

**Reproduction of a root-knot nematode population on flue-cured tobacco
homozygous for *Rk1* and/or *Rk2* resistance genes and the effect of soil temperature
on resistance gene efficacy**

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ABSTRACT

Utilizing resistant cultivars is a main control strategy for root-knot nematodes in flue-cured tobacco (*Nicotiana tabacum* L.). Most commercial cultivars possess the *Rk1* gene, providing resistance to races 1 and 3 of *Meloidogyne incognita* and race 1 of *M. arenaria*. This initiated a shift in root-knot populations to other species and races, creating a need for resistance to those populations. Numerous cultivars possess a second resistance gene, *Rk2*. Greenhouse experiments investigated whether possessing both *Rk1* and *Rk2* increases resistance to a variant of *M. incognita* race 3 compared to either gene alone, and if high soil temperatures impact their efficacy. Root galling, numbers of egg masses and eggs, and the reproductive index were compared from roots of Coker 371-Gold (susceptible), NC 95 and SC 72 (*Rk1Rk1*), T-15-1-1 (*Rk2Rk2*), and STNCB-2-28 and NOD 8 (*Rk1Rk1* and *Rk2Rk2*). The same data were analyzed from plants in open-top root zone cabinet growth chambers set to 25°C, 30°C, and 35°C to examine if resistance is temperature sensitive. Despite variability, *Rk1Rk2* entries conferred greater resistance than entries with *Rk1* or *Rk2* alone. Entries with *Rk1* alone reduced galling and reproduction compared to the susceptible control, whereas T-15-1-1 (*Rk2*) did not, but often suppressed reproduction. An apparent reduction in nematode reproduction was observed at 25°C and 30°C on entries possessing *Rk1* and *Rk1Rk2* compared to the control and *Rk2*. However, no apparent differences in reproduction occurred on *Rk1* and/or *Rk2* entries at 35°C compared to the control, indicating parasitism increased on resistant entries at higher temperatures.

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CHAPTER 1

Literature Review and Research Objectives

FLUE-CURED TOBACCO PRODUCTION

Tobacco importance in the United States and in Virginia. Tobacco (*Nicotiana tabacum* L.) is an important agricultural commodity grown worldwide. The United States is the fourth-largest producer of tobacco in the world, after China, Brazil, and India (FAO, 2015). Flue-cured tobacco makes up the largest portion of tobacco types grown in the United States, with an estimated 245,000 acres grown in 2014. In Virginia alone, 22,500 acres of flue-cured tobacco were grown in 2014, yielding 54 million pounds (USDA, 2015).

Root-knot nematodes on tobacco. Root-knot nematodes are a problem wherever flue-cured tobacco is grown and are responsible for estimated tobacco yield losses of 15% worldwide (Schneider, 1991a). Because flue-cured tobacco is generally grown in warm environments with light sandy or sandy loam soil, which is ideal for root-knot nematodes, root-knot parasitism is more severe in flue-cured tobacco than other types (Johnson, 1998). In Virginia, estimated yield losses in flue-cured tobacco due to root-knot nematodes range from 1% to 5% (Koenning et al., 1999); lower than the worldwide estimates, presumably due to the widespread use of soil nematicides (Johnson, 1998).

ROOT-KNOT NEMATODE EPIDEMIOLOGY

The genus *Meloidogyne*. Berkeley first described root-knot nematodes in the mid-1800s, but did not provide a name (Berkeley, 1855). In 1879, Cornu described root-knot nematodes using the name *Anguillula marioni* (Cornu, 1879). Shortly after, Müller illustrated perineal

patterns of root-knot nematodes, referring to them as *Heterodera radicum* (Müller, 1884). Then, Treub identified another root-knot species as *Heterodera javanica* (Treub, 1885). Göldi established the genus name *Meloidogyne* in 1887 (Göldi, 1892); however, root-knot nematodes weren't officially named *Meloidogyne* until 1949, when Chitwood reclassified the genus (Chitwood, 1949; Hunt and Handoo, 2009). Root-knot nematodes are obligate parasites that have an enormous host range, estimated to be pathogenic on over 3,000 plant species worldwide (Schneider, 1991a). Currently, there are approximately 100 species of root-knot nematodes described (Hunt and Handoo, 2009). However, the majority of the crop damage worldwide is attributed to only four *Meloidogyne* species: *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. javanica* (Treub, 1885) Chitwood, 1949, and *M. hapla* Chitwood, 1949 (Chitwood, 1949; Sasser, 1980); however, *M. enterolobii* Yang and Eisenback, 1983, *M. naasi* Franklin, 1965, *M. graminicola* Golden and Birchfield, 1965, and other species are increasingly being recognized as being responsible for a significant loss in crop production.

***Meloidogyne* species in Virginia tobacco.** Host resistance to *M. incognita* races 1 and 3 and *M. arenaria* race 1 is a widely used control tactic for root-knot nematodes in flue-cured tobacco (Koenning et al., 1999). Because of the widespread use of resistant cultivars, species and races that cannot be controlled by the cultivars are increasing in prevalence and importance: *M. incognita* races 2 and 4, *M. arenaria* race 2, and *M. javanica* (Fortnum et al., 1984; Barker, 1989; Eisenback, 2012).

Morphological identification. The life stages of root-knot nematodes differ dramatically in their morphology. The infective stage, second-stage juveniles (J2), are motile and vermiform in shape, averaging 400 µm by 15 µm (Webster, 1985). Similar to J2, males are motile and

vermiform, but much longer (an average of 1400 μm) and approximately 30 μm wide (Webster, 1985). On average, root-knot females measure 700 μm by 400 μm , are pyriform, and are sedentary within a plant root (Taylor and Sasser, 1978; Webster, 1985). The four most common root-knot nematode species on tobacco are most easily differentiated by the female perineal pattern, head shape, and stylet shape. The female perineal pattern is commonly used because species have differing characteristics of the dorsal arch, lateral ridges, striae, and the tail terminus (Eisenback et al., 1981; Eisenback, 1985). Species can also be distinguished by the male head shape and stylet morphology; however, males are generally rare in some populations. Juvenile head shape, stylet morphology, and tail length can also be used, but species differences in juvenile morphology are smaller than those between adults (Eisenback, 1985). Hunt and Handoo (2009) acknowledge that correct identification to species using morphology can be difficult due to the extent of intraspecific variation and morphological similarities between species. Utilizing PCR-based identification tools along with morphology and host range assays are crucial for proper identification. Proper identification to species is critical when utilizing species-specific control options such as some crop rotations and resistant varieties.

Molecular identification. PCR-based identification methods are a tool to aid in proper nematode identification, and using these methods together with traditional methods can be critical to achieving a more accurate species identification.

Morphology-based and host specificity-based identification have the potential for misidentification to a species level, due to the possibility of intraspecific variation in perineal patterns, host preference differences, and mixed populations (Agudelo et al., 2011).

PCR-based identification methods are beneficial in that a single nematode of any life stage can be used (Blok and Powers, 2009; Agudelo et al., 2011). Perineal patterns are most

often used for morphological identification; however, females are not always available, especially in soil samples. Soil samples most often contain J2, and J2 are generally impractical to use for species identification due to similar morphology.

One of the most common genetic regions used is ribosomal DNA (rDNA). This can be used to distinguish species using the 18S, 28S, 5.8S coding genes, and the internal transcribed spacer (ITS) region. It is important to note that even with rDNA, intraspecific variation has been observed (Blok and Powers, 2009). Additionally, the ITS regions are similar for the four common tobacco root-knot nematode species, creating a need for species-specific primers; many of which have been created (Blok and Powers, 2009; Agudelo et al., 2011).

Host identification. The North Carolina Differential Host Test is a common identification tool based on host susceptibility. It differentiates between the four most common root-knot nematodes (*M. incognita*, *M. arenaria*, *M. javanica*, and *M. hapla*), and helps identify *M. incognita* and *M. arenaria* to race (Table 1.1). The ability of a root-knot population to reproduce on certain cultivars of tobacco, cotton (*Gossypium hirsutum* L.), pepper (*Capsicum annuum* L.), watermelon [*Citrullus lanatus* var. *lanatus* (Thunb.) Matsum. and Nakai], peanut (*Arachis hypogaea* L.), and tomato (*Solanum lycopersicum* L.) is the basis of the host test (Taylor and Sasser, 1978).

Table 1.1. Differential host test for common *Meloidogyne* species and races.

<i>Meloidogyne</i> species and race	Differential host plant cultivars					
	Tobacco 'NC 95'	Cotton 'Deltapine 16'	Pepper 'California Wonder'	Watermelon 'Charleston Grey'	Peanut 'Florunner'	Tomato 'Rutgers'
<i>M. incognita</i>						
Race 1	–	–	+	+	–	+
Race 2	+	–	+	+	–	+
Race 3	–	+	+	+	–	+
Race 4	+	+	+	+	–	+
<i>M. arenaria</i>						
Race 1	+	–	+	+	+	+
Race 2	+	–	–	+	–	+
<i>M. javanica</i>	+	–	–	+	–	+
<i>M. hapla</i>	+	–	+	–	+	+

Variability among populations is a critical downside to the host test. For instance, some *M. javanica* populations can reproduce on pepper and peanut, even though the host test assumes that *M. javanica* is not pathogenic on either (Eisenback et al., 1981; Hartman and Sasser, 1985). Hartman and Sasser (1985) displayed data from almost 1000 populations, and found that 62% of *M. arenaria* race 1 populations parasitized tobacco, 85% of *M. arenaria* race 1 populations parasitized pepper, and 35% of *M. arenaria* race 2 populations parasitized pepper. The host test assumes that *M. arenaria* race 2 does not parasitize pepper, despite Hartman and Sasser establishing that over a third of populations do. Additionally, Barker and Melton (1990) noted that populations of *M. arenaria* derived from peanut have limited reproduction on tobacco NC 95, despite the host test noting that *M. arenaria* reproduces on it. Because there is a great deal variation between populations, it is risky to use the North Carolina Differential Host Test as a diagnostic tool alone without morphological or PCR-based methods as well.

More recently, authors have suggested adding new races to *Meloidogyne* species. Robertson et al. (2009) proposed new races of *M. incognita*, *M. arenaria*, and *M. javanica* in

Spain based on deviations from the host test. Similarly, López-Pérez et al. (2011) classified a number of populations on tobacco in Spain as *M. arenaria* race 3 because they parasitized pepper, but not peanut.

Life cycle: Hatch and movement. A female root-knot nematode lays up to several hundred eggs in a gelatinous egg mass, usually on the outside of the root (Curtis et al., 2009). Primarily dependent on soil temperature and moisture, the first-stage juvenile completes its first molt within the egg, becoming a second-stage juvenile (J2), and then hatches (Taylor and Sasser, 1978; Curtis et al., 2009). The J2 then moves through the egg membrane into the soil to find a root tip to penetrate (Eisenback and Hunt, 2009). Juveniles can move up to 40 to 100 cm vertically in the soil in ideal conditions (Eisenback and Hunt, 2009). A J2 is able to locate host roots by using chemosensory organs in its head (amphids) and tail (phasmids), which allow it to recognize chemical gradients and plant root-exudates in the soil (Hussey, 1985; Curtis et al., 2009). If conditions are not ideal for hatch, such as winter temperatures or host plant senescence, a J2 can remain quiescent and viable within an egg membrane for a period of time known as diapause. A diapause is a state of arrested development in which development will not resume until favorable conditions return, such as higher temperatures or the presence of a host. This is commonly how root-knot nematodes overwinter (Curtis et al., 2009).

Life cycle: Parasitism. After a J2 has found a root tip, it penetrates the host cortex above the root cap in the zone of elongation, and starts moving intercellularly towards the root cap, within the zone of elongation (Taylor and Sasser, 1978). Once migrating past the casparian strips, which may hinder a J2 from directly reaching the vascular system, the J2 turns around and starts migrating away from the root tip, inside the root vascular cylinder (Taylor and Sasser, 1978; Abad et al., 2003; Abad et al., 2009; Eisenback and Hunt, 2009). In the vascular cylinder,

the J2 pierces parenchyma cells with its stylet to begin feeding. The nematode secretes enzymes through its stylet that trigger increasing cell division in the host plant cells on which it is feeding (Taylor and Sasser, 1978; Abad et al., 2003). These cells enlarge into “giant cells” by dividing into multinucleate and hypertrophied cells without forming new cell walls. The J2 triggers the formation of three to eight giant cells, and feeds on them for three to eight weeks, depending on environmental conditions (Webster, 1985; Eisenback and Hunt, 2009). Cells surrounding the giant cells also exhibit hypertrophy, and hyperplasia (Caillaud et al., 2008). As the J2 feeds, it swells into a sausage-shape and molts quickly to a third-stage and fourth-stage juvenile, then adult (Fig. 1.1). Root-knot nematodes are usually only third-stage and fourth-stage juveniles for a combined four to six days before molting into an adult (Moens et al., 2009). During the fourth molt, a juvenile that will become a male will transform back into a vermiform shape, leave the root, and never resume feeding. Juveniles which will become female will remain sedentary inside the root, remain pyriform, will continue to feed, and eventually lay eggs in a gelatinous egg mass, within galled tissue that usually breaks through on the external surface of the host root (Webster, 1985; Abad et al., 2003; Eisenback and Hunt, 2009). Most root-knot populations reproduce by parthenogenesis and do not produce males, but juveniles will turn into males rather than females when populations are stressed; because males do not feed and thus compete with reproducing females for scarce resources (Triantaphyllou, 1960; Laughlin et al., 1969).

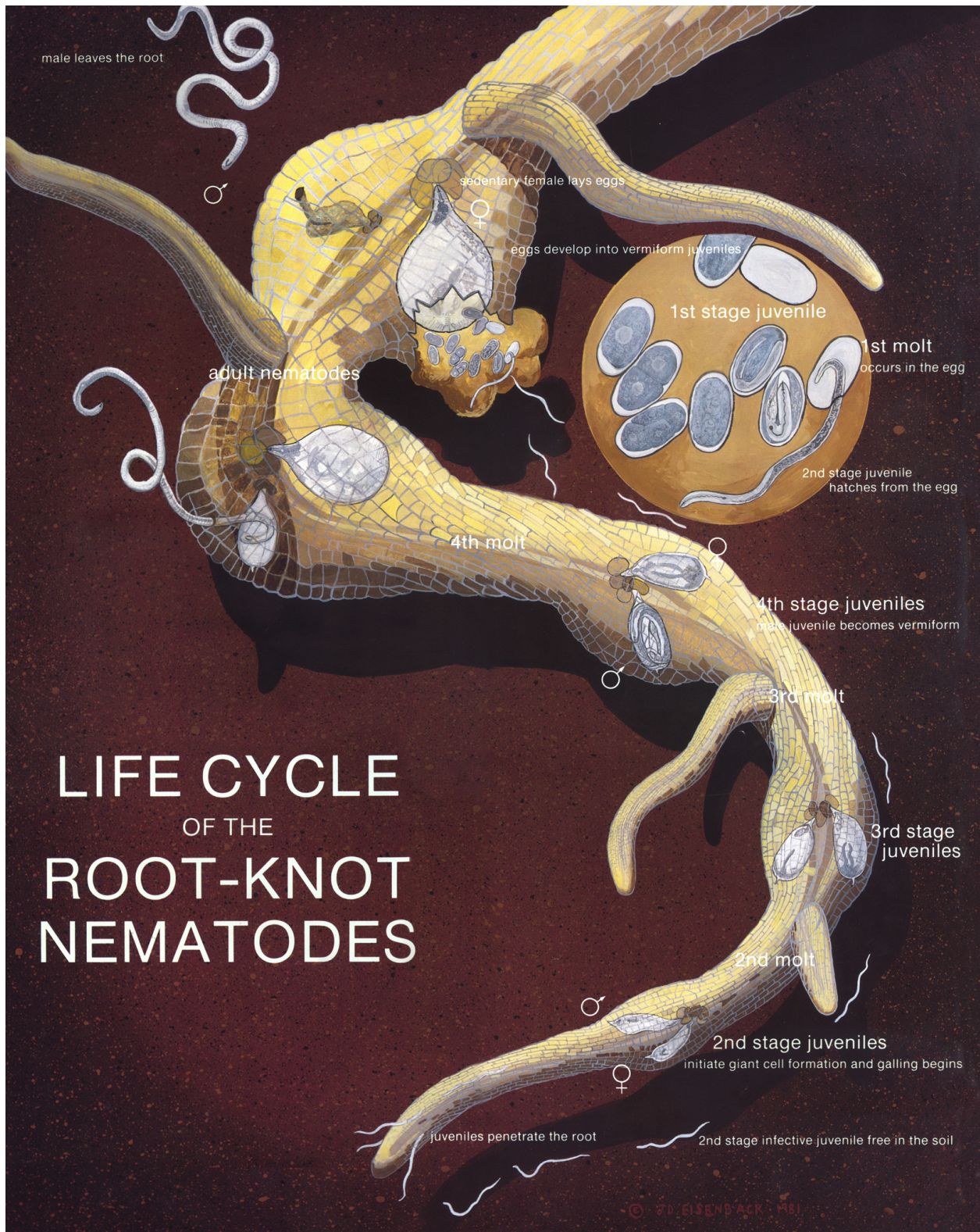


Fig. 1.1. Life cycle of a root-knot nematode. First-stage juvenile: J1. Second-stage juvenile: J2. Third-stage juvenile: J3. Fourth-stage juvenile: J4. Poster by J.D. Eisenback (Eisenback, 1981).

Symptoms. The primary aboveground symptoms displayed by tobacco plants parasitized by root-knot nematodes are stunting and leaf chlorosis. Plant growth is typically uneven in infested fields due to irregular areas with stunted plants (Fig. 1.2). This uneven growth occurs because root-knot nematode populations tend to be distributed in clusters or “hot spots”, where nematode population densities are high. Because of the plant’s reduced ability to uptake water and nutrients due to root-knot nematode parasitism, aboveground plant parts often exhibit symptoms of drought injury or nutrient disorders, such as nitrogen or potassium deficiencies. Drought injury causes plant wilting, tobacco nitrogen deficiencies are marked by leaf chlorosis, and tobacco potassium deficiency can cause marginal chlorosis and occasionally necrotic leaf spots. Severe root-knot infestations may cause loss of leaves or even plant mortality. The most noteworthy symptom of root-knot parasitism is the presence of galling on roots; the host plant response of cell hypertrophy where root-knot nematodes feed. The resulting galls can either be small and look like “beads on a string,” or amassed together and be several times the natural width of a root (Fig. 1.3) (Lucas, 1975; Shew and Lucas, 1991).



Fig. 1.2. A tobacco field in Southside Virginia showing symptoms of a nematode infestation.



Fig. 1.3. Typical root galling of tobacco (*Nicotiana tabacum* L.) infested with root-knot nematodes (*Meloidogyne* spp.).

Root-knot disease complexes. Root-knot nematodes are often associated with one or more other plant pathogens, causing more severe symptoms on a plant than either would alone. This association has been defined as a disease complex (Powell et al., 1971). Numerous studies have examined the incidence and severity of other tobacco pathogens when root-knot nematode species were present, such as black shank caused by *Phytophthora nicotianae* Breda de Haan, (Powell and Nusbaum, 1960), Fusarium wilt caused by *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen (Porter and Powell, 1967), and bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi et al. (Johnson and Powell, 1969). These diseases were more severe than they would have been alone, and in the case of black shank and Fusarium wilt, the fungal pathogens became the predominant pathogen on the co-infected plant (Powell et al., 1971).

It was initially thought that the root damage caused by nematodes enabled other pathogens to gain entry, but studies have indicated that the relationship between root-knot nematodes and other pathogens may be more complicated. A nematode infestation is thought to

weaken a plant's immunity, making it more vulnerable to attack by other pathogens (Webster, 1985). In some cases, the nematode can "break" a plant's resistance to fungi, which was found to be the case with *Meloidogyne* spp. and Fusarium wilt. *M. incognita*, *M. javanica*, and *M. arenaria* were noted to have predisposed resistant plants to *F. oxysporum* in tobacco, and *M. javanica* to *F. oxysporum* in tomato (*Solanum lycopersicon* L.) (Porter and Powell, 1967; Webster, 1985). Powell et al. (1971) proposed that that *M. incognita* altered the internal physiology of tobacco, which led to fungi being able to infect the plant. Similarly, Webster (1985) suggested that in bean (*P. vulgaris*) "*Meloidogyne* modifies the host's mineral composition and physiology and, consequently, slows host growth, and in many instances breaks the host's resistance to other parasites." Powell et al. (1971) observed that even some soil-borne fungi that are not pathogenic on mature tobacco became pathogenic when *M. incognita* was present. *Pythium ultimum*, *Rhizoctonia solani* J.G. Kühn, and *Trichoderma harzianum* Rifai were not a problem in adult tobacco plants except when there was a root-knot infestation, and then they caused root necrosis, plant stunting, chlorosis, and wilting (Powell et al., 1971).

ROOT-KNOT NEMATODE MANAGEMENT

Management strategies. There are numerous approaches to controlling root-knot nematodes in tobacco. Soil nematicides are one of the most consistent and beneficial methods, and commercial use of these products began around 1945 (Johnson, 1985). Soil nematicides are categorized into soil fumigants and non-fumigant nematicides. Soil fumigants are liquids, solids, or gases that vaporize once they are in the soil and move through soil pores and water, killing soil organisms. They are generally broad-spectrum and kill nematodes on contact (Spurr, 1985). Chloropicrin and 1,3-dichloropropene (1,3-D) are widely used volatile liquid (fumigant)

nematicides for tobacco and are often commercially used together in a mixture (Johnson et al., 2005). Methyl bromide is a broad-spectrum soil fumigant that was extensively utilized due to its efficacy, but has been phased out completely due to ozone-depletion effects (U.S. EPA, 2014). Non-fumigant (or contact) nematicides are liquids or solids that are incorporated into soil and spread through soil water, but do not volatilize. These generally have a narrower target spectrum than soil fumigants, and are commonly used as insecticides as well as nematicides. Non-fumigant nematicides can be classified into two chemical groups, carbamates and organophosphates (Spurr, 1985). Unlike fumigant nematicides, non-fumigant nematicides often paralyze nematodes instead of killing on contact (Spurr, 1985). Vydate® is a current, commonly used non-fumigant nematicide.

Crop rotation is a key cultural practice that can reduce root-knot nematode population levels or even switch populations from a more-aggressive to less-aggressive species. Since the host range for *Meloidogyne* spp. is variable, safe crops to rotate into are generally forage grasses and small grains, such as fescue, rye, corn, and sorghum (Fortnum et al., 2001; Johnson et al., 2005). However, some rotation crops are hosts for different *Meloidogyne* spp., such as soybean, cotton, peanut, and a number of vegetables, which is why species identification can be critical before choosing a cover crop.

Biocontrol is another option for root-knot nematode management. Some species of fungi that trap and kill nematodes have been looked into as biocontrol options for controlling root-knot nematodes. Similarly, plant growth-promoting rhizobacteria have been observed to increase tobacco resistance to *Meloidogyne* spp. (Johnson et al., 2005).

Resistant cultivars: History of *Rk1* and *Rk2* genes. Utilizing host resistance is one of the major root-knot nematode management strategies for tobacco. In plant nematology, true

resistance is defined as the inhibition of reproduction on a host (Roberts, 2002). The first known gene conferring resistance to root-knot nematodes was introduced into tobacco from *Nicotiana tomentosa* Ruis and Pav. in 1961 and was named *Rk* (Yi et al., 1998). This single dominant gene is now known as *Rk1* and is present in almost all commercial flue-cured tobacco cultivars available in the United States. *Rk1* imparts resistance to *M. incognita* races 1 and 3 and *M. arenaria* race 1 by inhibiting nematode feeding site establishment (Schneider, 1991b; Ng'ambi et al., 1999). According to Ng'ambi et al. (1999), the effect of the *Rk1* gene on conferring resistance to *M. incognita* races 2 and 4, *M. arenaria* race 2, *M. javanica*, and *M. hapla* is minimal or non-existent. However, Ternouth et al. (1986) noted that the *Rk1* gene conferred some resistance to *M. javanica*.

A second root-knot resistance gene in tobacco was identified in Zimbabwe in 1950, and labeled “T” (Schweppenhauser, 1975). This gene is now referred to as *Rk2* in the United States. The gene was discovered in local *Nicotiana tabacum* plants that had been grown along the Zambezi River in Zimbabwe in the Chimanimani or Nyoke area, in soil heavily infested with *M. javanica* (Schweppenhauser, 1975; Mackenzie et al., 1986; Ternouth et al., 1986). Four plant selections of the tobacco breeding material (numbered 17, 233, 256, and 260) were inoculated with *M. javanica*. At plant maturity, those inoculations resulted in only one to two immature female nematodes, no evident egg production, and negligible disruption of the vascular system (Schweppenhauser, 1975). As a result, those plant selections were determined to be “partially resistant” to *M. javanica*. Ternouth et al. (1986) observed that the resistance conferred by the “T” gene for *M. javanica* was greater than that provided by the *Rk1* gene (which the Zimbabwe publications referred to as “S”). Smeeton further observed very high resistance for *M. javanica* when both *Rk1* and the “T” gene were present together (Ternouth et al., 1986). Additionally,

Shepherd (1982) reported results from two trials in which juvenile root invasion on the “better breeding lines” experienced only 20% of that on susceptible cultivars, although subsequent nematode development was only slightly lower. Unfortunately, the paper does not specify what the better breeding lines were. If this is the case, the mode of action of “T” (or *Rk2*) would be very different from that observed for *Rk1*, which was determined to inhibit successful giant cell formation, but not penetration (Schneider, 1991b). Early reports on root-knot resistance and *Rk2* stated, “The genetic mechanism responsible for the very much higher resistance in the hybrid derivatives of [the plant selection] 256, over and above that of the parental strain, is not understood. Resistance is apparently controlled by multiple factors which are probably cumulative in their effect” (Schweppenhauser, 1968). However, after genetic linkages between root-knot resistance and leaf shape had been broken, “resistance to *M. javanica* appeared to be inherited as a monogenic dominant factor. . . . It is probable that one or more genes exerted a modifying influence and were able to induce a higher level of resistance” (Schweppenhauser, 1975).

A promising selection derived from strain 256 was crossed with Kostoff’s amphidiploid to create a breeding line with more desirable leaf morphology; the progeny from this cross was then crossed with the susceptible cultivar Kutsaga 51 to further improve the genetic background for agronomic traits, which resulted in the breeding line RKT15-1-1, which possessed the *Rk2* (or “T”) gene (Mackenzie et al., 1986). In 1979, Smeeton crossed RKT15-1-1 with flue-cured tobacco cultivars SC 72, and then NC 89, both of which possessed the *Rk1* gene. This created the STNC breeding lines: STNCA and STNCB, which thus possessed both *Rk1* and *Rk2* (Ternouth et al., 1986). The STNC breeding lines were subsequently crossed with cultivars Kutsaga 51E and Speight G-28 to further improve their flue-cured tobacco characteristics (Ternouth et al.,

1986).

This resistance to both *M. incognita* race 1 and 3 (*Rk1* gene), and *M. javanica* (*Rk2* gene) has been incorporated into multiple flue-cured tobacco cultivars developed in Zimbabwe, beginning with “*RKI*” (STNCB 2-28 x ms Kutsaga E1), released in 1993 (Way, 1994; Jack and Lyle, 1999; Jack, 2001). More recently, this resistance has been introduced into flue-cured tobacco cultivars released in the United States, such as CC 13, CC 33, CC 35, CC 37, CC 65, and PVH 2275 (Johnson, 2015).

Soil temperature effect on resistance. In many crops, soil temperature plays a critical role in the efficacy of resistance genes. In a number of plant-nematode systems, resistance has been noted to break down at higher temperatures. Schweppenhauser (1968) observed the root-knot resistance linked with *Rk2* appeared to be influenced by several environmental factors, noting specifically that nematode parasitism was consistently higher for the entries analyzed in experiments conducted at higher temperatures.

One of the most studied of these nematode resistance-temperature interactions is the *Mi* gene in tomato plants, which was noted to lose efficacy as temperature increased. Dropkin (1969) observed that only 2% of J2 developed in roots of tomato plants containing the *Mi* resistance gene at 28°C. At 33°C, however, 87% of J2 developed. Since then, studies have noted the breakdown of root-knot resistance with increasing temperature in a number of other crops. In common bean (*Phaseolus vulgaris* L.), resistance characterized by recessive genes failed in plant entries at 26°C to 28°C, and dominant resistance genes failed in plant entries at 28°C to 30°C (Omwega and Roberts, 1992). In sweet potato (*Ipomea batata* L.), the rate of *M. incognita* development and total population numbers increased in the resistant ‘Nemagold’ cultivar as temperatures increased from 24°C to 32°C (Jatala and Russell, 1972). In grape rootstocks,

resistance to *Meloidogyne* spp. started to break down at 27°C with increased root galling and egg mass formation, and further at 30°C and above (Ferris et al., 2013).

High temperatures also facilitated the formation of disease complexes on resistant cultivars of tomato. In a study by Abawi and Barker (1984), tomatoes containing the *Mi* gene were inoculated with *M. incognita* and *F. oxysporum* f. sp. *lycopersici*, at temperatures from 16°C to 35°C. *Fusarium* caused significantly more wilt and root necrosis at 35°C than at 16°C, presumably due to *M. incognita* predisposing the plant to attack by *Fusarium* at the higher temperature (Abawi and Barker, 1984).

However, Ammati et al. (1986) noted that apparent root-knot resistance failed at higher temperatures in some tomato plants, but not others. The tomato cultivar ‘VFN8,’ containing the *Mi* resistance gene, was resistant to *M. incognita* at 25°C and susceptible at 32°C. Three other genotypes: *Solanum corneliomulleri* J. F. Macbr. (previously known as *Lycopersicon glandulosum* C. H. Mull.) Acc. No. 126443, *Solanum peruvianum* L. Acc. No. 270435, and *Solanum peruvianum* L. Acc. Nos. 129152 and LA2157, retained resistance at 32°C. The authors suggested that gene(s) other than the *Mi* gene may have been present and maintained the resistance in those genotypes (Ammati et al., 1986). Additionally, it has been observed that tomato plants with the *Mi* gene grown at high temperatures remained susceptible for a few days even after decreasing the temperature, perhaps due to a time lag in induction of peroxidase activity and lignification (Zacheo et al., 1995). Zacheo et al. (1995) also noted that once nematodes started feeding on plants rendered susceptible by high temperatures, and the temperature was then reduced, the nematodes continued feeding and developing.

Study objectives. The objectives of this study were to investigate whether possessing both *Rk1* and *Rk2* increases resistance to a variant of *M. incognita* race 3 compared to either gene alone, and if increasing soil temperatures impact the efficacy of *Rk1* and/or *Rk2*.

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CHAPTER 2

Reproduction of root-knot nematodes on flue-cured tobacco homozygous for *Rk1* and/or *Rk2* resistance genes

ABSTRACT

Host plant resistance to root-knot nematodes is a principal control strategy in flue-cured tobacco. Most commercial cultivars possess the *Rk1* resistance gene to races 1 and 3 of *M. incognita* and race 1 of *M. arenaria*, causing a shift in population prevalence in Virginia tobacco fields towards other species and races, which created a need for resistance to those populations. An increasing number of cultivars now also possess the *Rk2* gene for root-knot resistance, but aspects of *Rk2* efficacy are unknown. Greenhouse experiments were conducted in 2013-14 to investigate whether possessing both *Rk1* and *Rk2* increases resistance to a variant of *M. incognita* race 3 compared to either gene alone. Trials were arranged in a completely randomized design including Coker 371-Gold (C371G; susceptible), NC 95 and SC 72 (homozygous for *Rk1*), T-15-1-1 (homozygous for *Rk2*), and STNCB-2-28 and NOD 8 (homozygous for both *Rk1* and *Rk2*). Each plant was inoculated with 5,000 root-knot eggs; data were collected 60 days post-inoculation. Data were collected for percent galling and egg mass and egg counts, the latter being used to calculate the reproductive index on each host. Despite variability, entries with both *Rk1* and *Rk2* together (*Rk1Rk2*) conferred greater resistance to a variant of *M. incognita* race 3 than plants with *Rk1* or *Rk2* alone. Entries with *Rk1* alone were successful in reducing root galling and nematode reproduction compared to the susceptible control. Entry T-15-1-1, with only the *Rk2* gene, did not reduce galling compared to the susceptible control but often suppressed nematode reproduction.

INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is an important agricultural commodity grown worldwide (FAO, 2015). Flue-cured tobacco makes up the largest portion of tobacco types grown in the United States, and in Virginia alone, 22,500 acres of flue-cured tobacco were grown in 2014 (USDA, 2015). Root-knot nematodes (*Meloidogyne* spp.) can cause significant yield losses in tobacco in the Southeast United States (Fortnum et al., 2001). In Virginia, yield losses in flue-cured tobacco due to root-knot nematodes are probably between 1% and 5% (Koenning et al., 1999). Utilizing tobacco varieties with root-knot resistance or tolerance genes is one of the principal control strategies for managing root-knot nematodes (Johnson et al., 2005).

In plant nematology, host resistance is defined as the inhibition of reproduction on a host (Roberts, 2002). Conversely, hosts with tolerance do not necessarily have inhibited nematode reproduction, but plant growth and yield are generally not affected (Roberts, 2002). The first root-knot resistance gene successfully introduced into a commercial tobacco cultivar in 1961 was from *N. tomentosa* Ruis and Pav., and it was called *Rk* (Yi et al., 1998). Now known as *Rk1*, this single dominant gene is present in all commercially grown flue-cured tobacco cultivars in the United States. *Rk1* imparts resistance to *M. incognita* (Kofoid and White, 1919) Chitwood, 1949 host races 1 and 3 and *M. arenaria* (Neal, 1889) Chitwood, 1949 host race 1 (Schneider, 1991; Ng'ambi et al., 1999b). According to Ng'ambi et al. (1999b), the effect of the *Rk1* gene on *M. incognita* races 2 and 4, *M. arenaria* race 2, *M. javanica* (Treub, 1885) Chitwood, 1949, and *M. hapla* Chitwood, 1949 is minimal or non-existent. However, Ternouth et al. (1986) noted that the *Rk1* gene conferred “some resistance to *M. javanica*.”

The first account of commercial tobacco in Zimbabwe containing a second tobacco root-knot resistance gene, along with *Rk1*, was in 1993 (Way, 1994; Jack and Lyle, 1999; Jack, 2001).

This gene was identified in Zimbabwe in 1950, and labeled “*T*” (Schweppenhauser, 1975). It was discovered in local *N. tabacum* plants that had been grown along the Zambezi River in Zimbabwe since the 1700s, in soil heavily infested with *M. javanica* (Schweppenhauser, 1975; Mackenzie et al., 1986; Ternouth et al., 1986). Plant selections were determined to be “partially resistant” to *M. javanica* after experimental inoculation resulted in only one or two females and no egg production (Schweppenhauser, 1975). Ternouth et al. (1986) observed that the resistance to *M. javanica* conferred by the “*T*” gene was greater than that provided by the “*S*” (or *Rk1*). The “*T*” gene is now often referred to as *Rk2* in the United States. Smeeton further observed very high resistance for *M. javanica* when both *Rk1* and the “*T*” (or *Rk2*) gene were present together (Ternouth et al., 1986). Additionally, Shepherd (1982) reported results from two trials in which *M. javanica* juvenile root invasion by on the “better breeding lines” was only 20% of that on susceptible cultivars, although subsequent nematode development was only slightly lower. If this is the case, the mode of action of “*T*” (or *Rk2*) would be very different from that observed for *Rk1*, which was determined to inhibit successful giant cell formation, but not penetration (Schneider, 1991). Although early reports on *Rk2* stated tentatively that the mechanism of the high resistance for *M. javanica* was apparently controlled by “multiple factors,” it was later concluded that the resistance was inherited as a monogenic dominant trait, probably also involving one or more modifying genes (Schweppenhauser, 1975).

The plant selections from the local Zimbabwe tobacco were crossed with cultivated tobacco entries to improve leaf morphology and agronomic traits, resulting in the breeding line RKT15-1-1 (Mackenzie et al., 1986). In 1979, Smeeton crossed RKT15-1-1 with flue-cured tobacco cultivars SC 72, and then NC 89, to create the STNC breeding lines: STNCA and STNCB, which thus possessed both *Rk1* and *Rk2* (Ternouth et al., 1986). The STNC breeding

lines were subsequently crossed with other commercial cultivars to further improve their flue-cured tobacco characteristics (Ternouth et al., 1986). This resistance was incorporated into multiple flue-cured tobacco cultivars developed in Zimbabwe, beginning with “*RK1*” (STNCB 2-28 x ms Kutsaga E1) released in 1993 (Way, 1994; Jack and Lyle, 1999; Jack, 2001). Most commercial tobacco cultivars planted in the United States are now homozygous for the *Rk1* gene (Koenning et al., 1999). Beginning in 2007, resistance or tolerance arising from combinations of *Rk1* and *Rk2* have been introduced into flue-cured tobacco cultivars released in the United States, such as CC 13, CC 33, CC 35, CC 37, CC 65, and PVH 2275 (Reed, 2007; Johnson, 2015).

Although *Meloidogyne incognita* has traditionally been considered the most common root-knot nematode species found on tobacco in Virginia (Johnson, 1989), a 2004 survey of 170 flue-cured tobacco fields in Virginia revealed that of the 43.5% of tobacco fields infested with root-knot nematodes, 56.7% were infested with *M. arenaria*, 25.0% with *M. hapla*, 16.7% with *M. incognita*, 11.7% with *M. javanica*, and 8.3% with unknown *Meloidogyne* species (Eisenback, 2012). A 2010 follow-up survey of 276 Virginia flue-cured tobacco fields identified a similar percentage of fields infested with root-knot nematodes (44.9%), with *M. arenaria* present in 58.8% of the infested fields, *M. hapla* in 22.3%, *M. incognita* in 11.1%, *M. javanica* in 11.1%, and unknown *Meloidogyne* species in 6.3% (Eisenback, 2012). *M. arenaria* was the most commonly detected root-knot species in these surveys, and the prevalence of *M. arenaria* increased from 56.7% in 2004 to 58.8% in 2010, whereas the prevalence of *M. incognita* decreased from 16.7% in 2004 to 11.1% in 2010. With this apparent shift in root-knot nematode populations in Virginia’s tobacco fields, cultivars with only the *Rk1* gene may not be as effective anymore in managing nematodes, depending on the root-knot populations present. It’s critical that farmers have cultivars that are effective against the nematodes in their fields. Therefore, it is

necessary to study the effects of the *Rk1* and *Rk2* genes on root-knot parasitism. The research in Zimbabwe alleged that *Rk1* and *Rk2* confer resistance to *M. javanica*, but effects of these genes on *M. incognita* races 2 and 4, and *M. arenaria* race 2 are largely undocumented. The objective of this work was to investigate whether or not possessing both *Rk1* and *Rk2* resistance genes in tobacco increased resistance to a variant of *M. incognita* race 3, compared to possessing either gene alone.

MATERIALS AND METHODS

Population source: A *M. arenaria* root-knot nematode population was requested in 2013 from Clemson University in Clemson, South Carolina. The nematode population received had been collected originally from a soybean field near Florence, South Carolina and identified as *M. arenaria* race 2 based on esterase (EST) and superoxide dismutase (SOD) isozyme patterns (P. Agudelo, personal communication). The identity of the population was later reconfirmed by perineal pattern morphology and species-specific PCR primers (Zijlstra et al., 2000), of which the population was positive for Mar/Rar and negative for Finc/Rinc (P. Agudelo, personal communication).

To re-verify the identity of the population, eight female stylets were excised following the procedure outlined by Eisenback (1985) and viewed using a scanning electron microscope to identify the population morphologically. Perineal patterns were cut from ten mature females following the technique of Eisenback (1985) and viewed using a compound microscope at $\times 630$. To additionally clarify the species identification, gel electrophoresis of esterase isozymes were performed on three females. Species-specific sequence-characterized-amplified-region (SCAR) primers (MiF/MiR, IncK14F/R, Rinc/Rinc) and PCR-DNA sequences on rRNA 18S, ITS, 28S,

D2/D3, histone and mitochondrial DNA COII-16S gene were then examined (W.M. Ye, personal communication). Additionally, three runs of a greenhouse differential host test were performed to potentially identify the population to a host race level (Taylor and Sasser, 1978).

Greenhouse trials: Five greenhouse experiments were conducted in 2013-14 to investigate the resistance efficacy of *Rk1* and/or *Rk2* genes in tobacco. Three experiments were carried out at the Virginia Tech campus in Blacksburg, VA and two at the Virginia Tech Southern Piedmont Agricultural Research and Extension Center (SPAREC) in Blackstone, VA. Each experiment was arranged in a completely randomized design with six replications, except for the April-June 2013 run in Blacksburg, VA, which had seven replications. Six plant entries were evaluated: Coker 371-Gold (C371G; susceptible to the four most common *Meloidogyne* species: *M. arenaria*, *M. incognita*, *M. javanica*, and *M. hapla*), NC 95 and SC 72 (homozygous for *Rk1*), T-15-1-1 (homozygous for *Rk2*), and STNCB-2-28 and NOD 8 (homozygous for both *Rk1* and *Rk2*). Seedlings with 4-6 true leaves (~5-10 cm tall) were planted in 15-cm-diam. clay pots with a 2:1 mixture of topsoil (53% sand, 40% silt, 7% clay; pH 5.5) to Profile® Greens Grade™ porous ceramic material. Plants were each inoculated with 5,000 root-knot nematode eggs 1-wk after transplant by pipetting or pouring the egg suspension into two 4-cm deep holes on either side of the plant. Plants were kept in a greenhouse at approximately 20-35°C, and grown without supplemental lighting.

Approximately 60 days after inoculation, trials were taken down. Root galling and numbers of egg masses and eggs from roots were compared among the entries. Roots were rinsed free of soil and the whole root system was weighed. Galled roots were separated from non-galled roots and root percent galling was calculated based on the fresh weight of galled roots versus the fresh weight of the entire root system. Roots were recombined, mixed, and divided in half by

weight. Half were stained with 0.15 g/liter Phloxine B for five minutes to define egg masses (Dickson and Struble, 1965). The numbers of egg masses from three 1-g subsamples per plant were counted using a dissecting microscope at $\times 10$ to estimate the number of females per gram root. Eggs were bleach-extracted from the surface of roots in the second half of each root system following the procedure by Hussey and Barker (1973). Extracted eggs were suspended in 500-ml water and counted in two 10-ml aliquots from each extraction using a compound microscope at $\times 40$. To assess the nematode reproductive capability on each entry, the reproductive index (P_f/P_i) was calculated by dividing the final number of eggs extracted per plant (P_f) by the initial number of egg inoculum (P_i) (Sasser et al., 1984).

Statistical analysis: Data from each trial were analyzed separately by analysis of variance (ANOVA) using the Statistical Analysis System-JMP® Pro 11 (SAS Institute, Cary, NC). Means for percent galling, counts of egg masses, and eggs were transformed [$\log_{10}(x + 1)$] before statistical analysis using the Tukey-Kramer honest significant difference (HSD) test ($P = 0.05$).

RESULTS

Species identification. The nematode population received had been identified as *M. arenaria* race 2; however, low reproduction on tobacco entry NC 95 suggested another identity. The morphology of the female stylets viewed using a scanning electron microscope (SEM) (Fig. 2.1), and perineal patterns (Fig. 2.2) were not consistent with either *M. arenaria* or *M. incognita*, but more similar to these two species than any other root-knot species.

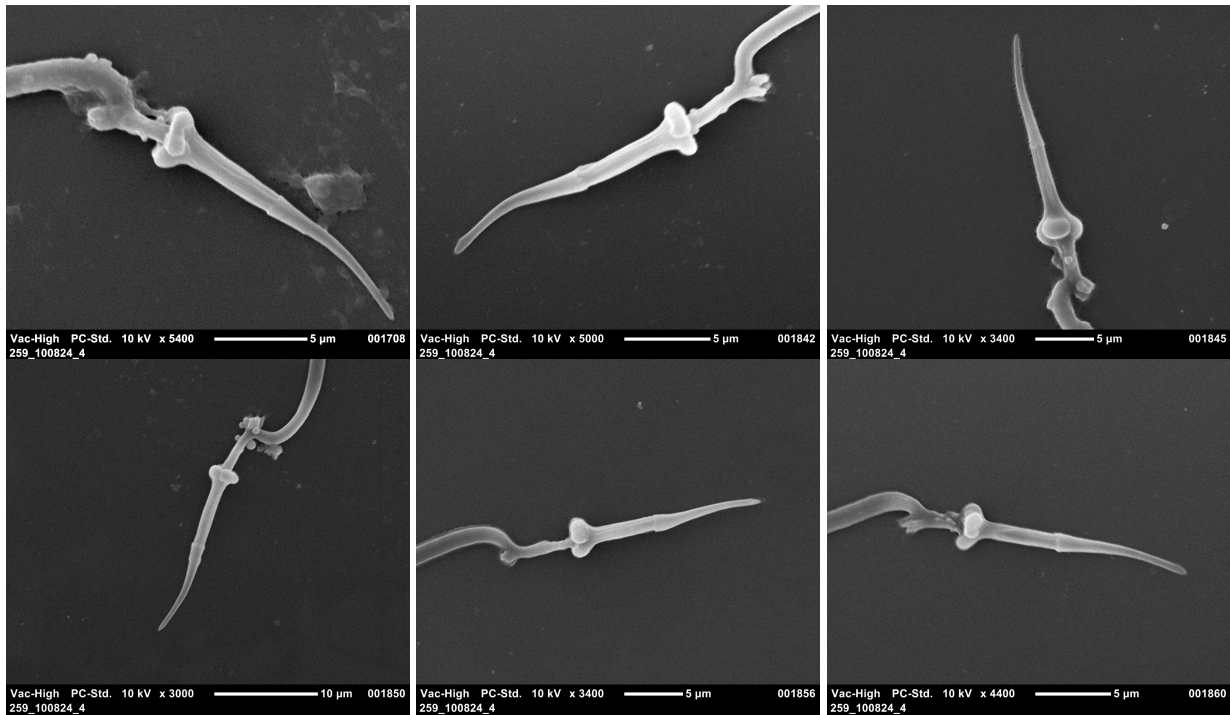


Fig. 2.1. Excised female root-knot nematode stylets, photographed using a scanning electron microscope (SEM).

The results from our gel electrophoresis of esterase isozymes suggested the population was *M. incognita*, as did PCR-DNA sequences on rRNA 18S, ITS, 28S, D2/D3, histone and mitochondrial DNA COII-16S gene (W.M. Ye, personal communication), results using the *M. incognita*-specific SCAR primer set MiF/MiR, and results from the differential host tests, which tentatively identified the population as *M. incognita* race 3 (Table 2.1, Fig. 2.3). However, results from the *M. incognita*-specific primer sets IncK14F/R and Finc/Rinc suggested that the population was another biotype of *Meloidogyne* (W.M. Ye, personal communication).

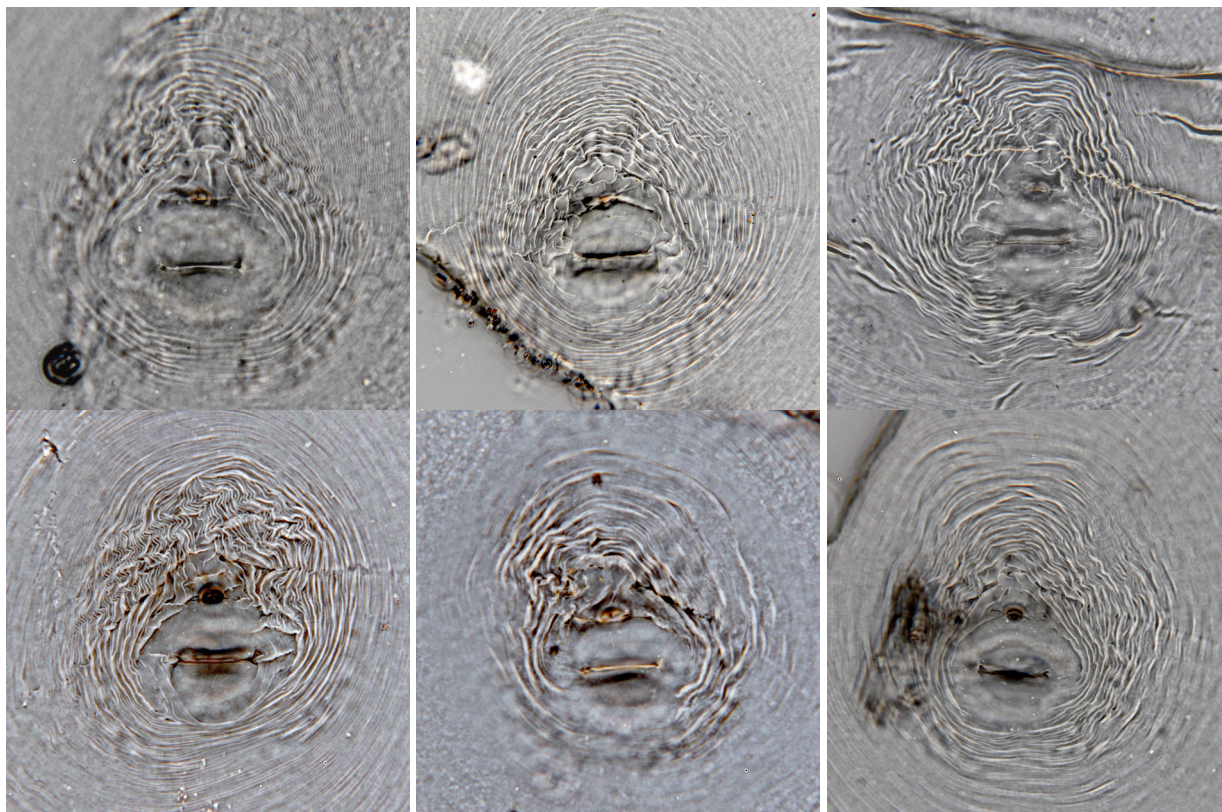


Fig. 2.2. Perineal pattern morphology of the population of root-knot used for the studies on resistance in tobacco.

Table 2.1. Results of the differential host test, identifying the population as *M. incognita* (Kofoid and White, 1919) Chitwood, 1949 host race 3. Note that cotton (*Gossypium hirsutum* L.) variety ‘FM 1944 GLB2’ is used instead of ‘Deltapine 16’ due to availability. Tobacco (*Nicotiana tabacum* L.), pepper (*Capsicum annuum* L.), watermelon [*Citrullus lanatus* var. *lanatus* (Thunb.) Matsum. and Nakai], peanut (*Arachis hypogaea* L.), and tomato (*Solanum lycopersicum* L.).

<i>Meloidogyne</i> species and race	Differential host plant cultivars					
	Tobacco ‘NC 95’	Cotton ‘FM 1944 GLB2’	Pepper ‘California Wonder’	Watermelon ‘Charleston Grey’	Peanut ‘Florunner’	Tomato ‘Rutgers’
<i>M. incognita</i>						
Race 1	–	–	+	+	–	+
Race 2	+	–	+	+	–	+
Race 3	–	+	+	+	–	+
Race 4	+	+	+	+	–	+
<i>M. arenaria</i>						
Race 1	+	–	+	+	+	+
Race 2	+	–	–	+	–	+
<i>M. javanica</i>	+	–	–	+	–	+
<i>M. hapla</i>	+	–	+	–	+	+

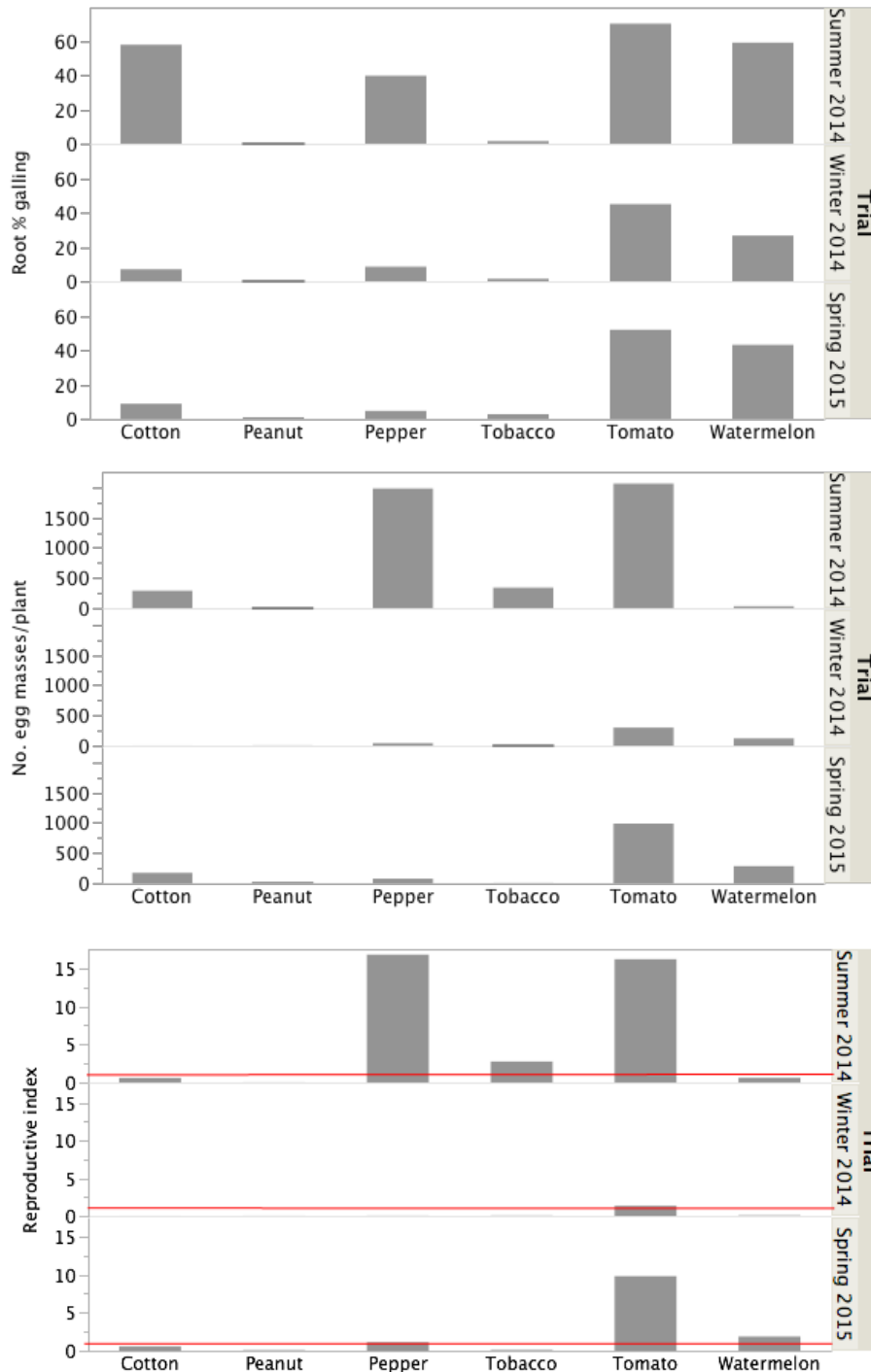


Fig. 2.3. Percent root galling, number of egg masses/plant, and the reproductive index of *M. incognita* race 3 on differential host plants, across three trials. The red line marks a reproductive index of 1. Tobacco (*Nicotiana tabacum* L.), cotton (*Gossypium hirsutum* L.), pepper (*Capsicum annuum* L.), watermelon [*Citrullus lanatus* var. *lanatus* (Thunb.) Matsum. and Nakai], peanut (*Arachis hypogaea* L.), and tomato (*Solanum lycopersicum* L.).

Total root weights. Root weights varied greatly among trials, ranging from an average of 19 g in the Blacksburg Nov 2013-Jan 2014 trial to 92 g in the Blackstone May-July 2014 trial (Fig. 2.4). There were no significant differences in total mean root weights among entries in the Blackstone Sept-Nov 2013, Blacksburg Nov 2013-Jan 2014, and Blackstone May-July 2014 trials, ($P = 0.5088$, $P = 0.6069$, and $P = 0.7319$, respectively). While the mean root weight for the *Rk2* entry T-15-1-1 was significantly larger than the *Rk1Rk2* entries NOD 8 and STNCB 2-28 in the Blacksburg Apr-Jun 2013 trial, root weights for these entries were not significantly larger in this study than those of susceptible C371G and the *Rk1* entries ($P \leq 0.05$). In the Blacksburg Apr-Jun 2014 trial, the mean root weight for the *Rk2* entry T-15-1-1 was significantly larger than one of the *Rk1Rk2* entries (STNCB-2-28) but not significantly larger than for any of the other entries ($P \leq 0.05$). T-15-1-1 had the highest mean root weight in every trial, except for Blacksburg Nov 2013-Jan 2014 when the susceptible entry was numerically higher. In all trials except Blacksburg Nov 2013-Jan 2014, at least one of the *Rk1Rk2* entries had the lowest mean root weight, and during the Blacksburg Apr-Jun 2013 and Blackstone May-July 2014 trials, both of the *Rk1Rk2* entries had the lowest mean root weights. Mean root weights for all entries in the Blacksburg Nov 2013-Jan 2014 trial were much lower than in every other trial.

Percent root galling. Significant ($P < 0.001$) differences were observed among entries in mean percent root galling in every trial (Fig. 2.5). Percent root galling for the entry with *Rk2* alone (T-15-1-1) was always between 17.0% and 73.1%, whereas galling of the susceptible entry (C371G) was always between 38.5% and 77.1%. Mean percent root galling for T-15-1-1 was never significantly different from that of C371G (Fig. 2.5). Entries containing the *Rk1* resistance gene alone (SC 72 and NC 95) displayed significantly lower galling than susceptible C371G in all trials, and galling was significantly lower than plants with *Rk2* alone in four of the five trials

($P \leq 0.05$). The mean percent root galling on the entries with the *Rk1* gene was always less than or equal to 7.3%. Plant entries containing both *Rk1* and *Rk2* resistance genes together (NOD 8 and STNCB) always exhibited significantly less galling than the susceptible entry and the *Rk2* entry ($P \leq 0.05$). Differences in galling between entries with *Rk1* and *Rk2* versus those with *Rk1* alone were only statistically significant ($P \leq 0.05$) in the Blacksburg Apr-Jun 2013 trial, but galling was always numerically lower for entries with both *Rk1* and *Rk2* compared to those with only *Rk1*. Root galling was always less than 1.0% in entries with *Rk1* and *Rk2* together.

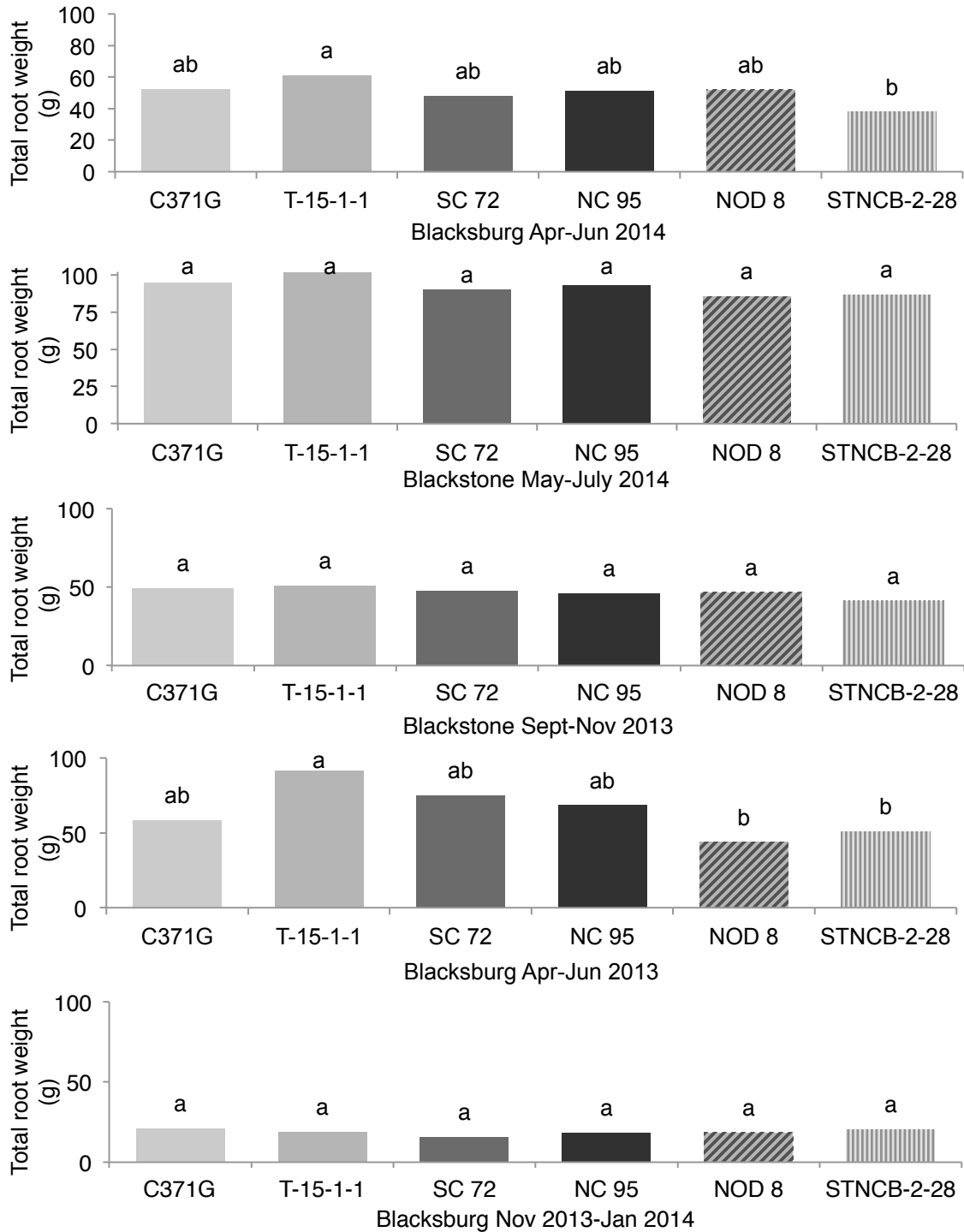


Fig. 2.4. Mean root weights (g) of various tobacco (*Nicotiana tabacum* L.) entries inoculated with a variant of *M. incognita* race 3 from five greenhouse trials conducted in 2013-14. Means followed by the same letter(s) are not significantly different according to statistical analysis of non-transformed data and the Tukey-Kramer honest significant difference test ($P = 0.05$). C371G is susceptible, T-15-1-1 has *Rk2*, SC 72 and NC 95 have *Rk1*, and NOD 8 and STNCB-2-28 have *Rk1Rk2*.

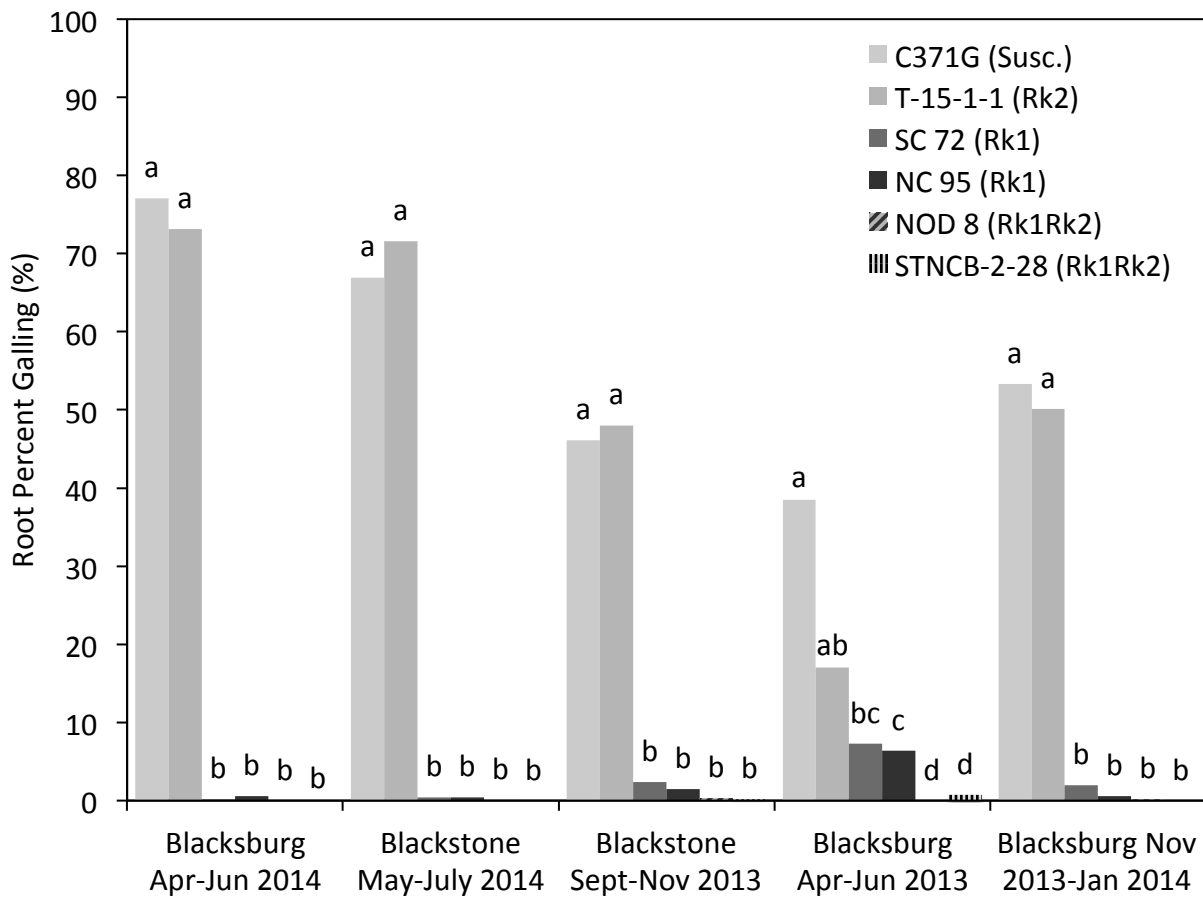


Fig. 2.5. Mean % root galling of six tobacco (*Nicotiana tabacum* L.) entries inoculated with a variant of *M. incognita* race 3 from five greenhouse trials conducted in 2013-14. Means followed by the same letter(s) are not significantly different according to statistical analysis of transformed $[\log_{10}(x+1)]$ data and the Tukey-Kramer honest significant difference (HSD) test ($P = 0.05$).

Egg mass counts. Mean egg mass counts were significantly different among the entries in every trial ($P < 0.001$). T-15-1-1, with *Rk2* alone, had significantly fewer egg masses per gram root than susceptible C371G in three of five trials, and egg mass numbers were always numerically lower for T-15-1-1 than for C371G ($P \leq 0.05$) (Fig. 2.6A). The number of egg masses for T-15-

1-1 ranged from 13 and 74 per gram root across all trials, while between 22 and 173 egg masses per gram root were found on susceptible C371G. Egg masses per gram root were always significantly lower on entries containing *Rk1* alone (NC 95 and SC 72) compared to susceptible C371G, while significantly fewer egg masses per gram root were observed on NC 95 and SC 72 than on T-15-1-1 (*Rk2*) in four of five trials ($P \leq 0.05$). The number of egg masses per gram root for NC 95 and SC 72 was always between zero and eight throughout the trials. NOD 8 and STNCB 2-28, with both *Rk1* and *Rk2*, always had significantly fewer egg masses per gram root than susceptible C371G, and in all trials except Blacksburg Apr-Jun 2013, fewer than the *Rk2* entry T-15-1-1 ($P \leq 0.05$). The *Rk1Rk2* entries exhibited significantly fewer egg masses per gram root than entries possessing *Rk1* alone in the Blackstone Sept-Nov 2013 and Blackstone May-July 2014 trials ($P \leq 0.05$).

Egg counts. Significant differences were observed in the mean egg count per gram root among the entries in every trial ($P < 0.001$). Although eggs per gram root were always numerically lower on T-15-1-1, containing the *Rk2* gene alone, compared to susceptible C371G, this trend was statistically significant in only the Blacksburg Apr-Jun 2013 trial ($P \leq 0.05$) (Fig. 2.6B). Significantly fewer eggs per gram of root were always noted on entries possessing *Rk1* gene alone compared to susceptible C371G, and significantly fewer than for T-15-1-1, with the *Rk2* gene, in three of five trials ($P \leq 0.05$). In the Blacksburg Apr-Jun 2013 trial, fewer eggs per gram root were extracted from T-15-1-1 than from roots of entries possessing only *Rk1*. Significantly fewer eggs per gram root were enumerated from the *Rk1Rk2* entries NOD 8 and STNCB 2-28 than from T-15-1-1 in three of five trials ($P \leq 0.05$). Significantly fewer eggs were also counted per gram root on NOD 8 and STNCB 2-28 compared to the *Rk1* entries NC 95 and SC 72 in the Blacksburg Apr-Jun 2014 and Blackstone May-July 2014 trials ($P \leq 0.05$).

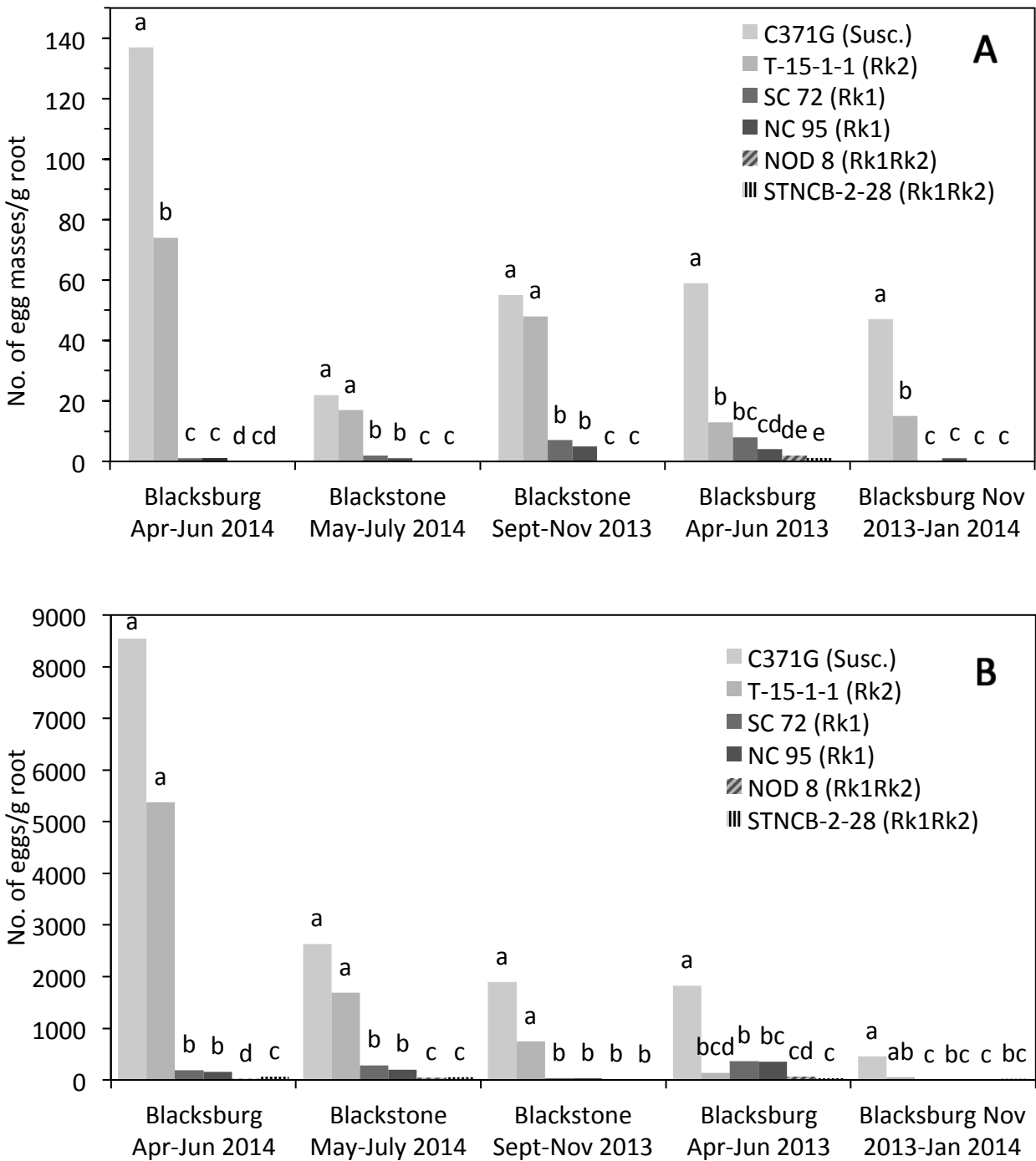


Fig. 2.6. (A) Egg masses per g root and (B) eggs per g root of *M. incognita* race 3 on six tobacco (*Nicotiana tabacum* L.) entries grown in five greenhouse trials conducted in 2013-14. Means followed by the same letter(s) are not significantly different according to statistical analysis of transformed [$\log_{10}(x+1)$] data and the Tukey-Kramer honest significant difference (HSD) test ($P = 0.05$).

Reproductive index. Mean reproductive indexes were significantly different among the entries in every trial ($P < 0.001$). The reproductive index on susceptible C371G was always greater than one (Table 2.2). The reproductive index for T-15-1-1, with *Rk2*, was greater than one in all trials except Blacksburg Nov 2013-Jan 2014, and was not significantly different from susceptible C371G in three of five trials ($P \leq 0.05$). In three of five trials, the reproductive index was less than one on the *Rk1* entries NC 95 and SC 72, and was always significantly lower than that on the susceptible C371G ($P \leq 0.05$). The reproductive indexes on the *Rk1Rk2* entries NOD 8 and STNCB 2-28 were always less than one and always significantly lower than that of susceptible C371G ($P \leq 0.05$). The reproductive indexes on NOD 8 and STNCB 2-28 were also significantly lower compared to the *Rk1* entries NC 95 and SC 72 in three of five trials, and compared to the *Rk2* entry T-15-1-1 in all trials except the Blacksburg Nov 2013-Jan 2014 experiment ($P \leq 0.05$), when reproductive indices were much lower on all of the entries tested compared to reproductive index results from all of the other trials.

Table 2.2. Reproduction of a variant of *M. incognita* race 3 on six tobacco (*Nicotiana tabacum* L.) entries in five greenhouse trials conducted in 2013-14.

Plant entry	Resistance genes	Reproductive index (P_f/P_i) ^{ab} by trial				
		Blacksburg Apr-Jun 2014	Blackstone May-July 2014	Blackstone Sept-Nov 2013	Blacksburg Apr-Jun 2013	Blacksburg Nov 2013- Jan 2014
C371G	None	88.5 a	48.3 a	15.6 a	18.9 a	2.1 a
T-15-1-1	<i>Rk2</i>	63.8 a	34.0 a	7.1 a	2.6 b	0.2 b
SC 72	<i>Rk1</i>	1.9 b	4.8 b	0.3 b	4.4 b	0.0 b
NC 95	<i>Rk1</i>	1.6 b	3.4 b	0.3 b	4.6 b	0.0 b
NOD 8	<i>Rk1Rk2</i>	0.2 c	0.7 c	0.1 b	0.5 c	0.0 b
STNCB-2-28	<i>Rk1Rk2</i>	0.4 c	0.9 c	0.0 b	0.3 c	0.1 b

^a Reproductive index = final population/initial population (P_f/P_i). Values less than 1.0 are accented with bold text.

^b Means followed by the same letter(s) are not significantly different according to statistical analysis of transformed [$\log_{10}(x+1)$] data and the Tukey-Kramer honest significant difference (HSD) test ($P = 0.05$).

DISCUSSION

Despite variability in our results, entries with both *Rk1* and *Rk2* (*Rk1Rk2*) conferred greater resistance to root-knot nematodes than entries with *Rk1* or *Rk2* alone, corroborating Smeeton's observations of that *Rk1* and *Rk2* together conferred higher resistance to *M. javanica* than either gene alone (Ternouth et al., 1986). Results of experiments performed at Virginia Tech in 2010-11 examining reproduction of *M. javanica* on the same flue-cured tobacco entries used in this experiment (C371G, T-15-1-1, SC 72, NC 95, NOD 8, and STNCB-2-28) support these results (Ma, W., Johnson, C. S., Eisenback, J. D., and Reed, T. D. unpublished data). In those experiments, as in our current investigations, root galling, egg masses per gram root, and eggs per gram root on NOD8 and STNCB 2-28 (with *Rk1Rk2*) were significantly reduced compared to those on NC 95 and SC 72 (with *Rk1* alone) or T-15-1-1 (with *Rk2* alone), in two of the three greenhouse trials. Similar results were also observed in a 2014 field study in a flue-cured tobacco field infested with *M. arenaria* in Mecklenburg County, VA, in which root galling was compared among cultivars and breeding lines varying in *Rk1* and/or *Rk2*. Cultivars used in the Mecklenburg County trial were the same used in our experiment. Galling was significantly lower on cultivars possessing both *Rk1Rk2* than on the susceptible control, C371G; cultivars with both *Rk1* and *Rk2* had the lowest percent galling of any entries in the experiment, which included entries possessing *Rk1* alone and *Rk2* alone (Pollok et al., 2015).

The role of *Rk2* in suppressing root-knot nematode reproduction may be quite different than that of *Rk1*. Ternouth et al. (1986) concluded that resistance to *M. javanica* conferred by the *Rk2* gene was greater than that provided by the *Rk1* gene. Results from a 2010-11 Virginia Tech greenhouse study support this to an extent, where *M. javanica* egg masses and eggs per gram root were significantly reduced in *Rk2* plants versus *Rk1* plants in one of three greenhouse trials

(Ma, W., Johnson, C. S., Eisenback, J. D., and Reed, T. D. unpublished data). In our results, *Rk2* alone often reduced parasitism and reproduction by a variant of *M. incognita* race 3 compared to the susceptible entry, but these reductions were generally less than those associated with *Rk1* alone. Ng'ambi et al. (1999b) noted that the amount of nematode reproduction was generally consistent with the amount of root galling, but we always observed galling on *Rk2* plants similar to that on the susceptible control. Galling of *Rk2* plants was also similar to that on the susceptible control in one of three *M. javanica* greenhouse trials in 2010-11 (Ma, W., Johnson, C. S., Eisenback, J. D., and Reed, T. D. unpublished work). T-15-1-1 (*Rk2*) did not significantly suppress galling by *M. arenaria* compared to the susceptible control in a 2014 field experiment (Pollok et al., 2015). The considerable root galling on *Rk2* plants, yet reduced reproduction, suggests that some nematodes are able to enter root tips, feed and develop, but not reproduce.

The mechanism of resistance provided by *Rk2* is not clear, nor is that of the increased resistance provided by *Rk1Rk2*. Shepherd (1982) reported that penetration of roots by juveniles was 80% less on “better breeding lines” than susceptible cultivars, yet the development of those that penetrated was only slightly lowered. The identity of the “better breeding lines” was not stated, but if this is the mechanism of resistance in *Rk2* plants, it would be very different from that of *Rk1*, which is a hypersensitive response that inhibits feeding-site formation (Schneider, 1991; Ng'ambi et al., 1999b). However, Schneider (1991) also observed that a small percentage of *Meloidogyne* populations were “able to establish feeding sites and continue development even in resistant cultivars” of tobacco possessing *Rk1*. The hypersensitive response mechanism is also that of the *Mi* root-knot resistance gene in tomato (Dropkin, 1969; Milligan et al., 1998), and of the *Ph_p* gene in tobacco, which confers resistance to the tobacco cyst nematode *Globodera tabacum* (Miller and Gray, 1972) Behrens, 1975 (Johnson et al., 2009). Comparing the

mechanisms involved in how *Rk1* inhibits galling and reproduction of races 1 and 3 of *M. incognita*, to how *Rk2* reduces reproduction of other *Meloidogyne* species, but not galling, could reveal important and useful aspects of host resistance to root-knot nematodes. Similar galling, with reduced reproduction, suggests that nematodes may initialize giant cell formation and start to feed, but are somehow inhibited afterwards. Perhaps something inhibits egg formation. Alternatively, feeding and/or reproduction may be simply slowed, which might explain why a number of nematodes were still able to produce egg masses and eggs. In tomato, a change in root exudates significantly reduced root penetration by *M. incognita* juveniles (Vos et al., 2012). However, the exudate change was attributed to root colonization by arbuscular mycorrhizal fungi, not a resistance gene. Conversely, Ye et al. (2009) observed a change in root structure in resistant rootstocks of *Prunus* spp. that prevented *M. incognita* from penetrating roots. Elucidating the specific mechanism(s) of resistance conferred by *Rk1/Rk2* as a hypersensitive response, modification of root exudates, or possibly an alteration of the root composition itself, could have significant implications for improving nematode management on tobacco.

The population used in these experiments was identified as a variant of *M. incognita* race 3 despite some inconsistent results. Our population reacted negatively to the IncK14F/R and Finc/Rinc *M. incognita*-specific primers, but Adam et al. (2007) noted that the primers IncK14F/R and Finc/Rinc did not always produce consistent results in their study. Similarly, variable results were also obtained from three host specificity assays to identify the species to race. Reproduction during the winter 2014 trial was low for all plant hosts, presumably due to winter low-light conditions (Witzenberger et al., 1988; Gislerød et al., 1989; Meng et al., 2015). Results between the Summer 2014 and Spring 2015 trials varied considerably, and reproduction was very low on pepper, cotton, tobacco and peanut in the Spring 2015 trial. Despite the

reproductive index of cotton and watermelon being less than one in at least two trials, those hosts were designated as susceptible based on egg mass numbers and the amount of root galling. Variation in host specificity exemplifies the difficulty in identifying root-knot populations to race. For example, Robertson et al. (2009) analyzed 140 root-knot populations from Spain and noted that six were *M. incognita* and able to reproduce on tomato, but not pepper, cotton, tobacco or peanut, and labeled it *M. incognita* race 5. Others have also noted a great deal of host variation between populations (Eisenback et al., 1981; Kirkpatrick and Sasser, 1983; Hartman and Sasser, 1985; Barker and Melton, 1990; Noe, 1992). Similarly, the 2004 and 2010 nematode surveys of tobacco fields in Southside VA resulted in 8.3% and 6.3% of unidentifiable *Meloidogyne* populations, respectively (Eisenback, 2012). Perineal pattern morphology can differ between and among populations, which might explain the variable results in the survey (Netscher, 1978; Eisenback et al., 1980).

The *Rk1* gene is effective in providing resistance to *M. incognita* races 1 and 3 and *M. arenaria* race 1 (Barker and Melton, 1990; Ng'ambi et al., 1999a; Ng'ambi et al., 1999b). The drastic reduction of reproduction on plants with *Rk1* compared to the susceptible entry confirms these results, since our population was established to be a variant of *M. incognita* race 3. Varying results of resistance to *M. javanica* caused by the *Rk1* gene have been reported. Ng'ambi et al. (1999b) noted that *M. incognita* races 2 and 4, *M. arenaria* race 2, and *M. javanica* caused significant galling on flue-cured tobacco cultivar Speight G 28 (*Rk1*), similar to Barker and Melton (1990) observation of a “slight level of resistance [in cultivars with *Rk1*] to *M. javanica* compared to susceptible cultivars.” Conversely, a significant reduction in *M. javanica* egg masses, eggs, and the reproductive index on plants with *Rk1* alone were observed in the experiments performed at Virginia Tech in 2010-11 (Ma, W., et al., unpublished data).

Total root weight appeared to have no impact on the results, except in the Blacksburg Nov 2013-Jan 2014 trial when root weights were less than half that of any other trial, and the number of eggs per gram root and the reproductive index were accordingly low. Low light conditions were presumed to have caused the small plant size and low root weights, due the experiment being performed over the winter (Witzenberger et al., 1988; Gislerød et al., 1989).

Studying the resistance efficacy of plants with *Rk1Rk2* on other species and races of *Meloidogyne* would be valuable, specifically on *M. incognita* races 2 and 4 and *M. arenaria* race 2. If the *Rk1Rk2* genes together are successful at suppressing reproduction of these nematode species, then tobacco cultivars will exist with almost complete resistance to the most damaging root-knot nematode species in flue-cured tobacco.

The results of this study show that having both *Rk1* and *Rk2* genes together in flue-cured tobacco cultivars provides greater resistance to root-knot nematodes than either gene alone. Managing root-knot nematodes in tobacco can be critical to producing a satisfactory crop. The shift in root-knot species toward previously less-frequent species and races increases the need for cultivars with multi-species and multi-race nematode resistance. Flue-cured tobacco varieties utilizing both *Rk1* and *Rk2* resistance will provide growers with a valuable tool for managing root-knot nematode populations.

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CHAPTER 3

The effect of soil temperature on reproduction of root-knot nematodes in flue-cured tobacco homozygous for *Rk1* and/or *Rk2* resistance genes

ABSTRACT

Most commercial flue-cured tobacco (*Nicotiana tabacum* L.) cultivars possess the *Rk1* resistance gene, which provides resistance to races 1 and 3 of the root-knot nematode *Meloidogyne incognita*, and race 1 of *M. arenaria*. An increasing number of cultivars now possess a second root-knot resistance gene, *Rk2*. High soil temperatures have been associated with a breakdown of root-knot resistance genes in a number of crops. The objective of this study was to investigate the effect of high soil temperature on the efficacy of *Rk1* and/or *Rk2* genes in reducing parasitism by a variant of *M. incognita* race 3. Three greenhouse experiments were performed from 2014-15 and were arranged in a randomized complete block design in open-top root zone cabinet growth chambers set at 25°C, 30°C, and 35°C. Plants were inoculated with 3,000 root-knot eggs and data collected 35 days post-inoculation. Galling, numbers of egg masses and eggs, and the reproductive index were compared from roots of Coker 371-Gold (C371G; susceptible), NC 95 and SC 72 (homozygous for *Rk1*), T-15-1-1 (homozygous for *Rk2*), and STNCB-2-28 and NOD 8 (homozygous for both *Rk1* and *Rk2*). Nematode reproduction was reduced at 25°C and 30°C on entries possessing *Rk1* and *Rk1Rk2* compared to the susceptible entry and the entry possessing *Rk2*. However, there were often no significant differences in reproduction at 35°C between entries with *Rk1* and/or *Rk2* compared to the susceptible control, indicating an increase of root-knot parasitism on resistant entries at higher temperatures.

INTRODUCTION

Flue-cured tobacco (*Nicotiana tabacum* L.) is an important agricultural crop grown throughout the world (Shepherd and Barker, 1990). Root-knot nematodes (*Meloidogyne* spp.) are a common pathogen wherever tobacco is grown, and *M. incognita* (Kofoid and White, 1919) Chitwood, 1949, *M. arenaria* (Neal, 1889) Chitwood, 1949, *M. javanica* (Treub, 1995) Chitwood, 1949, and *M. hapla* Chitwood, 1949 are the four root-knot species of economic importance that are regularly found in flue-cured tobacco (Johnson et al., 2005). Utilizing host plants with nematode resistance is a common control strategy for root-knot nematodes (Koenning et al., 1999). In Virginia, most commercial flue-cured tobacco cultivars possess the *Rk1* resistance gene, which provides resistance to races 1 and 3 of *M. incognita* and race 1 of *M. arenaria* (Ng'ambi et al., 1999a). An increasing number of cultivars also possess a second root-knot resistance gene, *Rk2* (Reed et al., 2015), which confers some degree of resistance or tolerance to *M. javanica* (Schweppenhauser, 1968; Schweppenhauser, 1975; Ternouth et al., 1986). Because of the widespread use of tobacco cultivars with the *Rk1* gene, root-knot species and races that cannot be controlled by cultivars with *Rk1* are increasing in prevalence and importance: *M. incognita* races 2 and 4, *M. arenaria* race 2, and *M. javanica* (Fortnum et al., 1984; Barker, 1989; Eisenback, 2012).

Soil temperature plays a critical role in the efficacy of resistance genes in a number of crops. One of the most studied of these nematode resistance-temperature interactions is the *Mi* gene in tomato (*Solanum lycopersicum* L.) plants. Dropkin (1969) observed that only 2% of root-knot juveniles developed in roots of tomato plants containing the *Mi* resistance gene at 28°C, whereas 87% developed at 33°C. In common bean (*Phaseolus vulgaris* L.), resistance to *M. incognita* due to recessive genes failed at 26°C to 28°C, while resistance due to dominant genes

failed in plant entries at 28°C to 30°C (Omwega and Roberts, 1992). Rate of nematode development and total population numbers increased on the *M. incognita*-resistant sweet potato (*Ipomea batata* L.) cultivar ‘Nemagold’ as temperatures increased from 24°C to 32°C (Jatala and Russell, 1972). In grape rootstocks, resistance to *Meloidogyne* spp. began to break down at 27°C with increased root galling and egg mass formation (Ferris et al., 2013).

Root-knot resistance does not always fail at higher temperatures in tomato. Ammati et al. (1986) noted that root-knot resistance failed at higher temperatures in some plants but not others. The tomato cultivar ‘VFN8,’ containing the *Mi* resistance gene, was resistant to *M. incognita* at 25°C and susceptible at 32°C. Three other genotypes: *Solanum corneliomulleri* J. F. Macbr. (previously known as *Lycopersicon glandulosum* C. H. Mull.) Acc. No. 126443, *Solanum peruvianum* L. Acc. No. 270435, and *Solanum peruvianum* L. Acc. Nos. 129152 and LA2157, retained resistance at 32°C. A suggested explanation for this occurrence was that that gene(s) other than the *Mi* gene may have been present and preserved the resistance in those genotypes (Ammati et al., 1986).

Baum et al. (1995) noted resistance to *M. incognita* race 3 starting to break down at 28°C in the flue-cured tobacco cultivar NC 95, containing the *Rk1* gene. Increased galling and numbers of egg masses were observed at 28°C, with further increases noted at 31°C and 35°C. Schweppenhauser (1968) observed that *M. javanica* infection on resistant (*Rk2* gene) and susceptible tobacco lines appeared to be greater in the summer compared to the winter in Zimbabwe, and assumed this apparent trend was due to approximate greenhouse air temperatures of up to 41°C in the summer in contrast to 21°C to 35°C in the winter. J. I. Way also noted increased root invasion and galling on resistant cultivars by *M. javanica* when soil temperature was increased from 25°C to 30°C, yet when soil temperature was raised again to 35°C, they

decreased (Shepherd and Barker, 1990). Unfortunately, Shepherd and Barker (1990) did not state whether the *M. javanica*-resistant cultivars mentioned contained the *Rk2* gene. A report from Japan also noted that root galling, rate of invasion, and nematode development by *M. javanica* increased on the cultivar Okinawa compared to susceptible cultivars as temperature increased from 28°C to 35°C; yet the mechanism of resistance in this cultivar is unclear (Fukudome and Kamigama, 1982). Because of these studies noting changes in resistance to *Meloidogyne* spp. associated with elevated temperatures, and the limited research on the effect of temperature specifically on the *Rk2* gene, alone and combined with *Rk1*, the objective of this study was to compare the effects of increasing soil temperature on resistance conferred by *Rk2*, *Rk1*, or both genes in combination against a variant of *M. incognita* race 3.

MATERIALS AND METHODS

Population source: A population variant of *M. incognita* race 3 was used in this experiment. A detailed account of the identification of the population to the species and race level is outlined in Chapter 2 of this thesis. In summary, a population of *M. arenaria* was received from Clemson University in Clemson, South Carolina, which had been identified as *M. arenaria* race 2 based on esterase (EST) and superoxide dismutase (SOD) isozyme patterns (P. Agudelo, personal communication), and later reconfirmed by perineal pattern morphology and species-specific PCR primers.

To re-verify the identity of the population morphologically, eight female stylets and 10 perineal patterns were evaluated, and gel electrophoresis of esterase isozymes were performed on three females. Species-specific sequence-characterized-amplified-region (SCAR) primers (MiF/MiR, IncK14F/R, Rinc/Rinc) and PCR-DNA sequences on rRNA 18S, ITS, 28S, D2/D3,

histone and mitochondrial DNA COII-16S gene were then examined (W.M. Ye, personal communication). Additionally, three runs of a greenhouse differential host test were performed to potentially identify the population to a host race level (Taylor and Sasser, 1978).

Greenhouse trials: Three greenhouse trials were conducted in 2014-15 to evaluate whether or not temperature influences root-knot resistance gene efficacy in flue-cured tobacco. The summer trial was performed from June to July 2014, the fall trial from September to October 2014, and the winter trial from December 2014 to January 2015. Each trial was conducted at the Virginia Tech Southern Piedmont Agricultural Research and Extension Center (SPAREC) in Blackstone, VA, and organized in a randomized complete block design. Six tobacco entries were evaluated: Coker 371-Gold (C371G), which is susceptible to the four common root-knot species on tobacco (*M. incognita*, *M. arenaria*, *M. javanica*, and *M. hapla*); SC 72 and NC 95, which are homozygous for the *Rk1* gene; T-15-1-1, which is homozygous for the *Rk2* gene; and STNCB-2-28 and NOD 8, which are homozygous for both *Rk1* and *Rk2*. Plants were grown in open-top root zone cabinet growth chambers (Environmental Growth Chambers, Chagrin Falls, OH). Nematode reproduction was investigated at three soil temperatures: $25 \pm 1.2^\circ\text{C}$, $30 \pm 1.6^\circ\text{C}$, and $35 \pm 2.3^\circ\text{C}$. Soil temperatures were taken with a digital stem thermometer two times per day and the range was averaged across all experiments (General Tools & Instruments LLC, Secaucus, NJ). At each soil temperature, there were four replications per treatment in the summer trial and six in the fall and winter trials. When seedlings had formed approximately four true leaves (~5-10 cm tall) each seedling was transplanted into a 10-cm-diam. clay pot filled with a 2:1 mixture of topsoil (53% sand, 40% silt, 7% clay; pH 5.5) and Profile® Greens Grade™ porous ceramic material (Profile Products, Buffalo Grove, IL). Each plant was inoculated with 3,000 root-knot nematode eggs one week after transplanting by pipetting an egg suspension into two holes

approximately 4-cm deep on either side of each plant. Plants were grown without supplemental lighting in the summer and fall trials, but for the winter trial, plants were grown with supplemental lights set to a 13:11 light:dark photoperiod using GE Plant and Aquarium Ecolux 40 W wide spectrum bulbs (General Electric, USA). Soil moisture was standardized between chambers based on a soil moisture deficit in a designated pot in each growth chamber using a soil tensiometer system (Irrometer Co. Inc., Riverside, CA).

Percent root galling, the number of egg masses per gram of root, and the number of eggs per gram of root were determined after 35 days. Soil was rinsed from roots and the fresh root system was weighed. Galled roots were separated from non-galled roots and percent root galling was calculated based on the fresh weight of galled roots versus the fresh weight of the entire root system. Galled roots were then recombined with non-galled roots and mixed. The mixed galled and healthy roots were then divided in half by weight, and half were stained with 0.15 g/liter Phloxine B (Dickson and Struble, 1965) to define egg masses. The numbers of egg masses from three 1-g subsamples per plant were counted using a dissecting microscope at $\times 10$ to estimate the number of females. Eggs were bleach-extracted from the second half of the root system using the procedure of Hussey and Barker (1973). Extracted eggs were suspended in 500-ml of water and counted in two 10-ml aliquots using a compound microscope at $\times 40$ magnification. To assess the nematode reproductive capability on each entry, the reproductive index (P_f/P_i) was calculated by dividing the final number of eggs extracted per plant (P_f) by the initial number of egg inoculum (P_i) (Sasser et al., 1984).

Statistical analysis: Data were analyzed separately by analysis of variance (ANOVA), and means were compared using the Tukey-Kramer honest significant difference (HSD) test ($P = 0.05$). Percent galling, egg mass counts, and egg counts were transformed by $\log_{10}(x + 1)$ before

statistical analysis. Non-transformed means are displayed. Data were analyzed using the Statistical Analysis System-JMP® Pro 11 (SAS Institute, Cary, NC).

RESULTS

Species identification. A nematode population received from Clemson, South Carolina had been identified as *M. arenaria* race 2, however, morphology of the female stylets and perineal patterns were not consistent with either *M. arenaria* or *M. incognita*, yet more similar to them than any other root-knot species.

The results from our gel electrophoresis of esterase isozymes suggested the population was *M. incognita*, as did PCR-DNA sequences on rRNA 18S, ITS, 28S, D2/D3, histone and mitochondrial DNA COII-16S gene (W.M. Ye, personal communication), results using the *M. incognita*-specific SCAR primer set MiF/MiR, and results from the differential host tests, which tentatively identified the population as *M. incognita* race 3, despite results from the *M. incognita*-specific primer sets IncK14F/R and Finc/Rinc suggesting that the population was another *Meloidogyne* biotype.

Total root weights. Differences in total fresh weight of roots were only observed among tobacco entries at 25°C in the summer and winter trials, at 30°C in the fall and winter experiments (Fig. 3.1). Mean root weight was greater at 25°C for NC 95 than for all other entries except NOD 8 in the summer trial ($P \leq 0.05$), while root weight of T-15-1-1 was greater than that of NC 95 at 30°C in the fall test ($P \leq 0.05$). In the winter study, root weight of NOD 8 was lower than that of all the other experimental entries at 25°C, and lower than that of the susceptible cultivar Coker 371-Gold and T-15-1-1 at 30°C ($P \leq 0.05$).

Rkl entries. In all trials, galling and mean numbers of egg masses and nematode eggs per g of root were significantly lower at 25°C in both cultivars possessing only *Rkl* compared to the susceptible control, Coker 371-Gold ($P \leq 0.05$) (Figs. 3.2-3.4). Galling and numbers of egg masses and eggs were also reduced by *Rkl* alone at 30°C versus the susceptible control in the summer and fall trials ($P \leq 0.05$), but not in the winter experiment. No differences were noted in galling among experimental entries at 30°C in the winter test (Fig. 3.2). Mean egg mass numbers were also similar for the *Rkl* cultivars and the susceptible control in that trial, and total numbers of root-knot nematode eggs per g of root were lower for NC 95, but not SC 72 ($P \leq 0.05$) (Figs. 3.3,3.4). Mean reproductive indices were significantly lower for cultivars with *Rkl* alone at both 25°C and 30°C in all trials compared to the susceptible cultivar ($P \leq 0.05$) (Table 3.1). In contrast, galling, mean egg mass counts and mean numbers of total eggs per g of root, and reproductive indices were all lower on the *Rkl* cultivars versus the susceptible control at 35°C in the fall study ($P \leq 0.05$), but not in the summer or winter tests (Figs. 3.2-3.4).

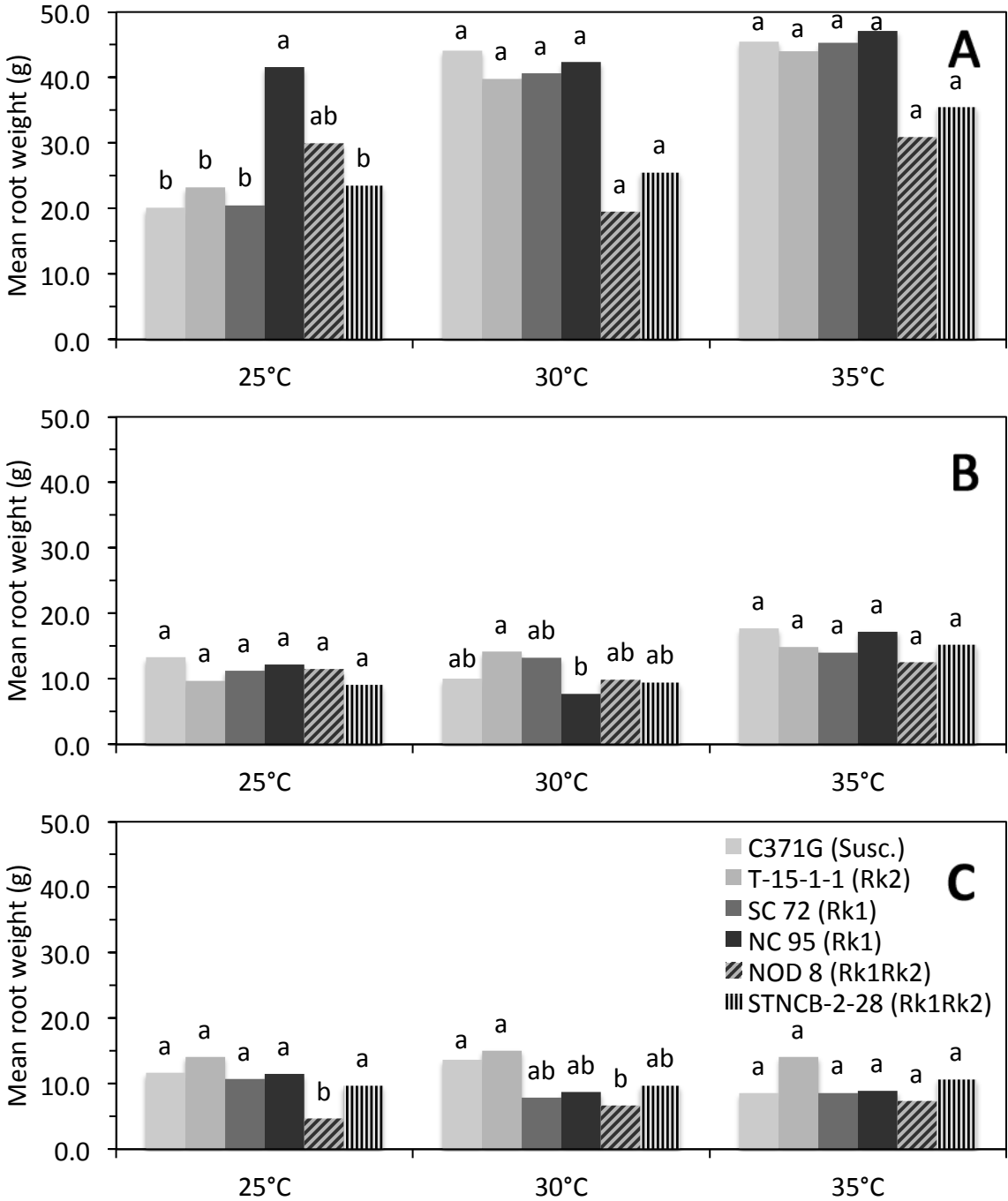


Fig. 3.1. Mean total fresh root weights (g) of six tobacco (*Nicotiana tabacum* L.) entries inoculated with a variant of *M. incognita* race 3 and grown at three soil temperatures in greenhouse trials (A) summer (June-July 2014), (B) fall (Sept-Oct 2014), and (C) winter (Dec 2014-Jan 2015). At each temperature within trials, means followed by the same letter(s) are not significantly different according to statistical analysis of transformed [$\log_{10}(x+1)$] data and the Tukey-Kramer honest significant difference (HSD) test ($P = 0.05$).

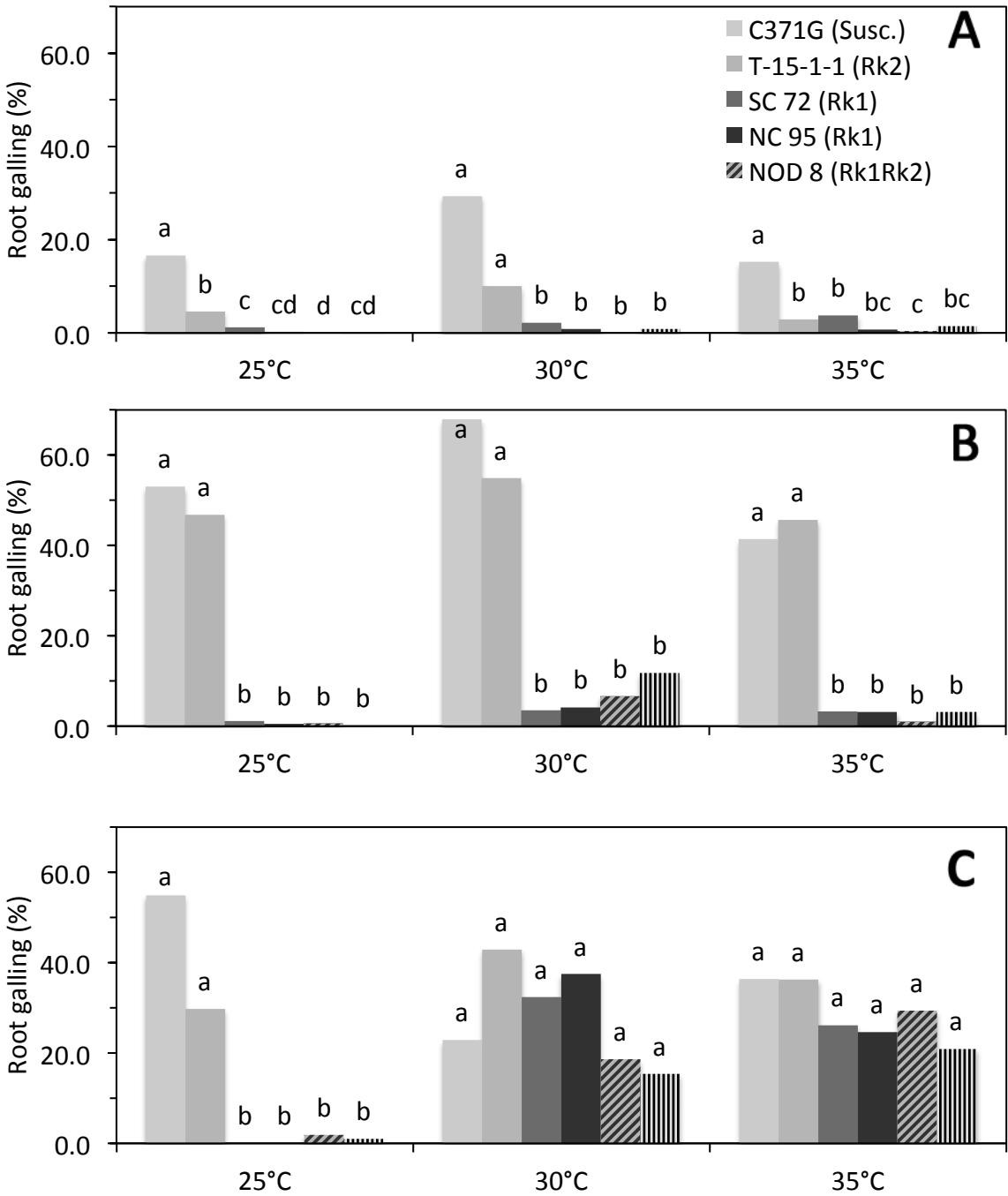


Fig. 3.2. Mean percent root galling of six tobacco (*Nicotiana tabacum* L.) entries inoculated with a variant of *M. incognita* race 3 and grown at three soil temperatures in greenhouse trials. (A) summer (June-July 2014), (B) fall (Sept-Oct 2014), and (C) winter (Dec 2014-Jan 2015). At each soil temperature within trials, means followed by the same letter(s) are not significantly different according to statistical analysis of transformed [$\log_{10}(x+1)$] data and the Tukey-Kramer honest significant difference (HSD) test ($P = 0.05$).

Rk2 entry results. *Rk2* did not reduce galling compared to susceptible Coker 371-Gold at any temperature in the fall and winter trials, but did reduce galling compared to Coker 371-Gold at 25°C and 35°C ($P \leq 0.05$), but not 30°C, in the summer trial (Fig. 3.2). While no differences were observed in egg masses between the *Rk2* entry and the susceptible control at 25°C in the summer trial or at 30°C or 35°C in any trial, fewer egg masses were observed per g of root on T-15-1-1 versus the susceptible control at 25°C in both the summer and fall trials ($P \leq 0.05$) (Fig. 3.3). Total numbers of eggs per g of root were similar for the *Rk2* entry and the susceptible control at all three temperatures in all trials, with the exception that fewer eggs were counted per g of root for T-15-1-1 than for Coker 371-Gold in the fall study ($P \leq 0.05$) (Fig. 3.4). However, reproductive indices were significantly lower at 25°C for the *Rk2* entry in all experiments ($P \leq 0.05$), but were always similar between these two entries at 30°C and 35°C (Table 3.1).

Galling and mean numbers of egg masses were significantly higher on the *Rk2* entry compared to the two *Rk1* cultivars at 25°C in all trials ($P \leq 0.05$), but only in the summer and fall experiments when soil temperature was maintained at 30°C ($P \leq 0.05$) (Figs. 3.2, 3.3). In contrast, *Rk1* appeared to significantly reduce galling and numbers of egg masses compared to *Rk2* at 35°C in the fall study ($P \leq 0.05$) but not the summer or winter tests. Total numbers of nematode eggs extracted per g of root were lower at 25°C for the two *Rk1* cultivars versus the *Rk2* entry in the winter trial ($P \leq 0.05$), but not in the summer test, while STNCB-2-28, but not NOD 8, exhibited fewer eggs per g of root compared to Coker 371-Gold at 25°C in the fall experiment ($P \leq 0.05$) (Fig. 3.4). Fewer egg masses per g of root were enumerated for both *Rk1* cultivars versus T-15-1-1 at 30°C in the summer and fall studies ($P \leq 0.05$), but not in the winter test (Fig. 3.3).

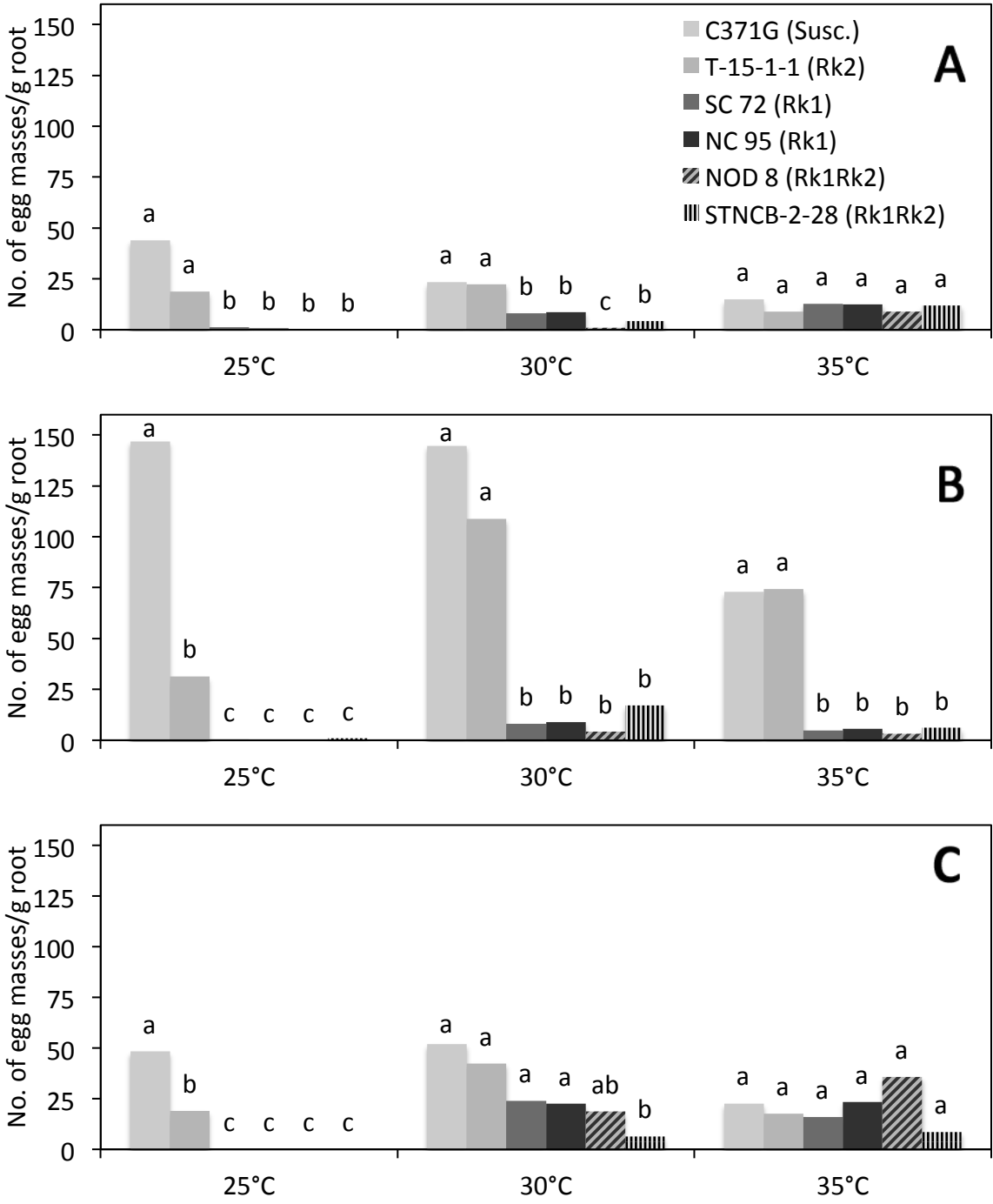


Fig. 3.3. Mean egg mass counts per g root of a variant of *M. incognita* race 3 on six tobacco (*Nicotiana tabacum* L.) entries grown at three soil temperatures in greenhouse trials. (A) summer (June-July 2014), (B) fall (Sept-Oct 2014), and (C) winter (Dec 2014-Jan 2015). At each soil temperature within trials, means followed by the same letter(s) are not significantly different according to statistical analysis of transformed [$\log_{10}(x+1)$] data and the Tukey-Kramer honest significant difference (HSD) test ($P = 0.05$).

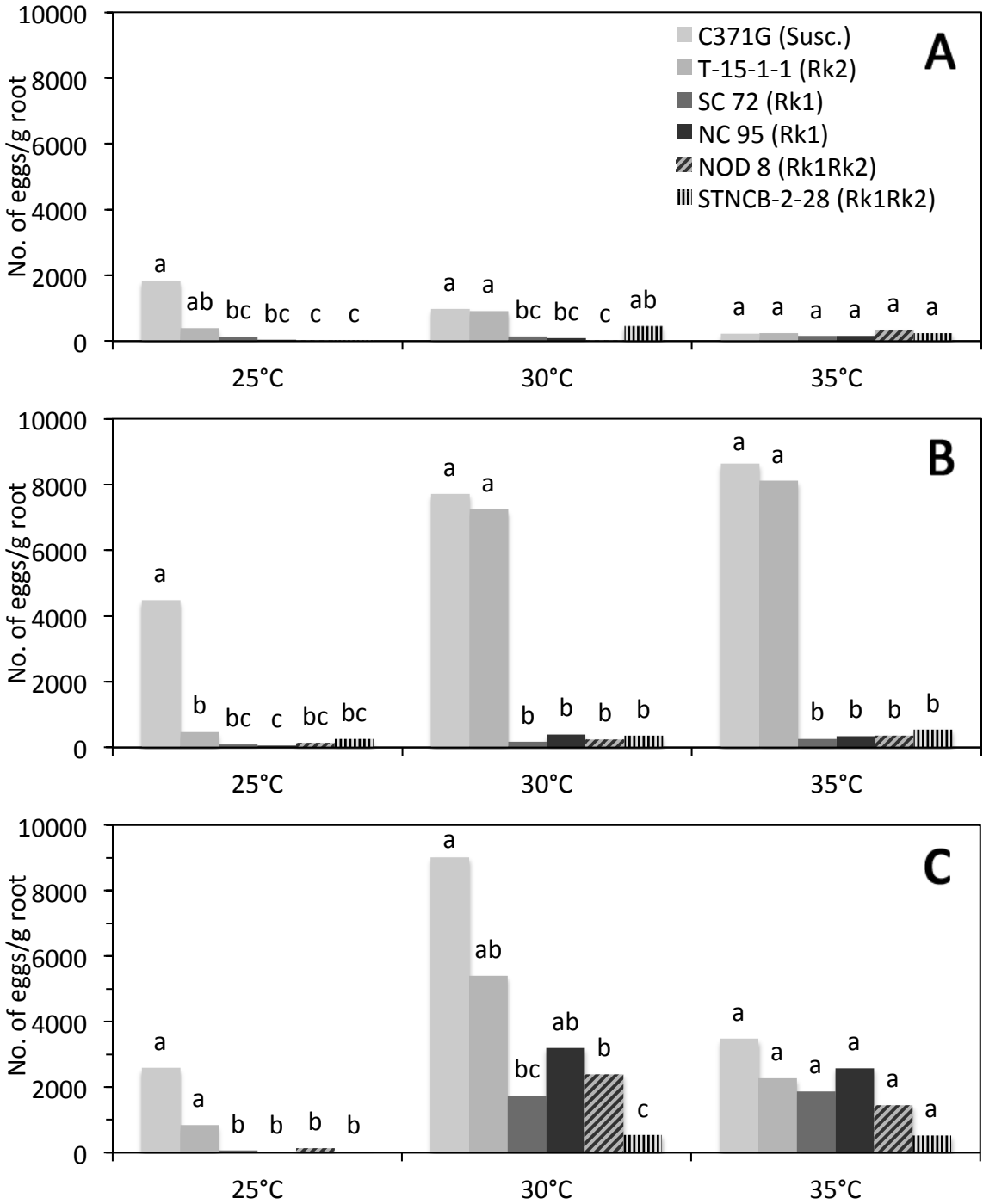


Fig. 3.4. Mean egg counts per g root of a variant of *M. incognita* race 3 on six tobacco (*Nicotiana tabacum* L.) entries grown at three soil temperatures in greenhouse trials. (A) summer (June-July 2014), (B) fall (Sept-Oct 2014), and (C) winter (Dec 2014-Jan 2015). At each soil temperature within trials, means followed by the same letter(s) are not significantly different according to statistical analysis of transformed [$\log_{10}(x+1)$] data and the Tukey-Kramer honest significant difference (HSD) test ($P = 0.05$).

Rk1Rk2 entries. Gallings and mean egg mass numbers per g of root were always significantly lower for entries with both *Rk1* and *Rk2* than for the susceptible control and T-15-1-1 (with *Rk2* alone) at 25°C ($P \leq 0.05$), at 30°C in the summer and fall trials ($P \leq 0.05$), and at 35°C in the fall trial ($P \leq 0.05$) (Figs. 3.2, 3.3). Mean numbers of eggs extracted per g of root were lower at 25°C for *Rk1Rk2* entries versus the susceptible control and T-15-1-1 in the summer and winter trials ($P \leq 0.05$), but not in the fall experiment (Fig. 3.4). At 30°C, mean numbers of eggs per g of root were lower for both *Rk1Rk2* entries compared to Coker 371-Gold and T-15-1-1 ($P \leq 0.05$), but in the summer trial egg numbers were similar among STNCB-2-2, T-15-11, and the susceptible control, while in the winter experiment numbers of eggs per g of root were lower for STNCB-2-2 versus Coker 371-Gold and T-15-1-1 ($P \leq 0.05$), but similar for NOD 8 and T-15-1-1. At 35°C in the summer trial, gallings were significantly lower for both *Rk1Rk2* entries versus the susceptible control cultivar ($P \leq 0.05$), but were lower for NOD 8 compared to the *Rk2* alone entry but not STNCB-2-28 ($P \leq 0.05$) (Fig. 3.2). Gallings were not significantly lower on entries with both root-knot resistance genes compared to the susceptible control and *Rk2* alone at 30°C and 35°C in the winter trial. Combining *Rk2* with *Rk1* never significantly lowered gallings compared to *Rk1* alone at any temperature in any trial (Fig. 3.2).

While entries with *Rk1* and/or *Rk2* reduced gallings at 35°C compared to susceptible Coker 371-Gold in the summer and fall experiments ($P \leq 0.05$), gallings were similar among all entries in the winter study (Fig. 3.2). Reproductive indices and mean numbers of egg masses and total nematode eggs extracted per g of root were similar among all entries at 35°C in the summer and winter trials, although lower reproductive indices and fewer egg masses and total eggs were observed at this temperature for entries with *Rk1*, or with *Rk1* and *Rk2*, compared to the susceptible and *Rk2* entries in the fall study ($P \leq 0.05$) (Figs. 3.3-3.4, Table 3.1).

Table 3.1. Reproduction of a variant of *M. incognita* race 3 on six tobacco (*Nicotiana tabacum* L.) entries grown at three soil temperatures in greenhouse trials conducted in 2014-15.

Plant entry	Resistance genes	Soil temperature (°C)	Reproductive index (P_f/P_i) ^{ab} by trial		
			June-July 2014 (summer)	Sept-Oct 2014 (fall)	Dec 2014-Jan 2015 (winter)
C371G	None	25	11.3 a	18.5 a	9.4 a
T-15-1-1	<i>Rk2</i>	25	3.0 b	1.9 b	3.6 b
SC 72	<i>Rk1</i>	25	0.7 c	0.3 b	0.2 c
NC 95	<i>Rk1</i>	25	0.6 c	0.2 b	0.1 c
NOD 8	<i>Rk1Rk2</i>	25	0.2 c	0.4 b	0.2 c
STNCB-2-28	<i>Rk1Rk2</i>	25	0.2 c	0.5 b	0.1 c
C371G	None	30	14.8 a	29.3 a	38.9 a
T-15-1-1	<i>Rk2</i>	30	11.8 a	35.4 a	25.3 a
SC 72	<i>Rk1</i>	30	1.9 b	0.7 b	3.8 bc
NC 95	<i>Rk1</i>	30	1.3 b	1.2 b	8.7 b
NOD 8	<i>Rk1Rk2</i>	30	0.1 b	0.9 b	3.3 bc
STNCB-2-28	<i>Rk1Rk2</i>	30	1.7 b	1.2 b	1.8 c
C371G	None	35	3.5 a	50.0 a	10.5 a
T-15-1-1	<i>Rk2</i>	35	3.5 a	37.0 a	10.8 a
SC 72	<i>Rk1</i>	35	2.3 a	1.1 b	3.4 a
NC 95	<i>Rk1</i>	35	1.9 a	1.7 b	5.8 a
NOD 8	<i>Rk1Rk2</i>	35	3.7 a	1.2 b	3.2 a
STNCB-2-28	<i>Rk1Rk2</i>	35	2.9 a	2.5 b	2.5 a

^a Reproductive index = final population/initial population (P_f/P_i). Values less than 1.0 are accented with bold text.

^b At each soil temperature within trials, means followed by the same letter(s) are not significantly different according to statistical analysis of transformed [$\log_{10}(x+1)$] data and the Tukey-Kramer honest significant difference (HSD) test ($P = 0.05$).

DISCUSSION

Results from these studies suggest that flue-cured tobacco resistance to a variant of *M. incognita* race 3 declined at 30°C and 35°C compared to 25°C. A drastic reduction in nematode reproduction at 25°C and 30°C was associated with *Rk1* alone and *Rk1Rk2* together compared to the susceptible entry. However, at 35°C, reproduction on *Rk1* and *Rk1Rk2* appeared similar to that of the susceptible entry. Additionally, resistance associated with *Rk1Rk2* together did not appear to be significantly more effective than that of *Rk1* alone in suppressing reproduction of this variant of *M. incognita* race 3. No consistent trends between *Rk1* and *Rk1Rk2* occurred in egg mass counts, egg counts, and the reproductive index across temperatures, perhaps because there was so little parasitism on these entries. The temperature-sensitive *Rk1* resistance results corroborate those by Baum et al. (1995), who observed *M. incognita* race 3 resistance began breaking down at 28°C, and further at 31°C and 35°C, in the *Rk1*-containing tobacco cultivar NC 95. The results of this study are also supported by results on other crops. Temperature-sensitive *Meloidogyne* resistance has been noted in tomato with the *Mi* resistance gene (Dropkin, 1969), common bean (Omweaga and Roberts, 1992), sweet potato (Jatala and Russell, 1972), grape (*Vitis* spp.) rootstocks (Ferris et al., 2013), and cotton (Carter, 1982).

Similarly, numbers of egg masses and eggs, and the reproductive index of plants with *Rk2* were almost always significantly lower than those of the susceptible control at 25°C. At 30°C and 35°C, however, there were frequently no significant differences in reproduction between the *Rk2* entry and the susceptible control. The *Rk2* gene appeared to inhibit reproduction less effectively at 30°C and 35°C than at 25°C, although *Rk2* alone seemed to provide only modest resistance to this population of *M. incognita* race 3 even at 25°C, especially compared to the effects of *Rk1* or *Rk1Rk2*. Schweppenhauser (1968) observed higher *M. javanica* infection on

resistant (*Rk2*) and susceptible lines in the summer, when approximate greenhouse air temperatures regularly reached 41°C, compared to winter infection with temperatures of approximately 21°C to 35°C. Ng'ambi et al. (1999b) observed that the cultivar Okinawa did not provide any resistance to *M. javanica* in their study at greenhouse temperatures of 27 ± 2°C and 30 ± 3°C. In contrast, Fukudome and Kamigama (1982) found that galls caused by *M. javanica* were rare on Okinawa at 28°C, but at 35°C there were no differences in galling, invasion rate, and development compared with the susceptible entry. In Zimbabwe, J.I. Way also noted increased *M. javanica* root invasion and galling on both resistant and susceptible tobacco when soil temperature rose from 25°C to 30°C, but parasitism actually decreased when soil temperature was further increased to 35°C (Shepherd and Barker, 1990). Shepherd and Barker (1990) did not state if the *M. javanica*-resistant cultivars contained the *Rk2* gene. Despite some variation, this same trend of increased galling at higher temperatures appeared across all entries, however, not in egg mass counts, egg counts, or the reproductive index.

Despite a reduction in reproduction, galling on the *Rk2* entry in most trials was not significantly different from that of the susceptible entry at all temperatures evaluated. This observation was consistent with results described in Chapter 1 of this thesis, and results from experiments performed at Virginia Tech in 2010-11 examining *M. javanica* reproduction on the same flue-cured tobacco entries used in this experiment (Ma, W., Johnson, C. S., Eisenback, J. D., and Reed, T. D. unpublished work). However, Ng'ambi et al. (1999b), noted that the amount of galling and reproduction were generally correlated. Consistent with our results, the galling by *M. incognita* and *M. javanica* without a corresponding increase in egg masses was noted in *Prunus* rootstocks (Lu et al., 2000). The authors determined that nematodes “can infect and partially develop in resistant root systems, but can’t complete their life cycles.”

Despite significantly lower reproduction on *Rk1* and *Rk1Rk2* entries than those of the control and *Rk2* at 30°C, the reproductive indices of those entries almost always increased from below one at 25°C to above one at 30°C and 35°C. A reproductive index above one, where the final nematode concentration, P_f , is higher than the initial concentration, P_i , suggests that a plant is somewhat susceptible. Our reproductive index results indicate that despite having lower reproduction compared to the susceptible entry and *Rk2* entry, reproduction was usually higher at 30°C for *Rk1* and *Rk1Rk2* entries than at 25°C, and further increased at 35°C.

A shorter average natural day length (11 h 42 m) during the fall trial presumably affected results compared to the summer (14 h 25 m) and winter (13 h) trials (U.S. Naval Observatory). The average natural day length during the winter trial (9 h 48 m) was supplemented to 13 h, which could have caused the similar results to the summer trial. The reason for differences in magnitude of total root weights and egg mass and egg counts between trials is unclear. Meng et al. (2015) determined that light quality had an effect on tobacco seedling root growth, which may have affected root growth in this experiment as well.

Nematode reproduction is limited by high temperature, which might naturally limit the plant growth consequences due to parasitism. *M. incognita* inhabits areas with an average annual temperature range of 18°C to 30°C, with an optimum warm-month temperature of 27°C (Eisenback et al., 1981). Gallings and egg mass numbers from the susceptible cultivar in this study usually decreased as soil temperature increased from 25°C to 35°C, presumably because 35°C is above the optimal temperature range for this population. Soil temperatures in Virginia are commonly above 30°C. Maximum daily soil temperatures logged at the Virginia Tech Southern Piedmont AREC were above 30°C for 8 days in May 2014, 15 days in June and July 2014, 11 days in August 2014, and 6 days in September 2014. May, June, and July also had

maximum temperatures above 35°C for one, two, and three days, respectively. The soil temperatures were taken below grass and likely field temperatures were even higher.

Soil temperatures fluctuate, and these fluctuations could influence the efficacy of resistance to root-knot nematodes in tobacco. Tobacco plants resistant to tobacco mosaic virus (TMV) become susceptible at temperatures above 28°C, but the hypersensitive response returns when temperatures drop, killing the plant (Malamy et al., 1992; Marathe et al., 2002; Zhang et al., 2009). Tomato plants resistant to *M. incognita* due to the *Mi* gene became susceptible after 48 h at 34°C, but remained susceptible for one-to-two days after temperatures were reduced to 27°C (Zacheo et al., 1995). Juveniles that had penetrated and started feeding when resistance was deactivated continued developing when temperatures decreased. Some cultivars of tomato (Ammati et al., 1986; Wang et al., 2013) and pepper (Thies and Fery, 1998; Djian-Caporalino et al., 1999; Djian-Caporalino et al., 2001) retained resistance to *Meloidogyne* species at high temperatures, even up to 32°C and 42°C in four pepper cultivars (Djian-Caporalino et al., 2001). Further research and breeding could yield positive results in eliminating the temperature-sensitivity of resistance to *Meloidogyne* species in flue-cured tobacco.

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