joining tree in Mega, using the Tamura-Nei model with 1000 bootstrapping repetitions and pairwise deletion used to address alignment gaps.

### ***Greenhouse experimental design***

The study was conducted at the University of Tennessee Knoxville North Greenhouse between November 2020 and May 2021. ‘BaOx2’ (South Central Growers, Springfield, TN, experiment 1) and ‘Sweetened’ (PWP Greenhouse, Pall Mall, TN, experiments 2 and 3), which are susceptible to powdery mildew, were used in the study. One plant (about 6-weeks-old) was planted per pot in 1-gallon plastic pots that were filled to about 2 inches from the top with Lambert Peat Moss potting mix (Quebec, Canada). Pots were arranged in a randomized complete block design with four replicates in the greenhouse. Experiments were conducted three times, with a varying number of fungicides tested. Greenhouse temperatures during Experiment 1 ranged from 17°C to 26°C, Experiment 2 ranged from 20°C to 27°C, and Experiment 3 ranged from 14°C to 31°C. The average relative humidity during the three trials was 51%, 48%, and 56%, respectively. Plants were maintained under a photoperiod of 16h light and 8h dark. Plants were watered at least once daily or as needed and fertilized once per week using Miracle-Gro Plant Food (The Scotts Company, Marysville, OH) at the rate of 1-tbs per gallon.

### ***Conidial inoculation***

Conidial suspensions containing  $2 \times 10^5$  conidia/ml were prepared using conidia collected from powdery mildew-infected plants ('Sweetened' cultivar) that had been growing in the greenhouse. Powdery mildew-infected leaves were collected from the infected plants and washed in deionized water. The conidia released into the water were then separated from plant debris by filtering through a four-layer cheesecloth. The concentration of the resulting conidial suspension was then estimated using a hemocytometer under a light microscope. The concentration was standardized to  $2 \times 10^5$  conidia/ml by dilution with deionized water. Suspensions were then sprayed on the leaves of each plant using a 1-L hand-held sprayer (Solo Inc., Newport News, VA). Plants were sprayed to achieve thorough coverage of both upper and lower leaf surfaces, with approximately 20 ml of conidial suspension applied to each plant.

### ***Fungicide applications***

Thirteen fungicide products, consisting of nine commercial and four experimental products, were evaluated in this study (Table 5.2). Most of the commercial products are considered organic bio-pesticides, labeled for controlling fungal diseases on several crops, and are registered for use on hemp in Tennessee. The products included Bonide sulfur (sulfur), Cinnerate (Cinnamon oil), Defguard (*B. amyloliquifaciens* D747), Exile (potassium salts of fatty acids), MilStop (Potassium bicarbonate), Regalia (extract of *Reynoutria sachalinensis*), Sil-Matrix (Potassium silicate), and Stargus (*Bacillus amyloliquifaciens* F727). Luna Experience is a commercial product containing fluoypram and tebuconazole. Although not registered for use on hemp, it was included in the study as a positive control because of its known efficacy against powdery mildew on other crops (Blundell et al., 2019; Proffer et al., 2013; Veloukas and Karaoglanidis, 2012; Vitale et al., 2016). The experimental products included IP-1, IP-2, IP-V1, and IP-V4 from industry partners (Table 5.2).

There were a total of 14 treatments across the three experiments, with each treatment tested in at least two experiments. Applications were made three times at 7-day intervals in each experiment, either as a prophylactic or post-inoculation treatment. The

first application of the prophylactic treatments was made 1-day before conidial inoculation, while that of the post-inoculation treatments was made 1-day or 7-days (Regalia\*) after conidial inoculation. Experiment 1 consisted of 11 prophylactic and 1 post-inoculation treatment. Experiment 2 consisted of 12 prophylactic and 2 post-inoculation treatments. Experiment 3 consisted of 5 prophylactic and 1 post-inoculation treatment. Regalia\* and IP-V1\* were the post-inoculation treatments. All treatments were applied to the plants the same day the treatments were prepared. The leaves of the plants were thoroughly sprayed with treatment solutions using 1-L hand-held sprayers (Solo Inc., Newport News, VA). Water was sprayed on plants as the control treatment.

### ***Disease assessments***

The plants were visually assessed for powdery mildew incidence and severity weekly at 7-days, 14-days, and 21-days after the first treatment application. The incidence represented the percentage of leaves exhibiting powdery mildew symptoms, and the severity represented the average percent leaf area exhibiting powdery mildew symptoms on each leaf with at least one powdery mildew lesion, rated between 0% to 100% (Tables 5.3-5.5).

### ***Data analysis and interpretation***

Disease index (DI) was calculated from the incidence and severity data to summarize disease levels at each disease rating date (Tables 5.3-5.5). The DI was calculated as shown:  $DI = (I \times S) / 100$ , where I=disease incidence, S = disease severity, and 100 represents the maximum possible incidence and severity scores. Also, the Area Under the Disease Progress Curve (AUDPC) was calculated to show disease progress during each experiment. The AUDPC was calculated as:  $AUDPC = \sum (\{ \text{average [rating \#0 + rating \#1]} \} \times \{ \text{days between rating \#0 + rating \#1} \} + \{ \text{average [rating \#1 + rating \#2]} \} \times \{ \text{days between rating \#1 and rating \#2} \} + \{ \text{average [rating \#2 + rating \#3]} \} \times \{ \text{days between rating \#2 + rating \#3} \})$ . To test for significant differences between treatments in all datasets (incidence, severity, disease index, and AUDPC), data were subjected to ANOVA in SAS (SAS Institute, 9.4, Cary NC), and means were separated

using the Tukey test ( $\alpha = 0.05$ ) with treatment as the fixed effect and replicate as the random effect. Furthermore, using disease index and disease index AUDPC means, percent disease reduction by each treatment compared to the control plants was calculated for each trial. The percent disease reduction was calculated using the equation:  $([\text{disease rating for untreated} - \text{disease rating for a treatment}] / \text{disease rating for untreated}) * 100$ . The overall disease reduction efficacy of each treatment was summarized as the average of all disease reduction percentages by each treatment across the trials. The average disease reduction percentage was then classified into different efficacy categories, ranging from excellent to fair. From 100% to 96% was “excellent”, 95% to 86% was “very good”, 85% to 76% was “good”, and 75% to 66% was “Fair” (Table 5.6).

## **Results**

### ***Molecular identification of powdery mildew pathogen***

The PCR resulted in an amplified region of about 381 bp for the ITS region, 337 bp for the IGS region, and 423 bp for the beta-tubulin gene. An NCBI BLAST search matched the sequences with that of *Golovinomyces ambrosiae* with a 100% query coverage and identity for ITS (accession No. MT355556.1), 99% for IGS (accession No. MK452567.1; MK452502), and 99% for beta-tubulin (accession No. MK389490.1). Likewise, the neighbor-joining tree constructed using these three genes and sequence data published in the Qui et al., (2020) phylogeny of *Golovinomyces* spp. showed the powdery mildew isolate from this study belongs to a monophyletic clade of *G. ambrosiae* isolates (Figure 5.1).

### ***Fungicide efficacy***

Fungicide treatments showed varying levels of powdery mildew suppression in the trials (Figure 5.2). Results related to disease incidence, severity, and index are presented in Tables 5.3-5.5. Disease index and index AUDPC data are discussed because the disease index is a summary statistic that includes disease incidence and severity.

## ***Experiment 1***

### ***Disease index***

Disease index for the control plants increased from 7 on the 7-day observation to 47 on the 21-day observation. Disease index for the fungicide-treated plants ranged from 0 (Bonide sulfur) to 8 (Regalia\*) on the 7-day observation, and from 0 (Bonide sulfur) to 18 (IP-2) on the 21-day observation (Table 5.3). All 12 treatments significantly reduced disease index at least once among the three disease rating times compared to the control plants. Four treatments (Cinnerate, Defguard, Milstop, and Bonide sulfur) significantly reduced the disease index two times, at 14-day and 21-day observations. The other eight treatments significantly reduced disease index only once, during the 14-day observation. None of the treatments significantly reduced the disease index on 11/24 (Table 5.3). There was no significant difference among the 12 treatments.

### ***Disease progress (AUDPC)***

Disease index AUDPC value was 529 for the control plants, while the value ranged between 1 (Bonide sulfur) and 184 (Regalia\*) for the fungicide-treated plants. All of the treatments had a significantly lower index AUDPC value compared to the control plants. There was no significant difference in index AUDPC values among the fungicide-treated plants (Figure 5.3a).

## ***Experiment 2***

### ***Disease index.***

Disease index for the control plants increased from 7 on the 14-day observation to 43 on the 21-day observation. Whereas disease index ranged between 0 (multiple treatments) and 9 (Defguard) on the 14-day observation, and between 0 (multiple treatments) to 16 (Defguard) on the 21-day observation (Table 5.4). All 14 treatments significantly reduced the disease index at least once among the three disease rating times compared to the control plants (Table 5.4). No disease was present at the 7-day observation. Significant disease reductions started on the 14-day disease observation. Eight treatments significantly reduced the disease index two times at the 14-day and 21-

day (3/23 and 3/30) observations compared to the controls, and four treatments (Defguard, Stargus, Regalia, and IP-V1\*) significantly reduced the disease index once at the 21-day (3/30) observation (Table 5.4). There was no significant difference among the 14 treatments at the 21-day disease index. Data were presented for two dates only in this trial because no disease was observed at the 7-day (3/16) disease rating.

### ***Disease progress (AUDPC)***

All treatments exhibited efficacy against powdery mildew index AUDPC. Disease progress was generally slow. The index AUDPC value for the control plants was 152, while the value for the fungicide-treated plants ranged between 0 (Bonide Sulfur, IP-2, Luna Experience, and Milstop) and 57 (Defguard). All of the treatments had a significantly lower index AUDPC value compared to the control plants. Bonide sulfur, Cinnerate, Exile, IP-1, IP-2, Luna Experience, Milstop, Regalia, Sil-Matrix, Stargus, IP-V1, and IP-V4 had similar index AUDPC values that ranged from 0 to 44, and they were not significantly different from each other. Defguard, with an index AUDPC value of 57, had a significantly higher AUDPC value than several of the best-performing treatments, but it was still significantly lower than the control (Figure 5.3b).

### ***Experiment 3***

#### ***Disease index.***

Disease index for the control plants increased from 14 on the 7-day observation to 45 on the 21-day observation (Table 5.5). Disease index for the fungicide-treated plants ranged between 0 (IP-1, Luna Experience, and Regalia) to 3 (IP-V4) on the 7-day observation, and from 0 (IP-V1\* and Luna Experience) to 12 (IP-2) on the 21-day observation (Table 5.5). All six treatments significantly reduced the disease index on all three disease rating days compared to the control. There was no significant difference among treatments (Table 5.5).

### ***Disease progress (AUDPC)***

All treatments exhibited efficacy against powdery mildew progress (Table 5.5). The index AUDPC value was 493 for the control plants, while the value ranged between 0 (Luna Experience) and 158 (IP-V4) for the fungicide-treated plants (Table 5.5). All of the treatments had a significantly lower index AUDPC value compared to the control (Figure 5.3c). Luna Experience, Regalia, IP-V1, and IP-1 had the lowest and statistically similar index AUDPC values. The value was 0 for Luna experience, 8 for Regalia, 28 for IP-V1, and 82 for IP-1. Treatments IP-2 having index AUDPC of 137 and IP-V4 having index AUDPC of 158 had the second-lowest index AUDPC values compared to the control (Table 5.5).

### ***Disease reduction percentage***

Overall disease reduction was calculated as the average of all index AUDPC reductions across trials (Table 5.6). Three treatments (Bonide sulfur, Luna Experience, and Milstop) had excellent overall disease reduction efficacy by reducing disease by over 95%. The overall disease index AUDPC reduction by Bonide sulfur was 100%, Milstop was 99%, and Luna Experience was 98%. Five treatments (Cinnerate, Exile, IP-1, Regalia, and Sil-Matrix) had very-good disease reduction efficacy by reducing disease by 86% to 95%. Regalia reduced disease by 94%, Exile reduced disease by 93%, Cinnerate reduced disease by 92%, and IP-1 and Sil-Matrix reduced disease by 88%. Five treatments (Defguard, IP-2, IP-V1\*, Regalia\*, and Stargus) had good disease reduction efficacy by reducing disease by 76% to 85%. IP-V1\* reduced disease by 84%, Regalia\* reduced disease by 82%, IP-2 reduced disease by 80%, Defguard reduced disease by 79%, and Stargus reduced disease by 76%. Treatment IP-V4 had fair disease reduction efficacy by reducing disease index AUDPC across trials by 70% (Table 5.6).

## **Discussion**

This study indicates that *Golovinomyces ambrosiae* is at least one of the pathogens responsible for greenhouse hemp powdery mildew in Tennessee. All of the sequenced genetic regions in this study matched those of *G. ambrosiae* with 99% to

100% homology, and they were in the same clade with previously reported *G. ambrosiae* in the phylogenetic analysis. This is the first report of *G. ambrosiae* causing powdery mildew on hemp in Tennessee. However, the *Golovinomyces* genus being identified as a hemp powdery mildew pathogen in this study is consistent with other studies in the United States and Canada (Scott and Punja 2021). Likewise, *G. ambrosiae* has been reported to cause hemp powdery mildew in Oregon (Wiseman et al., 2021) and Nevada (Schoener, 2021). Aside from hemp, *G. ambrosiae* is a known pathogen of on whorled sunflower (*Helianthus verticillatus*) in Tennessee (Trigiano et al., 2016). Findings from Kentucky, which neighbors Tennessee, as well as Ohio and New York, identified *G. spadiceus* as the hemp powdery mildew pathogen, which differs from the results of this study (Szarka et al., 2019; Farinas and Peduto, 2020; Weldon et al., 2020). Together, these findings suggest that multiple species are responsible for causing hemp powdery mildew in the U.S., and the fact that different species were identified in neighboring states implies that multiple pathogen species may be found in relative proximity.

Fungicides are important in developing management programs for powdery mildew disease. Among the nine commercial fungicides tested in this study, five (Defguard, Exile, Regalia, Sil-Matrix, Stargus) are approved by the Tennessee Department of Agriculture for hemp. Our results showed that all nine fungicides exhibited varying efficacy against hemp powdery mildew caused by *G. ambrosiae*.

Bonide sulfur, Luna Experience, and Milstop exhibited excellent efficacy. Luna Experience and Milstop are not in the list of approved products on hemp in Tennessee, indicating they are not yet allowed on hemp in the state. Bonide sulfur, however, has been historically used as an organic product to control many plant diseases for over 2,000 years. Bonide sulfur is a wettable powder sulfur treatment that is toxic to many pathogens including fungi. It is effective when used as a prophylactic treatment (Mangandi and Peres, 2009a, 2009b; Beckerman, 2008; Davis et al., 2008). Our study corroborates the prophylactic efficacy of sulfur against powdery mildew in the greenhouse. Luna Experience contains fluopyram and tebuconazole as the active ingredients. Fluopyram kills target pathogens by suppressing respiration. Tebuconazole, on the other hand, interferes with cell walls to prevent spore germination (Kwok and Loeffler, 1993;

Vargas-Pérez et al., 2020). Both compounds are systemic fungicides that are mobile within plant tissue and can protect against pathogen invasion. These modes of action may explain the high efficacy of Luna Experience in this study. Many researchers have shown that products containing either fluopyram or tebuconazole or both are effective against powdery mildew. Researchers reported that fluopyram-containing fungicides were effective against fungal diseases including powdery mildew, leaf spots, and grey mold on different crops (Proffer et al., 2012; Veloukas and Karaoglanidis, 2012; Vitale et al., 2016). Likewise, a product containing tebuconazole, otherwise known as a sterol demethylation inhibitor fungicide, was effective against fusarium head blight caused by *Fusarium graminearum* (Sun et al., 2014). These studies and the result of the present study implicate that Luna Experience is an effective fungicide against hemp powdery mildew. Even though Luna Experience is not registered for hemp, it will be useful for hemp researchers to be aware of a conventional synthetic fungicide with high efficacy as a potential control product in further research. Milstop is registered for use on hemp in the United States but it is currently not among the fungicides approved on hemp in Tennessee. Milstop is a “contact and kills” foliar fungicide with potassium bicarbonate as the active compound. Bicarbonate fungicides work by altering cellular pH, osmotic imbalance, and affecting cell membrane permeability; thus spore formation and mycelial growth (Ordóñez-Valencia et al., 2009). Potassium bicarbonate products have shown efficacy against varieties of diseases on different crops including apple scab on apples (Jamar et al., 2008), silver scurf on potato (Olivier et al., 1998), and powdery mildew on a gooseberry (Wenneker, 2015); gerbera daisies (Moyer and Peres, 2008); apples (Jamar et al., 2008); cantaloupe (Matheron and Porchas, 2013); and cucurbit (McGrath and Shishkoff 1999). These studies support our finding and implicate that potassium bicarbonate fungicides including Milstop exhibit promising potential as an effective organic product against hemp powdery mildew.

Cinnerate, Exile, Regalia, and Sil-Matrix exhibited high efficacy against hemp powdery mildew, indicating that they are great options for controlling powdery mildew caused by *G. ambrosiae* in greenhouse hemp production in Tennessee. Cinnamon oil is the active ingredient in Cinnerate, and cinnamon oil is known to inhibit fungal growth by

disrupting cell membranes and causing cellular dehydration (Perumal et al., 2016). This attribute may explain the efficacy of Cinnerate in this study whereby Cinnerate suppressed powdery mildew disease by 92%. In a similar report, cinnamon oil sprayed on the leaves of *Zinnia elegans* in a field study was found to decrease *Erysiphe cichoracearum* powdery mildew incidence and severity by up to 95% and 92%, respectively (Hegazi and El-Kot, 2010). Likewise, in another study, lauric acid (a major component of cinnamon oil) sprayed on barley seedlings was found to significantly reduce barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) infection by 33% to 84% at different concentrations (Walters et al., 2003). These reports are consistent with our findings and implicate that cinnamon oil products including Cinnerate could be used to control powdery mildew successfully in the greenhouse. Exile contains potassium salts of fatty acids, which is known to act through a “contact and kill” mode of action, whereby it disrupts the cell membrane and cellular function of target pathogens leading to their desiccation, suffocation, and cell death (Highland, 2010). Many earlier reports by other researchers have shown the efficacy of potassium salts of fatty acids against powdery mildew. Pasini et al., (1997) reported that potassium salts of fatty acids product significantly and consistently reduced the severity of rose powdery mildew (*Sphaerotheca pannosa* var. *rosae*) in a glasshouse. Emilia-Romagna and Elia (2000) showed that potassium salts of fatty acids treatments protected grape berries against grape powdery mildew (*Uncinula necator*). However, contrary to our finding, Pasini et al., (1997) found that the treatments exhibited a (rose flowers spotting) phytotoxicity, while Emilia-Romagna and Elia (2000) reported alterations of grape berry phytotoxicity. This may suggest that different crop varieties may exhibit different sensitivity to potassium salts of fatty acids, therefore caution should be observed before applying the products on new commercial crops. Regalia (extract of *Reynoutria sachalinensis* [giant knotweed]) exhibited very high efficacy (94% average disease reduction) when applied preventively, and good efficacy (80% average disease reduction) when used curatively. Extract of *Reynoutria sachalinensis* is known to contain compounds such as resveratrol, physcion, and emodin, all of which possess antimicrobial properties. The extract acts by way of inducing plant resistance via the induction of phytoalexins, production of phenolic

compounds, and increased production of defense-related proteins (chitinase, glucanase, and peroxidase) in plants (Margaritopoulou et al., 2020). These multiple modes of action may explain the excellent efficacy of Regalia in our study and *Reynoutria sachalinensis* treatments in many reports. A formulation of *Reynoutria sachalinensis* significantly reduced powdery mildew (*Podosphaera xanthii*) spore germination on courgette as early as 12-hours after inoculation as well as delayed hyphae and conidiophore formation (Margaritopoulou et al., 2020). Also, the treatment exhibited similar disease reduction activity against Zucchini powdery mildew (*Podosphaera xanthii*) on susceptible and moderately resistant genotypes. The treatment had improved efficacy against cucurbit powdery mildew (*P. xanthii*) when applied in combination with a wetting agent on a partially resistant cultivar, but efficacy on either susceptible or partially resistant without the wetting agent was similar. Furthermore, there was no difference in efficacy between 7-day and 14-day interval applications. Regalia demonstrated the most effective control activity against hemp powdery mildew disease caused by *Golovinomyces cichoracearum* (Scott and Punja, 2021). These findings support the result of this study and suggest that *Reynoutria sachalinensis* formulations exhibit great potential against powdery mildew and can be used to protect crops in the greenhouse against powdery mildew. In this study, however, we did not investigate efficacy at 14-day intervals. Investigating this may provide important information on the frequency of product application required for satisfactory disease control. Also, our results indicated that the preventive application had better efficacy compared to the post-inoculation application. This may suggest that *Reynoutria sachalinensis* should be applied as a preventive treatment for maximum protection. Sil-Matrix (potassium silicate) exhibited very good efficacy in this study. Silicon is known to protect plants using mechanical and physiological mechanisms. The mechanical modes of action may include the formation of a silicon matrix barrier beneath the cuticle of epidermal cell walls to inhibit pathogen penetration and regulate water loss (Kanto et al., 2007; Kim et al., 2002; Shetty et al., 2012). The physiological mechanisms may involve the initiation and accumulation of phenolic compounds and other host defense-related proteins around infection cells to induce pathogen resistance (Menzies et al., 1991; Kanto et al., 2007). The activity of plant defense-related compounds such as

chitinase, peroxidase, polyphenol oxidases, and flavonoid phytoalexins was induced in plants following treatment with silicon (Heckman, 2013). These mechanisms possibly explain the efficacy of Sil-Matrix against powdery mildew in this study and many studies reported by other researchers. Potassium silicate was reported to inhibit the growth of several plant pathogenic fungi and oomycetes including *Sclerotinia sclerotiorum*, *Pythium* F-group, *Mucor pusillus*, *Drechslera* sp., *Fusarium oxysporum*, *F. solani*, *Alternaria solani*, *Colletotrichum coccodes*, *Verticillium theobromae*, *Curvularia lunata*, *Stemphylium herbarum*, and *Phytophthora cinnamomi* (Bekker et al., 2006). Kanto et al., (2007) showed that the germination rate of *Sphaerotheca aphani* var. *aphani*, the strawberry powdery mildew fungus, was significantly reduced on silicon-treated leaves, with the fungus producing shorter germ tubes and secondary hyphae than that of untreated leaves. Fungal conidial production was suppressed by up to 60% due to silicon treatment. Similarly, a disease evaluation study on *Arabidopsis* showed that treatment significantly reduced powdery mildew (*Erysiphe cichoracearum*) severity as early as 8 days after inoculation. Furthermore, a scanning electron microscopy analysis revealed that mycelial development on the silicon-treated leaf was significantly reduced, whereas dense mycelial mats and conidial colonies were found on no-silicon control leaves (Ghanmi et al., 2004). Dallagnol et al., (2012) found that foliar and root application of potassium silicate on melon reduced powdery mildew (*Podosphaera xanthii*) progress by 65% and 73%, respectively. In the study, the application also significantly reduced conidial production, infection efficiency, and colony expansion and area. In another study, silicon treatment delayed rose powdery mildew (*Podosphaera pannosa*) development by 1-2 days and subsequently reduced disease severity by up to 49% (Shetty et al., 2012). These findings implicate the strong potential of silica products, such as Sil-Matrix, against powdery mildew diseases.

This study found that Defguard and Stargus exhibited moderate efficacy against hemp powdery mildew, with both treatments reducing disease by an average of 79% and 76% compared to control. Defguard and Stargus are bio fungicides formulated with *Bacillus amyloliquefaciens*, strains D747 and F727, respectively. Strains of *B. amyloliquefaciens* are beneficial rhizobacteria often reported with multiple modes of

action including the ability to colonize root hairs, leaves, and other plant surfaces to prevent the establishment of fungal and bacterial pathogens (Jiao et al., 2020). Also, they are known to inhibit spore germination and prevent mycelial growth and spore formation. There are reports that some strains of *B. amyloliquefaciens* can induce systemic resistance against pathogens or plant growth promotion (Akinrinlola et al., 2018; Jiao et al., 2020; Matzen et al., 2019). An endophytic *Bacillus amyloliquefaciens* strain YN201732 inhibited the germination of tobacco powdery mildew (*Erysiphe cichoracearum*) conidia by 50 to 87% *in-vitro*. The strain inhibited the growth of multiple plant pathogens in dual culture assays by up to 77%. The pathogens were *Phytophthora parasitica* var. *nicotianae*, *Fusarium avenaceum* f. sp. *fabae*; *Streptomyces scabies*; *Monilinia fructigena* F. *graminearum*, *Gaeumannomyces graminis*, *Setosphaeria turcica*, *Verticillium dahlia*, *Alternaria alternate*, *Fusarium oxysporum* f.sp. *lycoperisci*, *Botrytis cinerea*, and *F. solani* (Jiao et al., 2020). Molecular analysis showed that the bacterial strain induced the expression of genes that are indicated for jasmonic acid/ethylene signaling pathways, which may be related to defense against powdery mildew. The strain upregulated chitinase and polyphenol oxidase genes, which are closely related to induced systemic resistance in tobacco against powdery mildew. Preventive and therapeutic foliar spray of the strain on tobacco significantly suppressed powdery mildew severity in the greenhouse (Jiao et al., 2020). Matzen et al., (2019) reported that Serenade®ASO (*Bacillus amyloliquefaciens* [former *subtilis*] strain QST 713) moderately suppressed cereal powdery mildew (*Blumeria graminis* f.sp. *tritici*, f.sp. *hordei*, f.sp. *avenae*) by 20% to 65% following four weekly foliar applications. Disease reduction potential was reduced in later assessments and there was an insignificant yield increase following application. Best control was achieved when the application was done 0 to 2 days after inoculation. Except when applied at a high dose rate of 2 L/ha, treatment application at 4-day after inoculation had no disease reduction. Generally, the efficacy of most bacillus-based products can be improved by applying them regularly as a preventive treatment, or use in a rotation with or tank-mix compatible fungicides.

Overall, our study found that eight fungicides (Bonide sulfur, Cinnerate, Defguard, MilStop, Regalia, Sil-Matrix, and Stargus) exhibited varying levels of efficacy

against hemp powdery mildew caused by *G. ambrosiae*. Five of these products (Defguard, Exile, Regalia, Sil-Matrix, and Stargus) are registered for use on hemp in Tennessee, and they will be helpful for Tennessee greenhouse hemp growers in managing powdery mildew.

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## Appendix

**Table 5.1. Reference isolates used to construct the phylogenetic tree based on ITS, IGS, and beta-tubulin gene sequences. Source: Qiu et al., 2020.**

| Species                              | Isolate       | GenBank Accession Numbers |          |              |
|--------------------------------------|---------------|---------------------------|----------|--------------|
|                                      |               | ITS                       | IGS      | Beta-tubulin |
| <i>Golovinomyces ambrosiae</i>       | HMJAU-PM91819 | MK452590                  | MK452516 | MK452472     |
| <i>Golovinomyces ambrosiae</i>       | HMJAU-PM91820 | MK452591                  | MK452517 | MK452473     |
| <i>Golovinomyces ambrosiae</i>       | HMJAU-PM91836 | KX987303                  | MK452533 | MK389490     |
| <i>Golovinomyces ambrosiae</i>       | HMJAU-PM91842 | MK452612                  | MK452537 | MK452487     |
| <i>Golovinomyces ambrosiae</i>       | HMJAU-PM91855 | MK452626                  | MK452551 | MK452496     |
| <i>Golovinomyces circumfusus</i>     | HAL 3300      | MK452628                  | MK452555 | MK452459     |
| <i>Golovinomyces latisporus</i>      | HMJAU-PM91829 | MK452600                  | MK452526 | MK452482     |
| <i>Golovinomyces latisporus</i>      | HMJAU-PM91830 | MK452601                  | MK452527 | MK452483     |
| <i>Golovinomyces latisporus</i>      | HMJAU-PM91831 | MK452602                  | MK452528 | MK452484     |
| <i>Golovinomyces latisporus</i>      | HMJAU-PM91828 | MK452599                  | MK452525 | MK452481     |
| <i>Golovinomyces latisporus</i>      | HMJAU-PM91832 | MK452603                  | MK452529 | MK452485     |
| <i>Golovinomyces magnicellulatus</i> | HMJAU-PM91840 | MK452610                  | MK452534 | MK452486     |

**Table 5.2. Products evaluated against hemp powdery mildew in greenhouse trials**

| Product (Rate/A)           | Active ingredient                              | Source                                   |
|----------------------------|------------------------------------------------|------------------------------------------|
| Bonide sulfur (1.2% w/v)   | Sulfur                                         | Bonide Products, Inc., New York, USA     |
| Cinerrate (1 qt)           | Cinnamon oil                                   | SEIPASA, S.A, Spain                      |
| Defguard (6 qt)            | <i>Bacillus amyloliquefaciens</i> strain D747  | General Hydroponics, California, USA     |
| Exile (4 qt)               | Potassium salts of fatty acids                 | General Hydroponics, California, USA     |
| IP-1                       | Proprietary                                    | Industry partner                         |
| IP-2 (1% v/v)              | Proprietary                                    | Industry partner                         |
| IP-V1                      | Proprietary                                    | Industry partner                         |
| IP-V4                      | Proprietary                                    | Industry partner                         |
| Luna Experience (12 fl oz) | Fluopyram and Tebuconazole                     | Bayer CropScience Inc. New Jersey        |
| Milstop (5 lb)             | 85% potassium bicarbonate                      | BioWorks Inc. New York                   |
| Regalia (2 qt)             | 20% extract of <i>Reynoutria sachalinensis</i> | Marrone Bio Innovations. California, USA |
| Sil-Matrix (1% v/v)        | Potassium silicate                             | PQ Corporation, PA, USA                  |
| Stargus (4 qt)             | 96.4% <i>Bacillus amyloliquefaciens</i> strain | Marrone Bio Innovations.                 |

**Table 5. 3. Efficacy of fungicide treatments against powdery mildew in Experiment 1, 2020.**

| Treatment       | Incidence (%)   |                  |                  |                    | Severity (%)    |                  |                  |        | Disease index w |                  |                  |       |
|-----------------|-----------------|------------------|------------------|--------------------|-----------------|------------------|------------------|--------|-----------------|------------------|------------------|-------|
|                 | 11/24<br>7 days | 12/01<br>14 days | 12/08<br>21 days | AUDPC <sup>x</sup> | 11/24<br>7 days | 12/01<br>14 days | 12/08<br>21 days | AUDPC  | 11/24<br>7 days | 12/01<br>14 days | 12/08<br>21 days | AUDPC |
| Bonide sulfur   | 0.0 b           | 0.0 c            | 4.0 c            | 13.0 dz            | 0.0 b           | 0.0 c            | 2.0 d            | 5.0 c  | 0.0 c           | 0.0 c            | 0.0 b            | 1.0 b |
| Cinerrate       | 18 ab           | 33 bc            | 18 bc            | 424 bcd            | 25 a            | 12 bc            | 7.0 bcd          | 297 b  | 4.0 abc         | 4.0 bc           | 2.0 b            | 64 b  |
| Defguard        | 7.0 b           | 10 c             | 31 ab            | 227 cd             | 8.0 ab          | 8.0 c            | 10 bcd           | 151 bc | 1.0 bc          | 1.0 c            | 4.0 b            | 23 b  |
| Exile           | 13 ab           | 34 bc            | 45 ab            | 493 bcd            | 6.0 b           | 8.0 c            | 12 bcd           | 142 bc | 1.0 abc         | 3.0 c            | 7.0 ab           | 49 b  |
| IP-1            | 28 ab           | 50 abc           | 76 a             | 823 abc            | 10 ab           | 11 bc            | 15 abcd          | 204 bc | 3.0 abc         | 6.0 bc           | 11 ab            | 101 b |
| IP-2            | 28 ab           | 76 ab            | 74 a             | 998 ab             | 9.0 ab          | 17 bc            | 23 abcd          | 264 bc | 3.0 abc         | 14 bc            | 18 ab            | 177 b |
| Luna Experience | 1.0 b           | 0.0 c            | 20 abc           | 80.0 d             | 2.0 b           | 0.0 c            | 10 bcd           | 46 bcd | 0.0 c           | 0.0 c            | 8.0 ab           | 28 b  |
| Milstop         | 3.0 b           | 31 bc            | 2.0 c            | 256 cd             | 4.0 b           | 5.0 c            | 1.0 cd           | 61 bcd | 0.0 bc          | 2.0 c            | 0.0 b            | 12 b  |
| Regalia         | 18 ab           | 21 c             | 48 ab            | 447 bcd            | 10 ab           | 12 bc            | 18 abcd          | 215 bc | 2.0 abc         | 2.0 c            | 14 ab            | 78 b  |
| Regalia*        | 55 a            | 70 a             | 59 ab            | 1,105 ab           | 13 ab           | 19 bc            | 14 abcd          | 273 bc | 8.0 abc         | 14 bc            | 8.0 ab           | 184 b |
| Sil-Matrix      | 9.0 ab          | 20 c             | 76 a             | 471 bc             | 8.0 ab          | 5.0 c            | 12 bcd           | 133 bc | 1.0 abc         | 2.0 c            | 10 ab            | 52 b  |
| Stargus         | 31 ab           | 71 a             | 41 ab            | 878 abc            | 11 ab           | 14 bc            | 13 abcd          | 225 bc | 4.0 abc         | 10 bc            | 7.0 ab           | 127 b |
| Water (check)   | 57 a            | 89 a             | 85 a             | 1343 a             | 11 ab           | 48 a             | 53 a             | 601 a  | 7abc            | 45 a             | 47 a             | 529 a |

<sup>w</sup> Disease index (DI)= (I\*S)/100, where DI=disease index, I=disease incidence, S=disease severity, and 100 represents the maximum possible incidence and severity scores.

<sup>x</sup> Area under the disease progress curve (AUDPC) was calculated according to the formula:  $\sum_{i=1}^n [(DI_{i+1} + DI_i)/2] [t_{i+1} - t_i]$ , where DI = disease index at the *i*th observation, *t<sub>i</sub>* = time (days) since the previous rating at the *i*th observation, and *n* = total number of observations.

<sup>y</sup> Days after the first treatment application

<sup>z</sup> Means followed by the same letter(s) within columns are not significantly different (Tukey test, *P*<0.05).

\* Applied as a post-inoculation treatment

**Table 5.4. Efficacy of fungicide treatments against powdery mildew in Experiment 2, 2021**

| Treatment       | Incidence (%)    |                               |                    | Severity (%)     |                  |       | Disease index <sup>w</sup> |                               |       |
|-----------------|------------------|-------------------------------|--------------------|------------------|------------------|-------|----------------------------|-------------------------------|-------|
|                 | 03/23<br>14 days | 03/30<br>21 days <sup>y</sup> | AUDPC <sup>x</sup> | 03/23<br>14 days | 03/30<br>21 days | AUDPC | 03/23<br>14 days           | 03/30<br>21 days <sup>c</sup> | AUDPC |
| Bonide sulfur   | 0.0 c            | 0.0 c <sup>z</sup>            | 0.0 c              | 0.0 b            | 0.0 c            | 0.0 c | 0.0 b                      | 0.0 c                         | 0.0 c |
| Cinerrate       | 35 abc           | 21 bc                         | 320 bc             | 3.0 b            | 6.0 c            | 43 c  | 1.0 b                      | 2.0 c                         | 6.0 c |
| Defguard        | 70 a             | 66 ab                         | 722 a              | 13 a             | 23 b             | 168 b | 9.0 a                      | 16 bc                         | 57 b  |
| Exile           | 21 bc            | 28 bc                         | 248 c              | 3.0 b            | 7.0 c            | 45 c  | 1.0 c                      | 2.0 c                         | 7.0 c |
| IP-1            | 24 bc            | 9.0 c                         | 197 c <sup>d</sup> | 2.0 b            | 3.0 c            | 28 c  | 0.0 b                      | 1.0 c                         | 2.0 c |
| IP-2            | 0 c              | 1.0 c                         | 2.0 c              | 0.0 b            | 1.0 c            | 2.0 c | 0.0 b                      | 0.0 c                         | 0.0 c |
| IP-V1*          | 59 ab            | 61 ab                         | 626 ab             | 7.0 a            | 18 b             | 111 b | 5.0 ab                     | 11 bc                         | 40 bc |
| IP-V4           | 69 ab            | 63 ab                         | 700 a              | 10 a             | 18 b             | 133 b | 7.0 a                      | 13 bc                         | 44 bc |
| Luna Experience | 0.0 c            | 0.0 c                         | 0.0 c              | 0.0 b            | 0.0 c            | 0.0 c | 0.0 b                      | 0.0 c                         | 0.0 c |
| Milstop         | 0.0 c            | 0.0 c                         | 0.0 c              | 0.0 b            | 0.0 c            | 0.0 c | 0.0 b                      | 0.0 c                         | 0.0 c |
| Regalia         | 0.0 c            | 12 c                          | 42 c               | 0.0 b            | 3.0 c            | 12 c  | 0.0 b                      | 1.0 c                         | 3.0 c |
| Regalia*        | 5.0 c            | 15 c                          | 88 c               | 1.0 b            | 4.0 c            | 24 c  | 0.0 b                      | 1.0 c                         | 3.0 c |
| Sil-Matrix      | 30 bc            | 51 ab                         | 390 ab             | 3.0 b            | 10 c             | 55 c  | 2.0 b                      | 7.0 bc                        | 23 bc |
| Stargus         | 40 ab            | 54 ab                         | 469 ab             | 7.0 a            | 17 b             | 107 b | 3.0 ab                     | 11 bc                         | 37 bc |
| Water (check)   | 66 ab            | 80 a                          | 744 a              | 11 a             | 54 a             | 265 a | 7.0 a                      | 43 a                          | 152 a |

<sup>w</sup> Disease index (DI)= (I\*S)/100, where DI=disease index, I=disease incidence, S=disease severity, and 100 represents the maximum possible incidence and severity scores.

<sup>x</sup> Area under the disease progress curve (AUDPC) was calculated according to the formula:  $\sum_{i=1}^n [(DI_{i+1} + DI_i)/2] [t_{i+1} - t_i]$ , where  $DI$  = disease index at the  $i$ th observation,  $t_i$  = time (days) since the previous rating at the  $i$ th observation, and  $n$  = total number of observations.

<sup>y</sup> Days after the first treatment application

<sup>z</sup> Means followed by the same letter(s) within columns are not significantly different (Tukey test,  $P < 0.05$ ).

\*Post-inoculation treatment

**Table 5.5. Efficacy of fungicide treatments against powdery mildew in Experiment 3, 2021**

| Treatment       | Incidence (%)  |                 |                              |                     | Severity (%)   |                 |                              |        | Disease index <sup>w</sup> |                 |                              |        |
|-----------------|----------------|-----------------|------------------------------|---------------------|----------------|-----------------|------------------------------|--------|----------------------------|-----------------|------------------------------|--------|
|                 | 5/06<br>7 days | 5/13<br>14 days | 5/20<br>21 days <sup>y</sup> | AUDPC <sup>x</sup>  | 5/06<br>7 days | 5/13<br>14 days | 5/20<br>21 days <sup>c</sup> | AUDPC  | 5/06<br>7 days             | 5/13<br>14 days | 5/20<br>21 days <sup>c</sup> | AUDPC  |
| IP-1            | 3.0 c          | 63 ab           | 53 bc                        | 644 bc <sup>z</sup> | 2.0 c          | 13 de           | 14 cde                       | 152 bc | 0.0 b                      | 8.0 bc          | 8.0 b                        | 82 bc  |
| IP-2            | 8.0 c          | 65 ab           | 68 b                         | 763 b               | 5.0 c          | 19 cde          | 18 bcd                       | 241 b  | 1.0 b                      | 13 bc           | 12 bc                        | 137 bc |
| IP-V1*          | 13 c           | 10 c            | 3.0 d                        | 224 cd              | 8.0 bc         | 8.0 de          | 2.0 ef                       | 132 bc | 2.0 b                      | 1.0 bc          | 0.0 b                        | 24 bc  |
| IP-V4           | 20 ab          | 58 ab           | 58 b                         | 748 b               | 8.0 abc        | 25 bcd          | 19 bcd                       | 305 b  | 3.0 b                      | 14 c            | 11 b                         | 158 b  |
| Luna Experience | 0.0 d          | 0.0 c           | 0.0 d                        | 5.0 d               | 0.0 e          | 0.0 e           | 0.0 f                        | 5.0 c  | 0.0 b                      | 0.0 b           | 0.0 b                        | 0.0 c  |
| Regalia         | 1.0 c          | 4.0 c           | 25 c                         | 122 cd              | 1.0 c          | 2.0 e           | 7.0 def                      | 45 c   | 0.0 b                      | 0.0 b           | 2.0 b                        | 8.0 c  |
| Water (check)   | 58 a           | 86 a            | 89 a                         | 1,365 a             | 19 ab          | 39 ab           | 50 a                         | 595 a  | 14 a                       | 34 a            | 45 a                         | 493 a  |

<sup>w</sup> Disease index (DI)= (I\*S)/100, where DI=disease index, I=disease incidence, S=disease severity, and 100 represents the maximum possible incidence and severity scores.

<sup>x</sup> Area under the disease progress curve (AUDPC) was calculated according to the formula:  $\sum_{i=1}^n [(DI_{i+1} + DI_i)/2] [t_{i+1} - t_i]$ , where *DI* = disease index at the *i*th observation, *t<sub>i</sub>* = time (days) since the previous rating at the *i*th observation, and *n* = total number of observations.

<sup>y</sup> Days after the first treatment application

<sup>z</sup> Means followed by the same letter(s) within columns are not significantly different (Tukey test, P<0.05).

\*Post-inoculation treatment

**Table 5. 6. Disease (AUDPC) reduction by fungicide treatments across trials**

| Treatment     | Index AUDPC reduction <sup>y</sup> (%) |              |              |         | Efficacy <sup>z</sup> |
|---------------|----------------------------------------|--------------|--------------|---------|-----------------------|
|               | Experiment 1                           | Experiment 2 | Experiment 3 | Average |                       |
| Bonide sulfur | 100                                    | 100          | -            | 100     | Excellent             |
| Cinerrate     | 88                                     | 96           | -            | 92      | Very Good             |
| Defguard      | 96                                     | 63           | -            | 79      | Good                  |
| Exile         | 91                                     | 95           | -            | 93      | Very Good             |
| IP-1          | 81                                     | 99           | 83           | 88      | Very good             |
| IP-2          | 67                                     | 100          | 72           | 80      | Good                  |
| IP-V1*        | -                                      | 74           | 95           | 84      | Good                  |
| IP-V4         | -                                      | 71           | 68           | 70      | Fair                  |
| Luna          | 95                                     | 100          | 100          | 98      | Excellent             |
| Experience    |                                        |              |              |         |                       |
| Milstop       | 98                                     | 100          | -            | 99      | Excellent             |
| Regalia       | 85                                     | 98           | 98           | 94      | Very good             |
| Regalia*      | 65                                     | 98           | -            | 82      | Good                  |
| Sil-Matrix    | 90                                     | 85           | -            | 88      | Very good             |
| Stargus       | 76                                     | 76           | -            | 76      | Good                  |

<sup>y</sup> Index AUDPC reduction: disease reduction was calculated using the equation: ((disease rating for untreated – disease rating for a treatment)/disease rating for untreated) \*100.

<sup>z</sup> Efficacy categories ranged from an excellent to a fair efficacy category. Average index AUDPC reduction from 100% to 96% was “excellent”, 95% to 86% was “very good”, 85% to 76% was “good”, and 75% to 66% was “Fair”.

Hyphen (-): treatment was not tested’

\*Post-inoculation treatment

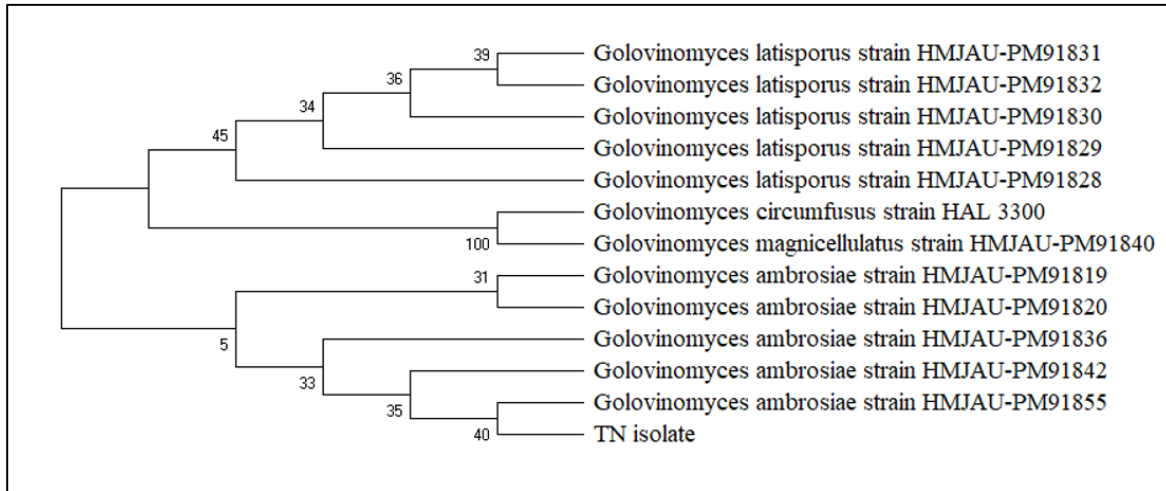


**Figure 5.1.** Molecular phylogenetic neighbor-joining tree based on the internal transcribed spacer region (ITS), intergenic spacer (IGS) and  $\beta$ -tubulin nucleotides sequences of 12 *Golovinomyces* species reported in the literature and one isolate collected from powdery mildew infected hemp leaf in Tennessee. Bootstrap values are shown at branch nodes. All sequences except the TN isolate were collected from Qiu et al. 2020.



**Figure 5.2.** Representative images showing differences in powdery mildew symptoms on water-sprayed (control) and select fungicide-treated leaves.

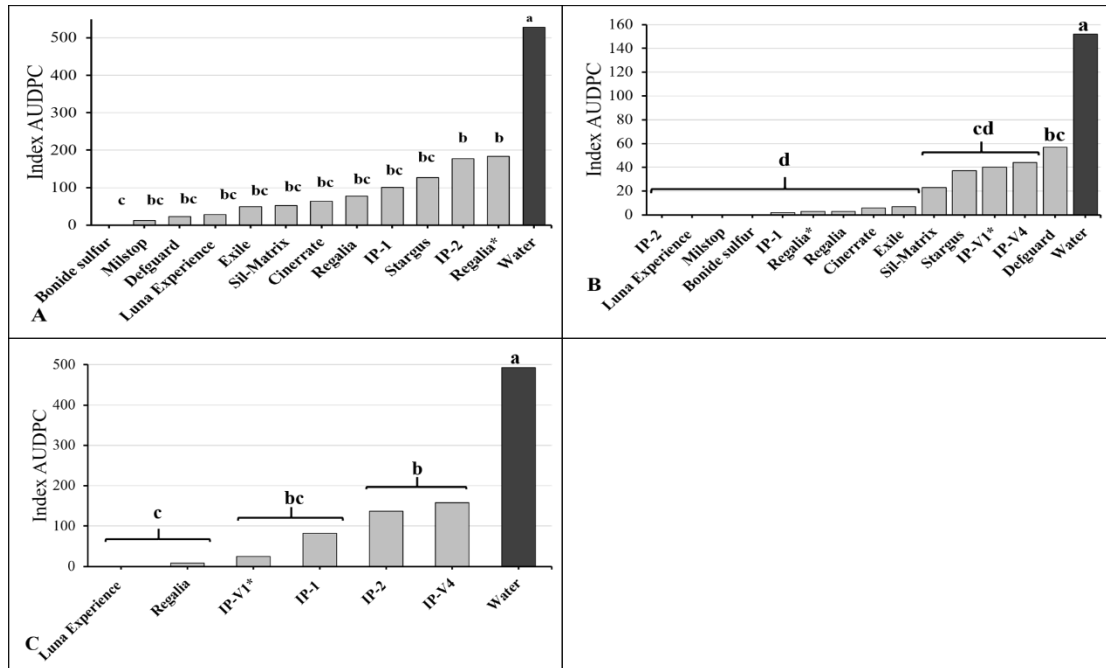


**Figure 5.3.** Index Area Under the Disease Progress Curve (AUDPC) for treatments in Experiment 1 (a), Experiment 2, (b) and Experiment 3 (c). Bars sharing any of the same letters within each experiment are not significantly different (Tukey test,  $\alpha = 0.05$ ). Asterisk (\*) = post-inoculation treatment

## CHAPTER VI. CONCLUSIONS

The first objective of the study reported in Chapter II was to assess the dynamics of plant-parasitic nematode populations across row-crop fields in Tennessee. This information helps facilitate improved management of nematodes and avoid field crop damage from the nematodes. Across all samples collected from 25 counties, fourteen nematode genera were found, suggesting the nematodes are potential threats to field crop production in Tennessee. Populations of spiral, lance, SCN, and root-knot nematodes were either most prevalent or occurred at high densities in samples, indicating that the nematodes demand close monitoring and may require drastic control measures to prevent crop loss. The second objective of the Chapter II study was to determine the virulence phenotypes of SCN populations in Tennessee fields. The information was to aid management recommendations for SCN populations. Findings suggest there are virulent SCN phenotypes prevalent in TN fields, with close to 100% of the populations tested able to feed and reproduce on PI 88788 SCN resistance type, and over 60% developed on the Peking resistance type. This suggests that cultivars developed from these resistance types could incur yield loss if grown in fields where these SCN populations were found and SCN population density could increase.

The objectives of the study reported in Chapter III were to evaluate the impact of SCN HG type 1.2.5.7 on soybean NF activity and growth on susceptible and resistant soybean genotypes. The study provides insights that can facilitate sustainable and improved management of SCN and prevent yield loss from the nematode. Results suggest that the impact of virulent SCN biotype (HG type 1.2.5.7) on NF activity can vary among susceptible soybean cultivars. The tested population decreased NF activity on one susceptible variety (Williams 82) but did not affect NF activity on another susceptible variety (Ellis) in this study. Results also indicate that NF activity on resistant soybean cultivars are not affected by virulent SCN activity. This was reflected in the NF activity of PI 88788 which was not affected by SCN in this study. Furthermore, results suggested that SCN activity will more likely affect soybean leaf color, leaf area, and root area than leaf nitrogen content and root length, as the latter group was not affected by SCN activity

in this study. Additionally, results also indicate that it is possible that SCN can develop highly on a soybean cultivar without diminishing the soybean growth and NF activity. This was reflected in Ellis that supported a high SCN population without diminishing NF activity and growth parameters. Overall, these results indicate that the effects of virulent SCN on susceptible cultivars can vary, but it has limited to no impact on resistant cultivars despite it being able to reproduce on the resistant cultivar tested.

The reports in Chapter IV were based on two objectives: (1) to identify the impact of microsclerotial inoculum density on charcoal rot disease severity of soybean, soybean growth and yield, and (2) evaluate the impact of two soybean varieties (susceptible and moderately resistant) on the resulting microsclerotial density in soils and plant tissues. Results indicated that densities of *M. phaseolina* inoculum that are equivalent 327 CFU/gram of soil can cause disease and yield loss in a greenhouse setting. Also, results suggested that yield loss can occur without an obvious growth reduction by charcoal rot. It is also indicated from the results that moderately resistant soybean varieties have the potential of preventing significant disease development by reducing the pathogen inoculum density DT97-4290, a moderately resistant variety, had a significantly lower microsclerotial density at harvest in this study. Further investigation of the highest microsclerotial density tested in this study in a microplot field trial would provide additional information and insights into the impact of *M. phaseolina* microsclerotial density on charcoal rot severity and subsequent soybean damage and yield loss.

Finally, in the Chapter V study, the objectives were to (1) identify the *Golovinomyces* species causing hemp powdery mildew in the greenhouse in Tennessee and (2) assess the efficacy of fungicides against the disease in the greenhouse. Results showed that *Golovinomyces ambrosiae* is the causative pathogen of hemp powdery mildew in Tennessee. This was shown through internal transcribed spacer (ITS) region, intergenic spacer (IGS) region, and beta-tubulin genes sequencing and based on a phylogenetic analysis containing sequence data from closely related species. Also, eight fungicides (Bonide sulfur, Cinnerate, Defguard, MilStop, Regalia, Sil-Matrix, and Stargus) exhibited varying levels of efficacy against hemp powdery mildew caused by *G. ambrosiae*, and five of them (Defguard, Exile, Regalia, Sil-Matrix, and Stargus) and

many other fungicides products that are now registered for use on hemp in Tennessee can facilitate effective control of hemp powdery mildew in the greenhouse in Tennessee.

These studies provide current data that can support the improved and sustainable management of plant-parasitic nematodes associated with field crops in Tennessee and identify the nematodes of growing concern in fields as the spiral, lance, SCN, and root-knot nematodes. Appropriate measures suggested earlier in the discussion session of Chapter II should be taken to prevent potential crop damage by the nematodes.

*Macrophomina phaseolina* microsclerotial density that can cause charcoal rot and soybean yield loss was identified through this study, and the potential of using moderately resistant cultivars to suppress field inoculum of the fungus was suggested, to support sustainable disease management. Finally, through this study, the pathogen causing hemp powdery mildew in the greenhouse in Tennessee was identified as *G. ambrosiae*. This information will guide further investigations of the pathogen in Tennessee. Finally, this research study provides fungicide efficacy data to support hemp powdery mildew control. Overall, the four research studies provided current data that can facilitate an improved and sustainable control of nematodes in field crops, charcoal rot disease on soybean, and powdery mildew disease on greenhouse hemp in Tennessee.

## **VITA**

Rufus J. Akinrinlola was born and raised in Ondo State, Southwest of Nigeria. He is obtaining a Ph.D. in Plant Pathology from the Entomology and Plant Pathology Department, University of Tennessee, Knoxville under the supervision of Dr. Heather Kelly and Dr. Zachariah Hansen. He obtained an a Master's degree in Plant Pathology from the University of Nebraska-Lincoln under the direction of Dr. Gary Yuen and Dr. Tony Adesemoye in May 2018, and Bachelor's degree in Microbiology from Adekunle Ajasin University, Akungba-Akoko, Ondo State Nigeria in 2013.