ABSTRACT

PHILBRICK, ASHLEY N. Advancing Chemical and Genetic Resistance-based Approaches for the Management of Root-knot Nematodes (*Meloidogyne* spp.) on Tomato. (Under the direction of Dr. Frank Louws and Dr. Adrienne Gorny).

Root-knot nematodes (RKNs), *Meloidogyne* spp., are considered the most economically damaging genus of plant-parasitic nematodes. This is mainly due to their broad host ranges, wide geographic distribution, and overall aggressiveness. There are approximately 100 species of RKNs; *Meloidogyne incognita*, *M. arenaria*, *M. hapla*, and *M. javanica* are the most commonly occurring worldwide. However, *M. enterolobii* is becoming an emerging threat to many crops throughout the world. Most of the reductions in yield and product quality associated with RKNs in North Carolina are caused by *M. incognita* and, more recently, *M. enterolobii*. RKNs infect many important crops including tomatoes, sweetpotatoes, soybean, and pepper. Management of RKNs rely on methods including sanitation, crop rotation, chemical control, and genetic resistance. Chemical nematicides are considered one of the most effective ways to manage RKNs; however due to environmental and health concerns, many of the fumigants previously used for RKN management, such as methyl bromide (an ozone depleting substance), have been or are currently being phased out. Alternately, genetic resistance is considered the most environmentally friendly way to manage RKNs; however current genetic resistance in tomato, mediated by the *Mi* gene, does not confer resistance to all RKN species. Thus, alternatives to fumigant nematicides and novel sources of genetic resistance are critically needed. Ideally, growers should utilize these techniques together as part of an integrated disease management system. Two experiments were conducted to evaluate chemical control and genetic resistance as management strategies for RKNs by 1) evaluating the non-fumigant nematicide NIMITZ (fluensulfone) and grafted tomato plants with resistant rootstock, on root galling severity, RKN

populations, and tomato yield in the field, and 2) evaluating diverse tomato genotypes for resistance to the RKN, *M. enterolobii*. From these studies, NIMITZ and the use of grafted plants were identified as useful alternatives to fumigants to utilize as part of an integrated disease management plan and several potentially resistant genotypes to *M. enterolobii* infection were identified.

Advancing Chemical and Genetic Resistance-Based Approaches for the Management of Root-knot Nematodes (*Meloidogyne* spp.) on Tomato

by
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DEDICATION

I would like to dedicate this paper to my family, who have shown great love and support for me throughout my life and academic journey- especially my grandparents, Dr. Charles Russell Philbrick and Jean Philbrick who have always been incredibly supportive of me.

BIOGRAPHY

Ashley Philbrick was born and raised near Raleigh, NC with dreams of attending NC State University. She graduated cum laude from NC State in May 2019 with a bachelor's degree in Biological Sciences. It was there she discovered her interest in plant pathology by having the opportunity to be an undergraduate research technician in Dr. Dorith Rotenberg's plant-virus-vector lab. Upon graduation, Ashley continued working as a research technician at NC State in Dr. Frank Louws' lab, mentored by Dr. Tika Adhikari, where she decided to pursue a master's degree in Plant Pathology with the lab.

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CHAPTER 1

Literature Review

Introduction to Root-knot Nematodes

Plant-parasitic nematodes (PPNs) are microscopic roundworms that feed on plant cells and are ubiquitous in agricultural soils. One common feature of PPNs is a stylet, which some use to pierce and kill plant cells, such as lesion (*Pratylenchus* spp.), sting (*Belonolaimus* spp.), and stunt (*Tylenchorhynchus* spp.) nematodes. Other PPNs use their stylet to pierce and establish permanent feeding sites in plant cells, such as root-knot nematodes (RKNs, *Meloidogyne* spp.) and cyst nematodes (*Heterodera* spp. and *Globodera* spp.) (Murillo-Williams et al., 2018). Root-knot nematodes (RKNs) are considered the most economically important genus of plant parasitic nematodes (Moens et al., 2009). Root-knot nematodes are widely distributed and have a very broad host range of over 2000 plant species- ranging from annuals to perennials and large acreage field crops to ornamentals (Agrios, 2005; Bleve-Zacheo et al., 2007; El-Sappah et al., 2019). Some important crops infected by RKNs include tomatoes, sweetpotatoes, soybean, and pepper. RKN infection is one of the leading problems faced by tomato growers (Seid et al., 2015; de O Silva et al., 2020). This can have huge implications for the vegetable industry as tomatoes are the second most common vegetable in global food production, with potatoes being the first (El-Sappah et al., 2019). There are approximately 100 species of RKNs; although the most common and widely distributed are: *M. incognita, M. hapla, M. arenaria, and M. javanica* (Moens et al., 2009). However, *M. enterolobii* is becoming an emerging threat to many crops in part due to its expanding geographic distribution and broad host range, but additionally its aggressiveness and lack of current genetic resistance (Philbrick et al., 2020). Current genetic resistance to RKN strains in tomato is mediated by the single dominant *Mi* gene which does not

confer resistance to *M. enterolobii* infection. RKN infection results in stunted growth, wilting, and characteristic galling or "knots" on the roots. These symptoms can reduce fruit quality and ultimately lead to reduced yields (Mitkowski and Abawi, 2003; Agrios, 2005; Moens et al., 2009).

Life Cycle

Root-knot nematodes are obligate biotrophs, thus require a host plant to feed and complete their life cycle (Bleve-Zacheo et al., 2007). Additionally, most of their life cycle takes place within the host plant as sedentary endoparasites. RKNs begin their life cycle as an egg where they undergo the first molt from a first stage juvenile (J1) to second stage juvenile (J2). The infective J2 hatches from the egg, then migrates to the root zone, and uses a combination of physical damage and secretion of enzymes to penetrate behind the root cap with its stylet. RKNs will then establish a permanent feeding site, creating giant cells, and inducing the formation of galls. Giant cells are enlarged, multinucleate cells that reallocate water and other nutrients from the host plant to the nematode. RKNs begin to swell as they molt an additional three times from a J2 to a third stage (J3), fourth stage (J4), then to an adult male or adult female. Adult males remain vermiform and are only formed in adverse conditions (extreme soil temperature, insufficient water, overcrowding, etc.) and will leave the root system to migrate to another host. Adult females remain sedentary and will eventually form as many as 600 eggs which are expelled in a protective, gelatinous matrix into the soil for the cycle to repeat, with several generations possible within a growing season (Mitkowski and Abawi, 2003; Agrios, 2005; Schwarz, 2019).

RKNs can reproduce via amphimixis, facultative meiotic parthenogenesis, or obligate mitotic parthenogenesis. *M. incognita* and *M. enterolobii* both reproduce by obligate mitotic parthenogenesis in which the nucleus splits into two daughter nuclei. This mode of reproduction results in eggs with the same genetic material as the parent. Since males are not required for reproduction and are only formed in adverse conditions, these RKNs are genetically disposed to become female.

Identification Methods

Accurate identification methods are critical for RKN management since they can have important implications in developing an appropriate integrated disease management plan. Conventional methods, like analyzing perineal patterns and other morphological characteristics, have been used in the past to distinguish RKN species. Perineal patterns are the flattened region between the anus and vulva of adult females. Morphological characteristics of adult males, adult females, and juveniles can also be used to distinguish between species. However, these techniques require special expertise and skill for proper identification between species as there have been many reports of misidentification through the years. For example, *M. enterolobii* was first misidentified as *M. incognita* due to similarities in perineal pattern (Moens et al., 2009). Another conventional method is the North Carolina Host Differential test which distinguishes RKN species and races by evaluating their response to various host plants (Moens et al., 2009; Schwarz, 2019). However, some recommend abandoning this technique due to the broad host range of RKNs (Moens et al., 2009). Advances in molecular techniques have provided new tools that are more readily able to identify RKN species.

Molecular techniques for distinguishing RKN species include isozyme analysis, polymerase chain reaction (PCR), and loop-mediated isothermal amplification (LAMP). Isozyme analysis is performed by the staining and visualization of three enzymes (esterase, cellulose acetate, and malate dehydrogenase) via electrophoresis. Root-knot nematode species are identified by which and how many bands can be detected. An advantage to this technique is that it can be utilized with a single adult female. Another effective technique is PCR which amplifies specific segments of DNA. By using species-specific primers, segments of DNA that are characteristic for each RKN species can be amplified and visualized through gel electrophoresis. If a band at the desired size is visible, that RKN species is present. An advantage to this technique is that there are many primers available for most RKN species. Additionally, LAMP assays have been developed to distinguish the 4 major species of RKN: *M. incognita, M. arenaria, M. javanica, and M. hapla* as well as *M. enterolobii* (Elling, 2003). LAMP assays use 4-6 primers that will recognize and amplify regions of the target DNA which can then be detected. An advantage to this technique is it can be used to identify species composition in field settings due to a faster protocol and easier visualization methods compared to PCR. Each of these techniques have advantages and disadvantages but tend to provide more accurate results than conventional methods.

Management Strategies

Spread of RKNs frequently occurs by water, tractors, and other farm equipment, as well as infected planting material (transplants, slips) (Agrios, 2005). Since most of the spread of RKNs is anthropogenic, sanitation is a key component to manage these nematodes. Sanitation is considered a cultural practice which involves preventing the spread of nematodes to non-infested

areas. Once RKNs become established in a field, it is not practically feasible to completely eradicate them (Schwarz, 2019). Another cultural management practice is the use of crop rotation with non-hosts; however, this can be difficult for most growers since they heavily rely on crops within RKNs extensive host range (Mitkowski and Abawi, 2003). When appropriate, some other cultural methods include the addition of organic amendments to encourage the growth of beneficial microorganisms, flooding, and soil solarization, where plastic film is used to cover and heat the soil to reduce RKN populations (Agrios, 2005).

Biologicals can be applied in the form of live microbes or secondary products of microbes with nematocidal properties to manage RKN populations. Several species of bacteria and fungi are known to have antagonistic effects on RKNs (Mitkowski and Abawi, 2003). For example, the bacterium *Pasteuria penetrans* and fungi *Trichoderma harzianum* are known to be obligate parasites of RKN species; the fungi *Dactylella oviparasitica* parasitizes the eggs of RKNs; and the mycorrhizal fungi *Gigaspora* and *Glomus* forms a symbiotic relationship with the host plant (Agrios, 2005). However, a problem associated with the use of biologicals is that it is often not cost-effective to generate and apply the large quantities of microbes needed for the field or efficacy is highly dependent on edaphic soil factors and weather patterns (Mitkowski and Abawi, 2003).

One of the most effective ways to manage RKNs is with the use of chemicals in the form of fumigants and non-fumigants (Agrios, 2005). Fumigants are formulated as either a liquid, gas, or granular particles that volatilize after application, and move through the open pore space in soil as a gas. Some fumigants currently used to manage RKNs are 1,3-dichloropropene (Telone II, Dow AgroSciences), metam sodium (Vapam, Cardinal), and metam potassium (K-pam, Cardinal). Each fumigant treatment typically allows for sufficient RKN management during a

growing season (Agrios, 2005). However, many effective fumigants, such as methyl bromide, have been or are currently being phased out due environmental and health concerns (Ristaino and Thomas, 1997; Martin, 2003). Non-fumigants are formulated as either a liquid or granule that are dissolved in water and are often applied through irrigation systems. Some non-fumigants used to manage RKNs are fluensulfone (NIMITZ, ADAMA, Raleigh, NC, United States) and fluopyram (Velum Prime, Bayer Crop Science, St. Louis, MO, United States).

Genetic resistance conferred by the host is the most economically and environmentally friendly RKN management strategy (Seid et al., 2015; Hajihassani et al., 2020). For RKNs, genetic resistance is defined as a gene or set of genes involved in reducing or preventing reproduction (Bleve-Zacheo et al., 2007). In tomato, the most common source of genetic resistance is conferred by the single dominant *Mi* gene (Mitkowski and Abawi, 2003; Bleve-Zacheo et al., 2007). A total of 10 Mi genes have been identified: *Mi-1, Mi-2, Mi-3, Mi-4, Mi-5, Mi-6, Mi-7, Mi-8, Mi-9, Mi-HT*. However, there are some challenges associated with the resistance in the *Mi* gene. One problem is that this gene is known to break down at high temperatures (above 28C°) (Mitkowski and Abawi, 2003; Bleve-Zacheo et al., 2007) at which RKNs tend to thrive. Another problem is that currently, only the *Mi-1* gene is available in commercially available tomato cultivars. Additionally, the *Mi* gene does not confer resistance to the emerging RKN species, *M. enterolobii,* leaving no known source of resistance to this species (Philbrick et al., 2020). It is also important to note that one source of resistance should not be over-utilized since it will eventually result in the evolution of RKN populations that can overcome this resistance (Barbary et al., 2015).

Integrated disease management involves the use of multiple strategies to keep plant diseases below the economic threshold. The evolutionary arms race occurring between RKNs,

and its host plant have a substantial impact on developing durable management plans. RKNs and other plant pathogens are constantly evolving ways to avoid detection and enhance virulence to the host plant; while the host plant is evolving ways to defend itself. Therefore, integrated disease management is key to preventing and managing RKN populations once they've been established. Due to current challenges with the phasing out of available fumigants, chemical control options are restricted and there is limited known genetic resistance in tomato. Thus, there is a critical need to evaluate new products and new sources of resistance. This thesis will focus on chemical control and genetic resistance further as integral parts of RKN management.

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CHAPTER 2

Meloidogyne enterolobii, a Major Threat to Tomato Production: Current Status and Future Prospects for its Management

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REVIEW
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Meloidogyne enterolobii, a Major Threat to Tomato Production: Current Status and Future Prospects for Its Management

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The guava root-knot nematode, *Meloidogyne enterolobii* (Syn. *M. mayaguensis*), is an emerging pathogen to many crops in the world. This nematode can cause chlorosis, stunting, and reduce yields associated with the induction of many root galls on host plants. Recently, this pathogen has been considered as a global threat for tomato (*Solanum lycopersicum* L.) production due to the lack of known resistance in commercially accepted varieties and the aggressiveness of *M. enterolobii*. Both conventional morphological and molecular approaches have been used to identify *M. enterolobii*, an important first step in an integrated management. To combat root-knot nematodes, integrated disease management strategies such as crop rotation, field sanitation, biocontrol agents, fumigants, and resistant cultivars have been developed and successfully used in the past. However, the resistance in tomato varieties mediated by known *Mi*-genes does not control *M. enterolobii*. Here, we review the current knowledge on geographic distribution, host range, population biology, control measures, and proposed future strategies to improve *M. enterolobii* control in tomato.

Keywords: root-knot nematode, population biology, disease management, RNA interference, gene editing

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INTRODUCTION

Root-knot nematodes are the most widespread soilborne plant pathogen (Agrios, 2005; Perry et al., 2009) and can cause several billion dollars of losses annually (Nicol et al., 2011; Elling, 2013). *Meloidogyne enterolobii* Yang and Eisenback, 1983, known colloquially as the guava root-knot nematode or the pacara earpod tree root-knot nematode (Yang and Eisenback, 1983), is an emerging threat due to its global distribution, wide host range, and the ability to reproduce on tomato genotypes carrying *Mi* resistance genes (Moens et al., 2009; Castagnone-Sereno, 2012). *Meloidogyne enterolobii* alone can cause up to 65% loss, which was higher than any of the other root-knot nematode species examined to date (Castagnone-Sereno, 2012; Castagnone-Sereno and Castillo, 2014). Many farmers may not even realize their fields are infected until the end of the season when crops are harvested, and they observe heavily galled root systems (Schwarz, 2019). Diagnosis of *M. enterolobii* infestation can be challenging due to morphological similarities between it and other root-knot nematode species (Blok and Powers, 2009; Castagnone-Sereno, 2012; Min et al., 2012). In the past, extensive research has been conducted under the International *Meloidogyne* Project (IMP), coordinated by leaders at North Carolina State University to assist developing

countries decrease crop loss attributed to root-knot nematodes. This effort has subsequently generated identification methods and disease management strategies (Sasser et al., 1983). Recently, major research was focused on identifying new sources of genetic resistance to *M. enterolobii* due to the ability of the species to successfully reproduce on crop varieties possessing currently available resistance genes (Hunt and Handoo, 2009; Castagnone-Sereno and Castillo, 2014). Sources of potential genetic and non-host resistance have been identified in tomato (Da Silva et al., 2019), peanut, garlic, grapefruit (Rodriguez et al., 2003), guava (Castagnone-Sereno and Castillo, 2014; Chiamolera et al., 2018), plum, peach (Castagnone-Sereno and Castillo, 2014), and sweetpotato (Schwarz, 2019). Here, we review recent advances in understanding the tomato – *M. enterolobii* pathosystem conducted throughout the world. We will discuss how this progress should facilitate *M. enterolobii* management in tomato production systems. This review will also provide species information and some directions for further research on this aggressive pathogen in tomato production systems.

TAXONOMIC COMPLEXITY

The genus *Meloidogyne* is comprised of approximately 100 species (Hunt and Handoo, 2009; Elling, 2013; Jones et al., 2013). The name "root-knot" refers to the large galls that these nematodes induce on their hosts (Jones et al., 2013). Worldwide, there are four 'major' species of root-knot nematode: *M. arenaria, M. incognita, M. javanica,* and *M. hapla* (Min et al., 2012; Elling, 2013; Jones et al., 2013; Suresh et al., 2019). *M. enterolobii,* initially identified as *M. incognita,* was first discovered in the Chinese pacara earpod tree (*Enterolobium contortisiliquum*) in 1983 (Yang and Eisenback, 1983; Castagnone-Sereno, 2012). In 1988, a species identified as *Meloidogyne mayaguensis* in Puerto Rico was thought to be a new species of root-knot nematode. However, based on morphological and molecular data it was reclassified as *M. enterolobii* in 2004 (Yang and Eisenback, 1983; Castagnone-Sereno, 2012; Elling, 2013; Da Silva and Santos, 2016). The common name, guava root-knot nematode (**Figure 1**), was given because of the significant damage this nematode has caused to guava fruit trees (*Psidium guajava*) in South America (Carneiro et al., 2001; Schwarz et al., 2020).

HOST RANGE AND GEOGRAPHIC DISTRIBUTION

Meloidogyne enterolobii has a broad host range (**Supplementary Table S1**). Only a few crop species (e.g., cabbage, corn, garlic, peanut) and several fruits (e.g., grapefruit, avocado, cashew, citrus, mango, strawberry) have been reported as non-hosts or poor hosts for *M. enterolobii* (Rodriguez et al., 2003; Brito et al., 2010; Freitas et al., 2017). This nematode has been reported worldwide (**Figure 2**) and established mainly in areas with a subtropical to tropical climate (Castagnone-Sereno, 2012; Elling, 2013; de Brita et al., 2018; Kirkpatrick et al., 2018; Da Silva et al., 2019; Overstreet et al., 2019; Schwarz et al., 2020). Although this nematode was originally detected in China, it has now been recorded in several African countries and South America (Elling, 2013; Da Silva et al., 2019). This nematode was also detected in commercial greenhouses in temperate regions in Switzerland (Kiewnick et al., 2009; Castagnone-Sereno and Castillo, 2014; Braun-Kiewnick et al., 2016). In the United States, *M. enterolobii* was first reported in Puerto Rico in 1988 and Florida in 2001 (Brito et al., 2004; Da Silva and Santos, 2016). It has since spread and has been reported in North and South Carolina (Rutter et al., 2019). In North Carolina, samples were originally collected and *M. enterolobii* was identified in 2011, but was not reported until 2013 (Ye et al., 2013). More recently, *M. enterolobii* was found in eight North Carolina counties: Johnston, Harnett, Sampson, Wayne, Greene, Wilson, Nash, and Columbus (Ye et al., 2013; Thiessen, 2018b; Schwarz et al., 2020). This nematode was also recently identified in sweetpotato in Louisiana.

BIOLOGY AND LIFE CYCLE OF *Meloidogyne enterolobii*

Meloidogyne enterolobii is an obligate biotrophic parasite and is not able to complete its life cycle without a living host (Eisenback and Triantaphyllou, 1991; Agrios, 2005; Elling, 2013). As with other root-knot nematodes, this species is an endoparasite, which feeds and matures to the adult stage of the life cycle fully inside host plant tissue (Elling, 2013; Suresh et al., 2019). *Meloidogyne enterolobii* can be distinguished from other *Meloidogyne* spp. based on the morphometrics of females, males, and juvenile stages. The most important diagnostic features are the form of a perineal pattern shape, stylet morphology of males and females, and position of the excretory pore in females; morphology of the head in the male; and the morphometrics of the head and hyaline tail in the second-stage juvenile (J2) (Yang and Eisenback, 1983).

The adult females have a white body and are pear or globe-shaped (Yang and Eisenback, 1983; Castagnone-Sereno and Castillo, 2014). Unlike adult males and J2s, the head of adult females is not distinctly set off from the neck (Yang and Eisenback, 1983; Castagnone-Sereno and Castillo, 2014). The morphometrics of *M. enterolobii* females recorded was average body length including neck 667.2 µm; body width 414.6 µm; neck length 264.8 µm; stylet length 13.4 µm; stylet knob height 2.7 µm; stylet knob width 4.3 µm; dorsal esophageal gland orifice to stylet base 3.7 µm; excretory pore not visible, and the distance from excretory pore to the head end was 178.2 µm (Yang and Eisenback, 1983; Rammah and Hirschmann, 1988). The adult males have a translucent white body and are vermiform, tapering at both ends (Yang and Eisenback, 1983; Castagnone-Sereno and Castillo, 2014). The morphometrics of males was average body length 1,496.4 µm; body width 37.0 µm; stylet length 23.6 µm; stylet knob height 2.6 µm; stylet knob width 4.6 µm; dorsal esophageal gland orifice to stylet base 4.9 µm; excretory pore to head end 165.4 µm; tail length 14.2 µm; and spicule length 28.3 µm (Yang and Eisenback, 1983; Rammah and Hirschmann, 1988). The chief features of the J2s bodies were translucent white and vermiform; truncate head region rounded; slender, and

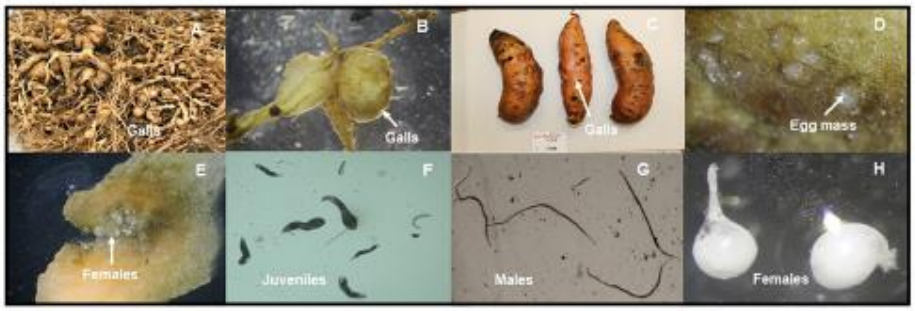

FIGURE 1 | *Meloidogyne enterolobii* individuals and symptoms on different crops in North Carolina, United States (Photos provided by Dr. W. Ye). Large galls and massive root swellings of tomato cv. "Rutger" in the greenhouse. The nematode was originally collected from Greene County in NC **(A)**. Galls on soybean from Johnston County, NC **(B)**. Galls on sweetpotato from Nash County, NC **(C)**. Egg masses on sweetpotato from Nash County, NC **(D)**. Adult females on sweetpotato from Nash County, NC **(E)**. Infective late second-stage juveniles (J2) from soybean in Johnston County, NC **(F)**. Males from soybean from in Wilson County, NC **(G)**. Females from sweetpotato in Johnston County, NC **(H)**.

FIGURE 2 | Geographic distribution of *Meloidogyne enterolobii* across the world. Numbers in parenthesis in each country indicate the year in which the nematode was reported.

narrow tails with pointed tips, and distinct hyaline tail termini (Rammah and Hirschmann, 1988; Brito et al., 2004).

Although variability in the morphometrics characters of J2 among of *M. enterolobii* isolates from different regions and countries were reported (Brito et al., 2004), the average measurements of J2s were body length 436.6 µm; body width 15.3 µm; tail length 56.4 µm; stylet length 13.0 µm; and excretory pore to head end 11.7 µm (Yang and Eisenback, 1983; Rammah and Hirschmann, 1988). Morphometrics obtained from juvenile specimens, and of the relative lengths of body, tail, and functional and replacement odontostylet (Yang and Eisenback, 1983; Rammah and Hirschmann, 1988), suggest the presence of four juvenile stages of *M. enterolobii* (**Figure 3**).

The life cycle of *M. enterolobii* is similar to that of other *Meloidogyne* spp. (Castagnone-Sereno and Castillo, 2014; Kirkpatrick et al., 2018; Overstreet et al., 2019). Adult females lay eggs in a protective gelatinous matrix which is usually expelled out of the root and into the soil (Moens et al., 2009; Perry and Moens, 2011; Elling, 2013; Jones et al., 2013; Castagnone-Sereno and Castillo, 2014; Overstreet et al., 2019). This matrix keeps the eggs together, protecting them from predation and extreme environmental conditions (Moens et al., 2009). After embryogenesis, the nematode develops into a first stage juvenile (J1), then undergoes a first molt to an infective J2, which hatches from the egg and is vermiform (Eisenback and Triantaphyllou, 1991; Chitwood and Perry, 2009; Moens et al., 2009; Elling, 2013; Jones et al., 2013). Hatching is dependent on both the

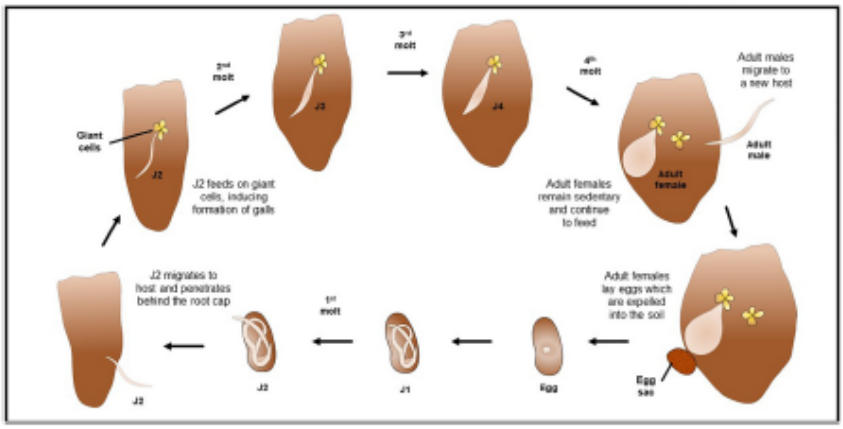

FIGURE 3 | Illustration of the life cycle and root galling of *Meloidogyne enterolobii*.

temperature and moisture conditions of the soil (Moens et al., 2009; Perry and Moens, 2011; Elling, 2013). J2s migrate to a new host's root system and penetrate the root epidermal tissues, usually behind the root cap (Eisenback and Triantaphyllou, 1991; Chitwood and Perry, 2009; Moens et al., 2009; Jones et al., 2013; Kirkpatrick et al., 2018; Overstreet et al., 2019). With a combination of physical damage by propelling their stylets as well as releasing cellulolytic and pectolytic enzymes, these nematodes migrate to the vascular cylinder where they establish permanent feeding sites (Eisenback and Triantaphyllou, 1991; Perry and Moens, 2011; Elling, 2013; Jones et al., 2013; Castagnone-Sereno and Castillo, 2014; Kirkpatrick et al., 2018). These feeding sites are comprised of 'giant cells,' which are responsible for the characteristic galls found on infected root systems (Moens et al., 2009; Elling, 2013; Jones et al., 2013; Castagnone-Sereno and Castillo, 2014; Overstreet et al., 2019). Giant cells are enlarged, multinucleated cells typically arising from plant vascular tissues that provide nutrition to nematodes by reallocating plant metabolites (Eisenback and Triantaphyllou, 1991; Moens et al., 2009; Mitchum et al., 2012). The J2 nematodes molt three more times, to the third (J3), fourth (J4) stages, and then to reproductive adults (**Figure 3**) (Eisenback and Triantaphyllou, 1991; Chitwood and Perry, 2009; Moens et al., 2009; Elling, 2013; Jones et al., 2013; Castagnone-Sereno and Castillo, 2014). The J3 and J4 stages do not feed as they lack a functional stylet (Chitwood and Perry, 2009; Moens et al., 2009; Jones et al., 2013).

Male *M. enterolobii* nematodes are vermiform and leave the root system, and do not feed as adults. However, males of many *Meloidogyne* spp. are only formed in unfavorable conditions, such as extreme soil temperatures, lack of sufficient soil moisture, or situations of overcrowding (Eisenback and Triantaphyllou, 1991; Chitwood and Perry, 2009; Elling, 2013; Jones et al., 2013). Females remain sedentary and continue to feed as they swell and become pear-shaped (Elling, 2013; Jones et al., 2013; Schwarz, 2019). Under favorable conditions, the life cycle of most *Meloidogyne* spp., including *M. enterolobii*, takes about 30 to 35 days to complete and each female can lay up to 600 eggs (Castagnone-Sereno and Castillo, 2014; Da Silva et al., 2019; Overstreet et al., 2019). Several generations of the life cycle may occur throughout the growing season (Chitwood and Perry, 2009).

Meloidogyne spp. can reproduce via amphimixis, facultative meiotic parthenogenesis, and obligate mitotic parthenogenesis (Chitwood and Perry, 2009). *Meloidogyne enterolobii* reproduces via obligate mitotic parthenogenesis or obligatory asexual reproduction (Chitwood and Perry, 2009; Castagnone-Sereno and Castillo, 2014), by which the nucleus separates into two daughter nuclei, having the same genetic material as the original (Chitwood and Perry, 2009). Males are not required for reproduction, but extreme environmental conditions may promote their development from individuals genetically disposed to become female (Eisenback and Triantaphyllou, 1991; Chitwood and Perry, 2009).

IDENTIFICATION METHODS AND POPULATION BIOLOGY

Traditionally, *Meloidogyne* spp. have been characterized through the morphology of adult females and males, including analysis of perineal patterns, which is the shape of cuticle folding around the vulva and anus of adult females. These methods of identification require considerable skill and experience and may otherwise lead to misidentification (Hunt and Handoo, 2009; Min et al., 2012; Elling, 2013). Some of the features of the perineal patterns were useful to separate *M. enterolobii* from other *Meloidogyne* species. In general, the perineal patterns of *M. enterolobii* are oval shape; the dorsal arch is high and round; phasmids are large, and weak lateral lines occasionally present (Karssen and van Aelst, 2001;

Brito et al., 2004). However, perineal patterns within the same species may also vary in individuals from the same population, making identification difficult (Humphreys et al., 2012; Da Silva and Santos, 2016; Suresh et al., 2019). Additionally, *M. incognita* and *M. enterolobii* can have very similar perineal patterns (Humphreys et al., 2012; Schwarz, 2019; Suresh et al., 2019) and *M. enterolobii* was originally thought to be *M. incognita* based on perineal pattern analysis. The perineal pattern of *M. enterolobii* females is an oval shape, dorsal arch usually high and round, weak lateral lines sometimes present, large phasmids and has occasional breaks of striation laterally, and a circular tail tip area lacking striae (Yang and Eisenback, 1983). In addition to their perineal pattern, female root-knot nematodes can be identified to greater taxonomic groups or species by stylet morphology, body shape, or neck length. Males and J2s can be distinguished through body morphometrics or by the morphology of the head and tail (Yang and Eisenback, 1983; Eisenback and Triantaphyllou, 1991; Hunt and Handoo, 2009). However, many *Meloidogyne* species share overlapping measurements and characteristics, making discrimination at the species level difficult (Eisenback and Triantaphyllou, 1991).

Isozyme analysis is a biochemical-based diagnostic method of staining and visualizing esterase, cellulose acetate, and malate dehydrogenase (*Mdh*) isozyme profiles after separation and migration with electrophoresis (Eisenback and Triantaphyllou, 1991; Blok and Powers, 2009). Inter-species variability gives rise to these isozymes, which provide similar catalytic function but diverge in their chemical properties, such as mobility during electrophoresis (Williamson, 1991). *Meloidogyne enterolobii* can be distinguished by the unique pattern of two distinct esterase bands and one malate dehydrogenase band (Brito et al., 2004; Hunt and Handoo, 2009; Pinheiro et al., 2015; Da Silva and Santos, 2016). This method was effective to differentiate and identify young adult females to species, but not for J2s, which are most predominantly found in soil samples (Castagnone-Sereno, 2012; Elling, 2013). Also, it is highly sensitive and can be performed with extracted protein from a single adult female (Eisenback and Triantaphyllou, 1991; Brito et al., 2004; Blok and Powers, 2009). Although isozyme analysis was widely used to identify *Meloidogyne* species (Eisenback and Triantaphyllou, 1991; Blok and Powers, 2009; Hunt and Handoo, 2009; Moens et al., 2009; Castagnone-Sereno, 2012; Elling, 2013; Da Silva and Santos, 2016), this technique requires more than one polymorphic enzyme to confirm the identity of some isolates and the signal of enzyme presence or absence can vary within and across samples.

Species-specific polymerase chain reaction (PCR) assays have been developed and used to differentiate *Meloidogyne* spp. (**Supplementary Table S2**) (Nunn, 1992; Blok et al., 1997, 2002; Zijlstra, 2000; Long et al., 2006; Kiewnick et al., 2013). A sequence characterized amplified region (SCAR) primer set, MK7/F and MK7/R, was used to identify *M. enterolobii* (Ye et al., 2013; Villar-Luna et al., 2015; Schwarz et al., 2020). However, Schwarz et al. (2020) found that the IGS2 primers, MeF/MeR were more specific than the MK7F/MK7R primers. In another study, internal transcribed spacer (ITS) region primers, TW81F/AB28R were used to detect *M. enterolobii* (Suresh et al., 2019). Multiplex PCR has been developed to identify and detect *M. enterolobii, M. incognita,* and *M. javanica* simultaneously using DNA extracted directly from individual galls at various stages of their life cycle (Hu et al., 2011; Elling, 2013). A new quantitative real-time PCR (qPCR) assay that quantifies the amount of nucleic acid present, was developed for the specific detection, identification, and potential quantification of *M. enterolobii* in soil and plant roots (**Supplementary Table S2**) (Toyota et al., 2008; Kiewnick et al., 2015; Sapkota et al., 2016). Additionally, the qPCR assay showed high specificity, sensitivity, and reproducibility (Braun-Kiewnick et al., 2016). A novel satellite DNA family, pMmPet, was discovered in *M. enterolobii*, allowing species-specific identification by PCR, as well as by Southern blot and dot blot analysis (Randig et al., 2009; Elling, 2013). It was shown the satellite repeat was stable among many populations of *M. enterolobii* and high abundancy, allowing for identification of a single individual, thus making it a strong diagnostic tool (Randig et al., 2009).

Loop-mediated isothermal amplification (LAMP) technique that amplifies DNA with high specificity, sensitivity, efficiency, and rapidity under isothermal conditions has been developed (Notomi et al., 2000). Furthermore, LAMP can amplify DNA under isothermal conditions within 1 h using either two or three sets of primers. LAMP assay has been developed and used to identify *M. enterolobii, M. arenaria, M. hapla, M. incognita,* and *M. javanica* (Niu et al., 2011, 2012; Elling, 2013) and has the potential to be used as a simple screening assay in the field (Elling, 2013). High resolution melting curve (HRMC) analysis is a new, post-PCR analysis method, which is simple, fast, and use a single-tube assay method-based on PCR melting (dissociation) curve technique and can discriminate DNA sequences based on their composition, length, and GC content (Reed et al., 2007). HRMC analysis was useful to differentiate different tropical species of *Meloidogyne* (Holterman et al., 2012; Elling, 2013). HRMC technique was also applied to *M. enterolobii* in 2-step nested PCR and single-tube assay and the results showed *M. enterolobii* isolates had different melting peak patterns, with one or two peaks with different heights centered on different melting temperatures, suggesting that the risk of using a fragment that produced multiple amplicons of different length in one species (Holterman et al., 2012). However, evaluating new single copy genes and gene regions in multiplex HRMC assays might be effective to differentiate among isolates of *M. enterolobii* or *M. enterolobii* from other *Meloidogyne* spp. (Holterman et al., 2012; Elling, 2013). Analysis of single nucleotide polymorphisms (SNPs) could be a beneficial low cost and high-throughput tool for *M. enterolobii* diagnosis (Davis et al., 2005; Holterman et al., 2012). Genotyping-by-sequencing (GBS) technique discovers SNPs to whole-genome profiling of association panels (Elshire et al., 2011) and has been used successfully to investigate the phylogenetic genetic relationships of *M. enterolobii, M. incognita,* and *M. javanica* populations in South Africa and identify 34 SNPs that were useful to discriminate between the three *Meloidogyne* species investigated (Rashidifard et al., 2018). The complete genomes of the root-knot species *M. incognita, M. hapla,* and *M. enterolobii* have been sequenced and reported (Abad et al., 2008; Opperman et al., 2008; Koutsovoulos et al., 2019).

Little genetic variation has been observed within the species of *M. enterolobii*, which is likely due to the mode of reproduction through mitotic parthenogenesis (Perry and Moens, 2011). DNA markers were used to test *M. enterolobii* isolates from different geographic regions and hosts and found this species was genetically homogenous (Tigano et al., 2010).

INTEGRATED DISEASE MANAGEMENT (IDM) STRATEGIES

Integrated disease management (IDM) is the simultaneous use of multiple disease management strategies to suppress disease severity or incidence and reduce the pathogen population below the economic threshold level (Ciancio and Mukerji, 2007). Although IDM is an economically and ecologically sound approach, once *M. enterolobii* populations become established, the pathogen can be difficult to manage (Schwarz, 2019; Schwarz et al., 2020). Thus, identifying effective measures and integrating these into disease management plans can delay disease epidemics, reduce disease intensity, and enhance yields. Several management strategies through soil solarization, biological soil disinfestation, biological control, soil amendments, soil flooding, fumigant and non-fumigant nematicide, and host plant resistance have been employed to minimize the effects of this pathogen in crop production worldwide (Zasada et al., 2010; Noling, 2015). Further, a robust and specific diagnostic method to detect *M. enterolobii* would increase food security and improve quarantine measures to support epidemiological studies and the decision-making process of management tactics on tomato worldwide.

In the United States, particularly in North Carolina, *M. enterolobii* is under an internal quarantine, and infected material, or the nematode in any life stage, cannot be moved out of the state. *Meloidogyne enterolobii* is not transferred by tomato seed but it can be spread through sweetpotato and potato "seed" as the seed pieces (parts of the roots or tuber stems) are in contact with the soil and may become infected (Thiessen, 2018b). Thus, growers need to avoid moving infected plant material, infested soil, and contaminated farm-equipment from infested fields with *M. enterolobii* to non-infested areas (Thiessen, 2018b; Schwarz et al., 2020). However, this may be difficult to accomplish due to the high level of agricultural trade between North Carolina and the surrounding states and even international locations. It is important not to plant infected tomato transplants, but planting non-infected clean transplants is essential to avoid infesting new planting fields.

Cultural Control

Cultural practices are non-chemical management tactics such as crop rotation with non-host crops or resistant cultivars, and these tactics are an economical method for nematode management. Crop rotation to non-host crops has a suppressive effect on *M. enterolobii* populations by inhibiting the reproduction and increase of populations through the absence of a favorable host. Rotation to non-hosts for at least 1 year can help reduce nematode populations (Schwarz, 2019). However, the rotation to non-hosts for a minimum of 3 years is recommended for tomato (Seid et al., 2015). Unfortunately, crop rotation has limits due to the broad host range of *M. enterolobii* (Thiessen, 2018a). Peanut, corn, and wheat have shown to be poor hosts for this nematode and can be utilized as rotation crops (Rodriguez et al., 2003; Brito et al., 2004; Elling, 2013; Castagnone-Sereno and Castillo, 2014; de Brita et al., 2018; Thiessen, 2018b; Schwarz et al., 2020). Weed management is another important prevention strategy because many weed species may serve as hosts to *M. enterolobii* (Rich et al., 2008; Thiessen, 2018b). Since nematodes can be easily transferred by water, farm equipment, and plant material, sanitation can prevent moving the pathogen to non-infested fields (Thiessen, 2018b). Other cultural methods such as fallowing soil, soil solarization, steaming, and flooding can be used under conducive circumstances (Seid et al., 2015; Schwarz, 2019). Additional targeted research in cultural control methods such at tillage, crop rotational plans, and soil amendments are needed to support optimal management of *M. enterolobii* in tomato. In addition, rotating tomato with non-hosts such as peanut (*Arachis hypogaea*), sour orange (*Citrus aurantium*), grapefruit (*Citrus paradise*), garlic (*Allium sativum*) (Rodriguez et al., 2003), and maize (*Zea mays*) (Guimaraes et al., 2003) could reduce *M. enterolobii* populations in soil.

Biological Control

Biological control or biopesticide is defined as an application of live microbes (bacteria and fungi) and their gene products, essential oils, plant extracts, individual and mixed acids such as organic and amino acids, natural bioactive substances, and industrial wastes (Seid et al., 2015; Forghani and Hajihassani, 2020). Some bacterial biocontrol agents that are commercially available include *Bacillus firmus* (Bio-Nem-WP/BioSafe, Agrogreen, Ashdod, Israel), *B. firmus* GB-126 (VOTIVO™, Bayer CropScience, Raleigh, NC, United States), *B. amyloliquefaciens* strain IN937a, *B. subtilis* strain GB03 (BioYield, Gustafson LLC, Plano, TX, United States), *Bacillus* spp. (Pathway Consortia, Pathway Holdings, NY, United States), and heat-killed *Burkholderia* spp. strain A396 (BioST™, Albaugh, LLC, IA, United States). These biopesticides have shown a bionematicide activity against eggs, juveniles, and adults and played an important role to manage *Meloidogyne* spp. (Stirling, 2014; Seid et al., 2015; Forghani and Hajihassani, 2020). The most prominent beneficial fungi for managing *Meloidogyne* spp. are *Arthrobotrys* spp. and *Monacrosporium* spp. (Cayrol et al., 1992; Bordallo et al., 2002). These beneficial microorganisms are hematophagous fungi that use sticky mycelia to capture nematodes (Nordbring-Hertz et al., 2006). Some endophytic fungi such as *Paecilomyces* and *Trichoderma* may also trap and kill *Meloidogyne* spp. in the soil or root systems. These beneficial fungi may act at different nematode life stages such as eggs, juveniles, or adults (Schouten, 2016). *Paenibacillus* spp., is one of the growth-promoting rhizobacteria (PGPR) which strongly caused J2 mortality and reduced hatching of several *Meloidogyne* spp. including *M. enterolobii* in tomato (Bakengesa, 2016). Recently, the effects of two egg-parasitic fungi, *Pochonia chlamydosporia* and *Purpureocillium lilacinum* against *M. enterolobii* were assessed *in vitro*. Two strains CG1006

and CG1044 of *P. chlamydosporia* and CG1042 and CG1101 of *P. lilacinum* were found to be the most effective and could be potential biocontrol candidates to manage *M. enterolobii* (Forghani and Hajihassani, 2020). Thus, future research on optimizing growth conditions, efficacy and broad-spectrum action, safety, and stability of beneficial endophytic bacteria or PGPR for commercialization and utilization in IDM need to be researched for ability to control *M. enterolobii*.

Arbuscular mycorrhizal fungi (AMF) are soil fungi that form a mutualistic symbiosis with the roots of plants (Baum et al., 2015; Schouteden et al., 2015). Importantly, AMF-mediated biocontrol mechanisms include altered root morphology, enhanced plant tolerance, competition for space and nutrition with plant-parasitic nematodes, induced systemic resistance (ISR), and altered rhizosphere interactions caused by abiotic and biotic factors, including plant pathogenic nematodes (Cayrol et al., 1992; Gianinazzi et al., 2010; Smith et al., 2010; Baum et al., 2015; Schouteden et al., 2015). In the past, research has been conducted on AMF-mediated biocontrol and their potential involvement in reducing *Meloidogyne* spp. populations (Schouteden et al., 2015). With the increase in microbiome research, the development of beneficial microbial agents for field application to *M. enterolobii* is imperative in the years to come. Also, some consideration should be given toward understanding the plant root interactions with beneficial microorganisms, their symbiotic relationships, and more detailed insights into the complex mechanisms underlying biocontrol agents-mediated effects on *M. enterolobii*. Direct effects of AMF on plant-parasitic nematodes and multiple benefits (Schouteden et al., 2015) suggested that AMF could be used as a biocontrol agent for managing *M. enterolobii* and to enhance nutrient bioavailability for superior tomato fruit quality and yield.

Chemical Control

Chemical nematicides have been used to combat *Meloidogyne* spp.; however, many of these products are being phased out due to environmental and health concerns (Elling, 2013). Two broad categories of nematicides to manage *Meloidogyne* spp., including *M. enterolobii*, are fumigants and non-fumigants. Soil fumigants are formulated as gases or liquids that quickly vaporize into gases and move through open-air spaces in the soil as a gas. Some common soil fumigants that are currently available are 1,3-dichloropropene (e.g., Telone II), metam sodium (e.g., Vapam, Sectagon-42) and metam potassium (e.g., K-Pam) (Zasada et al., 2010; Noling, 2019). Although fumigants are useful for managing *Meloidogyne* spp., they can be expensive, are subject to increased regulatory scrutiny, and do not eradicate an infested field (Zasada et al., 2010). Further, many fumigants are non-selective, also having activity on bacteria, fungi, and weed seeds in the soil. Non-fumigant nematicides are generally formulated as either granules or liquids and incorporated physically or when dissolved in water. These nematicides are either contact or systemic nematicides depending on whether they kill nematodes in soil by contact or are taken up by the plant first and then affect nematodes. Some common chemical non-fumigant nematicides used to control *Meloidogyne* spp. in the United States are fluensulfone (e.g., Nimitz, ADAMA, Raleigh, NC, United States), fluopyram (e.g., Velum Prime and Velum Total, Bayer CropScience, St. Louis, MO, United States), oxamyl (e.g., Vydate, DuPont, Wilmington, DE, United States), ethoprop (e.g., Mocap, AMVAC), and terbufos (e.g., Counter, AMVAC) (Noling, 2015; Watson and Desaeger, 2019). In the European Union and other countries in the world, fumigants metam sodium (AMVAC), and dazomet (e.g., Basamid G Certis, Columbia, MD, United States) were effective to control *M. enterolobii* populations in soil (Anonymous, 1987; Zasada et al., 2010). However, because of negative environmental side effects of these fumigants, metam sodium was recommended only be used with a minimum interval of 5 years[1].

Host Plant Resistance

Planting resistant varieties is the most environmentally and economically friendly method to combat root-knot nematodes in tomato (Seid et al., 2015). Plant resistance genes restrict or prevent nematode reproduction in a host. At least 10 plant resistance genes (*R*-genes; *Mi-1*, *Mi-2*, *Mi-3*, *Mi-4*, *Mi-5*, *Mi-6*, *Mi-7*, *Mi-8*, *Mi-9*, and *Mi-HT*) that confer resistance to *Meloidogyne* spp. in tomato have been identified (El-Sappah et al., 2019). Among them, only five genes (*Mi-1*, *Mi-3*, *Mi-5*, *Mi-9*, and *Mi-HT*) have been mapped. Compared with other *Meloidogyne* spp., *M. enterolobii* is pathogenic on crop genotypes possessing several sources of resistance genes. For example, *M. enterolobii* develops on crop genotypes carrying resistance to the major species of *Meloidogyne*, including resistant cotton, sweetpotato, tomatoes (*Mi-1* gene), potato (*Mh* gene), soybean (*Mir1* gene), bell pepper (*N* gene), sweet pepper (*Tabasco* gene) and cowpea (*Rk* gene) (Fery et al., 1998; Thies and Fery, 2000; Williamson and Roberts, 2009; Castagnone-Sereno, 2012; Quenouille et al., 2013).

The most common deployed gene, *Mi-1*, was originally identified in *Solanum peruvianum* and introgressed into *S. lycopersicum* (Da Silva et al., 2019). This gene is effective in providing resistance to *M. incognita*, *M. javanica*, and *M. arenaria* (Seid et al., 2015; Da Silva et al., 2019). One of the major concerns of *M. enterolobii* is that the *Mi-1* gene is not effective in controlling this species (Kiewnick et al., 2009; Seid et al., 2015; Da Silva et al., 2019). The resistance spectrum of the *Mi-1.2* gene was assessed against 15 populations of *Meloidogyne* spp. employing two contrasting tomato varieties, 'Santa Clara' (homozygous recessive *mi-1.2/mi-1.2*, susceptible) and 'Debora Plus' (heterozygous *Mi-1.2/mi-1.2*, resistant) (Gabriel et al., 2020). They found that the 'Debora Plus' hybrid possessing the *Mi-1.2* gene was susceptible only to *M. enterolobii* and *M. hapla* but exhibited resistance to the other 13 *Meloidogyne* spp. A great deal of effort has been put into finding new sources of resistance or tolerance to *M. enterolobii* in tomato. Da Silva et al. (2019) evaluated commercial and wild tomatoes and identified three varieties (*Solanum lycopersicum* 'Yoshimatsu' and 'CNPH 1246,' and *S. pimpinellifolium* 'CGO 7650' (= 'CNPH 1195') with tolerance to *M. enterolobii*.

Deployment of a new tomato variety by conventional breeding may take over 10 years. However, this process has been accelerated using PCR-based molecular markers linked to the

[1] http://www.ctb.agro.nl

R gene of interest, and marker-assisted selection (MAS) has been routinely used in tomato breeding programs (Foolad and Panthee, 2012; El-Sappah et al., 2019). In the absence of *M. enterolobii* resistant varieties, grafting tomatoes with resistant rootstocks could be an alternative strategy for this disease management (Louws et al., 2010; Schwarz et al., 2010; Baidya et al., 2017). Two tomato rootstocks, 'Brigeor' and 'Efialto,' showed lower reproduction for one isolate of *M. enterolobii*, but not for a second distinct isolate, indicating some differences in virulence of the isolates of *M. enterolobii* (Kiewnick et al., 2009). Yet within these breeding efforts (whether conventional or marker-assisted), special attention should be paid to genotype resistant or tolerant status to *M. enterolobii*. Plants tolerant to *M. enterolobii* are identified by minimal to no yield loss when infected, even under heavy infestation (Boerma and Hussey, 1992). However, they may still allow populations of the nematode to reproduce and increase, posing significant risk to subsequent susceptible crops and long-term *M. enterolobii* management. Although providing a robust option for avoiding short-term yield and economic losses, use of tolerant varieties should be assessed in the framework of holistic *M. enterolobii* management.

NOVEL APPROACHES TO ENHANCE DISEASE RESISTANCE TO *M. enterolobii* IN TOMATO

Management of *M. enterolobii* is challenging due to its broad host range, high reproductive rates, and its seemingly low economic threshold level. Recent advancements in genetic engineering have made it possible to incorporate and express indigenous and heterologous proteins from one organism to another and develop enhanced nematode resistance in plants. Strategies to engineer one or more natural resistance genes with synthetic resistance may be promising tools to suppress nematode infection and populations in tomato production systems (Gheysen et al., 1996; Jung et al., 1998; Opperman et al., 1998). However, lack of public acceptance of genetically modified organisms (GMO) tomatoes has a restricted deployment of this strategy into the market.

Harnessing Host Plant Resistance Through Marker-Assisted Selection (MAS) in Tomato Breeding Programs

A conventional breeding program involves successive crossing and extensive phenotyping, which make this procedure labor-intensive and time-consuming. Traditionally, bi-parental mapping populations have been used to detect and identify genes or quantitative trait loci (QTL) in tomato for resistance to *Meloidogyne* spp. including *M. incognita, M. javanica, M. hapla*, and *M. enterolobii* (Kiewnick et al., 2009; Foolad and Panthee, 2012; El-Sappah et al., 2019). Genome-wide association studies (GWAS) is a powerful technique to identify SNP markers associated with QTL in cultivated and wild tomato (Hirakawa et al., 2013). The integration of biotechnology techniques into a breeding program can greatly reduce this time to incorporate new resistance genes. Genomics-assisted breeding contributes to advance MAS for evaluating tomato germplasm collections, characterizing populations, finding markers linked to specific alleles of important genes, and stacking disease resistance genes for multiple pathogens including for root-knot nematode management (Arens et al., 2010). For example, the *Mi* region contains two *Mi1-1* and *Mi1-2* genes (Milligan et al., 1998). The *Mi1-2* gene, but not *Mi1-1*, has been suggested to confer resistance to *M. incognita, M. javanica*, and *M. arenaria* (Milligan et al., 1998). The PCR markers tightly linked to *Mi1-2* (Goggin et al., 2004) and *Mi-3* (Yaghoobi et al., 2005) were reported; however, the practical use of these resistance loci (*Mi-2* to *Mi-9*) has not yet been investigated thoroughly. These resistance genes should be assessed to different isolates of *M. enterolobii* and molecular markers linked to these *Mi*-genes as well as other disease resistance genes that are needed to evaluate for their stability in tomato (Arens et al., 2010). Recent advances in whole-genome sequencing have identified large numbers of SNPs and can facilitate the use of MAS more effectively in breeding programs. KASP (Kompetitive Allele Specific PCR), is a novel competitive allele specific PCR for SNP genotyping assay based on dual FRET (Fluorescent Resonance Energy Transfer) (Broccanello et al., 2018). Recently, sequences of SNP markers for the *Mi-1* gene for resistance to root-knot nematodes have been converted using KASP assay and used in tomato breeding (Devran et al., 2016). KASP assays are less expensive, highly reproducible, and flexible compared to other marker systems (Semagn et al., 2014). Thus, characterization of a large set of tomato varieties with SNP markers would be useful for the identification of markers linked to genes for resistance to *M. enterolobii* in tomato. Also, SNPs can be converted to KASP markers and used for the MAS gene pyramiding (Devran et al., 2016).

Developing Transgenic Plants Harboring Previously Cloned Resistance Genes

Genetic engineering offers an alternative to conventional breeding and is mainly focused on two strategies: (i) the transfer of the cloned resistance gene from other plants to tomato, and (ii) the transfer of the *Mi* resistance gene from resistant varieties to susceptible ones with highly desirable production qualities (El-Sappah et al., 2019). The *Ma* locus, which has been mapped to chromosome 7 of Myrobalan plum (*Prunus cerasifera*), has been cloned by a positional cloning approach (Claverie et al., 2011; Khallouk et al., 2011). The subsequent *Agrobacterium rhizogenes*-mediated hairy-root transgenic *Prunus* plants corroborated that the *Ma* locus conferred resistance to *M. arenaria, M. incognita, M. javanica, M. floridensis*, and *M. enterolobii* (Bosselut et al., 2011; Claverie et al., 2011). The *Ma* toll/interleukin-1 receptor-like nucleotide binding-leucine-rich repeat (TNL) gene confers high-level and wide-spectrum resistance to *M. incognita, M. arenaria*, and *M. javanica* and *M. enterolobii*, and TNL is possibly a candidate gene for the *Ma* locus (Bosselut et al., 2011; Claverie et al., 2011). Furthermore, the *Ma* – *M. enterolobii* interaction may provide a great opportunity

to decipher nematode effector recognition and TNL signaling (Claverie et al., 2011).

Proteinase inhibitors (PIs) are protein molecules secreted by pathogens, which inhibit the function of proteinases and proteases released by the pathogens (Ali et al., 2017). In *Meloidogyne* spp., PIs become active against all the four classes of proteinases from nematodes such as serine, cysteine, metalloproteinases, and aspartic. Transgenic expression of PIs is a method for managing *Meloidogyne* spp. (Hepher and Atkinson, 1992; Ali et al., 2017). For example, a modified rice cystatin gene (a cysteine proteinase inhibitor) in transgenic *Arabidopsis*, reduced nematode feeding, and fecundity of *M. incognita* females (Urwin et al., 1997). The pyramiding expression system produced synergistic effects by utilizing the two defense responsive genes: a plant *cysteine proteinase inhibitor* (*CeCPI*) and a fungal *chitinase* (*PjCHI-1*) in transgenic tomato and protected all growth stages of *M. incognita* infections (Chan et al., 2015). Future research to investigate interactions between these proteinases and *M. enterolobii* could be a novel approach to manage this nematode in tomato. However, concerns about the durability of such a transgenic resistance and the consumer's acceptance of transgenic tomato will need to be investigated.

Utilizing Host Generated RNA Interference (RNAi) to Silence Nematode Specific Effector Genes

RNA interference (RNAi) has emerged as a powerful strategy to downregulate gene activity and has also proven effective as a control tactic against *Meloidogyne* spp. (Elling, 2013). First described for *Caenorhabditis elegans*, RNAi has been used for silencing genes by suppressing their expression in a wide variety of organisms including plant-parasitic nematodes (Huang et al., 2006b; Ali et al., 2017). In this novel strategy, genes expressed in a range of cell types are silenced when nematodes take up double-stranded RNA (dsRNA) or short interfering RNAs (siRNAs) that elicit a systemic RNAi response (Lilley et al., 2012). These dsRNA molecules ranged from 42 to 1300 bp and were effective in inducing RNAi in both cyst and root-knot nematodes (Lilley et al., 2012). *Meloidogyne* spp. synthesizes effector proteins encoded by parasitism genes, and these effectors represent the molecular interface between the nematode and host (Elling, 2013). The nematode-secreted effectors produced within the esophageal glands play critical roles in parasitism (Davis et al., 2004; Baum et al., 2007; Haegeman et al., 2012). Such developments need to be coupled with an investigation of the mechanisms by which nematodes circumvent resistance (Williamson and Kumar, 2006). The feasibility of silencing nematode genes in the host plants using RNAi has been demonstrated in *Meloidogyne* spp. (Huang et al., 2006a; Yadav et al., 2006). For example, a secreted parasitism protein called 16D10, which is expressed in the subventral esophageal gland cells of multiple *Meloidogyne* spp. and interact directly with a host intracellular transcription regulator (Huang et al., 2006b). Furthermore, the silencing of the *16D10* gene by expressing dsRNA in transgenic *Arabidopsis* enabled the development of transgenic plants that were constitutively resistant to *M. arenaria*, *M. hapla*, *M. incognita*, and *M. javanica* (Huang et al., 2006a,b).

The translationally controlled tumor protein (TCTP) was first identified in mice (Yenofsky et al., 1982). A novel *M. enterolobii* TCTP effector, named *MeTCTP* was able to promote parasitism, probably by suppressing programmed cell death in the host (Zhuo et al., 2017). The silencing of the effector *MeTCTP* resulted in a reduction in parasitism and reproductive potential of *M. enterolobii*, providing evidence of the nematode effector gene as a target for host generated RNAi to achieve disease resistance (Zhuo et al., 2017). Recently, both genome sequence data and new bioinformatics tools have emerged for developing effective dsRNA constructs and stacking of dsRNA sequences to target multiple genes for nematode control (Banerjee et al., 2017). Identification and functional analysis of nematode effector targets using RNAi technology may hold great promise for enhancing plant resistance to *M. enterolobii* in tomato.

Exploiting Efficient Genome Editing Using the CRISPR-Cas9 Technique

The development of the clustered regularly interspaced short palindromic repeats (CRISPR) technology has become a powerful alternative to RNAi for gene silencing (Ali et al., 2019). The CRISPR/Cas9 technique incorporates foreign DNA sequences into host CRISPR loci to generate short CRISPR RNAs (crRNAs) that direct sequence-specific cleavage of homologous target double-stranded DNA by Cas endonucleases (Jinek et al., 2012). The CRISPR-Cas9 genome editing protocols have been established in the free-living nematode, *Caenorhabditis elegans* (Friedland et al., 2013; Dickinson and Goldstein, 2016) which creates DNA modification at specific loci and selects the T-DNA-free mutant (Banerjee et al., 2017). The recent availability of genome sequences for tomato (Sato et al., 2012) and *M. enterolobii* (Szitenberg et al., 2017; Koutsovoulos et al., 2019) could lead to the identification of both host and pathogen novel genes involved in the infection stage and help develop the CRISPR-Cas9 technique for enhancing the resistance to *M. enterolobii* in tomato.

CONCLUSION AND PERSPECTIVES

We have highlighted the progress made by several research groups in the biology and management of *M. enterolobii* in tomato using both conventional and modern technologies. Even with successes in managing other *Meloidogyne* spp. through host resistance, cultural, chemical, and biological control, the recent identification of highly virulent and aggressive nematode, *M. enterolobii*, poses a threat to tomato production globally. To manage this emerging pathogen, substantial investments are necessary to lead fundamental research focused on assessing the pathogen virulence and understanding the species identity, genetic diversity, population genetic structure, evolution, and parasitism mechanisms at a more detailed scale. Whole-genome sequences of *Meloidogyne* spp. will provide opportunities to identify the widespread occurrence of horizontally transferred genes encoding for unique effectors, contributing to successful plant parasitism in nematodes and in the modulation of the plant's defense system, the establishment of a nematode feeding

site, and the synthesis or processing of nutrients (Haegeman et al., 2011). Comparative genomic analyses across *Meloidogyne* spp. need to be exploited to advance understanding of the evolutionary relationships and population genetic structure of *M. enterolobii*. More importantly, the development of robust and specific diagnostic molecular markers is necessary to correctly identify *M. enterolobii* and prevent further spread of this highly destructive nematode. To ensure global food security, modern technologies in conjunction with classical methods should be a key priority for income generation, and sustainability to tomato growers and stakeholders (Barker, 2003). New insights into the current and future risks, supported by a more robust understanding of the interactions between tomato and *M. enterolobii* will enhance the opportunities for developing novel management tools as the ability to use chemical pesticides decrease and the need for food production continues to increase. Strengthening research collaborations and combining multidisciplinary experts working on *M. enterolobii* is required to combat this economically devastating nematode in tomato production systems.

AUTHOR CONTRIBUTIONS

TA and FL discussed and conceived ideas. TA designed the scope of the study. AP and TA wrote the manuscript. AG helped to revise the manuscript and offer additional discussion. All authors have read, edited, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.606395/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Table 2.1. Host ranges of *Meloidogyne enterolobii* reported worldwide (Rodriguez et al., 2003; Brito et al., 2010, Freitas et al., 2017).

Common name	Botanical name	Family
Arrow root	*Maranta arundinacea*	Marantaceae
Aubergine	*Solanum melongena*	Solanaceae
Banana	*Musa* spp.	Musaceae
Barbados cherry	*Malpighia glabra*	Malpighiaceae
Bell pepper	*Capsicum annuum*	Solanaceae
Black mulberry	*Morus nigra*	Moraceae
Bottle brush	*Callistemon citrinus*	Myrtaceae
Cape gooseberry	*Physalis peruviana*	Solanaceae
Carrot	*Daucus carota*	Apiaceae
Cassava	*Manihot esculenta*	Euphorbiaceae
Chinese date	*Ziziphus jujuba*	Rhamnaceae
Coffee	*Coffea arabica*	Rubiaceae
Common bean	*Phaseolus vulgaris*	Fabaceae
Cotton	*Gossypium hirsutum*	Malvaceae
Cucumber	*Cucumis sativus*	Cucurbitaceae
Guava	*Psidium guajava*	Myrtaceae
Ginger	*Zingiber officinale*	Zingiberaceae
Hedge cactus	*Cereus hildmannianus*	Cactaceae
Jackfruit	*Artocarpus heterophyllus*	Moraceae
Jamaican poinsettia	*Euphorbia punicea*	Euphorbiaceae
Japanese blueberry	*Elaeocarpus decipiens*	Elaeocarpaceae
Jerusalem cherry	*Solanum pseudocapsicum*	Solanaceae
Mulberry	*Morus alba*	Moraceae
Nanche	*Byrsonima cydoniifolia*	Malpighiaceae
Naranjilla	*Solanum quitoense*	Solanaceae
Papaya	*Carica papaya*	Caricaceae
Potato	*Solanum tuberosum*	Solanaceae
Soybean	*Glycine max*	Fabaceae
Sweet potato	*Ipomoea batatas*	Convolvulaceae
Tamboril	*Enterolobium contortisiliquum*	Fabaceae
Tobacco	*Nicotiana tabacum*	Solanaceae
Tomato	*Solanum lycopersicum*	Solanaceae
Watermelon	*Citrullus lanatus*	Cucurbitaceae
Yam	*Dioscorea rotundata*	Dioscoreaceae

Table 2.2. Diagnostic methods for the identification of *Meloidogyne enterolobii* infecting field crops.

Identifi -cation method	Primer code	Primer sequence (5'-3')	Amplicon size (bp)	References	Advantages	Disadvantages
Morphology (perineal patterns)				Yang and Eisenback, (1983); Eisenback and Triantaphyllo u, (1991); Hunt and Handoo (2009)	The traditional method of identification. A lot of data present on *Meloidogyne* morphology.	Requires experienced nematologists. Can lead to misidentificati on.
Isozyme analysis				Eisenback and Triantaphyllo u (1991); Moens et al. (2009)	Many *Meloidogyne* spp. have known, unique esterase and malate dehydrogenase patterns. Can use single adult female.	Limited to adult females.
Polymerase chain reaction (PCR) with universal primers	Universal primers (5S – 18S rDNA) Universal primers (D2/D3 28S rDNA)	TTAACTTGCCA GATCGGACG /TCTAATGAGCC GTACGC ACAAGTACCGT GAGGGAAAGT/ TGCGAAGGAAC CAGCTACTA	720 500	Blok et al. (1997) Nunn (1992)	Useful for phylogenetic and diversity studies More effective for taxonomic resolution at the genus level.	Less sequence reliability. Identification at lower taxonomic levels and classification accuracy is reduced due to nonspecific amplification among nontarget nematode species.

Table 2.2. (continued)

Identifi -cation method	Primer code	Primer sequence (5'-3')	Amplicon size (bp)	References	Advantages	Disadvantages
Polymerase chain reaction (PCR) with sequence characterized amplified region (SCAR)	SCAR primers SCAR primers (MK7-F/R SCAR primers (Me-F/ R) SCAR primers (Mi2F4/ Mi1R) SCAR primers (Fjav/Rjav)	GAAATTGCTTT ATTGTTACTAA G/ TAGCCACAGCA AAATAGTTTTC GATCAGAGGCG GGCGCATTGCG A/CGAACTCGC TCGAACTCGAC AACTTTTGTGA AAGTGCCGCTG / TCAGTTCAGGC AGGATCAACC ATGAAGCTAAG ACTTTGGGCT/ TCCCGCTACAC CCTCAACTTC GGTGCGCGATT GAACTGAGC/ CAGGCCCTTCA GTGGAACTATA C	322 520 236 300 670	Blok et al. (2002) Tigano et al. (2010) Long et al. (2006) Kiewnick et al. (2013) Zijlstra (2000)	Many species-specific primers are available for *Meloidogyne* spp. Simpler patterns than RAPDs (locus-specific) and codominant markers. Highly sensitive and robust assay. Labor non-intensive. Preformed with any life stage.	Requires expensive equipment and expense in designing specific primers. Sequence information of primers.
SYBR Green quantitative real-time PCR	RKNF/ RKNR	GCTGGTGTCTA AGTGTTGCTGA TAC/GAGCCTA GTGATCCACCG ATAAG		Toyota et al. (2008)	Monitor the amplification of any double-stranded DNA sequence. No probe is required	May generate false positive signals because the SYBR dye binds to any double-stranded DNA,

Table 2.2. (continued)

Identifi -cation method	Primer code	Primer sequence (5'-3')	Amplicon size (bp)	References	Advantages	Disadvantages
					which can reduce assay setup and running costs.	and it can also bind to nonspecific double-stranded DNA sequences.
TaqMan quantitative real-time PCR	Ment17F/R Probe	TGTGGTGGCTC ATTTTCATTA/ AAAAACCCTAA AAATACCCCAA A AGGAGCTG		Kiewnick et al. (2015)	Specific hybridization between probe and target is required to generate a fluorescent signal. Probes can be labeled with different dyes, which allows amplification and detection of two distinct sequences in one reaction.	The synthesis of different probes is required for different sequences. More expensive than SYBR Green assay.
	F/R Probe	TGGTTCAGGGT CATTTTTCTAT AAAGT/ CAAATCGCTGC GTACCAACA FAM-CCATTGGCACT ATAAC-MGB		Sapkota et al. (2016)	Detect and identify target nematode DNA in complex DNA backgrounds.	
Loop-mediated isothermal amplification (LAMP)						

Table 2.2. (continued)

Identifi -cation method	Primer code	Primer sequence (5'-3')	Amplicon size (bp)	References	Advantages	Disadvantages
	Me-LF/LB	GTTCGTTGCTT AACTTGCCAGA / TCTAAGGCAAA GTGGGCGGAGC TCTYTTTGCCTT AAACCATTCC AAGCACGCCAT CCCGTC/ TGTTGTTCGCT GTTCGC		Niu et al. (2012)	Could be detected by a cheap turbidity-meter and thus, does not require expensive equipment. High specificity and, sensitivity (up to 10 times more sensitive than PCR) under isothermal conditions using a set of four specially designed primers. Can be done quickly, in a field setting.	The usefulness of LAMP methods can be limited by the presence of inhibitors in the sample. Primer design is complex. Unable to perform multiplex amplification.
Whole-genome sequencing		*Meloidogyne incognita* *Meloidogyne enterolobii* *Meloidogyne hapla*		Abad et al. (2008) Koutsovoulos et al. (2019) Opperman et al. (2008)	Technical accuracy and reliable. Scientific information with genetic variants and potential values with future implications	Requires knowledge of the genome and complex bioinformatic tools Structural variants. Lack of laboratory validity.

CHAPTER 3

Evaluating Diverse Tomato Genotypes for Resistance to the Root-knot Nematode *Meloidogyne enterolobii*

ABSTRACT

There are approximately 100 species of root-knot nematode (RKN; *Meloidogyne* spp.), but most of the damage and yield loss associated with these nematodes worldwide can be attributed to four major species: *M. incognita, M. arenaria, M. hapla,* and *M. javanica*. However, an emerging threat to tomato production is *M. enterolobii*. This RKN species is becoming a significant concern due to its broad host range, increasing geographic distribution, and aggressiveness. Additionally, current genetic resistance in tomato (*Solanum lycopersicum*), mediated by the *Mi* gene, does not confer resistance to *M. enterolobii*. Therefore, new sources of genetic resistance are critically needed. A total of 31 tomato and wild relative lines were evaluated for their response to *M. enterolobii* infection in the greenhouse through inoculation screening trials. Each tomato line was evaluated for root galling severity and nematode reproduction by calculating a reproductive factor (RF). Tomato lines were compared to the susceptible control, Rutgers tomato. Typically, an RF greater than 1 indicates high reproduction and a susceptible interaction. An RF less than 1 indicates low reproduction and a potentially resistant interaction. In this experiment, four tomato lines were identified as highly resistant, while several others show moderate resistance to *M. enterolobii* infection. Further testing of these lines under field conditions could lead to the identification of novel sources of resistance to this highly damaging species of RKN, which could be used as resistant rootstock or be introgressed into commercially available tomato species through advanced breeding methods.

INTRODUCTION

Meloidogyne enterolobii (syn. *M. mayaguensis*), the guava root-knot nematode, was originally described by Yang and Eisenback in 1983 infecting the pacara earpod tree in China (Yang and Eisenback, 1983). Infection by *M. enterolobii* results in nutrient deficiency-like symptoms (chlorosis, stunting, wilting) and ultimately leads to a reduction in fruit quality and yield (Hajihassani et al., 2020). This species is considered subtropical and has been confirmed to be present in numerous countries across the world including parts of Asia, Africa, South America, Central America, Mexico, and the United States (Philbrick et al., 2020; Ye et al., 2021). *Meloidogyne enterolobii* was even detected in a greenhouse as far north as Switzerland (Kiewnick et al., 2009). *Meloidogyne enterolobii* can infect many different plant species and is becoming an emerging threat in part due to this broad host range (El-Sappah et al., 2019). Some important crops infected by *M. enterolobii* include tomato, sweetpotato, soybean, and pepper (Yang and Eisenback, 1983; Rodriguez et al., 2003; Schwarz et al., 2021). The broad host range of *M. enterolobii* can make management strategies like crop rotation difficult since growers tend to rotate with crops within this nematodes host range. Only a few crops, such as peanut, grapefruit, and garlic, have been identified as non- or poor hosts for *M. enterolobii* (Rodriguez et al., 2003).

Because genetic resistance is the most environmentally and economically friendly method for managing RKNs, it is an important tactic for many tomato growers (Hajihassani et al., 2020). However, current genetic resistance in tomato conferred by the *Mi-1* gene is not effective in managing *M. enterolobii*. The *Mi* genes confer resistance to 3 of the 4 major species of RKN- *M. incognita*, *M. arenaria*, and *M. javanica* (Hajihassani et al., 2020). There is a total of 10 *Mi* genes, but currently only *Mi-1* is commercially available. The *Mi-1* gene was first

identified in *Solanum peruvianum* PI 128657 and was introgressed into *S. lycopersicum* (Bleve-Zacheo et al., 2007; Philbrick et al., 2020). Additionally, the resistance conferred by the *Mi-1* gene is known to break down at high soil temperatures (above 28°C) which can be detrimental in warmer climates where RKNs are typically found (Mitkowski and Abawi, 2003; Agrios, 2005; Bleve-Zacheo et al., 2007).

There is currently limited knowledge on sources of genetic resistance to *M. enterolobii* infection in tomato, as previous screenings for resistance to this species have been unsuccessful in identifying novel sources of resistance (da Silva et al., 2019; de O Silva et al., 2020). Genetic resistance to *M. enterolobii* has been identified in related vegetable crops, such as sweetpotato (Schwarz et al., 2021) and pepper (Carillo-Fasio et al., 2020). However, the search for sources of resistance to *M. enterolobii* has expanded to wild relative species of tomato as many have displayed resistance to RKNs (Hajihassani et al., 2020).

Due to its broad host range, expanding geographic distribution, and lack of genetic resistance, *M. enterolobii* is becoming a significant threat to tomato production. Thus, the identification of novel sources of resistance to *M. enterolobii* are critically needed. An experiment was conducted in the greenhouse to evaluate diverse tomato genotypes for their response to *M. enterolobii* infection based on root galling severity and reproduction data. Four of the lines included in this experiment (Melody, Whitestar, Diamond, and Pion) were previously screened and identified as highly resistant to *M. enterolobii* infection (Hajihassani et al., 2020). These lines were used as a positive control to compare the potential resistance observed in this experiment.

MATERIALS AND METHODS

Tomato Germplasm

Thirty-one unique tomato and wild relative species were selected for this experiment based on reports for resistance to other RKN species (**Table 3.1**). Many of the lines contain the *Mi* gene. Seeds were obtained from four different sources: 'LA' lines were obtained from UC Davis's Tomato Genetics Resource Center, 'PI' lines were obtained from USDA ARS Germplasm Resources Information Network, 'NC' lines were obtained from Dr. Reza Shekasteband at NC State University, and the lines Melody, Whitestar, Diamond, and Pion were obtained from Dr. Abolfazl Hajihassani at University of Florida.

Planting

Seeds were soaked in a 1% bleach solution (7.03% available chlorine) for 30 minutes, rinsed thoroughly, and sown directly into potting mix (Sun Gro Horticulture growing mix) in germination trays. Germination trays were maintained in the greenhouse which was kept at 28°C or higher, plants were watered daily, and there was no supplemental lighting. Five weeks after sowing, plants were transplanted into individual 'Cone-tainer' pots filled with a steam sterilized 1:1 soil:sand mixture. Plants were allowed three days to recover prior to inoculation.

Inoculum Preparation and Inoculation of Plants

To prepare the inoculum, culture tomato plants maintained in the greenhouse were extracted for eggs using the 10% NaOCl method described by Hussey and Barker (1973). Briefly, plant roots were soaked in a 10% bleach solution for 45 seconds and were poured over a stack of sieves (top to bottom: 250µm, 75µm, 25µm). RKN eggs can pass through to the bottom collection sieve (25µm) while larger particles, such as root material and soil cannot. Plant roots were broken down by hand on the top sieve (250µm), while continuous water flow ensured

proper extraction of all eggs and wash them to the bottom sieve (25µm). A wash bottle was used to rinse the eggs into a tube with 35mL of water. Following extraction, approximately 15mL of a 70% sucrose solution was added to separate any remaining debris from the eggs by centrifuge for 10 minutes. This step allowed the eggs to float to the top of the solution while heavy debris, such as soil particles, was spun down to the bottom of the tube. Eggs were decanted from the sucrose-egg solution, collected again on the 25µm sieve, and were resuspended in 50mL of water. Eggs were counted using an inverted microscope (Nikon Instruments, Inc., Melville, NY) to calculate the correct volume of solution to inoculate each plant with approximately 2500 eggs.

To inoculate, eggs were pipetted directly into the soil at the base of each plant. Plants were fertilized with Osmocote® Smart-Release Fertilizer and were placed inside a BugDorm-6E1020 insect rearing cage in the greenhouse in a randomized block design. Due to the known presence of whiteflies in the greenhouse, plants were placed in this cage to prevent these insects from feeding on and transmitting other diseases to these plants. For each line, 5 plants (replications) were inoculated with eggs and 1 plant per line was not inoculated. The tomato cultivar Rutgers was used as a positive control due to its known susceptibility to *M. enterolobii*. Plants were assessed for disease 90 days post-inoculation and the experiment was conducted in the greenhouse twice (July - November 2021 and February – June 2022).

Disease Assessment

At 90 days post-inoculation, plants were carefully removed from ‘Cone-tainer’ pots, and the roots were rinsed thoroughly with tap water to remove soil particles. The root system of each plant was scored visually for root galling severity using a variation of the Zeck scale (Zeck, 1971) (**Figure 3.1**), in which a percentage of total root area infected by galling was assigned, thus allowing for greater flexibility when scoring for galling severity. Eggs were extracted from

individual root systems and separated from remaining debris using the same procedure as described above for preparing inoculum. To calculate the number of eggs in each sample, a 500µL aliquot was pipetted from the 50mL tube to a counting tray where eggs were counted using an inverted microscope. This was done three times to obtain an average number of eggs per 500µL of solution for each sample. To estimate the total number of eggs within the 50mL sample, or the total number of eggs extracted from each plant, the average number of eggs was multiplied using the following equation:

$$\text{Total \# of Eggs per Plant} = \frac{\#\ of\ Eggs\ Counted}{500\ \mu L} \times \frac{1000\ \mu L}{1\ mL} \times 50mL$$

This allowed us to calculate the reproductive factor (RF) for each plant:

$$\text{Reproductive Factor (RF)} = \frac{Final\ Nematode\ Population\ (Pf)}{Initial\ Nematode\ Population\ (Pi)}$$

In this equation, the final nematode population (Pf) is the total number of eggs in the sample after extraction and the initial nematode population (Pi) is the number of eggs the plants were inoculated with (in this case, 2500). An RF greater than one indicates that nematodes are reproducing at a high rate and this plant is susceptible. An RF less than one indicates that nematodes are reproducing at a lower rate and this plant is potentially resistant. Lines were scored as either resistant or susceptible based on the galling severity and RF.

Statistical Analysis

Root galling severity and RF data were subjected to an analysis of variance (ANOVA) using RStudio, (v. 3.6.2, R Core Team 2019). Where significant differences where identified, means were separated using Fisher's Least Significant Difference test (function 'LSD.test' within the 'agricolae' package). Data from the two replicate trials were analyzed separately.

RESULTS

In the first trial, there was a highly significant difference in root galling severity between lines ($P < 0.0001$) with 15 lines showing significantly less galling than the susceptible control, Rutgers (**Table 3.1**). There was also a significant difference in the RF value between lines ($P < 0.05$) with 6 lines showing significantly less reproduction than Rutgers and 18 lines with an RF value less than one (**Table 3.1**). A total of 8 lines: Melody, Whitestar, Diamond, Pion, NC 161L, PI 211840, PI 265957, and PI 415128 showed both significantly less galling when compared to Rutgers and an RF value less than 1. This indicates these lines are resistant to *M. enterolobii* infection. The lines PI 128657 (original source of *Mi-1*) and PI 126443 showed the most severe root galling, with values of 52% and 58% respectively, but both had RF values less than one. The lines Melody and Whitestar showed the lowest root galling severity with values of, 2% and 5% respectively and, with RF values close to zero. Rutgers showed a mean root galling severity of 23% and an RF of 1.65 (**Table 3.1**).

In the second trial, there was a highly significant difference in root galling severity between lines ($P < 0.0001$) with 17 lines showing significantly less galling than Rutgers (**Table 3.1**). There was also a significant difference in the RF value between lines ($P = 0.05$) with 7 lines showing significantly less reproduction than Rutgers and 5 lines with an RF value less than 1. A total of 3 lines (Whitestar, Pion, and PI 211839) showed both significantly less galling and an RF value less than 1 when compared to Rutgers, suggesting that these lines are resistant to *M. enterolobii* infection. Due to an unforeseen circumstance in the greenhouse, RF data was not able to be collected for 15 of the lines in the second trial. To confirm the conclusions of this experiment, these lines will need to be reevaluated. The lines PI 270435 and LA 2157 showed the most severe root galling, 86% and 69% respectively. In this case, PI 270435 had an RF value

of 1.67, however, like PI 126443 and PI 128657, LA 2157 had an RF value less than 1 (0.53). Rutgers showed a mean galling severity of 48% and an RF of 1.91 (**Table 3.1)**.

Although the first and second trials were statistically analyzed separately, there were similar trends in galling severity for each line tested (**Figure 3.2)**. The lines Melody, Whitestar, Diamond, and Pion displayed significantly less galling and very low reproduction in both trials; While the lines PI 211839, PI 211840, and PI 375937 would be beneficial to include in further screening trials based on low galling severity and/or low reproduction during this experiment.

A higher mean root galling severity was observed on the lines during the second trial with 25 of 31 lines demonstrating an average severity value 22% higher than in trial 1. The remaining 6 lines had a lower average severity value of 5% (**Figure 3.2**). The root galling severity values on the lines between trial 1 and 2 had Pearson Product Moment Correlation coefficient value of 0.43 ($P = 0.015$). In contrast, the RF for the 16 lines included in both trials was not correlated ($r = 0.11$) with 9 lines that had a higher RF in trial 2 (mean = 1.2 higher) and 7 had a lower RF (average value is 1.5 lower). Likewise, the galling incidence and the RF value for in both trials were not correlated.

DISCUSSION

Novel sources of resistance to *M. enterolobii* were affirmed and discovered in these greenhouse screening experiments. For example, lines Melody, Whitestar, Diamond, and Pion appear to be highly resistant to *M. enterolobii* infection with an average root galling below 10% (**Figure 3.3**) between both trials and RF values far lower than 1, affirming previous work (Hajihassani et al., 2020). These lines were also reported for resistance to the RKN *M. haplanaria*, suggesting that resistance to these two species may be based on the same gene or

genes (Hajihassani et al., 2020). Due to the low root galling severity and low RF of these lines, the resistance observed likely occurs pre-infection by preventing *M. enterolobii* to penetrate and enter the root system and post-infection by limiting the nematodes development and reproduction (Hamidi and Hajihassani, 2020). Nematode penetration and development assays could be conducted to confirm this by a series of staining and assessing root systems at different time points after inoculation and inspecting root galls for the number of egg laying females. The lines Melody and Pion have also been reported for resistance to potato cyst nematodes (*Globodera* spp.) and have shown utility as a trap crop to reduce plant-parasitic nematode populations in the field (Dias et al., 2017); therefore, utilizing these lines as trap crops may offer a beneficial alternative in fields with high nematode populations. The line PI 211840 had significantly less galling (**Figure 3.4**) in both trials compared to Rutgers and an RF of 0.8 in trial 1 but lacks RF data from trial 2. Given this data, it is reasonable to conclude that this line may have moderate resistance to *M. enterolobii*. The line PI 211840 has also been reported to be highly resistant to plant-parasitic nematodes and various wilt diseases by the Germplasm Resources Information Network (GRIN) and for resistance to diseases such as leaf mold caused by the fungal pathogen *Cladosporium fulvum* and tomato yellow leaf curl virus (TYLCV) (Kerr, 1983; Zakay et al., 1991). Tomato lines with multiple disease resistance are highly desirable to incorporate into tomato breeding programs. The greenhouse issues that arose in trial 2 taper the strength of conclusions in cases of missing data, however, these conclusions appear reasonable about the overall resistance observed from this experiment.

Lines PI 211839 and PI 375937 showed some inconsistent results between trials. Both lines showed significantly less galling compared to Rutgers in trials 1 and 2 but had a much higher RF in trial 1 than in trial 2. Due to this difference, these two lines may benefit from

further analysis as having moderate resistance to *M. enterolobii* infection, and it would be productive to circumscribe experimental factors that can help reduce variation from one trial to another. Lines PI 126443, PI 128657, and LA2157, exhibited significantly more root galling than the susceptible control (Rutgers) but had RF values close to or less than 1. This suggests that there may have been an incompatible reaction occurring in these lines where nematodes are able to enter the root system, establishing feeding sites, and form galls, but are not able to successfully reproduce. Specifically, resistance could be occurring post-infection by limiting the movement, development, and reproduction of *M. enterolobii* (Hamidi and Hajihassani, 2020). Nematode penetration and development assays could also be used to confirm this theory. While root galling did occur, this interaction could be very useful to investigate further as a source of resistance to *M. enterolobii* in tomato since resistance is often characterized by the ability of the host plant to prevent nematode reproduction (Hajihassani et al., 2020). The result also illustrates that data on galling severity alone is insufficient to conclude a lines host status, and full evaluation of both root galling and nematode egg production is needed. Additionally, the lines PI 270435 and PI 129152 (**Figure 3.5**) can be ruled out as candidates for resistance due to the high susceptibility to *M. enterolobii* observed in this experiment.

One factor that could have attributed to differences observed between trials could have been temperature or other environmental factors (light, humidity, etc.) in the greenhouse based on the time of year of trials the trials were conducted. The first trial was performed July – November 2021, and the second trial was performed February - June 2022. These conditions must be taken into consideration as some resistance genes, like the *Mi* gene, are known to break down at high soil temperatures (Mitkowski and Abawi, 2003; Bleve-Zacheo et al., 2007). It is unknown how this could have impacted the resistance observed in this experiment as soil

temperatures in the greenhouse reached up to 38°C at its hottest. It is important that sources of resistance remain effective at high temperatures since many RKN are known to thrive in tropical and subtropical regions (Mitkowski and Abawi, 2003; Agrios, 2005; Moens et al., 2009).

Several of the lines included in this trial would benefit from further testing under field conditions. Not only could this account for environmental factors observed in the greenhouse, but also show how lines respond to higher nematode populations over a longer period of time (Hajihassani et al., 2020). Another component a field trial would offer is yield data to show how the resistance observed in these lines effects the overall production of fruit. Determining lines resistant to *M. enterolobii* infection enables future research to identify the genes that confer this resistance and incorporate them into commercially available tomato cultivars by grafting with resistant rootstocks, traditional breeding methods, or by genetic engineering.

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Table 3.1. List of tomato and wild relative lines evaluated for response to *Meloidogyne enterolobii* infection. Dashes (-) for Trial 2 indicates unavailable reproductive factor data. Values followed by the same letter within the column are not significantly different at the $\alpha = 0.05$ level.

Line	Species Name	Root Galling Severity (0-100%)		Reproductive Factor (RF)	
		Trial 1	Trial 2	Trial 1	Trial 2
LA 1221	*Solanum lycopersicum*	23.0 ghij	48.0 cd	3.04 bc	2.50 abc
LA 2399	*S. lycopersicum*	39.6 bcdef	41.6 cde	1.21 bcd	-
LA 2940	*S. lycopersicum*	41.6 abcde	52.6 bcd	0.96 bcd	2.16 abcd
LA 2157	*S. arcanum*	44.0 abcd	68.8 ab	0.69 bcd	0.53 cd
LA 2172	*S. arcanum*	18.6 hijk	59.0 bc	3.49 ab	3.71 ab
NC 109	*S. lycopersicum*	24.6 fghij	22.4 efg	0.50 cd	-
NC 123S	*S. lycopersicum*	29.0 defgh	50.0 bcd	1.05 bcd	-
NC 161L	*S. lycopersicum*	16.0 hijkl	51.0 bcd	1.00 bcd	2.51 abc
NC 6GEM	*S. lycopersicum*	21.6 ghijk	51.0 bcd	1.30 bcd	3.81 a
PI 211838	*S. pimpinellifolium*	16.0 hijkl	25.8 ef	2.39 bcd	-
PI 211839	*S. pimpinellifolium*	21.7 ghijk	45.6 cde	3.59 ab	1.03 cd
PI 211840	*S. pimpinellifolium*	6.0 kl	39.6 de	0.80 bcd	-
PI 375937	*S. pimpinellifolium*	14.0 hijkl	43.0 cde	6.11 bcd	1.29 bcd
PI 128657	*S. peruvianum*	51.0 ab	59.0 bc	0.73 bcd	1.34 bcd
PI 129152	*S. peruvianum*	45.0 abc	53.8 bcd	2.42 bcd	-
PI 270435	*S. peruvianum*	15.2 hijkl	86.0 a	1.55 bcd	1.67 abcd
PI 265955	*S. lycopersicum*	31.0 cdefgh	25.6 ef	2.12 bcd	-
PI 265956	*S. lycopersicum*	39.3 bcdefg	40.2 cde	0.52 bcd	-
PI 265957	*S. lycopersicum*	21.0 hijk	56.5 bcd	0.63 bcd	-
PI 319695	*S. lycopersicum*	25.6 fghij	37.0 de	0.90 bcd	-
PI 349593	*S. lycopersicum*	28.2 efghi	47.5 cde	1.32 bcd	-
PI 415128	*S. lycopersicum*	21.4 hijk	44.0 cde	0.80 bcd	2.66 abc
PI 600919	*S. lycopersicum*	34.0 bcdefgh	48.3 bcd	0.26 d	2.84 abc
PI 645203	*S. lycopersicum*	15.3 hijkl	50.6 bcd	2.80 bcd	0.76 cd
PI 645267	*S. lycopersicum*	27.6 efghi	36.2 de	0.77 bcd	-
PI 126443	*S. corneliomulleri*	58.3 a	46.0 cde	0.57 bcd	-
Diamond	*S. sisymbriifolium*	10.6 jkl	7.0 fg	0.29 d	-
Melody	*S. sisymbriifolium*	2.0 l	7.4 fg	0.01 d	-
Pion	*S. sisymbriifolium*	13.0 ijkl	4.6 g	0.09 d	0.02 d
Whitestar	*S. sisymbriifolium*	5.0 kl	4.0 g	0.02 d	0.01 d
Rutgers	*S. lycopersicum*	22.6 ghij	48.0 cd	1.65 bcd	1.91 abcd

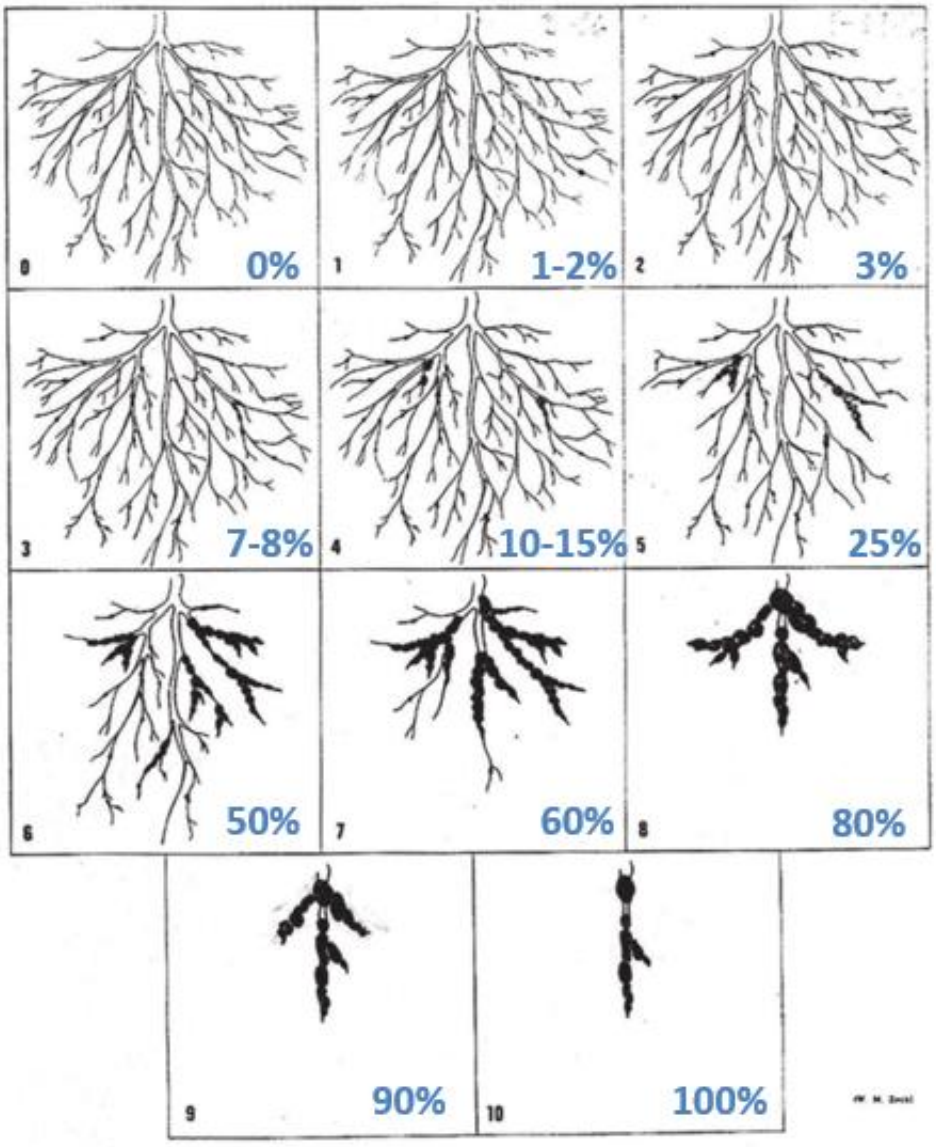

Figure 3.1. Root galling severity for each plant was assessed using a variation of this scale (Zeck, 1971). However, instead of using the 0-10 shown in black, plants were scored using a percentage as shown in blue. This allowed more flexibility when scoring for galling severity.

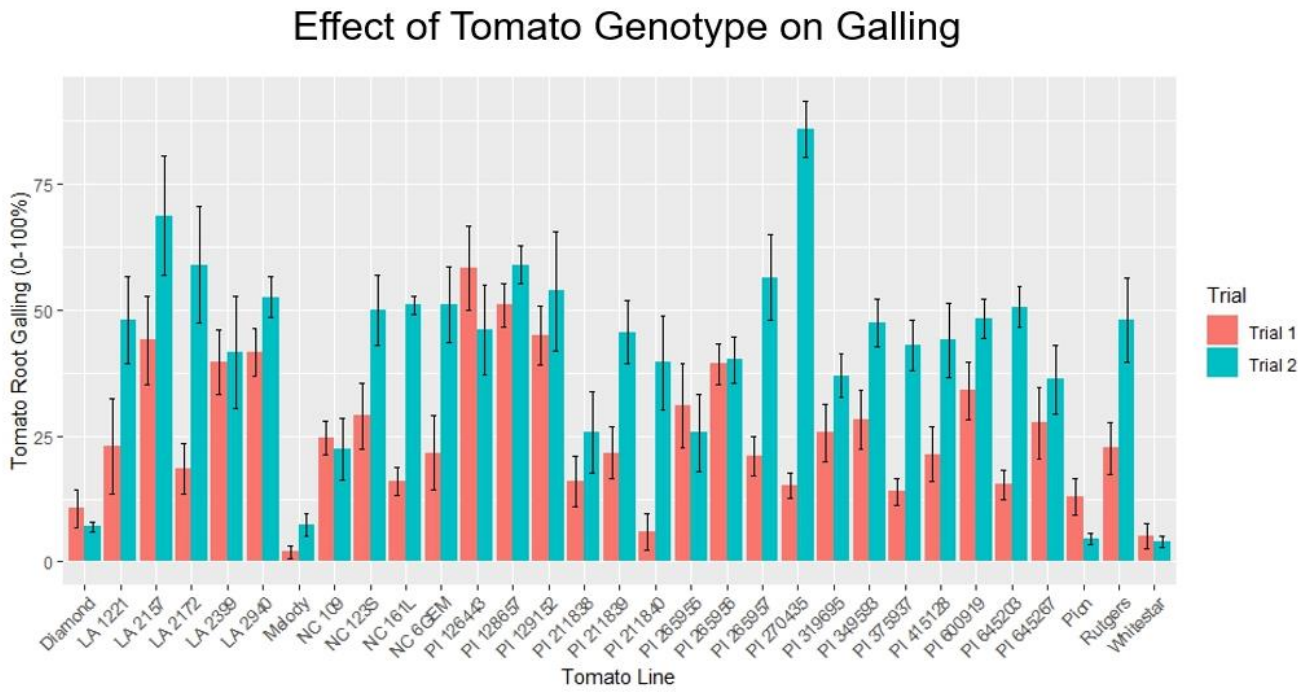

Figure 3.2. Mean root galling severity score for each tomato line evaluated in trials 1 (orange) and 2 (blue). Values are averages of 5 replicate plants. Bars represent standard error about the mean. The galling severity was 22% higher in trial 2 than trial 1.

Figure 3.3. Photos taken from trial 2 showing the roots of highly resistant lines Melody (row A), Whitestar (row B), Diamond (row C), and Pion (row D). Limited galling was observed on the inoculated plants (right) compared to the non-inoculated control plant (left) for each line. In addition to less severe galling, these plants also showed a total root mass similar to the non-inoculated control plant.

Figure 3.4. Photos from trial 2 showing the roots of the moderately resistant line PI 211840 (row A) compared to the susceptible control, Rutgers (row B). Significantly less galling was observed on lines PI 211840 than Rutgers, this line also had an RF less than 1 in the first trial (RF data unavailable for trial 2). The plant on the left shows the non-inoculated control plant with 3 inoculated plants to the right. In addition to less severe galling, PI 211840 also showed a total root mass similar to the non-inoculated control plant.

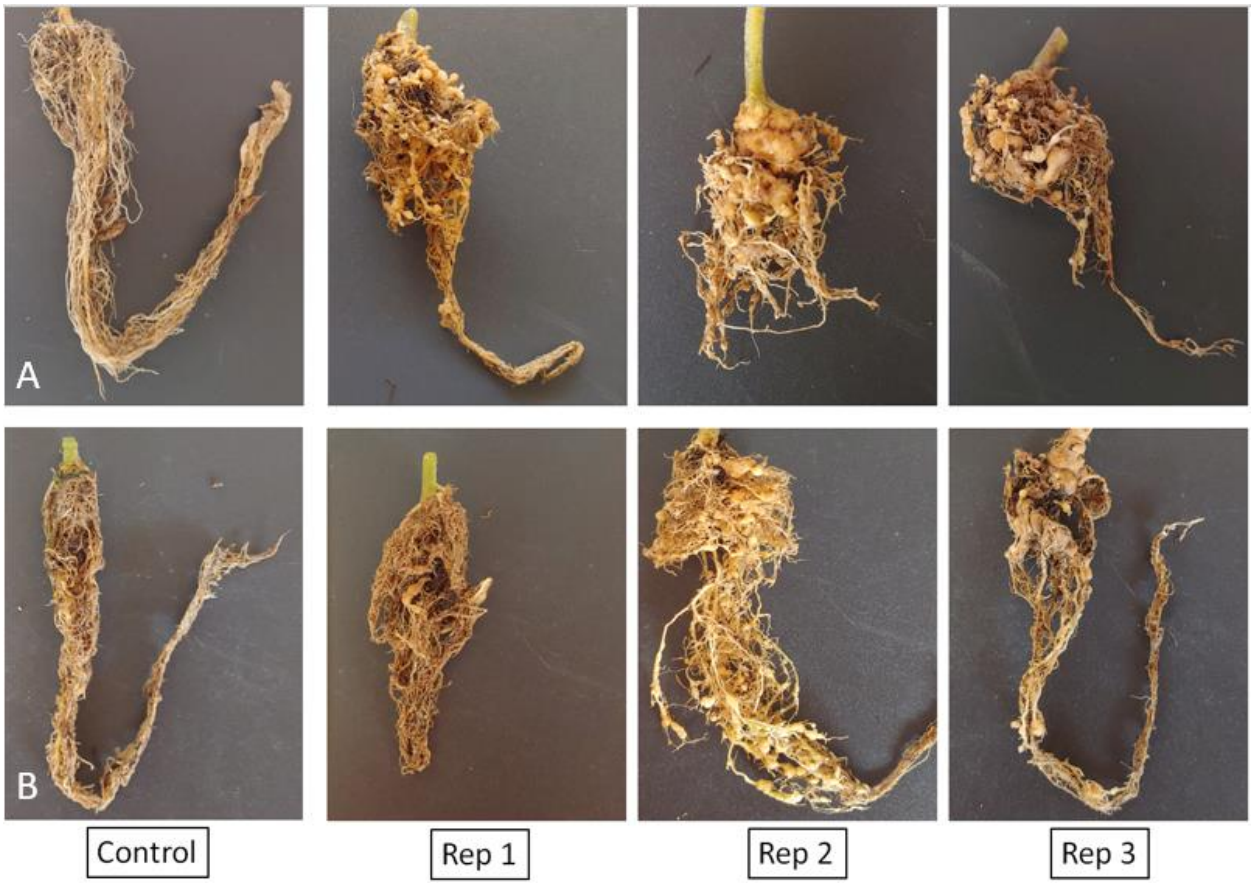

Figure 3.5. Photos from trial 2 showing the root systems of highly susceptible lines PI 270435 (row A) and PI 129152 (row B). Heavy galling was observed on the inoculated plants (right) compared to the non-inoculated control plant (left) for each line. Many of these plants, particularly PI 270435, also showed a loss in the total root mass compared to the control plant. In addition to severe root galling, these plants also had RF values much higher than 1.

CHAPTER 4

Evaluating the Non-fumigant Nematicide, NIMITZ® (fluensulfone), and Grafted Plants for Root-knot Nematode (*Meloidogyne* spp.) Management on Tomato

ABSTRACT

Root-knot nematodes (RKN; *Meloidogyne* spp.) pose a significant threat to many areas where tomatoes are grown. Fumigants, such as methyl bromide, have been used in the past for effective management of RKNs. However, due to environmental and health concerns, many of these fumigants have been or are currently being phased out. This creates the need for alternatives to fumigants for RKN management. Two other options for managing RKNs are the use of non-fumigant nematicides and the use of genetic resistance. A field-based experiment was conducted over the 2021 tomato growing season to evaluate the use of NIMITZ (fluensulfone, ADAMA) and grafted plants with RKN-resistant rootstocks on root galling severity, RKN populations, and yield. This experiment showed that grafted plants are very effective in managing all factors of RKN infection, while NIMITZ did not reduce root galling severity or RKN populations but did increase the total marketable yield. It is recommended that growers do not rely solely on one management tactic but utilize multiple tactics together as part of an integrated disease management plan. The results of this field trial indicated that grafted plants and NIMITZ are useful techniques for managing RKNs in tomato fields- particularly with the decline of available, effective fumigant products.

INTRODUCTION

Root-knot nematodes (*Meloidogyne* spp.) are a significant threat to tomato production, with yield losses as severe as 40% reported (Marques de Carvalho et al., 2015). There are several methods for reducing RKN infection in the field to keep damage below threshold levels, including sanitation, crop rotation, biological control, chemical control, and genetic resistance. Chemical control is the application of a chemical nematicide before, at, or after planting with the goal of reducing nematode populations. Chemical nematicides are available in the form of fumigants and non-fumigants. Fumigants are typically broad-spectrum and are formulated as either a liquid, gas, or granular particles (that volatilize), and move through the open pore space in soil as a gas. Non-fumigant nematicides typically have a narrower spectrum than fumigants and are formulated as either a liquid or granule that are dissolved in water. Some non-fumigant nematicides have systemic activity, where the active ingredient is taken up by the plant and translocated to other parts of the plant (Grabau, 2016). Chemical nematicides are considered one of the most effective ways to manage RKNs in tomato fields; however due to environmental and health concerns, many of the fumigants previously used for RKN management, such as methyl bromide (an ozone depleting substance), are or have been phased out (Ristaino and Thomas, 1997; Martin, 2003). Therefore, alternatives to these dangerous chemicals are critically needed. Some fumigants still available for RKN management in tomato production include chloropicrin (Pic100, Cardinal), 1,3-dichloroproene (Telone II, Dow AgroSciences), and a mixture of these two products (PicClor-60, Cardinal). Chloropicrin is not as effective for managing RKNs as 1,3-dichloropropene and PicClor-60 (Ristaino and Thomas, 1997; Martin, 2003). PicClor-60 is a mixture of 40% chloropicrin and 60% 1,3-dichloropropene, and the 1,3-dichloropropene contributes to the primary nematocidal activity of the formulation. Due to high toxicity and off-

target effects associated with broad-spectrum fumigants, they can also be costly, require specialized application, are highly volatile, and can result in phytotoxicity (Morris et al., 2016).

One alternative to fumigants are non-fumigant nematicides, such as NIMITZ (fluensulfone), which have lower mammalian toxicity, allowing for safer application (Morris et al., 2016). However, there have been many inconsistent reports on the efficacy of NIMITZ for the management of RKNs in tomato. Morris et al. (2016) reported efficient managements of RKNs by both drip and foliar applications of fluensulfone, while others have reported positive results only when NIMITZ was applied in combination with a fumigant compared to just the fumigant alone (Castillo et al., 2017). Other reports have found the fumigant, 1,3-dichloropropene, to be more effective than NIMITZ for managing RKNs (Driver and Louws, 2010). Therefore, further examination of the efficacy of this product is needed.

Another alternative to fumigants is genetic resistance, which is the most environmentally friendly method to manage RKNs (Seid et al., 2015; Hajihassani et al., 2020). Genetic resistance can be employed through the use of resistant cultivars as well as grafting with resistant rootstock. Grafting is a technique used to join two parts from different plants, so they grow as one single plant (Bilderback et al., 2014). The primary objective of grafting tomato plants is to provide resistance to soilborne pathogens (King et al., 2008; Louws et al., 2010; Ingram et al., 2021). This can be achieved by grafting the top portion of a plant (scion) to a resistant rootstock. Often the scion is chosen due to its desirable fruit qualities, such as flavor and appearance. Grafting plants with resistant rootstocks has been successfully utilized in the past on tomato and other vegetable crops (Ioannou, 2001; Lopez-Perez et al., 2006; King et al., 2008). The use of grafted plants is also becoming more economically competitive; as the price of fumigant products is increasing, the price of grafted plants is decreasing (Barrett et al., 2012). Although genetic

resistance for RKNs is species-dependent since the *Mi* gene only confers resistance to *Meloidogyne incognita, M. arenaria, and M. javanica,* it is a useful technique for locations where these species are a problem. To examine these fumigation alternatives further, an experiment was conducted to evaluate the use of the non-fumigant nematicide, NIMITZ, and RKN-resistant rootstock grafted tomato plants to manage disease caused by RKNs on tomato.

MATERIALS AND METHODS

Experimental Design

This experiment took place during the 2021 tomato growing season in collaboration with a grower-cooperator in north Georgia. In recent years, this grower have noticed increasing RKN pressure in areas of the field where they had been absent in previous growing seasons. The RKNs present in this field were diagnosed by the North Carolina Department of Agriculture and Consumer Services (NCDA&CS) as *M. incognita*. The treatments consisted of NIMITZ and RKN-resistant rootstock grafted plants and were arranged in a randomized block design, with all treatments applied within a single row of the field. Two non-treated controls were included in the trial, one within the same row as the treated plots (Control A), and another in the immediate adjacent row boarding the NIMITZ-treated plots (Control B). These two controls (Control A and Control B) only differ by their location in the field but were analyzed separately for this trial. Each of these treatments were replicated 4 times. Rows were pre-bedded by the grower on 28 May 2021 with each plot measuring 50 ft long and 5 ft wide. On 28 May 2021, the fumigant product, Chloropicrin 100, was also applied to the entire field and the plastic mulch, totally impermeable film (TIF), was laid directly after this application to slow the dissipation of the fumigant product. This fumigant was applied to manage other pathogens of tomato present in the

field as chloropicrin has minimal nematocidal activity (Ristaino and Thomas, 1997; Martin, 2003).

NIMITZ Application

NIMITZ was applied to plots on 29 June 2021 at a rate of 80 fl oz./Ac through the drip irrigation system via a small, pressurized tank system. NIMITZ product was mixed with a total of 1.7-gal water and applied at a pressure of approximately 3 PSI over 20 minutes. Because the plots were all within the same row and connected via the same drip tape system, to ensure NIMITZ was applied only to the correct plots in the field, the drip line was cut at the head of the NIMITZ treatment and a connector valve was added to tie in the tank containing the NIMITZ solution. An additional cut was made at the end of the treatment area and a butterfly valve in the off position was added to prevent the solution from continuing down the drip line to other treatments. Once NIMITZ had been applied to each treatment, the drip irrigation system was flushed with water for 15 minutes and was repaired to enable regular water flow. Applications were done a total of 4 times for each replication within the same day.

Planting

Planting for this trial took place on 12 July 2021 by a mechanical transplanter with the exception of the grafted plants, which were planted by hand. The tomato cultivar Loretta (Bejo Seeds, Inc.) was used with a total of 12 plants per plot. Loretta does not contain the *Mi-1* gene, which confers resistance to *M. incognita*. Since the grower preferred to grow certain cultivars (Loretta, Scarlet Red, and Plum Regal) which do not contain the *Mi-1* gene, grafted plants with resistant rootstocks (*Mi-1* gene) were also included in this trial. For grafted plants, the tomato cultivar Loretta was grafted onto the resistant rootstock Beaufort (De Ruiter, Now Bayer Crop Science). Grafting was done by TriHishtil, Mills River, NC.

Soil Sampling

Soil samples were collected on 5 November 2020 to obtain RKN population data for this field prior to treatment. This pre-season sample was collected before beds were formed, so bulk soil samples were collected by estimating the placement of each replication. However, because these soil samples were bulked by replication, they do not represent each individual treatment, only the entire replication. Therefore, RKN populations do not differ for the NIMITZ and grafted treatments, or Control A within the same replication since they were located within the same sample. Soil samples were collected again on 23 September 2021 at harvest. These soil samples were collected to represent each individual treatment and replication. All samples were sent to the NCDA&CS Nematode Assay Laboratory, in Raleigh, NC for processing to obtain nematode counts.

Harvest and Disease Assessment

Beginning on 23 September 2021, tomato fruit was harvested from each plot 3 times, approximately 1 week apart. For the first two harvests, tomatoes were harvested if they were red in color or at the 'breaker stage' where the fruit shows the first signs of changing color. At the last harvest, all fruit was harvested regardless of color. After each harvest, tomatoes were sorted by USDA standards (small, medium, large, extra-large, and jumbo) or as unmarketable and were weighed. The data for each size was combined to obtain a total marketable yield for each treatment and replication.

At the last harvest, three plants from each plot were dug from the ground, the stem and foliage were trimmed off, and the root system was brought back to the lab. The root system of each plant was rinsed thoroughly with tap water to remove soil particles and was scored visually for root galling severity using a variation of the Zeck scale (Zeck, 1971).

Statistical Analysis

Root galling severity, RKN populations, and yield data were subjected to an analysis of variance (ANOVA) using RStudio, (v. 3.6.2, R Core Team 2019). Where significant differences where identified, means were separated using Fisher's Least Significant Difference test (function 'LSD.test' within the 'agricolae' package). RKN population data was log transformed (log(x+1)) prior to analysis to normalize data because nematode populations were skewed. Due to flooding on the edge of the field where the first replication was located (**Figure 4.1**), ANOVA was performed with and without the first replication to get a more accurate representation of the data from this experiment.

RESULTS

Table 4.1 shows the root galling severity, RKN population at harvest, and the total marketable yield for each treatment with the data from replication 1 included. When the first replication was included in analysis, there was no significant difference observed in galling severity between treatments ($P > 0.1$). However, when the first replication was removed, there was a moderately significant difference observed in root galling severity between treatments ($P < 0.01$). The NIMITZ treatment and Control B were found to have significantly more galling than Control A and the grafted treatment (**Figure 4.2**).

Similar to galling severity, when the first replication was included in analysis, there was no significant difference observed in RKN populations at harvest between treatments ($P > 0.1$). However, when the first replication was removed, there was a significant difference observed in RKN populations at harvest between treatments ($P < 0.05$). The NIMITZ treatment and Control B were found to have a significantly higher RKN population than Control A, which had a

significantly higher RKN population than the grafted treatment (**Figure 4.3**). No significant difference in total marketable yield between treatments was observed (P > 0.1) (**Figure 4.4**).

DISCUSSION

Based on the root galling severity data from this experiment, grafted plants had an average of less than 5% root galling (**Figure 4.2**). In addition to reducing root galling severity, grafted plants reduced RKN populations, and had the highest numerical yield of all treatments. This conflicts with a previous study in which the same rootstock Beaufort did not significantly reduce root galling severity or RKN populations compared to non-grafted plants but did significantly increase yield, whereas in this study Beaufort was very effective in managing all aspects of disease caused by RKNs in this field (Lopez-Perez et al., 2006). Grafting with resistant rootstock has been reported to provide resistance for RKNs as well as several other major tomato diseases like bacterial wilt (*Ralstonia solanacearum*), fusarium wilt (*Fusarium oxysporum*), verticillium wilt (*Verticillium dahlia*), and tomato yellow leaf curl virus (TYLCV) (King et al., 2008). Control A also showed significantly less root galling than NIMITZ and Control B. Although the distribution of RKNs in the field was accounted for by the randomized block design, this could have been attributed to differences in nematode pressure within this treatment. This explanation is reflected by the difference in RKN populations observed at harvest for both controls- the final RKN population of Control A is significantly lower than Control B although they both received no treatment (**Figure 4.3**) except fumigation with chloropicrin.

While grafted plants reduced root galling severity, NIMITZ showed high root galling severity when compared to non-treated controls. Similarly, grafted plants decreased total RKN populations at harvest compared to the other treatments, whereas NIMITZ had the highest RKN

population at harvest. NIMITZ has been reported to have relatively fair efficacy for managing RKNs while other non-fumigants like Velum Prime (fluopyram) and Vydate L (oxamyl) have been reported to have good efficacy. Alternatively, fumigant nematicides like Telone II (1,3-dichloropropene) and PicClor-60 (1,3-dichloropropene and chloropicrin) have been reported to have relatively excellent efficacy for managing RKNs (Gorny and Schwarz, 2020). The fair efficacy of NIMITZ is corroborated by this experiment as well as another study which reported NIMITZ to reduce RKN damage on sweetpotato when compared to the non-treated control, while Velum Prime and Vydate were significantly more effective in reducing RKN damage than NIMITZ (Collins et al., 2017). Despite the high root galling severity and RKN population at harvest, NIMITZ had a total marketable yield numerically higher than either control, including Control A which showed significantly lower root galling and RKN populations at harvest compared to NIMITZ. This is similar to other reports that show a numerical increase in yield when NIMITZ was applied compared to non-treated controls (Collins et al., 2017; Rosado-Rivera and Thiessen, 2018). Additionally, the efficacy of NIMITZ is dependent on the host plant species. For example, NIMITZ has been reported to be very effective for managing RKNs on carrots (Morris et al., 2013; Becker, 2016). NIMITZ does not appear to be the most effective non-fumigant nematicide available but does provide some level of protection to the host plant by reducing disease caused by RKNs and numerically increasing yield at the end of the season (Collins et al., 2017).

Since NIMITZ was applied prior to planting, this likely subdued RKN populations at the beginning of the growing season, letting tomato plants grow and get ‘a head start’ on the nematodes. However, over the course of the growing season, NIMITZ began to dissipate from the soil, giving the remaining RKNs the opportunity to rebound with an abundance of tomato

roots available to infect. Therefore, NIMITZ may be more effective when applied at plant rather than pre-plant to reduce the window for nematode populations to rebound (Collins et al., 2017). This also explains why the grafted plants had significantly less root galling, RKN populations at harvest, and a higher yield than the NIMITZ treatment as genetic resistance offers season long management of RKNs.

Flooding in the field may have had adverse effects on RKN populations as well as tomato plant health. Although water is essential for plant and nematode growth, too much water creates an anaerobic environment that can be detrimental to plant establishment and nematode populations. While RKNs do not breathe oxygen through an advanced respiratory system as many other animals do, oxygen is required for processes such as gas exchange through the cuticle. This is why flooding can be a useful technique for managing RKN populations (Agrios, 2005). Plant roots also require oxygen for metabolic processes like nutrient uptake and respiration as roots will die without sufficient soil oxygen. Based on the results from removing the first replication, the flooding in this section of the field appeared to have a significant impact on root galling severity, RKN populations, and tomato yield. Data from the NIMITZ treatment and Control A, shows the first replication is very different numerically when compared to the rest of the data set (**Table 4.2**). Thus, taking out the first replication is more representative of the effects of each treatment in this study.

It is not known exactly how these nematodes were introduced to this part of the field, but this and other field-based studies highlight sanitation as a key management technique for RKNs. Proper sanitation techniques could have prevented the introduction of these nematodes to this part of the field (Thiessen, 2018). This also emphasizes the need for integrated disease management where several management techniques are utilized. Grafting tomato plants with

resistant rootstocks is very effective for managing root galling severity, RKN populations, and increasing the total marketable yield compared to no treatment. While NIMITZ may not have been successful in effectively reducing galling severity or RKN populations when faced with a high nematode pressure, it did numerically increase total marketable yield compared to the non-treated controls. Thus, both NIMITZ and grafted plants are useful tools to include in the integrated disease management toolbox for RKNs, especially with the steady decline of effective fumigants. The Covid-19 pandemic interfered with implementing year one of this study, this trial was conducted over year two, and the grower had to rotate to another field due to high RKN pressure by year three. Therefore, a second year of data could not be secured within the life of the grant.

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Table 4.1. The root galling severity, root-knot nematode (RKN) populations at harvest, and total marketable yield showing the variations observed for the first replication due to flooding in the field. Two non-treated controls were included in the trial, one within the same row as the treated plots (Control A), and another in the immediate adjacent row boarding the NIMITZ- treated plots (Control B).

Treatment	Replication	Galling Severity (0-100%)	RKN Population at Harvest	Total Marketable Yield (kg/ha)
NIMITZ	1	1.7	90	13557
	2	56.7	12120	23240
	3	32.7	10480	42177
	4	19.3	2440	51214
Grafted	1	2.3	970	30772
	2	0.3	1000	16785
	3	0.0	10	60683
	4	0.3	10	55948
Control A	1	80.0	17600	8392
	2	0.3	80	2797
	3	14.3	5450	66493
	4	3.3	480	32063
Control B	1	48.3	9600	13557
	2	16.0	1800	16569
	3	36.7	6200	40240
	4	43.3	3240	45404

Figure 4.1. Photo taken on 23 September 2021, showing the severe flooding that occurred in the first replication of treatments included in this trial. This section is located on the edge of the field in close proximity to a creek. Photo Credit: A. Philbrick.

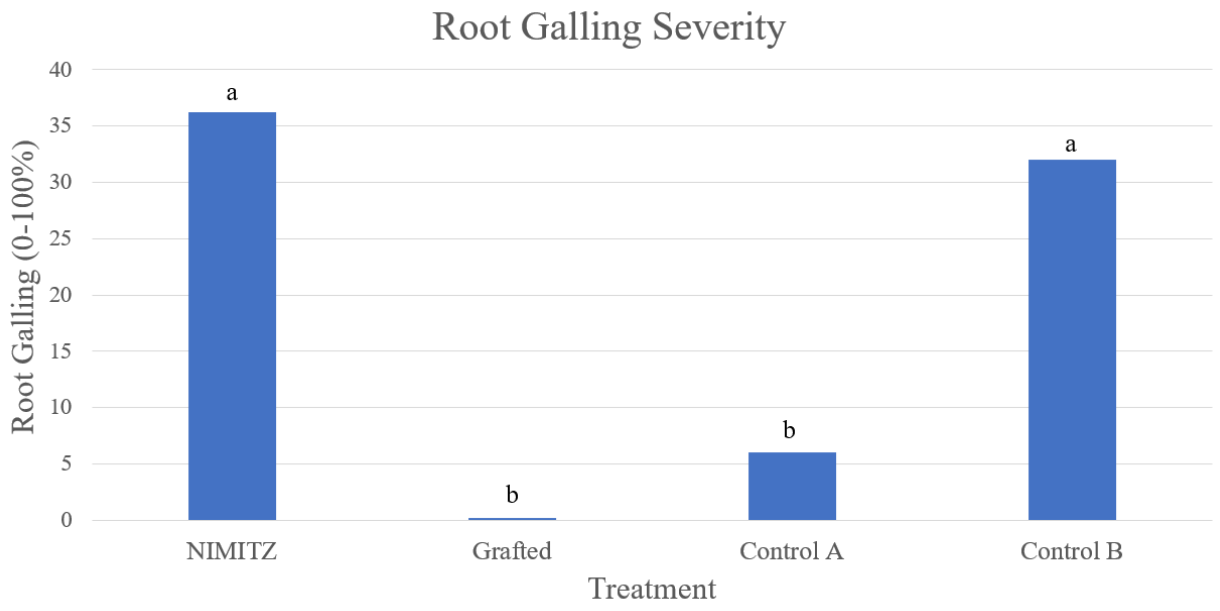

Figure 4.2. The average galling severity for each treatment with the first replication removed. Treatments with the same letter appearing above the bar are not significantly different at the $\alpha = 0.05$ level.

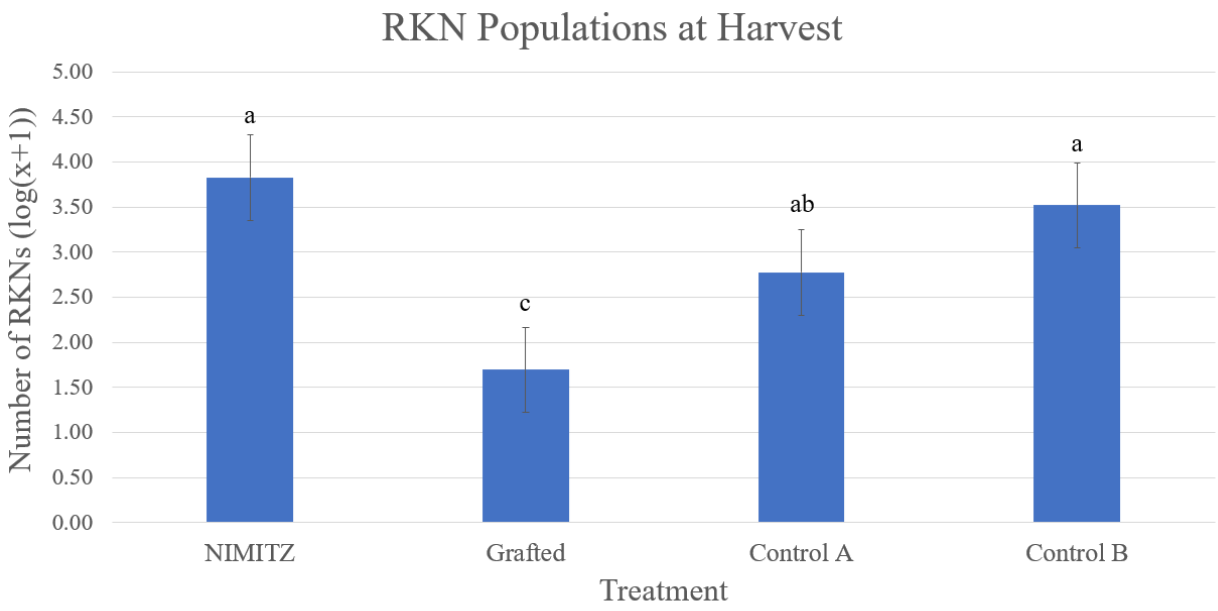

Figure 4.3. Root-knot nematode populations at harvest for each treatment with the first replication removed. Data were log transformed (log(x+1)) prior to analysis to normalize count data. Bars represent standard error about the mean. Treatments with the same letter appearing above the bar are not significantly different at the $\alpha = 0.05$ level.

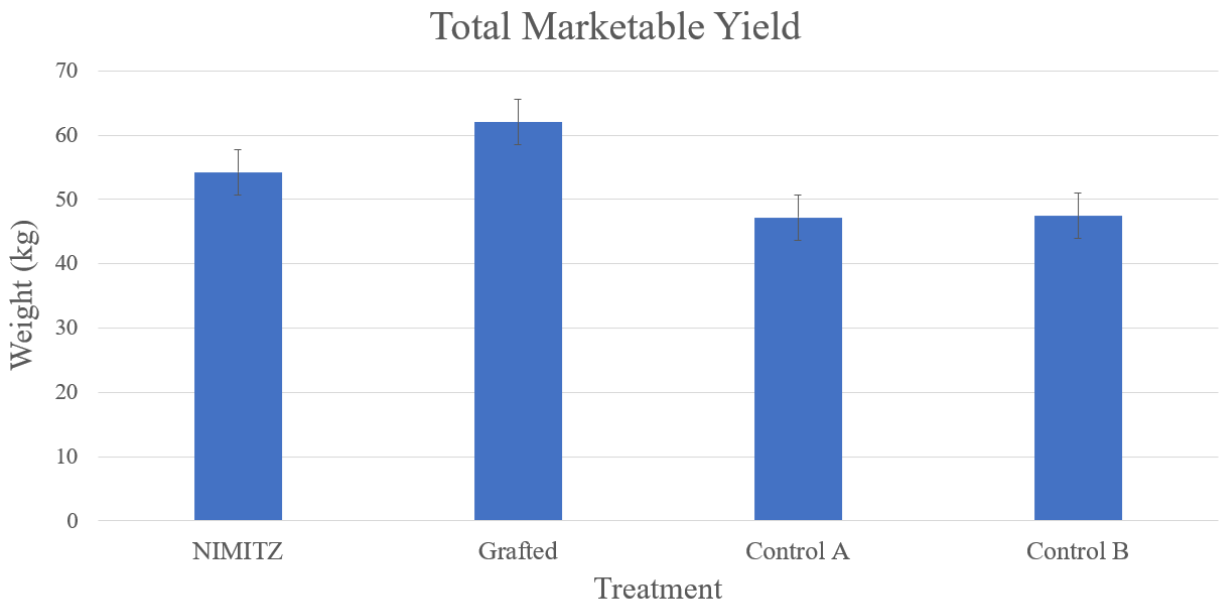

Figure 4.4. The total marketable yield for each treatment. Bars represent standard error about the mean. Both the grafted plants and NIMITZ treatments had a numerically higher total marketable yield when compared to the non-treated controls (Control A and Control B). Treatment groups were not significantly different from each other at the $\alpha = 0.05$ level.