ABSTRACT

SCHWARZ, TANNER. Investigations of the Root-Knot Nematode *Meloidogyne enterolobii* in North Carolina: Hosts and Resistance, Management, and Pathogen Interactions. (Under the direction of Dr. Adrienne Gorny).

Meloidogyne enterolobii is a devastating and highly impactful species of root-knot nematode (RKN). Its ability to infect a broad range of host plants and overcome RKN resistance genes has threatened crop production. Despite its characterization in 1983, there are knowledge gaps about biology, plant resistant genotypes, and pathogen interactions of *M. enterolobii*. *Meloidogyne enterolobii* is considered highly aggressive, causing more severe symptomology than other RKN species, although factors contributing to the aggression are relatively unknown. The proportion of eggs that successfully hatch into second-stage juveniles were evaluated among three RKN species, *M. enterolobii*, *M. hapla*, and *M. incognita* race 4 within *in vitro* laboratory experiments. It was found that *M. enterolobii* had a significantly greater proportion of hatched eggs compared to the other two species, while *M. incognita* had the lowest proportion. Based on these data, it is hypothesized that *M. enterolobii* may possess a biological advantage of significantly increased egg hatching when compared to *M. incognita* and *M. hapla*, potentially leading to the reported aggressiveness and severity of symptoms observed of *M. enterolobii*.

Nematode-resistant plant genotypes are an effective, environmentally sustainable, and cost-efficient tool for managing disease, yet *M. enterolobii* is capable of infecting currently deployed sweetpotato and soybean genotypes. Therefore, new sources of resistance are needed. Potential resistance to *M. enterolobii* was evaluated in 51 diverse sweetpotato genotypes, 72 *Glycine soja* and 44 *Glycine max* soybean genotypes in greenhouse bioassay experiments. Twelve sweetpotato genotypes and five *G. soja* soybean genotypes were identified as resistant, while none of the *G. max* genotypes were resistant. These results add to the knowledge of host

status and potential natural resistance to *M. enterolobii* in sweetpotato and soybean to improve management and aid plant breeding efforts.

Weeds that compete with crops can host RKN, acting as a source of nematode inoculum in a field and further damaging crops. The host status of ten weed species commonly found in North Carolina was determined to the RKN species *M. enterolobii* and *M. incognita* race 4 in a greenhouse bioassay. Differential host status of the weeds to these RKN species were documented. These results highlight the necessity of managing RKN through controlling weeds in order to protect crops.

Virulent isolates of RKN have been reported to overcome plant resistance through either naturally occurring populations or selection due to continuous exposure to a resistance gene. Yet it is unknown if this selection for virulence may occur among *M. enterolobii* populations. Phenotypically distinct isolates of *M. enterolobii* that reproduce on sweetpotato cultivars previously regarded as resistant were characterized at three time points across diverse sweetpotato germplasm. Isolates of *M. enterolobii* that were previously passed through certain resistant sweetpotato genotypes developed into 'break isolates', causing infection and root galling. This study aids to better understand host-nematode interactions and begin assessing risk of resistance breaking strains in the field.

Finally, a study was performed to determine if *M. enterolobii* and *Ceratocystis fimbriata*, the causal agent of sweetpotato black rot, may act in a synergistic way and contribute to a disease complex in sweetpotato. Results suggest that co-inoculation of *M. enterolobii* and *C. fimbriata* lead to increased root decay. However, nematode reproduction was adversely affected with root decay. Discolored, necrotic root tissue isolated from root galls caused by *M. enterolobii* were found to be infected with *C. fimbriata*. This is the first known study into the interaction between

M. enterolobii and *C. fimbriata*, and the interaction when both pathogens are present appears to be favorable to *C. fimbriata*, unfavorable for *M. enterolobii* life cycle completion, and can lead to reduced vegetative growth in sweetpotato.

Investigations of the Root-Knot Nematode *Meloidogyne enterolobii* in North Carolina: Hosts and Resistance, Management, and Pathogen Interactions

by
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DEDICATION

I would like to dedicate this dissertation to my family who have inspired me to pursue my aspirations. Thank you for your support, love, and belief throughout my life.

BIOGRAPHY

Tanner Schwarz was born on December 21, 1993 in Overland Park, Kansas. He completed his Bachelor of Science in Biology from the University of North Carolina Wilmington in 2016. Tanner received his Master of Science in Plant Pathology at North Carolina State University under the guidance of Dr. Eric Davis in 2019. He continued his graduate career at North Carolina State University to pursue a degree of Doctor of Philosophy in Plant Pathology under the guidance of Dr. Adrienne Gorny.

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CHAPTER 1

Literature Review

Root-Knot Nematodes

Root-knot nematodes (RKN; *Meloidogyne* spp.) are one of the most widespread pathogens reducing agricultural productivity (Sasser and Carter, 1985). Considered the most economically important of all plant-parasitic nematodes, RKN are soilborne pathogens that can infect nearly all plant species that the world relies upon for food, fiber, and fuel supply (Sasser and Carter, 1985; Munyaneza and Bizimungu, 2022). Root-knot nematodes attack plant roots in high numbers, and as a result of feeding inside roots, galls can form throughout the root system (Figure 1.1) (Mitkowski and Abawi, 2003). Although most of the diagnostic symptoms caused by RKN occur below ground, symptoms of RKN infection can also be observed above ground including wilting, chlorosis, stunting, and premature senescence (Mitkowski and Abawi, 2003).

Root-knot nematodes are obligate, sedentary endoparasites that begin their lifecycle as eggs in soil (Figure 1.2) (Sasser and Carter, 1985; Mitkowski and Abawi, 2003). In the egg (Figure 1.3), they undergo embryogenesis and one molt within the egg and hatch as vermiform second-stage juveniles (J2s), which is the only stage in the RKN lifecycle that can initiate infection (Sasser and Carter, 1985). The motile J2 penetrates plant roots at the zone of elongation, move intercellularly to the vascular cylinder, and secrete effectors through its stylet into 5 to 6 plant cells surrounding the head of the nematode (Mitkowski and Abawi, 2003; Atamian et al., 2012; Jones et al., 2013). In response to these effectors, these plant cells develop into giant cells, which are enlarged, multinucleate cells that serve as a permanent feeding site. The J2s feed and become sedentary as they progress through three additional molts to become adults, in which adult females are swollen and pear-shaped (Figure 1.1) (Williamson and Hussey, 1996). Under certain conditions such as a lack of available nutrition or overcrowding in the roots, juvenile RKN can develop into adult males, which exit roots without feeding (Chitwood, 2003). Formation of adult males that fertilize females is rare in many RKN species, and in most cases juvenile nematodes develop into females that reproduce by parthenogenesis (Chitwood, 2003). Females release eggs in a gelatinous matrix outside of the root, which may contain up to 1,000 eggs per egg mass (Curtis et al., 2009; Jones et al., 2013). Egg hatching is largely dependent on optimal temperature and moisture conditions, although root exudates can influence hatching response (Curtis et al., 2009). The J2s emerging from newly laid eggs may infect the same host plant or a neighboring host root system. The duration of the lifecycle for most RKN species is about 25 days under favorable conditions; thus, several generations can occur during a growing season (Lambert and Bekal, 2002).

There are approximately 100 described species of *Meloidogyne* (Mitkowski and Abawi, 2003). Although in general, RKN have a very broad host range, there exist some unique differences in host range among these species, and these differences in feeding habit have been leveraged for species and race identification purposes. The North Carolina Differential Host Test is a panel of six host plants, upon which the four ‘major’ RKN species (*M. hapla*, *M. incognita*, *M. javanica*, and *M. arenaria*) may be identified based on ability to infect and feed. Further, through the North Carolina Differential Host Test, unique sub-species physiological races have been identified based on differential infection of the panel host plants (Sasser and Carter, 1985). For example, *M. arenaria* has two races; race 1 infects and reproduces on peanut, while race 2 populations do not reproduce on peanut (Sasser et al., 1983; Dong et al., 2007). *Meloidogyne javanica* race 2 has the ability to reproduce on pepper, while race 3 infects peanut and not pepper (Robertson et al., 2009). It is important to identify which RKN species, and race, are present in a field to achieve successful management efforts. Crop selection can be crucial to avoid planting a susceptible host if nematodes are established in the field. If nematode infestations are not properly managed, a significant decline in quantity and quality of crop yield may result.

Root-Knot Nematode Management

There are numerous tactics to manage RKN, yet it can be difficult to eliminate or reduce nematode populations to non-threatening economic damage thresholds. Management of RKN is often most effective when implementing more than one tactic in an integrated management plan. Integrated pest management is the simultaneous use of multiple pathogen management strategies to suppress disease severity and reduce causal agent population densities to below economic damage threshold levels (Ciancio and Mukerji, 2007).

One management strategy is to avoid pathogen introduction into the field. Root-knot nematodes are limited in movement, moving no more than a meter per year in soil (Lambert and Bekal, 2002; Agrios, 2005). Most instances of nematode introduction to a field occur through anthropogenic activity (Agrios, 2005). Cultivation and practices that move infested plants and soil will transport nematodes further distances than the nematodes may move themselves (Mitkowski and Abawi, 2003). Tactics to help limit nematode introductions include sanitizing farm equipment and planting certified pathogen-free material. If proper precautions are not carefully performed, nematodes can be introduced into a field and reproduce to cause substantial economic crop loss (Lambert and Bekal, 2002).

In fields with established nematode infestations, management strategies are often designed to reduce inoculum. The use of nematicides is one tactic to help reduce populations to below their threshold of economic damage (Chitwood, 2003). Nematicides are chemical substances that are toxic to nematodes (Tsao and Yu, 2011). These chemicals can be categorized as either fumigants or non-fumigants in agricultural crop ecosystems. Fumigant nematicides are injected into soil before planting and move through the soil as a volatile gas to reach nematode populations (Chitwood, 2003). Non-fumigant nematicides are liquid or granular formulations that move through the soil with water (Chitwood, 2003). Nematicides can be effective in reducing large nematode populations, however, complete eradication is unlikely. Importantly, they can be harmful to the environment and pose health issues if not applied properly (Chitwood, 2003: Agrios, 2005). Many integrated pest management plans include the use of a nematicide in combination with other tactics to control nematode populations when the agricultural return on investment for nematicide use is predicted to be greater than the costs of nematicide application (Chitwood, 2003).

Crop rotation is one of the most common and effective cultural practices for pest management (Agrios, 2005). Rotation to resistant crops or non-host plants can help reduce nematode populations while maintaining yield (Agrios, 2005). Meadows et al. (2018) defined rotation as not growing a susceptible host in the same site for more than one year. Successful crop rotation is largely dependent on the knowledge of the nematode species present (Trivedi and Barker, 1986). The nematode species in a field will determine which plants can be implemented as resistant or non-hosts. However, this can be difficult to achieve as RKN have a broad host range and there are a limited number of economical RKN non-host or resistant crop cultivars (Lambert and Bekal, 2002; Wesemael et al., 2011). Despite this, crop rotation with non-hosts or nematode-resistant plant genotypes is an effective and environmentally sustainable management plan.

Other management tactics to help control nematodes include fallowing soil, soil solarization, steaming, and flooding (Stover, 1979; Cadet et al., 2003; Kokalis-Burelle et al., 2016). Field fallow is a technique in which a field is left unplanted and vegetation is removed for a length of time, reducing nematode populations through the absence of host plants needed for obligate parasite survival (Cadet et al., 2003). However, this tactic is not highly desirable as it provides no income to the farmer and increases the potential for soil erosion. Soil solarization is the practice of covering the soil with plastic mulching to raise the soil temperature and kill pathogens (Dwivedi and Dwivedi, 2020), but can be economically expensive and only partially effective against nematodes that are deeper in the soil profile. Steaming suppresses RKN in a similar manner to soil solarization, which use steam machines to increase soil temperatures (Grossman and Liebman, 1995). Flooding of a field requires saturation of soil for an extended period of time (Nelson et al., 2002). Both steaming and flooding require specialized equipment or water and soil conditions, and flooding may result in accumulation of additional diseases (Stover, 1979). Fallowing soil, soil solarization, flooding and steaming are only used in rare circumstances and have many limitations that make these tactics less worthwhile to manage nematode populations.

Natural enemies of nematodes can also be utilized for management. Biocontrols offer the potential to non-chemically reduce nematode populations which may offer long term management (Agrios, 2005; Nordbring-Hertz et al., 2011; Wilson and Jackson, 2013). The most common biological agents used to reduce plant pathogens are fungi and bacteria (Agrios, 2005). For example, nematophagous fungi such as *Arthrobotrys* species have evolved sticky mycelial structures to capture nematodes (Nordbring-Hertz et al., 2011), or *Purpureocillium lilacinus* which parasitizes nematode eggs (Wilson and Jackson, 2013). Bacteria also have been reported to control nematodes. For example, Poncho VOTiVO (Bayer CropScience) is a seed treatment using *Bacillus firmus* in which the bacteria colonize plant roots, creating a barrier of protection and increasing yield (Wilson and Jackson, 2013). However, biocontrols often require specific environmental conditions and may be difficult to establish in the field to survive for extended periods of time to maintain efficacy levels (Lambert and Bekal, 2002).

Nematode-resistant plant genotypes can be an effective tool to manage RKN. In a typical disease interaction on susceptible host plants, nematodes parasitize and establish feeding sites inside roots, and reproduce to sustain or increase the population. However, in plant genotypes with single-gene resistance, defense responses are triggered upon nematode root invasion and feeding which leads to cell necrosis (hypersensitive response) at feeding sites (Castagnone-Sereno, 2002). As a result, nematode development and reproduction is disrupted, leading to a decline in populations (Castagnone-Sereno, 2002; Agrios, 2005). For example, the tomato resistance gene *Mi-1* is effective in managing the RKN species *M. incognita*, *M. arenaria*, and *M. javanica* (Gururani et al., 2012), and has been documented to reduce nematode populations by 85% when compared to susceptible tomato varieties (Padilla-Hurtado et al., 2022). Planting varieties with genetic resistance is environmentally sustainable and cost effective for growers (Lambert and Bekal, 2002). This management tactic is often widely accepted and may allow exportation of plant products to countries that restrict chemical residues (Khanal et al., 2018). In addition, use of genetic resistance may be implemented in diverse crop production systems with otherwise limited management options including organic growers, subsistence farming, and farmers in developing countries (Roberts, 1992; Nuijten et al., 2018; Collett et al., 2021). However, the challenge to this management tactic is that resistance is frequently species specific to a target pathogen, and resistance has not yet been identified for numerous important cultivated plant species (Lambert and Bekal, 2002). Therefore, identification of new resistant plant genotypes is important for agricultural pathogen management.

Root-Knot Nematodes Parasitizing Resistant Plants

Although nematode-resistant plant genotypes are an effective management tactic, previous reports have provided evidence of RKN populations overcoming plant resistance, inducing infection, and developing inside the roots. These virulent populations can arise naturally or through selection by repeated cropping of the same source of genetic resistance. Tzortzakakis et al. (2005) reported populations of *M. incognita* and *M. javanica* infecting *Mi*-resistant tomato plants in Greece, with the nematode populations having occurred naturally without previous exposure to tomatoes with the *Mi-1* resistant gene. Hajihassani et al. (2022) found four naturally occurring virulent populations of *M. incognita* in a tomato field in Georgia, United States that had no history of *Mi-1* tomato cultivation. Virulence in nematode populations can also be selected by planting genotypes with the same resistance gene over consecutive seasons (Hajihassani et al., 2022). Kaloshian et al. (1996) observed populations of *M. incognita* were able to develop disease after continuous exposure to resistant tomato plants carrying the *Mi-1* gene. In a different study, Castagnone-Sereno et al. (1994) also determined that selection for virulent *M. incognita* isolates from nonvirulent populations occurred due to repetitive inoculation on resistant tomatoes. These results suggest that RKN-resistant plants must carefully be deployed in the field to reduce the risk of selection for virulence in the existing field RKN population. Since nematode-resistant plant genotypes are an important tool in integrated pest management strategies, it is vital that resistance remains durable for effective long-term management.

Root-Knot Nematode and Fungal Disease Complexes

Management of pathogens is often focused on plant responses to singular pathogen infections. However, plant infections can often involve multiple pathogen species, and interactions among pathogens may occur that further increase disease (Abdullah et al., 2017). Disease complexes can form among RKN and fungal pathogens, where disease severity and crop loss is often greater than what may result from infection by each individual pathogen. Nematodes and fungal pathogens have been documented to interact with one another for over a century (Atkinson, 1892). Synergistic relations have repeatedly been reported to negatively affect numerous crops (Zhang et al., 2020). A study found that *Rhizoctonia solani* infected okra and tomato plants only after *M. incognita* invaded the roots (Golden and Van Gundy, 1975). Root decay caused by *Rhizoctonia solani* was observed 4-5 weeks after infection, and fungal sclerotia were only formed on nematode gall tissue. The researchers suggested that the fungus was attracted to nematode galls, and the fungus only penetrated through wounds created by nematodes (Golden and Van Gundy, 1975). Mai and Abawi (1987) determined that certain species of *Fusarium* formed disease complexes with *M. incognita* and *M. javanica*, resulting in increased disease severity in several crops such as tomato and cotton when compared to single pathogen infection. Al-Hazmi and Al-Nadary (2015) concluded that wounding from *M. incognita* infection was an opportunistic opening for *Rhizoctonia solani* on green beans, increasing root damage and above ground symptoms. Other studies have documented similar interactions of nematodes and fungi leading to increased yield decline, such as *Phytophthora capsici* and *M. incognita* (Parkunan et al., 2016), *Verticillium dahlia* with *M. incognita* on cotton (Katsantonis et al., 2003), and *Ceratocystis fimbriata* with *M. incognita* in pomegranate (Kerakalamatti et al., 2019). Root-knot nematodes and fungi can be devastating to crop production individually, and further crop loss can occur with dual pathogen infections. It is important to identify and improve our understanding of these interactions for efficient integrated pest management.

Meloidogyne enterolobii

Meloidogyne enterolobii (Yang and Eisenback, 1983) is a very aggressive RKN species that is considered one of the most damaging RKN species in the world (Castagnone-Sereno, 2012). It was first described in China in 1983 on roots of pacara earpod trees (*Enterolobium contortisiliquum*) (Yang and Eisenback, 1983). In 1988, an RKN species called *M. mayaguensis* was characterized from eggplant (*Solanum melongena*) roots in Puerto Rico (Rammah and Hirschmann, 1988). In 2004, a study determined that *M. mayaguensis* is synonymous with *M. enterolobii*, and the name *M. enterolobii* was retained (Xu et al., 2004). Since its original detection, this species has been reported in numerous countries in Asia, Africa, North America, and South America (Brito et al., 2004; Iwahori et al., 2009; Kolombia et al., 2016; Silva et al., 2021). In the United States, *M. enterolobii* has been detected in Florida (Brito et al., 2004), North Carolina (Ye et al., 2013), South Carolina (Rutter et al., 2019), and Georgia (Hajihassani et al., 2023).

Meloidogyne enterolobii is considered a tropical to subtropical nematode species (Subbotin, 2019). Similar to other RKN, it has a broad host range and can infect many important cultivated crops, ornamentals, and weeds (Castagnone-Sereno, 2012; Subbotin, 2019), including sweetpotato, soybean, tobacco, tomato, guava, pepper, and coffee (Brito et al., 2007; Gomes et al., 2008; Onkendi and Moleleki, 2013; Anonymous, 2014). Due to its ability to cause substantial damage to guava, this nematode species is often referred as the guava RKN. There are only a few crop and fruit species identified as non-hosts or poor hosts, such as corn, garlic, peanut, avocado, grapefruit, cashew, mango, and strawberry (Rodriguez et al., 2003; Freitas et al., 2017; Philbrick et al., 2020). Although it has relatively limited distribution, *M. enterolobii* is considered one of the most important RKN due to its high virulence and ability to overcome resistance effective in managing other RKN species (Castagnone-Sereno, 2012). To illustrate, isolates of *M. enterolobii* have been reported to overcome the *Mi-1* gene in tomato (Kiewnick et al., 2009), the *N* gene in bell pepper (Kiewnick et al., 2009), *Mh* gene of potato (Castagnone-Sereno, 2012), and the *Mir1* gene in soybean (Cetintas et al., 2008).

Meloidogyne enterolobii is considered highly aggressive; anecdotally, it induces more severe root galling and has a higher infestation rate than other RKN species (Catagnone-Sereno, 2012). Although research is limited, it is suggested that *M. enterolobii* causes greater yield loss than other RKN. For example, a study by Cetintas et al. (2007) compared yield loss of tomato caused by several species of RKN; when compared to a non-inoculated control, *M. arenaria* caused a 42% crop loss, *M. incognita* and *M. javanica* each caused 46% loss, and *M. enterolobii* caused a 65% crop loss (Cetintas et al., 2007). Considering these results along with the ability of *M. enterolobii* to overcome RKN resistant plants, it is evident there is a need for research into effective management tactics.

Although there are tactics identified for managing *M. enterolobii*, due to its relatively recent emergence there is currently a limited number of options. A study by Alam et al. (2022) evaluated the suppression of *M. enterolobii* juveniles when treated with non-fumigant nematicides on tobacco in a growth chamber. It was determined oxamyl had 99% suppression, fluensulfone had 98%, and fluopyram had 94% less juveniles when compared to an untreated control (Alam et al., 2022). It was determined in a different *in vitro* study that two egg-parasitic fungi, *Pochonia chlamydosporia* and *Purpureocillium lilacinum*, reduced the number of juvenile *M. enterolobii* nematodes that hatched by 13 to 84% compared to controls in tissue culture plates (Silva et al., 2017). In addition, studies have identified plant genotypes resistant to *M. enterolobii*, including resistance in guava (*Psidium guajava*) (Chiamolera et al., 2018), pepper (*Capsicum annuum*) (Carrillo-Fasio et al., 2020), plum (*Prunus cerasifera*) (Claverie et al., 2011), cowpea (*Vigna unguiculata*) (Dareus et al., 2021), tomato (*Solanum sisymbriifolium*) (Hajihassani et al., 2020), and sweetpotato (*Ipomoea batatas*) (Rutter et al., 2021; Schwarz et al., 2021). However, most of these resistant genotypes are not yet suitable for commercial production and market consumption, as they lack other horticulturally desirable traits. More research is needed to identify management options effective against *M. enterolobii* for short and long-term success.

Sweetpotato: Global and Local Importance and *Meloidogyne enterolobii* Infection

Sweetpotato (*Ipomoea batatas* (L.) Lam.) is an important global crop. It is estimated that sweetpotato produces more nutrients per hectare than any other food crop (Ray and Ravi, 2005), which are rich in vitamins A and C, iron, potassium, and carbohydrates (El Sheikha and Ray, 2017). Sweetpotato is capable of producing yield without added fertilizer or irrigation (Loebenstein, 2009), making it an ideal crop for economically developing countries; globally, it is estimated that 23% of sweetpotatoes are grown in developing countries (FAOSTAT, 2021). The United States ranks within the top 10 sweetpotato producing countries; in 2022, an estimated 132,000 U.S. acres of sweetpotato valued at nearly $598.5 million was harvested (USDA-NASS, 2022a). As a nutrient rich and high economic value crop, sweetpotato production in the United States is important for both domestic and export markets. Production and exportation have increased over the past few years driven by nutrient benefits and value-added products, such as sweetpotato fries and chips, have led to increased consumption (El Sheikha and Ray, 2017). However, *M. enterolobii* represents a major threat to sweetpotato production. It is currently present in China, the world's largest producer, and also in other important sweetpotato producing countries such as Vietnam, Nigeria, and Brazil (Iwahori et al., 2009; Kolombia et al., 2016; Silva et al., 2021).

In the United States, *M. enterolobii* is present in several Southeastern states, including North Carolina, the nation's largest sweetpotato producing state, which represents about 64% of the nations harvested acres (USDA-NASS, 2022b). Importantly, the most commonly planted sweetpotato cultivars, 'Covington' and 'Beauregard', are susceptible to *M. enterolobii* infection (Brito et al., 2020; Rutter et al., 2021; Schwarz et al., 2021). Sweetpotato storage roots can be directly infected, causing severe galls and cracks that reduce the quality and quantity of yield (Lawrence et al., 1986; Overstreet, 2013; Schwarz et al., 2021). Due to the severity of symptoms and lack of strong management options, the North Carolina Department of Agriculture & Consumer Services declared an internal quarantine for all counties in the state to help reduce potential spread of this RKN species (NCDA&CS, 2018).

Previous research has been conducted to determine the host status of diverse sweetpotato genotypes to *M. enterolobii*. Rutter et al. (2021) found 19 out of 93 sweetpotato accessions screened were resistant, and Schwarz et al. (2021) determined 19 out of 91 genotypes tested were resistant. Although resistance was identified, these sweetpotato genotypes lack many horticulturally desired traits that prevent them from being an immediate management solution. Additional research is needed to evaluate the host status of sweetpotato genotypes and provide new sources of resistance for management and breeding purposes.

Soybean: Global and Local Importance and *Meloidogyne enterolobii* Infection

Soybean (*Glycine max* (L.) Merr.) is an economically valuable crop and important global source of food, high-quality protein, and oil (Bandara et al., 2020; Voora et al., 2020). Soybean has been linked to various health benefits, such as reducing coronary heart disease, breast and prostate cancer, and aiding to alleviate depression symptoms (Messina, 2016). Soybean is also an important source of animal feed, with 85% of world soybean production used for animal feed (Voora et al., 2020). It is estimated that soybeans are cultivated on 6% of global arable land (Bandara et al., 2020). Brazil is the largest soybean producer, producing nearly 5 million bushels of soybeans in 2020/21 (Colussi and Schnitkey, 2021). The United States is second in soybean production, and together with Brazil the countries account for over 80% of global exports (Gale et al., 2019). *Meloidogyne enterolobii* is present in both countries, and of concern, all commercially available varieties of soybean are susceptible to infection by this nematode species (Ye et al., 2013). In North Carolina, soybean is the most widely grown crop by acreage. In 2020, North Carolina soybean producers planted an estimated 1.6 million acres of soybean for a value of $673.8 million (USDA-NASS, 2021). *Meloidogyne enterolobii* was first reported in North Carolina in 2013 infecting roots of soybean plants, and reports of soybean infections are increasing (Ye et al., 2013).

Previous research of soybean’s host status to *M. enterolobii* is relatively limited. Gorny et al. (2021) reported that no commercially available soybean varieties tested had resistance to the nematode species. Cetintas et al. (2008) screened *G. max* varieties to four isolates of *M. enterolobii*. In the study, some of the soybean genotypes displayed resistance to one or two of the nematode isolates, however, they concluded that none of the soybeans were resistant to all four nematode isolates (Cetintas et al., 2008). The soybean gene *Mir1*, which confers resistance to *M.*

incognita, *M. arenaria*, and *M. javanica*, is ineffective against *M. enterolobii* (Cetintas et al., 2008; Castagnone-Sereno, 2012). Therefore, new management tactics are vitally needed, including the identification of resistant soybean genotypes to help mitigate economic loss caused by *M. enterolobii*.

Weeds: Impacts and *Meloidogyne enterolobii* Infection

Weeds are undesirable plants that compete with crops for valuable resources such as nutrients, water, space, and sunlight (Chauhan, 2020). Weeds are almost universally present during the growing season and after harvest in fallow periods (Rich et al., 2008). Weeds are a global issue that requires prodigious labor investments to mitigate their negative effects on crop production (Madden et al., 2021). It is estimated that weeds cause greater crop loss than either pathogens or insects (Fried et al., 2017), and if left uncontrolled a 100% crop yield loss can occur (Chauhan, 2020). Weeds often increase the need for crop production inputs, mainly through soil cultivation and the use of herbicides. Weeds competing for water requires additional irrigation inputs to maintain crops (Norris, 1996; Singh et al., 2022). In addition to abiotic constraints, weeds may attract predatory insects by providing food and habitat, in which the insects can subsequently damage crops and may vector dangerous pathogens (Madden et al., 2021). Weeds can also harbor or act as alternate hosts for pathogens, allowing reproduction and an increase in causal agent population numbers, making disease management more difficult (Dentika et al., 2021; Lopez et al., 2021).

Among the numerous pathogens that can harbor on weeds are plant-parasitic nematodes. There has been extensive research on the host status of weed species to RKN (Rich et al., 2008; Kokalis-Burelle and Rosskopf, 2012; Lopez et al., 2021). Root-knot nematodes can utilize weeds as hosts to survive and reproduce, increasing the inoculum levels in the field for subsequent crops and causing further damage. In addition, an increase in grower costs results from unmanaged weeds that subsequently increase nematode populations (Rich et al., 2008). Thomas et al. (2004) documented that weeds can provide protection for nematodes from nematicides and adverse environmental conditions, thus the presence of weeds may offset nematode control tactics. Different weed species can serve as hosts for different species of RKN, and reports of weed host status for *M. enterolobii* are relatively limited. Kaur et al. (2007) reported multiple weed species that are susceptible to *M. enterolobii*, such as *Amaranthus retroflexus*, *A. spinosus*, *Cnidoscolus stimulosus*, *Cucumis anguria*, and *Senna obtusifolia*. This study also determined nine weeds that were non-host to *M. enterolobii,* which included *Cassia occidentalis, Crotalaria spectabilis*, *Dactyloctenium aegyptium*, *Sorghum halepense,* and *Digitaria sanguinalis* (Kaur et al., 2007). Kaspary et al. (2022) evaluated six morningglory weed species inoculated with *M. enterolobii* and concluded that all six were susceptible. Growth et al. (2016) concluded that *Solanum pseudocapsicum* was susceptible to *M. enterolobii*. Additional research is needed to determine the RKN host status of weeds common in North Carolina, especially to *M. enterolobii*. A greater understanding of weed-nematode interactions will highlight the necessity of managing RKN through controlling weeds in order to protect valuable crops.

Concluding Remarks

Since its first characterization, *M. enterolobii* has repeatedly demonstrated how damaging it can be to agricultural productivity. Yet, for all of the economic losses that have resulted from this RKN, our knowledge of the species is not fully understood. To address these knowledge gaps, further studies of *M. enterolobii* should be explored. For example, it is considered that *M.*

enterolobii is a highly aggressive RKN species, able to cause more severe galling symptoms than other RKN, but factors contributing to the reported aggressiveness are relatively unknown. When RKN hatch from eggs, it is known that not every egg will hatch into an infective J2. The success rate at which nematodes hatch from eggs may be an important factor in causing disease. If a greater proportion of the eggs that are laid hatch, there may be an increase in potential success of J2s infecting plant roots. Comparing the egg hatching rates of *M. enterolobii* to other RKN species may provide insight into a biological attribute leading to greater disease symptoms.

Additionally, integrated pest management plans may often include implementing resistant plant genotypes, however, there are few commercially available resistant cultivars to control *M. enterolobii*. Sweetpotato and soybean are two important crops worldwide that currently lack sufficient resistance options. Research on resistant genotypes will help alleviate *M. enterolobii* disease pressure and improve management and plant breeding efforts. We can also expand our knowledge to improve management of *M. enterolobii* by understanding weed-RKN interactions. Weeds pose a serious threat to crop production individually, but if weeds are harboring nematodes, especially *M. enterolobii*, the combination of threats could be detrimental to crops. More research should be conducted to understand the host status of common weeds in North Carolina to *M. enterolobii*.

Furthermore, previous research has provided evidence of populations of RKN species that can overcome plant resistance genes. To illustrate, populations of *M. incognita* have developed disease after continuous exposure to tomato plants carrying the *Mi1* resistance gene (Kaloshian et al., 1996). This phenomenon has not been identified among populations of *M. enterolobii* to date. Due to the limited number of sweetpotato genotypes with resistance, it is important to identify if *M. enterolobii* can overcome resistance to help preserve these tools.

Lastly, disease complexes have been documented to form among pathogens, including RKN, that increase disease severity compared to single pathogen infection. There is currently limited knowledge of disease interactions involving *M. enterolobii*. *Ceratocystis fimbriata*, a fungus which causes black rot on sweetpotato, tends to form around predisposition wounds (Stahr and Quesada-Ocampo, 2020). If a pathogen interaction occurs between *C. fimbriata* and *M. enterolobii*, marketable sweetpotato yield may be compromised. Further research into the biological attributes, management, and interactions of *M. enterolobii* is required to reduce the threat of this pathogen to agricultural productivity.

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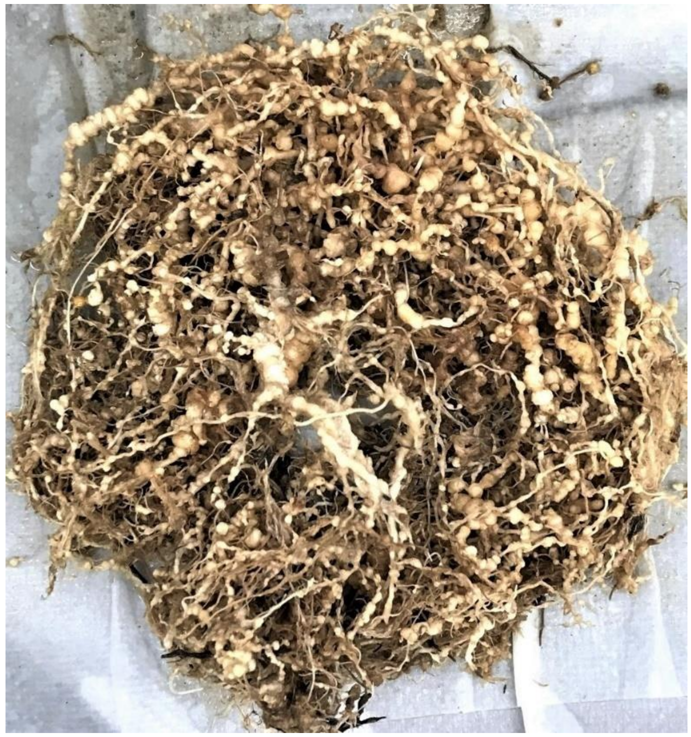

Figure 1.1: Root galling on ‘Rutgers’ tomato plant caused by *Meloidogyne enterolobii* infection.

(Photo, T. Schwarz)

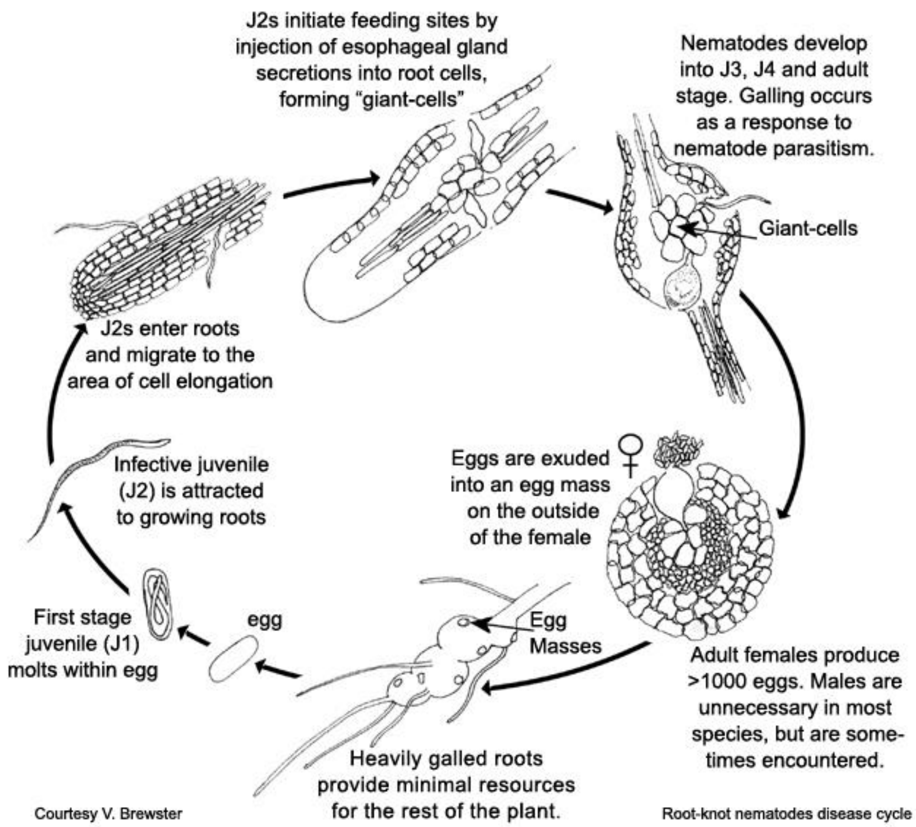

Figure 1.2: Life cycle of root-knot nematodes, *Meloidogyne* species. (Reproduced from Mitkowski and Abawi, 2003; V. Brewster).

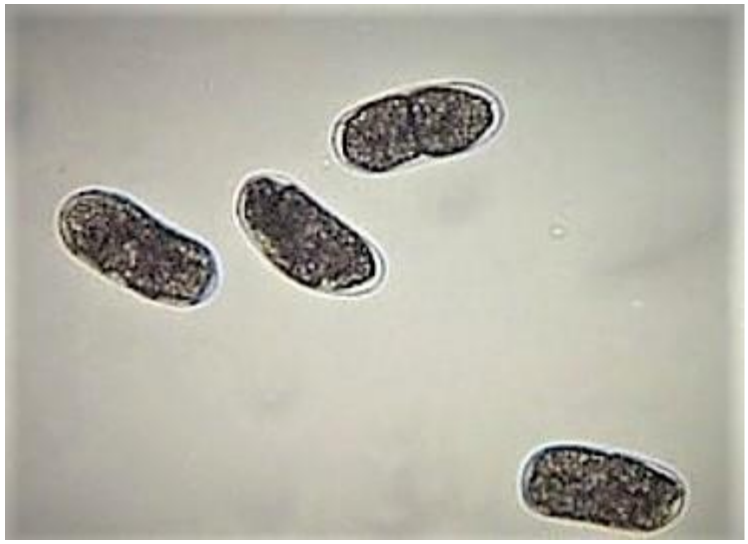

Figure 1.3: Eggs of the root-knot nematode *Meloidogyne enterolobii.* (Photo, T. Schwarz)

CHAPTER 2

Quantification and comparison of egg hatching rates among root-knot nematodes *Meloidogyne enterolobii*, *M. hapla,* and *M. incognita* race 4

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ABSTRACT

The proportion of eggs that successfully hatch into second-stage juveniles were evaluated among three root-knot nematode species, *Meloidogyne enterolobii*, *M. hapla*, and *M. incognita* race 4 within in vitro laboratory experiments. Eggs of each species suspended in a water solution were pipetted separately onto hatching screens and incubated at 27°C. Five days post inoculations, the number of hatched juveniles were counted. In addition, a study was conducted with a soil substrate in which eggs of each species were pipetted separately on to hatching screens augmented with 1 cc of sterilized soil, incubated at 27°C, then assessed for the number of juveniles hatched at 3, 5, and 10 days post initiation. It was found that *M. enterolobii* had a significantly greater proportion of hatched eggs, while *M. incognita* had the lowest proportion of eggs hatch in both water and soil substrates among all trials. In the water substrate trials, *M. enterolobii* had an average hatch rate of 29.55%, *M. hapla* with 26.24%, and *M. incognita* with 14.62%. In soil substrate trials, the percent of eggs hatched increased at each time point, with *M. enterolobii* hatching rate at 10 days post initiation ranging from 25.67 to 27.33%, *M. hapla* ranging from 13.40 to 14.80%, and *M. incognita* ranging from 3.60 to 4.33% across three trials. Based on these data, it is hypothesized that *M. enterolobii* may possess a biological advantage of significantly increased egg hatching when compared to *M. incognita* and *M. hapla*, potentially leading to the reported aggressiveness and severity of symptoms observed of *M. enterolobii*.

INTRODUCTION

Root-knot nematodes (RKN; *Meloidogyne* spp.) begin their life cycle in the egg stage and develop into first-stage juveniles (Carter and Sasser, 1985). The nematodes molt within the egg and hatch as motile second-stage juveniles (J2) in the soil (Carter and Sasser, 1985; Mitkowski and Abawi, 2011). Second-stage juveniles are the only life stage in which RKN can enter plant roots to cause infection (Sasser and Carter, 1985). Once a feeding site is established within the root, the juvenile will begin to swell and become sedentary and usually form a characteristic root gall. The nematode will undergo rapid molts through the third-stage and forth-stage juveniles, then a final molt to reach the mature adult stage. Once maturity is reached, a single swollen RKN female can typically lay between 200 to over 1,000 eggs, yet not every egg will remain viable or hatch an infectious J2 (Curtis et al., 2009). The success rate at which nematodes hatch from eggs may be important in nematode disease dynamics; if a greater proportion of nematodes hatch, there may be an increase in disease pressure, potentially resulting in increased J2 population levels within host plant roots.

Meloidogyne enterolobii is considered a RKN species of limited geographic distribution, yet the species can infect many economically important crops throughout the world (Castagnone-Sereno, 2012; Wesemael et al., 2011). It is considered a highly aggressive and pathogenic RKN species; anecdotally, it is regarded to induce more severe root galling and have a higher infestation rate than other RKN species (Castagnone-Sereno, 2012). However, comparatively little is known about the factors that contribute to making this species highly aggressive.

Comparisons of individual RKN species may provide insight to determine if a species has a biological advantage in egg hatching. *Meloidogyne incognita* and *M. hapla* are two of the most common species of RKN throughout the world (Ng'ambi et al., 1999). *Meloidogyne hapla*, the northern root-knot nematode, has the ability to survive at cold temperatures. Taylor et al. (1982) reported that *M. hapla* eggs can survive in regions that range in seasonal temperatures from 15°C to 27°C. Vrain and Barker (1978) determined that *M. hapla* eggs can hatch at low temperatures of 6.74°C. *Meloidogyne enterolobii*, in contrast, is considered a more tropical to subtropical species that thrives in warmer climates (Castagnone-Sereno, 2012; Philbrick et al., 2020). *Meloidogyne incognita*, the southern root-knot nematode, shares many common host crops and can be found to inhabit similar regions as *M. enterolobii* (Philbrick et al., 2020; Schwarz et al., 2020; Wendimu, 2021). A direct comparison of egg hatching rates between *M. incognita*, *M. enterolobii* and *M. hapla* could provide insights into distinct differences in successful egg hatching. Comparing egg hatching among species that occupy similar hosts and regions may provide insight into biological similarities or differences that could contribute to virulence and disease progression.

Previous reports have determined egg hatching rates in the presence of chemical and biological controls (Mendoza et al., 2008; Popal, 2020), and the ability of nematodes to hatch from eggs in cold temperatures (Daulton and Nusbaum, 1961; Morris et al., 2011; Starr and Jeger, 1985; Wu et al., 2018). However, direct comparison of egg hatching rates among different nematode species with the goal of quantifying differential hatching rates under controlled laboratory conditions, to our knowledge, has yet to be conducted. Therefore, the objective of this study was to evaluate the egg hatching rates of North Carolina isolates of *M. enterolobii, M. hapla,* and *M. incognita* race 4 to assess if differential hatching rates are occurring and hypothesize on the contribution of this biological attribute towards more severe symptomology observed among *M. enterolobii* populations.

MATERIALS AND METHODS

Water egg hatch trials. To perform egg hatching rate experiments, small hatching screens were created (Figure 2.1). The screens were created by cutting and connecting two sizes of plastic test-tube caps, cut to remove the closed end (cap 1: 1.4 cm tall and 2.3 cm diameter; cap

2: 1 cm tall and 2.2 cm diameter), which nested tightly within one another to create a firm seal. A 25 µm pore sized nylon mesh (ELKO Filtering Co., Miami, FL) (cut to approximately 3 cm by 3 cm in size) was stretched between the two test-tube caps. The mesh screen was tautly suspended across the open middle of the caps, and sandwiched between the nested test-tube caps, so that when standing, a gap existed of approximately 1 cm between the mesh and the bottom of the hatching screen assembly. The hatching screens were placed into individual wells of a 6-well tissue culture plate (Fisher Scientific, Waltham, MA) (Figure 2.2). When the experiment was fully assembled, the mesh hatching screen suspended unhatched eggs on top of the mesh, separate from the bottom of the well; once hatching occurred, J2 nematodes were small enough in diameter to migrate through the pores of the nylon mesh and collect at the bottom of the individual tissue culture plate well for quantification.

Two-month-old cultures of North Carolina isolates of each *M. enterolobii*, *M. hapla*, and *M. incognita* race 4, maintained separately on roots of ‘Rutgers’ tomato plants, were used to acquire eggs in each study. The identity of each single-species culture was previously confirmed using PCR and the North Carolina Differential Host Test. Tomato roots from cultures of each nematode species were subjected to egg extractions following the protocol of Hussey and Barker (1973) and collected in a volume of 50 mL with water. Aqueous egg suspensions of each separate nematode species were adjusted in density to approximately 250 nematode eggs mL^{-1}, and were then applied to hatch screens by pipetting 1 mL of water solution onto the top of each mesh screen. Six replicate hatching screen assemblies per nematode species were included. A small amount of water (approximately 1.5 mL) was added to the bottom of each well to maintain moisture; this volume of water never reached or surpassed the suspended mesh screen. The plastic cover of each culture plate was placed on top of the wells. The plates were then placed in an incubator maintained at 27°C with no light. Five days after initiation, the number of hatched nematodes were determined by removing the hatching screen assembly and counting the number of juveniles in the bottom of each culture plate well under an inverted microscope at 40x magnification. The percentage of eggs hatched was calculated by dividing the total number of juveniles in each well by the initial inoculum of 250 eggs. The full experiment was repeated three times.

Soil egg hatch trials. Egg hatching rates were also investigated within a soil substrate. The same protocols were performed as described above in the ‘water’ trials for creating the egg hatching screens and preparation of egg inoculum for each of the three RKN species. Soil (1:1 sand to field soil mixture of a sandy loam texture) was autoclaved and measured into aliquots of 1 cc. One cc of soil was then placed on top of each hatching screen mesh; this amount of soil was enough to completely cover the surface area of the mesh screens and avoid spilling over the top of the test tube cap (Figure 2.3). The 25 µm pore size screen retained the soil on top of the mesh within the cut test tube caps and prevented the soil from falling through to the bottom of the well. Approximately 250 eggs of each nematode species in 1 mL of water were introduced to each hatching screen by pipetting the egg solution to the top of the soil within the hatching apparatus. A small amount of water (approximately 1.5 mL) was added to the bottom of each well and plastic tops were placed on each plate. The plates were then placed into an incubator maintained at a temperature of 27°C with no lighting. Six replicate hatching screen assembly of each species were included. The number of hatched juvenile nematodes in each well were counted at 3, 5, and 10 days post inoculation under an inverted microscope at 40x magnification. The total number of juveniles in each well was divided by the initial inoculum quantity of 250 eggs to determine the percent hatch rate. The experiment was conducted a total of three times.

Data Analysis. Data was analyzed using SAS statistical analysis software version 9.4 (SAS Institute, Cary, NC). The water and soil substrate egg hatch experiments were analyzed separately. To determine if significant differences existed among egg hatch rate of nematode species, the percent of eggs that hatched were analyzed using a general linearized model (PROC GLM). Data from each of the three replicate experiments from the 'water inoculum' study were combined for analysis, as no differences in normality distribution or homogeneity of variances were found. In the 'soil inoculum' study, the three replicate experiments and each time point (3, 5, and 10 days post inoculation) were analyzed separately, as significant differences in normality distribution and homogeneity of variances were found. Means were separated using Fisher's Least Significant Difference (LSD) procedure at $\alpha=0.05$ where significant differences were identified.

RESULTS

Water egg hatch trials. Statistical analysis revealed there were non-significant differences among three replicate experiments ($P = 0.1176$) or among nematode replicates ($P = 0.9883$), therefore all three experiments were combined for analysis. There were significant differences ($P < 0.0001$) between egg hatching rates among nematode species (Figure 2.4). *Meloidogyne enterolobii* had the largest egg hatch rate with an average of 29.55%. *Meloidogyne hapla* was found to have a hatch rate of 26.24%. The species with the lowest hatch rate was *M. incognita* race 4, which had an average of 14.62% (Figure 2.4).

Soil egg hatch trials. Significant differences were found among trials ($P < 0.0001$) therefore each trial was assessed individually. There were significant differences ($P < 0.0001$) in egg hatching rates among each nematode species at each time point. Across all trials, *M.*

enterolobii had the largest percent of eggs hatch, and *M. incognita* consistently had the lowest percent hatch rate. In trial 1, 5.20%, 10.93%, and 25.67% of eggs hatched at 3, 5, and 10 days post inoculation respectively for *M. enterolobii*. *Meloidogyne hapla* had 2.00%, 7.40%, and 13.40% of eggs hatch at the respective time points. *Meloidogyne incognita* had hatch rates at 3, 5, and 10 days post inoculation of 0.80%, 1.07%, and 3.60%, respectively (Figure 2.5).

In trial 2, *M. enterolobii* had average hatch rates of 6.40%, 11.21%, and 27.27% at 3, 5, and 10 days post inoculation, respectively. The hatch rates of *M. hapla* were 3.60%, 7.07%, and 14.33% at 3, 5, and 10 days. *Meloidogyne incognita* had the hatch rates of 1.07%, 1.53%, and 4.33% at 3, 5, and 10 days post inoculation, respectively (Figure 2.6).

In trial 3, *M. enterolobii* had an average hatch rate of 6.00% at 3 days, 11.21% at 5 days, and 27.33% 10 days post inoculation. *Meloidogyne hapla* hatch rates were 2.60%, 7.79%, and 14.80% at 3, 5, and 10 days post inoculation. The lowest percent of eggs hatched was of *M. incognita*, with 0.93%, 1.80%, and 4.13% at 3, 5, and 10 days post inoculation (Figure 2.7).

DISCUSSION

The results indicated that a significant difference existed between egg hatching rates of *M. enterolobii*, *M. hapla*, and *M. incognita* race 4 when considered under the study test conditions. Among these species, *M. enterolobii* had the largest proportion of juveniles hatch from eggs when compared to those of *M. hapla* and *M. incognita*. Among all trials and time points, *M. enterolobii* had a significantly greater amount of eggs hatch in both water and soil substrates. *Meloidogyne incognita* race 4 had the lowest egg hatching rates in comparison to the other RKN species in each trial.

There were numerical differences in the percentage of eggs hatched among water and soil substrates, although it did not affect the order of which nematode species had the highest and lowest hatching rates. When comparing the water substrate trials at five days post initiation to the soil inoculations at five days, *M. enterolobii* had an average of 29.55% in the water and soil substrate averages of 10.93%, 11.20%, and 11.20% in trials 1, 2, and 3 respectively. Furthermore, *M. incognita* exhibited an average hatch rate of 14.62% in water and soil averages of 1.07%, 1.53%, 1.80% in trials 1, 2, 3. Although there was a disparity between substrates, this may have been due to the soil substrate acting as a barrier, slowing nematode movement over the study duration. The hatched juveniles may have remained suspended in the soil; whereas in water the juveniles would pass through the mesh screen and sink to the bottom of the tissue culture plate more easily. The results presented here should therefore not be interpreted that RKN hatch more readily in water than soil.

This study compared egg hatching rates of RKN species at single specific conditions of temperature and moisture. Khan et al. (2014) states that egg hatching is largely dependent on temperature and moisture, and these factors likely influenced egg hatching rates unique to this study; however, the objective of the present study was to compare the egg hatching rates of three nematode species at a constant temperature and moisture regimen. There is limited information about egg hatching rates among nematode species, especially that of the relatively recently emergent *M. enterolobii*. Damascena et al. (2019) performed a study in which egg hatch of *M. enterolobii* was analyzed in the presence of entomopathogenic nematodes, in which a non-treated control group was included that assessed the hatch rate in 25 mL of water, kept at 25°C, and counted every two days for a total of ten days. At four days an average hatch rate of 14.5%, and at six days a hatch rate of 32.9% was observed. When compared to our water substrate trial, *M.*

enterolobii had a hatch rate of 29.55% at five days post inoculation, which is relatively similar to the hatch rate observed by Damascena et al. (2019) at six days, despite both studies being conducted at different temperatures. Atamian et al. (2012) claimed that *Meloidogyne* species have egg hatching rates ranging from 5 to 50%, although no distinction between specific species, time, or temperature was mentioned. In a study of hatching rates after exposure to a biocontrol agent, Mendoza et al. (2008) found that non-treated control groups of *M. incognita* had a hatch rate of greater than 60% six days after incubation in water at 25°C. This is significantly higher than the hatching rate of *M. incognita* observed in the present study, perhaps suggesting this species is more sensitive to temperature differences. Alternatively, there may be differences in hatching rates for some RKN species across different races or isolates. Future studies that evaluate a greater range of temperature profiles and unique nematode isolates may be required to gain a more comprehensive understanding of differences in hatching rates.

Anecdotally, it is the author's experience that *M. enterolobii* causes more severe root galling symptoms on host crops than other species of RKN. Based on the data provided here, it is hypothesized that *M. enterolobii* may possess a biological advantage of significantly increased egg hatching when compared to other endemic species such as *M. incognita* and *M. hapla*, potentially leading to greater disease pressure and contributing to the reported aggressiveness of the species and severity of symptoms observed. In North Carolina, United States, all three of these RKN species are present (Schwarz et al., 2020; Ye et al., 2021). In field situations where all three of these species may be present and competing for feeding sites inside the host root system, it is speculated that *M. enterolobii* populations may have an epidemiological advantage due to its elevated egg hatching rate, if all species are starting from similar population sizes.

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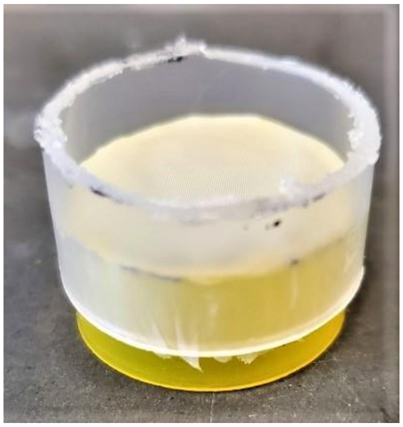

Figure 2.1: Egg hatching screen created by cutting and connecting two sizes of test-tube caps (clear cap: 1.4 cm tall and 2.3 cm diameter; yellow cap: 1 cm tall and 2.2 cm diameter), which nested tightly within one another to create a firm seal. A nylon mesh 25 µm pore sized screen was stretched between the two test-tube caps.

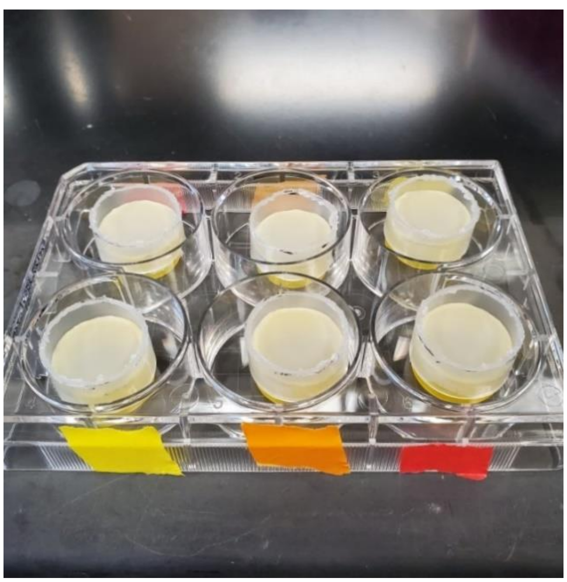

Figure 2.2: Image of six egg hatching screens in individual wells of a tissue culture plate. The culture plates containing hatching screens were then placed in incubators at 27°C.

Figure 2.3: Image of egg hatching screen with 1 cc of soil used for the ‘soil’ trials.

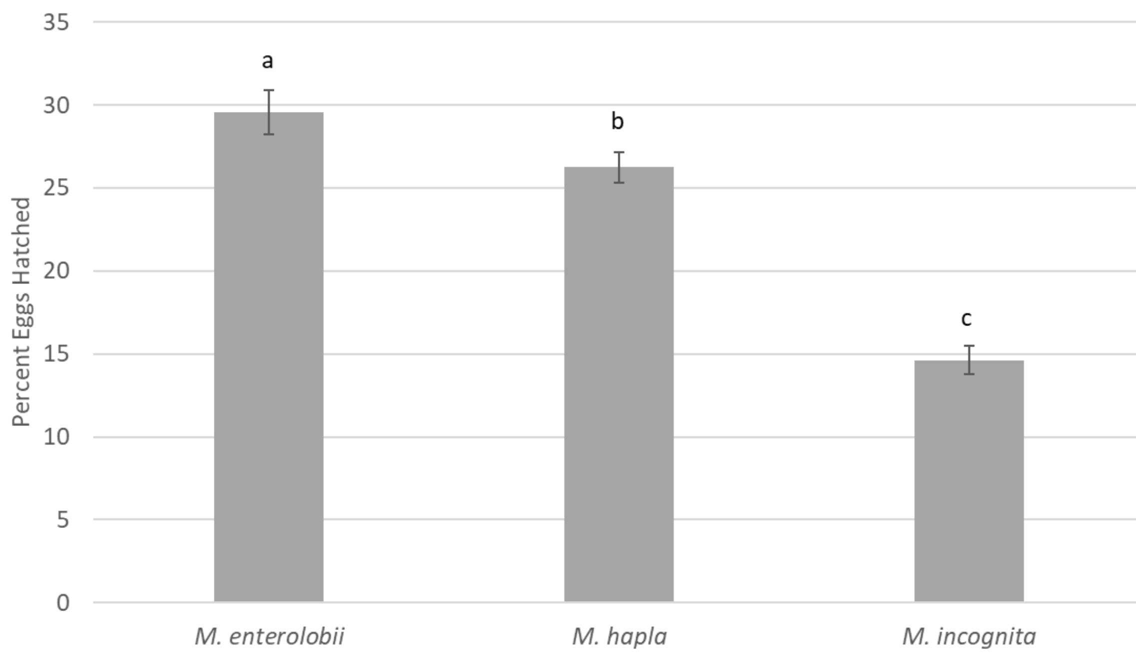

Figure 2.4: Percent of hatched eggs in water for root-knot nematode species *Meloidogyne enterolobii*, *M. hapla,* and *M. incognita* race 4. Each bar is an average of eighteen replications of three trials. Percent of eggs hatched was calculated based on an initial 250 eggs per replication. Egg hatch rates were determined five days post initiation on egg hatching screens containing 1 mL of water. Different letters denote significant differences at the $\alpha = 0.05$ level.

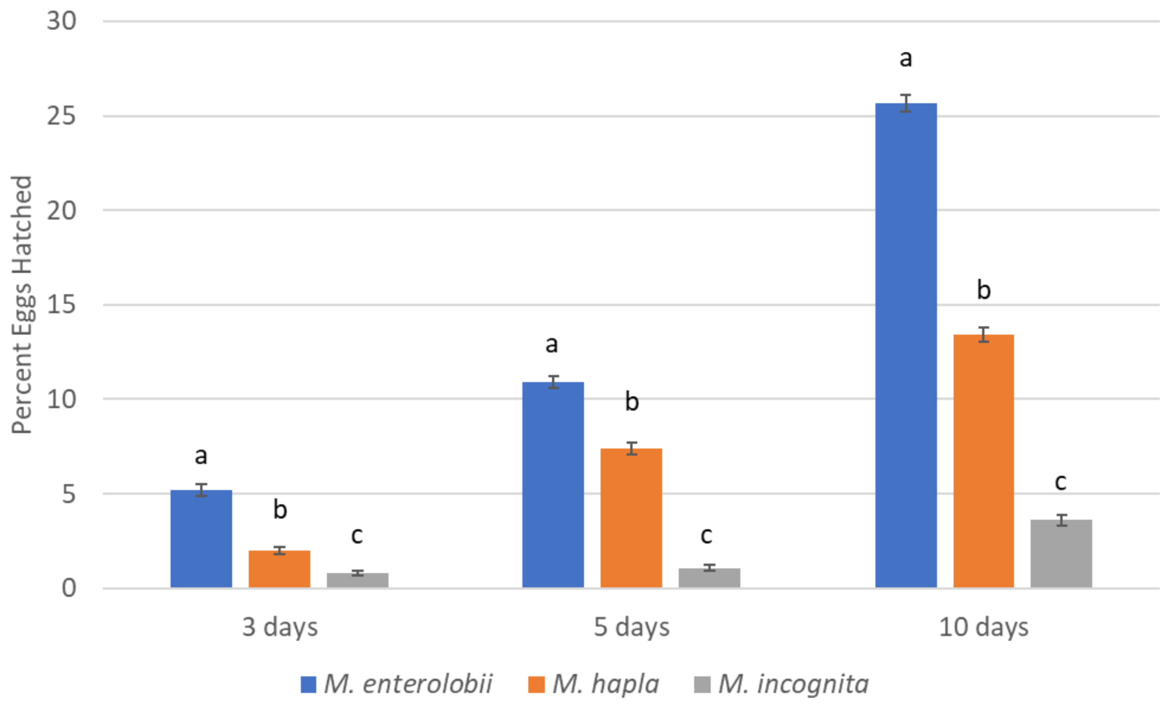

Figure 2.5: Percent of hatched eggs for root-knot nematode species *Meloidogyne enterolobii*, *M. hapla*, and *M. incognita* race 4 in a soil egg hatch study, Trial 1. Each bar is an average of six replications. Percent of eggs hatched was calculated based on an initial 250 eggs per replication. Egg hatch rates were determined 3, 5, and 10 days post initiation on egg hatching screens containing 1 mL of water solution pipetted to 1 cc of sterilized soil. Each time point was analyzed separately, in which different letters are significantly different at the $\alpha = 0.05$ level.

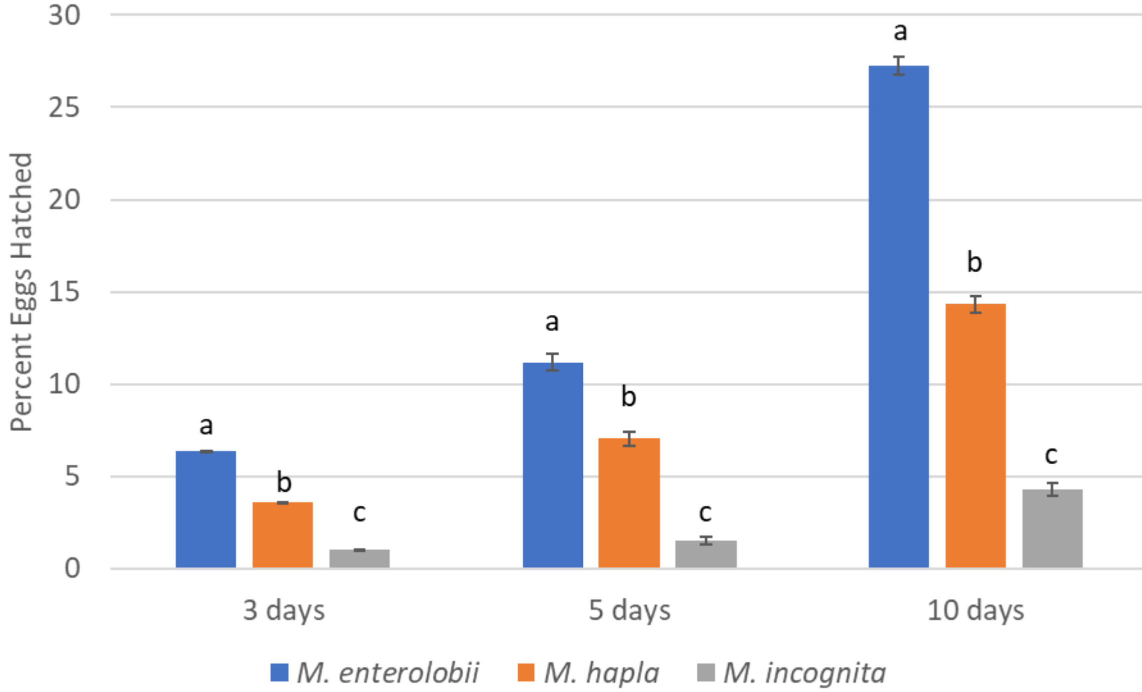

Figure 2.6: Percent of hatched eggs for root-knot nematode species *Meloidogyne enterolobii*, *M. hapla*, and *M. incognita* race 4 in a soil egg hatch study, Trial 2. Each bar is an average of six replications. Percent of eggs hatched was calculated based on an initial 250 eggs per replication. Egg hatch rates were determined 3, 5, and 10 days post initiation on egg hatching screens containing 1 mL of water solution pipetted to 1 cc of sterilized soil. Each time point was analyzed separately, in which different letters are significantly different at the $\alpha = 0.05$ level.

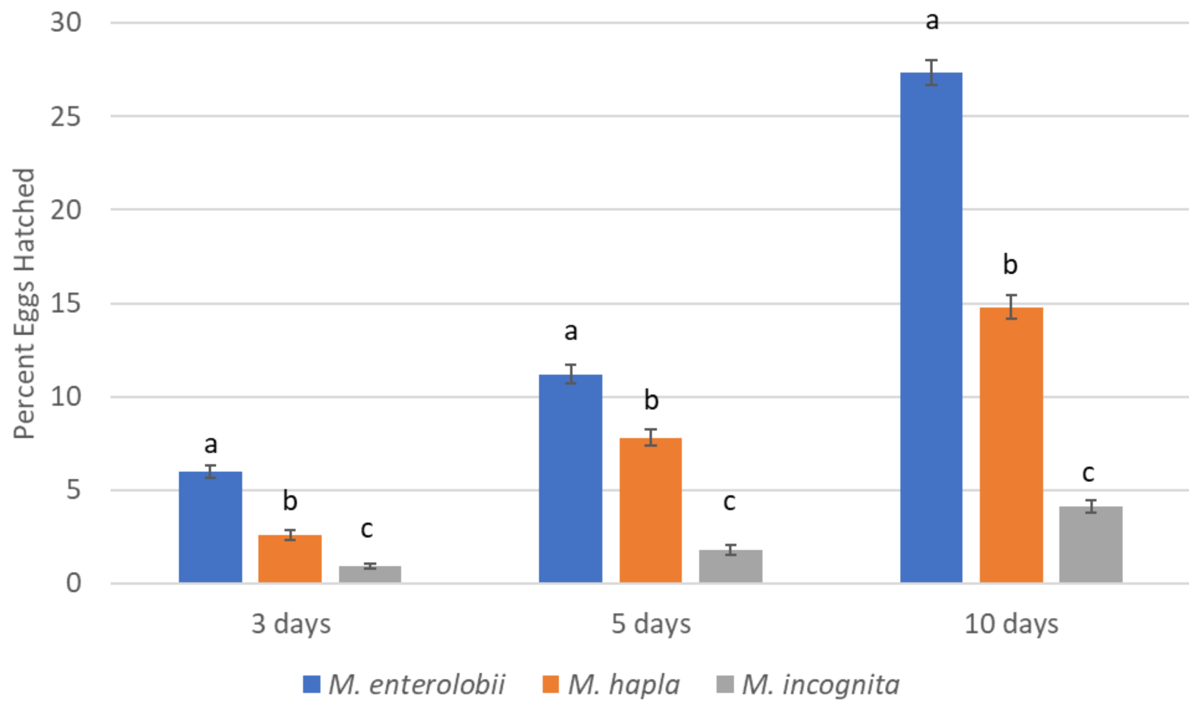

Figure 2.7: Percent of hatched eggs for root-knot nematode species *Meloidogyne enterolobii*, *M. hapla*, and *M. incognita* race 4 in a soil egg hatch study, Trial 3. Each bar is an average of six replications. Percent of eggs hatched was calculated based on an initial 250 eggs per replication. Egg hatch rates were determined 3, 5, and 10 days post initiation on egg hatching screens containing 1 cc of water solution pipetted to 1 mL of sterilized soil. Each time point was analyzed separately, in which different letters are significantly different at the $\alpha = 0.05$ level.

CHAPTER 3

Analyzing sweetpotato germplasm for potential resistance to the root-knot nematode *Meloidogyne enterolobii*

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ABSTRACT

Nematode-resistant plant genotypes are an effective, environmentally sustainable, and cost-efficient tool for managing disease and maintaining yield, yet the aggressive root-knot nematode species *Meloidogyne enterolobii* overcomes currently deployed host resistance in sweetpotato. Therefore, new sources of resistance are needed. Potential resistance to *Meloidogyne enterolobii* in fifty-one diverse sweetpotato genotypes was evaluated in a greenhouse bioassay experiment. Approximately 10,000 eggs of *M. enterolobii* were inoculated on each sweetpotato genotype, with three replicate plants per genotype, grown in a steam sterilized sand soil mixture. Sixty days post inoculation, root galling severity was determined, and nematode eggs were extracted from each whole root system. The reproduction factor (Rf = final total egg count / initial egg inoculum) was used to determine host status. Genotypes displaying an Rf value of less than one were considered resistant and poor host to *M. enterolobii*; genotypes with an Rf value greater than one were considered susceptible. Among the 51 genotypes tested, 12 were considered resistant, including: 'Tanzania', 'Jewel', 'Nancy Hall', 'Norton', 'Caromex', 'Regal', 'Beniko Machi', 'Whitestar', 'Hannah', 'Southern Delite', 'Carver', and 'NC09-1105'. Some of the susceptible genotypes included 'Beauregard', 'Covington', 'Gem', and 'Mugande'. The genotype 'NC07-0364' had the lowest Rf value of the susceptible hosts (Rf = 1.42), and 'NC17-0308' had the largest Rf value of 10.39, representing high level of reproduction in the nematode population. This study adds to the knowledge of sweetpotato resistance to *M. enterolobii* and aids in plant breeding efforts.

INTRODUCTION

In 2022, the United States harvested 132,200 acres of sweetpotato, valued at nearly \$598.5 million USD (USDA-NASS, 2022a). North Carolina is the nation's largest sweetpotato producing state, with 83,700 acres harvested at a production value of \$225 million USD (USDA-NASS, 2022b), representing about 64% of the nation's harvested acres. As a nutrient rich and high economic value crop, sweetpotato production in the United States is important for both domestic and export markets.

Root-knot nematodes (RKN), including *Meloidogyne incognita*, *M. arenaria*, *M. hapla*, and *M. javanica*, have been well documented for their ability to infect sweetpotato, decreasing the quality and quantity of yield (Cervantes-Flores et al., 2002; Lawrence et al., 1986; Overstreet, 2013). The relatively recent emergence of the guava root-knot nematode, *Meloidogyne enterolobii* (Yang and Eisenback, 1983), further threatens sweetpotato production. *Meloidogyne enterolobii* has limited known distribution in the United States, having been reported in Florida (Brito et al., 2004), South Carolina (Rutter et al., 2019), Georgia (Hajihassani et al., 2023) and North Carolina (Ye et al., 2013). This species has also been reported in numerous other sweetpotato producing countries, including China, Vietnam, Nigeria, and Brazil (Iwahori et al., 2009; Kolombia et al., 2016; Silva et al., 2021; Yang and Eisenback, 1983). *Meloidogyne enterolobii*, considered one of the most damaging RKN (Ye et al., 2021), attacks plant roots in high numbers causing reductions in plant vigor and yield (Castagnone-Sereno, 2012). Sweetpotato storage roots can be directly infected by *M. enterolobii* causing characteristic galls and cracks that reduce the value and marketability of harvested yield, and further allowing the nematodes to multiply and increase in field populations (Cervantes-Flores et al., 2002; Lawrence et al., 1986; Overstreet, 2013). Due to the severity of symptoms, lack of strong management tactics, and the vegetative propagative method through which sweetpotato planting material is generated and shipped, the North Carolina Department of Agriculture & Consumer Services declared a quarantine for all counties in the state (NCDA&CS, 2018) with the goal of minimizing risk of dissemination to other states and regions. Identification and optimization of management tactics for *M. enterolobii* is vital for efficient sweetpotato production to continue in infested regions.

Nematode-resistant plant genotypes are an effective, environmentally friendly, and cost-efficient method to reduce and manage RKN populations (Brito et al., 2020; Lambert and Bekal, 2002). A *M. enterolobii*-resistant sweetpotato cultivar could be incorporated into field rotation programs to manage nematode populations and preserve yield (Lambert and Bekal, 2002). Importantly, in certain parts of the world that lack other high-input management options such as chemical nematicides, resistant genotypes may be the only economically feasible form of management (Roberts, 1992). Of concern, *M. enterolobii* has been documented to overcome sweetpotato genotypes with resistance to other RKN, such as *M. incognita* (Brito et al., 2020; Cervantes-Flores et al., 2002; Schwarz et al., 2020). However, there is limited research regarding sweetpotato genotypes resistant to *M. enterolobii*, with only a handful of genotypes that display resistance (Brito et al., 2020; Rutter et al. 2021; Schwarz et al. 2021). Most of these resistant genotypes lack horticulturally desired traits for U.S. markets, and thus cannot immediately be deployed in the field (Brito et al., 2020; Rutter et al., 2021; Schwarz et al., 2021). Therefore, assessment of additional sweetpotato germplasm for resistance to *M. enterolobii* is needed. The goal of this research was to assess selections of sweetpotato germplasm from the North Carolina State University Sweetpotato and Potato Breeding and Genetics Program for resistance to *M. enterolobii* in a greenhouse bioassay.

MATERIALS AND METHODS

Sweetpotato material. Fifty-one sweetpotato genotypes, obtained from the North Carolina State University Sweetpotato and Potato Breeding and Genetics Program (Raleigh, NC), were evaluated for potential resistance to *Meloidogyne enterolobii* in a greenhouse setting (Table 3.1). Ten to fifteen-cm-long sweetpotato slips of each genotype tested were planted in 15 cm deep clay pots containing a 1:1 steam sterilized sand soil mixture. The roots of these sweetpotato plants were inoculated with *M. enterolobii* two weeks after transplanting, as described below.

Inoculum preparation and inoculation of plants. In 2017, a population of *M. enterolobii* was isolated from a sweetpotato field in Wilson County, North Carolina and identified via PCR (Schwarz et al. 2020), and has since been maintained in the greenhouse as a single species colony on 'Rutgers' tomato culture plants. *Meloidogyne enterolobii* eggs were extracted from two-month-old cultures of 'Rutgers' tomato plants following the 0.05% sodium hypochlorite method described by Hussey and Barker (1973) and collected in water. On the same day of egg collection, each potted sweetpotato plant was inoculated with 10,000 *M. enterolobii* eggs by pipetting 5 mL of inoculum at the base of each plant. Three replications per sweetpotato genotype were assessed, with each replication consisting of a single sweetpotato plant. Plants were arranged in a randomized complete block pattern on greenhouse benches, maintained in the greenhouse at 25-28°C with no supplemental lighting, and irrigated once per day.

Included among the 51 sweetpotato genotypes were four controls with known host status to *M. enterolobii* determined in a previous study (Schwarz et al., 2021). These were resistant genotypes 'Tanzania' and 'Jewel', and susceptible sweetpotato cultivars 'Covington' and 'Beauregard', with both positive (inoculated) and negative (non-inoculated) controls, replicated three times each. A single greenhouse trial (conducted June to August 2021) was considered sufficient in determining host status for breeding purposes.

Data collection and resistance evaluation. Host status of each plant was evaluated sixty days after *M. enterolobii* inoculation. Each plant was cut at the crown to isolate the roots. Roots were gently rinsed with water to remove soil. The weight of each root system was recorded, including storage roots if present. Nematode root galling severity was estimated based on the total percent of the root system that contained galls, following a modified protocol from Bridge and Page (1980), in which roots were scored on an integer scale from 0 to 100%, where 0% corresponded to no observable galls, and 100% corresponded to the entire root system covered in RKN galls. Each root system was then subjected to the sodium hypochlorite method detailed by Hussey and Barker (1973) to extract nematode eggs, which were recovered on a 25 µm-mesh sieve and collected in 50 mL tubes with water. The final total number of nematode eggs for each root system was estimated by averaging the count of three, 1 mL aliquots of eggs quantified via manual counting using an inverted compound microscope at 40x magnification (Nikon TM, Nikon Instruments, Melville, NY). The host status of each genotype was determined by calculating the nematode reproduction factor (Rf= final eggs per root system/ initial egg inoculum). An Rf value greater than one was considered as a susceptible host genotype; an Rf value less than one was considered as a resistant genotype. Gall ratings were used to support host status assessments, in which a root gall severity score >1% was not favorable for advancing sweetpotato genotypes for plant breeding purposes.

Statistical analysis. Data were analyzed using SAS statistical analysis software version 9.4 (SAS Institute, Cary, NC). To determine if significant differences existed between sweetpotato genotypes for the response variables of total eggs and gall rating, data were analyzed using a general linearized model (PROC GLM) (*n.b.*, Rf was not subject to statistical analysis as this value was calculated from the total eggs divided by a constant, 10,000). All total nematode egg data were $\log_{10}(x+1)$ transformed to satisfy the assumptions of normality and equal variances for statistical analyses. Where significant differences were indicated, means were separated using Fisher's Least Significant Difference procedure ($\alpha = 0.05$). Non transformed means are presented in Table 3.1, as the average of the three biological replicates.

RESULTS

Sweetpotato genotype had a significant effect on total *M. enterolobii* eggs ($P < 0.001$) and gall ratings ($P < 0.001$) recovered from inoculated root systems. Reproduction factors generated demonstrated both resistant and susceptible sweetpotato genotypes to *M. enterolobii*, and conclusions were supported by galling severity. Among the 51 genotypes tested, 12 had an Rf value of less than 1 and were considered resistant, including: 'Tanzania', 'Jewel', 'Nancy Hall', 'Norton', 'Caromex', 'Regal', 'Beniko Machi', 'Whitestar', 'Hannah', 'Southern Delite', 'Carver', and 'NC09-1105' (Table 3.1). Among these, 'Tanzania' and 'Jewel' were previously known resistant genotypes, included as controls, and upheld their resistance status in this trial. The smallest Rf value among resistant genotypes was 0.03 for 'Tanzania', and the highest was 'NC09-1105' at 0.97 (Table 3.1). All resistant (poor hosts) genotypes did not display any observable galls (0%) on any replicates with the exception of 'NC09-1105', which had a 1% average gall severity rating (Table 3.1), which translated to approximately 1-5 galls on the roots of all replicates of this genotype. The non-inoculated controls did not have any nematode gall formation and had zero total nematode eggs extracted from each root system (Table 3.1).

The susceptible controls, ‘Beauregard’ and ‘Covington’ preserved their host status with Rf values of 3.97 (39,711 total eggs) and 4.05 (40,500 total eggs) respectively. On average, ‘Beauregard’ had a root gall severity rating of 16.7% and ‘Covington’ 21.7%, representing a statistically significant increase in root gall formation compared to nearly all other susceptible genotypes observed, with the exception of ‘NC08-0036’ and ‘NC17-0308’. Of the genotypes determined to be susceptible, ‘NC07-0364’ had the lowest Rf value of 1.42 and a 3% gall rating. The genotype determined to be most susceptible to *M. enterolobii* was ‘NC17-0308’ with an Rf value of 10.39 and mean gall rating of 13.3% (Table 3.1).

DISCUSSION

The results from this study found both resistant and susceptible sweetpotato genotypes to *M. enterolobii*, which aids in advancing the understanding of sweetpotato-*M. enterolobii* interactions. Twelve out of 51 plant genotypes screened were considered resistant and overall, a poor host to *M. enterolobii*. ‘Tanzania’ and ‘Jewel’ were confirmed as resistant genotypes as determined in Schwarz et al. (2021) and served as effective controls in this test. However, Rutter et al., (2021), which also found ‘Tanzania’ as resistant, determined that ‘Jewel’ was susceptible in their study. It is hypothesized that this discrepancy may be due to different isolates of *M. enterolobii* or a difference in sweetpotato genetics. The other resistant sweetpotatoes in this study that confirm previous reports are ‘Carver’ and ‘Regal’, which both were found to be resistant to *M. enterolobii* by Rutter et al. (2021). The susceptible host status of ‘Covington’ and ‘Beauregard’ were also consistent with previous reports (Brito et al., 2020; Rutter et al., 2021; Schwarz et al., 2021). The other sweetpotato genotypes, to our current knowledge, have not been previously reported for their host status to *M. enterolobii*.

The sweetpotato genotypes that may be most useful in *M. enterolobii* resistance-breeding and management contexts are those with the lowest Rf value and 0% gall rating. All the resistant genotypes had low Rf values with the exception of ‘NC09-1105’. Although ‘NC09-1105’ had an Rf value of 0.97 and still in the threshold of resistance, it is not a strong recommendation as a genotype for breeding or growing purposes due to its average Rf value and minor nematode galling (1%) observed on the roots. The other 11 genotypes considered as resistant to *M. enterolobii* in this study have more potential as management tools, especially for incorporating the resistance trait into breeding programs. In addition, the identification of susceptible sweetpotato genotypes is essential for effective *M. enterolobii* management. Knowing which plant varieties are hosts to *M. enterolobii* will inform growers with at-risk fields that proper integrated pest management is needed to avoid potential crop damage.

Not every sweetpotato genotype is favorable for growing and consumption in all markets. Selection of sweetpotato genotypes is crucial for pathogen management, but also for their distinct horticultural characteristics; sweetpotatoes are diverse in taste, color, yield, disease resistance, and optimal climate growth parameters, and thus, not every genotype is adapted to grow in every region (Ddumba et al., 2014; La Bonte et al., 2008; Yencho et al., 2008). The sweetpotato genotypes in this study, with the exceptions of ‘Covington’ and ‘Beauregard’, are not horticulturally acceptable, field-ready cultivars preferred by regional growers and the U.S. market (Dr. Craig Yencho, personal communication). Therefore, the *M*. enterolobii-resistant genotypes determined in this study largely provide sources for breeding resistance genes into more desirable crops. Previous research has shown that the resistance trait to *M. enterolobii* can be inherited in sweetpotato (Schwarz et al., 2021); the sweetpotato breeding program at North Carolina State University produced a mapping population resulting from crossing the resistant sweetpotato ‘Tanzania’ and the susceptible genotype ‘Beauregard’ (‘TB’ population; Cervantes-Flores et al., 2008). A preceding greenhouse resistance screening experiment that included 8 of the ‘TB’ progenies determined that 5 ‘TB’ genotypes were resistant to *M. enterolobii* (Schwarz et al., 2021), providing evidence that the trait of resistance to *M. enterolobii* is segregating and can be successfully inherited. The resistant genotypes in this present study can be utilized to cross with desirable sweetpotato genotypes such as ‘Covington’ or ‘Beauregard’ to produce a favored crop that contains resistance to *M. enterolobii*. The results from this study are important to aiding in the advancement of resistance breeding.

This study used a population of *M. enterolobii* originating from an infected sweetpotato field in Wilson County, North Carolina and has since been maintained in the greenhouse on ‘Rutgers’ tomato plants. Results of host status of sweetpotato genotypes may be dependent on the origin of nematode population. Rutter et al. (2021) determined that two *M. enterolobii* populations from two different states in the Southeastern United States had pathotypically distinct responses on the same sweetpotato genotypes. As previously mentioned, ‘Jewel’ was found to be resistant in this study, yet Rutter et al. (2021) determined ‘Jewel’ as susceptible to *M. enterolobii*. The results presented in this current study may be unique to the nematode population used in our study. Observation of this phenomenon indicates that future work should evaluate differential responses among populations of *M. enterolobii* in sweetpotato to ensure host resistance is durable across unique *M. enterolobii* populations that may be present in distinct geographic regions.

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Table 3.1. Host status of sweetpotato genotypes to *Meloidogyne enterolobii* in a greenhouse resistance screening bioassay. Each sweetpotato was inoculated with 10,000 eggs of *M. enterolobii* and evaluated 60 days post inoculation. Values are the mean of three replicate plants. Values followed by the same letter within a column are not significantly different at the $\alpha = 0.05$ level. Control sweetpotato genotypes (‘Tanzania’, ‘Jewel’, ‘Beauregard’, and ‘Covington’) with (+) were inoculated with 10,000 nematode eggs; genotypes with (-) were not inoculated.

Sweetpotato Genotype	Nematode Eggs	Gall Rating (%)[a]	Reproduction Factor[b]	Resistant or Susceptible
Covington (-)	0$_{a}$	0$_{a}$	0.00	R
Beauregard (-)	0$_{a}$	0$_{a}$	0.00	R
Tanzania (-)	0$_{a}$	0$_{a}$	0.00	R
Jewel (-)	0$_{a}$	0$_{a}$	0.00	R
Tanzania (+)	333$_{ab}$	0$_{a}$	0.03	R
Nancy Hall	422$_{abc}$	0$_{a}$	0.04	R
Norton	1,156$_{abc}$	0$_{a}$	0.12	R
Caromex	1,278$_{abc}$	0$_{a}$	0.13	R
Regal	1,389$_{abc}$	0$_{a}$	0.14	R
Jewel (+)	1,756$_{abc}$	1.0$_{ab}$	0.18	R
Beniko Machi	2,089$_{abc}$	0$_{a}$	0.21	R
Whitestar	2,278$_{abc}$	0$_{a}$	0.23	R
Hannah	2,511$_{abc}$	0$_{a}$	0.25	R
Southern Delite	3,944$_{abcd}$	0$_{a}$	0.39	R
Carver	5,756$_{abcde}$	0$_{a}$	0.58	R
NC09-1105	9,656$_{abcdef}$	1.0$_{ab}$	0.97	R
NC07-0364	14,178$_{bcdefg}$	3.0$_{abcd}$	1.42	S
NC17-0101	14,433$_{cdefgh}$	2.3$_{abc}$	1.44	S
NC17-0129	17,089$_{defghi}$	2.3$_{abc}$	1.71	S
MD-88-116	17,744$_{defghij}$	3.7$_{abcde}$	1.77	S
NCP13-007	19,133$_{efghijk}$	3.0$_{abcd}$	1.91	S
NC17-0661	19,200$_{efghijk}$	3.7$_{abcde}$	1.92	S
NC12-1149	20,000$_{fghijkl}$	4.3$_{bcdef}$	2.00	S
MC16-0714	20,156$_{fghijkl}$	10.0$_{ghi}$	2.02	S
NC02-0350	20,389$_{fghijkl}$	3.7$_{abcde}$	2.04	S
NC14-0353	21,533$_{fghijklm}$	5.0$_{bcdef}$	2.15	S
Tainung 65	22,944$_{fghijklm}$	3.7$_{abcde}$	2.29	S
MC16-0200	23,344$_{fghijklm}$	4.3$_{bcdef}$	2.33	S

Table 3.1 (continued).

NC09-0188	23,567$_{fghijklm}$	6.7$_{defg}$	2.36	S
Diane #2M	24,278$_{ghijklm}$	1.7$_{ab}$	2.43	S
NC09-0136	26,278$_{ghijklmn}$	3.7$_{abcde}$	2.63	S
TC2	28,256$_{hijklmno}$	5.0$_{bcdef}$	2.83	S
NC17-0045	28,556$_{ijklmno}$	5.0$_{bcdef}$	2.86	S
Okinawa-100	29,833$_{ijklmno}$	4.3$_{bcdef}$	2.98	S
NCP13-005	30,089$_{ijklmno}$	3.7$_{abcde}$	3.01	S
MC15-0032	31,333$_{jklmnop}$	10.0$_{ghi}$	3.13	S
NC13-0821	33,122$_{klmnop}$	6.7$_{defg}$	3.31	S
MC14-0965	33,556$_{lmnop}$	6.0$_{cdefg}$	3.36	S
NC15-0418	33,833$_{lmnop}$	8.3$_{fgh}$	3.38	S
Gem	34,556$_{mnop}$	3.7$_{abcde}$	3.46	S
Beauregard (+)	39,711$_{nopq}$	16.7$_{j}$	3.97	S
Covington (+)	40,500$_{opq}$	21.7$_{k}$	4.05	S
MC17-0772	41,333$_{opq}$	4.3$_{bcdef}$	4.13	S
97A04	41,933$_{opq}$	11.7$_{hi}$	4.19	S
NC05-0408	45,333$_{pqr}$	6.7$_{defg}$	4.53	S
MC16-0260	51,467$_{qrs}$	6.7$_{defg}$	5.15	S
MC16-0298	57,844$_{rst}$	6.7$_{defg}$	5.78	S
NC12-0219	62,267$_{stu}$	10.0$_{ghi}$	6.23	S
NC07-0745	64,944$_{stu}$	10.0$_{ghi}$	6.49	S
Mugande	68,867$_{tu}$	6.7$_{defg}$	6.89	S
L22	70,700$_{tu}$	7.7$_{efgh}$	7.07	S
MC16-0062	72,611$_{u}$	8.3$_{fgh}$	7.26	S
NC17-0049	75,550$_{u}$	6.7$_{defg}$	7.56	S
NC08-0036	75,811$_{u}$	21.7$_{k}$	7.58	S
NC17-0308	103,856$_{v}$	13.3$_{ij}$	10.39	S
LSD[c]	14024	4.04		--
P-value	< 0.001	<0.001		--

[a] Gall rating was determined by estimating the percent of the root system containing galls, following the schematic by Bridge and Page (1980).

[b] Reproduction factor (Rf= final nematode eggs/initial inoculum of eggs). Initial inoculum of eggs in this study was 10,000 eggs of *M. enterolobii*. Rf values less than 1 are considered resistant (R); greater than 1 are considered susceptible (S).

[c] Fisher’s Least Significant Difference

CHAPTER 4

Evaluation of soybean genotypes (*Glycine max* and *G. soja*) for resistance to the root-knot nematode, *Meloidogyne enterolobii*

Submitted to *Plant Disease*; Accepted with revisions.

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ABSTRACT

Potential resistance to the root-knot nematode, *Meloidogyne enterolobii*, in 72 *Glycine soja* and 44 *Glycine max* soybean genotypes was evaluated in greenhouse experiments. Approximately 2,500 eggs of *M. enterolobii* were inoculated on each soybean genotype grown in a steam sterilized 1:1 sand to soil mixture. Sixty days post inoculation, plants were destructively harvested to determine the host status. Root galling severity was determined for each genotype and nematode eggs were extracted from roots. The host status of each soybean genotype was determined by assessing root galling severity and calculating the final eggs per root system divided by the initial inoculum, or the reproduction factor (Rf). Five *G. soja* soybean genotypes were identified as resistant to *M. enterolobii*: '407202', '407239', '424083', '507618', and '639621', which all had an Rf value of less than 1. None of the tested *G. max* soybean genotypes were identified as resistant to *M. enterolobii*, and all *G. max* Rf values were above 1. Some of the *G. max* genotypes determined to be susceptible to *M. enterolobii* include 'Hagood', 'Avery', 'Rhodes', 'Santee' and 'Bryan'. The genotype 'Bryan' had the lowest Rf values among the group at 5.06 and 6.67 in two independent trials respectively, which represents a 5- to 6-fold increase in reproduction of *M. enterolobii.* Plant genotypes resistant to root-knot nematodes are effective in managing the disease and preserving yield, are cost-efficient, and environmentally sustainable, and host resistance is often regarded as the most robust management tactic for controlling plant parasitic nematodes. Resistance to root-knot nematodes in soybean genotypes has been identified for other *Meloidogyne* species, yet there is currently limited data regarding soybean host status to the highly aggressive nematode, *M. enterolobii*. This study adds to knowledge of potential natural resistance to *M. enterolobii* in wild and cultivated soybean.

INTRODUCTION

Meloidogyne enterolobii (Yang and Eisenback, 1983) (syn. *M. mayaguensis*) is an aggressive species of root-knot nematode (RKN) that can cause significant damage on a broad range of host crops (Castagnone-Sereno, 2012). This soilborne pathogen attacks the roots of plants in large numbers, causing characteristic root galling and damage that leads to a decrease in quantity and quality of yield (Castagnone-Sereno, 2012; Ye et al., 2013). *Meloidogyne enterolobii*, considered one of the most damaging RKN species in the world (Castagnone-Sereno, 2012; Ye et al., 2021), was first described in China (Yang and Eisenback, 1983). It has since been detected in several countries in Africa, Asia, South America, and North America (Brito et al., 2004; Ye et al., 2013; Oyetunde et al., 2022). In the continental United States, *M. enterolobii* has been reported in Florida (Brito et al., 2004), North Carolina (Ye et al., 2013), South Carolina (Rutter et al., 2019), and Georgia (Hajihassani et al., 2023).

Once RKN is established in a field, it is difficult to reduce populations to non-threatening economic thresholds or eradicate them (Lambert and Bekal, 2002; Castagnone-Sereno, 2012). Resistant plant genotypes represent an effective, cost-efficient, and environmentally sustainable form of pest management that may be deployed worldwide (Roberts, 1992; Castagnone-Sereno, 2012). Plant genetic resistance inhibits or prevents nematodes from completing their life cycle and reproducing, subsequently reducing nematode populations in the soil (Castagnone-Sereno, 2012). However, RKN-resistant plant genotypes currently used by agricultural producers to manage other common *Meloidogyne* species are ineffective against *M. enterolobii* (Kiewnick et al., 2009; Castagnone-Sereno, 2012). To illustrate, isolates of *M. enterolobii* have been demonstrated to overcome the *Mi-1* gene in tomato (Kiewnick et al., 2009), the *Mir1* gene in soybean (Cetintas et al., 2008), and the *N* gene in bell pepper (Kiewnick et al., 2009). Due to the limited number of commercial RKN-resistant plant genotypes, restrictions on the use of many nematicides, and few non-host rotational crops for the wide host range of RKN, farmers have limited options for managing *M. enterolobii*.

Soybean is an economically valuable crop and an important global source of food, protein, and oil (Bandara et al., 2020; Voora et al., 2020), and it is estimated to be cultivated on 6% of arable land (Bandara et al., 2020). The United States is a leading country in soybean production, producing about 87.3 million acres in 2021 (USDA-NASS, 2021), although Brazil surpassed the United States in soybean production in 2021 (Colussi and Schnitkey, 2021). Both countries face agricultural production challenges where *M. enterolobii* is present and yield losses have been documented due to this pathogen (Castagnone-Sereno, 2012; Ye et al., 2013). In North Carolina, soybean is the most widely grown crop by acreage; in 2020, North Carolina producers planted approximately 1,600,000 acres of soybean yielding about $673.8 million in profit (USDA-NASS 2020). In North Carolina, *M. enterolobii* was first reported infecting soybean, and reports of *M. enterolobii* infection in soybean are increasing (Ye et al., 2013, 2021).

Resistance against *M. incognita*, *M. arenaria*, and *M. javanica* has been identified in some soybean varieties (Luzzi et al., 1987; Davis et al., 1998; Li et al., 2001). However, there is currently limited data of host status to *M. enterolobii* among soybean genotypes. Cetintas et al. (2008) screened *G. max* varieties to four isolates of *M. mayaguensis* (syn. *M. enterolobii*). In their study, a few plant genotypes displayed varying degrees of resistance to some of the *M. enterolobii* isolates tested, however, it was concluded that none of the soybean genotypes were resistant to all four *M. enterolobii* isolates. Therefore, it is vital to evaluate possible resistance in diverse soybean germplasm to the highly aggressive *M. enterolobii*. The objective of this work was to screen soybean genotypes, including those of wild soybean *Glycine soja* and cultivated soybean *G. max*, in the greenhouse for response and potential resistance to a North Carolina isolate of *M. enterolobii*.

MATERIALS AND METHODS

Source of Meloidogyne enterolobii and soybean seeds. The *Meloidogyne enterolobii* population used in this study originated from an infected sweetpotato field in Wilson County, North Carolina in 2017 and has since been maintained in the greenhouse. Polymerase chain reaction (PCR) was used to verify the nematode species as *M. enterolobii* (Schwarz et al., 2020). *Meloidogyne enterolobii* eggs used to inoculate each soybean plant in this study were obtained from two-month-old cultures grown on roots of 'Rutgers' tomato plants in the greenhouse. *Glycine soja* seeds were generously provided by Dr. Earl Taliercio (USDA-ARS), and seeds of *G. max* soybean seeds were obtained from the USDA-GRIN database. A total of 72 wild soybean (*Glycine soja*) genotypes and 44 cultivated soybean (*Glycine max*) genotypes were included in this study (Table 4.1 and 4.2).

Planting, inoculation, and evaluation of soybean for resistance or susceptibility to M. enterolobii. Due to differences in the number of seeds available for each genotype and variation in germination rates, *Glycine soja* genotypes were replicated three times each and *Glycine max* replicated five times each per trial, with a replicate composed of an individual plant. Soybean seeds were planted in plastic cone-containers (Stuewe & Sons, OR) of dimensions 25.4 cm deep by 3.8 cm wide at the top, with a 1:1 steam sterilized sand to soil mixture. Plants were maintained in the greenhouse at 25-28°C with no supplemental lighting. Three weeks after planting, the roots of each plant were inoculated with 2,500 *M. enterolobii* eggs. Eggs were extracted from tomato roots following the NaOCl extraction method of Hussey and Barker

(1973). On the same day the eggs were collected, eggs were inoculated to each soybean plant by pipetting 3 mL of inoculum solution into the soil at the base of each plant stem. At 60 days post inoculation, soybean plants were evaluated for resistance or susceptibility. The plants were cut at the crown to isolate the roots from the shoots. Each root system was gently rinsed free of soil with water. Visual gall ratings were scored based on the total percent of the root system containing galls (0-100%), following a modified protocol of the schematic by Bridge and Page (1980). Nematode eggs were then extracted from each individual root system following the NaOCl method described by Hussey and Barker (1973), and eggs were recovered on a 25 µm pore size sieve. A 1 mL aliquot of eggs from each root was counted using a compound inverted microscope (Nikon TM, Nikon Instruments, Melville, NY). The total final nematode egg count was calculated by multiplying the number of eggs counted in the aliquot by the total extraction sample volume. Host status of the soybean plants was determined based on the reproduction factor (Rf= final nematode egg count/initial inoculum egg count; initial inoculum herein 2,500 eggs) of the nematodes on each root system. An Rf value less than 1 was considered resistant; an Rf value greater than 1 was considered susceptible to *M. enterolobii*. For *Glycine soja*, all soybean genotypes identified as resistant in a first trial were retested in a subsequent trial to confirm resistance status. Additionally, a random selection of 10 susceptible *Glycine soja* genotypes from the first trial were included in the second trial. For *Glycine max*, all genotypes were retested in a second trial since no resistance was found in the first test.

Data analysis. Data was analyzed using SAS statistical analysis software version 9.4 (SAS Institute, Cary, NC) to explore possible differences in response between genotypes. Each trial was analyzed independently as trials were conducted at separate times, and data values were averaged across replications within each trial. Data were analyzed in SAS using a general linearized model (PROC GLM). Means were separated using Fisher's *t* test LSD procedure (alpha=0.05). Resistant soybean genotypes were classified by reproduction factor (final eggs per root divided by the initial inoculum), where an Rf value less than 1 is considered resistant and supported based upon Fisher's *t* test. Reproductive factor values were recorded in Tables 4.1 and 4.2; however, LSD significance groups separating these Rf values were calculated and reported according to total nematode eggs.

RESULTS

Glycine soja results. There were significant differences among *Glycine soja* genotypes ($P < 0.001$) for gall rating and total nematode eggs per root system in each of the trials performed (Table 4.1). Among the 72 wild soybean (*Glycine soja*) genotypes, five had an Rf value of less than 1 and were considered resistant to *M. enterolobii* (Table 4.1). These *G. soja* genotypes were '407202', '407239', '424083', '507618', and '639621'. All the resistant *G. soja* lines observed did not contain galls (0%) on root systems with the exception of soybean genotype '639621', upon which 1-5 galls (scored as 1%) were observed on the root system of each replicate (Table 4.1). The *G. soja* genotype with the lowest Rf values was '407239', with Rf values of 0.04 and 0.16 in the two trials (Table 4.1). Susceptible *G. soja* genotypes had visible gall formation, and more than 2,500 nematode eggs were extracted from each of the root systems. The *G. soja* genotype with the most severe gall rating was '479751' with an average of 75% of the root system containing galls among replicate plants (Table 4.1). Genotype '507624' had the highest Rf value of the *G. soja* genotypes, with a value of 21.94 in the first trial and 11.23 in the second trial (Table 4.1). A visual example of root galling comparisons of resistant genotype '407239' and susceptible '479751' in Figure 4.1 show the noticeable difference between no galling (0%)

and a high gall rating (75%) soybean root. Resistant genotypes identified in the first trial upheld their resistance in the second trial and the select susceptible soybean genotypes retested also maintained their susceptibility to *M. enterolobii* (Table 4.1).

Glycine max results. There were significant differences among *Glycine max* genotypes ($P < 0.001$) for gall rating and total eggs per root system in each of the trials evaluated (Table 4.2). All the 44 *Glycine max* soybean genotypes tested were identified as susceptible to infection by *M. enterolobii*; all genotypes had an Rf value of greater than 1 (Table 4.2). Root-knot nematode galls were observed on each root system (range of 3 to 75% gall rating) and all genotypes and replicates contained more than 2,500 nematode eggs. For example, 'Hagood' had an average gall rating of 75% and the largest Rf values of 39.35 and 31.56 in the two trials, representing a significant increase in nematode population in each root. 'Bryan' had the lowest Rf values of 5.06 and 6.67 in the two trials of *G. max* respectively, but still represented a significant increase in nematode population numbers and was determined as susceptible to *M. enterolobii* (Table 4.2). Figure 4.2 provides a visual of the different root galling severities seen among *G. max*. *Glycine max* genotypes remained susceptible to *M. enterolobii* in both trials (Table 4.2).

DISCUSSION

The results demonstrated that 5 out of 72 unique *Glycine soja* lines screened were resistant to *M. enterolobii*, while none of the *G. max* genotypes tested were identified as resistant. Analysis of *G. soja* genotypes indicated there were significant differences in *M. enterolobii* reproduction between resistant and susceptible plants. Determination of resistant genotypes through Rf values were aided by root galling severity. All resistant *G. soja* lines had no observable galls with the only exception being '639621'. Genotype '639621' had an average rating of 1%, corresponding to 1-5 galls in each replicate, although numerically these galls were significantly lower than those of susceptible lines. Susceptible soybean lines of *G. soja* and *G. max* all contained visible nematode galling damage. The research presented here provides the host status among selected genotypes of *G. max* and *G. soja* soybean for resistance or susceptibility to *M. enterolobii*.

These data highlight the high virulence of *M. enterolobii* and its ability to infect crops with resistance to other species of RKN. None of the tested *Glycine max* in this study were resistant to *M. enterolobii*, even those with previously reported resistance to other RKN species (USDA-GRIN). For example, genotype ‘G93-9009’ has reported resistance to *M. incognita*, *M. arenaria*, and *M. javanica* (USDA-GRIN), but was shown in this study to be highly susceptible to *M. enterolobii*. ‘Benning’ and ‘Boggs’, resistant to *M. incognita*, were susceptible to *M. enterolobii*. ‘Hagood’ has reported resistance to *M. incognita*, but in this study displayed the most severe galling symptoms and Rf value when infected by *M. enterolobii*.

The results from this study also corroborate with findings from Cetintas et al. (2008). Similar to this study, they reported that ‘G93-9009’, ‘Benning’, and ‘Boggs’ were considered susceptible to isolates of *M. mayaguensis* (syn. *M. enterolobii*); however, it was determined that ‘Boggs’ had resistance to two of four nematode isolates tested in Cetintas et al. (2008). The results suggest that resistance may be dependent on the isolate of *M. enterolobii*. Rutter et al. (2021) indicated that two *M. enterolobii* isolates from different states in southeastern United States had different phenotype responses when inoculated on the same sweetpotato genotypes. The findings in this present study may be unique to the isolate of *M. enterolobii* from North Carolina used.

Nematode-resistant plant genotypes are an environmentally sustainable and cost-efficient tool to manage RKN (Davis et al., 1998; Rutter et al., 2021; Schwarz et al., 2021). Plant genotypic resistance is often widely accepted and may allow exportation of plant products to countries that implement restrictions on chemical residues (Khanal et al., 2018). In addition, use of genetic resistance may be implemented in diverse production systems, including those with otherwise limited management options such as organic growers, subsistence farming and farmers in developing countries (Collett et al., 2021; Nuijten et al., 2018). In developing countries, resistant genotypes may be the only economically feasible form of RKN management (Roberts, 1992). However, *M. enterolobii* is known to overcome the *Mir1* resistance gene in soybean effective in controlling other RKN species (Kiewnick et al., 2009). Therefore, the identification of novel sources of resistance to *M. enterolobii* is important to soybean production, especially in regions where this nematode species is reported.

Although no lines of *Glycine max* screened in this study were identified as resistant, the results are still important for growers and researchers. If a grower has RKN present in their field, it is vital for the nematode species to be identified to determine if the soybean crop and particular genotype planted will be at risk to the RKN population present. If a susceptible soybean is planted in a field where *M. enterolobii* is present, the nematodes will be able to reproduce exponentially, leading to greater population numbers in the field and an increased disease pressure (Collett et al., 2021). The lack of resistance in cultivated *G. max* lines in the present study indicate that if *M. enterolobii* is confirmed present in a commercial soybean field, RKN management tactics other than host resistance will need to be deployed to protect yields.

These results may also serve as a foundation for breeding programs to create advanced genotypes with resistance to *M. enterolobii* (Rutter et al., 2021; Schwarz et al., 2021). In this study, 5 *Glycine soja* were resistant to *M. enterolobii*, yet these wild soybean genotypes are not suitable for agricultural production. These genotypes lack horticultural favored traits such as high yielding, drought tolerance, and vigor. However, the identification of new sources of resistance in wild *G. soja* lines lays the groundwork for future studies in host-nematode interaction and for breeding programs to incorporate the resistance trait into their crosses.

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Table 4.1. Galling and reproduction factor of *Meloidogyne enterolobii* observed in roots of *Glycine soja* soybean genotypes among two trials. All table values are the mean of three replications of *Glycine soja* plant roots inoculated with 2,500 eggs of *M. enterolobii* and evaluated at 60 days post inoculation.

***Glycine soja* Genotype**	**Trial 1 Rf[a]**	**Trial 2 Rf**	**Trial 1 GR[b]**	**Trial 2 GR**	**Susceptible or Resistant**
407202	0.06_{a}	0.32_{a}	0_{a}	0_{a}	R
407239	0.04_{a}	0.16_{a}	0_{a}	0_{a}	R
424083	0.85_{ab}	0.27_{a}	0_{a}	0_{a}	R
507618	0.63_{ab}	0.39_{a}	0_{a}	0_{a}	R
639621	0.37_{ab}	0.41_{a}	1_{a}	1_{a}	R
339871	6.41_{opq}	-	20_{defg}	-	S
366122	12.34_{ddee}	-	25_{ghi}	-	S
366124	6.08_{op}	-	25_{ghi}	-	S
378684	3.94_{ij}	-	10_{b}	-	S
378690	4.99_{lm}	-	15_{bcd}	-	S
378697	5.07_{lm}	-	15_{bcd}	-	S
378699	9.27_{xwz}	-	50_{k}	-	S
407020	4.30_{jk}	3.26_{b}	37_{j}	37_{e}	S
407052	2.85_{de}	2.82_{b}	13_{bc}	15_{bc}	S
407059	2.91_{def}	-	18_{cdef}	-	S
407085	9.42_{yz}	4.12_{c}	42_{j}	25_{d}	S
407096	7.55_{t}	-	15_{bcd}	-	S
407156	4.82_{klm}	3.11_{b}	23_{fgh}	25_{d}	S
407157	19.23_{hh}	-	50_{k}	-	S
407183	11.92_{ccdd}	-	42_{j}	-	S
407190	12.85_{ee}	-	25_{ghi}	-	S
407191	8.29_{uv}	2.92_{b}	25_{ghi}	32_{e}	S
407206	4.59_{kl}	-	18_{cdef}	-	S
407214	11.45_{bbcc}	-	42_{j}	-	S
407220	16.57_{gg}	15.96_{g}	25_{ghi}	23_{d}	S
407228	9.67_{zaa}	-	22_{efgh}	-	S
407231	19.08_{hh}	9.82_{e}	50_{k}	58_{f}	S
407240	7.37_{st}	-	17_{cdef}	-	S
407248	7.19_{rst}	-	20_{g}	-	S
407270	2.46_{cd}	-	10_{b}	-	S
407287	5.22_{mn}	-	13_{bc}	-	S
407300	11.11_{bb}	-	28_{hi}	-	S
407307	14.08_{ff}	10.77_{f}	50_{k}	25_{d}	S
407318	3.31_{efgh}	-	10_{b}	-	S

Table 4.1 (continued).

424004	2.08c	-	10b	-	S
424007	3.18efg	-	15bcd	-	S
424014	10.28aa	-	20defg	-	S
424035	3.89hij	-	27hi	-	S
424045	2.13c	-	13bc	-	S
424082	7.24st	-	15bcd	-	S
424123	7.39st	-	25ghi	-	S
464890	8.85vwxy	-	50k	-	S
468916	4.69klm	-	15bcd	-	S
479751	16.27gg	-	75l	-	S
479752	12.81ee	-	50k	-	S
507595	4.86klm	-	13bc	-	S
507609	8.67vwx	-	25ghi	-	S
507615	3.56ghi	-	10bc	-	S
507624	21.94ii	11.23f	20defg	20cd	S
507638	7.45t	-	50k	-	S
507656	3.21efg	-	10bc	-	S
507667	8.47vw	-	42j	-	S
507757	6.81qrs	-	25ghi	-	S
522226	7.48t	-	50k	-	S
549032	8.96wxy	-	30i	-	S
549046	2.78de	-	13bc	-	S
549047	6.09op	-	10b	-	S
562534	7.56t	-	17cdef	-	S
562547	7.37st	-	15bcd	-	S
562551	2.69cde	-	10b	-	S
562557	7.72tu	-	18cdef	-	S
562558	6.60pqr	-	16bce	-	S
562561	13.80ff	-	25ghi	-	S
562565	7.81tu	-	25ghi	-	S
597458	6.03op	5.68d	10b	13b	S
597460	14.33ff	-	42j	-	S
597461	7.60t	-	15bcd	-	S
639586	5.80no	-	10b	-	S
639588	7.75tu	-	17cdef	-	S
639590	7.38st	-	25ghi	-	S
639622	2.41cd	-	10b	-	S
639623	3.49fghi	-	18def	-	S

Table 4.1 (continued).

[a] Rf represents the reproduction factor (Rf= final total egg count/initial inoculum egg count). Rf values less than 1 are considered resistant (R); greater than 1 are considered susceptible (S).

[b] GR represents the gall rating, or the percent of the root system affected by galls, following the schematic of Bridge and Page (1980).

Table 4.2. Galling and reproduction factor of *Meloidogyne enterolobii* observed in roots of *Glycine max* soybean genotypes among two trials. All table values are the mean of five replications of *Glycine max* plant roots inoculated with 2,500 eggs of *M. enterolobii* and evaluated at 60 days post inoculation.

Glycine max **Genotype**	**Trial 1 Rf[a]**	**Trial 2 Rf**	**Trial 1 GR[b]**	**Trial 2 GR**	**Susceptible or Resistant**
LS94-3207	23.01_{nop}	20.96_{gh}	21_{l}	19_{i}	S
S96-2692	30.71_{st}	30.93_{opqr}	37_{s}	40_{p}	S
J87-233	15.56_{fghij}	14.15_{de}	18_{j}	13_{ef}	S
Avery	10.02_{bcd}	12.01_{cd}	14_{g}	14_{fg}	S
Rhodes	30.06_{rst}	29.70_{nopq}	30_{o}	30_{n}	S
S99-2281	11.31_{cde}	6.46_{a}	11_{e}	12_{de}	S
Prichard	30.78_{stu}	28.93_{mnop}	35_{r}	35_{o}	S
S11-20124C	13.77_{efgh}	21.21_{ghi}	6_{b}	9_{c}	S
Manokin	22.72_{mn}	25.09_{jkl}	31_{op}	40_{p}	S
Pershing	19.28_{kl}	20.39_{g}	32_{pq}	27_{m}	S
G01-PR16	24.91_{nop}	29.20_{nop}	35_{r}	40_{p}	S
560IT	35.47_{w}	35.66_{st}	60_{v}	60_{s}	S
JTN-4307	7.32_{ab}	7.41_{ab}	7_{c}	8_{b}	S
Hagood	39.35_{x}	31.56_{pqr}	75_{y}	45_{q}	S
DB04-10836	27.72_{pqr}	25.21_{jkl}	60_{v}	45_{q}	S
Bryan	5.06_{a}	6.67_{a}	3_{a}	7_{a}	S
Delsoy 5710	12.55_{defg}	11.79_{cd}	24_{m}	27_{m}	S
Fowler	31.76_{tuv}	33.18_{rs}	39_{t}	50_{r}	S
Boggs	15.88_{ghij}	13.82_{de}	13_{fg}	19_{i}	S
S99-3181	11.37_{cde}	16.05_{ef}	16_{i}	16_{h}	S
A6381	17.95_{jk}	23.71_{hi}	22_{l}	25_{kl}	S
N7002	34.69_{vw}	37.76_{t}	60_{v}	70_{u}	S
Nathan	9.79_{bcd}	12.27_{cd}	14_{g}	16_{h}	S

Table 4.2 (continued).

S01-9364	32.94tuvw	32.75qrs	50u	45q	S
Kuell	33.15tuvw	27.76lmno	50u	45q	S
Santee	32.81tuvw	27.00klmn	65w	35o	S
N8001	23.69mno	18.35fg	35r	40p	S
Desha	26.94opqr	29.86nopq	73x	70u	S
5002t	9.40bcd	20.64ghi	15h	10d	S
N6002	12.23def	13.37cde	10d	10d	S
Dillon	21.36lm	19.85g	25n	25kl	S
Lonoke	25.94nop	20.34g	35r	35o	S
Graham	26.64opq	31.14pqr	75y	65t	S
Osage	8.37abc	30.01oprq	26no	24k	S
TN4-94	16.77ijk	24.44ijk	15h	22j	S
G93-9009	18.12kl	21.41hi	22l	22j	S
G93-9106	14.36efghi	12.30cd	14g	12de	S
G93-9223	17.24ijk	10.16bc	13fg	10d	S
Benning	29.85pqrs	14.21de	50u	45q	S
A5980	18.42kl	16.40ef	13fg	10d	S
Carver	25.05nop	15.07def	37s	24k	S
Doles	22.94mn	30.43oprq	35r	60s	S
Musen	33.48uvw	25.71jklm	65w	65t	S

[a] Rf represents the reproduction factor value (Rf= final total egg count/initial inoculum egg count). Rf values less than 1 are considered resistant (R); greater than 1 are considered susceptible (S).

[b] GR represents the gall rating, or the percent of the root system affected by galls, following the schematic of Bridge and Page (1980).

Figure 4.1. Left image: Resistant *Glycine soja* genotype ‘407239’ root system inoculated with *Meloidogyne enterolobii*. Right image: Susceptible *Glycine soja* genotype ‘479751’ root system inoculated with *M. enterolobii*. Both images are roots photographed 60 days post inoculation. (Photos: T. Schwarz)

Figure 4.2. Left image: Susceptible *Glycine max* genotype 'Bryan' root system inoculated with *Meloidogyne enterolobii.* Right image: Susceptible *Glycine max* genotype 'Hagood' root system inoculated with *M. enterolobii*. Both images are roots photographed 60 days post inoculation. (Photos: T. Schwarz)

CHAPTER 5

Evaluation of weed species for host status to the root-knot nematodes *Meloidogyne enterolobii* and *M. incognita* race 4

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ABSTRACT

Weeds that compete with crops can also host plant-parasitic nematodes, acting as a source of nematode inoculum in a field and further damaging crops. The host status of ten weed species commonly found in North Carolina, USA was determined to the root-knot nematodes *Meloidogyne enterolobii* and *M. incognita* race 4 in the greenhouse. Each weed species was challenged with 5,000 eggs/plant of either *M. enterolobii* or *M. incognita* race 4, with five replicate plants per treatment in two separate greenhouse trials. Root galling severity and total number of nematode eggs per root system were recorded 60 days after inoculation. Reproduction factor (Rf = final nematode population/ initial nematode population) was calculated to determine the host status of each weed species to *M. enterolobii* and *M. incognita* race 4. Four weed species (*Datura stramonium, Digitaria sanguinalis*, *Senna obtusifolia*, and *Cyperus esculentus*) were poor hosts (Rf < 1) to both nematode species and roots of these weed plants did not display galling. Four weed species (*Ipomoea hederacea, Amaranthus palmeri, Portulaca pilosa,* and *Ipomoea lacunosa*) were hosts (Rf > 1) to both nematode species, and all had observable root gall formation. *Sida rhombifolia* and *Cyperus rotundus* were poor host to *M. enterolobii* but susceptible hosts to *M. incognita*. This study documents a differential host status of some common weeds to *M. enterolobii* and *M. incognita* race 4, and these results highlight the necessity of managing root-knot nematodes through controlling weeds in order to protect valuable crops.

INTRODUCTION

Weeds are non-desired plants that compete with crops for water, nutrients, sunlight, and space (Chauhan, 2020). Weeds are a global issue in agriculture that require proper management to prevent crop loss (Vila et al., 2021); a 100% yield loss can result if weeds are left uncontrolled (Chauhan, 2020). It is estimated that weeds cause greater crop loss than either pathogens or insects (Fried et al., 2017). Crops with sunlight or nutrient deficiencies caused by the presence of weeds are more prone to reduced yields and are more susceptible to infection by pathogens and disease (Reberg-Horton et al., 2019 Dentika et al., 2021). If weeds are already causing stress on crops, pathogen infection could be exacerbated (Dentika et al., 2021). Weeds can harbor plant pathogens and act as alternate hosts for pathogens, including nematodes, to reproduce and increase causal agent population numbers and subsequently the risk of disease in a succeeding crop, making effective field management more difficult (Rich et al., 2008; Dentika et al., 2021; Lopez et al., 2021).

The global crop loss due to plant-parasitic nematodes is estimated at $157 billion annually (Abdel-Sattar et al., 2020; Singh et al., 2015). The combined effects of weeds and nematodes in a field are detrimental to crop production. Previous research has shown that weeds can host many pathogens including plant-parasitic nematodes; species of *Meloidogyne*, *Heterodera*, *Pratylenchus*, *Helicotylenchus*, and numerous other nematodes can infect and multiply on weeds (Barlow, 2011; Lopez et al., 2021; Ntidi et al., 2012; Rocha et al., 2021). Nematodes can survive on weeds in the presence or absence of a crop, thus providing a source of inoculum for the following crops and increased disease risk (Kaur et al., 2007; Rich et al., 2008). Plant-parasitic nematodes are more difficult to control to an economic threshold at high population numbers (Castagnone-Sereno, 2012). Weeds allow nematodes to multiply, applying greater disease pressure on valuable crops. Methods such as plant genotypic resistance, crop rotation, soil tillage, organic amendments, soil solarization, and biocontrols have been documented to reduce nematode populations to varying success (Zasada et al., 2010); however, these tactics may be negligible if plant-parasitic nematode populations are surviving and increasing on weeds. In addition, nematicides are an effective tool to manage nematodes (Oka, 2020); yet concerningly, certain weeds have been documented to provide protection for nematodes from nematicides and adverse environmental conditions (Thomas et al., 2004). The presence of weeds as alternative hosts may offset control tactics. Therefore, weed management is vital for effective nematode management.

It is important to understand which weeds are host to plant-parasitic nematodes in order to improve management. *Meloidogyne enterolobii* (Yang and Eisenback, 1983) (syn. *M. mayaguensis*) is a highly virulent root-knot nematode (RKN) species that causes significant damage on a broad range of plants (Castagnone-Sereno, 2012). Considered one of the most damaging RKN species (Ye et al., 2021), *M. enterolobii* is a relatively recently emergent RKN species in the United States, with yet limited knowledge about its interactions with weeds. *Meloidogyne incognita*, the southern root-knot nematode, is widely distributed throughout the world and is one of the most common species of RKN in the United States, including in North Carolina (Schwarz et al., 2020). Like other RKN species, *M. incognita* has a broad host range that includes numerous important global crops. Weeds may be providing a secondary host refuge for these RKN species, causing further difficulties in nematode management and an increase in crop loss. Therefore, the objective of this study was to determine the host status of ten common weed species in North Carolina to *M. enterolobii* and *M. incognita* to identify differences in feeding habits and inform nematode management recommendations.

MATERIALS AND METHODS

Planting, inoculation, and evaluation of weed species for host or non-host status to Meloidogyne enterolobii and M. incognita. Ten species of weeds were evaluated for their host status to the root-knot nematodes *Meloidogyne enterolobii* and *M. incognita* race 4. The weed species tested included: Palmer amaranth (*Amaranthus palmeri*), yellow nutsedge (*Cyperus esculentus*), purple nutsedge (*Cyperus rotundus*), jimsonweed (*Datura stramonium*), large crabgrass (*Digitaria sanguinalis*), entireleaf morningglory (*Ipomoea hederacea*), pitted morningglory (*Ipomoea lacunosa*), pink purslane (*Portulaca pilosa*), sicklepod (*Senna obtusifolia*), and prickly sida (*Sida rhombifolia*). Seed of each weed species were planted in plastic cone containers of dimensions 25.4 cm deep by 3.8 cm wide at the top, containing a 1:1 steam sterilized sand to soil mixture in the greenhouse. Three weeks after planting, each plant was inoculated with either 5,000 eggs of *M. enterolobii* or 5,000 eggs of *M. incognita* by pipetting the inoculum solution into a 3 cm deep depression in the soil at the base of the stem. Each weed species was replicated five times for *M. enterolobii* inoculated plants, and five times for *M. incognita* inoculated plants in each of the two trials, with a single plant considered as a replicate. Plants were maintained in the greenhouse at 25-28°C with daily watering and no supplemental lighting. Eggs of *M. enterolobii* and *M. incognita* race 4 isolates (confirmed using species specific PCR; Schwarz et al., 2020) used as inoculum in each trial were extracted using the NaOCl extraction method of Hussey and Barker (1973) from the roots of 2-month-old cultures maintained on ‘Rutgers’ tomato plants in the greenhouse, quantified under an inverted compound light microscope at 40x magnification, and inoculated onto the weed plants the same day as extraction.

The host status of the weed plants was evaluated 60 days post inoculation. Each plant was destructively harvested and cut at the crown to isolate the roots to assess for root galling and total nematode eggs in each root system. Roots were gently removed from the pots and rinsed free of soil with cool tap water. Visual root gall severity ratings were assigned based on the total percent of the root system galled following the schematic of Bridge and Page (1980). RKN eggs were then extracted from each root system following the NaOCl method described by Hussey and Barker (1973), recovered on a 25 µm mesh sieve, and collected in a final volume of 50 mL with water. The total number of nematode eggs extracted from each root system was determined by counting three,1 mL aliquot of the egg extraction solution at 40× magnification using an inverted compound microscope (Nikon TM, Nikon Instruments, Melville, NY) and averaging the three counts. Reproduction factor (Rf = final egg count/ initial egg inoculum) was calculated and used to determine the host status of each weed. An Rf value less than or equal to 0.01 ($Rf \leq 0.01$) was considered a non-host. An Rf value greater than 0.01 and less than 1.0 ($0.01 > Rf < 1.0$) was considered a poor host. An Rf value greater than or equal to 1 but less than 5 ($1.0 \leq Rf < 5.0$) was considered a host plant, and an Rf value greater than 5 ($Rf > 5$) was considered a good host plant. Host and good host plants were considered susceptible, while non-host and poor-host plants were considered resistant.

*Data analysis***.** Data from Trial 1 and Trial 2 were evaluated separately and all statistical analyses were conducted within RStudio (v. 4.2.1; R Core Team, 2022). The assumptions of normality and homogeneity of variances were evaluated using the Shapio-Wilks (function ‘shapio.test’) and Levene’s test (function ‘leveneTest within the ‘car’ package), respectively. The tests indicated significant deviation from a normal distribution, so a $\log_{10}(x+1)$ transformation was applied to the Rf and eggs per gram of root, which rectified the assumption. All following statistical analysis were performed using the $\log_{10}(x+1)$ transformed data; however non-transformed data are presented in the results to aid in interpretation. To assess for statistical differences between weed species in Rf and eggs per gram of root, treatments were evaluated using analysis of variance with replicate as a random effect. Where significant differences were observed, Fisher's Least Significant Difference test was used to separate means (function 'LSD.test' within the 'agricolae' package).

RESULTS

Weed species response to Meloidogyne enterolobii. Among the ten weed species tested, there were both susceptible (host and good host) and resistant (poor host) weeds to *Meloidogyne enterolobii*. Jimsonweed, large crabgrass, prickly sida, purple nutsedge, sicklepod, and yellow nutsedge were all considered poor hosts to *M. enterolobii,* as each had an Rf value of less than 1.0 but greater than 0.01 (Table 5.1) and were significantly different ($P < 0.001$) from Rf values of weed species evaluated as susceptible hosts. Among these poor hosts, purple nutsedge had the highest Rf value at 0.53 and 0.44 in Trials 1 and 2, respectively (Table 5.1). Pricky sida had the lowest Rf value among the poor hosts at 0.07 and 0.05 in Trials 1 and 2 (Table 5.1). Gall ratings supported the poor host status; the root systems for all replicates determined as poor hosts contained zero observable galls, scored as 0% (Table 5.1). Palmer amaranth was considered a host plant ($1.0 \leq Rf < 5.0$), and entireleaf morningglory, pink purslane, and pitted morningglory were determined to be good hosts, as each had an Rf value greater than 5.0 (Table 5.1). Pink purslane had the highest Rf value (9.64) among susceptible hosts in Trial 1. The lowest Rf value among susceptible host weeds was 2.69 on Palmer amaranth, which still represents a significant increase to the initial inoculum. All weeds considered hosts contained observable root galling on all replicates. The highest gall rating was 50% on pink purslane, and 5% on Palmer amaranth was the lowest gall rating score determined among susceptible plants. The host status determined for each weed species in Trial 1 was consistent with the host status determined in Trial 2. There were no tested weed species considered as non-hosts (Rf $\leq$ 0.01) to *M. enterolobii*.

Weed species response to Meloidogyne incognita race 4. Among the ten weed species tested, there were both susceptible (host and good host) and resistant (poor host) weeds to *M. incognita* race 4. Jimsonweed, large crabgrass, sicklepod, and yellow nutsedge were considered poor hosts, as each had an Rf value between 0.01 and 1.0 (Table 5.2) and were significantly different ($P < 0.001$) from Rf values of the susceptible host plants. Yellow nutsedge had the largest Rf value among poor host weeds to *M. incognita*, with an Rf of 0.24 in both Trial 1 and 2. Jimsonweed had the lowest Rf value among the poor host weeds at 0.06 and 0.04 in Trials 1 and 2, respectively. No root galls were observed on any of the root systems identified as a poor host. Entireleaf morningglory, Palmer amaranth, and prickly sida were demonstrated to be and host plants ($1.0 \leq$ Rf < 5.0). Pink purslane, pitted morningglory, and purple nutsedge were considered susceptible and good hosts, as each had a significant Rf value greater than 5.0 (Table 5.2). Pitted morningglory had the highest Rf value among susceptible weeds at 14.23 in Trial 1 (Table 5.2). Prickly sida had the lowest Rf value among susceptible weeds at 1.80 and 2.04 in Trials 1 and 2, respectively (Table 5.2). Root galling severity ratings supported the designations of a susceptible host status, as all replicates contained observable root galling. Pink purslane had the highest root galling severity score at 60 and 35% in Trials 1 and 2, respectively (Table 5.2), and 5% on entireleaf morningglory, prickly sida, purple nutsedge was the smallest gall rating scored among susceptible host weeds to *M. incognita* (Table 5.2). No weed species tested was considered as a non-host (Rf $\leq$ 0.01) to *M. incognita* race 4.

DISCUSSION

Weeds, or any unwanted plant in a crop production system, are detrimental to agricultural production. For example, annual crop loss due to weeds in North America is estimated at $16 billion for soybean and $27 billion for corn (Soltani et al., 2016; 2017). Weeds may also impact agricultural production through their ability to host plant pathogens such as nematodes. The results obtained in this research indicate that several weeds are host to *M. enterolobii* and *M. incognita* race 4 and thus can increase populations of these nematode species in the soil, and may act as green bridges or pathogen reservoirs for present and future crops. Once RKN become established in a field, it is difficult to eradicate them or reduce their populations to below economic thresholds (Lambert and Bekal, 2002). By providing a refuge for nematodes, weeds can be devastating for effective nematode management, and through the results observed in this study, may have the possibility to "undo" management through other tactics such as chemical or cultural methods.

Two weed species, prickly sida and purple nutsedge, had differential host status depending on the nematode species. Prickly sida was considered a poor host to *M. enterolobii* (Rf values of 0.07 and 0.05; Table 5.1) but susceptible to *M. incognita* (Rf values of 1.80 and 2.04 Table 5.2). Although the Rf values for *M. incognita* on prickly sida are not considerably large, the values are still greater than one and therefore indicate some nematode reproduction occurred. Purple nutsedge was also identified as resistant and a poor host to *M. enterolobii* (Rf values of 0.53 and 0.44; Table 5.1), but susceptible to *M. incognita* (Rf values of 12.87 and 4.36; Table 5.2). The other eight weed species in this study were either a poor or susceptible host to both species of RKN. At a practical level, this differential host response highlights the importance of identifying both the nematode species and weed species present in a field management context, so that risk to the cash crop can be evaluated.

Results of the present study corroborate previous research regarding host status of certain weeds to *M. enterolobii* and *M. incognita*. Similar to this present study, Kokalis-Burelle and Rosskopf (2012) found that yellow nutsedge is a poor host to *M. incognita.* Prickly sida was also found to be susceptible to *M. incognita* (Davis and Webster, 2005; Belle et al., 2017), and Kaspary et al. (2022) also determined Palmer amaranth to be susceptible to *M. incognita*. Like in this study, Kaur et al. (2007) concluded that large crabgrass was resistant to *M. incognita* race 4 and *M. enterolobii* (published using the synonym *M. mayaguensis*) and sicklepod as a poor host for *M. incognita*.

However, some discrepancies between the present study and previous work were also identified. Kaur et al. (2007) found sicklepod to be a good host for *M. enterolobii* whereas our research determined sicklepod as a poor host. Belle et al. (2017) found purple nutsedge to be resistant to *M. incognita*, and Mendes et al. (2020) concluded that both yellow and purple nutsedge as susceptible to *M. enterolobii* and *M. incognita*, while the present work determined purple nutsedge as susceptible to *M. incognita* but resistant to *M. enterolobii* (Table 5.1).

Differences in host status of weeds between previous studies and the present study may be due to the genetic variability of weeds (Mangolin et al., 2012; Sterling et al., 2004). With potential for high genetic variability and short life cycles, it may be likely that the seeds from weed populations used in the studies differed genetically and could impact the ability of the nematode to infect the plant. In addition, nematode isolates may have differential phenotype characteristics depending on the origin of the population. For example, Rutter et al. (2021) found that two *M. enterolobii* population isolated from two different states in the Southeastern United

States had differential responses when inoculated to identical sweetpotato genotypes. Further, several previous research studies did not indicate which race of *M. incognita* was used in their study; physiological races of *M. incognita* are distinguished by the ability to infect a standard set of plant species and cultivars (Barker et al. 1985). Differences in the race of the population used may have contributed to varying weed host status results among studies. For example, Schroeder et al. (1993) found that yellow and purple nutsedge were susceptible to *M. incognita* race 3. This present study used the race 4 population and could be a major contributing factor to disparities observed in study conclusions.

Although certain weeds were considered resistant and poor hosts to either *M. enterolobii* or *M. incognita* race 4 in the present study, the weeds still supported a low level of nematode reproduction, as eggs were extracted and quantified from the root system. No weed was determined as a non-host for RKN, herein defined as displaying an Rf ≤ 0.01. Due to this finding, it is the authors' recommendation for producers to manage all weeds regardless of the weed or nematode species present in the field, as nematode reproduction may increase on these poor host weeds over time. To further illustrate the importance of weed management in the field, Barlow (2011) and Thomas et al. (2004) state that yellow and purple nutsedge weeds (*Cyperus* spp.) have a symbiotic relationship with root-knot nematodes. These nutsedge weeds increased production of tubers (the overwintering stage of the nutsedge plant) when infected by root-knot nematodes. The nematodes appear to be able to use these tubers to survive during unfavorable environmental conditions or during a period that lacks a primary host plant. An increase in nutsedge tuber size and multiplication of RKN populations increases the potential for further crop loss, potentially due to nutsedge pressure, nematode pressure, or both. Furthermore, Thomas et al. (2004) found that yellow and purple nutsedge tubers protected RKN from nematicides, allowing a greater proportion of the nematode individuals to survive the nematicide application and increase in population.

This present study reports the host status of ten weeds common in North Carolina to *M. enterolobii* and *M. incognita*, and identified differential host status among several of these weed species for the two RKN species tested. The study also highlights the importance of weed management in the field in order to also manage plant-parasitic nematodes and protect valuable crops. This research contributes to the knowledge of weed-RKN interactions and pathogen management.

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Table 5.1. Host status of ten weed species common in North Carolina, USA to the root-knot nematode *Meloidogyne enterolobii* in two separate greenhouse trials. Weed plants were inoculated with 5,000 eggs of *M. enterolobii* and evaluated for egg production and root galling 60 days post inoculation. Values are the mean of five replicate plants per trial. Values followed by the same letter within the column are not significantly different at the $\alpha = 0.05$ level.

Weed Species	**Rf**[a]		**Gall Rating**[b]		**R/S**
	Trial 1	**Trial 2**	**Trial 1**	**Trial 2**	
Palmer amaranth (*Amaranthus palmeri*)	2.69b	3.35b	5b	5b	S
Yellow nutsedge (*Cyperus esculentus*)	0.13a	0.09a	0a	0a	R
Purple nutsedge (*Cyperus rotundus*)	0.53a	0.44a	0a	0a	R
Jimsonweed (*Datura stramonium*)	0.08a	0.06a	0a	0a	R
Large crabgrass (*Digitaria sanguinalis*)	0.06a	0.07a	0a	0a	R
Entireleaf morningglory (*Ipomoea hederacea*)	5.36b	5.14b	14b	20b	S
Pitted morningglory (*Ipomoea lacunosa*)	8.26b	8.03b	19b	10b	S
Pink purslane (*Portulaca pilosa*)	9.64b	7.18b	50b	25b	S
Sicklepod (*Senna obtusifolia*)	0.15a	0.10a	0a	0a	R
Prickly sida (*Sida rhombifolia*)	0.07a	0.05a	0a	0a	R

[a] Reproduction factor, Rf= final total egg count/initial inoculum egg count. Rf values less than 1 are considered resistant (R) and a poor host; greater than 1 are considered susceptible (S) and a host or good host. No non-host weed species were observed in this study.

[b] Root gall severity rating, the visual estimate of the percent of the root system affected by galls, following the schematic of Bridge and Page 1980.

Table 5.2. Host status of ten weed species common in North Carolina, USA to the root-knot nematode *Meloidogyne incognita* race 4 in two separate greenhouse trials. Weed plants were inoculated with 5,000 eggs of *M. incognita* and evaluated for egg production and root galling 60 days post inoculation. Values are the mean of five replicate plants per trial. Values followed by the same letter within the column are not significantly different at the $\alpha = 0.05$ level.

Weed Species	**Rf**[a]		**Gall Rating**[b]		R/S
	Trial 1	**Trial 2**	**Trial 1**	**Trial 2**	
Palmer amaranth (*Amaranthus palmeri*)	2.84c	3.48c	9b	5b	S
Yellow nutsedge (*Cyperus esculentus*)	0.24a	0.24a	0a	0a	R
Purple nutsedge (*Cyperus rotundus*)	12.87c	4.36c	5a	5b	S
Jimsonweed (*Datura stramonium*)	0.06a	0.04a	0a	0a	R
Large crabgrass (*Digitaria sanguinalis*)	0.08a	0.05a	0a	0a	R
Entireleaf morningglory (*Ipomoea hederacea*)	4.14c	4.40c	5a	10b	S
Pitted morningglory (*Ipomoea lacunosa*)	14.23c	9.84c	10b	10b	S
Pink purslane (*Portulaca pilosa*)	8.79c	11.31c	60b	35c	S
Sicklepod (*Senna obtusifolia*)	0.08a	0.07a	0a	0a	R
Prickly sida (*Sida rhombifolia*)	1.80b	2.04b	5a	5b	S

[a] Reproduction factor, Rf= final total egg count/initial inoculum egg count. Rf values less than 1 are considered resistant (R) and a poor host; greater than 1 are considered susceptible (S) and a host or good host. No non-host weed species were observed in this study.

[b] Root gall severity rating, the visual estimate of the percent of the root system affected by galls, following the schematic of Bridge and Page 1980.

Figure 5.1: A) Sicklepod (*Senna obtusifolia*) resistant to *Meloidogyne incognita* race 4. B) Resistant large crabgrass (*Digitaria sanguinalis*) inoculated with *M. enterolobii* showing no nematode gall formation. C) Pink purslane (*Portulaca pilosa*) infected with *M. incognita* race 4; susceptible host displaying galling damage. D) Entireleaf morningglory (*Ipomoea hederacea*) susceptible to *M. enterolobii* with galling symptoms. (Photos: T. Schwarz)

CHAPTER 6

Characterization of sweetpotato resistance-breaking isolates of *Meloidogyne enterolobii* in a greenhouse bioassay

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ABSTRACT

Sweetpotato is an important global crop with a relatively limited pool of genotypes displaying resistance to the aggressive root-knot nematode, *Meloidogyne enterolobii*. Regarded as highly virulent, this nematode species has been previously reported to infect crops carrying resistance genes effective in managing other species of root-knot nematode. In the present study, phenotypically distinct isolates of *M. enterolobii* that reproduce on sweetpotato cultivars previously regarded as resistant were characterized at three time points across diverse sweetpotato germplasm to better understand host-nematode interactions and begin assessing risk of this resistance breaking strain in the field. Isolates of *M. enterolobii* were previously inoculated on certain resistant sweetpotato genotypes, subsequently developing into 'break isolates'. These were compared to a 'stock isolate' of *M. enterolobii* that had been maintained on susceptible 'Rutgers' tomato plants. Individual isolates were inoculated separately to eight sweetpotato genotypes at a rate of 10,000 eggs per plant to determine if 'break isolate' populations could overcome sweetpotato resistance. Three replicates of the same plant genotype per nematode isolate were evaluated at 60, 120, and 180 days post inoculation. The sweetpotatoes assessed were resistant genotypes 'Bwanjule', 'Centennial', 'Dimbuka-Bukulula', 'Murasaki-29', 'Tanzania', 'Tib-11', and susceptible genotypes 'Beauregard', and 'Covington'. When comparing the two nematode isolates, the 'break isolate' had significantly greater reproduction and galling severity on resistant sweetpotato genotypes than the 'stock isolate', especially at 120 and 180 days post inoculations, suggesting that these 'break isolates' of *Meloidogyne enterolobii* can overcome resistance in sweetpotato genotypes previously effective in inhibiting reproduction. In light of these results, and due to the limited number of sweetpotato genotypes resistant to this nematode species available to producers and plant breeders, the use of

host resistance as a management tool for *M. enterolobii* in sweetpotato should be carefully deployed in the field, in order to preserve the durability of resistance for the long term.

INTRODUCTION

Meloidogyne enterolobii (Yang and Eisenback, 1983) (the guava root-knot nematode) is regarded as one of the most damaging root-knot nematode (RKN) species in the world (Castagnone-Sereno, 2012; Ye, 2021). Tactics involving chemical nematicides to manage RKN can be limited in their use due to environmental concerns, government regulations, and/or economic constraints (Desaeger et al., 2020; Roberts, 1992). One robust solution to manage RKN is through the use of plant resistance genes (Roberts, 1992). In a typical disease interaction on susceptible plants, nematodes parasitize and establish feeding sites inside the roots, undergo development, and significant reproduction (egg laying) can occur to sustain or increase the nematode population (Lambert and Bekal, 2002; Sasser and Carter, 1985). In resistant plant genotypes, however, defense responses disrupt nematode development and reproduction, and populations decline as a result (Lambert and Bekal, 2002; Sasser and Carter, 1985; Williamson and Hussey, 1996). Of concern, *M. enterolobii* has been found to infect and reproduce on plants with resistance genes that are effective against other RKN species, such as the *Mi-1* gene in tomato and the *N* gene in pepper (Castagnone-Sereno, 2012; Kiewnick et al., 2009).

Resistant plant genotypes can be an effective tool to manage RKN in agriculture that is both environmentally sustainable and cost effective compared to other RKN management methods (Brito et al., 2020; Lambert and Bekal, 2002). Although nematode reproduction is greatly reduced on resistant crops compared to susceptible hosts, the resistant plants may still support a reduced quantity of nematode reproduction. Virulent, resistance-breaking nematode populations can arise that have the ability to successfully infect and develop inside the ‘resistant’ root system where maturation of fertile females and reproduction occurs. Virulent root-knot nematode populations have been reported, with populations arising either naturally or through selection. Hajihassani et al. (2022) reported virulent populations of *M. incognita* in Georgia, United States on *Mi*-tomato and Tzortzakakis et al. (2005) reported resistance-breaking populations of *M. incognita* and *M. javanica* on *Mi*-tomato in Greece, both of which were observed to have naturally occurred without previous exposure to resistant tomato plants carrying the *Mi* gene. Virulence can also be selected for in a RKN population through planting genotype or genotypes with the same resistance gene for consecutive seasons (Hajihassani et al., 2022). Kaloshian et al. (1996) found that select populations of *M. incognita* are ‘resistance-breaking’ populations that increase in numbers after continuous exposure to resistant tomato plants carrying the *Mi-1* gene. Castagnone-Sereno et al. (1994) claimed that selection occurred, under laboratory conditions, for virulent *M. incognita* isolates from nonvirulent isolates by repetitive inoculation on resistant tomatoes. It is unknown if selective pressure of resistance genes on nematode populations in RKN-resistant sweetpotato will result in similar virulent RKN populations.

Meloidogyne enterolobii reproduces by mitotic parthenogenesis, which typically leads to clonal offspring (Koutsovoulos et al., 2020). However, genetic variability has been observed within populations of RKN, including *M. enterolobii* (Castagnone-Sereno, 2002; Shao et al., 2020). Root-knot nematodes are polyploid species with diverged genome copies from ecologically distinct environments (Koutsovoulos et al., 2020). The genome structure contributes to diverged homologous gene copies and differential gene expression (Castagnone-Sereno, 2002). Previous research has also indicated that convergent gene copy losses were associated with resistance gene break-down in plants, indicating that RKN gene copy number variations are involved in adaptive processes (Castagnone-Sereno et al., 2019). For example, *M. incognita* isolates from independent lineages varied in gene copy number based on if the nematode population overcame tomato resistance (Castagnone-Sereno et al., 2019). Plant resistance may influence selection within the nematode population for traits that increase nematode virulence (Castagnone-Sereno et al., 1994; Castagnone-Sereno, 2002; Kaloshian et al., 1996), which over time may lead to significant difficulties in field management. To our knowledge, there currently have been no reports of virulent isolates of *M. enterolobii* overcoming resistance genes known to be previously effective against this nematode species. There are limited identified resistant plant genotypes to *M. enterolobii*, therefore the potential of overcoming host resistance is very concerning for management.

In a preceding study, a diverse panel of sweetpotato genotypes were screened for host status to *M. enterolobii* (Schwarz et al., 2021). Upon extraction of nematode eggs from the roots at the end of the study, it was found that minor amounts of eggs were recovered from those sweetpotato genotypes determined as resistant, ranging from 56 to 363 eggs per plant among the study. While the quantity of eggs produced on resistant plants was significantly smaller than the amount extracted from susceptible plants, nonetheless, eggs were recovered which indicated some reproduction occurred within the roots. It was the interest of the researchers to determine if the eggs recovered from these resistant sweetpotato plants were still viable, able to hatch, and cause subsequent infection on the same resistant sweetpotato genotypes. The goal of this present study was to determine if isolates of *M. enterolobii* previously inoculated on resistant sweetpotato genotypes could overcome sweetpotato resistance and cause significant infection and growth of virulent isolates of *M. enterolobii*.

MATERIALS AND METHODS

Source of potential 'break isolates'. In a preceding greenhouse study, sweetpotato genotypes (n=91) were assayed to determine their host status, including genotypes 'Bwanjule', 'Centennial', 'Dimbuka-Bukulula', 'Murasaki-29', 'Tanzania', 'Tib-11', 'Beauregard', and 'Covington' (Schwarz et al., 2021). Plants were inoculated with 10,000 eggs of a field-derived population of *M. enterolobii*, and sixty days post inoculation, the root system of each replicate plant was subjected to a standard NaOCl nematode egg extraction protocol (Hussey and Barker, 1973). Eggs were quantified through manual counting and the total number of eggs recovered from each plant estimated. Plants with less than 10,000 eggs recovered, or a reproduction factor (Rf, final nematode egg count/initial inoculum egg count) of less than 1 were considered resistant; those with an Rf greater than 1 were classified as susceptible. A large quantity of eggs (in some cases upwards of 600,000 eggs, corresponding to an Rf = 60) were extracted from susceptible plant genotypes, while in other cases a small number of *M. enterolobii* eggs were extracted from sweetpotato genotypes determined as resistant (56 to 363 eggs, corresponding to an Rf = 0.0056 and 0.0363, respectively). To test if the eggs recovered from the resistant sweetpotato genotypes were viable, these eggs were then inoculated in the greenhouse back onto healthy sweetpotato plants of the same plant genotype from which they were extracted. Due to the low concentration of eggs in the inoculum, plants were grown for 180 days to allow possible infection and multiplication to occur before being assessed for potential galling severity and egg production. Upon analysis, root galling was observed on all sweetpotato genotypes, indicating infection occurred and the inoculated eggs were indeed viable. The galled roots also suggested that these isolates of *M. enterolobii* were able to cause infection on resistant sweetpotato genotypes. Eggs were then extracted from these roots and maintained on the same sweetpotato genotypes (new, healthy plants) which they were extracted from. These eggs were used as inoculum for the controlled experiments described below.

Time point assay. To assess the ability of *M. enterolobii* populations to overcome sweetpotato resistance at different time points, two greenhouse studies were performed. The populations of *M. enterolobii* that were cultured on specific resistant sweetpotato genotypes (‘Bwanjule’, ‘Centennial’, ‘Dimbuka-Bukulula’, ‘Murasaki-29’, ‘Tanzania’, and ‘Tib-11’) and cultured separately on these same genotypes are referred to here as ‘break isolates’ (BI). A ‘stock isolate’ (SI) of *M. enterolobii*, which originated from an infected sweetpotato field in Wilson County, North Carolina in 2017 and since been maintained on susceptible ‘Rutgers’ tomato plants, was included in order to compare infection rates between nematode populations. The SI nematodes were not previously exposed to resistant sweetpotato genotypes.

The sweetpotatoes used to compare infection of nematode isolates include the resistant genotypes ‘Bwanjule’, ‘Centennial’, ‘Dimbuka-Bukulula’, ‘Murasaki-29’, ‘Tanzania’, and ‘Tib-11’, and two susceptible genotypes ‘Beauregard’ and ‘Covington’ were included as positive controls. Sweetpotato slips 12-15 cm in length were planted in a steam sterilized 1:1 sand: loam topsoil mixture in 15.2 cm diameter clay pots. Two weeks after initial planting of slips, each plant replicate was inoculated with 10,000 eggs of the respective nematode isolate. The plants were maintained in the greenhouse at 25-28°C with daily watering and no supplemental lighting. A set of replicate plants were assessed at 60, 120, and 180 days post inoculation in order to determine if timing is correlated to the ability of nematodes to cause infections. Each time point contained a total of 6 plant replicates for each sweetpotato genotype, with three of those replicates being inoculated with SI nematode eggs and three replications inoculated with BI eggs. At each time point, plants were destructively harvested by cutting at the crown to isolate the roots from the vegetative growth. Root systems were gently washed with water to remove soil. The whole root system of each replicate was weighed, and nematode galling severity was recorded. Galling severity was determined based on the percent of the total root system containing galls, scored from 0-100%. Nematode eggs were then extracted from each root following the NaOCl method of Hussey and Barker (1973). Eggs were collected on a 25 µm pore size sieve and resuspended in 50 mL of water for quantification. A 1 mL aliquot of egg suspension from each root was counted using a compound inverted microscope (Nikon TM, Nikon Instruments, Melville, NY). Total nematode eggs from each root system were calculated by averaging the count of three aliquots and multiplying by the total extraction sample volume (50 mL). Sweetpotato genotype host status was determined based on the reproduction factor (Rf). An Rf value greater than 1 was considered susceptible to *M. enterolobii*; an Rf value less than 1 was classified as resistant. Because sweetpotato genotypes 'Beauregard' and 'Covington' were susceptible in previous trials, there were no BI populations specific to these genotypes. Therefore, these genotypes were inoculated with the BI population from 'Tanzania' for the purpose of evaluating a potential resistance-breaking population on these susceptible plant replicates. The experiment was conducted twice (Trial 1 and Trial 2).

Data Analysis. Data were analyzed using SAS statistical analysis software version 9.4 (SAS Institute, Cary, NC). Data from Trial 1 and 2 were evaluated separately due to different seasonal timing of each trial. In addition, data were analyzed separately for each of the three time points. To determine if significant differences existed regarding the interaction of *M. enterolobii* isolates on sweetpotato genotypes, the response variables of reproduction factor and gall rating data were analyzed using a general linearized model (PROC GLM). Means were separated using Fisher's Least Significant Difference (LSD) procedure ($\alpha = 0.05$) where significant.

RESULTS

There were significant differences ($P < 0.001$) in the infection responses among *M. enterolobii* isolates on sweetpotato genotypes for reproductive factor and gall ratings in both trials, and at each of the three time points (Tables 6.1-6.4). The mean Rf and root galling severity of the three replicate plants for each sweetpotato genotype by nematode isolate are presented in Tables 6.1-6.4.

Sixty days post inoculation. Evidence of the ability of BI to overcome sweetpotato resistance was found at 60 days post inoculation. In Trial 1, the Rf values of BI on resistant sweetpotato genotypes were generally larger than the SI, however, only ‘Centennial’ was significantly different than the other resistant genotypes with an Rf of 1.03 for BI and 0.07 for SI (Table 6.1). Among the BI-inoculated plants in Trial 1, only ‘Centennial’ BI had an Rf value exceed the susceptible threshold of 1.0. Although all other resistant sweetpotato genotypes had Rf values of less than 1.0 for both nematode populations, in each case BI-inoculated plants had numerically more nematode eggs recovered compared to SI-inoculated plants of the same genotype, yet the difference was not statistically significant (Table 6.1). For example, ‘Murasaki-29’ SI had an Rf value of 0.01 compared to 0.23 of the BI. Among the susceptible sweetpotato genotypes ‘Beauregard’ and ‘Covington’, the SI-inoculated plants had a larger Rf value than BI-inoculated plants. ‘Beauregard’ and ‘Covington’ had SI Rf values of 7.44 and 6.67 respectively, while BI Rf value of 5.83 and of 5.61, respectively (Table 6.1). The gall ratings among resistant sweetpotato genotypes in Trial 1 were all zero, meaning that no galls were observed, with the exception of BI on ‘Centennial’, which had a gall severity rating of 5%. Both ‘Beauregard’ and ‘Covington’ were rated as 10% galling for each nematode isolate in Trial 1.

In Trial 2, there was a significant difference in Rf at 60 days between BI-inoculated plants compared to SI-inoculated plants for almost all resistant plant genotypes (Table 6.2). Each BI-inoculated plant had an Rf value greater than 1 with the exception of genotype 'Tib-11', which had an Rf value of 0.03 for 'stock' and 0.33 for 'break' isolates. For example, 'Dimbuka-Bukulula' had an Rf value of 0.04 for SI-inoculated plants and a value of 4.04 for BI-inoculated plants, representing significant multiplication of BI compared to SI. There was no significant difference between 'Beauregard' nematode populations among Rf values, in which SI had a value of 7.68 and BI value of 7.38. There was a significant difference for 'Covington' however, with 'stock' and 'break' isolates having Rf values of 12.40 and 7.71, respectively. In addition, each BI on resistant sweetpotato genotypes had gall ratings greater than that of SI. Zero galls were observed on resistant sweetpotatoes for SI, while each BI had root galling. For example, 'Bwanjule' had 0% galling for SI and 5% galling for BI. Similar to Trial 1, both 'Beauregard' and 'Covington' had 10% galling severity scores for each nematode isolate.

One hundred twenty days post inoculation. There were significant differences in the Rf values at 120 days between the nematode isolates in both trials assessed (Tables 6.1, 6.2). In Trial 1, the SI-inoculated resistant genotypes all had Rf values less than 1, with 'Centennial' having the largest value of 0.83. In comparison, each BI had significantly greater reproduction on sweetpotato roots previously considered resistant (Table 6.1, 6.2). For example, 'Tib-11' had Rf values of 0.09 and 52.67 for SI and BI isolates, respectively (Table 6.1, Figure 6.1). 'Tanzania' had Rf values of 0.01 for SI, and 96.83 for SI in Trial 1 (Figure 6.2). 'Dimbuka-Bukulula' had an Rf value of 195.72 for BI, while only 0.67 for SI (Table 6.1, Figure 6.3). Root galling severity ratings were also significantly greater in BI-inoculated resistant genotypes than SI. Root gall severity ratings of 0% were observed among SI-inoculated resistant plants, while galls were visible on BI-inoculated resistant plants (Table 6.3). The smallest gall ratings for BI were ‘Bwanjule’ and ‘Centennial’ at 5%; the largest gall ratings were 10% in ‘Dimbuka-Bukulula’, ‘Murasaki-29’, ‘Tanzania’, and ‘Tib-11’. There were no statistical differences of Rf values between BI and SI on the susceptible genotypes ‘Beauregard’ and ‘Covington’. ‘Beauregard’ had Rf values of 104.44 and 101.67 for SI and BI, and ‘Covington’ had Rf values of 86.33 and 77.06 for SI and BI, respectively (Table 6.1). However, there were significant differences in nematode gall ratings among the susceptible plants. An average gall rating of 33% was observed in ‘Beauregard’ SI-inoculated plants, while BI had a value of 58%. ‘Covington’ had gall ratings of 20% and 30% for SI and BI, respectively (Table 6.3).

Similar to Trial 1, Trial 2 had significantly more egg production in BI than SI-inoculated resistant genotypes. All resistant genotypes inoculated with SI had Rf values of less than 1.0 at 120 days post inoculation, meanwhile, Rf values among BI-inoculated resistant plants were greater than 1.0. For example, ‘Bwanjule’ had Rf values of 0.13 and 31.51 for SI and BI, respectively (Table 6.2). Gall ratings were significantly different between the nematode isolates; all SI-inoculated resistant plants had zero galling among the roots (Table 6.4). Galls were observed on all BI-inoculations, with ‘Murasaki-29’ having the largest rating of 10%; the other resistant genotypes had a 5% rating (Table 6.4). The Rf values of ‘Beauregard’ and ‘Covington’ were not significantly different between SI and BI-inoculated plants. ‘Beauregard’ had values of 26.52 and 26.26, and ‘Covington’ had Rf values of 34.52 and 37.73 for SI and BI, respectively (Table 6.2). Gall severity ratings were also significantly greater in BI than SI on susceptible genotypes. ‘Beauregard’ had average galling values of 25% and 33% for SI and BI, respectively, and ‘Covington’ had a galling value of 30% for SI and 42% for BI-inoculated plants (Table 6.4).

One hundred eighty days post inoculation. There were significant differences of Rf values between nematode isolates at 180 days post inoculation and at this time point was the first instance of SI having Rf values greater than 1.0 on resistant sweetpotato genotypes. In Trial 1, only SI on 'Tanzania' had an Rf of less than 1 (0.78). The other SI had values greater than 1.0; for example, 'Bwanjule' had a value of 2.06, 'Murasaki-29' with an Rf of 1.25, and 'Tib-11' had a value of 8.67 (Table 6.1). However, these values were significantly less than those of the BI-inoculated resistant plants. 'Bwanjule' BI had an Rf value of 86.56, 'Murasaki-29' with a value of 97.33 (Figure 6.4), and 'Tib-11' with a value of 78.72 (Table 6.1). At the 180 day time point in Trial 1, root galling was observed in nearly all the resistant genotypes inoculated with SI, yet root gall severity ratings were significantly different between nematode isolates (Table 6.3). Only 'Tanzania' had a root gall severity rating of 0% formed on roots for SI, while resistant genotypes 'Bwanjule', 'Centennial', 'Dimbuka-Bukulula', and 'Murasaki-29' were scored as 1%, and 'Tib-11' had a 5% gall rating for SI in Trial 1 (Table 6.3). All resistant plants inoculated with BI had gall ratings of 10%, with the only exception being 'Tib-11' with a 13% rating (Table 6.3). There were some significant differences between Rf values of susceptible sweetpotato genotypes (Table 6.1). 'Beauregard' had Rf values of 85.01 and 73.72, and 'Covington' with values of 94.94 and 81.11 for SI and BI, respectively (Table 6.1). Among root gall severity ratings in susceptible genotypes, 'Beauregard' SI-inoculated plants were significantly different than BI-inoculated plants, with the former having an average severity score of 25% and the later of 50%. However, gall severity ratings were not significantly different for 'Covington'; SI had an average galling rating of 33% and BI had a value of 37% (Table 6.3).

In Trial 2, all SI Rf values on resistant genotypes at 180 days post inoculation were less than 1.0 with the exception of 'Bwanjule' and 'Dimbuka-Bukulula', which had values of 1.11

and 1.49, respectively (Table 6.2). In comparison, all BI-inoculated resistant genotypes had Rf values greater than 1.0. Concerning the BI on the resistant sweetpotato genotypes, the smallest Rf value was 9.06 on ‘Tanzania’. When compared to the SI Rf value on ‘Tanzania’ of 0.11 there were statistically significant differences between the nematode isolates (Table 6.2). All SI-inoculated resistant genotypes had root gall severity ratings of 0% in Trial 2, while each BI-inoculated plant had significantly greater root galling (Table 6.4). The largest gall rating on BI-resistant genotypes was 20% for ‘Centennial’ and ‘Tanzania’, and the lowest rating was 7% for ‘Tib-11’ (Table 6.4). There were no significant differences between Rf values of either nematode isolate inoculated to susceptible sweetpotato genotypes (Table 6.2). ‘Beauregard’ had Rf values of 23.37 and 27.04, and ‘Covington’ had Rf values of 30.87 and 26.31 for SI and BI, respectively (Table 6.2). Similar to Trial 1, root gall severity ratings were significant different between nematode isolates on the two susceptible genotypes. ‘Beauregard’ had values of 50% and 67% (Figure 6.5), and ‘Covington’ had ratings of 50% and 58% for SI and BI, respectively (Table 6.4).

DISCUSSION

Results from this study indicate that populations of *M. enterolobii* can be selected for in a laboratory setting for virulence to overcome resistance in sweetpotato. In both trials, the *M. enterolobii* ‘break isolates’ (BI) had significantly more reproduction and infection on resistant sweetpotato genotypes than the population of *M. enterolobii* that had not previously passed through resistant sweetpotato genotypes (‘stock isolate’, SI). Each BI population exhibited the ability to reproduce and cause infection on the same plant genotypes in which they were previously collected from, especially notable at the later time points during the study. Root galling severity was also greater on sweetpotato genotypes inoculated with the BI, particularly at the 120- and 180-days post inoculation time points.

The two susceptible sweetpotato genotypes in this study, ‘Beauregard’ and ‘Covington’, yielded similar Rf values between both nematode populations. These susceptible sweetpotato genotypes had significantly greater root galling and *M. enterolobii* egg production compared to resistant genotypes regardless of isolate, although there were instances where it varied on which nematode isolate generated in a larger Rf value among timepoints. Root galling was more severe for susceptible plants infected with BI at 120 and 180 days, with ‘Beauregard’ having a root gall rating of 33% for SI and 58% for BI at 120 days in Trial 1. A report by Rutter et al. (2021) found that two populations of *M. enterolobii* collected from two different states in southeastern United States had differential infection rates when inoculated on the same sweetpotato genotypes, suggesting that differences in reproduction and galling among nematode isolates can occur. However, their study, along with reports by Brito et al. (2020) and Schwarz et al. (2021), had also determined ‘Beauregard’ and ‘Covington’ were susceptible to *M. enterolobii*. These results highlight that the two most widely grown sweetpotato genotypes grown in the United States are susceptible to infection among all *M. enterolobii* isolates screened.

There were instances at the 180-day evaluation time point in which SI reached Rf values greater than 1 in resistant genotypes; in Trial 1, there were 5 out of the 6 resistant sweetpotato genotypes, and 2 out of 6 genotypes in Trial 2 in which this was the case (Tables 6.1, 6.2). The reproduction of the SI on resistant genotypes at 180 days may have been due to age-related decline in plant defense. Alternatively, selection pressure may have led to SI overcoming resistance as observed at 180-days post inoculation in this study. In the field, sweetpotato is harvested typically around 90-120 days post planting (Dr. Craig Yencho, personal communication), and in this study at 120 days post inoculation, SI never reached the Rf threshold on resistant genotypes. Further, duration of the experiment may have influenced infection results. Generally, both SI and BI nematodes increased in Rf value over time, most notably when comparing values at 60 and 180 days. It is suggested that an increased time of exposure of the nematode population to a resistant plant genotype selects for virulence within the nematode population, allowing for infection on resistant gene-carrying plants to occur (Castagnone-Sereno et al., 1994; Castagnone-Sereno, 2002; Kaloshian et al., 1996). Future screening studies may need to perform assessments at longer time points than previously conducted due to increased nematode reproduction over time. For example, sweetpotato host status to RKN has previously been assessed at 56 days (Cervantes-Flores et al., 2002), 60 days (Brito et al., 2020; Rutter et al., 2021; Schwarz et al., 2021), and 90 days post inoculation (Brito et al., 2020). Although an extended time of analysis for host status to RKN may delay plant breeding efforts aimed at developing an elite cultivar with the resistance trait, the present study suggests that extending the duration of the resistance evaluation may lead to more robust host status assessment and ultimately better management of RKN.

Previous research has reported virulent populations of *M. incognita* and *M. javanica* capable of overcoming resistant tomato genotypes in both the field and controlled laboratory (Castagnone-Sereno et al., 1994; Hajihassani et al., 2022; Kaloshian et al., 1996; Tzortzakakis et al., 2005). To our knowledge, this is the first documentation and study of *M. enterolobii* overcoming host resistance previously identified in sweetpotato. Due to the broad host range and limited number of identified resistant germplasm, it is concerning that isolates of *M. enterolobii* are capable of overcoming resistance. Therefore, it is vital that stewardship of these and future resistant plant genotypes be deployed with an eye towards RKN resistance management in order to maintain the durability of these management tools. Crop rotation to non-host crops or to plant genotypes carrying different resistance genes can reduce selection pressure on nematode populations and preserve the efficacy of resistance genes.

In summary, we found that isolates of *M. enterolobii* exhibited differential phenotypic responses on sweetpotato. Future work may evaluate whether these differences in response have a genetic basis. Intraspecific genetic variations have been observed in populations of *M. enterolobii* (Castagnone-Sereno, 2002; Shao et al., 2020); however, these previous reports have not associated the genetic differences to differential phenotype infections on crops. Castagnone-Sereno et al. (2019) found that convergent gene copy losses in *M. incognita* was associated with the ability of the nematodes to overcome resistance in tomato; a genetic investigation may provide insight to whether a similar phenomenon is occurring in 'break isolate' populations of *M. enterolobii*. Furthermore, an experiment could be conducted to assess the ability of the individual break isolates (originating from and continuously maintained on each resistant sweetpotato genotype) to infect other resistant sweetpotato genotypes by assaying against the full panel of resistant sweetpotatoes. This may provide insight into the potential for a break isolate developed on a specific plant genotype to infect other resistant sweetpotato genotypes. Specific gene(s) in sweetpotato responsible for resistance to *M. enterolobii* have yet to be identified. Discovery and characterization of these resistance gene(s), in conjunction with greater knowledge of the potential for *M. enterolobii* to overcome resistance, will be vital in effective long-term management.

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Table 6.1: Reproduction factor (Rf) of *Meloidogyne enterolobii* isolates on sweetpotato genotypes in Trial 1 determined at 60, 120, and 180 days post inoculation. Values are the means of three replicate plants. Values followed by the same letter within each time point are not significantly different at the α = 0.05 level.

	Rf: 60 Days		**Rf: 120 Days**		**Rf: 180 Days**	
Sweetpotato Genotype	**SI**	**BI**	**SI**	**BI**	**SI**	**BI**
Bwanjule	0.06_{a}	0.25_{a}	0.01_{a}	37.67_{b}	2.06_{a}	86.56_{de}
Centennial	0.07_{a}	1.03_{b}	0.83_{a}	39.42_{b}	1.67_{a}	44.00_{c}
Dimbuka-Bukulula	0.01_{a}	0.10_{a}	0.67_{a}	195.72_{g}	8.50_{ab}	137.08_{g}
Murasaki-29	0.01_{a}	0.23_{a}	0.01_{a}	43.62_{b}	1.25_{a}	97.33_{f}
Tanzania	0.01_{a}	0.34_{a}	0.01_{a}	96.83_{e}	0.78_{a}	117.67_{g}
Tib-11	0.02_{a}	0.30_{a}	0.09_{a}	52.67_{b}	8.67_{ab}	78.72_{cde}
Beauregard	7.44_{d}	5.83_{c}	104.44_{f}	101.67_{f}	85.01_{def}	73.72_{cd}
Covington	6.67_{d}	5.61_{c}	86.33_{de}	77.06_{d}	94.94_{ef}	81.11_{cdef}

Reproduction factor: Rf= final nematode egg count/initial inoculum egg count; 10,000 eggs initial inoculum.

SI: 'Stock isolate' of *Meloidogyne enterolobii* collected from an infected sweetpotato field in Wilson County, North Carolina in 2017 and since been maintained on susceptible 'Rutgers' tomato plants; not previously exposed to resistant sweetpotato genotypes.

BI: 'Break isolate' of *M. enterolobii* that was cultured on each of the respective resistant sweetpotato genotypes.

Table 6.2: Reproduction factor (Rf) of *Meloidogyne enterolobii* isolates on sweetpotato genotypes in Trial 2 determined at 60, 120, and 180 days post inoculation. Values are the means of three replicate plants. Values followed by the same letter within each time point are not significantly different at the α = 0.05 level.

	Rf: 60 Days		**Rf: 120 Days**		**Rf: 180 Days**	
Sweetpotato Genotype	**SI**	**BI**	**SI**	**BI**	**SI**	**BI**
Bwanjule	0.01a	2.88b	0.13a	31.51de	1.11a	14.12b
Centennial	0.03a	2.35b	0.15a	12.72b	0.04a	22.47bc
Dimbuka-Bukulula	0.04a	4.04b	0.09a	16.33b	1.49a	9.26b
Murasaki-29	0.04a	3.06b	0.12a	20.06bc	0.02a	29.26cd
Tanzania	0.05a	3.60b	0.10a	30.50de	0.11a	9.06b
Tib-11	0.03a	0.33a	0.28a	13.96b	0.04a	10.42b
Beauregard	7.68c	7.38c	26.52cd	26.26cd	23.37bc	27.04c
Covington	12.40d	7.71c	34.52e	37.73e	30.87cd	26.31c

Reproduction factor: Rf= final nematode egg count/initial inoculum egg count; 10,000 eggs initial inoculum.

SI: 'Stock isolate' of *Meloidogyne enterolobii* collected from an infected sweetpotato field in Wilson County, North Carolina in 2017 and since been maintained on susceptible 'Rutgers' tomato plants; not previously exposed to resistant sweetpotato genotypes.

BI: 'Break isolate' of *M. enterolobii* that was cultured on each of the respective resistant sweetpotato genotypes.

Table 6.3: Root galling severity of *Meloidogyne enterolobii* isolates on sweetpotato genotypes in Trial 1 determined at 60, 120, and 180 days post inoculation. Values are the means of three replicate plants. Values followed by the same letter within each time point are not significantly different at the α = 0.05 level.

	GR: 60 Days		**GR: 120 Days**		**GR: 180 Days**	
Sweetpotato Genotype	**SI**	**BI**	**SI**	**BI**	**SI**	**BI**
Bwanjule	0_{a}	0_{a}	0_{a}	5_{b}	1_{a}	10_{bc}
Centennial	0_{a}	5_{ab}	0_{a}	5_{b}	1_{a}	10_{bc}
Dimbuka-Bukulula	0_{a}	0_{a}	0_{a}	10_{c}	1_{a}	10_{bc}
Murasaki-29	0_{a}	0_{a}	0_{a}	10_{c}	1_{a}	10_{bc}
Tanzania	0_{a}	0_{a}	0_{a}	10_{c}	0_{a}	10_{bc}
Tib-11	0_{a}	0_{a}	0_{a}	10_{c}	5_{ab}	13_{c}
Beauregard	10_{b}	10_{b}	33_{e}	58_{f}	25_{d}	50_{f}
Covington	10_{b}	10_{b}	20_{d}	30_{e}	33_{e}	37_{e}

Root gall severity rating (GR) severity rating, the visual estimate of the percent of the root system affected by galls, rated from 0-100%.

SI: ‘Stock isolate’ of *Meloidogyne enterolobii* collected from an infected sweetpotato field in Wilson County, North Carolina in 2017 and since been maintained on susceptible ‘Rutgers’ tomato plants; not previously exposed to resistant sweetpotato genotypes.

BI: ‘Break isolate’ of *M. enterolobii* that passed through each of the respective resistant sweetpotato genotypes.

Table 6.4: Root galling severity of *Meloidogyne enterolobii* isolates on sweetpotato genotypes in Trial 2 determined at 60, 120, and 180 days post inoculation. Values are the means of three replicate plants. Values followed by the same letter within each time point are not significantly different at the α = 0.05 level.

	GR: 60 Days		GR: 120 Days		GR: 180 Days	
Sweetpotato Genotype	**SI**	**BI**	**SI**	**BI**	**SI**	**BI**
Bwanjule	0a	5b	0a	5b	0a	10b
Centennial	0a	5b	0a	5b	0a	20c
Dimbuka-Bukulula	0a	5b	0a	5b	0a	10b
Murasaki-29	0a	5b	0a	10c	0a	20c
Tanzania	0a	7bc	0a	5b	0a	10b
Tib-11	0a	5b	0a	5b	0a	7b
Beauregard	10c	10c	25d	33e	50d	67f
Covington	10c	10c	30e	42f	50d	58e

Root gall severity rating (Gr) severity rating, the visual estimate of the percent of the root system affected by galls, rated from 0-100%.

SI: 'Stock isolate' of *Meloidogyne enterolobii* collected from an infected sweetpotato field in Wilson County, North Carolina in 2017 and since been maintained on susceptible 'Rutgers' tomato plants; not previously exposed to resistant sweetpotato genotypes.

BI: 'Break isolate' of *M. enterolobii* that passed through each of the respective resistant sweetpotato genotypes.

Figure 6.1: Sweetpotato genotype 'Tib-11' at 120 days post inoculations. Left image- break isolate of *Meloidogyne enterolobii* displaying root galling damage. Right image- stock isolate of *M. enterolobii* with no root galling symptoms. (Photos: T. Schwarz)

Figure 6.2: Sweetpotato genotype ‘Tanzania’ pictured at 120 days post inoculations. Left image- root galling of a break isolate of *Meloidogyne enterolobii*. Right image- stock isolate of *M. enterolobii* with no root galling symptoms. (Photos: T. Schwarz)

Figure 6.3: Sweetpotato genotype 'Dimbuka-Bukulula' pictured at 120 days post inoculation. Left image- break isolate of *Meloidogyne. enterolobii* displaying root galling damage. Right image- stock isolate of *M. enterolobii* with no root galling symptoms. (Photos: T. Schwarz)

Figure 6.4: Sweetpotato genotype ‘Murasaki-29’ pictured at 180 days post inoculations. Left image- galling induced by a break isolate of *Meloidogyne enterolobii*. Right image- stock isolate of *M. enterolobii* with no galling symptoms. (Photos: T. Schwarz)

Figure 6.5: Sweetpotato genotype 'Beauregard' pictured at 180 days post inoculation. The root system on the left represents *Meloidogyne enterolobii* stock isolate infection; root system on right is *M. enterolobii* break isolate infection. (Photos: T. Schwarz)

CHAPTER 7

Soilborne co-conspirators: Interaction between *Ceratocystis fimbriata* and the root-knot nematode *Meloidogyne enterolobii* on sweetpotato

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ABSTRACT

Sweetpotato (*Ipomoea batatas* (L.) Lam) is a nutrient rich, important global crop that can be greatly damaged by soilborne pathogens. In North Carolina, the fungal pathogen *Ceratocystis fimbriata* and root-knot nematode *Meloidogyne enterolobii* are both currently present. The objective of this study was to determine if *M. enterolobii* and *C. fimbriata* may act in a synergistic way to cause disease through a disease complex in sweetpotato. In two independent greenhouse trials, ten replicate plants of each sweetpotato genotype, 'Covington' and 'Murasaki-29', were subjected to the following pathogen treatment groups: no pathogen control, single inoculation of *C. fimbriata*, single inoculation of *M. enterolobii*, or simultaneous inoculation with *C. fimbriata* and *M. enterolobii*. A fifth treatment group of a time-staggered inoculation of *C. fimbriata* 30 days after *M. enterolobii* inoculation was included in the second trial. The sweetpotatoes were evaluated for *C. fimbriata* disease incidence, nematode root galling and egg count, and vegetative shoot and root weights sixty days after inoculation. Koch's postulates for *C. fimbriata* was completed on nematode galls collected from plants treated with *M. enterolobii*. After evaluation, plants inoculated with *M. enterolobii* prior to *C. fimbriata* in Trial 2 had a 100% *C. fimbriata* disease incidence, compared to 70-90% incidence when both pathogens were inoculated simultaneously. Additionally, root galling severity was found to be numerically greater in plants co-inoculated with both pathogens, yet nematode egg counts were significantly lower in co-inoculated plants when compared to plants inoculated with *M. enterolobii* alone. Vegetative growth weight was also significantly lower in co-inoculated plants, although few differences between the root weights of co-inoculated plants and control plants were found. Finally, completion of Koch's postulates confirmed that discolored, necrotic root tissue isolated from the root galls caused by *M. enterolobii* were infected with *C. fimbriata*. This is the first

known study into the interaction between *M. enterolobii* and *C. fimbriata* in any host, and the interaction when both pathogens are present appears to be favorable to *C. fimbriata*, unfavorable for *M. enterolobii* life cycle completion, and can lead to reduced vegetative growth in sweetpotato. Further research is necessary to fully understand the relationship of these pathogens when co-infecting sweetpotato host plants.

INTRODUCTION

Sweetpotato (*Ipomoea batatas* (L.) Lam) is a high value, nutrient rich, and important global crop. In 2022, the United States harvested 132,200 acres, valued at approximately $598.5 million (USDA-NASS, 2022a), for sale in both domestic and export markets. The nation's largest sweetpotato producing state, North Carolina, accounted for approximately 64% of the total annual harvest (USDA-NASS, 2022b). Disease management in sweetpotato is vital for efficient production, as sweetpotatoes can host numerous pathogens that may result in yield losses (Clark et al., 2009). Storage roots, which are the harvested product of sweetpotato plants, are formed underground and are especially at risk to soilborne diseases that directly decrease the quality and quantity of marketable yield. Among the risks, the soilborne fungal pathogen *Ceratocystis fimbriata* and the root-knot nematode *Meloidogyne enterolobii* can be devastating to sweetpotato production, and both are currently present in North Carolina (Schwarz et al., 2021; Scruggs et al., 2017; Ye et al., 2013).

Ceratocystis fimbriata is a soilborne ascomycete fungus that was first characterized in 1890 affecting sweetpotato plants in New Jersey (Halsted, 1890). This pathogen has a diverse host range and is known to infect hosts in more than 20 plant families and is present in over 50 countries (CABI, 2020). *Ceratocystis fimbriata* causes sweetpotato black rot, a disease that can compromise marketable yield. While black rot can be a severe postharvest issue where symptoms are more easily noticed on sweetpotato storage roots, the disease can begin during the growing season and symptoms may appear on sweetpotato slips, stems, and roots (Clark et al., 2009). Signs and symptoms of black rot on slips and stems include dark necrotic lesions on below-ground tissue of the plant, and above ground symptoms of stunting, wilting, chlorosis, and senescence may also be present (Clark and Moyer, 2013). When storage roots are infected, the roots will develop a firm rot in the form of shallow, expanding circular lesions ranging from gray to greenish-black in color, making the yield unmarketable (Clark and Moyer, 2013). Although not necessary for infection, *C. fimbriata* lesions tend to be formed around a wound, lenticel, or lateral roots (Lauritzen, 1926; Stahr and Quesada-Ocampo, 2020). Disease diagnostics data in North Carolina have shown an increase in sweetpotato black rot following the loss of methyl bromide; in 2015, *C. fimbriata* re-emerged and caused a devastating epidemic in the United States (Scruggs et al., 2017).

Meloidogyne enterolobii Yang and Eisenback, 1983, (syn. *M. mayaguensis*), an aggressive root-knot nematode (RKN) species, is a soilborne pathogen with relatively recent emergence in North Carolina and other areas of the Southeastern United States (Brito et al., 2004; Hajihassani et al., 2023; Ye, 2013). *Meloidogyne enterolobii* has a broad host range and is considered one of the most damaging RKN species in the world, causing characteristic root galls, chlorosis, reduced plant vigor, and decreases in quality and quantity of yield (Castagnone-Sereno, 2012; Ye, 2021). In sweetpotato, fibrous and storage roots can be infected by RKN, leading to galls and cracks in the storage roots that further reduce crop value (Lawrence et al., 1986; Overstreet, 2013). Concerningly, *M. enterolobii* is present in important sweetpotato producing regions where *Ceratocystis fimbriata* is also detected, and sweetpotato growers in

North Carolina anecdotally report higher yield losses to black rot in fields with *M. enterolobii* (Dr. Lina Quesada-Ocampo, personal communication). *Meloidogyne enterolobii* and *C. fimbriata* have been documented to be devastating soilborne pathogens to sweetpotato production when present individually; however, no studies have yet investigated the potential for increases in infection rate and disease severity when both pathogens are present, when compared to individual pathogen infection.

Previous studies have implicated several plant-parasitic nematodes and fungi in forming disease complexes, where crop loss is often greater than individual pathogen infection. A study found that *Rhizoctonia solani* was able to infect roots of okra and tomato plants only after *Meloidogyne incognita* invaded the roots (Golden and Van Gundy, 1975). Root decay by *Rhizoctonia solani* was observed 4-5 weeks after nematode infection and the fungus appeared only to penetrate through feeding wounds created by the nematodes, as fungal sclerotia were only formed on nematode gall tissue. The researchers indicated that the fungus was specifically attracted to nematode gall tissue and the sclerotia were selectively formed on nematode galls (Golden and Van Gundy, 1975). In a different study, *Fusarium* spp. occurred in synergy with the RKN species *M. incognita* and *M. javanica*, resulting in increased disease severity in several crops, including tomato and cotton, as opposed to when infected by a single pathogen (Mai and Abawi, 1987). Other studies have also documented interactions among fungi and nematodes leading to yield decline, including *Rhizoctonia solani* and *Ditylenchus dipsaci*, *Phytophthora capsica* and *M. incognita*, *Verticillium dahlia* with *M. incognita* (Hillnhiitter et al., 2011; Katsantonis et al., 2003; Parkunan et al., 2016; Zhang et al., 2020), and the recently discovered interaction of *Ceratocystis fimbriata* with *M. incognita* in pomegranate (Kerakalamatti et al., 2019).

Due to increased losses that can be caused by fungal-nematode disease complexes, it is important to identify and improve our understanding of such interactions for efficient integrated pest management. Two detrimental pathogens, *C. fimbriata* and *M. enterolobii*, have been studied for their individual effects on sweetpotato, however, co-infection has not yet been investigated to our knowledge. Therefore, the objective of this study was to determine if co-infection of sweetpotato with *M. enterolobii* and *C. fimbriata* leads to an increase in disease incidence or severity and whether there is a corresponding response in plant growth.

MATERIALS AND METHODS

Experimental design. Sweetpotato genotypes 'Covington' (*M. enterolobii* susceptible) and 'Murasaki-29' (*M. enterolobii* resistant; Schwarz et al., 2021), were used in this study. Both varieties are grown commercially in North Carolina and have known susceptibility to *C. fimbriata* (Scruggs et al., 2017). Two separate greenhouse bioassays were conducted, and in each twelve to fifteen-cm-long sweetpotato slips, sourced from the North Carolina State University Micropropagation and Repository Unit (Raleigh, NC), were planted in 15 cm deep, 15 cm diameter clay pots containing a 1:1 steam sterilized sand to soil mixture. Plants were maintained in the greenhouse with daily watering and no supplemental lighting. Three weeks after planting slips, each plant was randomly assigned to a treatment group. In the first trial, four treatment groups were included, with 10 replicate plants per sweetpotato cultivar in each treatment group. The pathogen treatments were as follows; no pathogen control, single inoculation of *C. fimbriata*, single inoculation of *M. enterolobii*, and simultaneous inoculation with *C. fimbriata* and *M. enterolobii*, For the second trial, a fifth pathogen treatment group was added to determine if inoculation timing could also influence an interaction between *C. fimbriata* and *M. enterolobii*, as done in other pathogen complex studies (Al-Hazmi and Al-Nadary, 2015; Castillo et al., 1998; Lakshman et al., 2017). In this fifth treatment group, separate inoculation of *C. fimbriata* was performed 30 days after *M. enterolobii* inoculation. Inoculum rates of each pathogen were consistent in each treatment and trial.

Pathogen material and inoculation. Eggs of *M. enterolobii* were used as inoculum and were extracted from 2-month-old roots of ‘Rutgers’ tomato plant cultures grown in a greenhouse, following the NaOCl method described by Hussey and Barker (1973). On the same day of egg collection, sweetpotato plants belonging to treatments containing nematodes were inoculated with 5 mL of aqueous inoculum suspension with 10,000 *M. enterolobii* eggs about 3 cm deep into the soil surrounding the stem of each plant. Ascospores and endoconidia of *C. fimbriata* were used as inoculum for all treatments that included the fungal pathogen. Mycelial plugs of *C. fimbriata* isolate ‘AS186’, kept in long-term storage at -80°C in a 25% glycerol solution (Thermo Fisher Scientific, Waltham, MA), were removed from storage and placed onto fresh carrot agar plates (16% Bolthouse Farms carrot juice, 16 g of agar per liter). The agar plates were kept under fluorescent light at 23°C until growth was observed, upon which hyphal tips of the new culture were transferred to a fresh agar plate. Following the methods of Stahr and Quesada-Ocampo (2021), a mixed suspension of ascospores and endoconidia was collected from 10-day-old *C. fimbriata* plates by adding 2 mL of 5% Tween 20 solution (Sigma-Aldrich, St. Louis, MO) and dislodging the spores using a sterilized metal bacterial spreader. The Tween 20 solution was then collected from the plate via a pipette and the spore concentration of the stock suspension was determined with a hemocytometer. The stock suspension was then diluted with sterile distilled water to 5×10^5 spores/mL, and 5 mL of spore suspension were applied to the soil surrounding the plant stem.

Disease assessment. Sweetpotato plants were assessed sixty days post inoculation. Plants were gently washed free of soil and cut at the crown to isolate the roots from the top vegetative growth (sweetpotato vines). Vegetative and root fresh weights of each plant were recorded. Visual nematode gall severity ratings were recorded based on the total percent of the root system galled, scored from 0-100%, following the schematic of Bridge and Page (1980). Visual ratings for black rot disease incidence were recorded based on signs and symptoms of *C. fimbriata* infection in the entire root system and stem (Stahr and Quesada-Ocampo, 2019). Eggs of *M. enterolobii* were then extracted from each whole root system following the NaOCl method described by Hussey and Barker (1973), recovered on a 25 µm mesh sieve, and collected in 50 mL plastic tubes with water. The total number of eggs per root system were determined for each replicate by averaging the count of three, 1 mL aliquots of egg extraction solution using an inverted compound microscope at 40x magnification (Nikon TM, Nikon Instruments, Melville, NY).

Koch's postulates. During the visual assessment of root systems for RKN gall severity ratings, discolored galls that appeared grey to black in color were observed only in the root systems of plants belonging to the co-inoculated treatment groups. To determine if it was *C. fimbriata* colonizing the galls, discolored galls (n=10) were excised using a scalpel from the root system of three randomly selected plants in both the co-inoculated and *M. enterolobii* only inoculated plants. The excised galls were surfaced sterilized in a 5% bleach solution for 30 seconds, immediately followed by rinsing with sterile distilled water. The sterilized galls were then placed onto carrot agar plates amended with ampicillin and rifampicin (20 mg/liter each) and were observed for fungal growth for up to 14 days. Fungal colonies recovered from the galls were cut from the agar plates, with preference given to colonies resembling *C. fimbriata* as described in Stahr and Quesada-Ocampo (2019). The agar plugs were placed approximately 3 cm deep in soil on the stem of one month old, healthy sweetpotato plants of both 'Covington' and 'Murasaki-29'. Thirty days post inoculation, plants were assessed for *C. fimbriata* infection through visual observation of symptoms.

Statistical analysis. To determine if an interaction between sweetpotato variety and pathogen treatment had any effect on the assessment variables (plant vegetative and root weight, gall rating, *M. enterolobii* eggs per plant, or *C. fimbriata* disease incidence) generalized linear models of each trial were run separately using the GLM procedure in SAS (version 9.4; SAS Institute Inc., Cary, NC). In the generalized linear models, individual assessment variables were treated as the response variable, with sweetpotato variety, pathogen treatment, and an interaction of the two as the fixed effects, and experimental replicate as a random effect. If no interaction between sweetpotato variety and pathogen treatment was found ($P > 0.05$), then the interaction statement was removed from the model and the analysis was run again. When significant interactions were present ($P < 0.05$), the statistical model with the interaction statement was run as a generalized linear mixed model using the GLIMMIX procedure in SAS, and SLICE and SLICEBY statements were used to analyze the simple effects within sweetpotato variety. Means for effects found to be significant ($\alpha = 0.05$) were separated using the LSMEANS statement to determine differences among treatments.

RESULTS

Ceratocystis fimbriata incidence. When assessing *C. fimbriata* incidence as a response variable, significant differences between pathogen treatment groups were found in both trials ($P < 0.0001$). However, the interaction between pathogen treatment and sweetpotato variety was insignificant in Trial 1 (P = 0.3988; Table 7.1) therefore, sweetpotato genotypes were pooled together during the statistical analysis. No *C. fimbriata* disease incidence was observed in the non-inoculated and *M. enterolobii*-only inoculated plants (Table 7.2), regardless of trial.

In Trial 1, 100% of plants inoculated with *C. fimbriata* alone and 95% of the simultaneously co-inoculated plants developed symptoms of *C. fimbriata* infection, although these incidence rates were not statistically different from one another. In Trial 2, where the interaction between pathogen treatment and sweetpotato genotype was significant (P = 0.0039; Table 7.1), incidence amongst plants was analyzed separately for each sweetpotato genotype. For 'Covington' plants, black rot disease incidence among simultaneously co-inoculation and time-staggered co-inoculated plants was 90% and 100%, respectively. This was significantly greater than 'Covington' plants inoculated with *C. fimbriata* only, where black rot disease incidence was 60%. Similar to 'Covington', 100% disease incidence was observed in 'Murasaki-29' plants that were time-staggered co-inoculated. Yet, unlike the 'Covington plants, 100% incidence was observed in the 'Murasaki-29' plants inoculated with *C. fimbriata* only, and significantly less incidence was observed in the simultaneously co-inoculated plants, with an incidence rate of 70%.

Meloidogyne enterolobii eggs and gall ratings. Significant differences between pathogen treatment groups were found when gall severity ratings and the number of nematode eggs extracted from each system were used as the response variable during the statistical analysis of both trials (P < 0.0001; Table 7.1). A significant interaction between pathogen treatment and sweetpotato genotype was also found for *M. enterolobii* egg count in both trials and gall rating in Trial 1. However, the interaction statement was not significant for gall rating in Trial 2 (P =

0.1099; Table 7.1), thus Trial 2 gall ratings from both sweetpotato genotypes were pooled together during statistical analysis.

In both trials, plants in treatment groups that did not include *M. enterolobii* (non-pathogen treatment and *C. fimbriata* only) did not contain any RKN galling or nematode eggs in the root systems, whereas both galling and eggs were observed in treatments which included *M. enterolobii* (Table 7.3). In Trial 1, the average number of nematode eggs recovered in 'Covington' plants simultaneously inoculated with both pathogens (183,430 eggs per root system) and plants inoculated with *M. enterolobii*-only (185,783 eggs per root system) were not statistically different (Table 7.3). However, there was a significant difference in gall ratings; co-inoculated 'Covington' plants had an average gall rating of 10%, while *M. enterolobii*-only 'Covington' plants had a gall rating of 6% (Table 7.3). For 'Murasaki-29' plants in Trial 1, plants simultaneously inoculated with both pathogens had an average of 49,527 eggs per root system and a 1.6% gall rating. When compared to *M. enterolobii*-only inoculated plants, significantly more eggs were found per root system (81,870 eggs per root system) yet the gall rating was statistically less at only 1% gall rating per plant (Table 7.3).

In Trial 2, no significant differences in root gall severity ratings were found between plants inoculated with *M. enterolobii*-only, both pathogens simultaneously, or both pathogens time-separated (Table 7.3). However, significant differences were found between egg counts collected from the different treatments for both sweetpotato genotypes. In 'Covington' plants, there was an insignificant difference of eggs among co-infected plants inoculated at separate times (234,194 eggs per root system) and *M. enterolobii*-only inoculated (248,697 eggs per root system), but significantly less eggs were recovered from co-infected plants inoculated simultaneously (145,480 eggs per root system). Yet, in 'Murasaki-29' plants, the *M. enterolobii*-

only treatment had significantly more eggs per root system (73,801 eggs per root system) when compared to co-infected plants inoculated simultaneously (41,803 eggs per root system) or time-separated inoculation (49,725 eggs per root system; Table 7.3).

Shoot (vegetative) and root growth weight. Significant differences between pathogen treatment groups ($P < 0.0001$), and an interaction between pathogen treatment group and sweetpotato genotype, were found when the weight of above ground vegetative growth was used as the response variable during the statistical analysis of both trials (Table 7.1). Therefore, the simple effects of shoot growth within a sweetpotato genotype were assessed. In Trial 1, non-inoculated 'Covington' plants had an average shoot weight of 23.47 g (Table 7.4), which was statistically greater than plants inoculated with *C. fimbriata* only (17.77 g) and plants that received simultaneous inoculations of both pathogens (14.98 g). Similarly, Trial 1 'Murasaki-29' plants that were not inoculated with any pathogen also had significantly greater shoot weights on average (37.64 g) than all other pathogen treatment groups (Table 7.4). In Trial 2, no significant differences were found between 'Covington' plants that were not inoculated (42.72 g), inoculated with *M. enterolobii* only (50.48 g), or both pathogens time-separated (43.74 g; Table 7.4). However, 'Covington' plants in Trial 2 inoculated with *C. fimbriata* only had significantly more shoot growth (63.06 g) than the non-inoculated plants, while the simultaneously inoculated plants had the least amount of shoot growth (29.82 g; Table 7.4). Among the Trial 2 'Murasaki-29' plants, the shoot growth of treatments inoculated with a single pathogen only were statistically equivalent to the non-inoculated plants which had a weight of 84.29 g (Table 7.4). Yet within the 'Murasaki-29' plants, the average shoot weight of plants inoculated with both pathogens simultaneously (52.28 g) and time-separated (55.79 g) were significantly lower than the shoot weight of the non-inoculated controls (Table 7.4).

When plant root weight was used as the statistical response variable, the interaction statement between pathogen treatment group and sweetpotato genotype was found to be significant only in Trial 1, thus findings from each sweetpotato genotype were pooled for analysis in Trial 2 (Table 7.1). In Trial 1, the root weight of ‘Covington’ plants inoculated with either pathogen individually was not statistically different from the weight of non-inoculated ‘Covington’ plants (73.96 g), while plants simultaneously inoculated with both pathogens had significantly lower root weights on average (54.72 g; Table 7.5). In contrast, roots weights of ‘Murasaki-29’ plants in Trial 1 and the pooled genotype plant weights in trial 2 had identical trends, in which the *C. fimbriata*-only treatment produced the lowest root weights (56.14 g and 74.85 g, respectively) of all pathogen treatments, which were found to be significantly lower than the non-inoculated plants (76.26 g and 88.81 g), respectively. The root weight of all other pathogen treatment groups were not found to be statistically different from the non-inoculated control in the Trial 1 ‘Murasaki-29’ plants or the trial 2 combined genotype plants.

Koch’s postulates. During visual assessment of the sweetpotato root systems, a portion of the nematode galls found in plants inoculated with both pathogens, simultaneously and time-separated, appeared grey or black in color and in some cases necrotic (Figure 7.1), symptoms which appeared consistent with *C. fimbriata* infected sweetpotato tissue. Galls with similar coloration were not observed in the non-inoculated, *C. fimbriata*-only, or *M. enterolobii*-only inoculated plants. When the discolored galls were removed from co-inoculated plants, surface sterilized, and then cultured on amended carrot agar plates, fungal colonies with grey to black mycelia and spore masses resembling *C. fimbriata* were observed growing from the galls within a 14-day period. Such fungal colonies were not observed in cultures prepared from galls collected from the *M. enterolobii* only inoculated plants. When agar plugs with fungal tissue recovered from the nematode galls of *M. enterlobii*-only plants were used to inoculate healthy sweetpotato plants, no symptoms consistent with sweetpotato black rot were observed after 30 days. However, all sweetpotatoes inoculated with fungal tissue recovered from the galls of co-inoculated plants developed classic symptoms of *C. fimbriata* including necrotic stem lesions and shallow, circular lesions on storage roots (Figure 7.2). These results completed Koch's postulates, confirming the presence of *C. fimbriata* within the nematode induced galls.

DISCUSSION

While many studies within plant pathology focus on the roles and responses of a singular pathogen infecting a host plant, there are also many instances of multiple pathogens interacting with one another to cause disease (Lamichhane and Venturi, 2015). For these disease complexes to occur, the interacting pathogens may be as closely related as two species within the same genus (Del Ponte et al., 2014) or can be as distantly related as pathogens from different phyla; as is the case for fungi and nematode interactions (Golden and Van Gundy, 1975; Lakshman et al., 2017). Fungi and nematodes have been documented to interact with one another in disease complexes for over a century (Atkinson, 1892) and the synergy of these pathogen interactions has repeatedly been reported to negatively affect many crops (Zhang et al., 2020). The evidence provided by the two independent greenhouse studies conducted herein demonstrate that an interaction between *M. enterolobii* and *C. fimbriata* on sweetpotato can occur and this interaction could have the potential to reduce sweetpotato yield quantity and quality at greater rates than individual pathogen damage.

There are many mechanisms in which fungi and nematodes can interact with one another and fungal utilization of nematode-induced wounds is one common method (Back et al., 2002).

In the current study, it is hypothesized that plants inoculated with *M. enterolobii* prior to *C. fimbriata*, as was done in Trial 2, would have had a greater number of nematode-induced wounds before fungal pathogen introduction. When plants were inoculated with both pathogens simultaneously, *C. fimbriata* disease incidence in ‘Covington’ and ‘Murasaki-29’ plants were 90 and 70%, respectively. However, when plants were inoculated with *M. enterolobii* first, the *C. fimbriata* incidence rate was 100% in both sweetpotato genotypes, which was found to be a significant increase of black rot disease incidence for ‘Murasaki-29’ plants. Considering the known opportunistic nature of *C. fimbriata* to infect at wound sites (Lauritzen, 1926; Stahr and Quesada-Ocampo, 2020) and the presence of *C. fimbriata* within nematode galls, it is suspected that this mechanism would be the most fitting for the observed pathogen interaction. Yet, the role of other mechanisms cannot be fully eliminated. Chemical exudates released from nematode infected sweetpotato roots and galls could be attracting *C. fimbriata* to the plants, similar to the earlier hypothesis described by Golden and Van Gundy (1975). Physiological changes to the plant may also play a role, as the presence of giant cells caused by *M. enterolobii* and other sedentary endoparasitic root-knot nematodes, can help enable fungal infection and plant decline even from secondary microbial invaders (Mayol and Bergeson, 1969). As *C. fimbriata* is already capable of infecting sweetpotato roots alone, such mechanisms may be not the primary driver for a *M. enterolobii*-*C. fimbriata* interaction but could instead be secondary contributors to the disease complex, though further *in vitro* assays would be required to fully understand the pathogen interaction dynamic.

Although the current study indicates that the interaction between *C. fimbriata* and *M. enterolobii* may be favorable for black rot infection, the opposite may be true for *M. enterolobii*. Instances of nematode populations becoming elevated during co-infection of fungi and nematodes have been recorded, but only in a limited number of studies (Taheri et al., 1994; Back et al., 2002). In the present study, the number of nematode eggs extracted from the root systems of co-inoculated plants was often lower, particularly within the 'Murasaki-29' plants, than when plants were inoculated with *M. enterolobii* alone. For example, in Trial 1, 'Murasaki-29' plants inoculated with *M. enterolobii* alone had an average of 81,870 eggs per root system, compared to 49,527 eggs per root system extracted from co-inoculated plants. Furthermore, in Trial 2, more eggs were extracted in 'Covington' plants inoculated with *M. enterolobii* prior to *C. fimbriata* (234,194 eggs) than when plants were inoculated with both pathogens at the same time (145,480 eggs). Such findings may indicate a negative relationship between nematode egg recovery and the length of time plants have been infected with *C. fimbriata,* which could be a result of *M. enterolobii* being directly affected by *C. fimbriata,* or the decay of roots and giant cells by the fungus preventing the completion of the nematode life cycle.

Other studies have also found that the timing of pathogen inoculation is important for nematode etiology on co-inoculated plants. Al-Hazmi and Al-Nadary (2015) observed a decrease in RKN galling and egg production when nematode inoculation of *M. incognita* preceded the fungus *Rhizoctonia solani* by two weeks. Griffin and Thyr (1988) also observed reduced nematode reproduction and root galling when *M. hapla* and *Fusarium oxysporum* f. sp. *medicaginis* were co-inoculated on alfalfa plants and hypothesized the two pathogens were competing for infection loci or feeding sites. Though not explored in this study, a deeper investigation into the timing of pathogen inoculation, as well as dose responses to varying levels of pathogen inoculum concentration, could help elucidate how *M. enterolobii* is being affected by *C. fimbriata* in sweetpotato roots.

Beyond noticeable pathogen presence among sweetpotato roots, the physiological impact these soilborne pathogens can have on the sweetpotato plants should not be overlooked. In this study, when compared to non-inoculated control plants, the co-inoculated plants consistently had significantly lower vegetative shoot weights, with only the Trial 2 separately inoculated 'Covington' plants as an exception. This was observed even when the shoot weights of sweetpotato plants inoculated with either *M. enterolobii* or *C. fimbriata* alone were found to be no different or significantly heavier than the non-inoculated plants. Part of what makes managing soilborne pathogens so challenging is that infection is often unnoticed until harvest or later (Stahr and Quesada-Ocampo, 2019). While shoot weight is not a consistent indicator for sweetpotato yield at harvest (Gobena, 2022), the observed reduction in vegetative growth for co-inoculated plants may help in detecting disease issues during the growing season if similar infections happen in the field.

Compared to shoot weight, the effect of *M. enterolobii* and *C. fimbriata* co-inoculation on sweetpotato root weight is less clear. Although found to be numerically lower in both trials, the root weight of co-inoculated plants was only statistically significantly lower than the non-inoculated control when comparing the Trial 1 'Covington' plants. However, it is important to consider that sweetpotato plants are generally harvested between 90-120 days after slip planting (Smith et al., 2009) and only storage roots with little to no disease symptoms are kept as the marketable yield. In this study, the plants were evaluated at 60 days post inoculation (approximately 80 days after planting) and full root systems were evaluated for nematode gall rating and root weight. While not quantified separately from the whole root system, there were also instances of black rot lesions directly on the primordial storage roots. It could be speculated that if the study were allowed to progress for between 90 to 120 days (more closely representing time until harvest in a field setting), then the differences in root weight between inoculum treatments could become more prominent, but further research that quantifies marketable weight and yield would be necessary to truly establish the full effect of these two pathogens.

Overall, this study demonstrates an interaction between *M. enterolobii* and *C. fimbriata* through the recovery of *C. fimbriata* from discolored nematode galls. Some variability in the measured growth responses of sweetpotatoes exposed to both pathogens was observed, along with the unexpected ability of *M. enterolobii* to infect previously determined resistant 'Murasaki-29' plants (Schwarz et al., 2021). This may be due to differences in sweetpotato genetics since the sweetpotato plants were obtained from different sources than that of Schwarz et al. (2021). The study findings also suggest that co-inoculation is favorable for *C. fimbriata* infection, unfavorable for *M. enterolobii* life cycle completion, and can lead to reduced vegetative growth. The interaction of these two severe sweetpotato pathogens acts as a reminder that integrated pest management is vital to ensure a marketable sweetpotato yield. This is also the first documentation of an interaction between *M. enterolobii* and *C. fimbriata* in any host, and as such, further research is necessary to truly understand the relationship of these pathogens when co-infecting host plants.

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Table 7.1. Significance levels (*F*- and *P*- values) from generalized linear model analysis for the effects of the interaction between sweetpotato varieties, 'Covington' and 'Murasaki-29', and pathogen inoculation treatment, *Meloidogyne enterolobii (Me)* and *Ceratocystis fimbriata* applied separately or simultaneously, for each assessment variable. Bolded values indicate significance at the α = 0.05 level. *Me*, *Meloidogyne enterolobii*. *Cf*, *Ceratocystis fimbriata*.

Assessment Variable		***Me* Egg Count**	**Root Weight**	**Shoot Weight**	**Gall Rating**	***Cf* Incidence**
Trial 1	F Value	**8.40**	**3.12**	**3.94**	**121.33**	1.00
	Pr > F	**<0.0001**	**0.0321**	**0.0122**	**<0.0001**	0.3988
Trial 2	F Value	**21.86**	1.85	**4.30**	1.95	**4.19**
	Pr > F	**<0.0001**	0.1268	**0.0033**	0.1099	**0.0039**

Table 7.2. *Ceratocystis fimbriata* (*Cf*) incidence for sweetpotato plants inoculated with *Meloidogyne enterolobii* (*Me*) and/or *Cf* separately or simultaneously. No interaction was found between sweetpotato variety and inoculation treatment in Trial 1, thus data collected from both sweetpotato varieties ('Covington' and 'Murasaki-29') were pooled for analysis. Means within a row followed by the same letter are not significantly different ($P \leq 0.05$).

Incidence of *Ceratocystis fimbriata* (%) by Sweetpotato Variety[a]		Inoculation Treatment: Non-inoculated	*Cf* only	*Me* only	*Cf* & *Me* simultaneously	*Cf* & *Me* time separated
Trial 1	Combined	0b	100.00a	0b	95.00a	-
Trial 2	Covington	0c	60.00b	0c	90.00a	100.00a
	Murasaki-29	0c	100.00a	0c	70.00b	100.00a

[a] An interaction was found between sweetpotato variety and inoculation treatment when assessing disease incidence for Trial 2, thus each variety was assessed separately to analyze the simple effects.

Table 7.3. *Meloidogyne enterolobii* (*Me*) egg counts per plant and gall ratings for sweetpotato plants inoculated with *Me* and/or *Ceratocystis fimbriata* (*Cf*) separately or simultaneously. No interaction was found between sweetpotato variety and inoculation treatment for the Trial 2 root gall severity ratings, thus data collected from both sweetpotato varieties ('Covington' and 'Murasaki-29') were pooled for analysis. Means within a row followed by the same letter are not significantly different ($P \leq 0.05$).

	Egg Count per Plant and Gall Rating by Sweetpotato Variety[a]	Inoculation Treatment				
		Non-inoculated	***Cf* only**	***Me* only**	***Cf* & *Me* simultaneously**	***Cf* & *Me* time separated**
Trial 1	Covington Egg Count	0b	0b	185783a	183430a	-
	Murasaki-29 Egg Counts	0c	0c	81870a	49527b	-
	Covington Gall Rating (%)	0c	0c	6b	10a	-
	Murasaki-29 Gall Rating (%)	0c	0c	1.0b	1.6a	-
Trial 2	Covington Egg Count	0c	0c	248697a	145480b	234194a
	Murasaki-29 Egg Counts	0c	0c	73801a	41803b	49725b
	Combined Gall Rating	0b	0b	8.5a	13.25a	10.75a

[a] An interaction was found between sweetpotato variety and inoculation treatment when assessing egg counts for both trials and gall ratings for Trial 1, thus each variety was assessed separately to analyze the simple effects.

Table 7.4. Vegetative shoot weights for the above ground growth of sweetpotato plants inoculated with *Meloidogyne enterolobii* (*Me*) and/or *Ceratocystis fimbriata* (*Cf*) separately or simultaneously. Means within a row followed by the same letter are not significantly different ($P \leq 0.05$).

Shoot Weight (grams) by Sweetpotato Variety[a]		Inoculation Treatment: Non-inoculated	*Cf* only	*Me* only	*Cf* & *Me* simultaneously	*Cf* & *Me* time separated
Trial 1	Covington	23.47a	17.77bc	21.37ab	14.98c	-
	Murasaki-29	37.64a	20.46b	23.44b	25.42b	-
Trial 2	Covington	42.72a	63.06a	50.48b	29.82c	43.74b
	Murasaki-29	84.02ab	74.35bc	99.39a	52.28d	55.79cd

[a] An interaction was found between sweetpotato variety and inoculation treatment when assessing shoot weights for both Trials, thus each variety was assessed separately to analyze the simple effects.

Table 7.5. Root weights for the below ground growth of sweetpotato plants inoculated with *Meloidogyne enterolobii* (*Me*) and/or *Ceratocystis fimbriata* (*Cf*) separately or simultaneously. No interaction was found between sweetpotato variety and inoculation treatment in Trial 2, thus data collected from both sweetpotato varieties (‘Covington’ and ’Murasaki-29’) was pooled for analysis. Means within a row followed by the same letter are not significantly different ($P \leq 0.05$).

Root Weight (grams) by Sweetpotato Variety[a]		Inoculation Treatment				
		Non-inoculated	*Cf* only	*Me* only	*Cf* & *Me* simultaneously	*Cf* & *Me* time separated
Trial 1	Covington	73.96a	72.31a	69.89ab	54.72b	-
	Murasaki-29	76.26a	56.14b	63.71ab	70.27ab	-
Trial 2	Combined	88.81a	74.85b	82.03ab	78.94ab	84.35ab

[a] An interaction was found between sweetpotato variety and inoculation treatment when assessing root weights for Trial 1, thus each variety was assessed separately to analyze the simple effects.

Figure 7.1. The root systems of **A.** ‘Covington’ and **B,** ‘Murasaki-29’ sweetpotato plants co-inoculated with *Meloidogyne enterolobii* and *Ceratocystis fimbriata*. The nematode galls appear necrotic and grey to black in color, consistent with symptoms of *C. fimbriata* infection. (Photos: T. Schwarz)

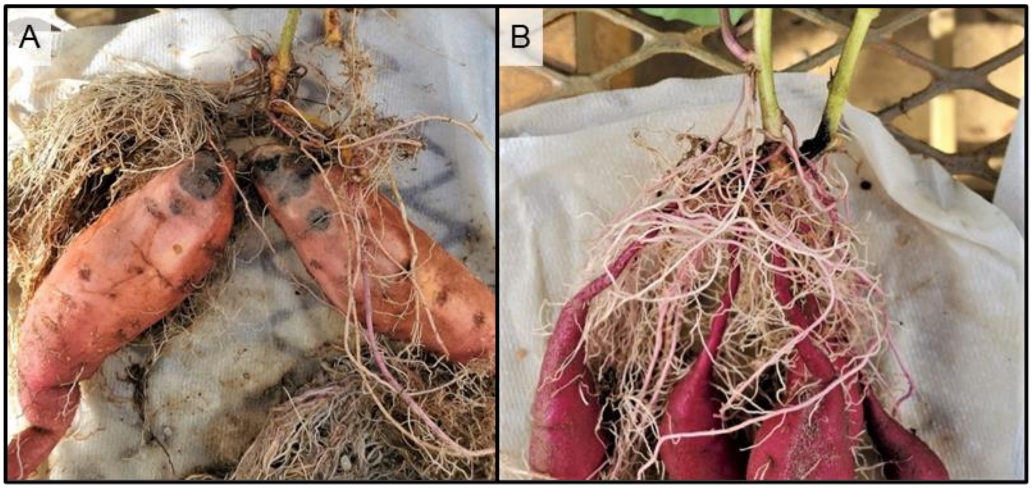

Figure 7.2. Sweetpotato plants 30 days after inoculation with the isolated fungus recovered from discolored *Meloidogyne enterolobii* galls, collected from plants co-inoculated with *M. enterolobii* and *Ceratocystis fimbriata,* to complete Koch's postulates. **A.** 'Covington' sweetpotato exhibiting characteristic grey, circular lesions consistent with sweetpotato black rot caused by *C. fimbriata*. **B,** 'Murasaki-29' sweetpotato plants with necrotic stem tissue at the root crown consistent with sweetpotato black rot. (Photos: T. Schwarz)